Int. J. Dev. Biol. 45: 405-414 (2001)

Original Article

Cell cycle phase, cellular Ca^{2+} and development in *Dictyostelium discoideum*

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ABSTRACT In *Dictyostelium discoideum,* the initial differentiation of cells is regulated by the phase of the cell cycle at starvation. Cells in S and early G2 (or with a low DNA content) have relatively high levels of cellular Ca^{2+} and display a prestalk tendency after starvation, whereas cells in mid to late G2 (or with a high DNA content) have relatively low levels of Ca^{2+} and display a prespore tendency. We found that there is a correlation between cytosolic Ca^{2+} and cell cycle phase, with high Ca^{2+} levels being restricted to cells in the S and early G2 phases. As expected on the basis of this correlation, cell cycle inhibitors influence the proportions of amoebae containing high or low Ca^{2+}. However, it has been reported that in the *rtoA* mutant, which upon differentiation gives rise to many more stalk cells than spores (compared to the wild type), initial cell-type choice is independent of cell cycle phase at starvation. In contrast to the wild type, a disproportionately large fraction of *rtoA* amoebae fall into the high Ca^{2+} class, possibly due to an altered ability of this mutant to transport Ca^{2+}.

KEY WORDS: *Dictyostelium, calcium, cell cycle, DNA content, cell fate, rtoA.*

Introduction

An unusual feature of the life cycle of the social amoeba *Dictyostelium discoideum* is that the multicellular state arises via aggregation. This makes it especially advantageous for investigations into the cellular correlates of differentiation and pattern formation. After starvation, free-living amoebae aggregate and form a cylindrical mass, the slug, which goes on to differentiate into two cell types - stalk cells and spores. Prestalk (or presumptive stalk) cells constitute nearly 20% of the slug and are located in its anterior; prespore (or presumptive spore) cells occupy the posterior 80% (Bonner, 1967). Early phenotypic differences with respect to cellular Ca^{2+} and cell cycle phase can bias post-aggregation cell fate (reviewed in Nanjundiah and Saran, 1992; Saran *et al.,* 1994; Azhar *et al.,* 1996). Cells that are in S or early G2 when starved have a different predisposition than those that are in mid to late G2. Similarly, cells that contain high levels of calcium at starvation differ in their fates from those that are relatively calcium-poor. The obvious question arises, are cell cycle phases and Ca^{2+} related, and if so, how?

Previous cell cycle studies in *D. discoideum* (reviewed in Weeks and Weijer, 1994) have been carried out on axenic strains rather than on the bacterially grown wild type. Not all workers are in agreement on the relative duration of phases. Some studies indicate a substantial G2 (46-60% of an 8 hr. cycle in axenically grown cells), a large S phase (19%-45%) and a short G1 (10%-25% of the cycle; Zada-Hames and Ashworth, 1978; McDonald and Durston, 1984). Other studies show that the G1 phase is absent (Durston *et al.,* 1984; Weijer *et al.,* 1984; Ohmori and Maeda, 1987). Both the cell cycle phase at starvation and progression through the cell cycle during development have been implicated in differentiation. Amoebae in S and early G2 at starvation exhibit a prestalk tendency whereas those in mid to late G2 tend to differentiate into prespores (Weijer *et al.,* 1984; McDonald and Durston, 1984; Gomer and Firtel, 1987). There are reports of both mitosis (Bonner and Frascella, 1952) and DNA replication (Durston, 1984) in the slug stage, and also of DNA replication without mitosis (Zimmerman and Weijer, 1993). In the latter situation prespore DNA, most likely mitochondrial DNA (Shaulsky and Loomis, 1995), replicates preferentially. Gomer and Ammann (1996) have shown that lengthening the S phase with a variety of inhibitors leads to a significant increase in the prestalk: prespore ratio. Wood *et al.*

Abbreviations used in this paper: BrdU, bromodeoxyuridine; FACS, fluorescence-activated cell sorting; PI, propidium iodide.

