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Free calcium increases explosively in activating medaka eggs

(aequorin/fertilization/ionophore A23187/*Oryzias*)

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ABSTRACT **We have used the calcium-specific light-emitting protein aequorin to follow changes in free calcium concentration during fertilization and cleavage of eggs from medaka, a fresh-water fish. Aequorin-injected medaka eggs show a very low resting glow before they are fertilized, indicating a low calcium concentration in the resting state. Upon activation by sperm, the calcium-mediated light emission increases to a level some 10,000 times the resting level with a 1 to 2 sec time constant for an *e*-fold increase, and then slowly returns to the resting level. Upon activation by the ionophore A23187, the early rise in luminescence is much slower, but once a threshold has been reached the subsequent rise becomes as rapid as the normal sperm-induced response. We infer that the explosive rise in calcium involves calcium-stimulated calcium release, and that a sperm normally triggers this rise by somehow inducing a more modest and localized rise in calcium.**

One of the most important aspects of fertilization is the activation of the previously resting egg by the entering sperm. This normal activation response of the egg is dramatic and self-sustaining, and has long been thought to involve and require an increase of free calcium in the cytoplasm. Early workers conceived of a calcium theory of activation because a variety of eggs can be activated parthenogenetically (i.e., without sperm) simply by exposure to calcium-rich solutions, or by various disturbances, e.g., pricking the egg, in (and only in) a calcium-bearing medium (ref. 1; also compare refs. 2–5). More recently, the calcium theory has been supported by experiments showing that the calcium-specific ionophore A23187 can activate eggs (6–9).

The results of previous efforts to measure a change in free calcium during activation have surely been consistent with a large increase, but are rather easily interpretable in other ways (2, 8, 10, 11). For example, the 10- to 100-fold increase in ^{45}Ca efflux during activation of sea urchin eggs (7, 11) might well be due to an increase in free cytoplasmic calcium, but might also represent the release of calcium from cortical vesicles.

In this paper, we demonstrate a dramatic rise in free calcium concentration within the cytoplasm of activating medaka eggs by injecting them with the calcium-specific photoprotein aequorin. This calcium indicator has been previously used to detect calcium transients in muscle (12) and nerve (13), and is presently, we believe, the most sensitive and reliable method for detecting changes in free calcium within cells. The egg we have used is from the medaka, a fresh-water fish. The advantages of this egg are: first, it is large (1.2 mm diameter); second, it can be injected without blocking subsequent fertilization (3); and third, it is transparent.

MATERIALS AND METHODS

Materials. Adult medaka (*Oryzias latipes*) were purchased from the Carolina Biological Supply Co., and the gametes were obtained by Yamamoto's method (3). Micromanipulation of the eggs indicated that a yolk membrane, like the one in *Fundulus* (14), separates its peripheral, 40 μm thick, cytoplasmic region from a 1 mm diameter central yolk compartment. Yamamoto's medium contains 128 mM Na^+, 2.6 mM K^+, 1.8 mM Ca^{++}, 134 mM Cl^-, and is buffered at pH 7.3 with bicarbonate. Ionophore A23187 was a gift of the Eli Lilly Co.

Aequorin Injection. To avoid activation during injection, each egg was first anesthetized in Yamamoto's medium plus 5 mM Chloretone (1,1,1-trichloro-2-methyl-2-propanol hydrate) for a half hour. About one nanoliter of a 1% aequorin solution (see ref. 13 for details of preparation) in 110 mM KCl plus 4 mM TES buffer (Sigma Chemical Co.) at pH 6.9 was injected through a beveled, 5 μm pipette. The injected material first formed a spherical, 0.1 mm diameter pocket bounded by distended yolk membrane, then spread out into the cytoplasm as the pipette was gently withdrawn. The egg was then allowed to recover in normal Yamamoto's medium for an hour.

