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REVIEW

Sources of Calcium in Egg Activation: A Review and Hypothesis

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A careful reanalysis of the literature indicates that the initial mechanism of activation in sea urchin eggs is remarkably similar to the mechanism established in medaka eggs: i.e., sea urchin eggs are activated by a qualitatively and quantitatively similar calcium explosion; one which is propagated in a wave sustained by the calcium-stimulated release of calcium from internal sources. These sources are probably in the endoplasmic reticulum. An exhaustive survey of the literature reveals that a wide variety of other activating eggs in the vertebrate line also exhibit secretory waves which are propagated at about 10 μm/sec, and can thus be assumed to reflect the same basic mechanism. Activating protostome eggs on the other hand do *not* exhibit such waves. This and other systematic differences from deuterostomes suggest that unlike deuterostome eggs, protostome eggs are primarily activated by calcium ions which enter the cytosol from the medium, and do so in response to depolarization of the egg's plasma membrane.

INTRODUCTION

Gilkey *et al.* (1978) have demonstrated that eggs of the medaka fish, *Oryzias latipes*, are naturally activated by a huge pulse of free cytosolic calcium; that the calcium in this pulse comes very largely from internal stores; and that it is released in a wave which is propagated across the cytosol by a chain reaction (or traveling "explosion") involving calcium-stimulated calcium release. The trigger level for this explosion proves to lie at a pCa of about 5.5 (i.e., 3 μM Ca^{2+}); the peak level, about 4.5 (i.e., 30 μM Ca^{2}); and the propagation rate about 10 μm/sec (Ridgway *et al.*, 1977; Gilkey *et al.*, 1978; Gilkey, in press.). These findings on the medaka egg raise the question of just how general the medaka mechanism is, and invite a reexamination of the whole literature on fertilization.

The key to this reexamination is the realization—which began with the work of Douglas in the 1960s—that exocytosis under natural conditions is always shortly preceded by a large increase in free calcium (Rubin, 1982). This does not mean that calcium pulses can not occur without obvious exocytosis. In some cells, few or no vesicles poised for fusion with the plasma membrane may be present during such a pulse. Moreover, an inhibitor of exocytosis may be present. For example, ammonia strong enough to activate urchin eggs somehow detaches their cortical vesicles from the plasma membrane and suppresses exocytosis, despite the induction of a calcium pulse (Hylander and Summers, 1981; Jaffe, 1980, p. 96). It does mean that in no natural case is exocytosis known to occur without a shortly preceding calcium rise.[1]

As a result, a large literature of morphological observations, extending back for more than 70 years, can now be reinterpreted in terms of changes in free calcium levels, with all that this implies. Some more historical aspects of this reexamination are presented elsewhere (Jaffe, in press). Here I will focus upon two main results of this reexamination: First, evidence that activation of the much studied sea urchin egg is quite similar to activation of the medaka egg; second—and more important—that while most or all eggs seem to be naturally activated by a calcium pulse, that the source and mechanism of this rise in cytosolic calcium seem to be generally different between deuterostome eggs on the one hand and protostome eggs on the other.

SEA URCHIN EGGS ARE APPARENTLY ACTIVATED BY A VERY SIMILAR EXPLOSION

A combination of diverse data indicate that sea urchin eggs are activated by a calcium explosion remarkably similar to the medaka egg explosion (Table 1).

First, observations began by Just (1919), and extended by Moser (1939a) and others (see Table 2) show that a sperm initiates an exocytotic wave in a sea urchin egg which traverses it at about the same average speed of 10 μm/sec. The kinetics of this wave do not fit a diffusion

[1] A probable exception to this generalization is the (exocytotic?) secretion of pathyroid hormone (Shobeck and Brown, in press).

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TABLE 1
CHARACTERISTICS AND APPROXIMATE PARAMETERS OF THE CALCIUM EXPLOSIONS IN MEDAKA EGGS AND IN SEA URCHIN EGGS

	Medaka	Sea urchin
Fills whole cytoplasm	Yes[a]	Yes[b,c]
Essential for development	Yes[d]	Yes[e,c]
Acts locally	Yes[f]	Yes[g]
Inherently decremental	Yes[h]	Yes?
Detonated by sperm calcium	?	Yes?[i]
Easier to trigger in animal half	Yes[h]	?
Faster in animal half	Yes[h]	?
Nearly independent of external calcium level	Yes[a]	Yes[j]
Independent of cortical granules	Yes[h,a]	Yes?[k]
Relatively independent of the membrane potential	Yes[l]	Yes?[m]
Average wave speed (at 20°C)	10 μm/sec[n]	10 μm/sec[n]
Pulse duration	15 min[o]	30 min?[p]
Peak calcium level	30 μM[a,o]	~10 μM[q,a]
Trigger level	3 μM[r]	~1 μM[s]

