A FREE CALCIUM WAVE TRAVERSES THE ACTIVATING EGG OF THE MEDAKA, *ORYZIAS LATIPES*

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ABSTRACT

Aequorin-injected eggs of the medaka (a fresh water fish) show an explosive rise in free calcium during fertilization, which is followed by a slow return to the resting level.

Image intensification techniques now show a spreading wave of high free calcium during fertilization. The wave starts at the animal pole (where the sperm enters) and then traverses the egg as a shallow, roughly 20°-wide band which vanishes at the antipode some minutes later. The peak free calcium concentration within this moving band is estimated to be about 30 μM (perhaps 100–1,000 times the resting level). Eggs activated by ionophore A23187 may show multiple initiation sites. The resulting multiple waves never spread through each other; rather, they fuse upon meeting so as to form spreading waves of compound origin.

The fertilization wave is nearly independent of extracellular calcium because it is only slightly slowed (by perhaps 15%) in a medium containing 5 mM ethylene glycol-bis[β-aminoethyl ether]N,N'-tetraacetic acid (EGTA) and no deliberately added calcium. It is also independent of the large cortical vesicles, which may be centrifugally displaced. Normally, however, it distinctly precedes the well-known wave of cortical vesicle exocytosis.

We conclude that the fertilization wave in the medaka egg is propagated by calcium-stimulated calcium release, primarily from some internal sources other than the large cortical vesicles. A comparison of the characteristics of the exocytotic wave in the medaka with that in other eggs, particularly in echinoderm eggs, suggests that such a propagated calcium wave is a general feature of egg activation.

KEY WORDS aequorin · calcium ion · fertilization · medaka egg · *Oryzias latipes* · wave of activation

Most unfertilized eggs are in a state of developmental arrest. One important function of fertilization is to trigger a complex set of biochemical

and biophysical events, collectively referred to as activation, which culminates in the continuation of development. Activation has long been thought to involve and require at least a transient increase in free calcium in the cytoplasm (55). Proof of this theory should have three parts: first, a measurement of the rise (if any) of free cytoplasmic calcium during activation; second, evidence that direct artificial induction of a comparable or smaller rise in free calcium will activate the eggs; and third, evidence that prevention of the natural rise will block activation.

The first direct and convincing evidence of point one—a natural rise in free calcium—was recently reported for the egg of the medaka (*Oryzias latipes*), a fresh water oriental killifish widely used in student laboratories (43). This investigation utilized the calcium-specific photoprotein, aequorin, a remarkable substance which luminesces at a rate that increases with the level of free calcium. Aequorin has been injected into a variety of living cells to continuously monitor their free cytoplasmic calcium over many hours (49). Medaka eggs that have been injected with aequorin show a transient, 15,000-fold increase in luminescence during activation, clearly indicating a large transient rise in free cytoplasm calcium.

In that study, we also presented evidence of point two: Treatment with ionophore A23187 initiates activation after passively raising free calcium to a level below the natural sperm-induced peak.

To clarify certain aspects of the present findings, we must first describe certain features of a medaka egg that are observable with a light microscope (Fig. 1). The egg has a large central yolk compartment surrounded by a thin layer of cytoplasm, which is in turn bounded by the plasma membrane and a tough protective membrane, the chorion. The chorion allows sperm entry only through a small funnel-shaped opening at the animal pole called the micropyle. The cytoplasm contains a closely packed layer of large cortical vesicles,[1] 10–40 μm in diameter, which are found everywhere except in a small region near the micropyle (and also where they are excluded by another prominent group of inclusions, the numerous, randomly distributed large oil droplets). Upon sperm entry through the micropyle (or artificial activation with a needle anywhere on the
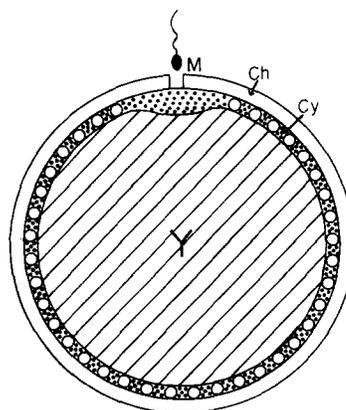
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[1] We call them vesicles rather than "alveoli" or "granules" because they are surrounded by a membrane.

FIGURE 1 Diagram of unfertilized medaka egg (1.2-mm diameter). A sperm will cross the chorion (*Ch*) via the micropyle (*M*), enter the cytoplasm (*Cy*) and initiate a wave of cortical vesicle secretion. Vesicles are indicated by small circles. The bulk of the egg is occupied by a membrane-bounded yolk compartment (*Y*). The cytoplasmic thickness (0.03 mm) is exaggerated, and oil droplets are omitted for clarity.

surface), the cortical vesicles fuse with the plasma membrane, fusion beginning at the site of stimulation and spreading over the cortex in a circular wave that eventually closes upon itself at the antipode; this fusion wave requires about 2 min to traverse the egg at room temperature (64).

On the basis of such observations, and other experimental manipulations, Yamamoto (61, 64) long ago postulated that an invisible "fertilization wave" propagates over the medaka egg during activation and gives rise to the observed vesicle fusion wave. Our previous observations of the time course of total light emission by activating aequorin-loaded eggs suggested that the process of calcium release is both explosive and propagated, i.e., it is calcium-stimulated and normally spreads over the egg from a site of localized increase in calcium which is somehow introduced by the sperm. Thus, they suggested that a free calcium wave is the material basis of Yamamoto's fertilization wave.

So bright is the glow of an activating, aequorin-loaded medaka egg that in a dark room we could easily see it with our naked eyes. We were therefore encouraged to observe the spatial distribution of the free calcium transient during activation with the aid of a microscope and an image intensifier. Our main finding is simple: There is, indeed, a calcium wave propagated through the activating egg's cytoplasm.

MATERIALS AND METHODS

Unfertilized medaka eggs were obtained and injected with aequorin as described previously (43). It should be emphasized that the luminescent reaction (between aequorin and free calcium within the egg) is so slow that very little of the aequorin is used up during activation of the egg (43). Hence the fall in luminescence seen after the peak of light (in our previous study) and behind the moving zone of light (in this one) indicates a fall in free calcium rather than a fall in aequorin.

The injected eggs were then placed in a clear plastic Petri dish and rotated to the desired orientation, using the micropyle as a landmark. Some successful experiments were done without further means to hold the eggs in position. However, this proved rather troublesome, so one of several means of accessory support was usually employed. These included the use of petroleum jelly; trapping between gold, silver, or stainless steel pins; or support in a close-fitting hole drilled in Plexiglas. Sperm were obtained by the method of Yamamoto (64).

Most experiments were carried out in Yamamoto's Ringer's solution. This medium contains 128 mM Na$^+$, 1.8 mM Ca^{++}, 132 mM Cl$^-$, and is buffered at pH 7.3 with bicarbonate. Some experiments were carried out in a modified calcium-free medium that contained 63 mM Na$^+$, 40 mM Mg^{++}, 2.6 mM K$^+$, 146 mM Cl$^-$, and 5 mM ethylene glycol-bis [β-aminoethyl ether]N,N'-tetraacetic acid (EGTA), and was likewise adjusted to pH 7.3 with NaOH. High magnesium was used to allow fertilization in the absence of calcium (61). Eggs were artificially activated by adding the Lilly ionophore A23187 (Eli Lilly and Company, Indianapolis, Ind.). Several microliters of a 0.1% solution of A23187 in dimethysulfoxide were gently pipetted into the medium near the egg, where a smoky precipitate of the drug formed immediately.

In experiments performed in Dr. Reynolds' laboratory, we used his previously described image intensifier system (42). In this system a magnified image of the object (in this case, the self-luminous medaka egg) is focused by a microscope on the cathode of an image intensifier tube (EMI 4 stage number 9694). The output phosphor of the intensifier is viewed by a Plumbicon vidicon (Phillips TV Plumbicon camera LDH 1051/01, control unit LDH 1060/01, fitted with Amperex Plumbicon vidicon XQ-1020 L). The camera image is presented in real time on a television monitor (concord VM 12) and simultaneously recorded on magnetic tape (Concord VTR 2000). Photographs for analysis and display were taken later of videotape playbacks. In experiments performed in Dr. Ridgway's laboratory, we used a similar system employing a Machlett (ML-8685) image intensifying "sniper-scope" kindly loaned to us by the Night Vision Laboratory of the U.S. Army. The output of this image intensifier was then directly photographed with a Nikon 35-mm camera (f 1.4 lens). The overall optical magnification of each system was calibrated with a stage micrometer.

