Organization of early development by calcium patterns

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Summary

This survey focuses on early or primitive developmental phenomena for which the location of a steady high calcium region or the direction of a calcium wave is critical and calcium is more than a trigger. It starts with the long studied roles of calcium in fucoid eggs and in *Dictyostelium* and progresses to newer work on high calcium regions in medaka fish, zebrafish, and *Drosophila* eggs. It then proposes that propagated, *ultraslow* developmental waves in six diverse systems indicate a new and important class of calcium waves. These include the morphogenetic furrow in *Drosophila* eye discs, floret formation in sunflowers, DNA replication waves in protozoan macronuclei, growth-cone like waves in hippocampal neurons, and two others. It then considers the possible organizing roles of *slow* calcium waves. Here, it emphasizes surface contractile waves during primary neural induction and elsewhere as well as the possibility of cellular peristalsis. Finally, it reviews the organizing roles of *fast* calcium waves in ascidian eggs. *BioEssays* 21:657–667, 1999.

Introduction

This is a time of massive advance in the molecular analysis of pattern formation; however, less effort has been devoted to its integrative and biophysical aspects. Here I review progress in understanding the developmental roles of one major player in adult physiology, namely cytosolic calcium. Moreover, I focus on phenomena that are truly organized by spatiotemporal calcium patterns.

In so focusing, I start with organization by relatively steady high calcium regions, move to organization by ultraslow calcium waves, and thence to organization by progressively faster kinds of calcium waves[1] (Fig. 1). I do this since progressively faster waves seem to play progressively lesser roles as organizers as distinct from triggers.

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High calcium regions in fucoid eggs

I start with such eggs since evidence for their organization by a high calcium region was obtained as early as 1975 and because they remain a valuable system for such analysis.

Newly fertilized fucoid eggs are practically apolar. A 1954 demonstration of this is the still remarkable fact that illumination of fucoid zygotes with polarized light induces half of them to develop as twin forms, which generate a rhizoid from two opposite poles rather than the usual one.[2] This indicates that various environmental vectors act by generating a new axis rather than by rotating some invisible, preformed one. Further, compelling evidence that fucoid zygotes are essentially apolar has been recently reviewed.[3]

Normally, two cell embryos have a grossly differentiated rhizoid cell at one end and a less differentiated thallus cell at the other. The zygotes are practically self-organizing; however, the region of rhizoid formation can be easily localized by many environmental vectors. Under natural conditions in the intertidal zone, the dominant vectors are probably unilateral light, powerful influences from various adult plants, as well as water flow.[4] An early study with ^{45}Ca[5] revealed calcium fluxes through photopolarizing eggs at an early stage and led to the hypothesis that the early feedback loops which

Figure 1. Main classes of natural calcium waves based upon speeds at room temperature. Fast waves through active cells refer to all such waves in this speed class except for the slightly slower ones that activate eggs. Fast waves are reaction-diffusion waves propagated by the short range diffusion of calcium ions between relay points in the ER or endoplasmic reticulum. Slow waves are a second, coherent class of calcium waves, which seem to be stretch propagated; whereas ultrafast calcium waves refer to those electrically propagated waves or action potentials that are primarily dependent upon an influx of calcium rather than sodium ions. Ultraslow waves refer to the developmental ones listed in Table 1. (From Ref. 1.)

localize rhizoid formation include a zone of high subsurface calcium.

The accumulating evidence for this model includes the ^{45}Ca flux study, a demonstration that injections of shuttle buffers (that are expected to even out calcium levels) are seen to block all easily visible signs of localization for 10 days or more[6,7] (Fig. 2), a recent one of the effects of external calcium levels and of calcium channel blockers during photopolarization[8] and much else. Those signs of localization which were suppressed by shuttle buffer injection included initial outgrowth of the rhizoid as well as the withdrawal of organelles from the forming rhizoid's tip. Yet, no effort has yet been made to observe the effects of such buffering on earlier and subtler processes such as local actin filament accumulation and polarized secretion; moreover until recently—repeated efforts to visualize high calcium at the rhizoidal pole had only succeeded in showing such localization at a relatively late, if still reversible, stage, well after outgrowth begins. Recently, however, Pu and Robinson have finally succeeded in visualizing high, subsurface calcium at the future rhizoid pole, at an early stage of photopolarization and long before outgrowth starts (See Fig. 3A from Ref. 9).

Beyond the need for a high calcium zone at the rhizoidal pole, the nature of early localization loops in fucoid eggs remains a subject of lively consideration. How might calcium induce its own localized increase? An early model based upon self-electrophoresis no longer seems viable. Moreover, purported evidence for the related idea of plasmalemmal calcium channel aggregation does not withstand critical examination.[10] However, the early idea that calcium induces its own increase by inducing the secretion of vesicles which bear calcium channels does remain quite plausible.