[a] Gilkey *et al.* (1978).
[b] Kiehart *et al.* (1977), Eisen (1982).
[c] Jaffe (1980).
[d] Gilkey, in press.
[e] Zucker and Steinhardt (1978).
[f] Sakai (1964).
[g] Allen (1954), Allen and Rowe (1958), Chambers and Hinkley (1979).
[h] Yamamoto (1961).
[i] Schackmann *et al.* (1978).
[j] Takahashi and Sugiyama (1973), Chambers (1980), Chambers and Angeloni (1981), Schmidt *et al.* (1982).
[k] Uehara and Sugiyama (1969).
[l] Nuccitelli (1980).
[m] MacKenzie and Chambers (1977), Lynn and Chambers (1982).
[n] See Table 2.
[o] Ridgway *et al.* (1977).
[p] Azarnia and Chambers (1976).
[q] Steinhardt *et al.* (1977).
[r] Gilkey, in press.
[s] Hamaguchi and Hiramoto (1981) (see also Baker *et al.* (1980)).

propagated process but do fit "some process involving autocatalysis" (Kacser, 1955, p. 457). Moreover, the activation wave which crosses a sea urchin egg—again like that across a medaka egg—can progress through a region devoid of cortical vesicles (Uehara and Sugiyama, 1969); can progress at nearly normal rates in the absence of extracellular calcium (Takahashi and Sugiyama, 1973; Chambers, 1980; Chambers and Angeloni, 1981; Schmidt *et al.*, 1982); but may peter out under various abnormal conditions, or in response to a subthreshold stimulus, so as to yield partially activated eggs (Allen and Rowe, 1958; Chambers and Hinkley, 1979). This latter result is presumably an extreme consequence of the inherently decremental character of the calcium chain reaction in the sea urchin egg just as it is in the medaka egg.

Nevertheless, complete waves can be initiated by pricking *Arbacia* eggs in (and only in) a calcium-bearing medium (Moser, 1939b), as well as by local application of the relatively specific calcium ionophore, A23187 to *Lytechinus* eggs (Chambers and Hinkley, 1979). These two results indicate that a wave can be initiated by a local rise in free calcium in the sea urchin egg just as it can in the medaka egg.

Injections of calcium buffers set at a free calcium level of 1 μM or more are found to completely activate eggs of the sea urchin, *Hemicentrotus;* while somewhat (say threefold?) lower levels only partially activate these eggs, as shown by local membrane elevation as well as a lack of monaster formation (Hamaguchi and Hiramoto, 1981). These data indicate a trigger level of about 1 μM, even though these authors did not observe their eggs for possible exocytotic waves.[2]

Measurements of the light emitted by aequorin-loaded eggs of the sea urchin, *Lytechinus* during fertilization provide an estimate of the peak free calcium level in these eggs (Steinhardt *et al.*, 1977). In their best synchronized batch, direct calibration of the aequorin indicated a peak free calcium level of about 5 μM, assuming equal distribution of the free calcium throughout the egg and thus somewhat underestimating it (see Gilkey *et al.*, 1978, p. 463). The experiments which indicate a lower trigger level were done on other species of sea urchin. Nevertheless, the estimated natural peak level in *Lytechinus* is so much higher than the estimated trigger level in *Hemicentrotus* as to provide evidence of regenerative calcium release in sea urchin eggs which is basically similar to—if somewhat less compelling—that available for medaka eggs.

Finally, the careful measurements of Azarnia and Chambers (1976) on *Arbacia punctulata* eggs show a sharp decline in *total* calcium within the egg after fertilization (Fig. 1). This decline is very rapid at first and then asymptotically approaches a nearly constant level at about 40 min after fertilization (and 25 min before first cleavage). About a quarter and a third of the total is lost at 15 and 30 min, respectively. Similar results were found with *Lytechinus* eggs. These results are also in good agreement with the pioneering measurements of Örström and Örström (1942) and of Monroy-Oddo (1946). The former inferred the loss in calcium within the fertilized *Paracentrotus* egg from careful measurements of the rise of net calcium within the seawater

[2] Moreover, the observations of Baker *et al.* (1980) on electroshocked eggs of the sea urchin, *Echinus*, can be taken to indicate a trigger level of a few micromoles per liter: In a medium containing 6 μM Ca^{2+}, the percentage of cortical vesicle fusion varied very sharply with field strength; going from about 10 to 85% as the field strength was doubled (their Fig. 5). When this observation is combined with the much more gradual change with field strength in the fraction of an egg's membrane area which is subjected to a given voltage, it suggests that electroshock triggered waves in these experiments. When external Ca^{2+} was varied at a fixed saturating field strength, the fraction of granule fusion rose sharply with external Ca^{2+} with a transition to a maximal response at a few micromoles per liter (their Fig. 6).

