Roles of Calcium and pH in Activation of Eggs of the Medaka Fish, *Oryzias latipes*

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ABSTRACT Unfertilized eggs of the medaka fish (*Oryzias latipes*) were injected with pH-buffered calcium buffers. Medaka egg activation is accompanied by a transient increase in cytoplasmic free calcium (Gilkey, J. C., L. F. Jaffe, E. B. Ridgway, and G. T. Reynolds, 1978, *J. Cell Biol.*, 76:448–466). The calcium buffer injections demonstrated that (*a*) the threshold free calcium required to elicit the calcium transient and activate the egg is between 1.7 and 5.1 μM at pH 7.0, well below the 30 μM reached during the transient, and (*b*) buffers which hold free calcium below threshold prevent activation of the buffered region in subsequently fertilized eggs. Therefore an increase in free calcium is necessary and sufficient to elicit the calcium transient, and the calcium transient is necessary to activate the egg. Further, these results are additional proof that the calcium transient is initiated and propagated through the cytoplasm by a mechanism of calcium-stimulated calcium release. Finally, a normal calcium transient must propagate through the entire cytoplasm to ensure normal development.

Unfertilized eggs were injected with pH buffers to produce short-term, localized changes in cytoplasmic pH. The eggs were then fertilized at various times after injection. In other experiments, unfertilized and fertilized eggs were exposed to media containing either NH_4Cl or CO_2 to produce longer term, global changes in cytoplasmic pH. These treatments neither activated the eggs nor interfered with the normal development of fertilized eggs, suggesting that even if a natural change in cytoplasmic pH is induced by activation, it has no role in medaka egg development.

The injected pH buffers altered the rate of propagation of the calcium transient through the cytoplasm, suggesting that the threshold free calcium required to trigger calcium-stimulated calcium release might be pH dependent. The results of injection of pH-buffered calcium buffers support this conjecture: for a tenfold increase in hydrogen ion concentration, free calcium must also be raised tenfold to elicit the calcium transient.

Dalcq, in 1928, first postulated that an increase in cytoplasmic calcium was necessary to activate egg development (8). Some fifty years passed, however, before a transient increase in cytoplasmic free calcium was directly observed to accompany egg activation, first in the eggs of the medaka fish (35). Subsequently, similar observations were made in sea urchin (44), frog (48), and mouse (7) eggs, suggesting that the phenomenon is universal. If this calcium transient is truly necessary for activation, two additional criteria must be met (19): increasing free calcium to a level less than or equal to that achieved during activation must activate the egg; and preventing an increase in free calcium must prevent activation. Here I report the results of calcium buffer microinjection experiments that address these criteria.

Work performed during the last few years has demonstrated that one of the consequences of the calcium transient in sea urchin (43) and frog (49) eggs is a moderate increase in cytoplasmic pH, and that exposure of sea urchin eggs to reagents that, among other effects, alter cytoplasmic pH will promote changes in metabolism similar to those occurring as a result of activation (10, 11). Further, direct buffering of pH and free calcium in sea urchin egg extracts has been shown to affect protein synthesis rates (53). These observations suggest that, in sea urchin eggs at least, some activation-induced changes in metabolism are caused not directly by the calcium transient, but indirectly by a transient-induced pH change.

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Such a mechanism may also operate in eggs of other organisms. In this paper I report the results of microinjection of pH buffers into medaka eggs, and of exposing the eggs to reagents that should alter cytoplasmic pH.

The main findings of the present work are that (*a*) the criteria listed above are fulfilled in medaka eggs, so the calcium transient is necessary for activation, (*b*) altering cytoplasmic pH does not affect or effect egg activation per se, and (*c*) altering cytoplasmic pH does change the threshold free calcium concentration required to activate the egg. Preliminary reports of this work have appeared elsewhere (17, 18).

