tion of buildings. A pH of 5.6 is often regarded as the acidity of natural rainwater and calculations show that even alkaline rain may also damage cemented surface considerably. Since Gibbs free energies for SO_2 interaction are more negative than for H^+ interaction, the deterioration is likely to be faster due to SO_2 reaction with buildings.

Thermodynamic calculations show that (1) interactions between major constituents of cement and SO_2 and H^+ are possible in the atmosphere, (2) cement deteriorates faster by reacting with SO_2 than with H^+ and the deterioration is possible under normal atmospheric conditions and even by natural rain and (3) acid deposition increases the rate of deterioration.

Finally, it should be stated that correct predictions regarding the deterioration of cemented surface by atmosphere require both thermodynamic analysis and kinetic study of the systems. From this angle the present report is only a half exercise.

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Spatial gradients of calcium in the slug of *Dictyostelium discoideum*

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Starved amoebae of *D. discoideum* aggregate and give rise to a long and thin multicellular structure called the slug. The cells within the slug eventually differentiate according to a simple anterior/posterior dichotomy. This motivates a search for gradients of putative morphogens along its axis. Calcium may be one such morphogen. On the basis of observations made by using the calcium-sensitive fluorescent dyes fura-2 and chlortetracycline, we report that there are spatial gradients in cytoplasmic and sequestered calcium in the slug. Anteriorly located and genetically defined prestalk cells (ecmA/pstA, ecmB/pstAB) contain significantly higher levels of calcium than the prespore cells in the posterior. However, the proportion of 'calcium-rich' cells in the slug is greater than that of the subset of prestalk cells defined by the expression of the ecmA or ecmB genes.

THE cellular slime mould *Dictyostelium discoideum* is an excellent system for the study of spatial patterning in multicellular development[1]. One of the main reasons for saying this is that the fate map at the 'embryonic' slug stage in the life cycle of *D. discoideum* is extremely simple, being based on the relative position of a cell along the slug axis (anterior prestalk versus posterior prespore).

Traditionally, there have been two broad approaches to the problem of pattern formation in multicellular development, and these are typified by the words 'regulative' and 'mosaic'[2]; the two approaches are not mutually exclusive[3]. Analogously, there are two conceptually distinct ways of looking at patterning in *D. discoideum*. One possibility is that starting from a homogenous aggregate, relative positions along the long axis of the slug cause the cells to adopt different fates. This could be the consequence either of local cell-to-cell interactions leading to a prepattern[4] or of relative position *per se* – as sensed via morphogenetic gradients, say ('positional information'[5]). A second possibility is that cell fates are predetermined before aggregation and the cells sort out within the slug according to these fates. It is becoming increasingly clear that *D. discoideum* does not follow any one of these strategies to the exclusion of the other. Instead, it adopts what may be called a policy of multiple insurance and, depending on circumstances, makes use of both[6]. There are feedback interactions regulating the relative proportions of presumptive cell types[7]; but there are also gradients of putative morphogens along the slug axis (though their interpretation is not straightforward[8]). On the other hand, it is known that differences in preaggregation phenotype can bias cell fate in heterogenous populations[9,10]. We have shown that naturally occurring variations in cellular calcium between preaggregation amoebae anticipate future prestalk–prespore differences[11]. Based on the findings reported here as well as other results, it appears that the calcium ion is a candidate morphogen at the slug stage.

It has long been hypothesized that calcium could be important for cell differentiation and morphogenesis in *D. discoideum*. A high concentration of externally added calcium has been claimed to induce differentiation of stalk-like cells[12], and we have substantiated this recently (R. Baskar and V. Nanjundiah, in preparation). Calcium

behaves as a second messenger in transducing external cyclic AMP signals[13–15]; in particular, it regulates intracellular contractile filament assembly[16]. On these grounds, we have previously[17] suggested that an anterior–posterior gradient of calcium could be responsible for the differential motive force driving slug movement[18] and, more generally, might play a role in the establishment of the prestalk–prespore pattern in the slug.