TABLE 2
VELOCITIES OF EGG ACTIVATION WAVES[a]

Group	Species	Habitat	Temperature (°C)	Diameter (μm)	Transit time (sec)	Rate (μm/sec)	Reference
Brown algae	*Cytoseira barbata*	Marine	18	200?	60	5	Knapp (1931)
Sponges	*Tetilla serica*	Marine	19	175	60	9	Kume (1952)
	T. japonica	Marine	?	130	15-60	7-27	Watanabe (1978)
Crinoids	*Comanthus japonicus*	Marine	24	250	68	6	Dan and Dan (1941)
Sea urchins	*Echinarachnius pama*	Marine	?	135	17	12	Just (1919)
	Arabacia punctulata	Marine	26	74	10	12	Moser (1939a)
	Psammechinus miliaris	Marine	18	100	20	8	Rothschild and Swann (1949); Kacser (1955)
	Clypeaster japonicus	Marine	?	120	15	13	Endo (1952)
	Strongylocentrotus drobechiensis	Marine	8	160	90[d]	3	Ginzburg (1964)
	S. purpuratus	Marine	16	75	20	6	Paul and Epel (1971)
Hemichordates	*Saccoglossus kowalevskyi*	Marine	23	400	60-120	5-10	Colwin and Colwin (1954)
Lampreys	*Entosphenus wilderi*	Freshwater	?	1000	300	5	Okkelberg (1914)
	Lampetra reissneri	Freshwater	18	1600	420	6	Yamamoto (1944a, 1947)
Cartilaginous fishes	*Acipenser guldenstadti*	Freshwater	18	3300	240[d]	22	Detlaf (1962)
	A. stellatus	Freshwater	22	2900	170[d]	27	Ginzburg and Nikiforova (1978)
Bony fishes	*Oryzias latipes*	Freshwater	28	1100	120	14	Yamamoto (1939, 1944b)
	O. latipes	Freshwater	28	1100	100	18	Iwamatsu (1965)
	O. latipes	Freshwater	10	1100	220	8	Gilkey *et al.* (1978)
	O. latipes	Freshwater	20	1100	140	12	Gilkey *et al.* (1978)
	O. latipes	Freshwater	30	1100	100	18	Gilkey *et al.* (1978)
	Perca fluviatilis	Freshwater[c]	17	800	180	7	Thomopolous (1953a)
	Gasterosteus aculeatus	Freshwater	20	960	180	8	Thomopolous (1953b)
	Ammodytes tobianus	Marine	17	710	160	7	Thomopolous (1954)
	Carassius auratus	Freshwater	21	1100	300	6	Yamamoto (1954)
	Hypomedusa olidus	Freshwater	21	730	120-180	6-10	Yamamoto (1954)
	Pungitius sp.	Freshwater	18	1000	180	9	Kusa (1956)
Amphibia	*Rana temporaria*	Freshwater[b,e]	15	2000	240[f]	10-15	Kasyanov *et al.* (1971)
	Xenopus laevis	Freshwater[b]	?	1300	180-240	9-11	Wolf (1974)
	X. laevis	Freshwater	21	1300	180	10	Hara and Tydeman (1979)
	Rana pipiens	Freshwater[b,e]	22	1800	150[g]	20	Goldenberg and Elinson (1980)

[a] Unless otherwise noted, observations were made on live, mature eggs activated by sperm.
[b] Prick activated.
[c] Activated by immersal in freshwater whether or not sperm are present and enter the egg.
[d] Observed on fixed eggs.
[e] Coelomic eggs.
[f] Estimated by tripling the observed animal pole to equator time.
[g] Estimated by multiplying the observed time from equator to antipode by 1.5-fold.

around the eggs and thus inferred a loss of 37% at 10 min after fertilization; the latter measured intracellular calcium (after a quick urea wash) and found losses of 38 and 48% at 15 and 30 min after fertilization, respectively. Furthermore, measurements of $^{45}Ca^{2+}$ fluxes give results in excellent agreement with these findings (Azarnia, 1971; Azarnia and Chambers, 1969). So, altogether, there can be practically no doubt about the basic facts.[3]

From these facts, Azarnia and Chambers (1969) concluded that "fertilization initiates the intracellular release of Ca, which is then extruded." One can further infer that intracellular free calcium remains well above the unfertilized level for about 30 min after fertilization;[4]

[3] Azarnia and Chambers also measured similar and parallel declines in the *magnesium* content of *Arbacia* and of *Lytechinus* eggs after fertilization. This raises interesting questions. Are there transient changes in free magnesium in the cytosol of these eggs? How are calcium and magnesium release related?

[4] Measurements of the light emitted by aequorin-loaded sea urchin eggs (by Steinhardt *et al.*, 1977) provide an estimate of the height *without* indicating its duration. The latter was not indicated because it proved impossible to detect the resting level of free calcium (i.e., the level in unfertilized eggs) so only the "top response" could be seen. When one considers that the photomultiplier current rose no more than 10-fold in the sea urchin experiments, while it rose more than 10,000-fold in the medaka experiments, one sees that only the very

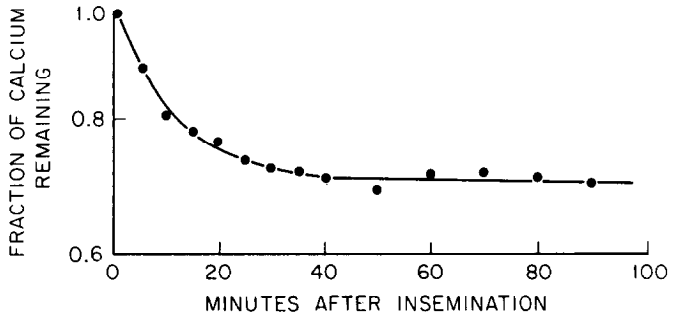

FIG. 1. The fall in total calcium content of *Arbacia* eggs following insemination. Before each measurement, loosely bound Ca was washed off by a 3-min wash in Ca-free seawater. The curve is a theoretical one, closely approximated by the expression 0.7 ± 0.3 ($e^{-.1t}$). From Azarnia and Chambers, (1976, Fig. 8).

a pulse duration which is close to that 15 min later seen directly in the medaka egg (Ridgway *et al.*, 1977). Altogether the data suggests that the sea urchin pulse has about twice the duration and half the amplitude of the medaka one. However, these are very uncertain differences. The important point is that the two seem to be so similar in magnitude.

