Development 121, 2291-2301 (1995)
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Patterns of free calcium in multicellular stages of *Dictyostelium* expressing jellyfish apoaequorin

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SUMMARY

To examine the patterns of high free cytosolic calcium or $[Ca^{2+}]_i$ during *Dictyostelium*'s development, we expressed apoaequorin in *D. discoideum*, reconstituted aequorin and observed the resultant patterns of calcium-dependent luminescence. Specific, high calcium zones are seen throughout normal multicellular development and are roughly coincident with those regions that later differentiate into stalk or stalk-like cells. A slug, for example, shows a primary high calcium zone within its front quarter and a secondary one around its tail; while a mound shows such a zone around the periphery of its base. Combined with previous evidence, our findings support the hypothesis that high $[Ca^{2+}]_i$ feeds back to favor the stalk pathway.

We also discovered several high calcium zones within the mound's base that do not coincide with any known prepatterns in *D. discoideum*. These include two, relatively persistent, antipodal strips along the mound's periphery.

These various persistent zones of high calcium are largely made up of frequent, 10 to 30 second long, semi-periodic calcium spikes. Each of these spikes generates a correspondingly short-lived, 200 to 500 μm long, high calcium band which extends along the nearby surface. Similar, but relatively large and infrequent, spikes generate cross bands which extend across migrating slugs and just behind their advancing tips as well as across the peripheries of rotating mounds and midway between their antipodal strips. Moreover, calcium has a doubling time of about a second as various spikes rise. This last observation suggests that the calcium bands seen in *Dictyostelium* may be generated by so-called fast calcium waves.

Key words: *Dictyostelium*, $[Ca^{2+}]_i$, calcium waves, aequorin

INTRODUCTION

Pattern develops through an interaction of localized transcriptional and cytoplasmic changes, the latter of which can be very rapid. These cytoplasmic or physiological changes depend upon the spatiotemporal arrangement of a small number of signalling substances among which $[Ca^{2+}]_i$ is particularly important. However, far less is known of the roles of $[Ca^{2+}]_i$ in pattern formation than of its roles in the physiology of mature organisms. The little that is known of the roles of $[Ca^{2+}]_i$ in pattern formation is largely restricted to very early, more-or-less unicellular stages. In the developing fucoid egg, symmetry is broken through the establishment of a high $[Ca^{2+}]_i$ zone at the future basal or rhizoidal pole. Suppression of this zone through the injection of calcium shuttle buffers, blocks basal rhizoid and apical meristem formation for 1-2 weeks (Speksnijder et al., 1989); moreover, similar findings are emerging for fish eggs (Fluck et al., 1992, 1994).

In *Dictyostelium*, a multicellular organism forms through the chemotactic aggregation of 10^3 to 10^5 individual amoebae towards regions of higher cAMP or adenosine cyclic 3′,5′-(hydrogen phosphate). Basic pattern is then established within the first multicellular stage, or mound, by the time that a tip or organizer region emerges. This pattern includes a large, centralized region of prespore cells surrounded by cells that will differentiate into 3 to 4 subtly different stalk or stalk-like cells. Many of these make up the tip while others lie scattered amidst the prespore region, within a pseudoepithelium that surrounds the whole system and in a rear-guard region opposite the tip (Kay and Insall, 1994).

There have been two schools of thought concerning the roles of $[Ca^{2+}]_i$ in directing *Dictyostelium* cells down the spore or stalk cell pathways. It was long ago proposed that the mechanisms that generate stalk or stalk-like cells both involve and require increases in $[Ca^{2+}]_i$ (Maeda and Maeda, 1973). The evidence included ^{45}Ca autoradiography, which showed that *total* calcium (including sequestered and extracellular calcium) was far higher in prestalk regions of the slug and the early culminant as well as observations that applications of two agents - 100 mM Ca^{2+} or of 7 μM Li^+ - which would be expected to raise $[Ca^{2+}]_i$, greatly favored stalk cell differentiation. However, another group has argued the opposite. Using

chlorotetracycline staining, they have confirmed that total, or actually sequestered, calcium is far higher in the slug's prestalk regions. However, they have reasoned that *low* $[Ca^{2+}]_i$ is associated with high sequestered calcium (Tirlapur et al., 1991).

Resolution of this issue has been hampered by the inability to visualize free calcium patterns within multicellular systems and we now report the first such observations in *Dictyostelium*. Saran et al. (1994a) took a key step towards such imaging by transforming *Dictyostelium* with jellyfish apoaequorin cDNA. Moreover, they mechanically disaggregated transformed slugs in EGTA-bearing solutions and separated the resultant suspensions into prestalk and prespore cells by density gradient centrifugation. The prestalk cells proved to contain twice the free calcium level of the prespore ones. This finding clearly suggested that the cells within the prestalk regions of intact slugs contain high free calcium as well as high sequestered calcium.

We have obtained similar transformants and have then visualized the resultant patterns of luminescence in intact mounds, slugs and culminants with an ultra-low light imaging system (Miller et al., 1994). Our observations show that high $[Ca^{2+}]_i$ does indeed colocalize with high sequestered calcium regions and with prestalk regions and does so in mounds as well as slugs. We have also reviewed considerable evidence that high extracellular cAMP and other treatments that raise $[Ca^{2+}]_i$ favor the stalk pathway and vice versa. So our findings favor the Maedas' hypothesis. We also find that high $[Ca^{2+}]_i$ zones are largely made up of $[Ca^{2+}]_i$ spikes, each of which generates a long, multicellular, transient band of high $[Ca^{2+}]_i$ along the nearby surface of the organism. In moving but not in static organisms, similar spikes generate transient cross bands. Moreover, our observations reveal a complex and unexpected pattern of high $[Ca^{2+}]_i$ within the mound during the stage that pattern is first established.

MATERIALS AND METHODS

Obtaining aequorin-loaded cells

KAx-3 cells were transfected with a vector expressing an ubiquitin/apoaequorin fusion protein from the Act15 promoter using vector Exp4(+) (Dynes et al., 1994). Initially, a *Dictyostelium* aequorin construct was made by amplifying the apoaequorin cDNA, kindly provided by Douglas Prasher (Prasher et al., 1985), adding appropriate cloning sites at the 5′ and 3′ ends (see Fig. 1). To improve its expression in *Dictyostelium*, the nucleotide sequence immediately 5′ to the ATG start codon was changed to the consensus optimal translational start sequence for this system (Firtel and Chapman, 1990). However, this construct provided less than optimal aequorin levels that decreased through development (data not shown). To overcome the apparent instability of apoaequorin in *Dictyostelium*, a ubiquitin-apoaequorin fusion protein was created (Bachmaier and Varshavsky, 1989; Varshavsky, 1992). The gene fusion was created using an ubiquitin PCR product that had a *Hin*dIII site at the C terminus, at the point at which ubiquinated proteins are cleaved. This was fused in-frame with the *Hin*dIII site at codon seven of aequorin. Cleavage of the putative fusion protein would produce an apoaequorin with an N-terminal valine residue in the aequorin cDNA. The ubiquitin/apoaequorin coding region was inserted into the EXP4(+) expression vector. Western blots confirmed that the fusion protein was correctly processed and produced a modified apoaequorin of the correct size (data not shown). High level expression of apoaequorin in *Dictyostelium* did not influence growth or development, consistent with other studies that have shown that injection of high levels of recombinant aequorins is non-perturbing (Shimomura et al., 1990).