Ionophore Application. In most cases, a few microliters of a 0.1% solution of A23187 in ethanol or dimethyl sulfoxide were gently pipetted into the medium near an egg, where it formed a smoky precipitate. In one case, a thin film of ionophore was deposited in a plastic petri dish by repeatedly evaporating droplets of an ethanol solution.

Recording Procedure. After recovery, the egg was centered in a petri dish lying on a well-shielded, end-window photomultiplier tube (EMI type 9635 A, see Fig. 1). A light-tight cover, having a highly reflective inner surface, fit over the top of both petri dish and photomultiplier. We estimate that about 70% of the total light emitted from the egg was collected by the photomultiplier. The photomultiplier was operated with the cathode at −800 V and the anode near ground. The dark current was 300 pA. See ref. 13 for details of the dynode chain. The anode current, which is directly proportional to the incident light, was recorded with a Keithley model 600B electrometer. A linear pen recorder was used to display the output as a function of time. The overall time constant of the recording system was roughly 0.5 sec.

A measurement was first made of the egg's "resting glow." Then the photomultiplier was turned off, the chamber was opened, sperm were added about 5 mm away from the egg, the chamber was closed, and the tube was turned back on. The calcium-mediated light emission from an egg could be followed for many hours. From time to time, we opened the recording chamber and observed the egg's development with a dissecting microscope.

RESULTS

Unfertilized Eggs. Fig. 2 shows several recordings which begin with the steady luminescence, or "resting glow," of some representative unfertilized eggs; the values of all the measured resting glows are given in Table 1 (in units of 100 pA.). They are subject to errors of about ±0.2 units, and are generally just barely measurable. A dozen resting glows lie between 0.8 and 2.5 units, two are smaller, and three are larger. A control egg (no. 17), which was not injected with aequorin, shows a glow

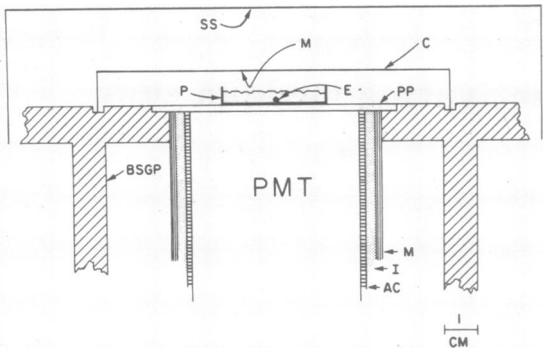

FIG. 1. Cross-section of the recording chamber. The aequorin-injected egg (E) lies in the center of a petri dish (P). The petri dish is supported by a thin clear plastic plate (PP) which is in turn optically coupled to the end-window of the photomultiplier tube (PMT). A metal cover (C) having a mirror-like inner surface (M) provides the primary light shielding, while several layers of black corduroy (not shown) and a second light shield (SS) provide additional protection. The photomultiplier tube is protected by an inner electrostatic shield at cathode potential (AC), an insulating layer (I), a mu-metal magnetic shield (M), and finally an outer electrostatic shield at ground potential (BSGP). Approximately to scale; scale bar = 1 cm.

of only 0.2 units. The low levels of light emission from aequorin-injected eggs at rest reflect a low level of ionized calcium in the cytoplasm (presumably in the vicinity of 0.1 μM), and are consistent with experiments on other tissues at rest (13).

The Calcium Transient on Activation by Sperm. After the addition of sperm (Fig. 2), there is a dramatic increase in calcium-mediated light output, which coincides with the activation of the egg. The main characteristics of this calcium transient on activation by sperm are: a delay, an explosive rising phase, a peak, and finally a slowly falling phase that eventually (over roughly 15 min) returns the luminescence at least approximately to its initial or "resting" level. The delay (0.8–3 min) between the addition of sperm and the onset of the calcium transient can probably be accounted for by the time required for the sperm to reach and penetrate the micropyle. The explosive rising phase is nearly exponential (see Fig. 3) with a time constant (for an e-fold increase) of only 1–2 sec at 20°. The peak of the response reaches a level some 10,000 times the resting level, and remains at half or more of this peak value for about a minute. The recovery (slow falling phase) is roughly exponential with a time constant on the order of 150 sec (see Table 1). Evidence is presented below that the kinetics of the slow falling phase represent a slow fall in the ionized calcium level and are not due to the exhaustion of the injected aequorin.