ALL DEUTEROSTOME EGGS MAY BE ACTIVATED BY A TRAVELING INTERNAL CALCIUM EXPLOSION

1. Similar Waves Cross Most Activating Deuterostome Eggs

By now, similar exocytotic waves have been described in about two-dozen species; including members of the brown algae, sponges, hemichordates, cartilaginous fishes, bony fishes, and amphibia as well as the originally observed lampreys and echinoderms. Altogether, such waves are now known in eggs from most of the main groups of deuterostomes with externally fertilized eggs (Table 2). Furthermore, this list includes eggs naturally activated by sperm in both salt- and freshwater, as well as eggs activated by spawning whether or not sperm are present and enter the egg. It also includes eggs ranging from 74 to 3300 μm in diameter and thus a range of nearly 100,000-fold or 10^5 in volume.

Over every case listed in Table 2 the range of average wave velocities is about 10-fold—going from 3 μm/sec (*Strongylocentrotus* at 8°C) to 27 μm/sec (*Acipenser* at 22°C). However, if one restricts one's attention to a moderate temperature range of 20 ± 5°C, and excepts the largest eggs—ones wider than 1.6 mm—than the range of reported velocities is less than threefold; going from 5 to less than 15 μm/sec. This narrow range suggests that the same basic mechanism is involved in every egg listed.

"tip of the iceberg" was detected; hence the few minutes duration of these pulses in no way modifies the 30-min estimate obtained from total calcium data.

So do several other similarities: first, as in eggs of the medaka, and of some other bony fish (Yamamoto, 1961), eggs of the lamprey (Yamamoto, 1944a, 1947), sturgeon (Detlaf, 1962), and frog (Goldenberg and Elinson, 1980) all show activation waves which are both easier to start and travel more quickly in their animal than in their vegetal halves. For example, using the same needle, it takes seven times less external calcium to successfully prick-activate a frog egg at its animal pole than at its vegetal one (Goldenberg and Elinson, 1980). Again, if a lamprey egg is prick-activated at its animal pole or its vegetal one, then the resulting waves take 2 1/2 and 4 1/2 min to reach the equator, respectively (Yamamoto, 1944a). As a result, when various eggs are activated by nonlocalized stimuli, such as heat or hypotonic media, the resulting wave always starts at the animal pole.

Moreover, Goldenberg and Elinson have made the interesting observation that in immature or somewhat CO_2-intoxicated frog eggs, that exocytosis may spread through the animal half and then stop sharply at the pigment boundary. This suggests some sharp discontinuity in the character or concentration of the wave-propagating components there. Such a discontinuity is also suggested by the results of iontophoretically injecting calcium into frog eggs (Cross, 1981). After injections into the animal half, a fertilization potential (followed by various other indicators of activation) appears within a few seconds; after injections in the vegetal half, within 1.0 min; in the grey zone, within 0.3 min. It would be interesting, and surely practical (Schroeder, 1980), to compare the excitability and wave speeds in the animal and vegetal halves of sea urchin eggs.

2. The Ascidian Egg

I would also like to consider the enigmatic, but very interesting, case of the activating ascidian egg. There is good evidence that the egg proper does not undergo the massive and rapid exocytosis found in so many other activating eggs; rather, there is some evidence in the older literature that membrane elevation may be produced by secretion from the peculiar test cells which are embedded in the egg's surface (Reverberi, 1971, p. 525). Nevertheless, within a minute or two after dechorionated *Ciona* eggs are inseminated, a *contractile* wave starts at the animal pole and then sweeps over the egg, reaching the vegetal pole in about 2 min (Sawada and Osanai, 1981).

This contractile wave progresses at a mean speed of about 2.5 μm/sec—close to the 5- to 15-μm/sec range characteristic of secretion waves in other eggs—and is reminiscent of the contractile wave long ago reported to accompany membrane elevation in the lamprey egg

(Okkelberg, 1914).[5] It seems to involve an irreversible contraction of an actin-rich cortical cytoskeleton toward the vegetal pole (Jefferey and Meier, 1983) and can be focussed on a region far from the vegetal pole if a calcium ionophore gradient is concentrated on this region (Jefferey, 1982). This suggest the possibility that the early contractile wave through the *Ciona* egg reflects a calcium wave similar to that in the medaka egg: In this view the focussing of contraction on the vegetal pole would result—at least in part—from a greater persistence of high free calcium at the vegetal pole similar to that seen directly in the medaka egg (Gilkey and Jaffe, unpublished).