Most of the information was necessarily gathered with the aid of objectives having a field just broad enough to image an entire 1.2-mm diameter egg. Specifically, two × 10 objectives were used: a Zeiss with a numerical aperture of 0.22 and a Leitz with one of 0.30. For comparison with previous results with a lensless system and a simple photomultiplier (43), it is important to realize that although the simple photomultiplier and the image intensifier should have comparable quantum efficiencies, the use of a lens radically reduces the quantum efficiency of the system as a whole. Specifically, one can estimate that only about 1% of the light emitted by an egg reaches the image intensifier through the above lenses: so the net quantum efficiency has been reduced about 100-fold in exchange for spatial information.

RESULTS

The Calcium Wave

Fig. 2 illustrates our main finding. A moving zone of free calcium is seen to start at the micropyle (where the sperm enters) and cross the egg in about 2 min. The front of this zone indicates the region in which a propagated rise in free calcium exceeds the threshold sensitivity of our intensifier system, whereas the rear boundary indicates the region in which free calcium has then fallen below this threshold. Table I lists some characteristics of all of the calcium waves that we observed. The wave front took an average of 138 s to go from pole to pole (in those six experiments that were done at about 26°C and that yielded the most reliable values). Inasmuch as medaka eggs average about 1,100 μm in diameter (exclusive of the chorion), this transient time yields an average propagation velocity (at 26°C) of 12.5 μm/s.

The moving zone has the form of a shallow band about 20° wide. This band's depth is seen to be about 0.05-0.1 mm in the lower power views illustrated in Fig. 2. However, its radial bounds are better seen in the higher power views illustrated in Figs. 3 and 4. Fig. 3a shows a high-power view of the moving zone at it just begins, in the region beneath the micropyle. The moving zone seems to start significantly below the plasma membrane, but then soon spreads outward just to this membrane as well as inward to a level about 60-70 μm below it. As Figs. 3b and c show, the fully illustrated case is representative of the three available. Fig. 4 shows a high-power view of the free calcium zone as it moves through the egg's equator. Again, its outer boundary seems to be at the plasma membrane, and it extends inward

about 70–80 μm. The apparent depth of the luminescent zone is greater than the measured depth of the cytoplasm in the unfertilized egg, which we find to be about 20–40 μm at both the micropylar and equatorial regions. However, we are not sure whether some luminescence really originates below the yolk membrane (which seems unlikely) or whether the apparently greater depth of the luminescent zone is an artifact arising from various optical phenomena including scattering, refraction, reflection, and light originating in planes far above or below the plane of focus.

It can be seen in Fig. 2a that the zone's propagation velocity falls markedly as it crosses the egg. This is a consistent feature. To measure it reliably, we compared the time taken by each wave to cross the micropylar or animal half of the egg with that taken to cross its vegetal half. These determinations could be done most accurately in the four cases in which the egg's axis was horizontal, and these half-transit times are shown in Table I. Each wave took about 30–60% longer to cross the vegetal half than the animal one. In absolute terms, the average time taken to cross the animal hemisphere at about 26°C was 59 s. This yields an average propagation rate of 14.6 μm/s. The corresponding values for the vegetal hemisphere are 85 s and 10.2 μm/s. A comparable slowing of the wave of vesicle exocytosis has been reported by Iwamatsu (24), and our own
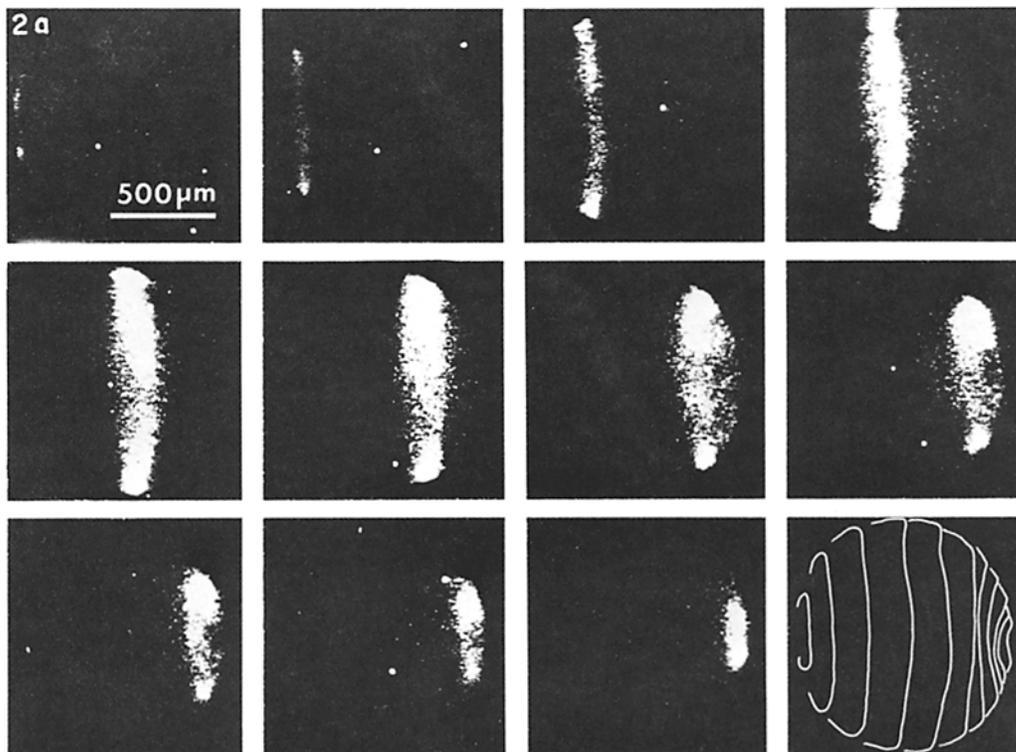

FIGURE 2 a   A free calcium wave propagating across a sperm-activated medaka egg. Successive photographs are 10 s apart. Egg axis horizontal with micropyle to the left. Last frame is a tracing showing the leading edges of the 11 illustrated wave fronts. Egg number 4 of 5/7/76 in Yamamoto's Ringer's. Bar, 500 μm.

FIGURE 2 b   Same as 2a except that the axis is vertical with the micropyle up. Egg number 2 of 5/5/76.

FIGURE 2c   Same as 2b except that a calcium-free Yamamoto's Ringer bearing 5 mM EGTA was used. Egg number 4 of 5/8/76.