Here I would propose an additional mechanism which I will call "*soft wall feedback.*" Early calcium zones induce secretions which locally soften the wall and allow turgor pressure to locally expand the wall and thus open stretch-sensitive, calcium channels in the plasma membrane. According to this model, these would be the channels which render fucoid eggs polarizable by osmotic pressure gradients of less than 5%[11]; lead to multicellular but apparently apolar embryos in grossly hyperosmotic media[12]; induce electrogenic ion pulses in response to small osmotic pressure reductions[13] and underlie the responses to stretch seen in cell-attached patches on fucoid protoplasts.[14] This model could also account for the tendency of eggs to form rhizoids towards those from a nearby egg (the cis group effect) and the tendency of rhizoids to form downstream in response to ultralow (0.1–1 µm/second) flow rates.[4] I would also suggest that these wall-softening secretions may include expansions—those potent, reversible, proteinaceous, but nonenzymatic softeners of plant cell walls that were recently discovered by Cosgrove.[15]

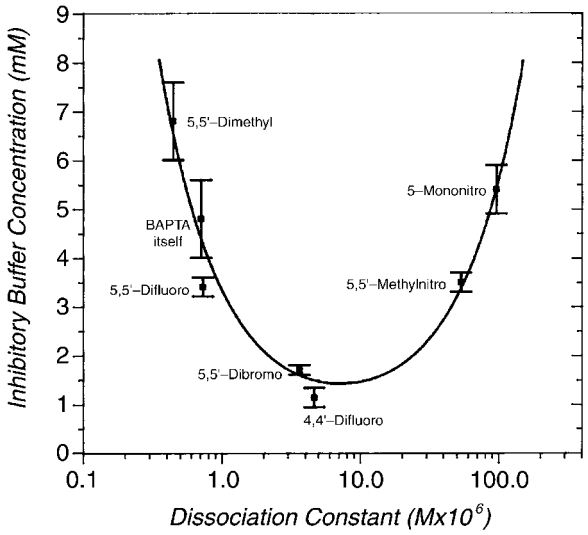

Figure 2. Critical calcium buffer concentrations found to freeze the development of fucoid zygotes for days to weeks. Final cytomatrix concentration is plotted against buffer dissociation constant for seven different BAPTA-type calcium buffers. The curve is a theoretical one for buffers, which shuttle back and forth between a high calcium zone, where they swiftly bind calcium, and a low calcium one, where they swiftly release it. In this way they are thought to facilitate diffusion between a source, here believed to lie just below the plasma membrane in the nascent growth region, and a sink, here believed to lie just above the subsurface ER. Overly weak buffers (like 5-mononitro BAPTA) take up calcium from the source ineffectively; overly strong ones (like 5,5′ dimethyl BAPTA) release calcium to the sink ineffectively; whereas near optimum strength ones (like 4,4′-difluoro BAPTA) half dissociate at the pCa found midway between the calcium source and the calcium sink.[6] Remarkably similar data has been reported for the inhibition of germinal vesicle breakdown in *Xenopus* oocytes.[7]

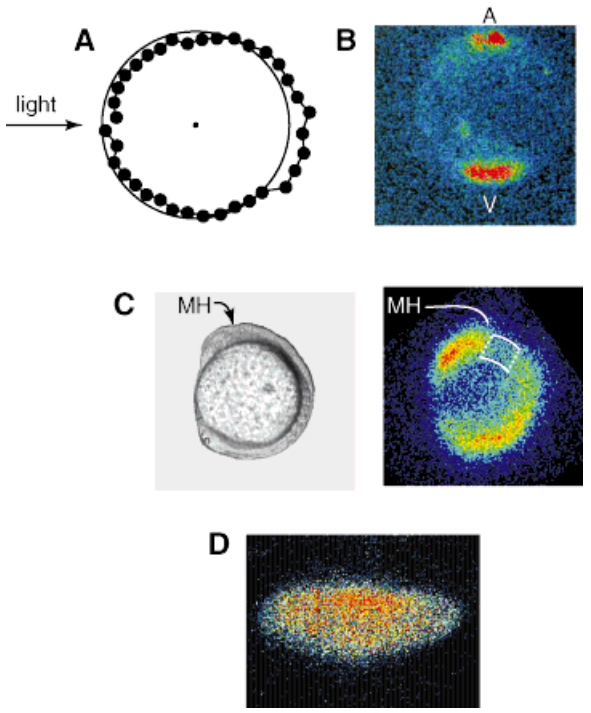

Figure 3. Some calcium gradients during early pattern formation. A: Polar coordinate plot showing the cortical calcium level in fucoid zygotes 4 hours before photolocalized outgrowth starts. Outgrowth starts in the high calcium zone that formed away from the light. The circle indicates the uniform, base calcium level before the application of localizing light. Via ratios of Calcium Crimson to Rhodamine B dextrans. See text and reference 9. B: High calcium zones at the animal and vegetal poles of medaka fish eggs from 4 to 6 hours after fertilization. Comparable images were seen from soon after fertilization through gastrulation, a day later. Pseudocolor via injected aequorin. See text and reference 31. C: A low calcium zone co-localizes with the (outlined) future hindbrain region of the zebrafish embryo in the 6-somite stage. Note the sharp calcium gradient at the midbrain/hindbrain transition (MH). Comparable images were seen from the 3-somite to the 14-somite stage. Pseudocolor via injected aequorin. See text and reference 34. D: High calcium region in the upper, dorsal side of a wild type *Drosophila* embryo during cellularization. Pseudocolor via injected aequorin. See text and Reference 38.

