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Original Article

A cell type-specific effect of calcium on pattern formation and differentiation in *Dictyostelium discoideum*

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ABSTRACT Spatial gradients of sequestered and free cellular calcium (Ca^{2+}) exist in the slug of *Dictyostelium discoideum* (Maeda and Maeda, 1973; Tirlapur *et al.*, 1991; Azhar *et al.*, 1995; Cubitt *et al.*, 1995). When we vary intracellular Ca^{2+} with the help of calcium buffers and the ionophore Br-A23187, there are striking effects on slug morphology, patterning and cell differentiation. In the presence of a calcium ionophore, high external Ca^{2+} levels lead to an increase of intracellular sequestered and free Ca^{2+}, the formation of long slugs, a decrease in the fraction of genetically defined prespore cells and 'stalky' fruiting bodies. Conversely, a lowering of external Ca^{2+} levels results in a decrease of intracellular Ca^{2+}, the formation of short slugs, an increase in the prespore fraction and 'spory' fruiting bodies. We infer that Ca^{2+} plays a significant morphogenetic role in *D. discoideum* development, by selectively promoting the prestalk pathway relative to the prespore pathway.

KEY WORDS: *Development, differentiation, pattern formation, calcium.*

Introduction

Because of its unusual mode of development, the cellular slime mould *D. discoideum* is ideal for probing the links between single cell physiology and multicellular patterning. The multicellular stage of *D. discoideum* begins after starvation. Free-living amoebae aggregate via chemotaxis to cAMP and form a polarized mass, the slug, and go on to differentiate into a fruiting body consisting of a dead stalk with a spore mass on its top. The slug exhibits a simple anterior-posterior pattern of cell fates. Prestalk cells are located in approximately the anterior 20% and prespore cells occupy most of the posterior (Bonner, 1967). The anterior-posterior pattern is influenced by many factors, and there are grounds for thinking that the Ca^{2+} ion might be one of them (reviewed in Nanjundiah, 1997; Jaffe, 1997).

Three sets of facts motivate an investigation into a possible morphogenetic role for Ca^{2+} in *D. discoideum*. Firstly, Ca^{2+} functions as a 'second messenger' following extracellular stimulation by cAMP (Newell *et al.*, 1995). This makes it plausible that Ca^{2+} changes accompany cAMP-mediated cell-to-cell signaling in the slug. Secondly, there is suggestive evidence, some of it indirect, that Ca^{2+} can influence cell differentiation in *D. discoideum* (Maeda, 1970; Blumberg *et al.*, 1989; Kubohara and Okamoto, 1994). Finally, studies with disaggregated cells (Abe and Maeda, 1989; Saran *et al.*, 1994a) as well as whole slugs (Maeda and Maeda, 1973; Tirlapur *et al.*, 1991; Saran *et al.*, 1994b; Azhar *et al.*, 1995; Cubitt *et al.*, 1995) show that there are spatial gradients of sequestered and cytoplasmic (free) Ca^{2+}: in respect of both, the Ca^{2+} level in anterior prestalk cells is significantly higher than that in posterior prespore cells. Taken together, these observations lead us to the hypothesis that Ca^{2+} is an important mediator of cell type differentiation and pattern formation in *D. discoideum*. In what follows, we test this hypothesis by monitoring the relative proportions and spatial dispositions of genetically defined cell types after increasing or decreasing cellular Ca^{2+} levels with the help of an ionophore.

Results

$[Ca^{2+}]_{seq}$ and $[Ca^{2+}]_{cyt}$ in the tip, middle and end region of slugs developed in altered calcium environments

Fluorescence was measured in dissociated cell monolayers obtained after teasing apart the cells that comprised the slug. The measurements for $[Ca^{2+}]_{seq}$ are depicted in Fig. 1. $[Ca^{2+}]_{seq}$ in the cells of the tip (prestalk), middle (prespore) and end (rear-guard) region of control slugs is 448.91 $\pm$ 250.31, 225.57 $\pm$ 125.31 and 277.15 $\pm$ 165.29 μM respectively. 100 μM $CaCl_2$ or 7 μM Br-A23187 individually do not alter $[Ca^{2+}]_{seq}$, but when applied together, they

Abbreviations used in this paper: D. discoideum, Dictyostelium discoideum; cAMP, 3'5' cyclic-Adenosine monophosphate; Prestalk, Presumptive stalk; Prespore, Presumptive spore; Br-A23187, 4-bromo-ionophore; NR, Neutral Red; CTC, Chlortetracycline; MES, 2-[N-Morpholino] ethanesulfonic acid; sfe, spore forming efficiency; $[Ca^{2+}]_{seq}$, cellular level of sequestered calcium; $[Ca^{2+}]_{cyt}$, cellular level of cytosolic calcium.

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raise its level significantly. $[Ca^{2+}]_{seq}$ in cells from the tip, middle and end region of slugs developed on $CaCl_2$ + Br-A23187 is 1280.11 ± 194.73 ($p<0.001$), 450.27 ± 173.53 ($p<0.001$) and 470.26 ± 302.02 μM ($p<0.01$) respectively. Conversely, when applied together, EGTA and Br-A23187 lower $[Ca^{2+}]_{seq}$ significantly. $[Ca^{2+}]_{seq}$ in cells from the tip, middle and end region of slugs developed on EGTA + Br-A23187 is 69.19 ± 17.13, 63.15 ± 17.13 and 59.79 ± 17.45 μM respectively ($p<0.001$). Controls showed that EGTA alone, but neither of $CaCl_2$ nor Br-A23187 by itself, had a slight $[Ca^{2+}]_{seq}$-lowering effect.