FIGURE 2 d   Same as 2a except that the axis is oblique with the micropyle downward. The last three photographs include after-images in the intensifier tube resulting from external illumination of the egg. Egg number 1 of 6/11/76.
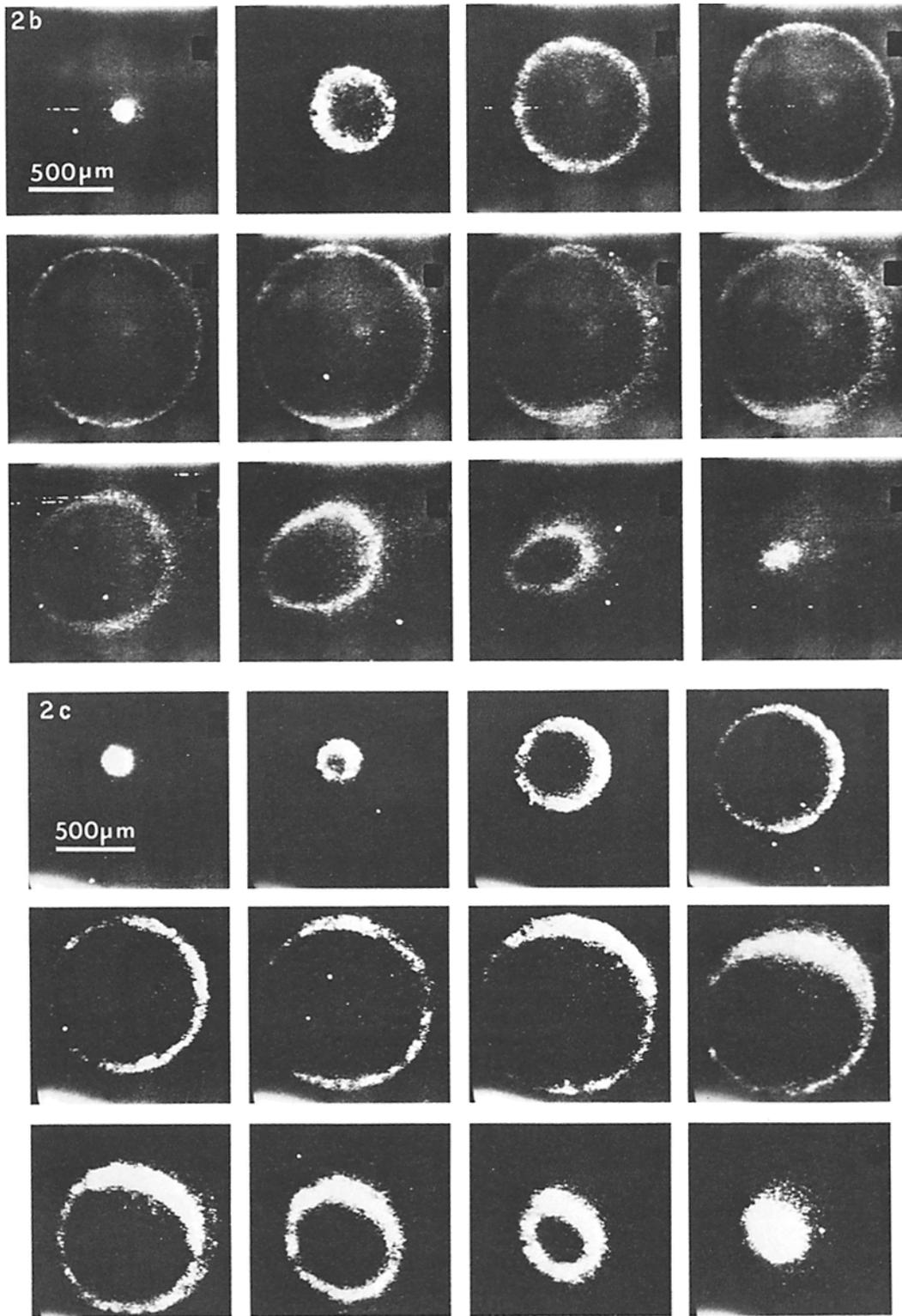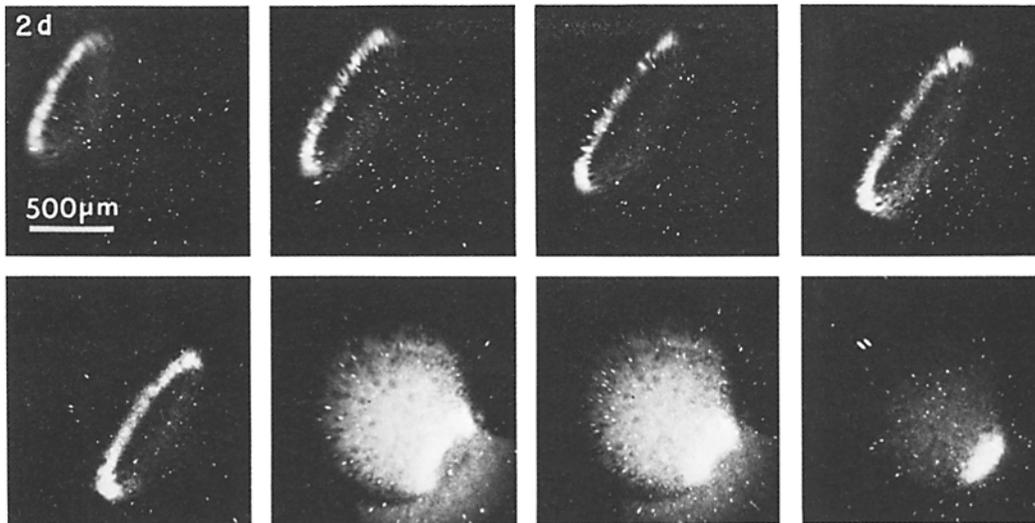
preliminary observations also indicate such a deceleration, though of somewhat smaller degree. It may also be noted in Fig. 2 that the moving zone both broadens and brightens as it traverses the egg. However, these latter changes are quite variable in degree, may even be absent, and even when present may be partly artifactual because the aqueorin was injected somewhat closer to the vegetal than to the animal pole.

Relation to the Vesicle Fusion Wave

If the free calcium wave is in fact Yamamoto's "fertilization wave," i.e., if it initiates all of the subsequent events of activation, then it should precede the wave of fusion of vesicles with the plasma membrane. To examine this question, we alternated photographs of the vesicle fusion wave with photographs of the free calcium wave. To make each photograph of the fusion wave, we interrupted the light path to the intensifier by manually inserting a prism, and thus redirected the light to an auxiliary camera. Then the egg was externally illuminated, a photograph taken, the external light shut off, and the prism manually withdrawn. It proved difficult to attain the desired records with this awkward technique. Nevertheless, one such effort succeeded, and the results are shown in Fig. 5. Within the obvious limitations of this experiment, it appears that the calcium wave does, indeed, precede the fusion wave. In the three comparisons derivable from this experiment, the delay between the detectable front of the calcium wave and the detectable beginnings of vesicle fusion varied from about 5 to 15 s.

Requirements for a Calcium Wave

Fig. 2c shows a free calcium wave traversing an egg (5/8, number 4, Table I) immersed and fertilized in a calcium-free, EGTA-bearing medium. (We estimate the free calcium level in this medium to be 10^{-9} M or less.)[2] There is no apparent qualitative difference between this wave and waves in the usual calcium-bearing medium. The same was true for a duplicate experiment on the egg of 5/7, number 5. There was, however, a small but apparently significant increase in the transit times found in calcium-free medium. Both of the two transit times recorded in this medium happened to be 161 s, whereas the nine relatively reliable transit times recorded in the usual calcium-bearing medium varied from 102 to 159 s, and averaged 138 s with a standard deviation of ±18 s. These results indicate that most or all of the calcium needed for propagation is released from internal sources.

Fig. 6 shows a calcium wave traversing an egg fertilized after being centrifuged hard enough to drive the large (≥10 μm) cortical vesicles to the centrifugal pole and the oil droplets to the centripetal pole. In this particular egg, the micropyle lies halfway between its centrifugal and centripetal poles and, therefore, the wave begins halfway between these poles. The wave appears to traverse

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[2] This was done by using published binding constants for EGTA (taking into account the pH, Mg^+, and ionic strength of our solution; 50) as well as the manufacturer's estimates of the maximum amount of calcium present as an impurity in the reagents used.

TABLE 1

Transit Times and Other Characteristics of the Activating Eggs Observed

Date*	Number	Temperature	Special conditions	Orientation	Objective	Number of waves	Transit times		
		°C					A half	V half	Total
								s	
A. Sperm-activated eggs									
5/5/76	1			Horizontal	×10	1	65	90	145
	2			Vertical	×10	1			159
	3			Vertical	×50‡	1			
	5			Horizontal	×10	1	58	78	136
	6			Horizontal	×40	1			
5/6	1			Vertical	×10	1			(115?)§
	2	25.8		Vertical	×10	1			(135?)§
	3	26.3		Vertical	×10	1			102
	4	26.6		Horizontal	×10	1	55	75	130
	5			Horizontal	×40‡	1			
5/7	1			Horizontal	×40‡	1			
	2			Horizontal	×40‡	1			
	3			Horizontal	×40||	1			
	4	27.4		Horizontal	×10	1	59	97	156
	5	27.2	Ca free	Vertical	×10	1			161
5/8	1	24.8	Centrifuged	Oblique	×10	1			143
	3			Oblique	×40	1			
	4	25.8	Ca free	Vertical	×10	1			161
6/9	1	21		Oblique	×10	1			
	2	21		Vertical	×10	1			
	3	21		Horizontal	×10	1			
	4	21		Horizontal	×10	1			155
	5	21		Horizontal	×10	1			
6/10	1	21		Oblique	×10	1			120
6/11	1	21	Relate to exocytotic	Oblique	×10	1			
	2	21	wave	Oblique	×10	1			
6/12	1	21		Oblique	×10	1			
	2	21		Oblique	×10	1			
B. Ionophore-activated eggs					×10				
5/5	4			Vertical	×10	1			98
5/6	6	26.9		Vertical	×10	≥6			
	7	26.5		Horizontal	×10	4			
	8	26.5	Ca free	Vertical	×10	2			141
5/7	6	27.3		Horizontal	×10	1			128
5/8	2	25.3	Centrifuged	Oblique	×10	1			151

*May experiments were done at Princeton; those in June were done at Richmond.