Maeda and Maeda[19] made use of atomic absorption spectroscopy, ^{45}Ca labelling and alizarin red staining to demonstrate an anterior–posterior difference in total (= free + sequestered) calcium within the slug, but their methods left open the possibility of differential uptake of ^{45}Ca or alizarin red between the two cell types. Tirlapur *et al.*[17] showed with the help of calcium–chlortetracycline fluorescence that there is an *in vivo* anterior-to-posterior spatial gradient of sequestered calcium in the slug. With the help of the calcium-sensitive fluorophores quin-2 and fura-2 and cells dissociated from the slug, Abe and Maeda[20] inferred the existence of a cell-type-specific difference in cytoplasmic calcium levels. This was confirmed by Saran *et al.*[15] by monitoring light emission from amoebae that had been transformed with the cDNA for jelly fish apoaequorin and allowed to form slugs. Schlatterer *et al.*[21] used a fura-2–dextran conjugate to measure cytoplasmic Ca^{2+} but did not report any cell-type-specific differences.

A direct demonstration of an *in vivo* spatial gradient of cytoplasmic (free) calcium in the slug would be important because the cytoplasmic pool is the best candidate for a mediator of the downstream effects of calcium in the cell (a nuclear pool would also be a candidate, but we do not know whether one exists). We demonstrate below that within intact slugs, both cytoplasmic and sequestered calcium are higher in prestalk cells than in prespore cells. However, as judged by cell counts, some calcium-rich cells, also falling within the prestalk category, do not express the prestalk-specific ecmA or ecmB genes.

The experiments involved the wild-type strain NC4 and the axenic strain Ax-2 of *D. discoideum.* Fura-2 was obtained from Molecular Probes; all other chemicals were of analytical grade and purchased locally. Haploid NC4 amoebae were raised on pregrown lawns of *E. coli* K 802, starved and allowed to develop on nutrient-free agar plates as previously described[17]. For monitoring free Ca^{2+} we loaded amoebae by electroporation with the Ca^{2+} -sensitive fluorescent indicator fura-2 (ref. 22). Fura-2 was added at a final concentration of 90 μM to a 100 μl aliquot of a freshly starved cell suspension ($1–3.10^7$ cells/ml in 50 mM sucrose solution) and electroporation performed in a BioRad Gene Pulser using a single low-voltage electric pulse (0.09 kV/mm, 25 μF, 200 ohms, cuvette width 1 mm). Amoebae were revived in HMK buffer (20 mM Hepes–NaOH, 5 mM $MgCl_2$, 100 mM KCl, pH 6.9) at room temperature immediately thereafter. Excess dye was removed by centrifugation and the cells were dispersed on non-nutrient agar plates. The slugs which eventually developed were photographed under bright-field or UV illumination (Zeiss Axioskop fluorescence microscope filter set 02; broad band excitation filter around 365 nm, high-pass emission filter beyond 420 nm) using a Kodak 400 ASA film and automatic exposure control.

A second set of experiments involved staining with neutral red (NR) and chlortetracycline (CTC). NR is a vital dye that stains autophagic vacuoles present in prestalk cells, both those in the anterior region as well as those within anterior-like cells in the posterior region of the slug[23,24]; CTC fluorescence is an established marker of membrane-associated Ca^{2+} (ref. 17). For visualizing a subclass of genetically defined prestalk cells we made use of the transformants pDd63 and pDd56. These carry the *Escherichia coli* lacZ gene under the control of cell-type-specific promoters for the *Dictyostelium* genes which encode extracellular matrix proteins EcmA and EcmB, respectively[25,26]. The transformants, kindly made available by Dr J. Williams, are derived from the haploid axenic strain Ax-2, whose developmental phenotype is for all practical purposes identical to that of NC4. Both transformants carry a gene for neomycin resistance and were grown on neomycin-resistant *Klebsiella aerogenes* (a gift from Dr D. Welker) on a 2% agar medium containing the neomycin analogue G418 at a concentration of 80 μg/ml. In order to simplify the terminology, we refer to slugs derived from the respective transformants as 'ecmA slugs' and 'ecmB slugs'. Staining with 0.005% NR or 40 μM CTC was carried out on freshly starved amoebae as previously described[17].