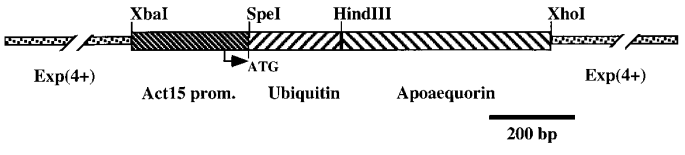

Fig. 1. Restriction map of the ubiquitin/AEQ fusion vector.

The transformed cells were grown in HL-5 media (Sussman, 1987) containing G418 at 40 μg/ml and shaken with 50 ml of cell suspension in 250 ml flasks at 240 revs/minute. One ml of such a suspension at a density of 5×10^5 cells/ml were transferred into a 3.5 cm diameter Petri dish. 10 ml of coelenterazine (2 mg/ml, in methanol) was added and well distributed to give a final coelenterazine concentration of 50 μM. To attain optimal reconstitution of intracellular aequorin, this dish was incubated for 24 hours in the dark. Starvation and development was then begun by plating out washed cells on a 1 mm thick layer of 1% agar bearing 12 mM (KH_2/Na_2H)-PO_4, 0.23 mM $CaCl_2$ and 0.1 mM $MgCl_2$ at pH 6.1 in a 3.5 cm Petri dish. All this was done at room temperature (20-22°C).

Measuring and imaging luminescence

This was done with a system based upon an imaging photon detector (Miller et al., 1994). For the purely temporal measurements illustrated in Fig. 2, we used a 5×/0.16 plan-apochromat Zeiss objective and development was followed with video images obtained every 2.0 minutes with biologically ineffective red light.

However, imaging was done with a Zeiss Fluor10×, 0.5 NA, 2.0 mm working distance objective so as to gather as much light as possible while still seeing an entire organism in one field. With one exception, shown in Fig. 6g-h, the objective was focussed just above the substratum. This system is far from confocal. It blurs but does not discard out-of-focus light, so the light intensities recorded from different regions of an evenly luminescent specimen would be expected to be nearly proportional to region thickness. 'Burnout' experiments - done by slowly killing the organism with a stream of dry air to slowly release calcium and discharge all of the aequorin - showed no apparent variations in net releasable luminescence along slugs or around mounds. Altogether, the relative light intensities from different regions should have been roughly proportional to the 2.5 power of local $[Ca^{2+}]_i$ (Blinks et al., 1982) and nearly the first power of local thickness.

The system records light as a list of individual photon coordinates; so images of the same experiment can be and were reconstructed by accumulating photons over various periods. Except for Fig. 2, the pixels were 20×20 μm. The collection of luminescent light was interrupted for five seconds every 5.0 minutes to allow capture of an image with a video camera via biologically ineffective far red light. When such a video view was from the side, as when it recorded images of a nascent tip, the illumination came in horizontally and sideways to the camera.

RESULTS

$[Ca^{2+}]_i$ rises during aggregation as cells make increasing contact with each other

The net luminescence from a single aggregation field rises 10- to 20-fold between the initiation of development by starvation and the attainment of a tight mound some 8 to 10 hours later (Fig. 2). This rise in luminescence corresponds to a rise in $[Ca^{2+}]_i$ to a level that is three times the resting one of 0.05 μM found in vegetative amoebae (Blinks et al., 1982; Schlatterer et al., 1992). More important may be the dramatic rise in the

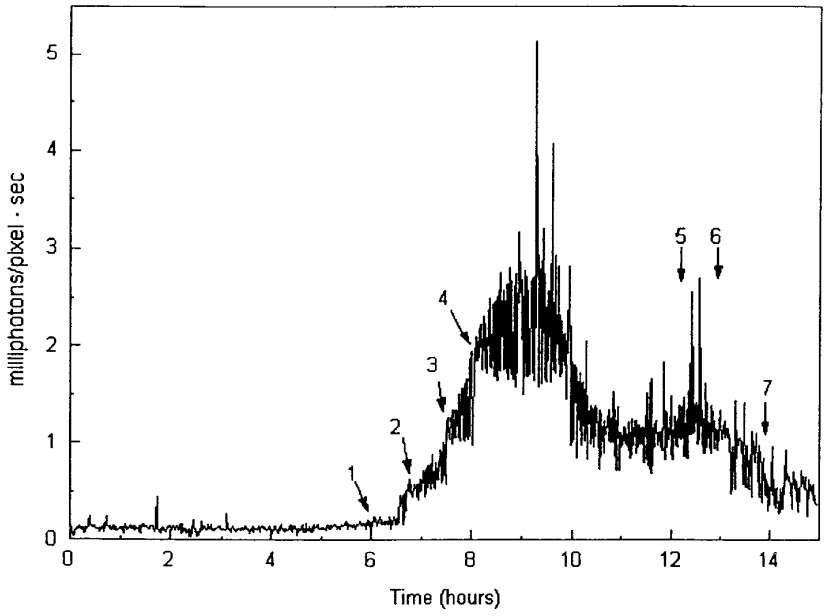

Fig. 2. $[Ca^{2+}]_i$ levels and $[Ca^{2+}]_i$ spiking rise with increasing cell contact. The graph shows the average luminescence from a single *D. discoideum* aggregation field (and thence mound and slug) versus time after starvation. In this experiment, luminescence rose 21 fold from the starting level of 0.1 milliphotons/pixel·second to the first peak. At time 1, the first sign of a multicellular mass was seen in the video image; at times 2-3, increasing streaming was seen; at 4, streaming had largely ceased and a tight aggregate or mound had formed; at 5-6, a secondary peak of luminescence accompanied tip formation; at 7, the slug was falling over and starting to move out of the optical field. Up to point 7, the measured luminescence came from a fixed mass of cells.

frequency and size of the pulses of luminescence and hence of $[Ca^{2+}]_i$ that we see. Both average free calcium and calcium spiking increase concomitantly with increasing cell contact and the first discernible zones of high luminescence clearly coincide with aggregation streams. The luminescence seen before stream formation was too weak to show the weak $[Ca^{2+}]_i$ waves that should accompany cAMP waves. However, these same experiments did show a secondary peak of both time-averaged $[Ca^{2+}]_i$ and of calcium spiking about 3 to 7 hours after the first one and at about the time of first finger formation. In three such experiments, the luminescence within this secondary peak rose about 1.3 fold, 1.7 fold and 3 fold above the plateau recorded between the two peaks.

