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Calcium buffer injections block fucoid egg development by facilitating calcium diffusion

[calcium gradients/polarity/tip growth/1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid]

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ABSTRACT **The polarity of fucoid eggs is fixed either when tip growth starts or a bit earlier. A steady flow of calcium ions into the incipient tip is thought to establish a high calcium zone that is needed for its localization and formation. To test this hypothesis, we have injected seven different 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid (BAPTA)-type calcium buffers into *Pelvetia* eggs many hours before tip growth normally starts. Critical final cell concentrations of each buffer prove to block outgrowth (as well as cell division) for up to 2 weeks. This critical inhibitory concentration is lowest for two buffers with dissociation constants or K_d values of 4–5 × 10^{-6} M and increases steadily as the buffers' K_d values shift either below or above this optimal value to ones as low as 4 × 10^{-7} M or as high as 9.4 × 10^{-5} M. To analyze these results, we have derived an equation (based on the concept of facilitated diffusion) for the effects of diffusable calcium buffers on steady-state calcium gradients. The data fit this equation quite well if it is assumed that cytosolic free calcium at the incipient tip is normally kept at about 7 μM and, thus, far above the general cytosolic level.**

The localization of rhizoidal tip growth in the fucoid eggs is a prototype of the symmetry-breaking problem (1). The tip's locus can be determined by various external stimuli that may leave quite different traces in the egg; however, all are thought to be amplified by the same positive feedback loop. This inner loop is thought to include an influx of calcium ions into the nascent tip, and this influx, in turn, is thought to raise the free calcium there to a level far above the general cytosolic one (2). The evidence for this hypothesis includes preferential $^{45}Ca^{2+}$ influx into the dark and future growth pole of photopolarizing eggs (3), an early electrical current entry there (4), and preferential tip formation towards a calcium ionophore source (5).

Recently Brownlee and Wood have used calcium microelectrodes to measure a steady free-calcium level of about 3 μM within the growing tips of fucoid rhizoids—a level that is, indeed, far above the general cytosolic one (6). Nevertheless, there has been relatively little evidence of either the existence or the need for a high calcium region within the nascent tip before its position is fixed and its growth begins. As a result, the whole calcium hypothesis of tip localization has been seriously questioned (7).

Therefore, we have begun to reinvestigate the problem by injecting fucoid eggs at a stage well before tip localization with various calcium buffers. Our purpose was not to change the average free-calcium level within the whole cytosol; rather we aimed to oppose the later development of calcium gradients and particularly the later formation of a high-calcium region within the nascent tip. We reasoned that if such a high-calcium region does indeed form and is needed, then appropriate buffer injections should block tip formation as long as the buffer remains in the cytosol. In particular, we used various calcium buffers of the 1,2-bis(*o*-aminophenoxy)-ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid (BAPTA)-type introduced by Roger Tsien (8). They have the important advantage that their dissociation constants can be widely varied by substitutions on their aromatic rings.

MATERIALS AND METHODS

Ripe thalli of *Pelvetia fastigiata* were collected near Monterey, CA, sent to us by Sea Life Supply (Sand City, CA), and stored for up to 2–3 weeks at 4°C before use. Their gametes were obtained by dark-shock (9) and were cultured in filtered natural sea water at 15°C. BAPTA-type buffers were obtained from Molecular Probes. Eggs were injected with various solutions by the quantitative method of Hiramoto (10). In this method, eggs are pushed into a wedge-shaped space and then injected from the side. We introduced a row of about two dozen eggs into this space at 2–3 hr after fertilization, injected them at 5–9 hr, and observed their later development in this same chamber. Before injection, the eggs were illuminated with strong, horizontally directed white light. This served to favor horizontal growth and thus eased later observations of development. After injection with most buffers, eggs were either again illuminated with white light or by similar red light: a choice without apparent consequences. However, in the case of the blue light-absorbing nitro-BAPTAs, we always used red light after injection.

To minimize damage during withdrawal of the injection pipette, the eggs' turgor was reduced during injection by using sea water made hypertonic with 370–500 mM mannitol. The injection pipette was cautiously pushed into each egg's plasma membrane until it was visibly dented. Then the injection system was tapped to rupture the plasma membrane and impale the egg. Control injections of up to ≈16% of an egg's volume did not visibly disturb development. So to minimize injection damage, we used buffer volumes of 8% or less. After injection we waited a few minutes before withdrawal to allow the buffer to diffuse evenly through the cytosol and thus minimize loss of buffer through the injection wound. The injection pipette was then slowly withdrawn over a period of another few minutes.