3. The Mammalian Egg

Relatively little is yet known about egg activation in internally fertilized organisms such as mammals and higher plants. However, there is one hint of an activation wave in mammalian eggs. Some years ago, it was reported that "in [rat and mouse] eggs that contain two sperms, the points of entry through the zona were seen much more frequently in opposite hemispheres than in the same hemisphere, an observation which suggests that the zona reaction is a propagated change" (Braden *et al.*, 1954). Moreover, hamster eggs show two phenomena which seem characteristic of deuterostome eggs, which are generally wave-activated, but not of protostome eggs, which are synchronously activated (see below). They can be prick-activated (Uehara and Yanagimachi, 1976) and they can be more easily activated by a calcium ionophore in low-calcium media than in normal ones (Steinhardt *et al.*, 1974).

It is also true that relatively slow but large rises in free calcium (from <0.1 μM to >5 $\overline{\leq} M$) have been reported in aequorin-loaded mouse eggs when they are artificially activated with alcohols (Cuthbertson *et al.*, 1981). However, these results are very hard to interpret since exhaustion of the aequorin made it impossible to follow the subsequent fall (if any) in free calcium, and because the injected cells were all sacrificed before cleavage might have started. Thus there is a serious possibility that these results were obtained with dying eggs.

4. What are the Sources of Calcium in Activating Deuterostome Eggs?

It is clear that the primary sources of activation calcium in medaka eggs and in sea urchin eggs are internal ones. Presumably, this is likewise true for most or all other deuterostome eggs: but just what are these internal sources? We suggested that they are specialized regions of the egg's endoplasmic reticulum analogous to those of muscle cells (Gilkey *et al.*, 1978). The main basis for this suggestion was the fact that in some (and perhaps most) muscle cells, natural contraction is mediated by "regenerative" or calcium-stimulated calcium release from specialized subsurface cisternae of the endoplasmic reticulum.

There is compelling evidence for an important natural role for such regenerative release in adult mammalian and avian heart muscle (Fabiato and Fabiato, 1979; Fabiato, 1982) and good evidence for it in mammalian skeletal muscle (Barrett and Barrett, 1978; Fabiato, 1982) as well as amphioxus skeletal muscle (Henkart *et al.*, 1976, pp. 342–344). In adult rat ventricle—a tissue in which regenerative calcium release is especially well-developed and well-studied—the minimum trigger level is at a pCa of about 7.1 (0.08 μM Ca); the optimum trigger level at a pCa of about 6.25 (0.6 μM Ca); and the peak free Ca^{2+} is at a pCa of about 5.4 (4 μM Ca^{2+}). Even in mammalian fast skeletal muscle—a tissue in which a natural role for regenerative calcium release has been harder to prove—Fabiato has succeeded in triggering calcium release (in skinned fibers) by raising the external pCa from 7.0 to 5.8 (2 μm Ca^{2+}) within 0.2 seconds. Thus there is a clear precedent for natural regenerative release of calcium from the endoplasmic reticulum (ER); but to my knowledge, from no other source.

One of the key findings on regenerative calcium release in muscle is its sharp dependence upon the *speed* of the change in free cytosolic calcium. This striking rate dependence probably results from the shorter response time of the regenerative release mechanism than of the closely associated (and counteracting) uptake mechanism in the endoplasmic reticulum. In any case it could well provide an explanation for the fact that suboptimal local calcium increases in eggs sometimes induce local exocytosis without inducing a spreading wave of exocytosis.

Recently, Gardiner and Grey (1983) have discovered similar subsurface cisternae in ripe *Xenopus* eggs. Their distribution in both space and time strongly suggests that they are indeed a source of activation calcium in these eggs. They are two to three times more concentrated in the cortex of the animal hemisphere than in the cortex of the vegetal hemisphere of these eggs; a distribution that might provide, or help provide the greater wave speed and excitability of the animal hemisphere. They become about 10-fold more abundant during oocyte maturation; disappear locally within a minute after the exocytotic wave reaches their vicinity; and show some continuities with deeper portions of the ER, which were reported earlier to shrink after fertilization

[5] Schroeder (1975) has reviewed several cases of contractile waves in prostostomes which start (at the animal pole) long after activation of the egg. *These late contractile waves are all much slower*—0.3 to 0.6 μm/sec rather than 5 to 15 μm/sec—and therefore may well be propagated by a rather different mechanism, e.g., a calcium-mediated chain reaction which is propagated mechanically rather than by diffusion (cf. Odell *et al.*, 1981).

(Campanella and Andreucetti, 1977). Since there is evidence that subsurface cisternae in other cells swell and shrink as their calcium content rises and falls (Henkart *et al.*, 1976, pp. 342-344), such appearance, growth, shrinkage and disappearance may well correspond to a gross storage and release of calcium.

Thus the evidence for an ER source of activation calcium in deuterostome eggs has grown strong. However, evidence for another—or is it another?—source should not be forgotten. Yamamoto has reported the existence of tiny "a-granules" around the large cortical vesicles of ripe medaka eggs: vesicles of about 0.3 to 0.5 μm in diameter which are barely visible under high-power light microscopy, are dark red—at least in the orange-red strains that he studied—and are seen to fade out during exocytosis (1962).