Regions of high $[Ca^{2+}]_i$ are roughly coincident with regions of future stalk or stalk-like cells

A peripheral ring of high luminescence appears at the base of the early mound and persists until the mound's tip develops some hours later (Figs 3, 4). Burnout experiments showed aequorin to be uniformly distributed within the mounds. Moreover, correction for thickness would increase the relative luminescence from the shallower ring. So one can safely infer that the early ring of high luminescence indicates a corresponding ring of high $[Ca^{2+}]_i$.

The literature indicates that this early ring is a zone destined to form stalk or stalk-like cells as early as the early mound stage. The best evidence for this lies in the expression of the prestalk-specific gene, *ecmA*. Recent observations using a labile-β-gal system (Detterbeck et al., 1994) show a striking localization of *ecmA* expression within a peripheral ring near the base of mounds well before tip formation (H. MacWilliams, personal communication). Similarly Fig. 4A-C in Williams et al. (1989) shows a similar expression pattern for *ecmB* and Fig. 6 in Howard et al. (1992) shows such expression of *PTP1*, another indicator of future stalk or stalk-like cells. In contrast, prespore specific proteins are first expressed within this 'doughnut's' hole as is shown by Figs 4 in Krefft et al. (1984), 2B in Williams et al. (1989) and I-II in Haberstroh and Firtel (1990).

Clear and striking patterns of luminescence are then seen in both migrating slugs (Fig. 5) and in stationary ones prior to culmination (Fig. 6). Again, burnout experiments show a uniform distribution of aequorin during these stages. So, with some correction for specimen thickness, one can safely infer that the observed patterns of luminescence indicate corresponding patterns of $[Ca^{2+}]_i$. In both cases, an anterior zone that roughly coincides with the well-known prestalk region has by far the highest calcium; while a central one that contains most of the prespore cells has the lowest, and a posterior one shows a secondary zone of high calcium. But unlike the anterior zone, which dims at its edges, the rear one brightens there. While the anterior zone presumably dims at its edges because they are thinner, the bright edges of the posterior one indicate a high calcium cap. This high calcium cap may well arise from rearguard cells destined to form stalk-like basal disc cells (Jermyn and Williams, 1991). Compare Fig. 1D and 1H of Jermyn and Williams, which shows secondary *ecmA* localization at a slug's posterior end, with Plate 1 of Takeuchi et al. (1977) which shows exclusion of a prespore antibody there.

Moreover, in one favorable case, we were able to observe free calcium in the maturing fruiting body and to do so over a period of 10 hours. Luminescence was largely restricted to the sorus, presumably because the aequorin had burned up as other regions died. Within the sorus, one sees high calcium zones at both ends (Fig. 7). Once again, *ecmA*, *ecmB* and *rasD* staining shows stalk-like gene expression in the corresponding upper and lower cups of non-spore cells at the upper and lower ends of the sorus. (Jermyn and Williams, 1991, Esch and Firtel, 1991).

Pulses, bands and waves of $[Ca^{2+}]_i$

Much or most of the high calcium seen in various prestalk regions of the mounds (Figs 3, 4), of the migrating slugs (Fig. 5) and of the static slugs (Fig. 6) as well as of the maturing culminant (Fig. 7) consists of 10 to 30 second long spikes. When these could be imaged, they were seen to extend through 200 to 500 μm long bands that were either parallel to a slug's long axis (Figs 5, 6) or parallel to a mound's surface (Fig. 3k).

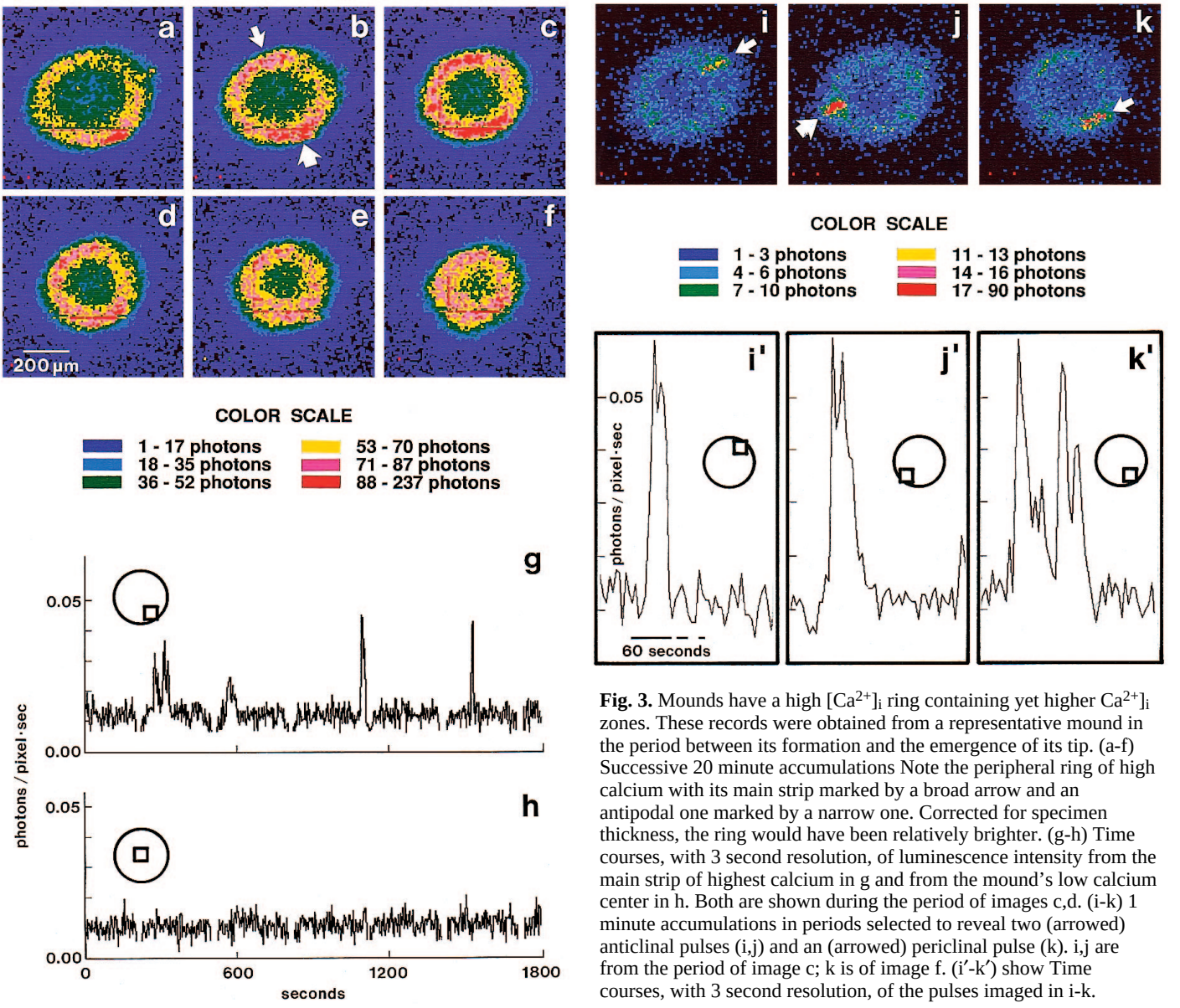

Fig. 3. Mounds have a high $[Ca^{2+}]_i$ ring containing yet higher $Ca^{2+}]_i$ zones. These records were obtained from a representative mound in the period between its formation and the emergence of its tip. (a-f) Successive 20 minute accumulations Note the peripheral ring of high calcium with its main strip marked by a broad arrow and an antipodal one marked by a narrow one. Corrected for specimen thickness, the ring would have been relatively brighter. (g-h) Time courses, with 3 second resolution, of luminescence intensity from the main strip of highest calcium in g and from the mound's low calcium center in h. Both are shown during the period of images c,d. (i-k) 1 minute accumulations in periods selected to reveal two (arrowed) anticlinal pulses (i,j) and an (arrowed) periclinal pulse (k). i,j are from the period of image c; k is of image f. (i′-k′) show Time courses, with 3 second resolution, of the pulses imaged in i-k.