Control eggs were injected with a background medium containing 550 mM mannitol, 200 mM KCl, and 5 mM Hepes buffer at a pH of 7.0. In experimental eggs, the injection

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Abbreviation: BAPTA, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid.

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solution also contained 4–40 mM calcium buffer together with an appropriate amount of $CaCl_2$. (The mannitol was correspondingly reduced to avoid changes in osmolarity.) With some practice, practically 100% of the eggs injected with background medium developed in a manner indistinguishable from uninjected controls except for a small oil droplet introduced for identification and (sometimes) a small blob of cytoplasm that had oozed out of the injection wound. In this way we were able to reliably and quantitatively inject about a dozen eggs within a few hours.

The final cytosolic concentration of buffer was separately calculated for each injected egg by taking the measured volumes of injectate and egg into account and correcting for noncytosolic volume as well as for water of hydration in the buffers. The noncytosolic volume was (somewhat arbitrarily) estimated to be about half of the whole on the basis of direct measurements of *Pelvetia* eggs' water content (11). Water of hydration estimates were taken from ref. 12. Eggs given an overdose of buffer die within 1 or 2 days. Death is generally obvious via gross mottling and browning, a gross layer of exudate, and numerous saprophagous bacteria and protozoa (Fig. 1*A Right*); however, we occasionally confirmed it with sea water made hypertonic with 0.5 M mannitol: unlike live eggs, dead ones fail to plasmolyze in this medium.

RESULTS AND ANALYSIS

Qualitative Observations. When eggs were injected with a critical final cytosolic concentration of calcium buffer, germination (i.e., the initiation of rhizoidal tip growth) was grossly delayed. In a population of control eggs cultured at 15°C, the germination of various eggs starts in about 12–18 hr and is quite obvious in all eggs by the end of day 1 (Fig. 1*D Left*), whereas in almost all critically injected eggs, it had not started by the end of day 1 (Fig. 1*D Right*). Except for 4,4′-difluoro-BAPTA (discussed below), only a quarter of those eggs that were ungerminated on day 1 ever resume development. Those that do resume development generally do so normally. However, most eggs that are ungerminated on day 1 remain dormant (but alive) for long periods. Four eggs, that were still ungerminated on day 2, were followed for 2 weeks or more. In one, shown in Fig. 1*B*, germination finally began after 15 days; in another shown in Fig. 1*E*, it began on day 13; in a third, it had not yet begun on day 14; while a fourth was still ungerminated on day 11 and dead by day 14. Of those that failed to germinate on day 2, about 3–10% nevertheless divided to form one or more inner cell walls. On the other hand, we observed no injected eggs that formed substantial outgrowths yet failed to divide.

Above the critical inhibitory buffer concentration, eggs not only failed to germinate but died within 1 or 2 days (Fig. 1*A Right*). Below this concentration, eggs developed entirely normally. In one experiment, about half of the uninjected controls had begun apical differentiation in 11 days as marked by the formation of characteristic apical papillae, while one or two of four eggs injected to reach 1–2 mM BAPTA had likewise formed such papillae at this time.

Eggs inhibited with 4,4′-difluoro-BAPTA (unlike the other six buffers tried) quickly resumed development. All 15 of such eggs, which were more or less inhibited on day 1, had largely recovered by the end of day 2. However, particularly at higher final buffer concentrations, they showed a curious tendency to form two rhizoidal growth tips (Fig. 1*C*). Of nine eggs injected to reach 3–6 mM buffer, three developed into such twins; yet such twinning was rare in controls injected with background media.