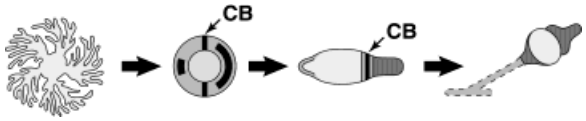

Figure 4. Cartoon of the high intracellular calcium regions during the multicellular development of *Dictyostelium*. These are made up of brief, tangential bands except for the cross bands (CB) and the peripheral ring of steady, somewhat raised calcium in the mound. Via strains transfected with apoaequorin. (From Refs. 19, 20.)

Other interesting fucoid localization mechanisms involve the localization of actin filaments at the rhizoidal pole. Such f-actin localization is first detectable long after unilateral light (or other vectors) can polarize eggs but well before outgrowth starts. Moreover, it is somehow needed to localize poly(A)$^+$ RNAs at the antirhizoid or thallus pole—again well before outgrowth starts.[16,10] Here I would propose that actin filament growth is started by the high, subsurface calcium zone and is driven by g-actin addition at the outer, barbed ends of these filaments. I do this on the basis of what seem to be similar actin dynamics in growing axons[17] and elsewhere. In such ways, the ever more irreversible localization of rhizoid cells may be established through mechanisms dependent upon calcium localization.

High calcium regions in *D. discoideum*

Maeda's model

I next consider this cellular slime mold since as early as 1973, the Maeda's advanced the idea that its development is organized by high calcium within prestalk regions and because it remains a valuable multicellular system for such analysis.[18]

Subsequent observations of *D. discoideum* strains that were transfected with aequorins (or really apoaequorins) revealed a clear association of high cytosolic calcium in prestalk as opposed to prespore regions from the earliest multicellular stages through the end of development (Fig. 4). Moreover, a wide variety of manipulations which should have raised cytosolic calcium favor stalk as opposed to spore cell formation and visa versa.[19,20] As one striking example of such manipulations, as long ago as 1975, a variant with reduced, extracellular cAMP phosphodiesterase was found to form numerous fruiting bodies—called "stalk cell bumps, which consist of a sphere of stalk cells with no spores at all."[21] Chia's intensive analysis demonstrated that the mechanism which so greatly favored stalk cell formation was mediated by modest increases in extracellular cAMP and such increases are now well known to raise cytosolic calcium. As a more recent example, the fraction of stalk cells formed in a monolayer was increased from 2% to 80% by adding 10 mM thapsigargin—a treatment well known to raise cytosolic calcium.[22] As yet another example, development in a 10 mM solution of a low calcium buffer, tetrapotassium BAPTA was seen to radically *decrease* the ratio of stalk to spore cells (Matsuyama and Jaffe, unpublished). This "sporogenous" medium was thought to act by blocking the massive calcium uptake that normally accompanies aggregation. Not only would the BAPTA have reduced the calcium concentration

gradient that drives in calcium but the 40 mM potassium should have substantially depolarized the cells' plasma membranes and thereby much reduced the electrical force that also drives in calcium. One can conclude that high cytosolic calcium is a needed part of those cycles which forward stalk cell as opposed to spore cell development in *Dictyostelium* and this raises the question of what starts and limits the inferred, stalkifying, high calcium cycles.

Calcium waves in Dictyostelium

In addition to high steady calcium, the very highest calcium regions seen with aequorin exhibited large, semiperiodic, 10 to 30 second long calcium pulses each of which generated a 200 to 500 μm long, multicellular band of high calcium (Fig. 4). These data also strongly suggested that each pulse generated a band by spreading as a fast calcium wave.

It was therefore suggested that calcium pulses are restricted to prestalk regions and fail to spread to prespore ones because of the larger intercellular spaces found in the latter. The idea is that intercellular wave transmission is effected by secreted cAMP and that this is diluted to ineffective levels by the large intercellular spaces seen in prespore regions.[20] In time, this fast block to wave spread would be reinforced by the low levels of cAMP receptors 2 and 4 in prespore cells.[23]

Moreover, periodic, often spiral, *slow* (≈1.5 μm/second), dark field waves are seen to traverse *D. discoideum* during its mound and slug stages.[24] They are thought by their discoverers to somehow direct the movement of prestalk cells towards the organizer at the organism's tip. Moreover, they are thought to be propagated between cells by cAMP since they seem to develop continuously from aggregation waves, which are known to be propagated in this way. Nevertheless, it has been proposed that they are similar to other slow calcium waves and are likewise stretch propagated.[20]

First because their observed speed of about 1.5 μm/second at room temperature is within the range of other slow calcium waves (Fig. 1). Second because 1.5 μm/second fits a stretch propagation model if one assumes that waves of cell rounding underlie the observed dark field waves: *D. discoideum* cells round up or ''cringe'' about 10 seconds after stimulation by cAMP. If stretching also induces cringing after 10 seconds, then a stretch propagated wave would move about one cell length in 10 seconds. If one takes 15 microns as the cell length along the direction of movement within aggregates one gets 1.5 μm/second. Third, because they often spread along long, multi-armed spirals and thus along (slightly curved) lines within a three dimensional mass of cells. The inherently directional process of stretch propagation offers an obvious explanation of near linear spread; whereas diffusional propagation does not. Fourth, because the periodic, multicellular, calcium bands seen in these same stages seem to be generated by fast (≈30 μm/sec) waves. How could two kinds of waves with essentially the same propagation mechanism but greatly different speeds coexist in the same system?