THE ACTIVATION OF PROTOSTOME EGGS INVOLVES SYNCHRONOUS AND PROLONGED CALCIUM PULSES

The limited evidence on this point is summarized in Table 3. The eggs of two polychaetes, of *Limulus*, of two barnacles, and of a squid all exhibit an episode of extended exocytosis during activation which indicates a prolonged (≥10 min) period of high cytosolic calcium. On the other hand, there are a considerable number of spiralian eggs (all of which are shed into the sea in the germinal vesicle stage) which exhibit virtually no exocytotic reaction either when shed or when fertilized. These include eggs of the bivalve molluscs *Mytilus*, *Spisula*, and *Barnea;* and the echiuroid worms *Urechis* and *Ikedosoma* (Austin, 1968, p. 102). In all of these cases, there is no morphological basis for inferring the presence (or absence) of a calcium increase during activation. However, in the best-studied members of this group—*U. caupo* and *B. candida*—$^{45}Ca^{2+}$ flux and net calcium data strongly support the existence of such an increase (work of Johnston and Paul, 1977 reviewed by Jaffe, 1980 as well as Jaffe *et al.*, 1979; Dubé and Guerrier, 1982); so altogether, the available evidence, while rather limited, nevertheless supports this same conclusion for protostome eggs generally.

However, in all cases where the cortical reaction has been closely observed in protostome eggs, it is reported

TABLE 3
EVIDENCE FOR CALCIUM PULSES IN ACTIVATING PROTOSTOME EGGS

Group		Activated by	Wave?	Evidence for a calcium pulse[i]
Polychaetes	*Nereis*[a]	Sperm	No	Rapid jelly secretion *in vivo.* Slow (5 min) exocytosis in e.m.
	Sabellaria[b]	Shedding and sperm	No?	Rapid jelly secretion and exocytosis *in vivo;* then slow (5 min) exocytosis in e.m.
Echiuroids	*Urechis*[c]	Sperm	No	Ca^{45} flux; net Ca change; electrophysiology
Xiphosura	*Limulus*[d]	Sperm[h]	No	Pitting *in vivo* at 5 min; exocytosis of 0.5-μm vesicles within 3 to 6 min when fixed; of 4-μm vesicles, 10-60 min
Crustaceans	Barnacles[e] *Balanus*, and *Pollicipes*	Sperm	?	Slow (20 min) membrane elevation *in vivo;* slow exocytosis in crude e.m.
Pelecypods	*Barnea*[f]	Sperm and shedding	?	Ca^{45} flux
Cephalopods	*Loligo*[g]	?	?	E.m. shows slow but extensive exocytosis

[a] Lillie (1911), Fallon and Austin (1967).
[b] Novikoff (1939), Pasteels (1965), Wilson (1968).
[c] Johnston and Paul (1977), Jaffe *et al.* (1979).
[d] Bannon and Brown (1980), Brown and Clapper (1980).
[e] Walley *et al.* (1971), Lewis (1975), Klepal *et al.* (1979).
[f] Dubé and Guerrier (1982).
[g] Arnold and Williams-Arnold (1976).
[h] But shedding alone will work albeit more slowly (G. G. Brown, unpublished).
[i] Clark *et al.* (1977) have reported a cortical reaction in öocytes of the shrimp, *Penaeus*, which is magnesium rather than calcium dependent. This remarkably violent and synchronous reaction is initiated by spawning of the öocytes into seawater rather than fertilization. However, close examination of the process clearly shows that the membranes of the öocyte's cortical vesicles fuse with the öolemma prior to spawning so as to form stable crypts. The magnesium in the seawater acts on these crypts to induce swelling and dissolution of the large rods within them, rather than membrane fusion (Clark *et al.* (1980)).

to start in different regions of the egg surface at about the same time rather than starting at one point and spreading in a wave. In *Nereis*, for example, Lillie reported in 1911 that "the effect of the localized stimulus of the attached spermatozoon is practically instantaneously effective over the entire extent of the membrane; it is more like an electrical discharge or some other physical disturbance than a chemical effect." Furthermore, activating eggs of *Urechis* and of *Spisula*, while exhibiting no exocytotic reaction, do elevate a protective membrane starting about 4 min after fertilization and this process again does not start at one point and spread in a wave but starts everywhere more or less simultaneously (Tyler, 1931; Gould-Somero and Holland, 1975; Allen, 1953). So the inferred increases of free calcium within activating protostome eggs can be called *synchronous*.

However, within any one region of a protostome egg, the fusion of different vesicles occurs over a period of the order of 100 times longer than in deuterostone eggs. In sea urchin and in medaka eggs the exocytotic wave front is relatively sharp—that is why the waves are so obvious!—and fusion of almost all of the vesicles in a given zone occurs within 1 to 10 sec, respectively (for references, see Table 1 and also Iwamatsu and Ohta, 1976, plate 2B). Yet as early as 1939, Novikoff compared his observations on *Sabellaria* with those of Moser on *Arbacia* and noted that the "disappearance . . . [of these granules] . . . requires but 10 seconds [for the whole wave in Arbacia] as compared with . . . 5 to 10 minutes in Sabellaria." Table 3 lists subsequent similar observations. Most recently, Brown and Clapper (1980) have observed the cortical reaction in *Limulus* eggs. Fusion of even the small (0.5 μm) outermost cortical vesicles is not completed until 3 to 6 min after shedding and insemination, while fusion of the larger (4 μm) and deeper vesicles is not completed for 10 to 60 min. One can infer that cytosolic calcium in activating protostome eggs does not rise as far as it does in deuterostomes but remains near its peak levels for a much longer period. Moreover, there is nothing in these or other observations to suggest that the inferred increases of calcium within any protostome eggs are regenerative. So it seems best to refer to them as *prolonged pulses* rather than explosions.