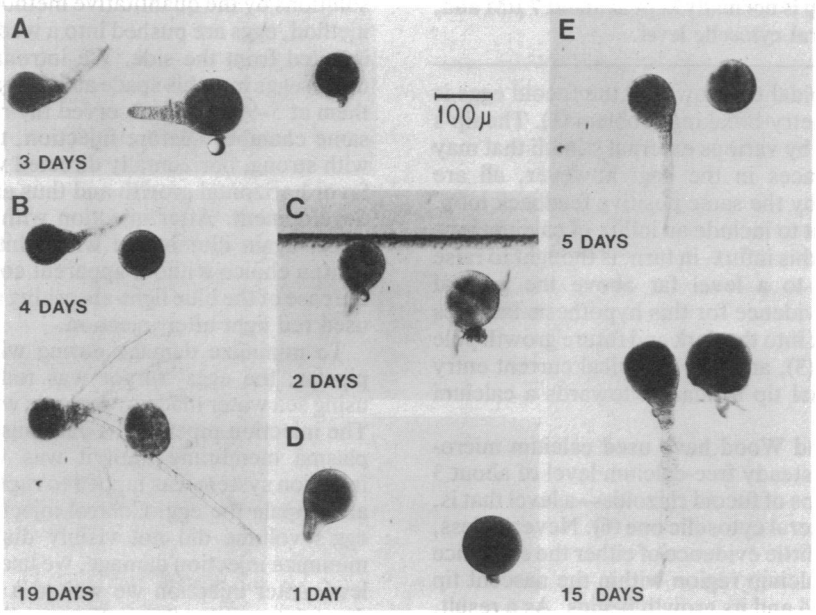

FIG. 1. Effects of injected calcium buffers on the development of *Pelvetia* eggs, photographed on the days indicated. In each case the buffer was dissolved in a background medium that also contained 0.1 mol of calcium per mol of buffer; the growth polarizing white light came from the top of the page. (*A*) Experiment 17 on March 20, 1987. Middle egg (with 556-pl total volume) injected at 6 hr with 29 pl of 15 mM 5,5′-dibromo-BAPTA to give a final cytosolic buffer concentration of 1.6 mM. Germination of this egg was delayed about 6 hr. The right egg (with a 333-pl volume) was injected at 7 hr with 29 pl of the same buffer to give 2.6 mM; it was dead by day 1. The left egg was uninjected. (*B*) Experiment 16 on March 19, 1987. The right egg was injected at 8 hr with 17 pl of 15 mM 5,5′-dibromo-BAPTA to give a final concentration of 1.3 mM; the left egg was uninjected. Both subsequently were cultured in red light. In the injected egg, an inner cell wall first formed at 8 days, and a minute outgrowth formed after 15 days. (*C*) Experiment 32 on April 14, 1987. The right egg was injected at 7 hr with 16 pl of 50 mM 4,4′-difluoro-BAPTA to give a final concentration of 3.0 mM; the left egg was impaled but not injected. In this case, the control egg was impaled but not injected, and both showed a small exudate at the injection site. (*D*) Experiment 36 on April 22, 1987. The right egg was injected at 6 hr with 24 pl of 50 mM 5,5′-difluoro-BAPTA to give a final concentration of 4.3 mM; the left egg was uninjected. The injected egg died on day 2. (*E*) Experiment 29 on April 8, 1987. The right egg was injected at 7 hr with 7 pl of 150 mM 5,5′-dimethyl-BAPTA to give a final concentration of 3.8 mM; the left egg was uninjected. The injected egg first showed inner walls on day 2 and a small outgrowth after day 13.

Quantitative Results. Fig. 2 displays all of our reliable observations (which were made 2 days after fertilization) of the germination or death of eggs injected with 5,5'-dibromo-BAPTA. Since injected eggs would be expected to reset their average calcium concentrations within minutes, yet eggs injected with critical concentrations of buffer remained inhibited for weeks, it seems unlikely that the buffers act by resetting average calcium levels. Nevertheless, we checked and confirmed this important point by determining the mean inhibitory buffer concentration at different levels of coinjected calcium. As Fig. 2 shows, this critical level remained the same whether 0, 0.1, or 1 mol of calcium was coinjected with each mol of buffer. Fig. 2 also helps to convey the fact that the inhibitory concentration ranges are quite narrow: below a roughly 2-fold range, development is entirely normal; above it, eggs die in 1 or 2 days.