The measurements for $[Ca^{2+}]_{cyt}$ are depicted in Fig. 2. As judged by Ca^{2+}-fura-2 fluorescence, the $[Ca^{2+}]_{cyt}$ in cells from the tip, middle and end region of control slugs is 128.45 ± 29.19, 89.67 ± 24.11 and 107.9 ± 21.57 nM respectively. 1 mM EGTA alone or with Br-A23187 lowers $[Ca^{2+}]_{cyt}$ significantly. $[Ca^{2+}]_{cyt}$ in cells from the tip, middle and end region of EGTA slugs is 71.93 ± 7.61 ($p<0.001$), 66.8 ± 7.61 ($p<0.05$) and 66.8 ± 8.88 nM ($p<0.001$) respectively. In EGTA + Br-A23187 slugs, it is 44.81 ± 5.08, 43.68 ± 2.54 and 43.29 ± 5.08 nM respectively ($p<0.001$). $CaCl_2$ by itself does not alter $[Ca^{2+}]_{cyt}$ significantly. $[Ca^{2+}]_{cyt}$ in cells from the tip, middle and end region of $CaCl_2$ + Br-A23187 slugs is 195.25 ± 51.60 ($p<0.01$), 138.30 ± 49.49 ($p<0.01$) and 142.8 ± 41.88 ($p<0.02$) nM respectively. Interestingly, Br-A23187 by itself lowers $[Ca^{2+}]_{cyt}$ slightly. $[Ca^{2+}]_{cyt}$ in cells from the tip, middle and end region of Br-A23187 slugs is 88.63 ± 31.72 ($p<0.01$), 71.93 ± 25.38 ($p<0.1$) and 66.8 ± 19.03 ($p<0.001$) nM respectively.

NR staining pattern and prestalk cell counts

When amoebae are stained with NR, a vital dye specific for prestalk, rear-guard and anterior-like cells (Bonner, 1952; Sternfeld and David, 1981), and made to develop on 5 mM Tricine-buffered agar (controls), strong positive staining is visible in the anterior of the slugs (Fig. 3A). Anterior-like cells are stained too but in terms of the fraction of slug length, their contribution is not significant. On the other hand, when development occurs in the presence of elevated Ca^{2+} (100 μM Ca^{2+} + 7 μM Br-A23187), the NR staining pattern is strikingly different from normal. The main reason for the difference is a significant contribution from cells situated in the very posterior of the slug (Fig. 3B; Table 1). Counts made after slugs are disaggregated show that the fraction of cells that stain with NR (not shown) is approximately the same as the % prestalk tendency (the relative percentage of NR stained length in the slug, Fig. 3C). Ca^{2+} by itself or Br-A23187 by itself does not affect the % prestalk tendency (the relative percentage of NR stained length in the slug, Fig. 3C, Table 1). However, when used in combination, that is, under conditions of elevated Ca^{2+}, the fraction of NR-positive cells goes up from 19.6 ± 4.9% to 42.4 ± 2.8%, and the % prestalk tendency rises from 16.9 ± 6.0 to 41 ± 17.1 (Fig. 3C, Table 1).

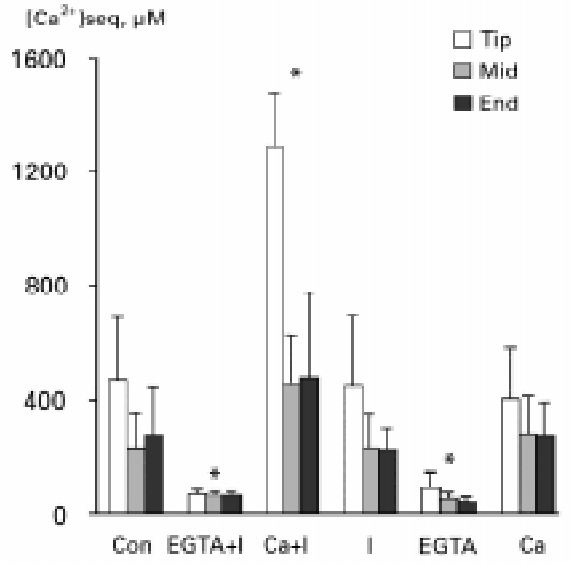

Fig. 1. Gradient of sequestered Ca^{2+} in the slug. *Levels in μM (mean ± SD) measured in cells taken from the from the tip, middle and end of slugs formed after development on agar containing: 10 mM MES (control; n=103), 1 mM EGTA + 7 μM Br-A23187 (n=23), 100 μM $CaCl_2$ + 7 μM Br-A23187 (n=11), 7 μM Br-A23187 (n=14), 1 mM EGTA (n=11) and 100 μM $CaCl_2$ (n=32). Asterisks denote values significantly different from the corresponding control carried out on cells taken from the tip, middle or end of slugs (t-test, p<0.001).*

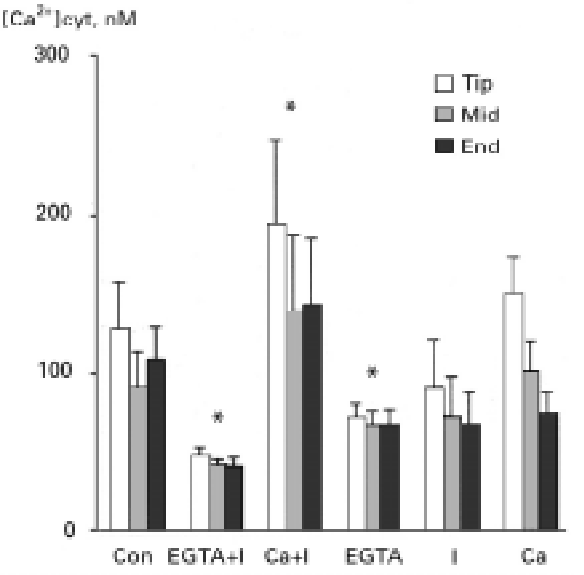

Fig. 2. Gradient of free Ca^{2+} in the slug. *Levels in nM (mean ±SD) measured in cells taken from the tip, middle and end region of slugs formed after development on agar containing: 10 mM MES (control; n=15), 1 mM EGTA + 7 μM Br-A23187 (n=8), 100 μM $CaCl_2$ + 7 μM Br-A23187 (n=11), 7 μM Br-A23187 (n=5), 1 mM EGTA (n=13) and 100 μM $CaCl_2$ (n=6). Asterisks denote values significantly different from the corresponding control carried out on cells taken from the tip, middle or end of slugs (t-test p<0.001).*

Ca^{2+} influences the extent and spatial pattern of genetically defined prestalk cells

In the *ecmA-lacZ* (prestalk-specific) transformant, a positive correlation was observed between the levels of cellular Ca^{2+} and the spatial extent of gene expression. In the controls, as expected the anterior prestalk portion alone gets stained (Fig. 4A) and this complements the NR staining pattern (Fig. 3A). Development under increased Ca^{2+}conditions (Williams *et al.*, 1989) leads to the appearance of staining in both the front and the back of the slug leaving the middle portion unstained (Fig. 4B). Again, this observation conforms to the NR staining pattern of slugs formed in elevated Ca^{2+} environments (Fig. 3B). (We did not always obtain slugs when