Protostome Eggs May Be Primarily Activated by the Entry of Calcium through Voltage-Gated Channels

It is clear that activating protostome eggs differ from those of deuterostomes in lacking waves and in undergoing a far slower process of exocytosis. A survey of the literature reveals four other striking and systematic differences between egg activation in these two groups (Table 4). All six of these striking differences can be understood if one postulates that protostome eggs are primarily activated by calcium which enters the cytosol from the medium in response to a shift in membrane potential. Indeed, this is the main new hypothesis put forward in this paper.

(1) Since membrane depolarization occurs over an entire egg practically at the same time, this mechanism would explain the absence of activation waves in protostome eggs. (2) The relative slowness of exocytosis in protostome eggs can be understood if the underlying increase in calcium is not explosive. By the postulated mechanism, calcium could continue to enter the cytosol as long as the fertilization potential continued. This lasts about 9 min in *Urechis* (Jaffe *et al.*, 1979). (3) The easy reversibility of activation in *Urechis* and *Spisula* eggs can likewise be understood if the underlying calcium rise is not regenerative. So can its remarkable repeatability in these forms; by reversing the process with acid seawater, it is possible to sperm-activate the same *Urechis* egg at least three times in a row (Tyler and Schultz, 1932) and the same *Spisula* egg at least twice (Allen, 1953). Unlike an intracellular calcium source, an external one cannot be exhausted. (4a) The reversal of activation in *Spisula* and *Urechis* eggs by early reductions in external calcium can also be easily understood if the activating calcium rise in these eggs comes from the medium and is not regenerative. (4b) The inhibition of ionophore A23187 action on *Chaetopterus* and on *Spisula* eggs by reduced external calcium is in striking contrast to the stimulating effect of such a reduction on sea urchin and on hamster eggs. It can be understood if *Chaetopterus* and *Spisula* eggs lack the large stores of easily releasable calcium found in deuterostome eggs. (5) The 30% *increase* in net calcium in activating *Urechis* eggs is likewise in striking contrast to the corresponding decrease in sea urchin eggs. It can obviously be understood if the activation calcium in *Urechis* enters the cytosol from the medium instead of internal stores. Furthermore, *Urechis* eggs can be sperm-activated in the presence of enough D-600 to reduce calcium entry to a tenth of its normal value but no lower (Gould-Somero *et al.*, 1979); while in *Barnea*, enough D-600 to reduce calcium entry to a seventh reduces the number of activated eggs to control levels (Dubé and Guerrier, 1982). Again, this can be understood if the primary natural source of activation calcium in *Urechis* and *Barnea* eggs is assumed to be external. (6) The activation of *Spisula* eggs by small increases in extracellular potassium—half the eggs are activated by raising external K^+ less than threefold, from about 9 to 25 μM—can be understood if they are naturally activated by calcium entry through voltage-gated channels. Fur-

TABLE 4
CONTRASTS IN THE ACTIVATION OF DEUTEROSTOME AND PROTOSTOME EGGS

	Deuterostomes	Protostomes
1. Activation wave?	Always?[a]	Never[b]
2. Duration of local fusions:	1 to 10 sec[a]	≥300 sec[b]
3. Effects of early inhibition:	Local activation (M[c], Su[d])	Reversal (Sp[e], U[f])
or of too small a stimulus:	Local activation (M[g], Su[h,i])	Reversal (Sp[e], U[f])
4. Effects of reducing external Ca^{2+} on activation by:		
(a) Sperm	None (M[j], Su[k])	Reversal (B[s]?, Sp[e], U[f])
or by:		
(b) A23187	Stimulation (H[l], Su[h])	Inhibition (C[m], P[t], Sp[n])
5. Change in net internal Ca^{2+}:	30% Fall (Su[o])	30% Rise (U[p])
6. Effects of high external K^+:	None (Su[q])	Activation (A[u], B[s], C[r], P[t], Sp[e], U[v])

Note. A = *Amphitrite;* B = *Barnea;* C = *Chaetopterus;* H = hamster; M = madaka; P = *Pectinaria;* Sp = *Spisula;* Su = sea urchins; U = *Urechis.*