I feel that this issue calls for experiments involving appropriate mechanical intervention and measurement.

Rapid patterning of cell masses with restricted gas exchange

Within minutes after confinement in a tube[25] or between two plates,[26] a population of *D. discoideum* cells forms an outer, 100 μm wide zone, near the air, which starts down the stalk path plus an inner zone, which starts down the spore path. This fast formed outer zone resembles the one that forms more slowly during natural mound formation. There already seems to be a consensus that attributes such rapid differentiation to hypoxia within the inner, prespore zone. One reason is the large increase in the prestalk fraction effected by small increases in oxygen above the natural level around unconfined cells.[27] Another is the sharp increase in outer zone width when the oxygen level is raised around confined cells.[27] A third are calculations showing that *if* respiration continued unabated after confinement, that oxygen would swiftly fall to zero about 50 μm in from the surface.[20]

How could hypoxia possibly *reduce* the level of cytosolic calcium within the inner, prespore zone? I have proposed that *Dictyostelium* is a so-called hypoxia tolerant organism whose cells react to hypoxia by sharply reducing their respiratory rate and thus avoid anoxia in the inner zone. According to this model, inner zone cells would survive hypoxia by throttling energy turnover via responses (discussed by Hochachka et al.[28]) that would include closure of calcium channels to reduce $[Ca^{2+}]_i$.

The sharp boundary between these zones implies some highly nonlinear and interactive process and new, unpublished work of Yasuji Sawada et al. has confirmed this. By confining *D. discoideum* cell masses confined between plates, one of which is made of porous glass, they have induced remarkable (they would say Turing-like) patterns of equally spaced prestalk spots rather than a simple outer annulus of prestalk cells. Moreover, by using an array of extracellular platinum electrodes applied to cells that are confined by *non*porous glass, they have also revealed that the inner, prespore cells have extracellular voltages, which are up to 150 or even 200 millivolts (!) higher than the outer, prestalk ones. I would suggest that these observations may be understood if low calcium *D. dictyostelium* cells have the very high (up to 400 millivolt), membrane potentials that are characteristic of unexcited fresh water or terrestrial fungi and higher plants and that are generated by electrogenic proton pumps (or proton ATPases as the biochemists say); whereas high calcium cells have their membrane potentials reduced to about the potassium equilibrium level of about 70 millivolts. Indeed, to some extent such voltage patterns have been directly measured in *D. discoideum* itself.[29]

Moreover, the 100 to 200 millivolt standing voltage differences seem to be maintained along extracellular distances of the order of 30 microns, which would correspond to steady voltage gradients or fields of the order of 5,000 mV/mm. There is good evidence that natural organismal fields of only 20 to 40 mV/mm help to organize early development in amphibians and birds.[30] This then suggests that self-electrophoresis (ultimately generated by steady calcium differences) is an important mechanism in swiftly sharpening the early differentiation of prestalk from prespore cell regions.

By self-electrophoresis I refer to the transport of integral proteins along the plasma membranes and of various macromolecules through the extracellular matrix of *Dictyostelium* by natural, transorganismal voltage gradients. Such transport through the extracellular matrix would be comparable to gel electrophoresis in vitro. Recent discussions of such mechanisms can be found in References 30 and 30a.

High calcium regions as organizers of early animal development

Interesting initial studies of this have been done on medaka fish eggs, on zebrafish eggs, and on *Drosophila* eggs (Fig. 3).

1. Aequorin-injected medaka zygotes develop into normal fish and show striking, steady zones of high calcium at their vegetal poles. These appear as soon as the fertilization wave gets there and persists for about a day and thus up to the beginning of gastrulation. High calcium zones also appear at the animal pole but not until about half an hour after fertilization. These oscillate three to four times during the first six hours before disappearing and probably represent the slow calcium waves that accompany the first three to four cell divisions[31] (Fig. 3B).

Moreover, injections of shuttle buffers that are comparable—in dissociation constant and concentration to those that suspend the development of fucoid eggs inhibit the formation of the high calcium zones in medaka eggs *and* concommitantly inhibit pattern formation in these eggs.[32] Thus they inhibit the movements that form the blastodisc at the animal pole and the movements of oil droplets towards the vegetal pole, as well as inhibiting cytokinesis and obvious embryonic axis formation This indicates that the high calcium zones seen in medaka eggs are likewise needed to organize developmental pattern. One could easily imagine that these essential high calcium zones act via calmodulins to drive microfilament and microtubule-dependent transport as well as the action of the microtubule organizing center that forms at the animal pole. Indeed, shuttle buffer injection has been seen to disrupt the array of microtubules that converges on this center.[33]

2. Aequorin-injected zebrafish eggs likewise develop normally and display complex calcium patterns. The early ones are dominated by cell division so their discussion is beyond the scope of this article. Later ones, however, include a sharp boundary between high Ca^{2+} in the presumptive forebrain and midbrain vs. low Ca^{2+} in the presumptive hindbrain. This calcium boundary first appears a bit before 11 hours of development and thus the 3-somite stage and remains until about 20 hours and thus the 22-somite stage (Fig. 3C). The midbrain/hindbrain boundary region is known to be an organizer of brain pattern, which suggests that this calcium boundary is such an organizer. Moreover, a recent article[34a] reports a rhythmic series of intense calcium waves that circumnavigate zebrafish embryos during gastrulation as well as segmentation and arise every 5–10 minutes. These remarkable waves travel at 4–5 microns/per second at 28°C and thus are clearly in the slow (mechanically propagated?) range. However, they were only reported in dechorionated embryos and were not detected in the chorionated and minimally disturbed embryos studied by Creton et al.[34] This suggests that they are injury waves which do not normally accompany development.

