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Spatial patterning of the distribution of Ca^{2+} in *Dictyostelium discoideum as* assayed in fine glass capillaries

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Abstract. We have shown previously that the Ca^{2+}-specific fluorescent dyes chlortetracycline (CTC) and indo-1/AM can be used to distinguish between prestalk and prespore cells in *Dictyostelium discoideum at a* very early stage. In the present study, pre- and post-aggregative amoebae of *Dictyostelium discoideum* were labelled with CTC or indo-1 and their fluorescence monitored after being drawn into a fine glass capillary. The cells rapidly form two zones of Ca^{2+}-CTC or Ca^{2+}-indo-1 fluorescence. Anterior (air side) cells display a high level of fluorescence; the level drops in the middle portion of the capillary and rises again to a lesser extent in the posteriormost cells (oil side). When bounded by air on both sides, the cells display high fluorescence at both ends. When oil is present at both ends of the capillary, there is little fluorescence except for small regions at the ends. These outcomes are evident within a couple of minutes of the start of the experiment and the fluorescence pattern intensifies over the course of time. By using the indicator neutral red, as well as with CTC and indo-1, we show that a band displaying strong fluorescence moves away from the anterior end before stabilizing at the anterior-posterior boundary. We discuss our findings in relation to the role of Ca^{2+} in cell-type differentiation in *Dictyostelium discoideum.*

Keywords. ***Dictyostelium;*** **indo-1/AM; chlortetracycline; capillary; calcium; cell differentiation.**

1. Introduction

The developmental cycle of the social amoeba *Dictyostelium discoideum* begins with the emergence of amoebae from spores, proceeds through growth and cell division and ends with the aggregation of starved cells to form a multicellular fruiting body consisting of a spore mass held up by a stalk of dead cells (Bonner 1967). Terminal differentiation into stalk and spore is preceded by a phase in which the long and thin aggregate or slug exhibits a clear spatial pattern of presumptive cell types with pre-stalk amoebae in the anterior one-fifth and prespore amoebae forming the bulk of the posterior four-fifths. Studies on *D. discoideum* have long been plagued by the question whether prestalk and prespore tendencies are primarily the result of spatially patterned cues, or whether cells with pre-existing tendencies sort out and give rise to the spatial pattern in the slug (Bonner 1992; Nanjundiah and Saran 1992). There is substantial evidence in favour of both points of view (Leach *et al* 1973; McDonald and Durston 1984; Gomer and Firtel 1987; Saran *et al* 1994a; Early *et al* 1995; Azhar *et al* 1996).

A remarkable discovery made recently by Bonner *et al* (1995) offered a fresh insight into the problem. They found that agglomerated amoebae of *D. discoideum* could be drawn up into fine glass capillaries by surface tension. Having entered the capillary, the amoebae proceeded spontaneously to organize themselves into a quasi-1-dimensional

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tissue that mimicked features of supracellular organization found in the course of normal development in the slug, in particular those of polarity and anterior-posterior differentiation. The onset of patterning was rapid (ca. a few minutes), did not require cell movement and apparently depended only on which end of the tissue was exposed to air and which end was relatively anaerobic. The origin—meaning prior state of differentiation—of the amoebae seemed to have no bearing on the outcome. Clearly, the implication was that (at least under certain circumstances) positional cues could override all prior tendencies and suffice for the establishment of the normal spatial pattern. We and others have earlier shown that a pre-aggregation heterogeneity in respect of cellular Ca^{2+} mirrors future prestalk and prespore tendencies and that prestalk cells in the slug have markedly higher levels of both sequestered and free Ca^{2+} than prespore cells (Maeda and Maeda 1973; Tirlapur *et al* 1991; Saran *et al* 1994b; Azhar *et al* 1995). In view of the importance of Ca^{2+} in the development of *Dictyos telium* (Newell *et al* 1995) the obvious question arose whether these spatial patterns can be found in the capillary arrangement as well. We show here that they can.