We therefore call them *periclinal* pulses or bands. Taken over a whole organism, such periclinal pulses occurred every 1 to 2 minutes.

It is clear that pulses tend to rise far more quickly than they fall. However, only four pulses were big enough, and thus included enough photons, for their rise rates to be reliably measured. In these favorable cases, the main and steepest part of a pulse showed a rise in luminescence of about 2- to 3-fold per second. Corrected for the fact that aequorin's luminescence rises with about the 2.5 power of calcium (Blinks et al., 1982) this corresponds to a doubling time for calcium of 1.2±0.2 seconds (s.e.m.). Calcium pulses that rise at that rate indicate so-called fast calcium waves that spread at 15 to 30 µm/second at room temperature (Jaffe, 1991, 1993, 1995). However, in only one favorable case, were we able to (barely but clearly) see a pulse's spread. In this one case, a pulse that generated a crossband within a moving slug, the luminescence and hence high calcium was seen to spread out laterally from a starting point in the midventral region towards both sides at about 27 µm/second.

Moreover, inspection of the time courses of groups of periclinal pulses revealed a number of obviously periodic runs with periods of about two minutes or (less frequently) of about one minute (Fig. 8). To check these observations, we subjected the time courses of luminescence from various high calcium regions to autocorrelation analyses. The clearest results were found in the anterior zone of high calcium within a static slug and within the main high calcium strip (described below) within a mound. These showed clear peaks at 2.4 minutes and at 1.7 minutes, respectively, but also showed clear peaks at 7.4 minutes and at 6.9 minutes.

Calcium cross bands recur in fixed places with respect to the substratum

In addition to spikes that fill periclinal bands of free calcium, we discovered a remarkable class of spikes that form

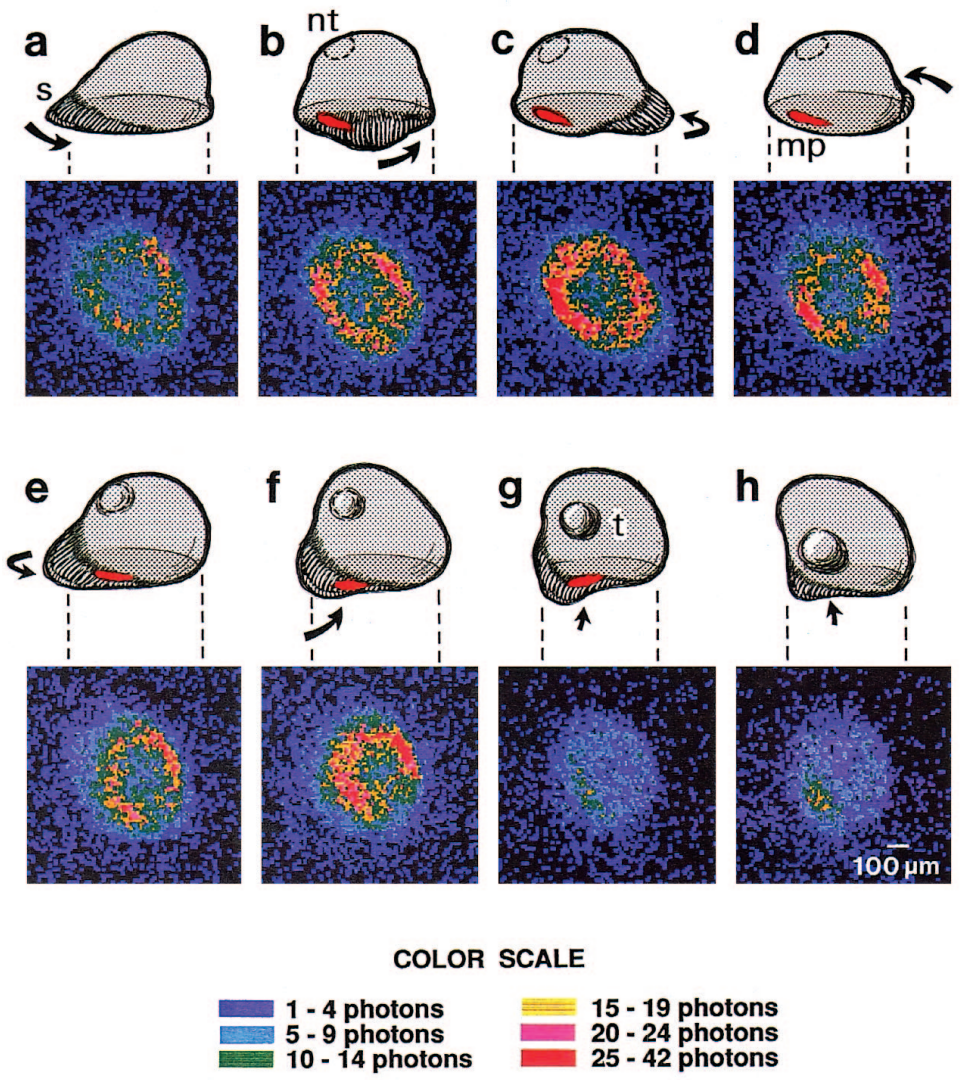

Fig. 4. Mounds also rotate and show a nascent tip plus high $[Ca^{2+}]_i$ in the early tip. These images were obtained from experiment number 28, which fortuitously included several novel features. The pseudocolor images show successive 5 minute accumulations of luminescence while the drawings above them were made from video images. The latter showed a skirt (S) whose slow rotation and subsequent resorption are indicated by black arrows. They also showed a nascent tip (nt), which later became the definitive tip (t). Both tips were above the the main patch of high calcium (mp), *that is diagrammed in red*. Between f and g, the microscope objective was refocussed from its usual plane just above the substratum to the definitive tip. The latter is seen to be a high calcium region that is arrowed in g and h.

anticlinal or cross bands in both slugs and mounds. These 'anticlinal spikes' show the same time course as do periclinal ones but tend to be larger. They cross a moving slug behind its tip (Fig. 5d,g,j,j′,m) and cross a mound's periphery mid way between the high calcium strips discussed below (Fig. 3i,j). Moreover, they show a surprising tendency to recur at the same place with respect to the substratum over relatively long times. Thus the bands that cross the slug shown in Fig. 5c-m recurred three or four times at each of three different positions with respect to the substratum within periods of 6 to 11 minutes. To be more exact, cross bands recurred 4 times at position 1 of Fig. 3m within 11.2 minutes; 3 times at positions 2 in 6.4 minutes; 3-4 times at 3 in 10.2 minutes. The cross bands within the mound shown in Fig. 3 recurred five times at an 8 o'clock position and 3 times at a 2 o'clock position within 1-2 *hours*. To be more exact, the 8 o'clock band illustrated in Fig. 3j-j′ was preceded by a much weaker one in the same place 38 minutes earlier and followed by three somewhat weaker ones in this same spot 2, 5 and 8 minutes later; while the 2 o'clock one shown in Fig. 3i-i′ was preceded by a similar size one 13 minutes earlier and followed by one 49 minutes later. While cross bands are repeatedly seen in moving slugs and in (the always rotating) mounds, they are never seen in static slugs.