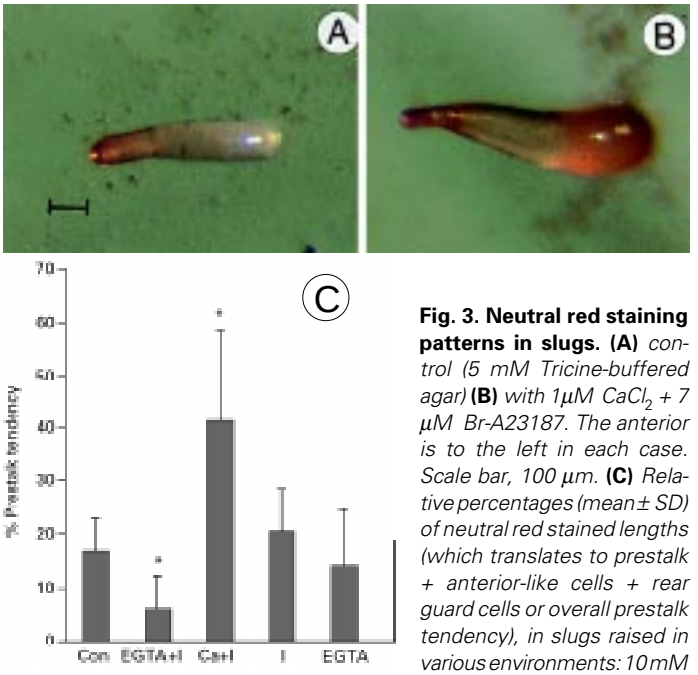

Fig. 3. Neutral red staining patterns in slugs. (A) *control (5 mM Tricine-buffered agar)* **(B)** *with 1μM $CaCl_2$ + 7 μM Br-A23187. The anterior is to the left in each case. Scale bar, 100 μm.* **(C)** *Relative percentages (mean ± SD) of neutral red stained lengths (which translates to prestalk + anterior-like cells + rear guard cells or overall prestalk tendency), in slugs raised in various environments: 10 mM MES buffered agar (control; n=25), 1 mM EGTA + 7 μM Br-A23187 (n=49), 100 μM $CaCl_2$ + 7 μM Br-A23187 (n=13), 7 μM Br-A23187 (n=33), 1 mM EGTA (n=56) and 100 μM $CaCl_2$ (n=26). Asterisks denote values significantly different from control; (t-test p<0.001). Also see Table 1.*

development occurred in a Ca^{2+}-depleted environment; most of the time fruiting took place immediately after aggregation).

Ca^{2+} influences the extent and spatial pattern of genetically defined prespore cells

In the *D19-lacZ* (prespore-specific) transformant, we find a complementary correlation of the pattern of gene activity with cellular Ca^{2+} levels. As expected, only the posterior region of the slug exhibits *lacZ* activity in controls (Fig. 4C, Dingermann *et al.*, 1989). When Ca^{2+} levels are raised, a central region of the slug stains strongly for β-galactosidase leaving the anterior prestalk region and a sizeable fraction of the posterior unstained (Fig. 4D). At times a stalk tube is visible behind the slug (Fig. 4D; vacuolated stalk cells can be seen in the tube at high power). In a Ca^{2+}-depleted environment, the entire length of the slug gets stained, if a slug is formed at all (Fig. 4E). In short, the pattern of prespore distribution in the slug is complementary to that of prestalk cells in all Ca^{2+} regimes (compare Figs. 4 C,D,E with Figs. 3 A,B and Figs. 4 A,B). Cell counts taken after staining disaggregated slugs with a FITC-conjugated antibody specific for prespore cells (Takeuchi, 1963) confirmed that the increase in prestalk cell fraction caused by Ca^{2+} was accompanied by a decrease in the fraction of prespore cells (not shown).

High [low] levels of intracellular Ca^{2+} lead to the appearance of 'stalky' ['spory'] fruiting bodies and cause a decrease [increase] in the fraction of amoebae that differentiate into spores

A dramatic increase can be seen in the average size of the slugs that are formed in an environment containing 1 μM or 100 μM $CaCl_2$ and 7 μM Br-A23187 (compare Fig. 5A with 5B; also see Fig. 6). On the other hand, slugs that are formed under conditions of lowered Ca^{2+} were much smaller than normal (compare Fig. 5A with 5C; also see Fig. 6). This can be explained by an effect of Ca^{2+} on aggregation territory size, or equivalently, on the number of amoebae that enter an aggregate (initial cell densities being the same). There is a significant enhancement in territory size when the Ca^{2+} level is raised and correspondingly, a decrease when the Ca^{2+} is lowered.

The size and shape of the stalk make it difficult to count individual stalk cells and so to estimate stalk:spore ratios in fruiting bodies. Instead, we have calculated the sfe, defined as the number of spores formed relative to the number of amoebae that were dispersed on the plate. The sfe was calculated after discounting for a small increase in cell number subsequent to plating (this increase was estimated independently under each environmental condition and varied between 3.47 % to 13.26 %, not shown). Compared to controls (Fig. 5D), fruiting bodies display a 'stalky' appearance under conditions that raise intracellular Ca^{2+} (Fig. 5E) and a 'spory' appearance when development occurs under Ca^{2+}-depleted conditions (Fig. 5F).

A qualitative feature of the 'stalky' fruiting bodies that develop under raised Ca^{2+} conditions is that they have unusually large basal discs. This stands out even on casual observation (Fig. 5E) and is confirmed by measuring basal disc diameters (Table 2). Control populations developing on MES agar have a sfe of 71.42 ± 3.67 %; 100 μM $CaCl_2$ leaves this essentially unchanged at 74.78 ± 11.4 % ($p>0.1$, Fig. 7). 7 μM Br-A23187 alone lowers the sfe to 59.65 ± 3.50 % ($p<0.1$) whereas when applied simultaneously, 7 μM Br-A23187 and 100 μM $CaCl_2$ cause the sfe to fall all the way to 50.2 ± 3.37 % ($p<0.05$). Conversely, 1 mM EGTA by itself causes an increase in sfe to 81.93 ± 1.40 % ($p<0.1$, Fig. 7). When provided together with 7 μM Br-A23187, EGTA raises the sfe to 85.8 ± 3.37 % ($p<0.1$, Fig. 7). These values are the average of two experiments done on different days.