MATERIALS AND METHODS

Gametes of the medaka fish (*Oryzias latipes*) were obtained as described previously (35). The gametes were kept, and the experiments carried out, in either Yamamoto's Ringer's (56) containing 128 mM NaCl, 2.56 mM KCl, 1.83 mM $CaCl_2 \cdot 2H_2O$ and adjusted to pH 7.3 with 0.1 M $NaHCO_3$, or modified Yamamoto's containing 127.3 mM NaCl, 2.56 mM KCl, 1.83 mM $CaCl_2 \cdot 2H_2O$, and 1 mM morpholinopropane sulfonic acid (MOPS; Sigma Chemical Co., St. Louis, MO), adjusted to pH 7.3 with 1 M NaOH. The experiments were performed at temperatures ranging from 22 to 28°C.

pH buffering solutions were made with 40 mM MOPS or PIPES (Sigma Chemical Co.) plus sufficient KCl to make the solution isotonic with the egg's cytoplasm (equivalent to 133.33 ... mM NaCl; see reference 48). Media for buffering both free calcium and pH contained 40 mM MOPS or PIPES, 20 mM EGTA (Sigma Chemical Co.), sufficient $CaCl_2 \cdot 2H_2O$ to achieve the desired free calcium concentration, and sufficient KCl to achieve isotonicity; most of the buffers were within 3% of the desired osmolality, as determined with an osmometer (model 3DII; Advanced Instruments, Needham Heights, MA). The total calcium concentration required to achieve the desired free calcium at a given pH was determined with a calculator programmed with a set of equations derived from the four proton, two calcium, and two magnesium binding equilibrium expressions for EGTA. The binding constants used were published in 1974 (32). I followed the common practice of using hydrogen ion activity, derived from pH measurements, in my early calculations. However, as explained in the Introduction to reference 32, the binding constants are expressed in terms of proton concentration, rather than activity. One must therefore either use corrected binding constants, or convert proton activity to concentration before performing the calculations. Because this did not come to my attention until after the calcium buffer injections were nearly complete, I continued to use hydrogen ion activity in the remainder of the work for consistency. Table I lists the total calcium/EGTA ratios used to achieve the desired free-calcium concentrations at the three pH values used in the experiments. The top row of the table lists two sets of values for free calcium. The upper set, in parentheses, is my original set of values used with uncorrected constants to obtain the total calcium/EGTA ratios. The lower set was derived from the calcium/EGTA ratios using corrected constants, and so should be more accurate.

The pH of all buffers was adjusted to within ±0.005 U of the desired value using KOH and HCl; the accuracy of the pH standards (Coleman certified buffer tablets; Perkin-Elmer, Oak Brook, IL; made up fresh weekly) used to calibrate the meter was ±0.02 U. All buffers were filtered through 0.4 μm nuclepore (Nuclepore Corp., Pleasanton, CA) before use. A solution containing 100 mM K_2 EGTA, made with EGTA, KOH, and distilled deionized water (containing about 1 μM calcium), was determined to have 16 μM total calcium by atomic emission spectrophotometry; this is a negligible quantity for the purposes of this study.

The buffers were injected through a bevelled micropipette of ≤5 μm tip diameter. The mechanics of the injection procedure were as follows (refer to Fig. 1): During pre-injection maneuvers, the buffer in the pipette was kept under slightly more than atmospheric pressure, causing a continuous outward flow of buffer and thereby avoiding contamination of the pipette contents by the Ringer's. The medaka egg has a large central membrane-bound 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 (Fig. 1*a*). During insertion, the pipette penetrated the chorion, plasma membrane, and cytoplasm, and then distended the yolk membrane (a lipid bilayer) several hundred micrometers (Fig. 1, compare *a* and *b*). If the pressure on the pipette contents was insufficient, it was increased slightly until the injectate began to inflate the yolk membrane like a balloon (Fig. 1*c*). The volume of injectate could be estimated from the diameter of the balloon. When the pipette was withdrawn, the recoiling yolk membrane pressed the bolus of injectate against the somewhat gel-like cytoplasm (Fig. 1*d*); the injectate then spread slowly by convection and diffusion. The eggs were injected in the region between 60° and 120° from the animal pole.