High $[Ca^{2+}]_i$ strips and other novel phenomena in mounds

Strips of yet higher luminescence and hence $[Ca^{2+}]_i$ appear within the mound's early ring within an hour after its formation. Both well-studied mounds showed two, antipodal and more or less persistent strips of high calcium of which one was clearly larger and more persistent. One of the two mounds in which early strips were studied (IPD28) was also intermittently observed from the side. These views revealed a small but unmistakeable spot or bump, which only slowly moved over the surface of the mound. We call it a nascent tip since it finally became the definitive one. It may be comparable to the 'red-eye' seen in *Dictyostelium* mounds (Carrin, 1990) or even to the 'biochemical tip' seen in *Polysphondelium* (Paterno and O'Day, 1981; Byrne and Cox, 1987). When the positions of the main early strip and of the nascent tip in IPD28 are compared, one sees that the tip was approximately above the main strip. Moreover, when we focussed up to about the level of the nascent tip, a region of high calcium was recorded and later observed in or near the tip region (Fig. 4g-h).

Since the nascent tip at the top of the mound was above the main strip of luminescence seen at its base and since the mound

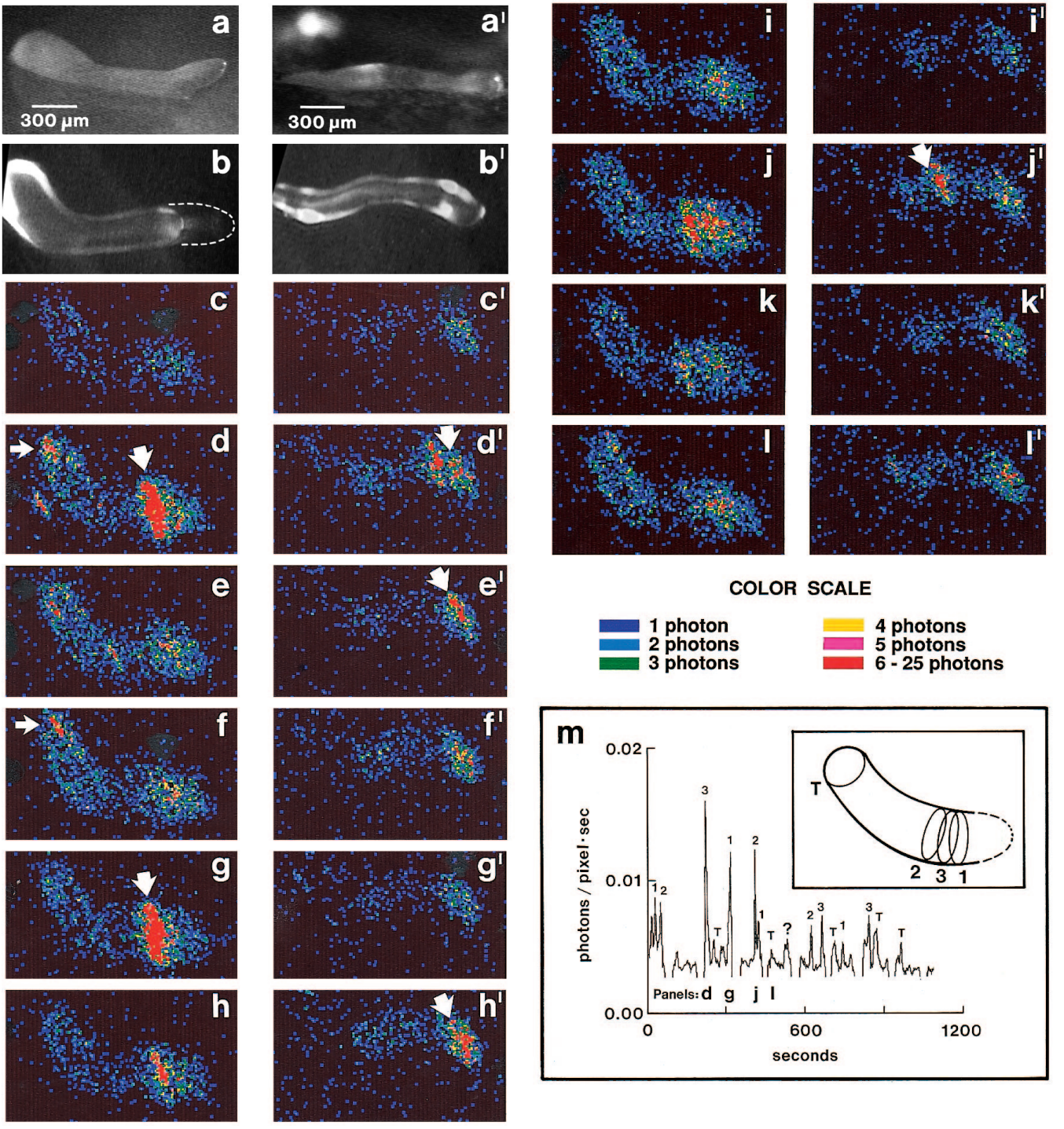

Fig. 5. Migrating slugs also exhibit transient crossbands of high $[Ca^{2+}]_i$. (a-l) A migrating slug with its head raised up above the substratum; (a′-l′) another slug with its head down on it. (a,a′) Video side views; (b,b′) bottom views. The pseudocolor images show successive 30 second accumulations. The broad arrows point to cross band pulses while the narrow ones point to tail pulses. (m) The locations and relative times of the cross band pulses and the tail pulses (T) seen in c-l, as well as ones imaged before and after the 300 second period shown in c-l.

was developing in its normal aerial milieu, it occurred to us that the strange strips of high basal luminescence might be mirages generated by light coming from the nascent tip and focussed downward by reflection from the mound's curved aerial surface. We therefore investigated this possibility by classical ray tracing (Data not shown.) We can report that such reflection artifacts do exist but could not account for the existence of two antipodal strips nor for their high relative intensity. The high calcium strips are real if still enigmatic. To our knowledge, neither they nor the cross bands between them resemble any heretofore described pattern within *Dictyostelium* mounds.