‡ Micropylar region.

§ Poor focus.

|| Edge of equatorial region.

the egg in an essentially normal manner, crossing the egg in about 143 s. Thus, it traverses the vesicle-free region in and near the oil droplets (seen as holes within the advancing front). Indeed, for some reason, the wave is particularly persistent in the oil droplet region. Evidently, the main source of calcium needed for propagation is not the large cortical vesicles themselves.

Ionophore-Initiated Calcium Wave

Fig. 7 shows calcium waves traversing two eggs that were activated with the calcium ionophore A23187 instead of sperm. Fig. 7a shows an egg activated with a relatively small dose of ionophore. The wave resembles a sperm-initiated wave

except for the fact that it began about 45° from the micropyle and crossed the egg relatively quickly. In fact, it took only 98 s, which compares with times of 136, 145, and 159 s for the three sperm-activated eggs studied on the same day. In crossing the micropylar region, the wave front is curiously perturbed. The wave front first falls behind the rest of the wave (in a section about 300-μm wide) and then catches up with it.

Fig. 7b shows an egg activated by a somewhat larger dose of ionophore. In this case, seven separate calcium waves were initiated at seven different points. Each of these waves was 45°-90° from the micropyle (which was upward, i.e. facing the reader): three began almost simultaneously
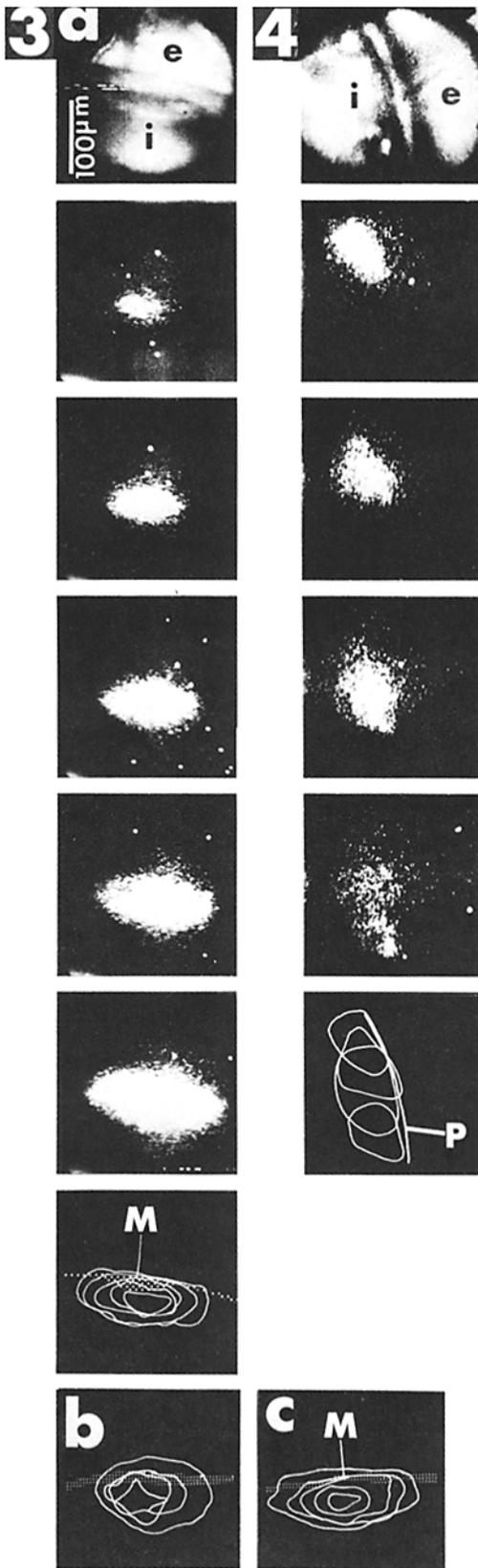
FIGURE 3 High-power views of initiation of calcium waves (made with a ×40 water-immersion objective). (a) View of micropylar region of egg number 1 of 5/7/76. Top photograph, made via transmitted light, of the unfertilized egg: e indicates extracellular region; i, intracellular. Bar, 100 μm. Middle five photographs show the luminescence from the cytoplasm beneath the micropyle during activation. (2 s elapsed between successive photograph.) Bottom photograph shows a tracing (taken directly from the negatives) of the boundaries of the spreading luminescent region superimposed on a stippled zone indicating the range of possible plasma membrane positions. The micropyle (M) is not evident in the photographs, but its position was observed with reference to the two chorionic hairs visible on the original videotape. (b and c) Tracings of egg number 5 on 5/6/76 and egg number 2 on 5/7/76, respectively. (Micropyle position unknown in 3b).

FIGURE 4 High-power view of propagation of calcium waves through the equatorial region of egg number 3 on 5/7/76. This egg's axis was horizontal with the micropyle toward eleven o'clock, but the micropyle is far out of the field of view. Again, the top photograph is a transmitted light view of the unfertilized egg. The middle four photographs show the luminescense emitted during passage of the calcium wave through the region viewed. (4 s elapsed between each successive photograph.) Bottom figure shows a tracing (taken directly from the negatives) of the boundaries of the spreading luminescent region superimposed on a tracing of the inner chorion boundary which should indicate the plasma membrane's position (P) in this case. Scale same as in Fig. 3.
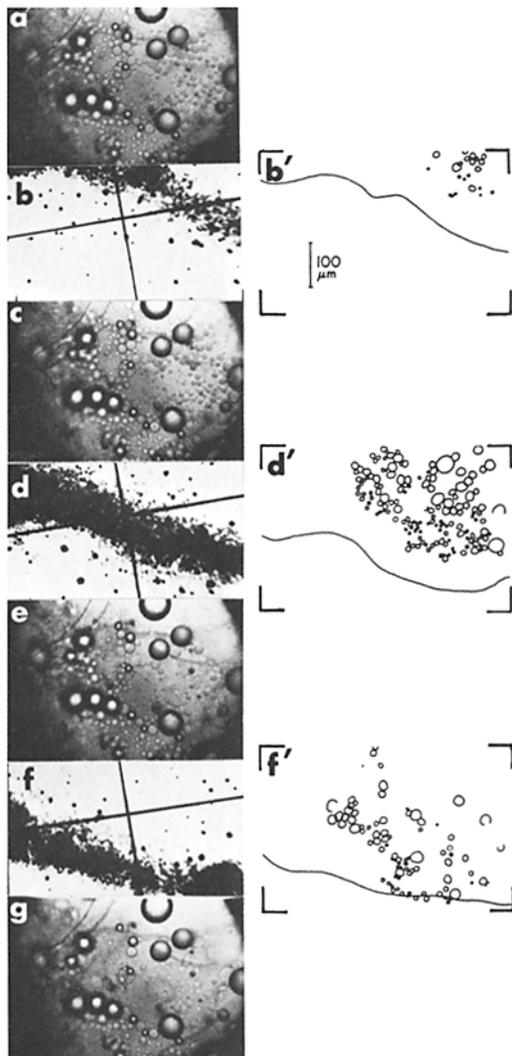
FIGURE 5 Relationship between calcium and vesicle fusion waves. The left-hand column shows alternate photographs (via transmitted light) of the egg's cortex, and reverse contrast, aequorin-mediated images of the calcium wave. Scale bar in b' indicates 100 μm. The right-hand column shows tracings of each calcium wave front superimposed on those cortical vesicle images that had changed or disappeared between the preceding and succeeding transmitted light photographs. For example, tracing b' represents the front of the calcium wave in photograph b superimposed on those vesicle images in photograph a that changed or disappeared in photograph c. (Note that the most prominent bodies in the photographs are oil droplets, structures that do not change during passage of the fertilization wave.) Each calcium wave front is taken to be the line at which the grain density first rises above background. About 13 s elapsed between successive transmitted light images. Each luminescent light image was produced by a 4-s exposure which began about 5 s after the last transmitted light photograph and ended about 4 s before the next one. Hence, each tracing of newly fused vesicles includes a group that had fused during a period which extended about 4 s later than the corresponding calcium image. Considering that the propagation rate of the wave in the equatorial region observed is about 10 μm/s, the vesicle fusion front at the end of the calcium wave exposure should actually have been about 40 μm behind its indicated location.

and then four others began about 8 s later. When these seven waves met, they did not propagate through each other. Rather, they fused to eventually form two separate rings, one of which rose to die out near the micropyle and the other of which fell to die out about 140° away. The waves propagated relatively quickly through the upper, animal half, taking 24 s from first initiation to upper closure but 112 s from first initiation to lower closure. In part, this can be attributed to the 30–60% greater average propagation velocity found in the animal half; in part, it can be attributed to the roughly 50% greater distance traversed to reach lower closure. However, the lower-half transit time was about fourfold greater,

so another factor must be invoked. We believe this second factor to be the action of the iono- phore which had direct access to the upper half but not to the lower half because this egg was embedded in petroleum jelly for support.