The same amoebae were stained with both NR and CTC and allowed to develop until the slug stage in order to form an estimate of the degree to which there was an overlap in the corresponding patterns (of red colour and fluorescence). Using a standard protocol[26], β-galactosidase activity was visualized in other slugs that were approximately of the same age. For the purpose of counting, single slugs were squashed under cover slips and individual cells observed under high power. It would have been desirable to carry out all three procedures – NR staining, CTC staining and staining for β-galactosidase enzyme activity – on the same slug. Unfortunately, this was not possible because NR staining disappeared upon fixation.

Essentially all amoebae were successfully electroporated and showed a diffuse cellular fluorescence, indicating that the dye was not sequestered (Figure 1 *a*). This was the typical outcome after incubation at room temperature. On the other hand, amoebae that were incubated in cold HMK buffer after electroporation showed punctate

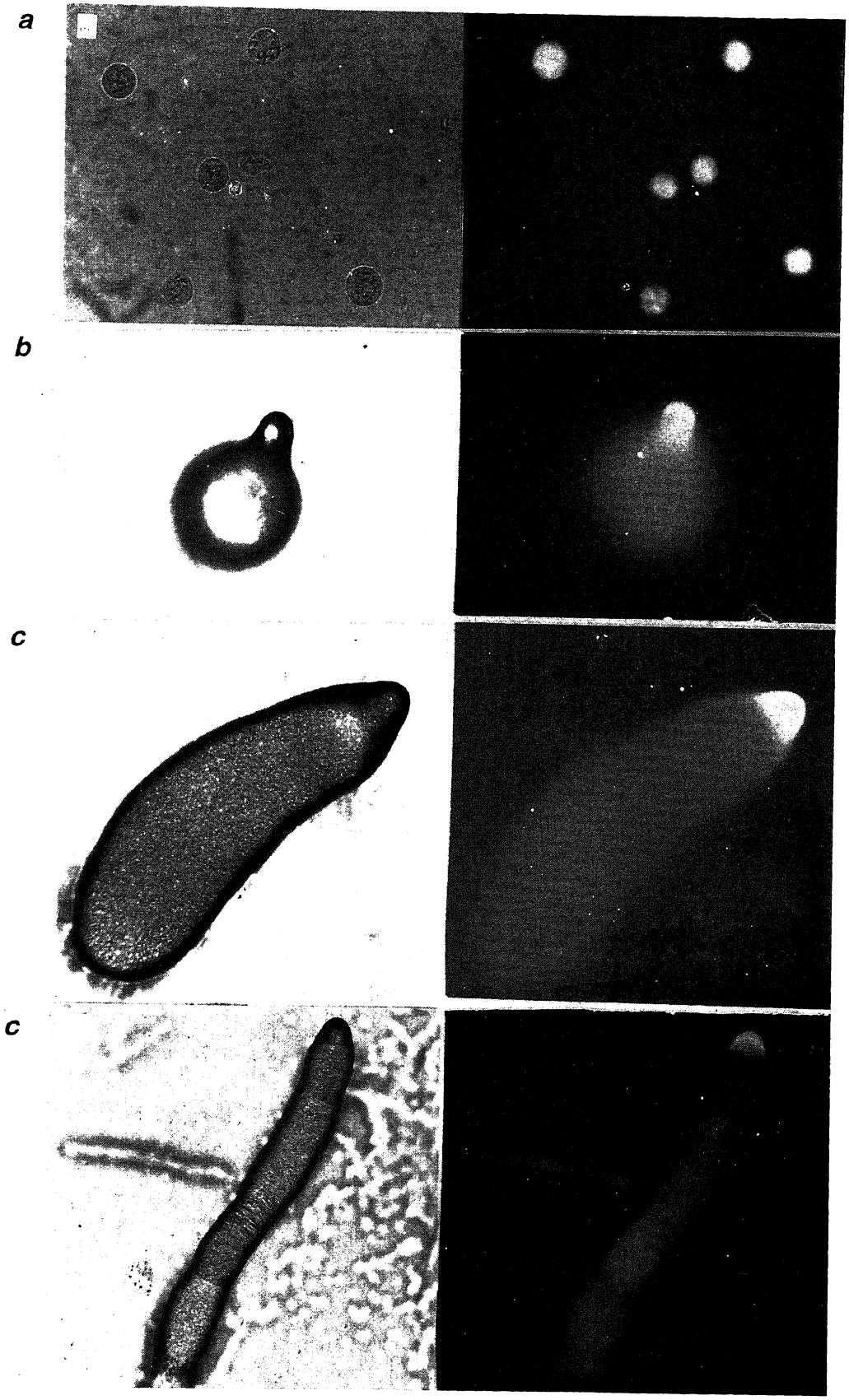

Figure 1. Bright field and fluorescence photographs: *a*, of amoebae freshly electroporated with fura-2; *b*, of late tipped aggregates; and *c*, of slugs (both pairs). An individual amoeba is about 10 μm in diameter; linear dimensions in the remaining photographs are approximately 0.35 mm, 0.75 mm and 1 mm, respectively.