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0214-6282/2000/$20.00

Printed in Spain
www.ehu.es/ijdb

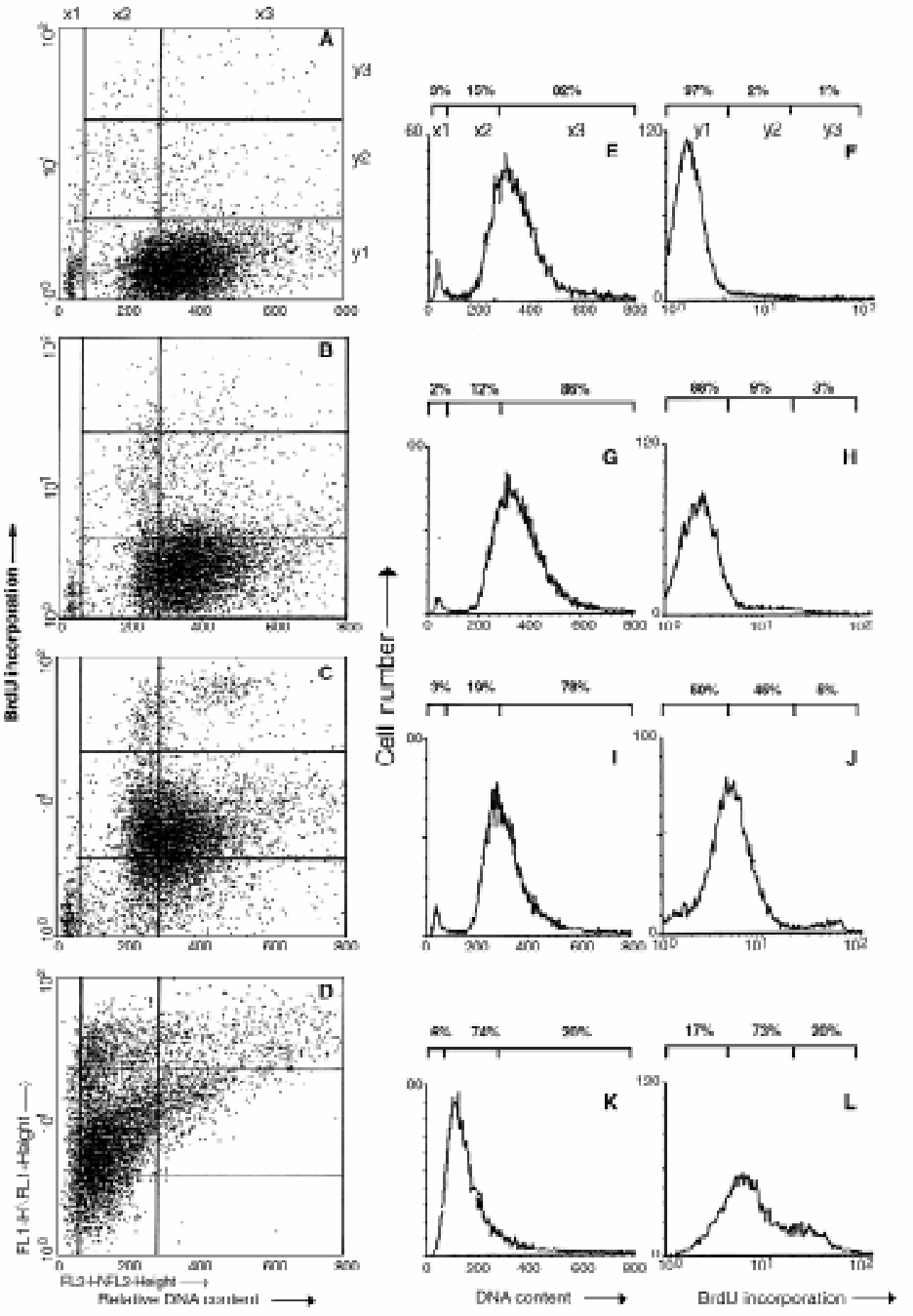

Fig. 1. Cell cycle phases in NC4 amoebae. *A representative bivariate FACS analysis of relative DNA content and BrdU incorporation in asynchronously growing amoebae. Vegetative cells were processed as described in Materials and Methods to simultaneously monitor BrdU incorporation and relative DNA content.* **(Abscissa)** *relative DNA content as judged by propidium iodide fluorescence;* **(Ordinate)** *BrdU incorporation as seen by fluorescein-isothiocyanate (FITC) fluorescence. (A), (B), (C) and (D) show two parameter dot plots.* **(A)** *amoebae without BrdU pulse;* **(B)** *amoebae after a 5 min BrdU pulse;* **(C)** *amoebae after a 30 min BrdU pulse;* **(D)** *amoebae after a 30 min BrdU pulse following 8 h hydroxyurea treatment. (E,F), (G,H) and (I,J) and (K,L) are corresponding one-dimensional distributions of relative DNA content and BrdU incorporation of (A), (B), (C) and (D) respectively. x1, x2, x3 and y1, y2, y3 denote windows depicting different classes of DNA content and BrdU incorporation used for cell cycle phase assignment (see text). The cutoff for both nuclear and mitochondrial BrdU incorporation was based on microscopic observations after cell sorting. Amoebae below the cutoff have no nuclear BrdU incorporation (y1 and y2) whereas those above do (y3). Also, a small number of cells (lying within rectangle x2,y3) show significant BrdU incorporation into both nuclear and mitochondrial DNA. Note the higher fraction of cells in rectangle (x2,y3) after hydroxyurea treatment (D) as compared to control (A).*

(1996) have isolated a mutant, ratio A (*rtoA*), in which the link between the cell cycle phase at starvation and cell-type choice has broken down. This defect is associated with the appearance of an abnormally high percentage of stalk cells in the fruiting body.

Cellular Ca^{2+} too plays an important role in cell-type differentiation and pattern formation in *D. discoideum* (reviewed in Newell *et al.,* 1995). There is a difference in total Ca^{2+} levels between presumptive stalk and spore cells in the slug (Maeda and Maeda, 1973; Saran *et al.,* 1994a; Azhar *et al.,* 1995; Cubitt *et al.,* 1995). When measured following dissociation, the concentration of Ca^{2+} in prestalk amoebae of the slug is seen to be approximately twice that in prespore amoebae (Abe and Maeda, 1989; Saran *et al.,* 1994b). We have shown previously that freshly starved or pre-aggregation amoebae anticipate the prestalk-prespore distinction in respect to calcium. Amoebae in the L or 'low Ca^{2+}' class exhibit a prespore tendency and those in the H or 'high Ca^{2+}' class exhibit a prestalk tendency (Azhar *et al.,* 1996). Adding weight to the evidence that Ca^{2+} is a mediator of prestalk and stalk differentiation, (a) the prestalk inducer DIF can elicit an increase in cellular Ca^{2+} (Azhar *et al.,* 1997) and (b) artificially raising or lowering the Ca^{2+} level can induce cells to differentiate in the desired direction (Baskar *et al.,* 2000). Clearly, then, both the cell cycle and cellular calcium can exert a determinative influence on differentiation in *Dictyostelium*, even though the influence is reversible. The regula-

tion of events in the cell cycle by Ca^{2+} has been the subject of investigation in other systems. Through the interplay of Ca^{2+} and several proteins, cells regulate key steps in the cell cycle such as reentry of quiescent cells into proliferation and the transition through the G1/S, G2/M and the metaphase/anaphase border (Laskey *et al.,* 1989; Whittaker and Patel, 1990; Takuwa *et al.,* 1995). Recently, by using flow cytometry, it has been shown that Ca^{2+} levels vary in different cell cycle phases in rat fibroblasts (Pande *et al.,* 1996). This hints that there may be a link between Ca^{2+} and cell cycle phase in *D. discoideum* too. We have previously shown a link between cell cycle phase and *sequestered* Ca^{2+} (Azhar *et al.*, 1998). Are cytoplasmic (free) Ca^{2+} levels and the cell cycle phase also correlated, and if so, can one influence the other in a manner relevant to the development of *D. discoideum*? The present study demonstrates that the answer to both questions is yes. Further, there are cell cycle-dependent and independent mechanisms for initial cell-type choice, both mediated by Ca^{2+}.

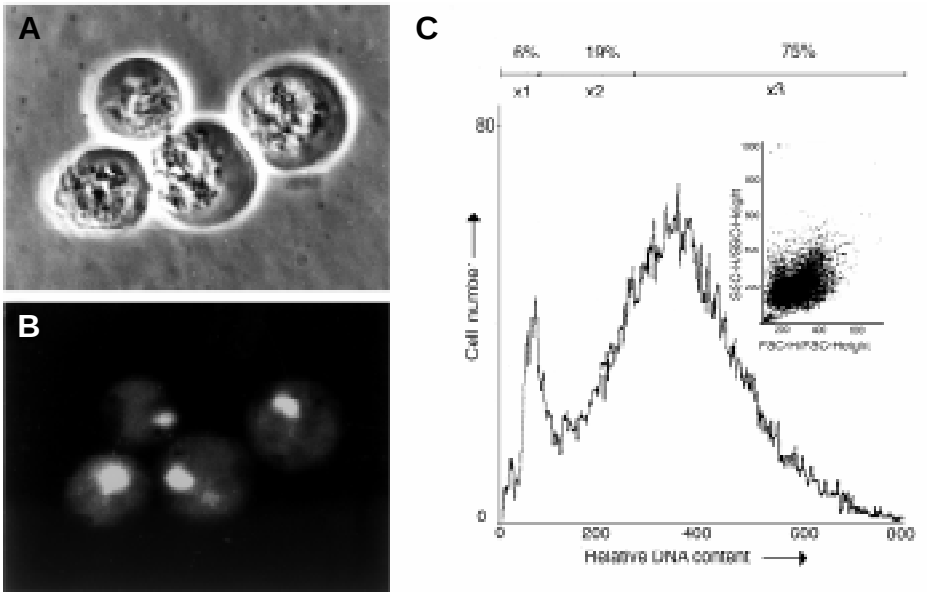

Fig. 2. DNA fluorescence and FACS analysis. **(A)** *Phase contrast and* **(B)** *the corresponding fluorescence image of SYTO-11 labeled, freshly starved amoebae of D. discoideum (Magnification X440).* **(C)** *Fluorescence intensity distributions of relative DNA content as revealed by SYTO-11 labeling. The bars indicate the defining range of percentage of amoebae in G1 (window x1), S (window x2) and G2 (window x3) phases as based on Fig. 1 A-L. Inset shows a dual parameter dot plot depicting a correlation of forward scatter and side scatter of SYTO-11 labeled cells.*

Results

Figures 1 and 2 illustrate the basis on which we have established a link between cellular DNA content and cell cycle phase, initially by making use of simultaneous labeling with BrdU and PI (Fig. 1) and next with the help of SYTO-11; see Materials and Methods for details. Depending on how we assign cell cycle phases, we find that bacterially grown NC4 amoebae have a G1 phase that can occupy anywhere from 2.6% to 13% of the cycle (Table 1). We have earlier shown that indo-1/AM labeled freshly starved amoebae display a bimodal distribution of Ca^{2+}-indo-1 fluorescence (Azhar *et al.,* 1996). When such amoebae are simultaneously labeled with indo-1/AM and SYTO-11, a clear correlation is seen. As Fig. 3 A-C shows, amoebae containing relatively high levels of Ca^{2+} (approximately 9%; Ca^{2+}-indo-1 fluorescence intensity falling above channel 20, Fig. 3 A,B) have a relatively low DNA content (ordinate channels 70-320, Fig. 3A) whereas the majority of the population (approximately 91%), consisting of cells containing relatively low levels of Ca^{2+}, overlaps with the entire range of DNA content distribution. From the results displayed in Figs. 1 and 2, it follows that amoebae containing relatively high Ca^{2+} levels tend to fall in the G1, S and possibly early G2 phases of the cell cycle; whereas amoebae with low Ca^{2+} levels occur in all phases of the cell cycle.