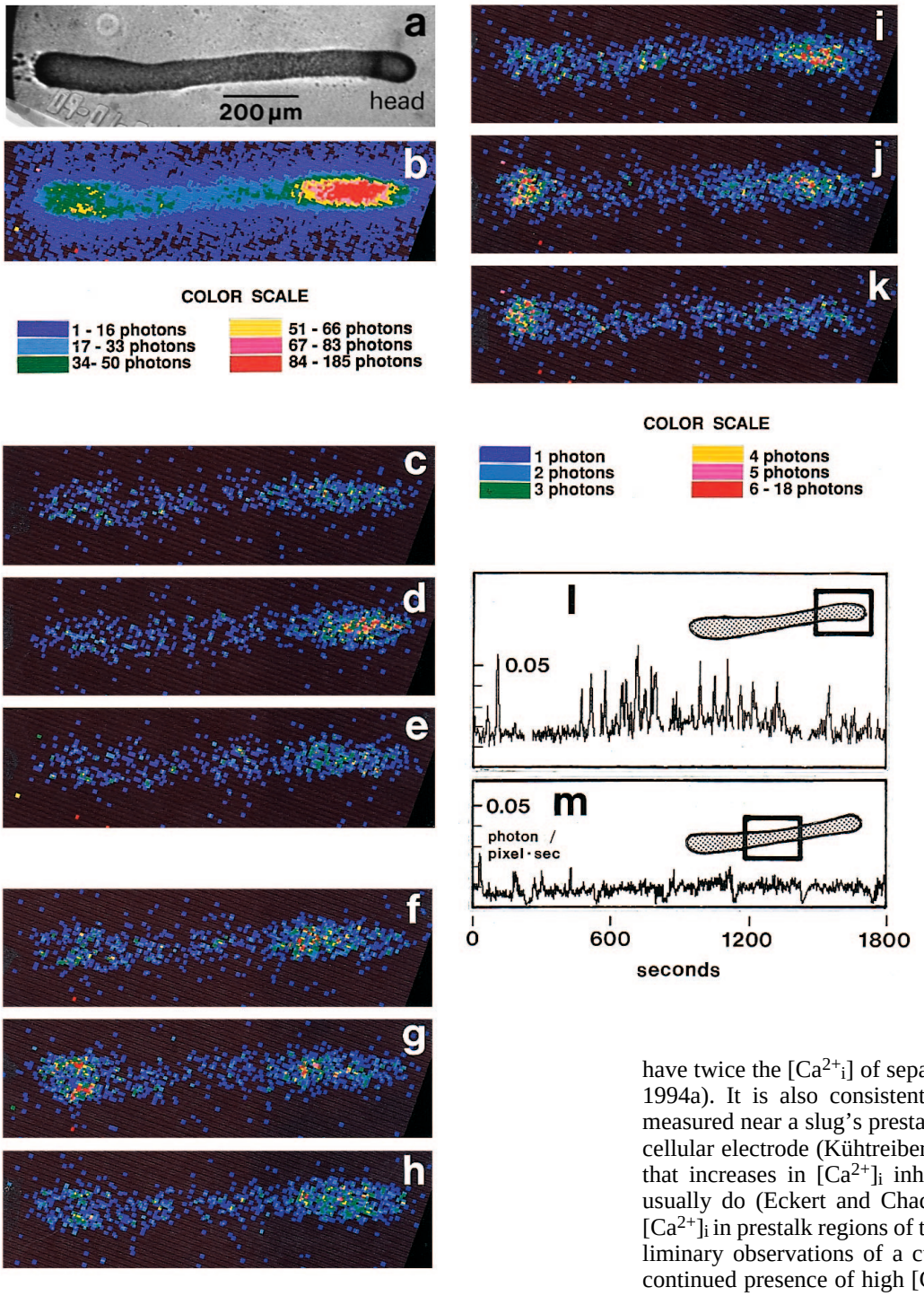

Fig. 6. The prestalk zones of stationary slugs contain high $[Ca^{2+}]_i$ made up mainly of spikes. These records were obtained from a stationary slug about an hour before it began to culminate. (a) Video image; (b) pseudocolor image of luminescent light accumulated for 10 minutes. Note the main, centrally focussed, high calcium zone in the slug's front quarter, which presumably consists of prestalk cells as well as the secondary (and peripherally focussed) one in its rear, which presumably consists largely of cells destined to become stalk-like disc cells. (c-e) Three successive 10 second accumulations that show a head pulse during image b's period; (f-h) Three images that show a peripherally focussed tail pulse; (i-k) images showing a terminally focussed tail pulse. (l,m) detailed temporal records from the (boxed) anterior and central zones in this static slug.

DISCUSSION

In a pioneering paper, Knight et al. (1993) showed images of $[Ca^{2+}]_i$ patterns from the surface of cold-shocked or wounded seedlings. However, our report is the first study of free calcium patterns within a whole, developing multicellular organism. Its most significant finding may be the early and continued appearance of high free calcium zones in the regions destined to form stalk or stalk-like cells as opposed to spore cells. This result is especially clear in the slug (Figs 6, 9) and is consistent with a recent report that prestalk cells that are separated from slugs have twice the $[Ca^{2+}_i]$ of separated prespore cells (Saran et al., 1994a). It is also consistent with the lower influx of Ca^{2+} measured near a slug's prestalk regions with a vibrating extracellular electrode (Kühtreiber and Jaffe, 1990) if one assumes that increases in $[Ca^{2+}]_i$ inhibit Ca^{2+} influx, as indeed they usually do (Eckert and Chad, 1984). Our evidence for high $[Ca^{2+}]_i$ in prestalk regions of the mound is also strong. Our preliminary observations of a culminant are consistent with the continued presence of high $[Ca^{2+}]_i$ in prestalk regions and the death that accompanies final stalk cell differentiation surely involves a rise in $[Ca^{2+}]_i$. Moreover, a recent report shows that freshly starved amoebae fall into high and low sequestered calcium classes and that the former tend to enter prestalk regions of the slug (Saran et al., 1994b). If the colocalization of cytosolic and sequestered calcium begins soon after starvation, this finding would extend the prestalk localization of high $[Ca^{2+}]_i$ all the way back to the earliest, preaggregative stage of *Dictyostelium*'s development. So from first to last, the differentiation of cells down the prestalk pathway may well involve a rise in $[Ca^{2+}]_i$.