Discussion

Our results fall into two categories: (a) Measurements of $[Ca^{2+}]_{seq}$ and $[Ca^{2+}]_{cyt}$ in prestalk and prespore cells after perturbing Ca^{2+}; (b) Observations of the effects of Ca^{2+} on pattern formation and

TABLE 1

RELATIVE PERCENTAGES (MEAN ± S.D.) OF NEUTRAL RED-STAINED LENGTHS OF SLUGS DEVELOPED IN VARIOUS CALCIUM ENVIRONMENTS

		(% PRESTALK TENDENCY)		
Parameter	**n**	**Slug anterior**	**Slug posterior**	**Total**
10 mM MES (control)	25	16.6±5.7	0.3±1.3	16.9±6.0
1 mM EGTA + 7 μM Br-A23187	49	6.0±6.2*	0	6.0±6.2*
100 μM $CaCl_2$ + 7 μM Br-A23187	13	27.6±11.8*	13.5±12.6*	41.0±17.1*
7 μM Br-A23187	33	17.2±6.5	3.3±4.9**	20.5±7.9
1 mM EGTA	56	13.5±10.3	0.4±1.6	13.9±10.6
100 μM $CaCl_2$	26	17.9±4.8	0.8±3.0	18.7±5.4

(t-test, *p<0.001; ** p<0.01, in all cases in comparison with the appropriate 'control' value).

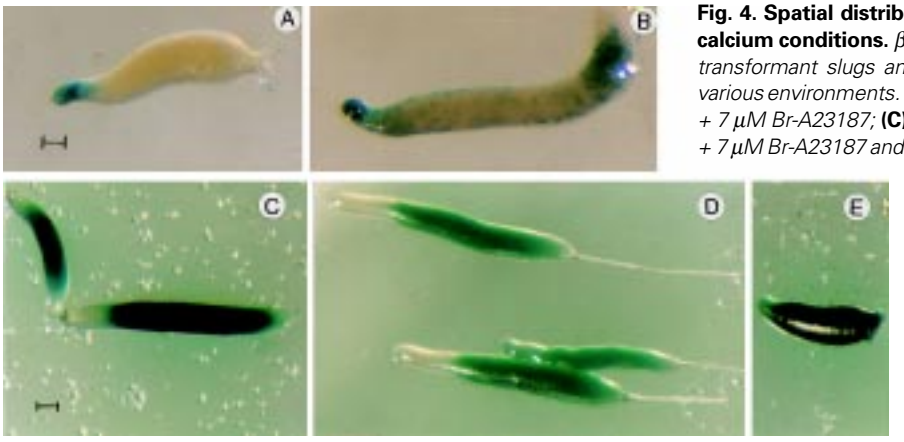

Fig. 4. Spatial distribution of prestalk and prespore cells under altered calcium conditions. *β-galactosidase expression patterns in* **(A,B)** *ecmA-lacZ transformant slugs and* **(C,D,E)** *D19-lacZ transformant slugs developed in various environments.* **(A)** *5 mM Tricine-buffered agar (control);* **(B)** *1 μM $CaCl_2$ + 7 μM Br-A23187;* **(C)** *5 mM Tricine-buffered agar (control);* **(D)** *1 μM $CaCl_2$ + 7 μM Br-A23187 and* **(E)** *1 mM EGTA + 7 μM Br-A23187. Scale bar, 100 μm.*

differentiation. In all experiments, including controls, aggregation and post-aggregative development took place over a comparable time course.

The treatments that caused a change in $[Ca^{2+}]_{cyt}$ did not always cause a change in $[Ca^{2+}]_{seq}$ and vice-versa, implying that EGTA or Br-A23187 might have partially selective effects. The application of Br-A23187 alone resulted in no difference in $[Ca^{2+}]_{seq}$ levels when compared to controls though $[Ca^{2+}]_{cyt}$ levels were lowered (see Fig. 2). This may be on account of Ca^{2+} release into the external medium (Wick *et al.*, 1978); it is known that in the presence of A23187, cells attempt to equilibrate $[Ca^{2+}]_{cyt}$ levels within with those outside (Pressman *et al.*, 1976). In the light of these observations, we assume that where Br-A23187 alone has been used as a control, it tends to partially equilibrate the extracellular Ca^{2+} level with $[Ca^{2+}]_{cyt}$. Observations made by others support this hypothesis (Aeckerle and Malchow, 1989). The important point is that these two pools of intracellular Ca^{2+} showed an increase or decrease in parallel when the treatment involved EGTA or extracellular Ca^{2+} in combination with Br-A23187. Note that in our experiments, Ca^{2+} has been measured in cells that have been teased out of slugs. These cells do not experience the normal intercellular environment; thus it remains a theoretical possibility that the actual values reported here differ from those *in vivo*.

At what stage does Ca^{2+} act? Starved amoebae developing on filter papers placed on 5 mM Tricine buffered agar were transferred at different time points to an environment consisting of 1 μM $CaCl_2$ + 7 μM Br-A23187 or 1 mM EGTA + 7 μM Br-A23187. Transfers made until the late aggregate stage succeeded in lowering - or, respectively, raising - the sfe exactly as in the experiments reported in the present work (the size of the structures formed was not monitored; data not shown). This accords with the fact that the state of determination in *D. discoideum* is reversible until terminal differentiation occurs. To summarize our observations pertaining to $[Ca^{2+}]_{seq}$: there is a spatial gradient of $[Ca^{2+}]_{seq}$ with the level being higher in the anterior (prestalk region); the high Ca^{2+} treatment both raises levels uniformly and steepens the gradient. The low Ca^{2+} treatment lowers levels uniformly and flattens the gradient. A similar spatial gradient is observed for $[Ca^{2+}]_{cyt}$, with a higher $[Ca^{2+}]_{cyt}$ level in the anterior prestalk region than in the posterior prespore region. As with $[Ca^{2+}]_{seq,}$ high Ca^{2+} treatment raises $[Ca^{2+}]_{cyt}$ levels uniformly and steepens the gradient; low Ca^{2+} treatment lowers levels all along the slug and flattens the gradient. The calcium level of prespore cells in slugs developed in a 'high calcium' regime is higher than the calcium level of prestalk cells in control slugs, a feature that we will return to at the end.