3. Work in progress on dorsoventral pattern formation in Drosophila embryos likewise indicates a role for high calcium regions. This work was encouraged by a 1986 analysis of the data on the ventral activation process in Drosophila zygotes.[35] It suggested that this process depended upon a high calcium region on the ventral side—one which in more modern terms would allow the dorsal protein to enter ventral nuclei by activating dorsal/cactus protein complexes. This model could explain the still puzzling fact that transfer of cytoplasm from *anywhere* in the eggs of wild type flies into the eggs of *Toll*$^-$ flies induced a locally ventralized region.[36] It would do so on the theory that the transfer process exposed the cytoplasm to high calcium, which is normally absent in *Toll*$^-$ flies. It would have done so by exposing this cytoplasm to the high calcium perivitelline gel during transfer.

Subsequent work on appropriate *Drosophila* cell lines shows that an artificial increase of cytoplasmic calcium (via ionomycin treatment) does indeed destabilize *Drosophila* cactus protein and dephosphorylate the dorsal protein—processes that would be expected to allow nuclear entry of the dorsal transcription factor.[37] In further pursuit of this model, Robbert Créton has injected *Drosophila* zygotes with aequorins and found that they too develop normally after such injections. During the syncitial blastoderm stage, when ventral activation occurs, one does see a weak if reasonably clear high calcium region on the ventral side.

Then, during the subsequent stage of cell formation, one sees a *reversal* of the dorsoventral calcium pattern with a stronger and unmistakable high calcium region now appearing on the dorsal side[38] (Fig. 3D). This period coincides with establishment of the dorsal region by a process that includes action by dpp proteins that are secreted into the dorsal part of the perivitelline gel and then act back on dpp receptors there. Moreover, our preliminary work shows that an artificial increase in ventrolateral calcium during cell formation induces

TABLE 1. Some Propagated Ultraslow, Developmental Waves

System	Process	°C	nm/second[a]		Ref.
Volvox Rouselleti					
sperm globoid	inversion	room		10	40
V. aureus	″	room		10	41
V. carteri	″	28?		30	42
Drosophila	morphogenetic				
eye disc	furrow forwards	25	1[b]		43, 44
	″ sideways	25		10[b]	
Sunflower	floret formation	28	6[c]		45, 46
Euplotes					
macronucleus	DNA replication early	22	1.3		47
	″ late	22		8	47
Rat axon	growth cone-like				
	waves	35		60	48
Rat lens	mitosis	37		60	49

[a]The two columns reflect emerging evidence of two subclasses of ultraslow waves.
[b]"As the furrow moves [forward] . . . [5 µm wide] ommatidia are added . . . every 2 hours . . . [but new clusters. . . are formed to each side at 10 minute intervals." (44)
[c]Obtained by multiplying the formation of 4–5 floret rings per day under optimal conditions (Ref. 46, p. 176) by a ring width of 110 µm.(45)

ectopic transcription of a Kruppel-lacZ construct, which is normally expressed in the dorsal-most embryonic tissue, the amnioserosa. We would therefore suggest that the dorsal high calcium region is a needed part of the mechanism, which establishes the dorsal region in Drosophila. This dorsal calcium could act upstream of dpp protein action by favoring its dorsal transcription and/or secretion as well as downstream of it on the pathway that leads to dorsally localized *zen* transcription.(39)

Ultraslow waves and the organization of development

Actively propagated calcium waves have been classified into four main groups according to their natural velocity as shown in Figure 1. The evidence for fast (≈30 µm /second), slow (≈1 µm /second), and ultrafast (≈1 meter/second) calcium waves has been presented elsewhere.(1)

Here, for the first time, I compile evidence for a natural class of ultraslow calcium waves, which move at only 1 to 60 nanometers/second. All of the cases listed in Table 1(40–49) are developmental waves, which can be induced to start ectopically and to move against their usual direction. For this fundamental reason, one can infer that they are actively propagated through an excitable system. However, the evidence for them being calcium waves in the sense of requiring a moving rise in calcium in order to be propagated is still very limited. It consists of little more than the fact that these waves are accompanied by gross shape changes of a sort that are elsewhere known to require increases in calcium. Nevertheless, I would propose that they will prove to be calcium waves in the same sense that the other classes are. Here I will discuss four of the six systems listed in Table 1. However, I will not discuss somite formation waves even though they generally move at ultraslow rates; this because of serious doubt as to whether these waves are propagated ones or are even replays of propagated ones.(50)

The morphogenetic furrow in Drosophila *eye discs may be a reaction-diffusion wave propagated by several secreted proteins*

A 1 to 10 nm/second wave of surface contraction sweeps across each of Drosophila's eye discs during the third larval instar so as to generate a close packed array of ommatidia in the retina. Although this wave normally starts in a disc's midposterior margin, genetic intervention can induce it to start and spread from other places, particularly the anterior margin as well as the interior of the disc.(51,52,44,43) Thus each third instar eye disc acts like an excitable medium that will form a retina when organized by an ultraslow wave that can start almost anywhere within it. These waves somehow orient as well as space the 800 ommatidia that will comprise each retina.