Altering the cell cycle phase concomitantly alters cellular Ca^{2+}

Exponentially growing amoebae were pre-incubated with hydroxyurea for various time periods, then double-labeled with indo-1/AM and SYTO-11, analyzed by FACS (Fig. 4 A-D). Hydroxyurea treatment caused the bimodal DNA distribution to become unimodal and more or less restricted to channels 75-250, the bulk of the cells falling in the early part of window x2, that is, to the G1-S boundary (Fig. 4C; compare with Fig. 1D). The percentage of cells in window x2 rose from 33% in the control (Fig. 4A) to 70% in hydroxyurea treated cells (Fig. 4C). Secondly, at the same time, the fraction of cells containing relatively high levels of Ca^{2+} rose from 38% to 60% (compare Fig. 4B with 4D). In this experiment, the fraction of "high Ca^{2+}" cells in the control was higher than the average usually observed, which lies within the range of 18.47±11.01% as reported by Azhar *et al.* (1997). What is striking is that the percentage of cells falling within the DNA content range corresponding to window x2 (33%) was also higher than the average value of 24% mentioned earlier; this reinforces the belief that the two are correlated. Observations taken at intermediate time points indicate the existence of transition states with increasing fractions of amoebae coming to lie within window x2 and a corresponding shift from the low to the high Ca^{2+} class (not shown).

The complementary experiment involved treatment with nocodazole, a mitotic inhibitor (Capuccinelli, 1979). This led to a change in the opposite direction: a preferential accumulation of cells within channels 250-720 (window x3) (compare Fig. 4A, 4E). At the same time, nocodazole caused a displacement of amoebae from the 'high Ca^{2+}' class to the 'low Ca^{2+}' class (Fig. 4 B,F).

Cell cycle phase at starvation correlates with post-aggregative cell fate

Exponentially growing amoebae were labeled with SYTO-11 and sorted on the basis of relative DNA content into two sub-populations. The first consisted of amoebae from channels 75-250 (window x2, S phase) while the second comprised amoebae from channels 250 to 720 (window x3, G2 phase). Amoebae from one of the two sub-populations were additionally labeled with DAPI, mixed with unlabeled amoebae from the other sub-population and

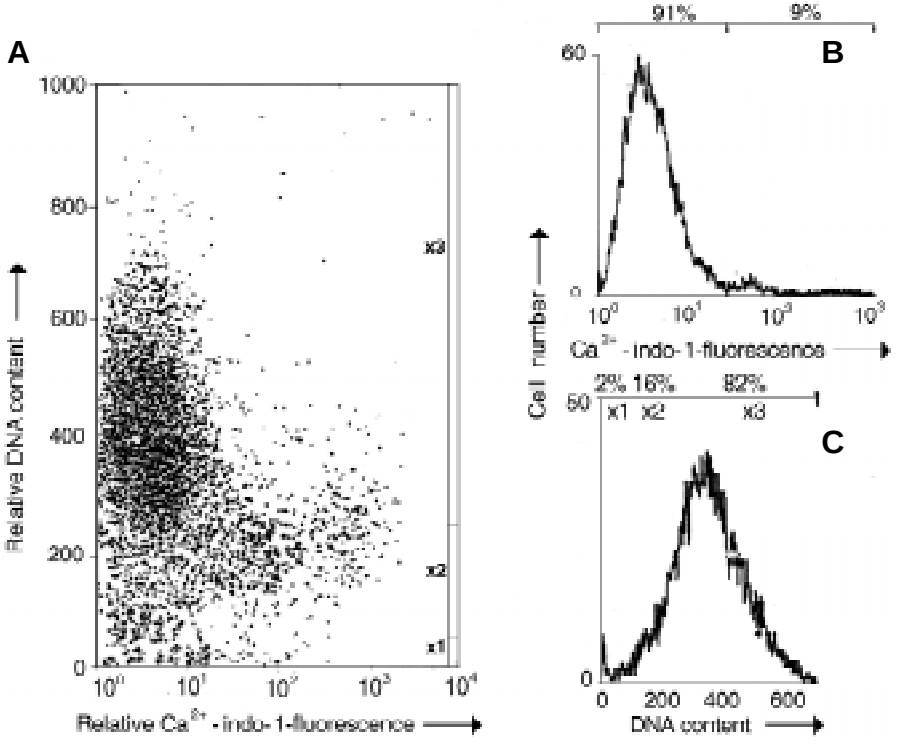

Fig. 3. Simultaneous monitoring of cell cycle phase and calcium. (A) *Representative dual parameter FACS dot plot of indo-1/AM and SYTO-11 double-labeled, freshly starved amoebae of D. discoideum.* **(Abscissa)** *Ca^{2+}-indo-1 fluorescence;* **(Ordinate)** *relative DNA content as revealed by SYTO-11. The bars indicate the defining range of percentage of amoebae in G1 (x1), S (x2) and G2 (x3) phases. Note that amoebae from x2 (or S) with relatively low DNA content predominantly contain high cellular Ca^{2+}.* **(B)** *and* **(C)** *show 1-D histograms of Ca^{2+}-indo-1 fluorescence and relative DNA content, respectively, from the same population displayed in (A).*

followed through development. DAPI fluorescence monitored in slugs showed that S phase amoebae were confined to the anterior prestalk zone whereas G2 phase amoebae ended up in the prespore zone (Fig. 5 A-D).

rtoA *mutant cells are enriched in the fraction that falls in the high Ca^{2+} class and display significantly enhanced levels of cellular Ca^{2+}*

As previously reported (Wood *et al.,* 1996), there is a significant enhancement of the stalk:spore ratio in the proportioning mutant *rtoA*. (Fig. 6). Earlier work has shown that vegetatively growing amoebae of *rtoA* have an unchanged distribution of cell cycle phases (Wood *et al.,* 1996). We find that freshly starved amoebae of *rtoA* contained on average 54.0±1.6% (n=3) amoebae which fall in the high cellular Ca^{2+} class compared with a figure of 20.6±2.8% in the parental DH1 strain (Fig. 6). Ion mass spectroscopy also indicated that *rtoA* cells have a 1.360±0.003-fold higher average Ca^{2+} content compared to DH1 cells. To examine the basis for the increased percentage of cells with high cytosolic Ca^{2+} in *rtoA*, we measured the ability of these cells to transport Ca^{2+} from outside into live cells. The Ca^{2+} influx into *rtoA*, normalized to that of the parental DH1 line, was 0.31±0.13 (Average +/- SEM, n=3). In digitonin-permeabilized cells, the uptake of *rtoA* cells normalized to that of DH1 cells was 0.61±0.02 (Average +/- SEM, n=3). This indicated that there is, paradoxically, a lower Ca^{2+} influx rate into *rtoA* cells, even though these cells have a greater total amount of Ca^{2+} inside them. However when we examined Ca^{2+} efflux, the *rtoA* cells had a much lower efflux: 0.08±0.11 (Average±SEM, n=9) relative to the parental DH1 strain.