In Fig. 3, mean inhibitory concentration is plotted against the buffer dissociation constant for all seven buffers tested. One sees that the two buffers with K_d values of about 4 or 5 $\times 10^{-6}$ M (5,5'-dibromo- and 4,4'-difluoro-BAPTA) were most effective, only taking about 1 mM to inhibit germination. As the K_d of the buffers moves above and below this optimal value, the critical inhibitory level increases steadily, reaching about 6 mM for the strongest buffer tested (5,5'-dimethyl-BAPTA with a K_d of 0.4×10^{-6} M) as well as the weakest one (5-mononitro-BAPTA with a K_d of 9.4×10^{-5} M).

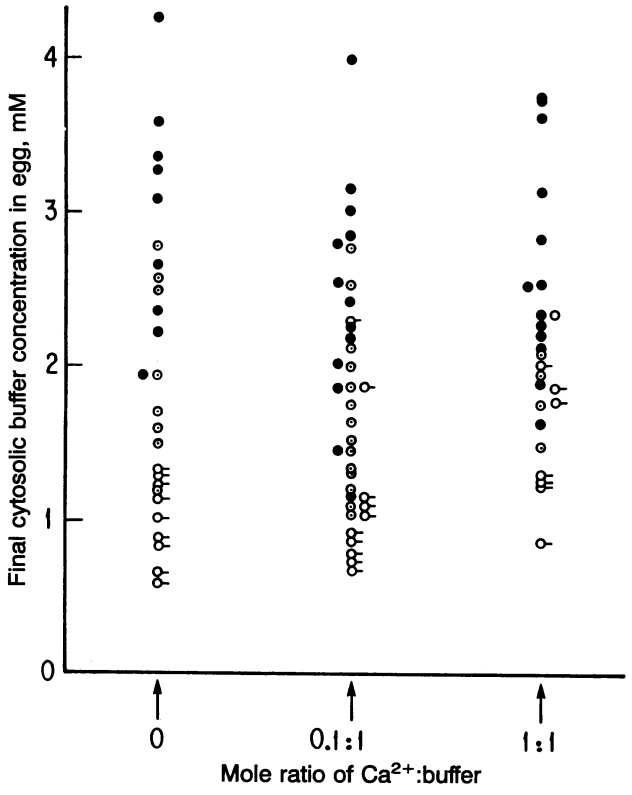

FIG. 2. The states of all *Pelvetia* eggs that were injected with 5,5'-dibromo-BAPTA (at 6–9 hr) observed two days after fertilization: ●, dead; ⊙, inhibited; and ○–, normal. Eggs were coinjected with 0.0, 0.1, or 1.0 mol of calcium per mol of buffer ($K_d = 3.6 \times 10^{-6}$ M) as shown. In each case, the mean inhibitory concentration was defined as the mean value of the final cytosolic buffer concentrations in all inhibited eggs. These values (± SEM) were 1.6 ± 0.1, 1.4 ± 0.1, and 1.5 ± 0.1 mM, respectively. Eggs were scored as inhibited if they showed no outgrowth. Death was generally obvious via texture and color as well as saprophagous bacteria and protozoa; however, we occasionally confirmed it via a failure to plasmolyze in hypertonic medium.

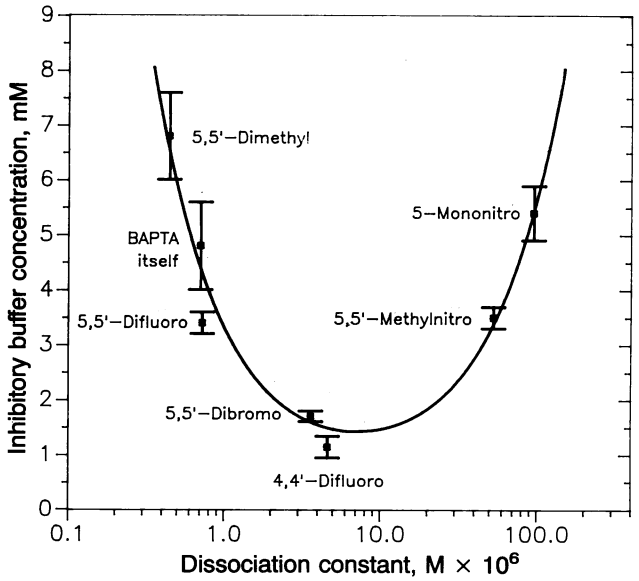

FIG. 3. Mean inhibitory concentrations (± SEM) versus buffer dissociation constant for seven BAPTA-type buffers. Inhibitory concentrations were obtained from the data shown in Fig. 2 plus similar data for the other six buffers tested. In all cases, these data were obtained from eggs coinjected with 0.1 mol of calcium per mol of buffer. In one case—that of 4,4'-difluoro-BAPTA—the eggs were scored on day 1 instead of day 2 (see text). K_d values were measured in 300 mM KCl/1 mM Mg^{2+} by Pethig *et al.* (12). The curve plots Eq. 7 with $D_{Ca}/D_B = 17$, C = 7.2 μM, and $r = 2.9$.