Control Experiments. Eggs that have not been injected with aequorin show neither a resting glow nor a calcium transient on activation. Eggs that have been aequorin injected but fail to activate (as judged by subsequent visual examination) do show a resting glow (for six cases: 0.5, 0.5, 4, 4, 5, and 11 units) but do not show a calcium transient.

When the aequorin is injected into the yolk (as a vesicle bound by yolk membrane) instead of into the cytoplasm, then, as shown in Fig. 2 (egg no. 16) and Table 1, the magnitude of the activation transient is drastically reduced. The residual response observed in this "yolk-injected control" is probably due to the escape of a small fraction (0.3%) of the injected aequorin from the yolk to the cytoplasm during the injection maneuvers. This result is consistent with the conclusion that the main calcium transient on activation is a cytoplasmic, rather than a yolk, event. Further, if we compare the response of the yolk-injected control (egg no. 16, which presumably has a low

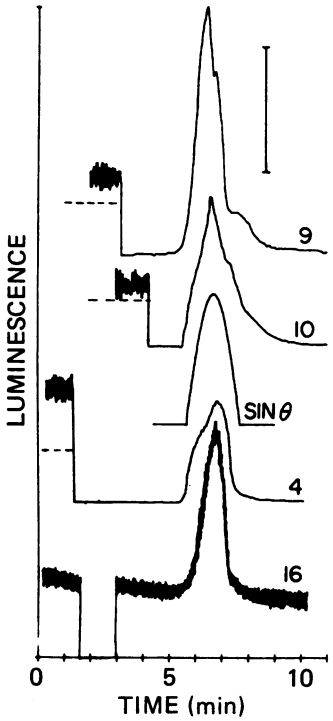

FIG. 2. Pulses of luminescence from fertilizing aequorin-loaded medaka eggs. The records begin with a determination of the resting glows, which are recorded at high gain. They are barely above the dark current (broken line). During the break in each record, the photomultiplier is shut off, sperm are added, and the photomultiplier is turned back on. In most cases it is turned on at much lower gain. The vertical scale mark indicates 10 units of luminescence before the break; afterwards, it indicates 10,000 units for egg records 9 and 10; 100,000 for 4 (which was injected with about 10-fold more aequorin than usual), and 10 units for 16 (which received far less). The central curve is the function sin θ, from 0 to π. It has the shape of a moving zone source (see *text*).

cytoplasmic aequorin concentration) to egg no. 4 (which was injected with roughly 10 times the usual amount of aequorin, and hence has a high aequorin concentration) it will be seen (Fig. 2) that although the magnitude of the activation transient depends on the aequorin concentration, the shape of the activation transient does not. It is therefore reasonable to suppose that the aequorin itself is not seriously distorting the activation response.

Because the calcium transient during activation is so large, it is quite natural to ask if this response consumes an appreciable fraction of the injected aequorin. To answer this question, we simply "burned" the remaining aequorin after activation by treating an egg (no. 4) with Triton X-100 and calcium ionophore (A23187). The ratio of light emitted during the activation to the total light emitted during the activation and the "burn" was less than 0.1; therefore, we estimate that less than 10% of the total aequorin is consumed during the activation response.

The Calcium Transients on Activation with Ionophore A23187. A wide variety of eggs can be activated by treatment with the calcium-specific ionophore A23187 (6–9); medaka eggs are no exception. Some minutes after introduction of ionophore near an egg it is seen to undergo a fairly typical activation response, and a coincident calcium transient. The bottom curves of Fig. 3 show two such transients and the data in Table 1 serve for further comparison of sperm- and ionophore-induced transients.