Discussion

Most studies of Ca $^{2+}$ variation during the cell cycle have utilized chemical methods or cell lysis followed by fluorimetry (Brooks-Frederich *et al.,* 1993; Wahl *et al.,* 1993). Depending as they do on measurements of populations of cells, these methods make it impossible to uncover any cell-to-cell heterogeneity in Ca^{2+} that may exist between cells at the same stage of the cell cycle. On the other hand, phenotypic heterogeneity is a central feature of all stages of development in *D. discoideum.* This meant that we had to make use of an alternative method for evaluating the cellular Ca^{2+} status, one that would permit the monitoring of single cells. As validated by previous investigations on *D. discoideum*, FACS is a possible alternative (Saran *et al.,* 1994a; Azhar *et al* 1996). But the use of FACS for making Ca^{2+} and cell cycle measurements on whole cells causes other problems. The presence of mitochondrial DNA - which can constitute a third of total cellular DNA in *D. discoideum* - interferes with the signal of interest to us, which emanates from nuclear DNA. Further, working with isolated nuclei would have defeated our aim of monitoring cell cycle phase and calcium at the same time; besides, it is extremely difficult to synchronize the growth of amoebae that are raised on a suspension of bacteria. It is for these reasons that we had first to establish a correlation between DNA content (as judged by PI fluorescence) and DNA synthesis (as judged by BrdU incorporation) in fixed cells. Only after that could we go onto study

TABLE 1

CELL CYCLE DISTRIBUTION IN BACTERIALLY GROWN NC-4 AMOEBAE

Strain	Cell cycle phase (relative duration) mean values, %			Doubling time (hr)	Reference
	G1	S	G2+M		
AX-2	19.1	26.0	55.0	8.0	Zada-Hames and Ashworth (1978)
AX-2	11.0	43.0	46.0	8.5	McDonald and Durston (1984)
AX-2	absent	6.9	90.2±2.7	7.2	Weijer *et al.* (1984)
AX-4	absent	9.4	89.7±0.9	8.0	Gomer and Amman (1996)
RtoA*	absent	8.9	90.7±0.9	9.3	Wood *et al.*(1996)
NC4-H	13.0	27.0	60.0	4.0	This study#

*Percentages relative to AX-4 (Wood *et al.*, 1996)
Based on Syto-11 fluorescence. From the PI+BrDU experiments on the other hand, the percentages are 2.6, 15.3 and 82.0; see Fig. 1.

the correlation between the Ca^{2+} status (using Indo-1) and DNA content (with the help of SYTO-11) in live cells. As an extra precaution, we took care to verify that cells that were identified as being in the S phase showed a predominantly nuclear incorporation of BrdU (not shown).

Assignment of cell cycle phases

The distribution of DNA content in dividing mammalian cells is a reliable indicator of their position in the cell cycle (Darzynkiewicz and Tragnos, 1990). In *D. discoideum*, however, making the link is subject to ambiguities because of the difficulty of distinguishing nuclear from mitochondrial DNA (Weijer *et al.,* 1984). We used two types of fluorescent dyes that bind to nucleic acid, propidium iodide and SYTO-11, for measurement of DNA content by flow cytometry. Essentially similar results were obtained vis-à-vis relative fluorescence intensities with both dyes (compare Figs. 2C, 1E). After combining data from BrdU incorporation we are able to identify the position of cells in cell cycle based on their relative DNA content. These assignments were reinforced by the effects of hydroxyurea and nocodazole (compare Fig. 4A, 4C, 4E). The relationship between DNA content and cell cycle in *Dictyostelium* does not follow the same pattern as in mammalian cells. Specifically, because of interference from mitochondrial DNA, the DNA content of G2 + M cells is not exactly twice that of G1 cells. Also, our data indicate the presence of a small G1 phase (Table 1).

Cellular Ca^{2+} and relative DNA content or cell cycle phases are correlated

Indo-1/AM and SYTO-11 double-labeled vegetative (or freshly starved) amoebae display a quasi-bimodal distribution of both Ca^{2+} content (Azhar *et al.,* 1996) and relative DNA content (Fig. 3 B,C). As shown in Results, amoebae with high cellular Ca^{2+} are preferentially in S and early G2;. on the other hand, cells with relatively low cellular Ca^{2+} tend to occur in all phases of the cell cycle (Fig. 3). Thus, G2-phase cells are heterogeneous with respect to their calcium content. In rat fibroblasts intracellular Ca^{2+} varies in a systematic way in different phases of the cell cycle but the significance of the variation is not clear (Pande *et al.,* 1996); interestingly, there too Ca^{2+} levels are maximal at the G1/S border and drop when the cells are in G2/M.

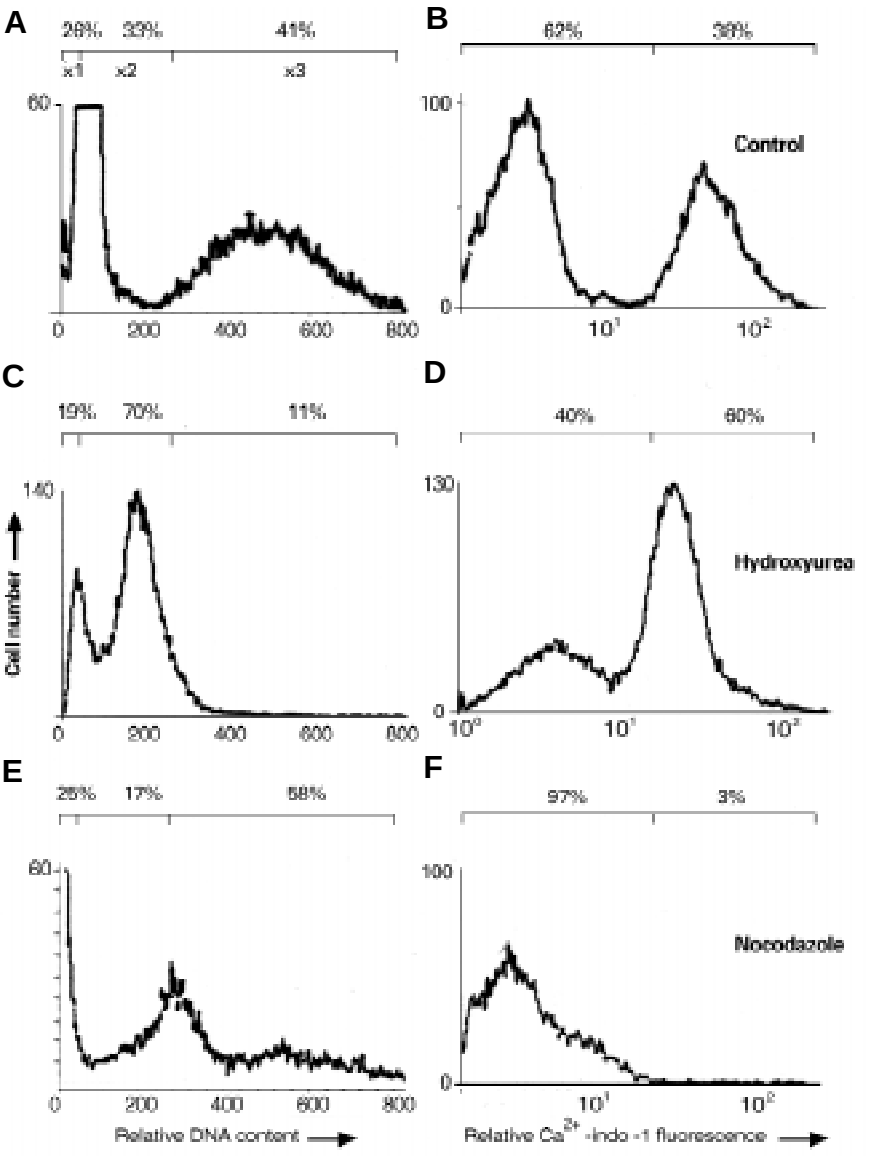

Fig. 4. Effect of cell cycle phase inhibitors on calcium. *Fluorescence intensity distributions of indo-1/AM and SYTO-11 double-labeled amoebae of D. discoideum.* **(Left)** *relative DNA content distribution as judged by SYTO-11 and* **(Right)** *relative Ca^{2+} content.* **(A,B)** *control amoebae;* **(C,D)** *after 8 h of hydroxyurea treatment;* **(E,F)** *after 4 h of nocodazole treatment. Note that both hydroxyurea and nocodazole lead to a change in relative DNA content and Ca^{2+} content as expected on the basis of the correlation shown in Fig. 4 A-C.*