The NR staining pattern, the spatial extent of genetically defined prestalk and prespore cells' distribution in *ecmA-lacZ* and *d19-lacZ* transformant slugs, sfe, slug and fruiting body morphologies all show that Ca^{2+} affects differentiation and patterning in *D. discoideum* by promoting the prestalk and stalk pathway, or by inhibiting the prespore or spore pathway or both. Besides affecting cell-type proportions, an increase in Ca^{2+} leads to an increase in slug length and the formation of 'stalky' fruiting bodies; correspondingly, a decrease in Ca^{2+} leads to the formation of shorter than normal slugs and 'spory' fruiting bodies (Figs. 4 A-C, 4 D-F). The reported Ca^{2+}-dependent increase in expression of the cell adhesion molecule gp80 (Kamboj *et al.*, 1990), and the consequent formation of very long slugs, might partially account for these observations. Also, Saito (1979) noted that slug formation was inhibited at high levels of EGTA in the agar and that the fruiting bodies that resulted were smaller than normal. The effects on slug and fruiting body sizes can be explained partially by the fact that

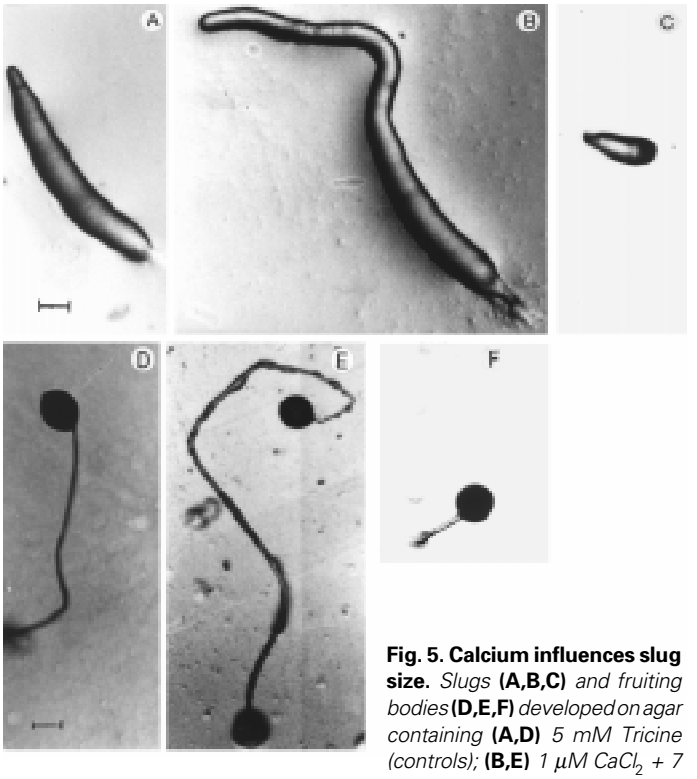

Fig. 5. Calcium influences slug size. *Slugs* **(A,B,C)** *and fruiting bodies* **(D,E,F)** *developed on agar containing* **(A,D)** *5 mM Tricine (controls);* **(B,E)** *1 μM $CaCl_2$ + 7 μM Br-A23187; and* **(C,F)** *1 mM EGTA + 7 μM Br-A23187. Scale bar, 100 μm. So as to emphasize the effect, extremes of the ranges are shown (See Fig. 6). Long slugs often tend to break up into two or more smaller ones.*

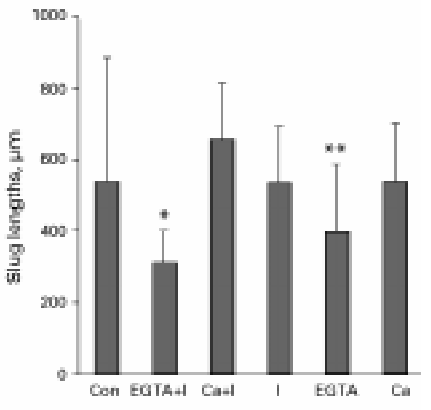

Fig. 6. Slug lengths under altered calcium conditions. *Slug lengths (µm, mean ± SD) of slugs developed on agar containing: 10 mM MES (control, n=25), 1mM EGTA + 7 µM Br-A23187 (n=40), 100 µM $CaCl_2$ + 7 µM Br-A23187 (n=20), 7 µM Br-A23187 (n=25), 1mM EGTA (n=58) and 100 µM $CaCl_2$ (n=25). Asterisks denote values significantly different from the corresponding control (t-test, *p<0.001, **p<0.05).*

under increased Ca^{2+} conditions there is an approximately 1.3-fold decrease in aggregation territory density (not shown) and a 1.6-fold increase in slug volume. It is noteworthy that in *D. discoideum* cells that have a disrupted *countin* gene, there is no detectable secretion of an extracellular '(cell number) counting factor' and aggregations streams do not break up, resulting in huge (up to 2 x 10^5 cells) fruiting bodies (Brock and Gomer, 1999). Interestingly, it appears that the 'stalky' or 'spory' character of the fruiting body is determined by variations in the number of cells allocated to the stalk-plus-basal disc pathway than by changes in the number allocated to the spore pathway: Figs. 5 D-F show that there is very little change in the size of the spore mass under varying Ca^{2+} regimes (the size of an individual spore remains the same in all conditions). Table 2 provides quantitative support to this inference. Unfortunately this point cannot be tested under normal developmental conditions because prestalk and prespore cells can interconvert and restore their relative proportions (Raper, 1940; Sakai, 1973).