Moreover, there is good, older evidence (Maeda and Maeda,

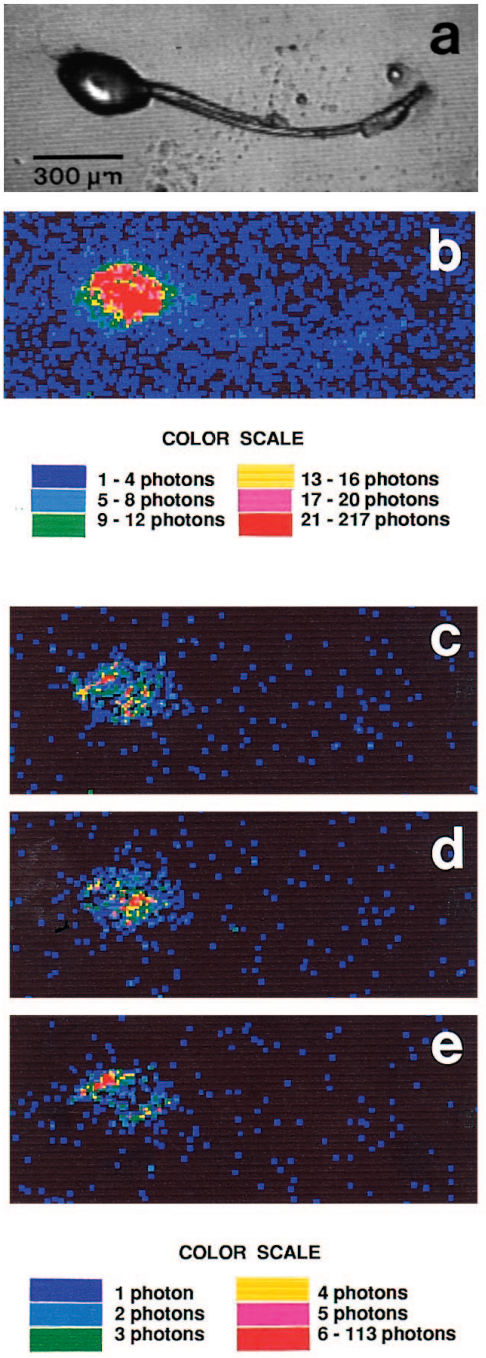

Fig. 7. Sori have high $[Ca^{2+}]_i$ at both ends. These images were obtained from a horizontal fruiting body. (a) Video image; (b) a pseudocolor image of a 10 minute accumulation; (c-e) selected 30 second accumulations over a 2.5 hour period.

1973; Tirlapur et al., 1991) that sequestered calcium is likewise high in the prestalk zones of the slug. This colocalization of cytosolic and sequestered calcium rules out models in which high levels of sequestered calcium are associated with low $[Ca^{2+}]_i$ via more efficient sequestration. Instead, such colocalization suggests a cycle in which high $[Ca^{2+}]_i$ is pumped into organelles which then overload to release it so as to start periodic calcium waves (Jaffe, 1991, 1995).

Table 1. Effects of $[Ca^{2+}]_i$ changes on stalk/spore ratios in *D. discoideum

	%Stalk	%Spore	ref.
A. Effects of raising $[Ca^{2+}]_i$ via:			
100 mM Ca^{2+}	25 → 90	75 → 10	1
10 µM Li^+	25 → 100	75 → 0	1-2
100 µM cAMP	25 → 90	75 → 0	3
low cAMPase mutant	25 → 25 to 100	75 → 75 to 0	4
150 µM TMB8†	40 → 70	50 → 15	5
10 µM thapsigargin†	2 → 80	0?	6
7 µM A23187	25 → 40	75 → 60	7
B. Effects of lowering $[Ca^{2+}]_i$ via:			
7 µM A23187 + 1 mM EGTA	25 → 10	75 → 90	7
low cAMP relay mutant	25 → 7	75 → 93	8
5 mM caffeine	25 → 14	75 → 86	9

Arrows indicate estimated changes in stalk or spore percentages induced by the listed treatment.

*Developing at an air/water interface unless otherwise indicated.

†Submerged monolayer.

References:

1. Maeda, 1970.

2. Sakai, 1973. Lithium was the only cation in the medium. It probably raised $[Ca^{2+}]_i$ in the *Dictyostelium* cells exposed to it since replacement of $[Na^+]_o$ by $[Li^+]_o$ is inferred to raise $[Ca^{2+}]_i$ in frog ventricles (Niedergerke, 1963), in *Chironomus* salivary glands (Rose and Loewenstein, 1971), in squid axons (Baker, 1972), in frog neuromuscular junctions (Crawford, 1975) and in toad skin (Aboulafia et al., 1983) and - to our knowledge - wherever the matter was studied. Moreover, it probably raises $[Ca^{2+}]_i$ by a mechanism - cytosolic accumulation followed by mitochondrial depolarization - which should operate in *Dictyostelium*. However, $[Li^+]_i$ can also mimic $[Ca^{2+}]_i$. Thus its interruption of the polyphosphoinositide cycle (Maslanski et al., 1992) seems to occur by mimicking high $[Ca^{2+}]_i$'s inhibition of a magnesium dependant inositol monophosphatase (Hallcher and Sherman, 1980).

3. Chia, 1975. The added cAMP was separated from the cells by a dialysis membrane.

4. Chia, 1975. These P-4 mutants contained a much reduced amount of cell-bound phosphodiesterase. Under Chia's conditions, 48% of the aggregates gave pure stalk cell bumps; 13%, thick stalk culminants; 39%, normal ones.

5. Abe and Maeda, 1991. A rise in $[Ca^{2+}]_i$ in response to TMB8 was seen via fura-2.

6. Kubohara and Okamoto, 1994. With a DIF-deficient strain. Thapsigargin specifically inhibits the calcium-ATPase in endoplasmic reticula and should raise $[Ca^{2+}]_i$.

7. R. Baskar and V. Nanjundiah, personal communication.

8. Wang and Schaap, 1985. Poor relay indicates inhibited cAMP secretion and would be expected to inhibit the intercellular propagation of fast calcium waves.

9. Wang and Schaap, 1985. 5 mM caffeine cuts aggregation wave speeds by 40% (Siegert and Weijer, 1989) presumably by inhibiting cAMP synthesis and secretion.

Our analysis of the pulse trains from both slugs and mounds did show a substantial degree of periodicity with periods of about 2 and 7 minutes. The 2 minute periods are comparable to ones reported for individual cell velocities in both the tips (Durston and Vork, 1979) and the prespore zones (Siegert and Weijer, 1991) of moving slugs and to one for the rates of calcium influx into a moving slug's tip (Kühtreiber and Jaffe, 1990). They are also comparable to the aggregation wave and individual cell velocity periods seen after cell contact and stream formation occurs (Gross et al., 1977; Siegert and Weijer, 1989). In contrast, the 7 minute component of pulsing is comparable to those in the oscillations of extracellular $[Ca^{2+}]$ observed in suspensions of starved cells soon after they attain