Observations of the Exocytotic Wave Via Transmitted Light

To better compare the relationship of the cal- cium and exocytotic waves, we measured the times taken for the exocytotic wave to cross our medaka eggs as a function of temperature. Eggs, with their micropyles upward, were observed at ×100 with a Leitz stereoscope. Timing began
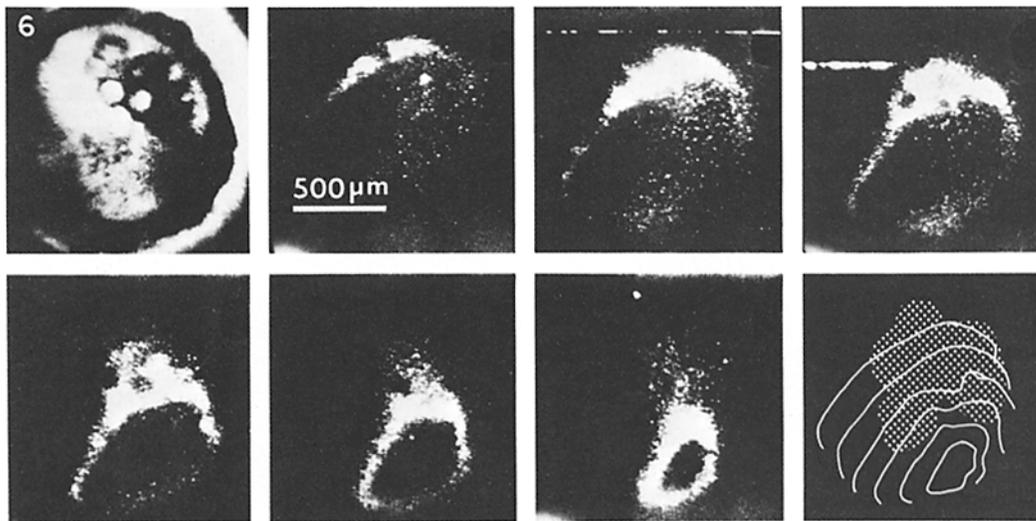
FIGURE 6 Free calcium wave propagating over a sperm-activated, centrifugally stratified egg. Successive photographs are 15 s apart. The egg was centrifuged so as to displace large (≥ 10 μm) cortical vesicles and all oil droplets to antipodal regions about 90° from the micropyle, leaving the remainder of the cytoplasm free of large inclusions. The first photograph is a transmitted light image of the egg, oriented with oil droplet-bearing region upward. Subsequent photographs show the calcium wave propagating through the cortical vesicle-free cytoplasm in and around the oil droplet region. Tracing shows the leading edges of the six illustrated wave fronts superimposed upon the (stippled) oil droplet region. Bar, 500 μm.

when exocytosis of large (10–40 μm) vesicles was first seen near the micropyle. Then, each egg was inverted, and we recorded the time when the exocytotic wave closed at the antipode. The results are shown in Fig. 8. The transit times for exocytosis fall from values of 200 s or more at 10°C to about 80 s at 33°C. This high temperature dependence indicates that the propagation rate of the exocytotic wave, and hence of the underlying calcium wave, is not exclusively limited by diffusion between calcium sources.

Fig. 8 also shows that the transit times for the calcium wave at 26°–27°C tend to be about 25% longer than those for exocytosis at this temperature. The significance of this difference is not clear at this time. We know that it is not caused by exposure of the eggs to anesthetic or by mere aging; so it may indicate some injury produced by injecting aequorin into the eggs. As was noted before, the development of aequorin-injected eggs arrests in the late blastula stage (43).

To better understand the beginnings of the fertilization wave, we also observed the micropylar region of activating eggs with Nomarski differential interference optics. On the basis of ordinary bright-field optics, Yamamoto (61) had previously

described a small region around the micropyle of the unfertilized egg as devoid of cortical vesicles. We can confirm that there is indeed a specialized micropylar region, about 100 μm in diameter, that is devoid of the large (10- to 40-μm diameter) vesicles found throughout the rest of the cortex. However, using Nomarski optics, we soon discovered that this region contains a considerable concentration of what seem to be miniature cortical vesicles fixed just beneath the plasma membrane. They are 1.5–5 μm in diameter and are one to several vesicle diameters apart in the micropylar region. Furthermore, they are also found, albeit at a lower and variable concentration, throughout the rest of the cortex interspersed between previously described larger vesicles. Although the increase in maximum vesicle size occurs rather abruptly around the micropylar region, we have not made counts sufficient to determine whether the distribution of vesicle sizes in the egg as a whole is truly bimodal.

We could clearly see the very first sperm enter the micropyle if we looked directly down it soon after insemination. If the egg had been rotated slightly so as to tilt the micropyle off the vertical, we could also see this first sperm dart through
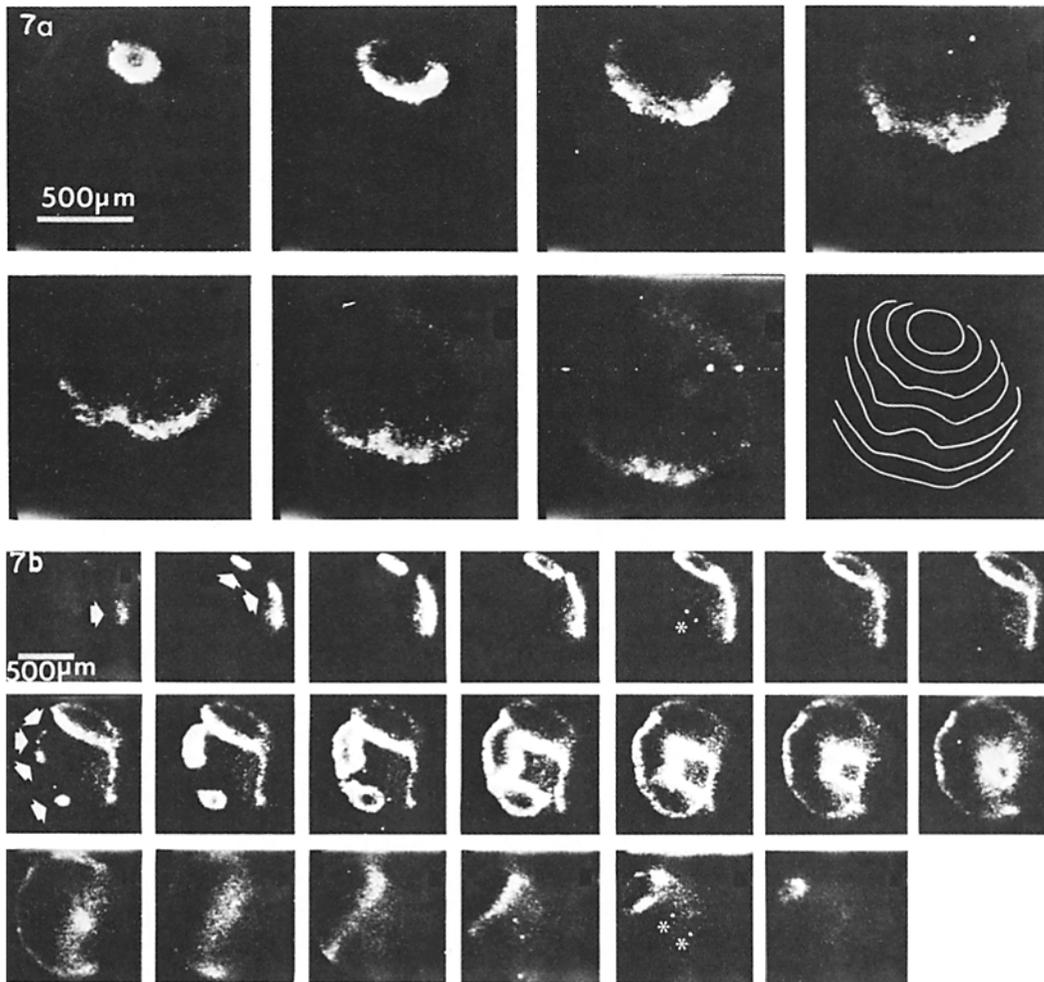
FIGURE 7 Ionophore-initiated calcium waves. (*a*) One initiation point. 5 s elapsed between successive photographs. Egg number 4 of 5/5/76 with micropyle upward. (*b*) Seven initiation points (indicated by arrows). Ion noise spots, e.g. those indicated by asterisks, can be readily distinguished from initiation sites in the original records by their failure to persist and grow, as well as by their characteristically sharp outlines. 2 s elapsed between photographs in the first two rows whereas those in the last row are 15 s apart. Egg number 6 of 5/7/76 with micropyle upward. Bars, 500 µm.