Both Abe and Maeda (1991) and Kubohara and Okamoto (1994), using non-physiological (monolayer) conditions, showed that Ca^{2+} was likely to play a role in cell differentiation in *D. discoideum*. This had been reported much earlier as an observation by Maeda (1970) who however did not provide any supporting data. By incubating cells with Ca^{2+} antagonists or blockers to Ca^{2+} entry, Blumberg *et al.* (1989) demonstrated that the cAMP-induced accumulation of prespore mRNA could be blocked. Schaap *et al.* (1996) and Azhar *et al.* (1997) found that the application of DIF, a prestalk inducer, led to an increase in cellular Ca^{2+}, and the latter study made it plausible that the increase was paralleled by an increase in the ratio of prestalk to prespore cells. In contrast to practically all earlier studies, in which cells were in the form of suspensions or monolayers, the present study shows that under conditions of normal development in *D. discoideum*, an increase in cellular Ca^{2+} promotes the prestalk pathway and a decrease in cellular Ca^{2+}, the prespore pathway; always in a relative sense, that is, at the expense of the other pathway. The advantage of our approach is that it enables us to say something about patterning in the context of normal development. One can of course never rule out the possibility that the action of Ca^{2+} is indirect. When we perturb Ca^{2+} levels, we are also interfering with intercellular signaling, and the effect of Ca^{2+} may be mediated by some other chemical (cAMP, for example; see Schaap *et al.*, 1995).

Using DNA probes, Pinter and Gross (1995) monitored gene transcript levels in varying Ca^{2+} environments after incubating amoebae in shaken suspensions for two hours. They concluded that Ca^{2+} plays a role in post-aggregative gene expression in both prespore and prestalk cells but does not have a cell type-specific role. One reason for the difference between the present findings and theirs is that in our experiments the perturbing environment was present throughout development. Also, it may be that the crucial effect of raised or lowered Ca^{2+} levels is on the spatial pattern of transcription and not on cellular mRNA concentrations. Also, intercellular heterogeneity (even in clonal populations grown in the same medium) is known to be a characteristic feature of *D. discoideum* (Saran *et al.*, 1994b; Azhar *et al.*, 1996). Unless accounted for, this fact will invariably obscure the interpretation of quantitative responses measured in large populations under shaken culture conditions. Cubitt *et al.* (1998) expressed a constitutively active form of a human erythrocyte plasma membrane calcium pump in *Dictyostelium* cells. Transformed and wild-type cells both showed similar induction patterns of the prestalk-specific gene *ecmA* and the prespore-specific gene *SP60/cotC* although the level of expression of *ecmA* was reduced about two-fold in the transformants. They also found that the expression of the prestalk marker *ecmB*, whose DIF-induced expression is sensitive to intracellular calcium chelators (Schaap *et al.*, 1996), was affected by the expression of the Ca^{2+} pump; extended treatment of cells with EGTA blocked induction of both prestalk- and prespore-specific genes equally. On the basis of their findings, Cubitt *et al.* (1995) suggested that intracellular Ca^{2+} controls cellular morphogenesis through effects on cell movement and sorting during tip formation. However, their results are equally consistent with the possibility that an increase of intracellular Ca^{2+} is a positive stimulus for stalk cell differentiation.

The absolute $[Ca^{2+}]_{seq}$ and $[Ca^{2+}]_{cyt}$ in the prespore cells of slugs developed in high Ca^{2+} regimes falls within the respective range of $[Ca^{2+}]_{seq}$ and $[Ca^{2+}]_{cyt}$ measured in prestalk cells of control slugs (Fig. 8). The implication is that cells with comparable levels of $[Ca^{2+}]$ can occupy different spatial positions in the slugs. Therefore it cannot be the absolute $[Ca^{2+}]$ that determines cell type-specificity in a cell-autonomous fashion, but rather, the relative differences in calcium between cells (which could perturb intercellular signaling). This reinforces the points that calcium is one of the regulators of cell-type proportion, but not the only one, and that both individual cellular properties and intercellular signaling are important for the determination of cell fate in *D. discoideum* (Atzmony *et al.*, 1997).

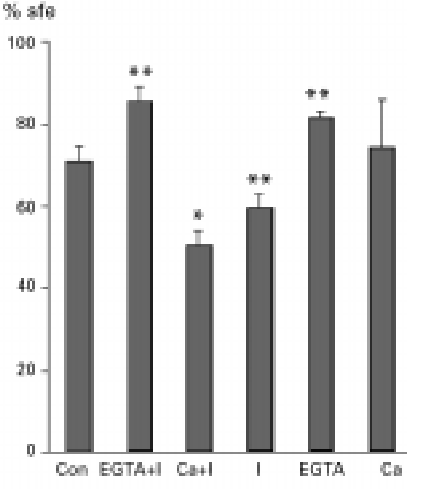

Fig. 7. Percentage spore forming efficiency (sfe). *sfe after development on agar containing: 10 mM MES (control) , 1mM EGTA + 7 µM Br-A23187, 100 µM $CaCl_2$ + 7 µM Br-A23187, 7 µM Br-A23187, 1mM EGTA and 100 µM $CaCl_2$. Asterisks denote values significantly different from control (t-test *p<0.05, **p<0.1).*

A number of questions remain. Chief among them are (a) the interaction between Ca^{2+} and previously hypothesized morphogens in *D. discoideum* such as cAMP and DIF, (b) the route whereby Ca^{2+} acts in order to promote cell type-specific differentiation, (c) whether its action is restricted to a subset of prestalk and/or prespore cell types and (d) the relation between Ca^{2+} and other aspects of early cellular heterogeneity based on nutritional status (Leach *et al.*, 1973) or cell cycle phase (McDonald and Durston, 1984).

Materials and Methods

Growth and development of cells

D. discoideum NC-4H amoebae were grown in association with *Klebsiella aerogenes* and harvested using standard procedures (Tirlapur *et al.*, 1991) except that the buffer was a mixture of potassium phosphates (KK2, pH 6.2). In experiments where calcium levels were quantified using fluorescent dyes, either 5 mM Tricine buffer, pH 7.0 or 10 mM MES buffer, pH 6.2 was used. Amoebae were washed free of bacteria by centrifugation at 500 rpm for 5 min and developed at 22°C in the dark on 2 % KK2 agar or MES agar. All chemicals were of analytical grade and obtained from Difco or Sigma except Fura-2 acetoxymethyl ester (Fura -2/AM) and Pluronic F-127 (Molecular Probes Inc., USA) and dry dimethylsulphoxide (anhydrous DMSO, Aldrich, USA).