2. Materials and methods

2.1 *Growth and development of cells*

D. discoideum (wild type strain NC-4H) amoebae were grown on SM agar plates with *Klebsiella aerogenes* bacteria and harvested using standard procedures (Tirlapur *et al* 1991) except that the buffer was a mixture of potassium phosphates (KK_2) at pH 6·5. Amoebae were washed free of bacteria by centrifugation at 400 *g* for 10 min and developed at 22°C in the dark on 2% KK_2 agar. All chemicals were of analytical grade and obtained from Merck or Sigma, except for indo-1/AM, Pluronic F-127 (Molecular Probes Inc., USA) and dry dimethylsulphoxide (anhydrous DMSO) (Aldrich, USA)

2.2 *Chlortetracycline/indo-1/ AM/neutral red labelling of cells*

1-2 × 10^6 amoebae were incubated for 30–40 min with 80 µM of chlortetracycline (CTC) or 2 µM indo-1/AM in the presence of mild detergent (0·2% Pluronic F-127) at 22°C while being gently shaken. Similarly, amoebae were stained with 0·005% neutral red at 22°C while being shaken gently for 20 min. They were later washed twice with KK_2, plated for development on KK_2 agar and used at whichever developmental stage was examined.

2.3 *Capillary setup*

The methods of Bonner *et al* (1995) were followed more or less identically. Our capillaries had a diameter of 30 µm to 170 µm. After being drawn in, the length of the cellular mass varied from about 0·3 mm to 0·8 mm. CTC-stained or indo-1/AM and neutral red (NR)-stained amoebae from the aggregate centre were pulled into the capillary simply by surface tension. In experiments involving freshly starved amoebae, the amoebae were first compacted by centrifugation into a tight pellet. The capillary was gently pushed into the pellet and a mass of cells soon rose up. This end of the capillary (with amoebae) was then placed in a drop of mineral oil on a glass slide, with the other end opened to the air. In a

few seconds the cells moved up the capillary pulling the oil behind them. When the mass had risen sufficiently, the air end of the capillary was broken under oil and sealed with vacuum grease. As a result there were three compartments in the capillary: air, cells, oil.

In experiments involving 'double anterior' or 'double posterior' arrangements a slight modification was made. The amoebae were allowed to rise into the lumen and both capillary ends sealed with grease; this gave rise to a 'double anterior' (air-cells-air) system. When a 'double-posterior' (oil-cells-oil) arrangement was desired, a drop of oil was pulled first into the capillary and cells drawn in immediately thereafter. Some more oil was drawn up after the cells so that there was no air present between the cells and the oil. Cells from aggregates and from prestalk and prespore regions of CTC and NR-stained slugs were drawn into the capillaries similarly. Each experiment was repeated five times unless indicated otherwise.

2.4 *Microscopy*

CTC-stained and NR-stained amoebae and capillaries were observed in a Zeiss Axioscope fluorescence microscope. CTC fluorescence was monitored using a standard Zeiss UV filter set (G365, FT395, LP 420). Ca^{2}+-indo-1 fluorescence was monitored with the help of