The ionophore transients show a relatively prominent starting

Table 1. Characteristics of pulses of increased calcium within activating medaka eggs

Run	Egg no.	Activator	Amplitudes[a]							Durations			Time constants[b]		
			Resting glow	Explosive phase		Peak glow	Peak/ resting ratio	Peak/ start ratio	Return level	Early rise (sec)	Half peak (sec)	Fall (min)	Explosive phase (sec)	Fall (sec)	Second rise (sec)
				Start	End										
I	17[c]		0.2												
	1	Sperm	0.7	4	10,000	16,000	20,000	4,000	Resting	12	61	11	2.9	102	
	4[d]	Sperm	4.9	80	5,000	78,000	16,000	1,000	Resting	11	84		0.6		
	7	Sperm	0.2	~0.2	300	7,000	30,000		Resting	None?	60	14	1.4	123	
	8	Sperm	1.9	3	60	12,000	6,000	4,000	Resting	8	57		1.4		
	9	Sperm	2.1			20,000	10,000		Resting		60	18		148	
	10	Sperm	1.3			12,000	9,000		Resting		82	17		130	
	12	Sperm	0.9			16,000	18,000		Resting		70	12		115	
	13	Sperm	1.1			11,000	10,000		Resting		56	12			
	14	Sperm	0.8			14,000	18,000		Resting		80	12			
	15	Sperm	0.8			8,000	10,000		Resting		75	12			
	16[e]	Sperm				15					54				
	6	Ionophore	2.5	300	6,000	57,000	20,000	200	380	105	66	4	1.7		50
II	19	Ionophore	0.5	1	100	1,500	3,000	1,000	2	70	76	13	0.8	188	1700
	20[f]	Ionophore	2.9	6	250	710	200	100		70	117		2.1		
	21[f]	Ionophore	6.3	20	300	780	120	40	74	12	83	2	1.5		250
	23[g]	Ionophore	1.5	8	700	2,400	1,600	300	45	45	129	5	0.8		
	24[h]	Ionophore	0.9	20	400	2,900	3,000	150	59	55	84	12	0.8	255	1600

[a] Amplitudes are in uncalibrated units representing light quanta per second emitted by the aequorin reaction in each egg. One unit actually equals 100 pA delivered by the photomultiplier above its dark current. The eggs used in runs I and II were injected with different, uncalibrated batches of aequorin (and also came from different batches of fish) so the absolute values of their luminescence are not necessarily comparable.
[b] Times to rise or fall *e*-fold.
[c] Control egg not injected with aequorin.
[d] Injected with about ten nanoliters instead of the usual one nanoliter of aequorin solution.
[e] Only a trace of the aequorin solution entered the cytoplasm (see *text*).
[f] Locally activated in a small region around the aequorin injection site.
[g] Activated by being placed, vegetal side down, on a thin ionophore layer.
[h] Surrounded by a Vaseline barrier and open to ionophore entry only at its vegetal end.

phase (which is only barely detectable in the sperm transients) as well as a more or less inhibited recovery phase which eventually leads to a secondary rise. However, the three main phases are remarkably similar to the sperm-induced transients. The explosive rise time constants average 1.6 ± 0.6 sec for four sperm transients and 1.3 ± 0.2 sec for six ionophore ones; ionophore transient no. 6 (which was obtained in the same experimental run as the ten sperm ones) reaches a peak level 23,000 times its base glow compared to an average ratio of 15,000 for the sperm ones and has a half peak duration of 66 sec compared to an average of 69 sec for the comparable sperm ones; and even the recovery phase of no. 19 (the one least inhibited by ionophore) is quite similar to the natural recoveries.

Several other points may be noted about the ionophore-activated eggs. One of them, no. 23, was activated by being placed (vegetal side down) on a thin film of ionophore; another, no. 24, was activated with the usual solution (qua suspension), and while surrounded by a thick coating of Vaseline open only at the vegetal pole. These two cases suggest that ionophore, unlike sperm, can initiate activation far from the micropyle. Two other eggs, no. 20 and no. 21, suffered a small but visible degree of local activation during injection. This may somehow account for their relatively anomalous transients.