Mild hydroxyurea treatment has been shown to lengthen the S phase and increase the percentage of prestalk cells in a differentiation assay (Gomer and Ammann, 1996). Here, in addition to inhibiting progression through the cell cycle, hydroxyurea simultaneously shifts cells to the high cellular Ca^{2+} class, a class that is known to exhibit a prestalk tendency (compare Figs. 4 A-B; 4 C-D; Table 1; Azhar *et al.,* 1996). At intermediate times (4 and 7 hr) hydroxyurea treatment leads to a trimodal pattern of Ca^{2+}-indo fluorescence, suggesting that even though two broad classes are seen, the variations of Ca^{2+} during the cause of the cell cycle are quasi-continuous. Nocodazole, which causes amoebae to accumulate at the G2/M boundary, shifts them to the low cellular Ca^{2+} class which is known to display a prespore tendency (compare Fig. 4 A-B with 4 E-F; also with 4 C-D; Azhar *et al.,* 1996). In sum, perturbation of the cell cycle phase by hydroxyurea and nocodazole leads to a change in cellular Ca^{2+} as expected on the basis of the correlation noted previously, thereby indicating that it reflects a causal link between the two.

As pointed out, a fraction of amoebae from the S and early G2 phases also display low cellular Ca^{2+}; these can amount up to about 50% of the S/early G2 class, and therefore about 10% of the total number of amoebae. Using an antibody against a prestalk-specific antigen (CP2) in a low-density monolayer differentiation

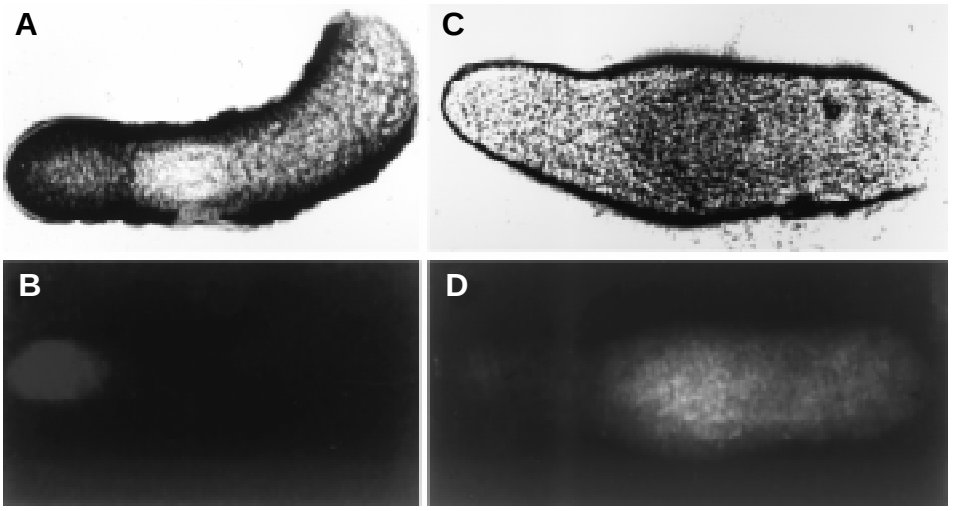

Fig. 5. Cytoplasmic calcium and cell fate. *Amoebae of D. discoideum from one of the two sub-populations - either from* **(A,B)** *x2 (or S) (enriched with high Ca^{2+} cells) or* **(C,D)** *x3 (mid to late G2 phase) (enriched with low Ca^{2+} cells) - were additionally labeled with DAPI and reconstituted with unlabeled amoebae from the other class as explained in methods. (A,C) show bright field and (B,D) are fluorescence images of migrating slugs (x44). The slug anterior is to the left. Note that highly fluorescing amoebae are localized predominantly in the prestalk region (A,B) and posterior prespore region (C,D) of the migrating slug.*

assay, Gomer and Firtel (1987) found that antigen-positive cells constituted just one member of each sister-pair of cells starved when in S or early G2; in their assay the other sister exhibited neither prestalk nor prespore characteristics. This raises the intriguing possibility is that the 'low Ca^{2+}' cells and the 'high Ca^{2+}' cells that occur in the same cell cycle class consist of pairs of sisters.

Cell cycle phases and cellular Ca^{2+} together predict post-aggregative cell fate

Cell sorting and subsequent cell fate studies show that amoebae belonging to the S phase (enriched with cells of high Ca^{2+} class) display a prestalk tendency (Fig. 5 A-B). Similarly, amoebae from the mid to late G2 phase (enriched with cells of low Ca^{2+} class) exhibit a prespore tendency (Fig. 5 C-D). Sorting and mixing experiments, based separately on Ca^{2+} and the cell cycle, have previously given similar results (Saran *et al.,* 1994; Azhar *et al.,* 1996; McDonald and Durston 1984; Weijer *et al.,* 1984; Gomer and Firtel, 1987). Ca^{2+} thus appears to act conjointly with the cell cycle to predispose amoebae to one or the other fate. How the two influences combine remains uncertain.

What happens to the correlation between Ca^{2+} and cell fate when Ca^{2+} and the cell cycle are de-linked from one another? To explore this we used the mutant *rtoA* which has a disproportionately high proportion of stalk cells but is reported to display a normal cell cycle distribution as compared to its parental type DH1 (Wood *et al.,* 1996). FACS analysis shows that freshly starved amoebae of *rtoA* contained a larger fraction of cells in the high cellular Ca^{2+} class than the parental DH1 strain (Fig. 6). We have previously shown that artificially raising or lowering cellular Ca^{2+} leads to opposite effects. A rise in Ca^{2+} enhances the proportion of prestalk cells in the slug and generates a 'stalky' fruiting body morphology whereas a decrease in Ca^{2+} increases the fraction of prespore cells and causes a 'spory' morphology (Baskar *et al.* 2000) The multiple phenotypes of *rtoA* support such a correlation and point to the existence of a Ca^{2+}-dependent but cell cycle-independent initial cell-type choice mechanism in this mutant. However, the increase in the number of high Ca^{2+} cells as reported here - 52% versus 24% - exceeds the observed increase in the number of CP2-positive prestalk cells - 15% versus 11% - found by