Analysis. Our conception of how a mobile calcium buffer acts to inhibit development is shown in Fig. 4. We reason that it acts by facilitating the diffusion of calcium away from a needed point of high subsurface calcium. The buffer does this by: (1) picking up free calcium at the high end of a steady gradient, (2) diffusing with its bound calcium to the low end, (3) releasing the calcium there, and (4) then diffusing back to the high end to pick up more calcium. This shuttle provides an alternate route for the diffusion of calcium down a gradient and thus effectively increases its mobility of diffusion. Note that this cycle requires the buffer to be weak enough to release calcium at the low end of the gradient (step 3) as well as strong enough to pick it up at its high end (step 1). That is the essential reason why the buffer should be most effective when its K_d matches the (average) calcium level within the gradient, rather than simply becoming more effective as it grows stronger. Injection of too strong a buffer will initially extract calcium from sources within and without the cell; but thereafter it will sit, unable to release its bound calcium and thus will remain inert as yolk.

Because of the high reaction rates of BAPTA-type buffers (13), we assume that they are everywhere in near local

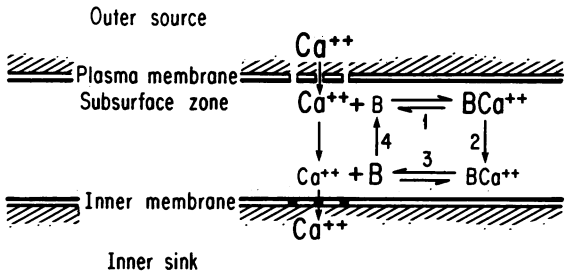

FIG. 4. Model of how a mobile calcium buffer, B, acts to speed the steady diffusion of calcium ions down a gradient in the nascent growth zone. Size of symbols suggests their relative concentrations. Calcium ions are assumed to leak into (and be pumped out of) this zone at constant rates.

equilibrium with free calcium. Thus, such equilibrium is assumed for steps 1 and 3 of our model. We may then ask how large the buffer-mediated or facilitated calcium flux, J_B, is between any two points as compared to the direct, unmediated flux, J_{Ca}, of free calcium. Presumably the buffer will begin to affect the cell when these two fluxes become comparable.

Let r be the ratio, J_B/J_{Ca}, of facilitated-to-direct calcium flux down a gradient over a small distance, dx, in the region considered. Let C be the concentration of free calcium, B of free buffer, BC of its calcium complex, and $\overline{B}$ of total buffer. Let D_{Ca} and D_B be the diffusion coefficients of free Ca^{2+} and buffer, respectively. By Fick's first law:

$$J_{Ca} = D_{Ca} \cdot dC/dx. \quad [1]$$

Similarly step *ii* of the shuttle gives:

$$J_B = D_B \cdot d(BC)/dx. \quad [2]$$

In the steady state, the flux "up" step *iv* equals that of the "down" step *ii*. Hence,

$$dB = d(BC) \quad [3]$$

and

$$J_B = D_B \cdot dB/dx. \quad [4]$$

Hence,

$$r = D_B/D_{Ca} \cdot dB/dC. \quad [5]$$

The term dB/dC is equivalent to the change in net calcium added to a buffer solution (during a titration) per change in free calcium. This is well known to be greatest when the buffer's dissociation constant, K_d, equals C; moreover, an exact expression for it (in a slightly different form) has been called van Slyke's buffer value (14):

$$dB/dC = (\overline{B}/C) \cdot [f/(1 + f)^2], \quad [6]$$

where $f = K_d/C$. Putting Eq. 6 in Eq. 5 yields:

$$\overline{B} = rC \cdot (D_{Ca}/D_B) \cdot [(1 + f)^2/f]. \quad [7]$$

To apply Eq. 7 to our data, we must assume that our system is in a relatively steady state—i.e., that the free calcium in the cytosol turns over many times during the transitions considered. The following calculations indicate that this is a safe assumption. The 6- to 7-hr-old *Pelvetia* egg is known to turn over its calcium at 0.11 pmol/hr (15, 16). The total amount of free calcium in the cytosol may be estimated to be 0.3 μM times the cytosolic volume of 200 pl or 6×10^{-5} pmol. Dividing this by the turnover rate yields an estimated free calcium turnover time of only 2 sec, whereas the transitions involved in establishing a point of outgrowth seem to be on the order of 5 min to 1 hr and thus far longer.

We take D_B to be 3.8×10^{-7} cm^2/sec, the average of values for the BAPTA-type buffer, fura-2, in frog muscle [3.9×10^{-7} cm^2/sec (17)] and in barnacle muscle [3.6×10^{-7} cm^2/sec (18)]. These values are uncorrected for binding to the cytoskeleton but so is B in Eq. 4, so they are appropriate. On the other hand, D_{Ca} refers to free calcium in Eq. 7; so we take D_{Ca} to be 6.4×10^{-6} cm^2/sec, the average for free calcium in a metabolically poisoned marine worm's axoplasm [5.3×10^{-6} cm^2/sec (19)] and an aqueous medium of similar ionic strength [7.5×10^{-6} cm^2/sec (20)]. D_{Ca}/D_B, then, is taken to be 17.

Finally, the curve in Fig. 3 is the one computed to select values of C and of r that generate a least-squares fit of Eq. 7 to the data. With values of 7.2 μM and 2.9-fold for C and r, the fit is good.

DISCUSSION

Facilitated Diffusion Theory. Recent work on transcellular transport by facilitated diffusion has focused on the calbindins, small calcium-buffering proteins found within intestinal cells and elsewhere (21). Our analysis provides the first clear equation to relate the degree of such facilitation to all of the relevant concentrations and constants. However, Kretsinger *et al.* have provided some revealing computer-generated steady-state calcium profiles during such transport (22). A comparison of these to Eq. 7 suggests that their steep and shallow parts are ones where free calcium levels are far from and close to K_d, respectively.

Tip Growth. We obtain an excellent fit of our data to a facilitated diffusion theory on three main assumptions.

First, we assume that the ratio D_{Ca}/D_B in fucoid eggs can be obtained from the ratio of D_{Ca} in metabolically poisoned axoplasm to D_B for fura-2 in myoplasm. Some readers may feel that D_{Ca} is best obtained from the classical studies of $^{45}Ca^{2+}$ movements within metabolically active cells. However, the rates of $^{45}Ca^{2+}$ movement within such cells is limited by active uptake into various membrane-bound compartments. Hence, they provide no information about the diffusion of calcium within the cytosol. In our model (Fig. 4), it is just such true diffusion—across the cytosol between membranes—that is pertinent: hence, our use of Donahue and Abercrombie's more recent results (19).

Second, we take 7 μM to be the average free calcium level normally present within a nascent outgrowth region. The chlorotetracycline images of Kropf and Quatrano, particularly their figure 2*a* (7), suggest that the high cytosolic calcium within this region may be largely restricted to a thin intermembrane space between the plasma membrane and various submembrane cisternae or vesicles. This may be why direct measurements of cytosolic calcium within a nascent outgrowth have not been achieved yet. However, as noted in the Introduction, Brownlee and Wood have measured a free calcium level of about 3 μM somewhat below—say 10 μm below—the tips of growing fucoid rhizoids (6).

Third, we infer that r is about three—i.e., we infer that the buffer must speed cytosolic calcium diffusion by about 4-fold to permanently block the initiation of an outgrowth. This too seems plausible in view of the following findings. The buffer-mediated reduction in subsurface calcium, which blocks outgrowth, would be expected to induce adaptive responses, including the opening of calcium channels to speed calcium influx (23). However, if this influx exceeds an egg's ability to pump calcium out, it must eventually become overloaded with calcium and die. In fact, eggs do die in a day or two when the injected buffer levels exceed inhibitory ones by only a few fold. This indicates that an egg's ability to adapt to buffer injection by speeding the flow of calcium through its surface is comparably limited. Hence, it seems reasonable to infer that a few-fold acceleration of internal calcium diffusion suffices to inhibit development.