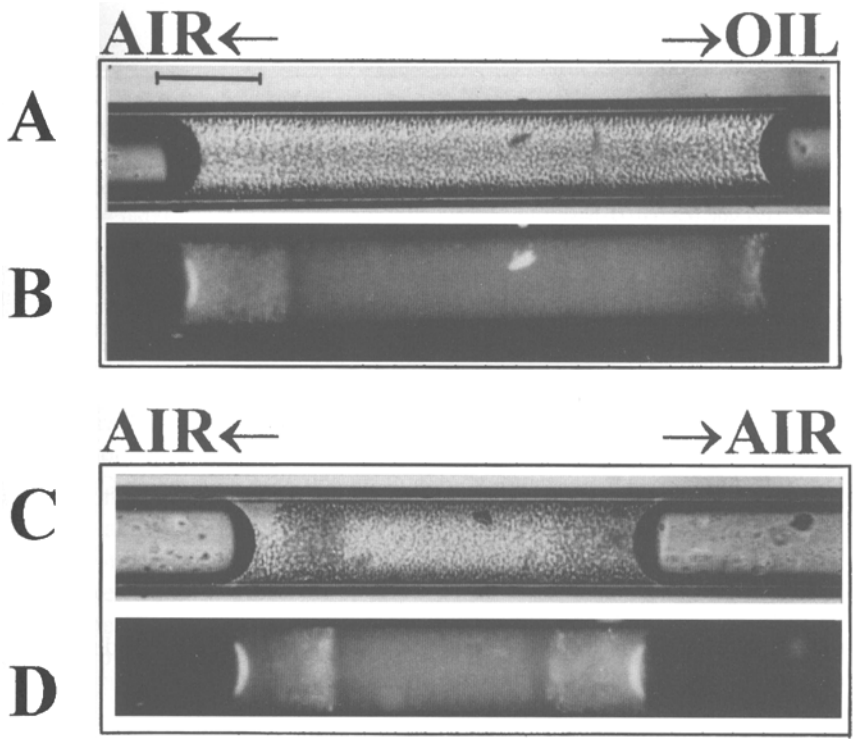

Figure 1. CTC-stained amoebae from an aggregate centre observed within 5 min in the capillary. (**A**) and (**C**), bright field and **(B)** and (**D**), fluorescence micrograph of the same capillary as the one immediately above it. Air end (anterior) on the left, oil end (posterior) on the right in (**A**) and **(B) (C)** and **(D)** show double anterior symmetry (air at both ends). Note intense band of Ca^{2}+ -CTC fluorescence at both ends of the capillary. The capillary meniscus also fluoresces in all photographs. (Bar = 100 µm).

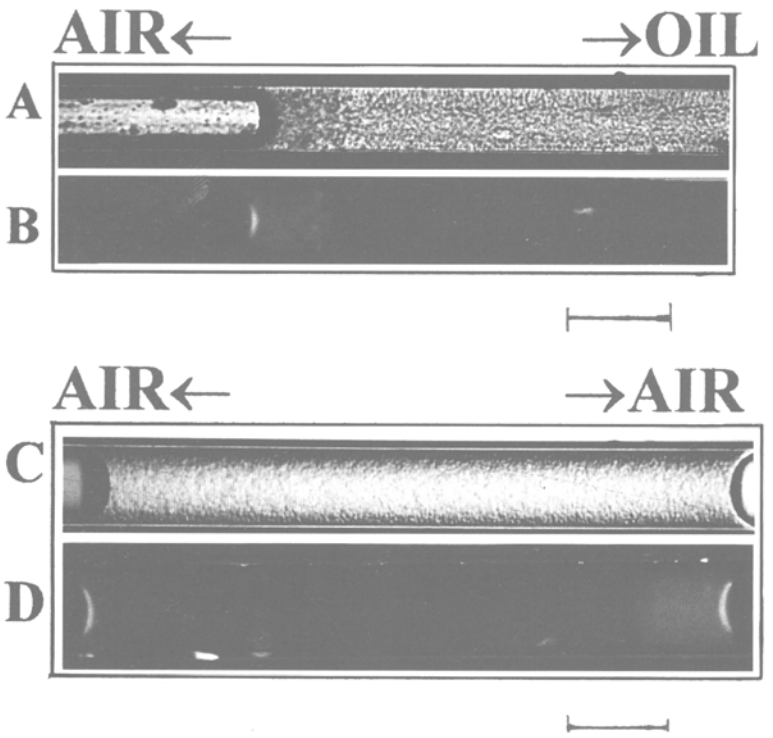

Figure 2. Indo-1/AM stained freshly starved amoebae observed within 5 min in the capillary. (**A**) and (**C**), bright field and (**B**) and (**D**), fluorescence. (**A**) and (**B**) are with an air-oil polarity. Air end is at left side. (**C**) and (**D**) have a double anterior symmetry (air at both ends). (Bar = 100 μm).

a special filter set from M/s 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 at 450 nm and emission filter 405 DF20. NR-stained amoebae were observed under fluorescence using the Zeiss blue filter set (BP 450-490, FT 510, LP 520). We note that NR fluoresces (Kirk 1970) but there is only one previous instance known to us wherein its fluorescence has been monitored in *D. discoideum* (Lokeshwar 1983). The NR-staining pattern is especially striking when viewed in fluorescence optics with blue light excitation. Photography was with Kodak Gold 400 ASA colour and Orwo 125 ASA black and white print films using an automatic exposure control.

3. Results

In conformity with the notation of Bonner *et al* (1995) we refer to the end nearest air as the anterior and the one next to mineral oil as the posterior.