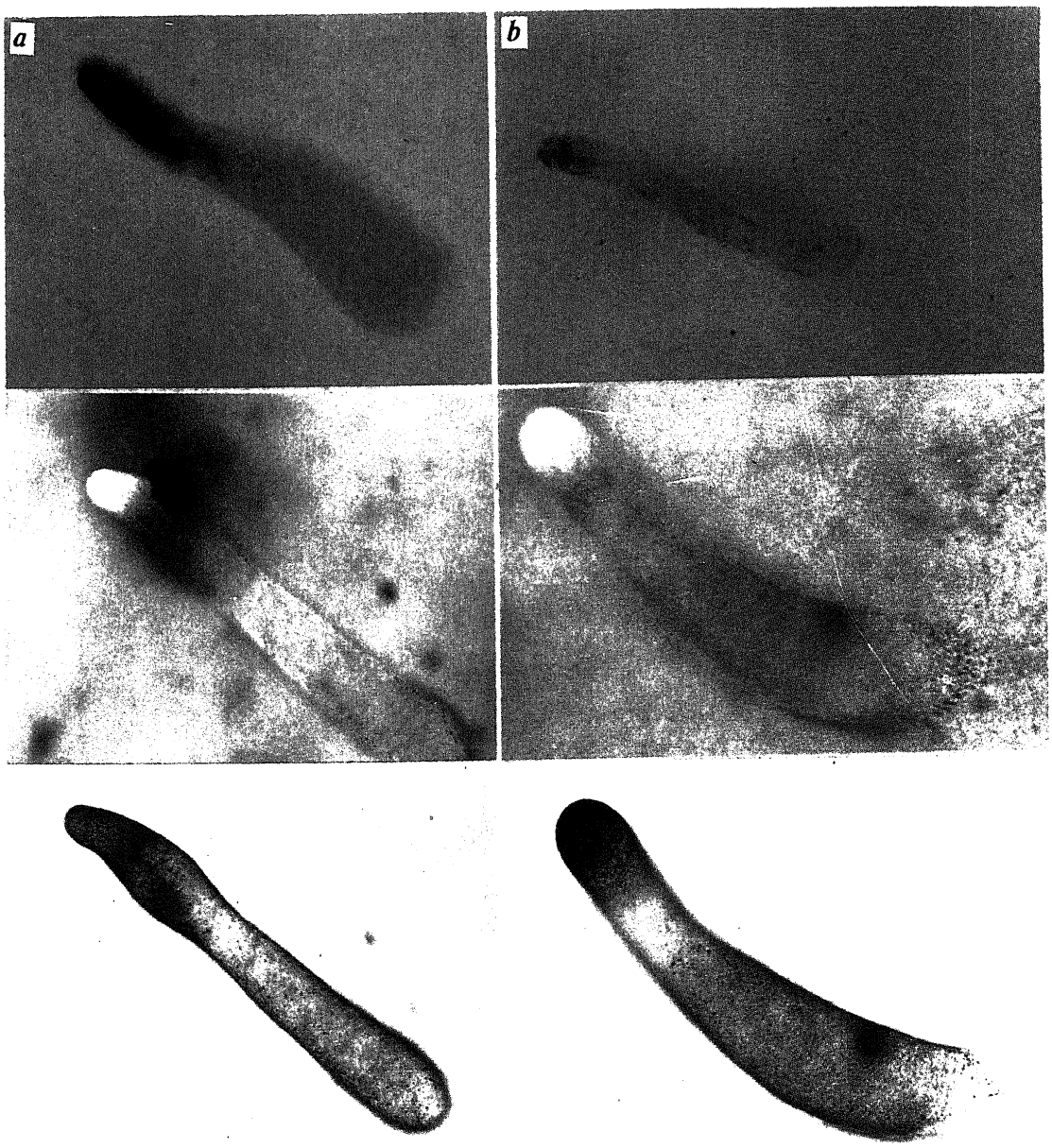

Figure 2. ecmA (*a*) and ecmB (*b*) slugs stained with neutral red (NR; a marker of prestalk cells), chlortetracycline (CTC; a fluorescent indicator of sequestered calcium) and β-galactosidase activity (in colour; an indicator of the spatial distribution of expression patterns of the ecmA and ecmB genes, respectively). In each case, NR staining and CTC fluorescence patterns show the same slug.

fluorescence, suggesting sequestration of fura-2 within organelles (not shown). As judged by trypan blue exclusion and ethidium bromide staining, approximately 70% of the amoebae remained viable following electroporation.

The earliest slugs were observed about 15 h after starved amoebae were dispersed on agar. Fluorimetric ratio measurements were carried out on lysates of equal numbers of pooled cells dissociated from anterior or posterior slug fragments after adding 1 mM $CaCl_2$. There is no difference in fura-2 content between prestalk and prespore cells: with 10^6 cells/ml before lysis, the ratio of fluorescence emission at excitation wavelengths 340 nm and 380 nm was the same in both cases (not shown). Development was followed by carrying out intermittent observations under fluorescence optics. Here we note that the filter combination used by us does not distinguish between free and Ca^{2+}-bound fura-2, but in view of the fact that the total fura-2 content was the same in both cell types, this does not affect any of the interpretations to follow.

One first notices a clear spatial segregation of fluorescence after aggregation is completed, at the stage of tip formation: the tip fluoresces more intensely than the rest of the aggregate (Figure 1 *b*). By the time a slug has formed, the contrast intensifies and Ca^{2+}–fura-2 fluorescence appears to be confined to the anterior prestalk portion of the slug (Figure 1 *c*). The extent of

Table 1. Percentages of amoebae staining with neutral red (NR) and displaying Ca^{2+}–CTC fluorescence and of amoebae staining for β-galactosidase activity

	NR	Ca^{2+}–CTC	β-gal
ecmA slugs			
Slug 1	28.57	28.57	
	20.00	16.67	
			15.20 ± 2.75
Slug 2	18.75	12.50	
	26.67	16.67	
Average	23.50	18.60	
ecmB slugs			