Since the assumptions needed to fit Eq. 7 seem reasonable and the fit is good, we conclude that: (*i*) These buffers act by facilitating calcium diffusion; (*ii*) the nascent growth zone within fucoid eggs bears a high-calcium region of the order of 10 μM; and (*iii*) this high-calcium region is needed to initiate local growth. However, it seems less clear whether this region is needed for "symmetry breaking" (1) or "axis fixation" (7) in the sense of an irreversible commitment to outgrowth before outgrowth begins. This question remains

cloudy because it is unclear whether such commitment, in fact, becomes irreversible before outgrowth occurs.

Cell Division. With few exceptions, *Pelvetia* eggs that failed to germinate likewise failed to divide, while those that did germinate divided normally. There is no reason to believe that the germination of fucoid eggs induces their division or vice versa. Normally, they germinate long before they divide; however, when germination is osmotically suppressed with high sugar concentrations, they will nevertheless undergo numerous divisions (24). Therefore, we would infer that injected BAPTA-type buffers block cell division by blocking the formation of some high-calcium region that is quite separate from the one in either the nascent or active growth tip.

We know nothing of the (localized) calcium transients involved in fucoid cell division. However, Hepler and Callaham have observed a substantial one within dividing *Tradescantia* stamen hair cells. This gradient lasts about 30 min (25). We have calculated above that the turnover time for free cytosolic calcium in the whole *Pelvetia* egg is only about 2 sec. So we should be quite safe in assuming that the cell division calcium transient in fucoid eggs takes many turnover times and, therefore, is a steady-state phenomenon analyzable by Eq. 7. Therefore, we propose that the peak inner calcium level needed to support cell division in *Pelvetia* is comparable to that needed at the nascent growth tip—i.e., on the order of 10 μM.

It is interesting to consider application of this same analysis to two recent reports that injection of enough BAPTA into sea urchin eggs inhibits nuclear envelope breakdown (26, 27). In the former, less BAPTA was injected and breakdown was only delayed so our analysis is inapplicable; however, in the latter, more was injected, breakdown seemed to be permanently blocked, and our steady-state analysis may well be applicable. In the latter, injection of BAPTA to reach "1.8 mM" blocked nuclear-envelope breakdown in half of the injected eggs. Correcting "1.8 mM" for noncytosolic volume (as well as buffer water of hydration), as we did for the fucoid egg, one obtains about 2.8 mM. This figure is similar to the 4.8 mM figure we find for developmental arrest by BAPTA in fucoid eggs. Therefore, we propose that the peak inner calcium level needed to support nuclear-envelope breakdown in sea urchin eggs is likewise about 5–10 μM.

Cell Death. Why do eggs that are overloaded with calcium buffer die in a day or two? The same reduction in subsurface calcium that presumably blocks germination should also speed calcium influx (23). If this accelerated influx exceeds the egg's ability to pump calcium out, the cell will ultimately become overloaded with calcium and die. What is known of calcium turnover in *Pelvetia* eggs is quite consistent with this hypothesis: Thus, it can be calculated from the data of Allen *et al.* that a *Pelvetia* egg contains a total of about 1.3 pmol of intracellular calcium (11), while we calculated above that this egg turns over its calcium at about 0.11 pmol/hr. The ratio yields a normal turnover time for total calcium of about half a day. Therefore, it is reasonable to suppose that a limited excess of influx over efflux would take 1 or 2 days to so overload the egg's calcium reservoirs as to kill it.

Possibilities and Limitations. Our results indicate that injections of BAPTA-type buffers, together with Eq. 7, provide an effective, general test for the actions of relatively steady, cytosolic calcium gradients. Much as cytochalasin immersion serves to test for actions of an actin cytoskeleton, so "baptism" may test for the actions of such calcium gradients.

This test need not work for calcium gradients that last too short a time to approach a steady state—say, less than a few seconds. Moreover, it should give a false positive for slow transients that involve no gradients on a whole-cell level; however, we imagine that such relatively delocalized transients will prove to be rather rare events within living cells.

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