3.1 *Pre- and post-aggregative amoebae exhibit an anterior-posterior gradient of Ca^{2+}-CTC or Ca^{2+}-indo-1 fluorescence*

Fluorescence from the anterior end appears to be from a fairly broad zone and is also more intense than that from the posteriormost end, which has the appearance of a thin band (figures 1A, B and 2A, B). The gradient is set up quite rapidly, certainly within 2 to 5 min

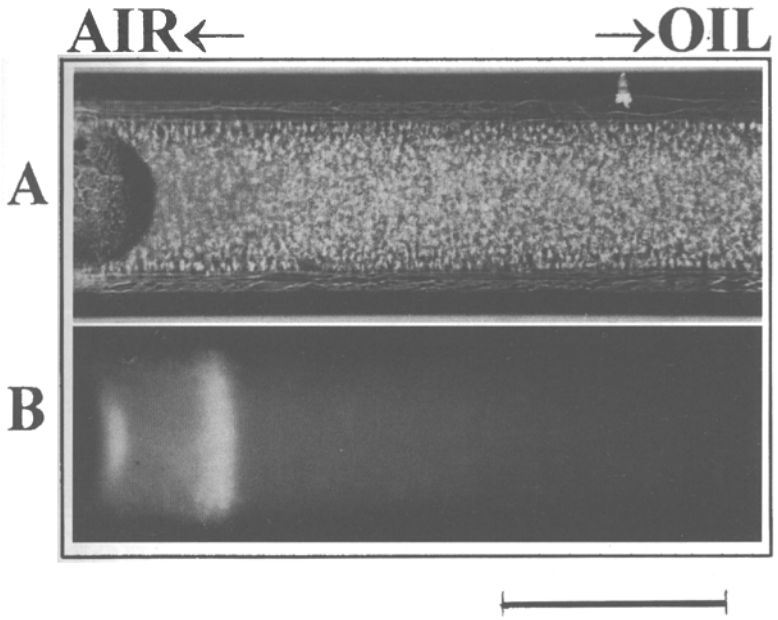

Figure 3. CTC-stained amoebae from aggregate centre, air-oil polarity; only anterior portion shown. (A), bright field and (B), fluorescence. Photographs taken after 20 min from start. (Bar = 100 μm).

(i.e., as soon as the manipulations for proper focussing etc., are complete). Since unstained amoebae also showed some autofluorescence, they were examined in controls and it was verified that autofluorescence was significantly less intense than fluorescence from stained amoebae (not shown). Ca^{2+}-CTC (as also Ca^{2+}-indo-1) fluorescence at the anterior-posterior boundary intensified markedly after about 20 min in the dark (figure 3A, B). CTC-stained amoebae from the prestalk and prespore regions of migrating slugs exhibited a similar pattern of Ca^{2+}CTC fluorescence as freshly starved amoebae and aggregates but the pattern appeared to take somewhat longer to emerge, roughly 10 min or so instead of within 5 min.

NR-stained pre- and post-aggregative amoebae were also examined with an air-cells-oil polarity. Confirming the findings of Bonner *et al* (1995), an anterior zone showed intense NR-staining as compared to the posterior. This anterior-posterior distinction in NR-staining was visible well within 5 min after the cells were pulled up into the capillary. When cells were taken from the prestalk or prespore regions of NR-stained slugs they showed no anterior-posterior difference at first but, as with Ca^{2+}-CTC fluorescence, a clear difference was visible 10-15 min after the cells were drawn in (not shown).

3.2 *Pre- and post-aggregative amoebae display a symmetric pattern of fluorescence in a double anterior* (*air-cells-air*) *design*

Within about 5 min, cells in the double anterior setup exhibited high Ca^{2+}-CTC or Ca^{2+} -indo-1 fluorescence at both ends and a significantly lower level of fluorescence in the middle region (figures 1C, D and 2C, D). Amoebae from prestalk or prespore