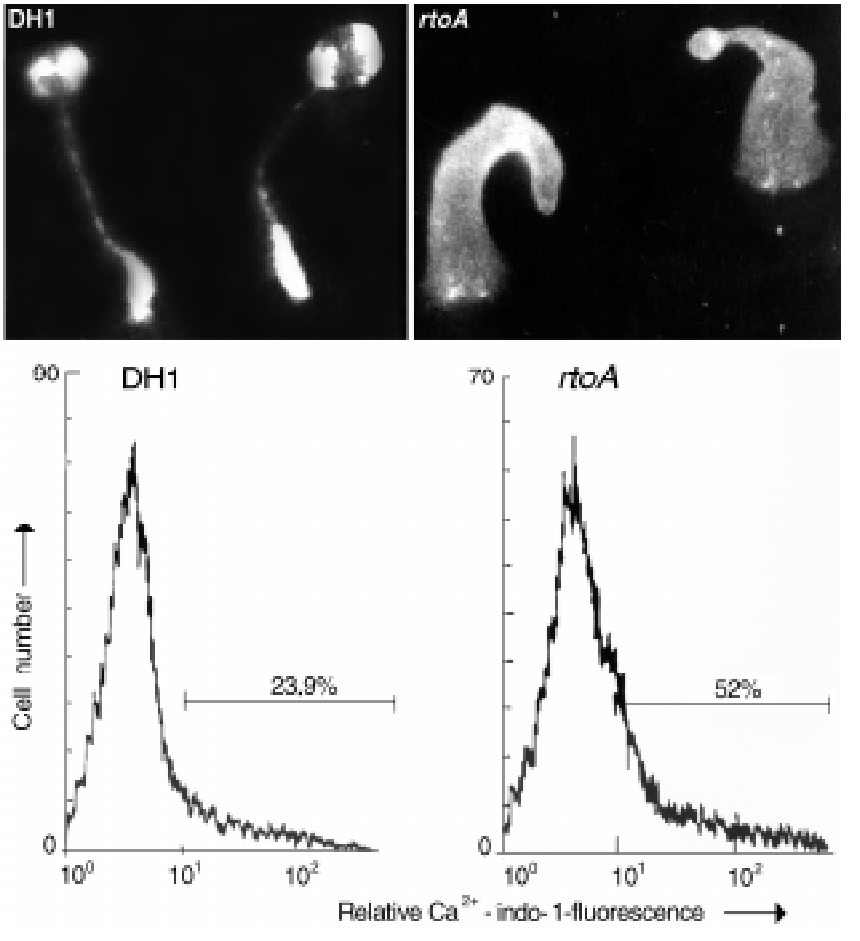

Fig. 6. Calcium distribution in *rtoA*. *Representative fluorescence intensity distributions of indo-1/AM loaded, freshly starved amoebae of the D. discoideum rtoA mutant. The bars indicate the percentages of the amoebae falling into the 'high' cellular Ca^{2+} class, assigned on the basis of setting an autofluorescence cutoff from the parental wild type DH1. Top panel shows bright field images of the morphology of DH1 and rtoA fruiting bodies.*

Wood *et al.* (1996). Even if we allow for the fact that CP2-positive cells constitute only a part, perhaps one-half, of the prestalk cell population, the difference between the two sets of figures is striking. This suggests three possibilities. Firstly, there may be a non-linear relationship between Ca^{2+} levels and initial cell fate. Secondly, there may be a cell fate - homeostasis mechanism which potentially counteracts the effect of the elevated Ca^{2+} levels. And thirdly, one or more additional influences may modulate the initial cell type choice. Indeed, previous work has shown that cell size (Bonner *et al.,*1971) and levels of metabolizable sugars at the time of starvation (Leach *et al.*, 1973) are two early pointers to cell fate. Finally, stimulation of freshly starved amoebae by DIF, a putative prestalk inducer, causes a Ca^{2+} increase in only a subset of amoebae belonging to the low Ca^{2+} class (Azhar *et al.*, 1997). This shows a DIF-dependent aspect of heterogeneity that may also play a role in terminal differentiation.

Conclusions

A subset of growing amoebae that are early in the cell cycle, and so have a low DNA content, contain high levels of Ca^{2+} and exhibit a prestalk tendency. In contrast, amoebae that are late in the cell cycle and have a high DNA content contain low levels of Ca^{2+} and tend to become prespore cells. Inevitably, as with many other cellular properties in *D. discoideum*, 'high' and 'low' are relative, not absolute, attributes; and the tendencies are merely that, not indications that the amoebae are 'determined' in an irreversible sense. Calcium acts 'downstream' to the cell cycle in the sense that the Ca^{2+} status can be altered by blocking the cell cycle early, which leads to a progressive increase in the fraction of cells falling in the high Ca^{2+} class, or blocking it late, which makes amoebae progressively enter the low Ca^{2+} class. Accordingly, the fruiting bodies that develop following such treatment exhibit 'stalky' or 'spory' phenotypes respectively (not shown). These results show that Ca^{2+} is yet another early aspect of functional heterogeneity between cells of *D. discoideum*. The list of such aspects keeps growing and, as of today, includes cell size (Bonner *et al.*, 1971), nutritional status (Leach *et al.*, 1973), cell cycle phase (McDonald and Durston, 1984; Weijer *et al.*, 1984), sequestered Ca^{2+} (Azhar *et al.,* 1996) and the sensitivity of freshly starved amoebae to DIF (Azhar *et al.*, 1997). From an evolutionary point of view, these can be thought of as so many correlates of the phenotype with relative cellular fitness (what we have previously termed 'qualities'; Atzmony *et al.*, 1995). Phenotypic selection at the level of the individual cell ensures that following aggregation, high-quality amoebae display traits of prespore cells on the whole and low-quality amoebae display prestalk traits (again, on the whole). Seen thus, it appears that while the notion of morphogens and positional information is a useful way of describing collective behaviour during the later stages of differentiation and pattern formation in *D. discoideum*, the essence of that behaviour must be sought in the myriad and interlocking ways in which cells differ from each other at all stages of the life cycle.

Materials and Methods

Growth and development of cells

D. discoideum (wild type strain NC4-H), mutant *rtoA* and DH1 amoebae were grown on standard nutrient media agar with *Klebsiella aerogenes* and harvested using standard procedures (Sussman, 1987) except that the buffer was a mixture of potassium phosphates (KK_2) at pH 6.4. Amoebae were washed free of bacteria by centrifugation at 400x g for 5 minutes and developed at 22°C in the dark on 2% non-nutrient agar. All chemicals were of analytical grade and obtained from Merck or Sigma, except for SYTO-11, indo-1/AM and Pluronic F-127 (Molecular Probes Inc., USA.) and dry dimethylsulfoxide (anhydrous DMSO) (Aldrich, USA.).

Pulse-labeling of bacterially grown NC4-H amoebae with 5'-bromo 2'-deoxyuridine (BrdU) and its immunocytochemical detection

Exponentially growing amoebae of *D. discoideum* were incubated with BrdU (100 μM) for 30 min and then fixed in 70% chilled ethanol while vortexing vigorously to avoid clumping of cells. Amoebae were washed free of bacteria with 1x phosphate-buffered saline (PBS) at pH 7.4 and processed according to the protocol of Dolbeare and Selden (1994). Amoebae were incubated with primary anti-BrdU monoclonal antibody (Becton-Dickinson) for 30 min, washed twice with 1x PBS and subsequently incubated with anti-mouse IgG-FITC secondary antibody for 30 min. After washing twice with 1x PBS, amoebae were finally stained with propidium iodide for their DNA content using standard protocol (Dolbeare and Selden, 1994). BrdU incorporation of amoebae was monitored simultaneously with the measurement of DNA content by flow cytometry.