Calcium Transients Later in Development. At the conclusion of the activation transient, the luminescence returns to the resting level. The next detectable event is a small transient which was seen to occur at 20 min after fertilization in two out of six eggs (see Table 2 and Fig. 4). These are followed by a consistent small transient at about 27 min which was observed in five out of six eggs (Table 2 and Fig. 4). This event, at 27 min, may be coincident with the initiation of oil droplet movement.

The luminescence of egg no. 18 was monitored for another 5–6 hr. The first event seen after the transient at 27 min was a small transient at 2.7 hr (Fig. 4B). Visual observations, made 9 min after the transient, showed division into two daughter cells which were rounded up but had not yet returned to the usual interphase contact. Thus, division was nearly completed. The next transient occurred at 3.8 hr (Fig. 4C). Visual obser-

Table 2. Characteristics of pulses of increased free calcium during ooplasmic segregation in medaka eggs

Egg no.	Times[a] (min)		Amplitudes[b]		
	First pulse	Second pulse	Base glow	Pulses	
				First	Second
1		27.5	½		2½
10		28.6	1½		½
12		27	1		1
13	21	27	1	1	1
14	20.3	27.2	1	1½	1
15		34	1		trace

[a] After the activation pulse began.
[b] In arbitrary scale units (see Table 1). Pulse heights are measured above the basal level.

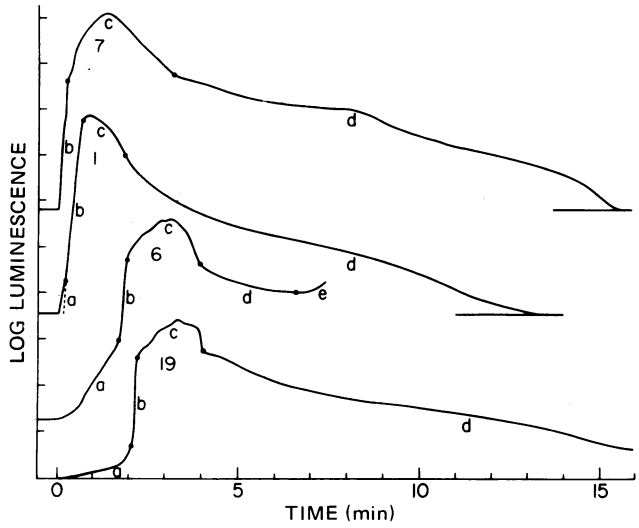

FIG. 3. Illustrative semilogarithmic plots of activation responses. Vertical scale units indicate tenfold increases in luminescence relative to each egg's resting level. The first detectable increase in luminescence defines zero time. Eggs no. 7 and no. 1 (above) were sperm activated; no. 6 and no. 19 (below) were ionophore activated. The curves are divisible into three to five more or less well-defined phases; (a) the early rise, (b) the explosive rise, (c) the peak phase, (d) the recovery phase, and (e) the secondary rise. The just-detectable early rise in egg no. 1 is brought out by extending its second phase. A secondary rise in egg no. 19 can be seen if the record is extended to 23 min.

vation made 10 min later showed the second cell division to be complete.

Development. Following fertilization, eggs injected with aequorin-free vehicle, like uninjected controls, develop normally (3, 15); i.e., they activate (as shown by cortical vesicle fusion and chorion elevation), segregate, cleave, and go on to form normal embryos which finally hatch. Aequorin-injected eggs show normal activation, segregation, and cleavage but eventually arrest in the late blastula stage. Ionophore-activated eggs show normal activation and anomalous segregation and then arrest.

DISCUSSION

Our main conclusions from these results are as follows: prior to fertilization, the ionized calcium level is relatively low in the cytoplasm of the medaka egg; upon activation, either by sperm or by ionophore, there is a dramatic increase in the cytoplasmic calcium concentration; following activation the cytoplasmic calcium concentration returns to virtually resting levels; and, finally, subsequent development proceeds with occasional small calcium transients.