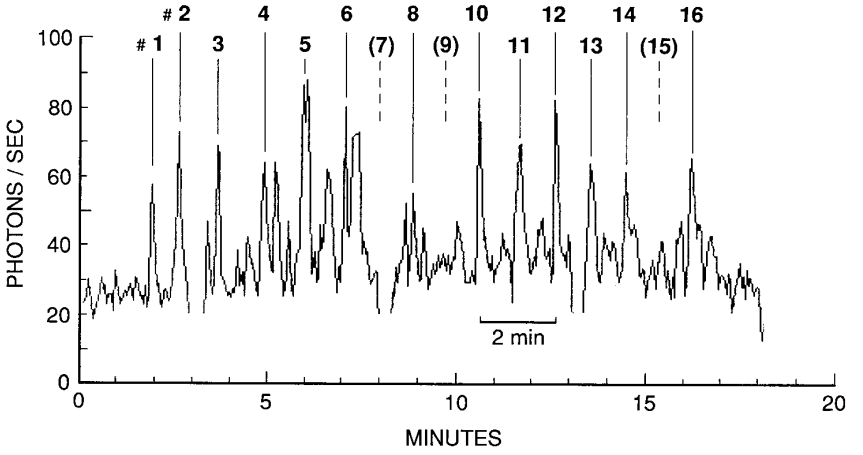

Fig. 8. Calcium spiking shows some clear periodicity. This figure expands part of Fig. 6l, which shows the time course of luminescence from the anterior high calcium zone of a static slug. The eight spikes labelled with even numbers have an average period of 2.0 minutes; while the six marked 1, 3, 5, 11, 13 and (15) can be thought of as an incomplete run of eight others, which have an average period of 1.9 minutes. Together, these 13-14 spikes can be thought of as defining a second harmonic with a period of 1.0 minutes.

the aggregation-competent state (Bumann et al., 1986) as well as the periods of wave initiation during early, prestreaming aggregation (Devreotes, 1982). Taken together, these data suggest that changes in $[Ca^{2+}]_i$ are part of the control loops that generate various fundamental oscillations in *Dictyostelium*.

Cross bands may generate slugs' collars and 'footprints'

The remarkable anticlinal or cross bands of calcium are somehow associated with movement. They occur repeatedly in migrating slugs but are never seen in static ones. Moreover, they occur repeatedly in the mound, a structure known to rotate steadily at 15 to 30°/minute (Clark and Steck, 1979; Siegert and Weijer, 1989; Elliott et al., 1993; Breen and Williams, 1994; our Fig. 4). The shapes and positions of the cross bands seen behind the tips of migrating slugs clearly suggest that they occur in or near the annular grooves or 'collars' that are seen in migrating slugs, and which may serve to improve their grip on the substratum (Breen et al., 1987). Indeed, calcium pulses so generally induce contraction as to suggest that these cross bands induce annular contractions that generate the collars. Perhaps they also induce the crossbands of local secretion seen as proteinaceous 'footprints' in their slime trails (Vardy et al., 1986; Breen et al., 1987; Zhou-Chou et al., 1995). Since similar, if less frequent, anticlinal pulses also appear in mounds, it would be of great interest to look for similar patterns in the extracellular matrix below mounds. In any case, cross bands recur in the same positions over such long times as compared to mound rotation speeds as to suggest that different cells generate successive pulses - perhaps by feedback from the non-rotating extracellular matrix.

Calcium bands may be generated by fast calcium waves

Cross bands generally formed too fast for their formation to be seen with the few photons available. Nor did we ever discern the process of forming the generally weaker periclinal bands. Homogeneous solutions of certain peculiar reagents are well known to exhibit initially synchronous chemical oscillations (Ross et al., 1988). Moreover, there is at least one credible - if still questionable - report of synchronous calcium oscillations within the confines of a single, 100 μm diameter living cell (Carroll et al., 1994). Nevertheless, the vast majority of calcium oscillations within living systems larger than about 30 μm in diameter are known to take the form of propagated calcium waves. The well-established absence of gap junctions in *Dictyostelium* (Johnson et al., 1977; Ingalls et al., 1986) rules out electrically propagated waves. On the other hand, so-called slow (mechanically propagated?) calcium waves move no faster than 3 μm/second (Jaffe, 1995). The propagation of such slow waves through even half the lengths of the observed calcium bands would have taken much longer than the observed spikes. Hence slow waves can also be ruled out as band formers. This leaves so-called fast calcium waves as the most probable mechanism for generating the observed bands.

Moreover, all that we know about calcium bands in *Dictyostelium* is consistent with their generation by fast waves. In those cases where pulse rise rates could be measured, $[Ca^{2+}]_i$ doubled in about a second, a value that is quite characteristic of fast calcium waves (Jaffe, 1993). Moreover, in the one case where a wave could be made out, it moved at about 27 μm/second and thus in the range expected for fast waves. We would therefore propose that the transient, high calcium bands seen in *Dictyostelium* are generated by fast, multicellular calcium waves analogous to those seen to traverse astrocyte networks in rat brain slices (Dani et al., 1992). Finally, if one asks what could transmit such waves between *Dictyostelium* cells, cAMP provides a plausible answer. This is because cAMP released into the minute intercellular spaces characteristic of the prestalk regions of *Dictyostelium* could easily reach the 100 μM levels needed to generate large, fast rising calcium spikes (Schlatterer et al., 1994).

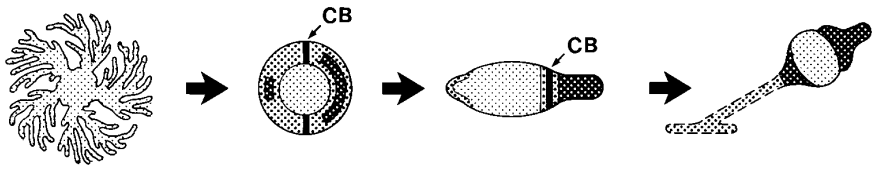

Fig. 9. Cartoon of the high $[Ca^{2+}]_i$ regions during multicellular development. These are made up of brief, periclinal bands except for the anticlinal or cross bands (CB).

High $[Ca^{2+}]_i$ favors the stalk pathway over the spore one

Finally, Table 1 summarizes the effects on the stalk/spore ratio of ten agents or mutations that have known or probable effects on $[Ca^{2+}]_i$ in *Dictyostelium*. One sees that all seven manipulations that should raise $[Ca^{2+}]_i$ markedly increase the stalk/spore ratio; while all three manipulations that should lower $[Ca^{2+}]_i$ markedly lower this ratio. Moreover, a recent, molecular genetic study provides further, strong evidence that the inhibition of increases in $[Ca^{2+}]_i$ favors the prespore pathway. In particular, *cAR4*-null cells, which should be deficient in late, cAMP-driven calcium pulsing, are clearly shifted towards the prespore as opposed to the prestalk pathway: Prestalk gene expression is significantly reduced while prespore-specific markers are both overexpressed and detected in zones normally restricted to prestalk cells. In addition, similar shifts are seen in terminally differentiated fruiting bodies (Louis et al., 1994). Thus there is strong and growing evidence that manipulations that raise $[Ca^{2+}]_i$ increase the stalk cell to spore cell ratio and vice versa. Hence high free calcium is more than an indicator of future stalk differentiation. It seems to be an early and essential part of the stalk differentiation cycle.

Some of this work forms part of G. F.'s Diplomarbeit at Universität Konstanz. J. Kunkel provided critical help with the autocorrelation analysis. The work was supported by National Institutes of Health grant GM37830 to R. A. F., National Science Foundation grants DCB-9103569 to L. F. J. and DIR-9211855 to A. L. M. and L. F. J., and an MBL Bernard Davis Fellowship to G. F.

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(Accepted 19 April 1995)