DNA synthesis profile of bacterially grown amoebae

We correlated the incorporation of BrdU (an analogue of deoxythymidine) in relation to the total DNA content of the cells by flow cytometry. Representative experiments are shown in the form of two-parameter dot plots (Fig. 1 A-D) and corresponding one-dimensional histograms displaying relative DNA content and BrdU incorporation (Figs. 1 E,F with 1A; G,H with 1B; I,J with 1C; K,L with 1D). In Fig. 1 A-D, each 'dot' represents a cell; the abscissa (linear scale) indicates relative propidium iodide fluorescence, i.e. relative DNA content, and the ordinate (logarithmic scale), BrdU incorporation per cell. Sorting and microscopic observations were carried out to verify that none of the recordings shown were due to bacteria or cellular debris. In Fig. 1A, which refers to labeling with propidium iodide alone, the spread of points along the ordinate represents non-specific background fluorescence; this provides the cut-off level for identifying BrdU-specific fluorescence in subsequent experiments. With reference to relative DNA content, the bulk of the cells in Fig. 1A (82%) fall within channels 250-720 (denoted by window x3), about 15% within channels 75-250 (x2) and approximately 3% within channels 0-75 (x1). (The logic behind demarcating the windows is explained below). In three independent experiments the percentage of cells (mean +/- SD.) was 2.6±0.4% in x1, 15.3±2.8% in x2 and 82±3.2% in x3. When propidium iodide treatment was preceded by a (brief 5 min) incubation with BrdU (Fig. 1B), only a few cells (3%) incorporated BrdU very much above background levels and they fall in window y3 on the ordinate (channels 18 to 80); the bulk lie within windows y1 (88%, channels 1 to 4) and y2 (9%, channels 4 to 18). On the other hand, upon 30 min BrdU incubation, (Fig. 1C) the fraction of cells incorporating BrdU is 50% in y1, 45% in y2 and 5% in y3. The basis of the distinction between windows y2 and y3 in Figs. 1B and 1C is that the cells from region y3 displayed BrdU incorporation into nuclear as well as mitochondrial DNA, in contrast to those in y2, which showed incorporation of BrdU only into mitochondrial DNA. Thus, 60% of the cells that incorporate BrdU into nuclear DNA after 30 min incubation have already done so after 5 min, whereas the corresponding fraction in the case of mitochondrial DNA is just one-fifth.

Hydroxyurea is supposed to block DNA synthesis and arrest cells at the G1/S boundary (Tobey and Crissman, 1972). When we incubated amoebae for 8 h in growth medium containing hydroxyurea prior to the BrdU and propidium iodide treatments, there was a change in the distribution of staining with respect to both parameters (Fig. 1D). A majority of the cells (74% as against 15% in the absence of hydroxyurea) were now distributed in x2 and the percentage of cells in x1 increased from 3% to 6%, with those in x3 falling from 82% to 20%. The implication is that hydroxyurea treatment does not block DNA synthesis (nuclear or mitochondrial) completely, because a significant number of cells still fall within the x3 window (20%, Fig. 1D). This is not unexpected: mitochondrial DNA, which can constitute up to 50% of total cellular DNA, replicates throughout the cell cycle (Weijer

et al., 1984; unpublished observations of measurements of nuclear and mitochondrial DNA content by microscope photometry by Azhar and Nanjundiah), and previous study has also shown that cells can escape the hydroxyurea block and proceeded through DNA synthesis (Gomer and Ammann, 1996).

Taken together, these data on DNA content and BrdU incorporation permit us to correlate relative DNA content with the position in the cell cycle. Those amoebae that fall in x1 have a low level of DNA (Fig. 1A); since they do not incorporate BrdU either (Fig. 1 B,C), they are also not synthesizing DNA. Thus the G1 phase is defined by the rectangle (x1, y1) (Fig. 1B). Those amoebae whose DNA content falls within window x2 (Fig. 1A) have a slightly higher level of DNA. Among them, the ones that incorporate BrdU (Fig. 1 B,C) - as indicated above, into nuclear as well as mitochondrial DNA - are clearly in S phase, meaning that S phase includes the rectangle (x2,y3) (Figs. 1B, C). There are other cells falling in x2 (rectangles x2,y1 and x2,y2) which do not incorporate BrdU within 30 min, but we tentatively assign them to S phase too. The reasons for this are two. Firstly, in terms of DNA content they overlap with the previously assigned S phase cells. And secondly, even though these cells did not incorporate BrdU into nuclear DNA after a 30 min pulse, the G1/S arrest using hydroxyurea shows that they can indeed do so under other conditions (see rectangle x2,y3 in Fig. 1D). The large majority of amoebae, about 82% of the total, falls within window x3 (Fig. 1A) and contains a significantly higher amount of DNA than the S phase amoebae. We assign them to G2 (Fig. 1B, rectangles x3,y1 and x3,y2). Some of these (about 34%) incorporate BrdU after a 30 min incubation (Fig. 1C, rectangle x3,y2), but only into mitochondrial DNA.

Overall, then, with reference to Fig. 1C, these assignments indicate that about 2.6±0.4% (n=3) of exponentially growing amoebae feeding on bacteria are in G1 (rectangle defined by x1,y1), 15.3±2.8% are in S (rectangles x2,y1; x2,y2; x2,y3) and 82±3.2% are in G2 (rectangles x3,y1; x3,y2). The relative percentages are similar to published values for axenically grown Ax-2 amoebae except that in our experiments S phase occupies a slightly higher fraction, and G2 phase a slightly lower fraction, of the cell cycle then previously published estimates (McDonald and Durston, 1984; Weijer *et al.,* 1984; Ohmori and Maeda, 1987). About 0.1% of cells show high DNA content as well as significant BrdU incorporation (Figs. 1C, rectangle x3,y3) but remain unaccounted for. These could either be cells containing unusually large numbers of mitochondria or multinucleate cells. Neither of them were seen in the microscope, probably because their numbers were so few.

Double labeling of freshly starved amoebae with indo-1/AM and SYTO-11

Stock solutions of indo-1/AM (1 mM), SYTO-11 (5 mM) and the non-ionic detergent Pluronic F-127 (200 μg/μ1) were prepared in dry DMSO. Exponentially growing amoebae were incubated at a density of 1-2x10^6 cells/ml at 22°C for 30-40 minutes with 2 μM indo-1/AM and 1 μM SYTO-11 in the presence of 0.2% Pluronic F-127 under gentle stirring. The amoebae were washed thrice in ice-cold KK_2 buffer after dye loading and resuspended in KK_2 buffer for FACS analysis. Although the biochemical effects of SYTO dyes have not been fully characterized, no toxicity has been noted under our staining conditions; SYTO-11 labeled amoebae develop exactly as unlabeled controls do when they are deposited on plates (not shown).

Flow cytometry using Indo-1 and SYTO-1

The monitoring of *D. discoideum* amoebae by FACS has been reported by us previously (Azhar *et al.,* 1996). After passing cells through a mesh to remove clumps and aggregates, amoebae were analyzed using a $FACS_{tar}$ plus cell sorter (Becton-Dickinson, San Jose, California, USA.) tuned to 333-363 nm at 200 mW. Ca^{2+}-indo-1 fluorescence from cells was collected at the FL-32H position through a filter combination consisting of 400 LP and 424 DF 44 filters. SYTO-11 fluorescence was monitored at 520 nm after passing through a 475 DF 25 filter at the FL-1H position. Outputs from 20,000 cells in each sample were analyzed using the LYSYS II software (Becton-Dickinson).

For sorting, amoebae were simultaneously labeled with indo-1/AM and SYTO-11 and later sorted based on SYTO-11 fluorescence into two subpopulations: S and early G2 and mid to late G2 phase. Amoebae were collected with sorting gates set according to the standard procedures recommended by the manufacturer. A difference of 20 fluorescence units was maintained in order to avoid contamination of the amoebae with different class. Samples were collected in batches so as to ensure purity, stored on ice every 15 min and plated after the sorting procedure was completed. About 1x10^6 amoebae belonging to each fluorescence class were collected in a typical session.