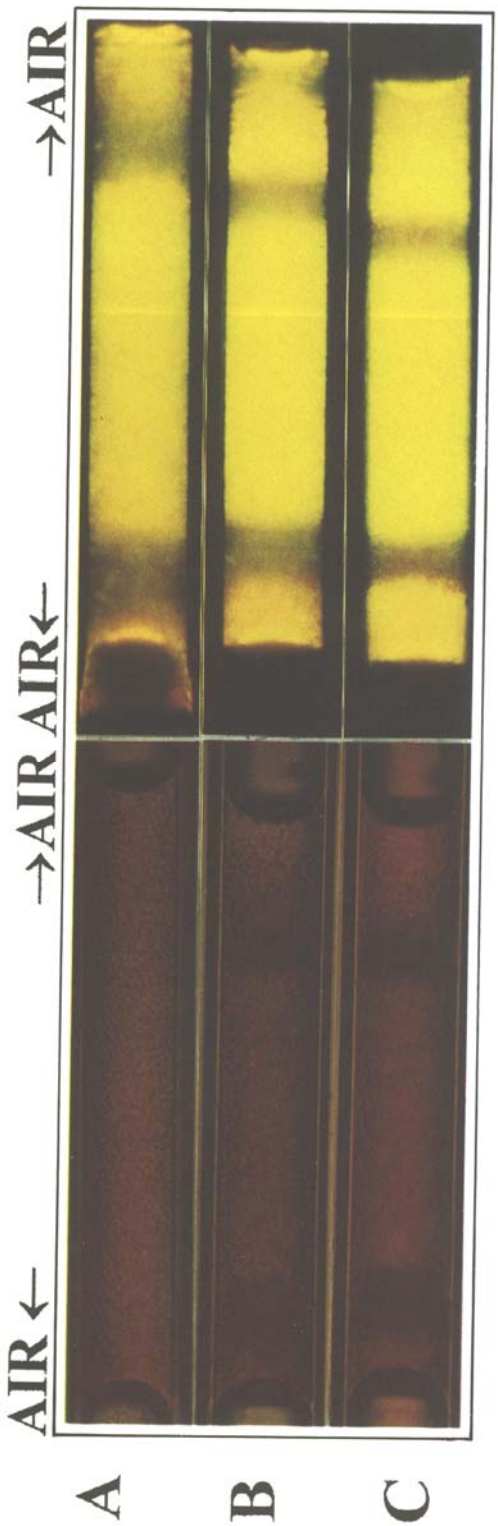

Figure 4. NR-stained amoebae from aggregate centre; double-anterior symmetry. The left panel shows bright field and the right panel, the corresponding fluorescence micrograph. (**A**), (**B**) and (**C**) show the same capillary at 2, 5 and 20 min from the start respectively. The NR-positive bands visible at both ends at 5 min have moved inwards and sharpened with the formation of two distinct zones. One zone is at either end of the capillary well inside the terminus (especially clear in fluorescence pictures in the right side panel). Note that in fluorescence images the "NR-positive" region does not fluoresce because of very low pH as reported earlier (Kirk 1970). (Bar = 100 μm).