The Explosive Rise. We interpret the massive calcium transient during activation as an explosive, i.e., autocatalytic, phenomenon involving calcium-stimulated calcium release. This interpretation rests on several considerations. First, pricking any part of the egg's surface can induce activation if, and only if, calcium is allowed to enter the egg through the wound (2).

Second, our experiments (Fig. 3) show that in ionophore-activated eggs there is a slow rise in luminescence followed by a sharp transition to the usual explosive rise. This suggests, but does not prove, that there is a surprisingly low threshold calcium level required to trigger an endogenous autocatalytic response. And finally, of course, as shown in Figs. 2 and 3, the main rising phases of both sperm-induced and ionophore-induced calcium transients are exponential.

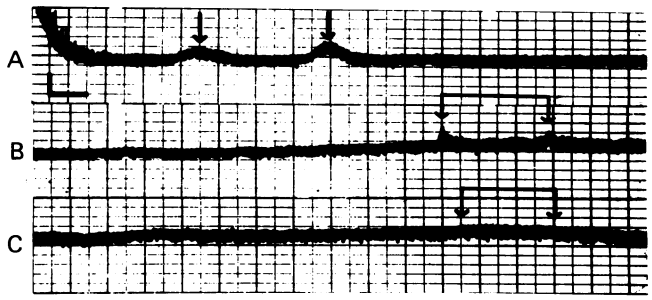

FIG. 4. Calcium pulses later in development. (A) During ooplasmic segregation of egg no. 14. (B and C) During first and second cleavages of egg no. 18. Scales: two luminescent units and 2 min.

A Calcium Wave? Although our results describe the magnitude and time course of the calcium transient on activation, it is important to understand that our methods (see Fig. 1) detect the integrated "global response" and do not directly correspond to "local" activation events either in space or in time. Yet, activation of the medaka egg is marked by a spectacular wave of cortical vesicle secretion which can easily be seen in a low power microscope (3). In a normal, sperm-activated egg, this secretion starts at the micropyle (where the sperm enters) and in the course of several minutes spreads to the opposite, or vegetal, pole. Moreover, in an egg that is activated by pricking [or is dechorionated so as to allow sperm entry far from the micropyle (16)], this secretion wave need not start at the micropyle but starts wherever the needle or sperm penetrates the egg. From such facts, Yamamoto long ago deduced that activation of the medaka egg spreads via some sort of self-propagating wave or chain reaction (3).

Our global results (Fig. 2) are consistent with the idea of a calcium wave which during activation spreads from pole to pole in the form of a narrow active zone. From geometrical arguments, the expected signal would first wax as the calcium wave moves from the pole to the equator and then wane as the wave moves on to the vegetal pole, tracing out the positive portion of a simple sine wave. The expected global signal from such a local calcium wave has been drawn in Fig. 2, assuming: (*i*) that the calcium wave is fairly narrow, (*ii*) that the magnitude of the signal will be proportional to the active surface area (because the wave should move through the egg's thin shell of cytoplasm but not through its central yolk compartment), and (*iii*) that the velocity of propagation is constant. At present, further speculation on the nature of the local calcium activation is not worthwhile. But visualization of the local events using image-intensification techniques promises to resolve this question, as well as others, in the future.

Consequences. The calcium transient on activation may be the cause of later effects. During activation, a great deal of heretofore compartmentalized calcium becomes free in the cytoplasm, and the obvious question then becomes, free to do what? Part of the released calcium is evidently required to trigger continued calcium release. Part may be required to bring about secretion of cortical vesicles. But much will be free to somehow bring about the further development of the egg. Development may be effected by reactions in the cytoplasm which occur while the free calcium is still high. In addition, the fact that the free cytoplasmic calcium level eventually returns to the resting level need not mean that the cytoplasmic calcium distribution has returned to the pre-activation state. Indeed, one of the most long-lasting effects of the calcium transient may well be the translocation of calcium from one site to another.

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