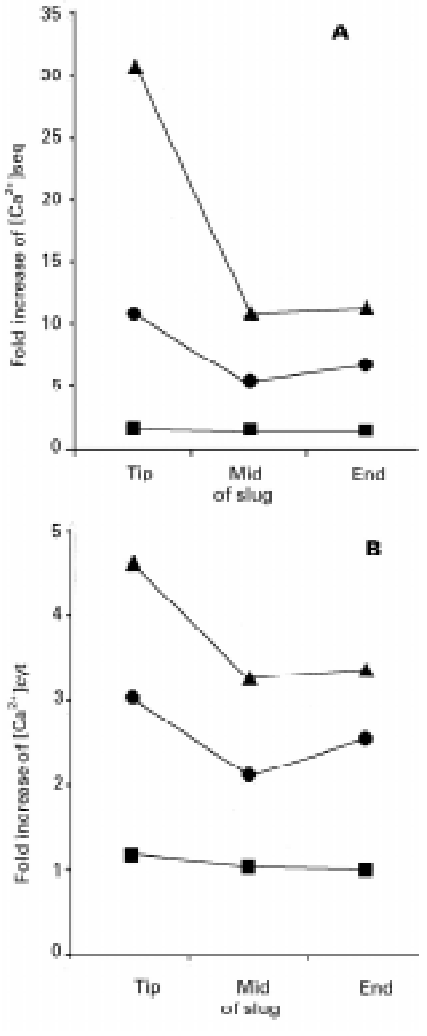

Fig. 8. Ca^{2+} gradients in the slug in different environments. *The fold increase in* **(A)** *$[Ca^{2+}]_{seq}$ (relative to 41.65 μM, which was the lowest value, measured in prespore cells from the posterior end region of slugs developed under EGTA) and* **(B)** *$[Ca^{2+}]_{cyt}$ (relative to 42.39 nM, which was the lowest value, measured in prespore cells from the posterior end region of slugs developed under EGTA + Br-A23187) in the tip, middle and end region of slugs formed after development on agar containing: (●) 10 mM MES (control), (▲) 100 μM $CaCl_2$ + 7 μM Br-A23187 and (■) 1mM EGTA + 7 μM Br-A23187.*

TABLE 2

FRUITING BODY DIMENSIONS (MEAN ± S.D., IN μm) OBTAINED IN DIFFERENT CALCIUM ENVIRONMENTS

Parameter	MES buffer control	7 μM Br-A23187 +1mM EGTA	7 μM Br-A23187 +100 μM $CaCl_2$	7 μM Br-A23187	1 mM EGTA	100 μM $CaCl_2$
n	23	14	15	20	18	13
Sorus diameter	114.3± 11.2	130.4± 25.4	100.9± 12.4	125.4± 22.4	99.8± 12.5	95.3± 13.3
Stalk length	1040.9± 95.7	389.3±* 118.2	1745.6±* 43.6	1348.1± 114.5	621.9±* 37.7	1075.9± 48.0
Basal disc diameter	128.6± 18.7	77.6±* 12.7	279.6±* 62.7	134.3± 20.0	85.3±* 1.3	102.0± 28.0
Mean ratio of stalk length to sorus diameter	9.3± 0.2	2.7±** 0.2	19.1±* 0.3	12.5± 0.4	6.3± 1.2	12.2± 1.8

(t-test, m*p<0.005; **p< 0.05, in all cases in comparison with the appropriate 'control' value).

The *ecmA-lacZ* and *D19-lacZ* transformants carry plasmids containing prestalk- and prespore- specific promoters respectively, fused to a bacterial *lacZ* reporter gene (Dingermann *et al.*, 1989; Williams *et al.*, 1989). They were grown on antibiotic resistant *K. aerogenes* in the presence of 20 μM G418.

Varying cellular calcium

Growth-phase amoebae were washed off agar plates with ice-cold 5 mM Tricine buffer to remove bacteria and spun down twice in the same buffer. $CaCl_2$ and EGTA were added along with Br-A23187 so as to get a final suspension of 1.8×10^7 amoebae/ml. 200 μl of this suspension was pipetted on to 3 cm dishes containing non-nutrient 2 % agar, allowed to settle for 30 min and excess fluid gently decanted; the plates were then incubated at 22°C in the dark. The agar was washed prior to use a number of times in double-distilled de-ionized water together with the de-ionizing resin TMD8; Br-A23187 was added just before pouring. Br-A23187 was maintained as a 1 mM stock solution in DMSO or ethanol. We settled on 7 μM as a suitable concentration for the ionophore after a number of trials with both lower and higher concentrations. The plates used for development were made up with agar containing the same combination of $CaCl_2$ (or EGTA) and Br-A23187 as in the solution that was spread on the surface. In the experiments reported below in which 1 μM $CaCl_2$ was used, the Ca^{2+} level was buffered with the help of EGTA in 5 mM Tricine (made up to pH 7.0 with KOH and supplemented with 3 mM KCl) according to the recipe of Bumann (1986). Buffering of calcium was not attempted when the concentration used was 100 μM. Identical results were obtained when 1 μM or 100 μM Ca^{2+} was used. In some experiments, amoebae were incubated in 0.005 % NR for 15 min following which they were allowed to develop as before on agar made up with the appropriate $CaCl_2$ / EGTA. In all the experiments reported in this paper, we confirmed by trypan blue exclusion that no cell death occurred prior to the terminal stalk cell differentiation.

Patterning in the slug and spore forming efficiency (sfe)

Slugs were disaggregated mechanically by flooding the plates with 5 ml of 10 mM cold EDTA and pipetting repeatedly. *ecmA-lacZ* and *D19-lacZ* transformant slugs were fixed in 4 % glutaraldehyde for 30 min (or in 1% glutaraldehyde in Z buffer for 15 min as in Dingermann *et al.*, 1989) and washed thrice with Z buffer (60 mM Na_2HPO_4/ 40 mM NaH_2PO_4 / 10 mM KCl/ 1 mM $MgSO_4$, pH 7.0). Freshly prepared X-gal staining solution was added directly to the plates, which were incubated at 37°C for 12-24 hrs. In order to calculate the sfe, fruiting bodies were allowed to mature fully for 3 days. Plates were then rinsed with 1 ml cold KK2 buffer , spores washed out, spun down, resuspended and counted in a haemocytometer. It was verified that no spores were left on the plates.