Slug 1	33.33	29.62	
	26.32	31.58	
			15.63 ± 2.52
Slug 2	34.48	20.69	
	30.77	23.08	
Average	31.23	26.24	

NR, neutral red; β-gal, β-galactosidase.
Data in the first two columns were derived from counts on about 100 cells from each of two different slugs.
β-gal in slugs derived from ecmA and ecmB transformants.
β-gal-positive cells were counted from other slugs (last column, mean ± s.d. from about 150 cells observed from each of the four slugs).

the fluorescence-positive region varies from experiment to experiment and constitutes about 5–20% of the total slug length. Independent support to this figure was obtained from flow cytometric analysis of cell suspensions derived from fura-2-labelled slugs. Approximately 17% of the cells exhibited fluorescence levels in excess of the background fluorescence of unstained amoebae (not shown). Figure 2 *a* displays the similarities between ecmA gene expression, NR staining and Ca^{2+}–CTC fluorescence, and Figure 2 *b* shows the same in the case of ecmB gene expression.

NR stains all the prestalk cells in the slug's anterior as well as the anterior-like cells in the slug's posterior[23,24]; the latter are not clearly visible in Figures 2 *a* and *b*. Table 1 shows that based on NR staining, the overall fraction fated to form stalk is 23.50% (ecmA slugs) and 31.23% (ecmB slugs). *Among* these, between 79% and 84% are positive for Ca^{2+}–CTC fluorescence. The visual appearance of fluorescence distribution in slugs formed from CTC-labelled amoebae is sufficiently consistent for us to believe that whatever the actual percentages involved, this is a general result. In short, some cells that are positive for NR do not exhibit Ca^{2+}–CTC fluorescence. In contrast to this, barring just one exception, every cell in our sample that was positive for Ca^{2+}–CTC fluorescence was also stained by NR. About 65% of the NR-positive cells are positive for ecmA gene expression; the corresponding percentage in the case of ecmB gene expression is about 50% (Table 1). In 'ecmB slugs' the percentages of NR-positive and β-galactosidase-positive cells are on the high side, but in our experience not outside the range of variation normally encountered.