[a] See Table 2 and text.
[b] See Table 3 and text.
[c] Sakai (1964).
[d] Allen (1954), Allen and Hagstrom (1955), Allen and Rowe (1958).
[e] Allen (1953).
[f] Tyler and Schultz (1932), Gould-Somero (1975).
[g] Yamamoto (1961).
[h] Chambers and Hinkley (1979).
[i] Millonig (1969), Rossignol *et al.*, in press.
[j] Gilkey *et al.*, (1978, p. 453).
[k] Chambers (1980), Chambers and Angeloni (1981), Schmidt *et al.* (1982).
[l] Steinhardt *et al.* (1974).
[m] Brachet and Donini-Denis (1978), Eckberg and Carroll (1982).
[n] Schuetz (1975).
[o] Örström and Örström (1942), Monroy-Oddo (1946), Azarnia and Chambers (1976).
[p] Johnston and Paul (1977).
[q] Jaffe (1976), Hagiwara and Jaffe (1979, pp. 411–412).
[r] Ikegami *et al.* (1976), Hagiwara (1979).
[s] Dubé and Guerrier (1982).
[t] Anstrom and Summers (1981).
[u] Mead (1896).
[v] M. Gould-Somero, unpublished.

thermore, the relatively long (≥4 min) exposure to high K^+ needed can be understood if they are naturally activated by a prolonged fertilization potential. Similarly, *Barnea* eggs are completely activated if external K^+ is raised from about 9 to 61 m*M* in as little as 1 m*M* Ca^{2+}. It takes about 2 min of exposure to such a solution to activate all eggs (Dubé and Guerrier, 1982).

Confirmation and extension of this inference to *Urechis* eggs is furnished by a penetrating study of their fertilization potential (Jaffe *et al.*, 1979). It shows that the large calcium influx which follows fertilization goes largely through voltage-gated channels within the egg's membrane. These channels, in turn, are opened by a radical (up to 85 mV) shift in membrane potential produced by sodium leakage through or near the (inserted) sperm membrane. This last was elegantly shown by the fact, that up to at least 50 sperm per egg, the amount of ^{24}Na which enters polyspermic eggs is directly proportional to the number of sperm which enter (Jaffe *et al.*, 1979, Fig. 7b). It was later confirmed by more direct experiments (Gould-Somero, 1981).

Here, yet another contrast between protostomes and deuterostome eggs presents itself. In *Urechis*, it appears that sodium leakage through or near the inserted sperm membrane is responsible for the fertilization potential and thus for letting in calcium. By contrast, in frog eggs, there is good evidence for a reverse relationship; the rise in cytosolic calcium seems to open chloride channels and thus produce the fertilization potential (Cross, 1981).

WHY DOES EGG ACTIVATION DIFFER IN PROTOSTOMES AND DEUTEROSTOMES?

The differences displayed in Tables 2–4 are striking and consistent—particularly the presence of activation

waves in deuterostome eggs (as well as brown algae and sponges) and their absence in protostomes. What does this mean?

One might wonder if these differences are actually associated with the meiotic stage at the time of fertilization rather than protostomy. It is true that the organisms with egg activation waves tend to be fertilized at later meiotic stages than those without such waves. However, there is just enough overlap in this regard as to suggest that meiotic stage is irrelevant: Among eggs displaying activation waves, *Saccoglossus* and *Ciona* are fertilized in metaphase I and *Tetilla* in the germinal vesicle stage (Costello and Henley, 1971; Rothschild, 1956). On the other hand, among organisms that appear to have the activation properties of protostomes, *Pectinaria* and *Chaetopterus* are likewise fertilized in metaphase I (Costello and Henley, 1971; Rothschild, 1956; Anstrom and Summers, 1981).

One might also wonder if these differences are actually associated with egg size rather than protostomy. It is true that the organisms with egg activation waves tend to have larger eggs than those without such waves. This tendency is particularly clear if one restricts one's attention to the better characterized eggs listed among protostomes in Tables 3 and 4. Nevertheless, there is again enough overlap between the two groups as to suggest that size is irrelevant or at least secondary: Among eggs displaying activation waves, *A. punctulata* and *Stronglyocentrotus purpuratus* are only 75 μm in diameter. While among the better characterized protostome eggs, *U. caupo* has a maximum diameter of 130 μm (Gould-Somero, 1975) and *N. limbata* a maximum diameter of 140 μm (Costello and Henley, 1971).

In considering the contrasts between the two groups, one should distinguish evidence concerning propagated vs synchronous activation on the one hand, from evidence concerning internal vs external calcium sources on the other hand. Synchronous activation certainly suggests a mechanism which is electrically mediated, and more specifically one which is mediated by calcium entry through voltage-sensitive channels. However, one could easily imagine cases in which synchronous calcium entry in turn triggered calcium release from internal stores. Indeed, the initial violent synchronous exocytosis observed in *Nereis* and in *Sabellaria* eggs might suggest a mechanism of such an "intermediate" character. It is only in *Urechis*, and to a lesser extent in *Spisula* and *Barnea* eggs that we have relatively direct and substantial evidence for an external source of the bulk of the activation calcium. So at present the clearest general difference between the two groups is the presence of propagated activation in deuterostomes (and some lower forms) versus synchronous activation in protostomes.

Since this difference seems to be rather general and not connected with either meiotic stage or egg size, one wonders whether it is somehow connected with fundamental differences in mechanisms of pattern formation. Is that not what deuterostomy and protostomy refer to?

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Note added in proof. Using aequorin, Eisen *et al.* have at last seen calcium waves crossing sea urchin eggs during fertilization.

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