Two considerations suggest that these organizing waves are reaction-diffusion ones. One is their well discussed dependence upon the synthesis of numerous, more-or-less diffusible extracellular proteins, particularly those encoded by *hedgehog* and *decapentaplegic* as well as *wingless*, *spitz*, and *scabrous*.(51,53) Moreover, experiments with the temperature sensitive Notch allele indicate that the Q_{10} of furrow progression is about two, a relatively low value that is characteristic of reaction-diffusion waves.(54) Application of the Luther equation(55) indicates that if the propagators' limiting diffusion constant, D, were comparable to that of hh or dpp size proteins in water then the forward propagation velocity (of about 1 nm/second) would require an impossibly low limiting reaction rate, k; however, if D were that of hedgehog, then its binding to cholesterol(56) could easily reduce D to a value compatible with a reaction-diffusion mechanism.

What roles may calcium play in these waves? No doubt local calcium increases will prove needed to effect such phenomena as the apical contractions, which presumably help generate the famous morphogenetic furrow. Less certain and more interesting are its possible roles in propagating the wave. Since secretion is usually driven by a rise in subsurface calcium, secretion by the protein products of *hh*, *dpp*, etc. will probably prove to be driven in this way. Since *dpp* is clearly required for wave initiation and is probably needed for wave propagation, these considerations suggest a loop in which

calcium induces secretion of *dpp*'s protein product and this secreted protein then acts back to raise subsurface calcium.

Plant floret formation waves

A wave of floret formation moves from the edge to the center of the maturing sunflower disc at speeds of up to 6 nm/second.[45,46] Until this wave is about halfway in, wounding the still unpatterned inner region can induce the formation of organs, which are normally peripheral (such as involucral bracts) followed by reverse or centrifugal waves of disc floret formation; while at later stages, similar wounds have little consequence. One can infer that the disc acts like an excitable medium in which ultraslow waves of patterning or commitment normally move in from the periphery but can move out from wounds which presumably raise $[Ca^{2+}]_i$.[57]

Moreover, Paul Green long argued that initial patterning of the arrays of organs generated in plant shoots and flowers is generated by incipient buckling within the outer epithelium or tunica in response to lateral constraints on growth. Presumably, such incipient buckling would induce calcium release from stretch sensitive channels within the plasma membranes and/or the ER of incipient humps in the buckling epithelium.[58,59]

Progression of the DNA replication band in ciliate macronuclei

The macronuclei of hypotrichous ciliates are enormous, horseshoe shaped ribbons. Thus in the best studied one, *Euplotes eurystomus*, they are about 170 µm long and 6 µm wide.[60] Their DNA consists of highly endoreplicated, gene-sized DNA molecules, which are locally duplicated as two grossly visible replication bands progress through them over several hours. These bands start from their tips in *Euplotes* or from their center in the very closely related *Aspidisca*.[61] This fact indicates that these macronuclei are excitable systems—a conclusion that is confirmed by the fact that ectopic initiation and reverse progression can be induced in *Euplotes* by simple heat shock.[62] I propose that these replication waves involve and require calcium released from the macronuclear envelope since this envelope does not break down during passage of these waves, since nuclear envelopes are known to be regions of the endoplasmic reticulum and since these waves also induce gross contraction of the macronuclei.

Growth-cone-like or lamellipodium-like waves in axons

Ruthel and Banker have recently reported their discovery of periodic, anterograde, waves of growth-cone-like projections along the axons of isolated, embryonic, rat hippocampal neurons[48] (Fig. 5C). These waves start at the axon's base every half hour. They then move from soma to axon tip at steady rates of 60 nm/second, taking about an hour to complete the few hundred micron long trips. Spontaneous retrograde waves are also occasionally seen; moreover retrograde waves can be somehow induced by nocadazole treatment. This indicates that these ultraslow waves are somehow actively propagated along an excitable system.

Occasionally, these waves move at the same 60 nm/second rate in a covert form, then reappear along the axon as a renewed wave or at the growth cone as shown by a growth spurt. In visible waves, the underlying axon become grossly thickened, starting within the wave's rear. Altogether, I see a curious similarity between these ultraslow axonal waves and ultraslow macronuclear ones; even though the visible consequences of the underlying (calcium?) waves are radically different.

Although their discoverers call these slowly moving processes ''growth-cone-like'', it may be better to call them ''lamellopodium-like''. I suggest this since they are shaped like growth cones *without filopodia* yet contain four proteins that are more-or-less characteristic of growth cones.