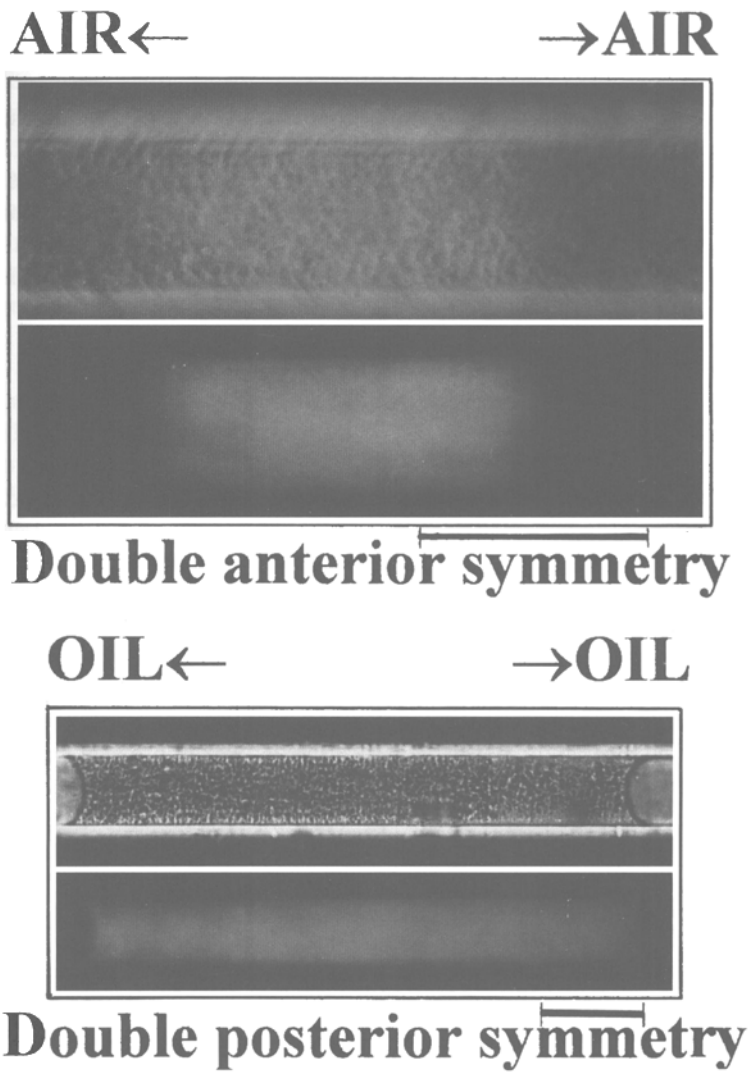

Figure 5. NR-stained amoebae taken from an aggregate centre and viewed in bright field (top) or fluorescence (bottom). Top, double-anterior arrangement (air at both ends) and bottom, double-posterior (oil at both ends). Note that amoebae exhibit strong staining (and very weak fluorescence) at the ends in the double-anterior arrangement but no significant spatial differences in staining (and uniform fluorescence) in the double-posterior case. (Bar = 100 μm).

regions of slugs displayed a similar symmetric pattern of fluorescence as freshly starved amoebae (not shown). With NR-stained pre- or post-aggregative amoebae, both ends of the capillary showed strong staining, and no fluorescence, within 5 min [figures 4 (left) and 5 (top)]. These NR-stained regions had moved inwards by 15-20 min and stabilized their positions in approximately 20 min (compare figure 4A, B and C). The rest of the capillary contained cells that were weakly stained with NR but fluoresced. Cell movement did not appear to be responsible for any of the shifts in staining pattern.

3.3 *Pre- and post-aggregative amoebae do not exhibit significant differences of fluorescence when monitored in a double posterior* (*oil-cells-oil*) *arrangement*

Unlike the previous situations, no marked difference in Ca^{2+}-dependent fluorescence could be seen along the length of the cell mass except for a few cells which fluoresced intensely at either end of the capillary (not shown). By and large there appeared to be a uniform emission of Ca^{2+}-indo-1 fluorescence along the length of the capillary. The higher than average Ca^{2+}-CTC fluorescence at both posterior ends was similar to that seen at (the single) posterior end in the normal (i.e., air-cells-oil) experimental design (see figure 1B). Also, NR-stained pre- and post-aggregative amoebae showed a uniformly weak staining pattern combined with a spatially uniform pattern of fluorescence in the double posterior setup (figure 5, bottom).

4. Discussion

We confirm the observations of Bonner *et al* (1995) that soon after being drawn into a capillary—in our observations, within 5 min at *most*— *Dictyostelium* amoebae display both patterning and polarity; that is, they spontaneously organize themselves into distinct zones that resemble the prestalk and (possibly) prespore portions of slugs. The existence of a prestalk zone is inferred from the pattern of cellular compaction and neutral red staining (figures 1A, 4 and 5) and, what is new, also from the differences in Ca^{2+}-specific fluorescence as seen along the length of the capillary (figures 1A, B and 2A, B). The existence of the prespore zone is inferred by default: on the basis of this study alone it is no more than a non-prestalk zone. However, preliminary experiments with the mitochondrial activity stain rhodamine 123 indicate that the cells in question might fall in the prespore class (not shown). The prestalk zone is first seen as a localized group of amoebae near the air end of the capillary that soon begins to take up the appearence of a bright transverse band of fluorescence. The band starts at the very end and slowly moves posteriorly, stabilizing at a distance of 65·1 ± 8·8 μm from the anterior end; the prestalk zone constitutes a fraction of about 18·42 ± 3·59% of the whole length. We wish to point out that prestalk-prespore proportioning may infact not be exact, and that the level of ambient oxygen may determine the position of the boundary between the two zones (this possibility was suggested by a referee). The width of the band is approximately 15·3 ± 28 μm (mean ± SD) from 5 measurements in all cases; the band forms about 19·20% of the prestalk zone. Thus it appears reasonable to identify the entire anterior zone as comprising the prestalk region with the band marking the prestalk-prespore boundary. We note that a stationary boundary of high Ca^{2+} concentration has not been seen so far in normal slugs, though Cubitt *et al* (1995) report moving cross band pulses. In the 'double anterior' configuration, two zones of high Ca^{2+} are seen, one at each end (figure 1C, D). These zones are about the same size. When summed, the two prestalk zones in the double-anterior situation add up to about 43·6% of the entire length.