Staining with fluorescent dyes

Based on our previous experience with CTC (Tirlapur *et al*., 1991) and fura-2 (Azhar *et al*., 1995), methods were further standardized after trying out various dye concentrations, incubation times and temperatures. A 0.01 M stock solution of CTC was prepared by dissolving 5.2 mg of CTC in 1 ml anhydrous DMSO. The stock was diluted with MES buffer just before use to get a 200 μM solution, which was used for staining slugs. Incubation with 200 μM CTC for 30-45 min had no toxic side effects as confirmed by the formation of normal fruiting bodies by labeled cells. Stock solutions of fura-2/AM (1 mM) and the non-ionic detergent pluronic F-127 (20 %, 200 μg/μl) were prepared in anhydrous DMSO and diluted with MES buffer just before use to get final concentrations of 90 μM and 0.02 % respectively.

13-16 hrs old migrating slugs were lifted off the agar onto a slide containing a drop of MES buffer containing the same $CaCl_2$/EGTA plus Br-A23187 combination as used for development. Excess buffer was removed from the slide using absorbent tissue and immediately replaced with either 200 μM CTC or 90 μM fura-2/AM along with 0.02 % pluronic F-127. The slugs were incubated with CTC for 30-40 min and with fura-2/AM for 1 hr. Extra dye was then removed with absorbent tissue and the slugs were gently rinsed twice in the appropriate Ca^{2+}-EGTA buffer. Using a dissection needle, slugs were teased open to yield a monolayer of cells. Fluorescence intensities were then measured separately in cells originating from the tip (anterior 5 % by length), middle (25 %-75 %) and end (posterior 5 %) of the slug. We verified that Ca^{2+}-fura-2 fluorescence emanated from the cytoplasm (i.e. the dye was not sequestered).

Microscopy

Amoebae and migrating slugs were observed using a Plan NeoFluar 10X objective and a 30 μm x 30 μm window in a Zeiss Axioscop with phase optics and fitted with epifluorescence attachments. For fluorescence measurements, the system was equipped with a photomultiplier tube (PMT; *PTI* Model 810 Photomultiplier detection system, Photon Technology International, New Jersey). The set-up consists of a high pressure 75 W power arc Xenon lamp that has a high continuous light output over the spectral region 200-900 nm. This feeds a scanning monochromator (*PTI* Model RM-M) containing adjustable slits for controlling brightness and wavelength bandwidth (a 6 nm band pass was selected). Using the monochromator, a single excitation wavelength of 410 nm was chosen for CTC. For the dual excitation wavelength probe fura-2/AM, alternate excitation wavelengths of 340 nm (for bound dye) and 380 nm (for free dye) were selected by slewing the monochromator between the two wavelengths at 4 sec intervals. Outputs at the desired fluorescence emission wavelength were collected by sliding a filter holder containing the appropriate emission filter in the path of the fluorescence light emitted from the sample. Zeiss filter sets used for monitoring Ca^{2+}-CTC and Ca^{2+}-fura-2 fluorescence intensities were # 09 (BP 450-490, FT 510, LP 520) and # 21 (BP 340/10 or 380/10, FT 425, BP 500-530) respectively. The PMT sends a continuous record that can be stored and analyzed using standard data acquisition and processing software. For photography, Ilford 100 ASA or Konica 400 ASA black and white film was used with an automatic shutter exposure.

Quantification of Ca^{2+} levels in single cells

$[Ca^{2+}]_{seq}$ were calculated according to the single wavelength calibration equation of Tsien *et al.* (1985)

$$[Ca^{2+}]_{seq} = K_d\ (F - F_{min})/(F_{max} - F)$$

where F = observed fluorescence intensity; F_{min}, F_{max} = fluorescence intensities determined by exposing the sample to Ca^{2+}-depleted and Ca^{2+}-saturated conditions respectively as described in the calibration procedures below; K_d = 4.4 x 10^{-4} M is the dissociation constant assumed for CTC-Ca^{2+} binding (Caswell and Hutchinson, 1971).

$[Ca^{2+}]_{cyt}$ were calculated as described by Grynkiewicz *et al.* (1985),

$$[Ca^{2+}]_{cyt} = K_d\ (Sf_2 / Sb_2) \times (R - R_{min} / R_{max} - R)$$

where R is the measured fluorescence ratio (F_1/F_2), the ratio of fura-2 fluorescence intensities obtained with excitation at $\lambda_1 = 340$ nm and $\lambda_2 = 380$ nm and proportional to the Ca^{2+}-bound and Ca^{2+}-free dye levels respectively. R_{min}, R_{max} are the respective $F1/F_2$ ratios obtained after exposing the sample to Ca^{2+}-depleted and Ca^{2+}-saturated conditions respectively as described in the calibration procedure below. Sf_2 / Sb_2 = the ratio of fluorescence outputs measured at 380 nm (the excitation peak of the Ca^{2+} free indicator) before and after $CaCl_2$ addition. K_d = 224 nM is the dissociation constant assumed for fura-2-Ca^{2+} binding (Haugland, 1996).

The fluorescence output from these calcium indicators was calibrated using two methods. The first was by releasing the indicator into a surrounding medium of known Ca^{2+} concentration by detergent lysis of cells. The second was by manipulating Ca^{2+} levels inside cells using an ionophore. Using the former approach, 1 x 10^7 amoebae in MES buffer were incubated with 600 μM CTC for 30-45 min. Cells were washed and intracellular CTC released by disrupting cells with 0.01 % (v/v) Triton X-100 into MES buffer containing 5 mM EGTA or 5 mM $CaCl_2$ so as to yield F_{min} and F_{max} respectively. In the second approach, intracellular Ca^{2+} was manipulated by the use of 7 μM Br-A23187 in the presence of 5 mM EGTA or 5 mM $CaCl_2$. $[Ca^{2+}]_{cyt}$ were calibrated by the cell lysis method as described above except that 1 x 10^7 amoebae in MES buffer were incubated with 90 μM fura-2/AM for 1 hr following which cells were washed and incubated for a further 30 min. Calibration was also performed using 0.5 μM fura-2 penta-potassium salt in a cell-free method (Thomas *et al*., 1991); in this method 5 mM EGTA or 5 mM $CaCl_2$ are added to 0.5 μM of the penta-potassium salt of fura-2. This gives R_{min} and R_{max} respectively. Autofluorescence was subtracted from all readings.

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