this funnel so as to reach the plasma membrane in less than 1 s. The first visible response of the egg to sperm entry was the sudden disappearance of one or two miniature vesicles lying within 10 µm of the micropyle. However, a distinct delay, 5.5–12 s (at a room temperature of 23°–25°), intervened between sperm entry and this first visible response (Table II A). Then, other miniature vesicles proceeded to vanish in a process that spread out from the micropyle at an average rate of 13 ± 3 µm/s. Thus, even the earliest measurable propagation rate, beginning 10 µm or less

from the micropyle, is about the same as the average rate over the whole animal hemisphere. However, it should also be noted that, at any one point, large vesicles begin exocytosis about 5 s after miniature vesicles do. Indeed, even though no exact measurements have been made, it seems clear that this delay increases continuously with vesicle size.

Finally, about 55–70 s after sperm entry, we regularly observed the start of a process of early cortical contraction marked by the obvious movement of all cytoplasmic inclusions (i.e., oil drop-
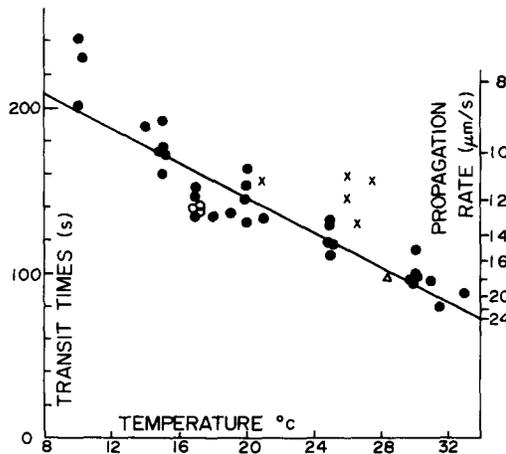
FIGURE 8 Transit times of vesicle fusion waves in medaka eggs as a function of temperature. Each time was taken as that elapsed between the first large (>10 μm) vesicle fusion near the micropyle to the fusion at its antipode. At any given temperature, each solid circle is an average of two to three or more observations on a separate batch of eggs. The triangle (at 28.5°) indicates the only comparable measurement found in the literature (24). Open circles are transit times measured on eggs that underwent a period of anesthesia similar to that endured by the eggs used to study the free calcium waves. Crosses indicate the average transit times for the free calcium waves (as seen in eggs with horizontal axes [Table I]). Right-hand ordinates indicate average propagation rates, assuming an egg diameter of 1,100 μm.

lets, surviving cortical vesicles, etc.) toward the micropyle. This movement (at least within the 200-μm diameter field being observed) then ceased about 90–110 s after sperm entry. The inclusions at the field's edge had moved about 20–40 μm toward the micropyle during this process.

DISCUSSION

Wave Propagation Via Calcium-Stimulated Calcium Release

The main conclusion to be drawn from the results is that a wave of increased free calcium propagates through the cytoplasm of the medaka egg during activation (Fig. 2). We further conclude that propagation is brought about by a process of calcium-stimulated calcium release. Concretely, we imagine a chain reaction as follows: a sperm (or ionophore) raises free calcium in some restricted location(s). This free calcium induces the release of additional free calcium

from some nearby, preexisting source(s). Calcium ions then diffuse out to sources in adjoining regions to release still more calcium which diffuses farther, etc. This chain reaction would explain the advancing front of the observed free calcium wave. The decline observed some distance behind this front would be attributed to exhaustion of the local sources together with slow uptake of the released calcium by some set of distributed sinks (e.g. mitochondria), or to export into the medium or the yolk.

Two alternatives to a propagative mechanism for the observed calcium wave might be consid-

TABLE II

Characteristics of Changes Near the Micropyle of Activating Medaka Eggs*

Egg		Time after sperm entry		
			Contraction‡	
A. Batch	Num-ber	First small vesicle vanishes	Starts	Ends
		s	s	
1	1	9	85	
	2	12	61	
	3	11½	61	107
	4	10	61	104
2	5	11	61	99
3	6	6½	67	98
	7	5½	67	92
4	8	9	61	107
	9	10	57	105
5	10	8		
	11			

		Distance from micropyle of vanishing vesicles			
B.		First seen	Second noted	Delay	Propagation rate
		μm	μm	s	μm/s
6	12	20 μm	119 μm	7.8	13
	13	34	112	6.0	13
	14	14	136	7.2	17
7	15	9	68	4.8	12
	16	9	68	3.7	16
	17	7	34	3.0	9

* Micropyle observed directly from above with Zeiss Nomarski differential interference optics and a ×40 water-immersion objective. Eggs 1–7 were observed on 4/9/77 at a room temperature of 23°C; 8–11, on 4/13 at 25½°C; 12–17, on 5/18. Eggs 1, 3, and 6–9 were observed by Lionel Jaffe; eggs 2, 4–5, and 10–17 were observed by John Gilkey.

‡ Observed at the edge of the microscope field, about 100 μm from the centrally positioned micropyle.

ered a priori. In a diffusive mechanism, the sperm would somehow introduce under the micropyle some substance that would then diffuse to the opposite pole of the egg and induce calcium release as it spread. Arguments against a diffusive mechanism have been presented by Yamamoto (64). One cogent argument relies on the fact that eggs may be artificially activated by pricking with a needle. By varying the size of the needle, or the number of pricks, it is possible to apply a graded stimulus. In response, either all of the vesicles break down or none do. If the mechanism were diffusive, one would expect that a stimulus that is not quite large enough to induce breakdown of all of the vesicles might nevertheless induce breakdown of some vesicles near the site of stimulation; but, in fact, none do break down.

Alternatively, the membrane depolarization that occurs about 10-20 s after fertilization (36) might imaginably stimulate calcium release from sources distributed over the whole cortex. All sources would thus be electrically stimulated at practically the same time,[3] but their response times might be graded so as to simulate true propagation. In fact, the animal region of the cortex is known to respond to various artificial stimuli substantially faster than the vegetal region (64). Nevertheless, such mock propagation can be ruled out by the results of artificially activating medaka eggs at their vegetal poles, opposite the micropyle. This can be done either by pricking (64) or by local insemination of dechorionated eggs (47). In each of these cases, a wave of exocytosis starts at the vegetal pole and ends at the animal pole, rather than vice versa.

In this paper, we have observed two additional facts that support the concept of propagation: (a) the peak level of free calcium does not diminish (and may even rise) as the wave traverses the egg. (b) A calcium wave may be initiated anywhere in the peripheral cytoplasm by ionophore treatment. Because a calcium wave can apparently be initiated by a rise in calcium anywhere in the egg's cortex, it is difficult to imagine how the natural rise in the free calcium could fail to be propagated.

Reasons for inferring calcium-stimulated calcium release during activation have been pre-

sented in the previous paper (43). First, most of the 15,000-fold increase in light that follows the fertilization of aequorin-loaded eggs has an exponential time course. Presumably, this represents an autocatalytic or regenerative process near the sperm. Second, in ionophore-activated eggs, a relatively slow rise in luminescence precedes such an exponential rise. Presumably, the transition from slow to rapid phases indicates the triggering of a regenerative process by passively introduced calcium ions. In this study, we have observed two additional facts that support the concept of calcium-stimulated calcium release. First, as noted above, an ionophore can initiate calcium waves anywhere on the egg's cortex. This is evidence that calcium can induce calcium release anywhere on the egg's cortex as well as the region under the micropyle. Second, ionophore-initiated waves move faster than comparable sperm-initiated ones, at least in the region of the egg exposed to ionophore. Presumably, ionophore treatment raises free calcium in most of the cortex above the resting level (thus closer to the triggering level), and in this way speeds the chain reaction.