Their speed and apparent contractile accompaniment suggests that they involve ultraslow calcium waves. So does a recent observation that lamellopodium-like processes can be induced far behind the growth cone of cultured *Aplysia* bag cell neurons by local application of a calcium ionophore.[63]

Slow calcium waves and the organization of development

Slow surface contraction waves and cellular peristalsis

Evidence that such slow waves are calcium waves has been presented elsewhere.[1] The concept of surface contraction waves (SCWs) was introduced by Koki Hara and his colleagues decades ago.[64] They used dark field microscopy to show that two successive circular SCWs start where cleavage will later start in axolotl eggs[64] (Fig. 6A) and in *Xenopus* eggs[65,66] and at least the very first of these travels all the way to the vegetal pole.

Then five years ago, Savage and Danilchik reported a powerful new way to observe SCWs in the vegetal hemisphere of *Xenopus* eggs. They labeled components of the subcortical germ plasm with a fluorescent dye and observed their movements via confocal microscopy.[67] During successive SCWs, these germ plasm islands move toward the vegetal pole at the conserved slow wave speed of 0.5 µm /second, do so for up to a minute or more and thus 40 µm and do not later regress. These observations suggest that these islands are driven towards the vegetal pole by *cellular peristalsis*.

Moreover, soon after Hara's discovery, Lewis et al. independently discovered so-called ''peristaltic constrictions'' in barnacle zygotes[68] (Fig. 6B). These repeated, gross SCWs occur throughout the 4–6 hour period between fertilization

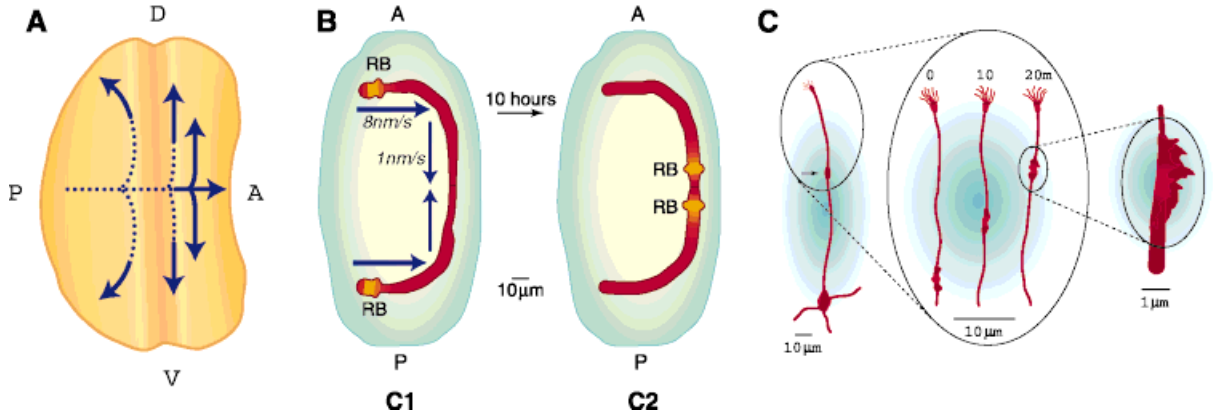

Figure 5. Some ultraslow (1 to 100 nm/second) developmental waves. A: Pattern formation in Drosophila's eye disc proceeds in a wave, which moves at about 1 nm/second from posterior to anterior along the disc's equator. Its most obvious feature is the morphogenetic furrow. This central wave continues in a succession of faster (≈10 nm/second) ones, which spread from the equator to dorsal and ventral edges so as to cover the disc. B: Movement of the DNA replication band (RB) along the macronucleus of the ciliate, *Euplotes eurystomas* (schematic ventral view). Behind the replication band, the macronucleus grossly shortens and thickens. C: Lamellipodium-like waves along the axons of neurons that were recently isolated from embryonic, rat hippocampi. (See Table 1 and text.)

and second polar body formation. The ooplasm changes from visible homogeneity to gross segregation along the AV axis during this period. The challenge remains of testing whether barnacle egg waves act to segregate the ooplasm by cellular peristalsis.

The Gordon/Brodland wave of neural induction

A 1987 article by Gordon and Brodland[69] presents a mechanical model of primary neural induction in amphibians. This argues for a mechanical instability that differentiates competent ectoderm into a tangentially contracting preneural hemisphere and tangentially expanding preepidermal one:

> "Our hypothesis for the bistability of the ectodermal cells is very simple: The microfilament ring tries to constrict the apical end of the cell, whereas the apical mirotubules, through their force of polymerization, together with archenteron and blastocoel pressure, try to expand it. If the microfilaments overcome the microtubule and pressure forces, the cell becomes a neuroepithelial cell. If the apical microtubules and pressure overcome the microfilaments, it becomes an epidermal cell."[69]

It further argues that this process starts with a rapid (5 minute) essentially mechanical induction by the involuting notochord of a small preneural region, which then somehow mechanically spreads through a hemisphere of peripheral tissue. A 1994 article by Brodland et al.[70] then describes a remarkable, more-or-less predicted, over 100 micron deep, SCW, which in fact accompanies primary neural induction in the axolotl. We then proposed that this apparently neurogenic SCW is a slow calcium wave basically similar to others in this class.