The Ca^{2+}–fura-2 fluorescence distribution in the slug appears to be restricted to a slightly smaller fraction of the slug's anterior than the NR- or CTC-stained regions (compare Figure 1 with Figures 2 *a, b*). Whether this difference is real or an artefact arising from different strains and different experimental conditions remains an open question for the present. It may be that cells with a relatively high level of cytoplasmic Ca^{2+} constitute a subset of all cells with relatively high levels of sequestered Ca^{2+}, but such an inference would be in conflict with data derived from other experiments based on the use of a different Ca^{2+}-sensitive fluorescent dye, Indo-1 (Azhar *et al.*, in preparation). On the whole, it is our impression that cells that contain high levels of sequestered Ca^{2+} also contain high levels of cytoplasmic Ca^{2+}, and we are in the process of verifying this.

As explained, we could not carry out staining for NR (or CTC) and β-galactosidase activity in the same slugs. Therefore, it is difficult to quantitate the relative percentages of NR-positive (or Ca^{2+}–CTC-fluorescence-positive) and β-galactosidase-positive cells. Going by the numbers in Table 1, the indication is that ecmA- or ecmB-positive cells belong to a larger class of 'calcium-rich' cells, and further that the latter are, in turn, members of a still larger class of all NR-positive cells.

The qualitative picture is clearer: there is a significant overlap between patterns of NR staining, Ca^{2+}–CTC fluorescence and gene expression. This feature is especially striking in the case of the ecmA pattern. A comparison of Figure 2 *a* with Figure 1 further shows that in the case of ecmA gene expression (pstA cells) the overlap also extends to the distribution of Ca^{2+}–fura-2 fluorescence. Close examination of Figure 2 *b* shows that the ecmB pattern (pst AB cells) is, as expected[26], restricted to a roughly cylindrically shaped portion within the prestalk region. This cylindrical core is not picked out as a special region by any of the other stains (NR, CTC or fura-2).

Our conclusions are as follows:

(a) As judged by fura-2 fluorescence, there is a spatial gradient of cytoplasmic Ca^{2+} between the anterior (prestalk) and the posterior (prespore) regions of *Dictyostelium* slugs and the gradient is first perceptible at the tipped aggregate stage. The apparent all-or-none aspect to Ca^{2+}–fura-2 fluorescence (Figure 1) suggests that the cellular distribution of calcium is bimodal. To put it differently, the ranges over which Ca^{2+} levels vary in prestalk and prespore cells do not overlap. These observations were reported at a meeting[27] at which Cubitt

et al.[28] also presented similar data (derived independently from the use of aequorin fluorescence as a monitor of cytoplasmic Ca^{2+}).

(b) The Ca^{2+}–CTC fluorescence pattern indicates that cell-type-specific differences in sequestered calcium are similar to those in cytoplasmic calcium. Flow cytometry on CTC- and fura-2- or Indo-1-stained amoebae reinforces this feeling (not shown). A possible inference is that the same cells have high (or low) levels of sequestered and cytoplasmic Ca^{2+}. This needs to be rigorously substantiated.

(c) There is a partial overlap between those cells in the anterior of the slug that belong to the prestalk category (as judged by NR staining), those that have high levels of sequestered Ca^{2+} (as judged by Ca^{2+}–CTC fluorescence) and those that constitute genetically defined prestalk cells expressing the ecmA or ecmB genes (as judged by staining for β-galactosidase activity in transformants).

(d) The prestalk class is known to be heterogenous with regard to spatial patterns of gene activity and not all cells that are visibly stained with NR express the ecmA or ecmB genes[29]. By showing that essentially all Ca^{2+}-rich cells fall within the prestalk category but some do not belong to the ecmA or ecmB classes, our data highlight a different aspect of prestalk cell heterogeneity (Table 1).

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