An important precedent for the phenomenon of calcium-induced calcium release was reported some years ago in muscle. In skinned muscle cells, increases in external calcium were shown to release more calcium from the sarcoplasmic reticulum (13, 18). There is a serious question as to how physiological this phenomenon is in skeletal muscle (12). However, in skinned cardiac muscle segments, the minimum effective concentration seems to be far lower, about 10^{-7} M, which strongly suggests that regenerative calcium release is a natural component of the heart's contractile control mechanism (17). It is also very interesting that Natori (34) has noted "slow" spontaneous contraction waves within skinned skeletal muscle fibers in physiological media. These waves move at 90 μm/s and are accompanied by "internal" potential transients of only 1-2 mV. Natori speculated that they result from calcium-induced calcium release propagated along the sarcoplasmic reticulum.

What is the Peak Level of Free Calcium in the Activation Wave?

No completely certain basis for estimating the absolute peak level of free calcium is available. Nevertheless, it seems to us that a useful first estimate of $10^{-4.5}$ M (30 μM) can be made. To do this, we make use of two main data.

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[3] The time constant governing the passive and electronic spread of depolarization from the point of sperm entry over the rest of the egg cortex can be estimated to be about 1-10 ms.

First, our earlier observation showed that the total luminescence of fertilized, aequorin-loaded medaka eggs rises to a peak level about 15,000 times that at the resting level (43; Table I). Second, a recent study by Allen et al. (1) (a) shows that at very low free calcium levels aequorin luminescence in vitro is calcium independent, and (b) measures the ratio of aequorin's luminescence in vitro to the calcium-independent level at various higher levels of free calcium. We can calibrate the measured in vivo luminescence ratio via the in vitro ratios, by assuming that the resting level in the egg is at or near the calcium-independent one.

The data of Allen et al. indicate that aequorin luminescence in vitro reaches a level near—to be specific, two times—the calcium-independent one at $10^{-7.3}$ M. However, these data were obtained from experiments done in the absence of magnesium, an ion known to competitively inhibit aequorin luminescence (7). A correction for magnesium inhibition, based on a study of this effect by Baker et al. (6), and estimates of free magnesium in other cells—namely, squid axons (8) and barnacle muscles (4)—shift the "near-independence" level from $10^{-7.3}$ to $10^{-6.8}$ M.

Two considerations suggest that the free calcium in unfertilized medaka eggs is at or below $10^{-6.8}$ M: (a) microinjection of medaka eggs with Ca-EGTA buffers indicates that activation is initiated in different individual eggs at free calcium levels between about 10^{-7} and 10^{-6} M (J. C. Gilkey, unpublished data). Hence, the resting levels should be substantially below this range to avoid spontaneous activation. (b) Free calcium values in various other resting cells are estimated to be about 10^{-7} M or less (2, 3, 5).

We will also assume that the peak of the total luminescence of activating eggs occurs at about the time that the calcium wave has reached its greatest circumference (cf. Fig. 2 and reference 43). At this time, the wave has an apparent band width of about one-tenth of an egg's diameter. Hence, it can be calculated that the band occupies about one-tenth of the egg's surface. Let us make the approximation that all the measured light at this time came from this band. Then, the resting glow from this band should have been about one-tenth of the measured resting glow, and the peak to resting ratio in this band would be 10 × 15,000 or 150,000. Finally, we may now apply this value to the curve of Allen et al., measuring luminescence vs. free calcium in vitro. We lower their

calcium-independent level by twofold to correct for free magnesium, then read from the curve the level of free calcium needed to give a luminescence rate 150,000 times this calcium-independent one. This yields the above-mentioned estimate of $10^{-4.5}$ M. Thus, if the resting concentration of free calcium is about 10^{-7} M, it rises about 300-fold during activation.

Source of the Released Calcium

We infer that a calcium wave crosses any particular region of the egg only once. We infer this from two observations: First, the large calcium transient which accompanies activation is never repeated. At least it is not repeated during the first few hours after fertilization, a period which includes the first few cell divisions (43). Second, even in cases where several waves are initiated more or less simultaneously at different points, they annihilate each other when they meet, i.e., they fail to propagate through one another. This suggests that the sources of calcium released during activation become refractory or are irreversibly exhausted or are even destroyed during this process. It is as if the unfertilized egg's cortex were like a field of dry grass and the sperm like a match. The match, by this analogy, initiates a ring of fire that spreads outward, leaving a burnt-out zone in its wake.

The large cortical vesicles themselves are normally destroyed during activation. Nevertheless, several facts indicate that they are not the main sources of released calcium. First, it has been known for some time that medaka eggs can be centrifuged so as to drive these vesicles off the surface monolayer and into a close-packed mass at the centrifugal pole. In this centrifugal mass, most of the vesicles are now located well inside the egg. Such stratified eggs can be fertilized, activated, and induced to develop; but their interior cortical vesicles remain visibly intact (62, 64). Second, we now find that during the activation of such a stratified egg, the calcium wave proceeds unimpeded through the large regions of the egg's cortex which are free of large cortical vesicles (Fig. 6). Third, we find that the normal calcium wave (in uncentrifuged eggs) significantly precedes the vesicle fusion wave (Fig. 4).

Although the main source(s) of calcium are evidently not the large cortical vesicles themselves, most of the calcium does seem to enter the cytoplasm from some internal source(s) rather than the external medium, for the calcium wave

proceeds in a normal manner (albeit a bit more slowly) in a medium whose calcium has been reduced by the order of 1,000,000-fold, from 2 × 10^{-3} M to about 10^{-9} M, and held low with the aid of 5 mM EGTA (Fig. 2c). One possible internal source of the calcium released during activation is Yamamoto's "a-granules" (65). These particles were reported to be about 0.1–0.3 μm in diameter, deep red in color, attached to the outside of the cortical vesicles (and perhaps the oil droplets), and — most important — were said to dissolve just before vesicle fusion starts. It must be noted, however, that our preliminary observations with the light microscope, as well as the electron microscope observations of Iwamatsu and Ohta (26), have so far failed to confirm the existence of such granules.

Another — and we think quite plausible — source could be some modified region of the endoplasmic reticulum analogous to the sarcoplasmic reticulum of muscle cells. The available fine structural studies of medaka eggs are of limited value (26). However, a recent study of another large vertebrate egg, that of *Xenopus*, shows an extensive endoplasmic reticulum in the cortex that may well be a source of the calcium released during activation (9). In the unfertilized *Xenopus* egg, the cortical vesicles are surrounded by cisternae reminiscent of the terminal cisternae of vertebrate skeletal muscle (38). Like the latter, they are interconnected through other elements of the smooth endoplasmic reticulum. During activation, a wave of vesicle secretion progresses around the egg in about 5 min (60). During this same period, the extent of the cortical endoplasmic reticulum is somehow substantially reduced (9), a structural event that might correspond to the nonrepeatability of a calcium wave. Furthermore, as discussed above, the natural release of calcium from the sarcoplasmic reticulum of contracting heart muscle cells may well be induced by calcium itself (17). The analogy with muscle action is further strengthened by the evidence of cortical contractions soon after vesicle secretion. Other imaginable sources might include the mitochondria, the miniature cortical vesicles, or even the yolk compartment. The slow process of calcium reduction that follows its explosive increase might imaginably be brought about by any of the structures mentioned as possible sources.

The above discussion concerns the possible sources of calcium released during wave propagation. One may also wonder where the calcium

that starts the chain reaction comes from. One appealing possibility is that this starting calcium is first released within the sperm during its final movements[4] and then, in turn, is carried into the egg during gamete fusion.

Are There Calcium Waves Through Other Eggs?

This paper reports the first demonstration of a calcium wave through an activating egg. However, vesicle fusion waves are well known in other fish eggs, in the *Xenopus* egg and in various echinoderm eggs (Table III). Moreover, exocytosis (without evidence of its spatiotemporal course) seems to be a nearly universal concomitant of egg activation from algae (39, 48) through mammals (35, 53), and even man (45). One, therefore, wonders whether calcium waves may not accompany egg activation quite generally.

Besides being studied in the medaka egg, activation has been most closely studied in echinoderms, particularly in sea urchin eggs (15). Our review of this literature indicates that the known characteristics of the exocytotic wave, and thus of the inferred fertilization wave in sea urchin eggs, are quite similar to those in medaka eggs.