We know from previous studies that intracellular heterogeneity in respect of Ca^{2+} levels is both a predictor of the prestalk-prespore dichotomy, when Ca^{2+} is assayed in freshly starved cells (Saran *et al* 1994a; Azhar *et al* 1996), as well as its correlate, when Ca^{2+} levels are monitored in the slug (Azhar *et al* 1995; Cubitt *et al* 1995). In the latter situation, a spatial gradient of Ca^{2+} is seen, with prestalk cells containing about twice as much Ca^{2+} as prespore cells. Previously we have found that the population of

NR-positive or 'Ca^{2+} -rich' cells in the slug is significantly higher than the population of cells in which the promoters of the prestalk-specific genes *ecmA* or *ecmB* are active. The respective percentages are approximately 23·5% (NR) and 18·6% (Ca^{2+} -CTC fluorescence) versus 15·2% *(ecmA* activity) in *ecmA* slugs and 31·2% (NR) and 26·2% (Ca^{2+} -CTC fluorescence) versus 15·6% *(ecmB* activity) in *ecmB* slugs (Azhar *et all* 995). Here also we find differences in the size of the prestalk zone as defined by NR. CTC and indo-1 staining— 22·7 ± 4·3% (n = 7), 184 + 3·5% (n = 5) and 14·5 ± 3·4% (n = 5) respectively relative to the total cell mass in the capillary. As can be seen, these figures are comparable to the ones found for slugs. On the other hand, in the capillary set up, we see a band of enhanced Ca^{2+}-dependent fluorescence (figure 3) and NR staining (figure 4) at the boundary between the inferred prestalk and prespore zones. It seems reasonable to assume that the bands displaying strong Ca^{2+}-CTC fluorescence (figure 3), Ca^{2+}-indo-1 fluorescence (not shown) and NR staining (figure 4) represent the same cells. However, the differences in the relative sizes of the anterior prestalk zone as defined by various markers (just discussed) may indicate otherwise.

Our conclusions are as follows. (i) As first shown by Bonner *et al* (1995), the capillary setup is an excellent model for longitudinal patterning in the slug. (ii) Polarity depends on external cues, with the anterior, prestalk region appearing at the air end. If there are two air ends, two mirror-imaged anterior zones form. The outcome is not as clear with a 'double posterior' arrangement. (iii) As judged by CTC or indo-1 fluorescence, a region of increased cellular Ca^{2+} is set up towards the anterior end, with the maximum levels of Ca^{2+} present in a thin band towards the back of the anterior zone, significantly lower levels in the bulk of the cell mass and a smaller secondary maximum at the very posterior. This exactly parallels the pattern seen with CTC in the case of normal slugs (Tirlapur *et* al 1991). (iv) The basis of the distinct Ca^{2+} zones is unclear, but the rapidity of onset of the phenomenon makes it evident — because signaling over the entire cell mass must be involved—that fast intercellular signals must play a role. A rough speed would be 100 µm/min (0·5 mm/5 min). The observation that the band of enhanced Ca^{2+} moves posteriorly (without any accompanying cellular movement) suggests the propagation of a slower wave of Ca^{2+} with a speed of about 3·25 µm/min (65·1 µm/20 min) that stabilizes at the prestalk-prespore boundary. These speeds should be compared with the speeds of propagation of cAMP waves during aggregation that range from 350 µm/min (Alcantara and Monk 1974) or from 144 µm/min to 696 µm/min (Nanjundiah 1976) and of the one clear case of a calcium wave that spread at 1620 µm/min reported by Cubitt *et al* (1995). Whether Ca^{2+} itself is the signaling agent, or whether an extracellular signal (e.g., cAMP) is the primary cause for the phenomena we observe in the capillary, is unknown. Recent findings of Bonner (private communication) suggest that cAMP signaling does not seem to be involved in rapid zone formation. (v) The entire phenomenon is indicative of a process of spontaneous self-organization; the prior state of differentiation of the amoebae in the capillary is immaterial. It is interesting that freshly starved amoebae organize themselves into the Ca^{2+} classes on a spatial basis. Thus, given the right conditions, purely spatial cues can override preexisting tendencies to differentiate into prestalk or prespore. Under other circumstances, pre-aggregation biases, including those related to cellular Ca^{2+} (Saran *et* al 1994a; Azhar *et al* 1996), have been shown significantly to influence the course of differentiation (also see Leach *et* al 1973; McDonald and Durston 1984; Gomer and Firtel 1987). Evidently evolution has so moulded the *Dictyostelium* life cycle that cellular behaviour is determined, not in a fixed fashion, but rather in a manner that is strongly dependent on contingencies (`multiple insurance'; Nanjundiah and Saran 1992).

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