This then raises the question of whether the Gordon/Brodland wave is mainly propagated by a planar mechanism within the ectoderm as is believed to be true in *Xenopus laevis or* mainly by a vertical one (from the involuting mesoderm below it) as is believed to be true in *Rana pipiens*.[71] My view of it as a typical slow calcium wave suggests that it will prove to be largely planar.

It also revives the question of whether a rise in calcium is a necessary part of the wave of neural determination regardless of just how it spreads. Observations in the 1930s, by Holtfreter, of neural induction by "sublethal cytolysis" are highly reminiscent of comparable old work on parthenogenesis and therefore suggest a needed role for calcium and indeed for calcium waves in neural induction. A 1994 article from Marc Moreau's laboratory provides strong recent support for the idea that "increased internal Ca^{2+} mediates neural induction in the amphibian embryo."[72] Now the challenge is to image the predicted slow calcium waves in more-or-less intact amphibian embryos. Moreover, a 1997 article from Moreau's laboratory uses targeted aequorin in a manner that points toward this goal and also implicates the noggin protein in the process of primary neural induction.[73]

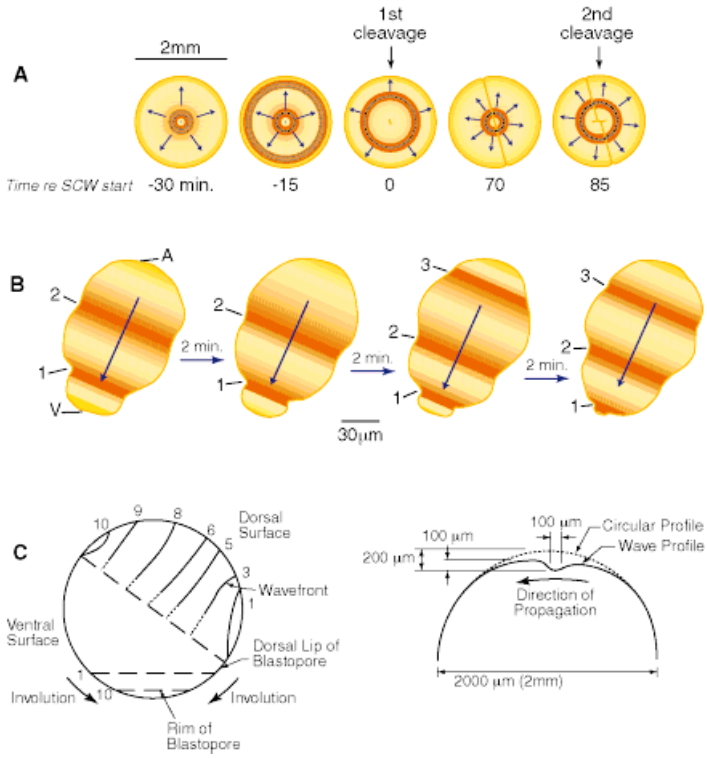

Figure 6. Some surface contraction waves involved in pattern formation. A: Prior to first and second cleavage of the axolotl. (After Ref. 64.) B: Prior to the first mitotic cleavage of a barnacle. (After Ref. 68). C: During primary neural induction in axolotls. Left: Diagram of a lateral view showing the wave front with time after time of initiation shown in hours. Right: Diagrammatic section during wave propagation. (After Ref. 70.)

Fast postfertilization calcium waves may establish and then clearly emanate from the ascidian organizer

In the beautifully clear *Phallusia* egg, a fertilization wave is followed by two fast calcium wave groups, which precede first and second polar body formation respectively.[74] The early waves start at the point of sperm entry, are refracted towards the vegetal pole, but end at[75,76] and presumably generate[77] the more-or-less separate *contraction pole* by somehow eccentrically shrinking a preformed basket of cortical actinomyosin.[78] The contraction pole becomes the center of polar lobe like bulges, of a cortical concentration of e.r., and a subcortical one of mitochondrion-rich myoplasm,[76,79] the origin of the second group of fast postfertilization calcium waves,[80] the forerunner of the gastrulation site, and the ascidian organizer as well as the dorsal side of the larva.[81,82]

These observations of development in creatures near the roots of the vertebrate line raise a question about mammals including man. Does the direction of postfertilization waves in mammals matter as well as their frequency?

Conclusions

Intracellular calcium patterns are as important in organizing development as they are in maintaining adult function. However, far slower mechanisms, ones that take hours rather than milliseconds, predominate. Moreover, far less is known of these matters.

In pursuing them, it will be essential to connect the needed revival of developmental physiology with the flourishing progress in developmental genetics. However, the emergence of pattern is an inherently integrative phenomenon in which the reductionism of molecular genetics will have to be complemented by biophysical thinking. Thinking which encompasses integrative phenomena such as calcium waves, self-electrophoresis and tensegrity. It will also be essential to develop better techniques for imaging calcium patterns with minimal disturbance of development as well as better methods to subtly manipulate calcium patterns. Nondisturbing calcium imaging of development calls for advances in chemiluminescent methods such as genetically targeted apoaequorins[83] and electroporated aequorins.[84] Subtle enhancement of calcium increases may be attained by further application of uncaging methods to developing systems[85]; whereas subtle suppression of calcium increases may be attained by genetically introducing natural, calcium shuttle buffers such as the calbindins.[86]

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