Simultaneous monitoring of DNA content and cellular Ca^{2+}

Labeling of DNA with propidium iodide requires prior fixation of cells. Since the aim of this study was simultaneously to estimate relative DNA content (or cell cycle phases) and cytoplasmic calcium levels, we needed a method of estimating cellular DNA in live cells. For this we used the nucleic acid stain SYTO-11 (Frey 1995; Chenn *et al.,* 1995). Freshly starved or vegetative amoebae displayed an intense nuclear fluorescence along with a diffuse cytoplasmic fluorescence on staining with SYTO-11 (Figs. 2 A,B). When fluorescence was monitored using a cell sorter, the profile was essentially the same as that displayed by fixed and RNAse-treated amoebae that were labeled with propidium iodide (compare Figs. 2C, 1E). This tells us that the relative fluorescence intensity profiles are not qualitatively affected either by fluorescence emanating from dye bound to RNA or by binding to mitochondrial DNA. As expected on the basis of the previous results, the SYTO-11 fluorescence distribution was quasi-bimodal, with a small number of amoebae falling within a low intensity mode and the bulk in a high intensity mode (compare Figs. 2C, 1E) and the channel distributions were more or less as in Fig. 1. Also, simultaneous monitoring of forward scatter (cell size) and side scatter (cell granularity) of amoebae ruled out the possibility that cells in the low fluorescence intensity class could be debris (see *inset* in Fig. 2C). Accordingly, we have used the same window settings as before to demarcate different cell cycle phases but this time on the basis of relative DNA content alone. On the basis of comparing the DNA distribution profiles in Fig. 1C with those in Figs. 2C, 1E, we deduce that cells whose SYTO-11-specific fluorescence falls between channels 0-75 (window x1) must be in G1 phase. Among cells whose SYTO-11-specific fluorescence intensities ranges between channels 75-250 (window x2), a subset must be in S; and those with fluorescence outputs lying within channels 250-720 (window x3) must be in G2. In neither of these studies did we make an attempt to distinguish between the M and G2 phases. With the help of four independent experiments using SYTO-11, our best estimate is that about 11.7±8.2% of exponentially growing amoebae are in G1, 24.0±4.9% in S and 64.2±13.0% in G2. Because the SYTO-11 stain could not be used simultaneously with BrdU - the fluorescence spectra overlap - these percentages are clearly less reliable than those made previously using propidium iodide and BrdU simultaneously. Nevertheless the broad overlap in relative cell cycle phase assignments indicates that SYTO-11 can reliably be used to monitor DNA distribution and cell cycle phases in living cells of *D. discoideum*, albeit with a small margin of error. Our conclusions do not depend on a very precise assignment of cell cycle phases based on SYTO-11 fluorescence.

Cell cycle alteration of amoebae by hydroxyurea and nocodazole

Cell cycle phases of bacterially growing amoebae of *D. discoideum* (NC4-H) were altered by arresting cells in S or at G1/S border by 150 μM hydroxyurea. Hydroxyurea (150 μM) slows down the growth and does not affect viability (as judged by propidium iodide dye permeability test; not shown) or development. Alternatively, cells were arrested in mitosis or towards G2/M boundary by 10 μg/ml nocodazole treatment. Nocodazole (10 μg/ml) also slows down the growth and has no effect on cell viability. Amoebae were shaken independently with hydroxyurea or nocodazole in two different experiments and labeled simultaneously with indo-1/AM and SYTO-11 at different time intervals. Hydroxyurea or nocodazole was

present continuously in the staining buffer. Samples were finally analyzed by FACS. Control experiments have ruled out the possibility of any immediate and non-specific effect of hydroxyurea and nocodazole on Ca^{2+}-indo as well as SYTO-11 fluorescence.

Secondary labeling with 4',6-diamidino-2-phenylindole (DAPI)

After indo-1/AM and SYTO-11 loading and sorting were completed, amoebae from S and early G2 phase and mid to late G2 were collected and labeled with 1 µg/ml DAPI for 45 min while being shaken continuously at 22°C. Labeled amoebae were washed thrice in cold KK_2, resuspended in the same buffer and shaken for further 20 min at 22°C. After two more washes in ice-cold KK_2, these DAPI labeled amoebae were mixed with unlabeled amoebae of different class in a 1:4 ratio. In a second set of experiments amoebae from mid to late G2 phase were labeled similarly with DAPI after sorting and reconstituted with unlabeled counterpart in a 1:1 ratio. The reconstituted mixture was allowed to develop on 2% KK_2 agar plates for about 15-20 h at 22°C. DAPI labeled amoebae in both of these experiments were observed during development under a fluorescence microscope using appropriate DAPI filters. In order to verify that the DAPI label did not diffuse from cell to cell, freshly starved but unsorted amoebae were labeled in a similar way and mixed in various ratios with unlabeled amoebae.

Calcium flux assays

To measure total cellular calcium and influx and efflux of Ca^{2+}, cells were grown as described above and harvested and washed in ice-cold 0.5 g/l streptomycin. All subsequent procedures were at room temperature. To measure total cellular calcium, cells were additionally washed twice in deionized water, collected by centrifugation and the pellets were lyophilized. The pellets were then extracted with 1 N nitric acid and the ionic composition of the extract was measured with the Notre Dame inductively coupled plasma mass spectrometer. For influx measurements, multiple aliquots of cells were resuspended at 5 x 10^6 cells/ ml in M45 (1 mCi 45Ca /ml in 20 mM MES, pH 6.1), or M45/ 1 mM $CaCl_2$. After gently shaking for 1 minute or 20 minutes, cells were harvested by centrifugation, resuspended in 100 mM $CaCl_2$/ 20 mM MES, pH 6.1, and collected again by centrifugation. The pellets were resuspended in 100 ml of 1 M acetic acid. After mixing with 1 ml scintillation fluid, the pellets were counted. To measure gross Ca^{2+} efflux, the harvested cells were washed additionally in 20 mM MES, pH 6.1 and resuspended at 5 x 106 cells / ml in M45 / 20 mg glucose / ml. The cells were shaken for 2 h to load the cells with 45Ca and then washed twice in MG (20 mg glucose/ml in 20 mM MES, pH 6.1). The cells were resuspended at 5 x 106 cells/ml in MG and 0.5 ml was centrifuged, and 0.1 ml of supernatant was counted. After 30 min, the other 0.5 ml was centrifuged and 0.1 ml was counted. Ca^{2+} influx into internal vesicles was measured with the method developed by Milne and Coukell (1989) using cells permeabilized by 0.01% digitonin for 20 min. Samples were taken at 0, 1, 2, 3 and 4 min and the rate of Ca^{2+} uptake defined as the increase in $45Ca^{2+}$ present in the cell per minute.

Microscopy

Amoebae and migrating slugs were observed using a Leica DMRB3 and Zeiss Axioskop fluorescence microscope fitted with phase contrast objectives. Ca^{2+}-indo-1 fluorescence was monitored with the help of a special filter set from Omega Optical, Vermont, USA (Cat No. X F-07) meant for detecting Ca^{2+}-bound dye; the set consists of an excitation filter 360 HT25, dichroic mirror 390 DCLP 02 and emission filter 405 DF20. SYTO-11 labeled amoebae were monitored by Zeiss standard fluorescein (BP450-490; FT510; BP515-565) filter set. DAPI fluorescence of amoebae and slugs was monitored using Zeiss standard UV (G365; FT395; LP420) filter set. Photography was carried out with Kodak 400 ASA black and white print film using an automatic exposure control.

Acknowledgements

This work was supported by grants from CSIR and the Alexander von Humboldt Stiftung. We thank Dr. Preeti Chhabra for considerable help with data analysis and preparation of the manuscript.

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Received: March 2000
Revised by Referees: June 2000
Modified by Author and Accepted for publication: February 2001