(a) In medaka eggs, the existence of an invisible fertilization wave, which is independent of the large cortical vesicles, can be easily demonstrated by centrifuging these vesicles to one pole (64). In sea urchin eggs, these vesicles cannot be centrifugally detached from the cortex (22, 33). Nevertheless, Uehara and Sugiyama (57) have elegantly demonstrated the propagation of the fertilization wave across a ring of the urchin egg surface which had been freed of vesicles by localized treatment with an anionic detergent.

(b) The average propagation rate of vesicle fusion in these small marine invertebrate eggs is remarkably similar to its rate in the medaka and other large, fresh-water, vertebrate eggs (Table III). In all cases this rate is about 5–15 μm/s.

(c) There may be a similar delay between effective sperm attachment and the first visible changes in both sea urchin and medaka eggs. Moser (33) directly observed a delay of 10–20 s between the time that he applied highly concen-

TABLE III

Average Propagation Rates of the Vesicle Fusion Wave through a Variety of Eggs

Habitat	Tempera-ture	Species	Diameter	Transit time	Rate	Year	Reference
	°C		μm	s	μm/s		
Fresh water	?	*Xenopus laevis* (toad)	1,300	320	6.4	1974	(21, 60)
	15	*Oryzias latipes* (medaka)	1,100	180	9.1	1977	This study
	26	*Oryzias latipes* (medaka)	1,100	110	15.7	1977	This study
	18	*Acipenser sp.* (sturgeon)	1,000	180	9	1962	(11)
	18	*Pungitius sp.* (stickleback)	1,000	180	9	1956	(32)
Marine	24	*Comanthus japonicus* (cri-noid)	250	68	5.8	1941	(10)
	18	*Cystoseira barbata* (brown alga)	200?	60	5	1931	(30)
	?	*Clypeaster japonicus* (heart urchin)	120	15	13	1952	(14)
	18	*Psammechinus miliaris* (sea urchin)	100	20	8	1949 1955	(29, 44)
	16	*Strongylocentrotus purpura-tus* (sea urchin)	75	20	5.9	1971	(37)
	26	*Arbacia punctulata* (sea ur-chin)	74	10	11.7	1939	(33)

trated sperm to one region of the *Arbacia* egg (at 26°C) and the time that exocytosis began. (He could clearly see it start because he inseminated a region that was cleared via centrifugation.) This is close to the 6- to 12-s delay that we have observed (using Nomarski optics) between entry of the sperm into the micropyle and the first dissolution of miniature cortical vesicles in medaka eggs.

(d) Fresh-water fish eggs can be fertilized in artificial media made up without added calcium, and we have found the free calcium wave through medaka eggs to be nearly independent of external calcium. Echinoderm eggs, on the other hand, cannot usually be fertilized in calcium-deficient sea water. However, Takahashi and Sugiyama (54) have reported that external calcium is only needed to induce the acrosome reaction of sea urchin sperm; sperm that are preactivated in calcium-bearing media can then readily fertilize eggs in artificial media made up without the addition of calcium. Furthermore, sperm of the Japanese heart urchin, *Clypeaster japonicus*, were reported to undergo the acrosome reaction in calcium-free sea water and also to fertilize eggs in a calcium-free medium (31, p. 33). These facts suggest that the fertilization wave through echinoderm eggs, like that through medaka eggs, is largely main-tained by internally released calcium rather than calcium entering from the medium.

(e) Sea urchin eggs are too small to yet allow aequorin visualization of any free calcium wave

during activation. Nevertheless, Steinhardt et al. (52) recently succeeded in demonstrating a cal-cium transient in the activating sea urchin egg by measuring the light emitted from a group of about 10 activating, aequorin-loaded *Lytechinus* eggs. Furthermore, the peak free calcium level reached in *Lytechinus* eggs is comparable to the 30 μM estimated for medaka eggs. In the best synchro-nized batch, direct calibration of the aequorin indicated a peak free calcium level of about 5 μM, assuming equal distribution of the free cal-cium throughout the egg. If one assumes that the peak calcium in *Lytechinus* eggs is restricted to about one-tenth of the cytoplasmic volume, as it is in medaka eggs, then the peak calcium within this active region would be higher than 5 μM. Inasmuch as the aequorin luminescence rises with the square of free calcium in the calcium range being considered, it would be $\sqrt{10}$-fold higher or about 15 μM.

Do Sperm Trigger Development by Raising Free Calcium within the Egg?

We may formulate a simple calcium hypothesis of fertilization by stating that a sperm normally initiates egg development by only three actions: it introduces a set of chromosomes and a centriole (19, 23) and it either introduces or somehow induces a local rise in free calcium. Our obser-vations of aequorin-loaded medaka eggs show that a sperm does indeed somehow raise local

free calcium far above the resting level; and a comparison of the characteristics of fish egg and echinoderm egg activation suggests that such a rise may well be a general feature of the fertilization process. But is this rise a sufficient trigger for all that follows?

There is little doubt that an increase in free calcium suffices to induce exocytosis of the cortical vesicles. There is abundant evidence that secretion by exocytosis of all sorts of cell vesicles is triggered by a rise in free calcium (46); and we find the calcium wave to just precede the exocytotic wave in the particular case of activating medaka eggs. However, an alternate theory must be considered as an explanation of the other (and developmentally more significant) aspects of egg activation—phenomena such as the initiation of cell division and the acceleration of DNA and protein synthesis. It has been suggested that the sperm may initiate some of these other events by somehow independently lowering the concentration of cytoplasmic H^+ ions rather than by raising free Ca^{++} ions (16, 28).

It seems to us that the presently available evidence favors the simpler theory (that calcium ions suffice as a primary trigger), at least for vertebrate eggs, but that this evidence is not decisive. One simple, but we believe substantial argument for the simple calcium theory is that medaka eggs (63), Xenopus eggs (61), hamster eggs (58), and probably even sea urchin eggs (33) can only be prick-activated in (or after transfer to) a calcium-bearing medium. Furthermore, prick-activation of medaka eggs (25; as well as frog eggs [19]) sometimes initiates development all the way to hatching or beyond, provided the pricking is supplemented by a cleavage-initiating factor which is very probably centriolar (23). It is true that pricking a hole in the plasma membrane should open a new path for proton carrier movement as well as for calcium ion movement. However, considering that the pH's of the medaka, Xenopus, hamster, and Rana media were 7.3, 7.2, 7.4, and 7.2, respectively, together with the probability that a substantial membrane potential opposes proton efflux even from these pricked cells, it is by no means certain that protons would even leave through a leak rather than enter through it. Furthermore, the ubiquitous, small, lipid-soluble molecule CO_2 should be a rather effective proton carrier across the plasma membrane of undamaged eggs. So it is difficult to imagine how a small hole could substantially speed proton equilibration. Certainly, an opening for direct proton movement would have relatively little consequence, considering that the internal proton concentration (of about 0.1 μM) is about four orders of magnitude lower than the external calcium ion concentration.

On the other hand, the levels of calcium present in the effective prick-activation media were so much higher than the estimated natural activation levels in the cytoplasm as to raise some possibility that the natural mechanism was bypassed rather than simulated in these experiments: The medaka, Xenopus, and sea urchin media contained 1,800, 400, and 10,000 μM Ca^{++}, respectively, which may be compared with our estimate of only 30 μM for the natural peak activation level in medaka eggs. Furthermore, the general effectiveness of the ionophore A23187 in activating eggs (51) is not a significant argument for a simple calcium theory because this drug seems to carry Ca^{++} ions across cell membranes largely in exchange for H^+ ions (40, 41).

It seems to us imaginable that the natural activation process also involves an exchange of Ca^{++} ions for H^+ ions (say, across the mitochondrial membranes) and thus simultaneously raises Ca^{++} and lowers H^+ within the cytoplasm. We could also imagine that the transient depolarization, which accompanies effective sperm attachment in the medaka egg (36) as well as in the sea urchin egg (27, 56), both initiates internal calcium ion release (as in muscle cells) and drives H^+ ions out across the plasma membrane. Again, a rise in Ca^{++} ions and a fall in H^+ ions in the cytoplasm would be independent consequences of fertilization. Because of such residual ambiguities and possibilities, we are now studying the effects on activation of controlling Ca^{++} and H^+ ion levels within the cytoplasm of the medaka egg by more direct, quantitative, and specific means. These results will be reported elsewhere.

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