The quantity of buffer injected was 1–10 nl, with most eggs receiving ~1 nl. The quantity of buffer injected had a quantitative but not a qualitative influence on the result. As the yolk membrane returned to its normal position, the injected buffer initially interacted with, and therefore should have strongly affected, a roughly equivalent volume of cytoplasm (estimated from the diameter of the spreading injectate bolus and the thickness of the cytoplasm). Buffer injections, and control injections of isotonic KCl produced no damage attributable to alteration of the composition of the affected volume of cytoplasm.

CO_2-Ringer's was made by equilibrating normal Ringer's with 5% CO_2 in air while readjusting the pH with 1 M $NaHCO_3$ as necessary; experiments were performed under a 5% CO_2/95% air atmosphere to maintain equilibrium. 10 mM NH_4Cl-Ringer's was made by adding NH_4Cl to normal or modified Ringer's and readjusting the pH with 1 M NaOH.

Initial Buffered Hydrogen and Calcium Ion Concentrations: The actual buffered hydrogen ion concentration in the egg cytoplasm is of course uncertain, especially for buffers with pH far from 7.0, since the buffers must contend with the egg's passive and active proton buffering systems. The pH was undoubtedly displaced toward normal cytoplasmic pH, since nonmuscle cells have ~10–20 mM passive buffering capacity (38), and the injected 40 mM buffers were rapidly diluted by about twofold. Further, the effects of pH buffers were observed at the earliest ~2 min after injection, since ~1 min was required to fertilize the eggs and ~1 min for the calcium wave (see below) to reach the injected region, so the egg's active buffering systems had significant time to act. Nevertheless, as described below, it is clear that the

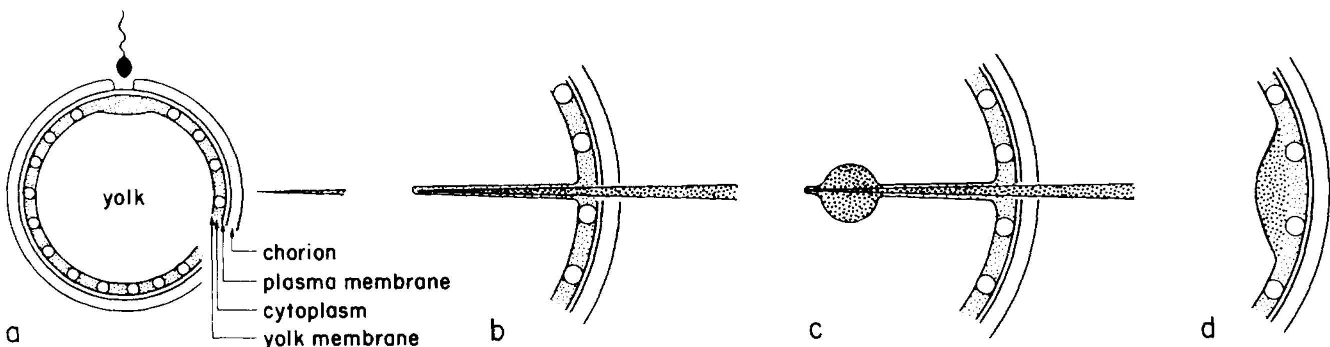

FIGURE 1 Mechanics of the injection procedure. (a) Schematic cross-section of an unactivated medaka egg with the micropipette at right, poised for injection. The 1.2-mm diameter egg has a large central membrane-bound yolk compartment surrounded by a thin (~30 μm) layer of cytoplasm, which is in turn bounded by the plasma membrane and a tough protective membrane, the chorion. The chorion has a tiny opening at the animal pole, the micropyle, which is the site of sperm entry. The cytoplasm contains a large number of cortical vesicles (open circles) and large oil droplets (omitted for clarity). (*b*) The pipette has penetrated the chorion, plasma membrane, and cytoplasm and has distended the yolk membrane. (c) The distended yolk membrane has been inflated with injectate. (*d*) The pipette has been withdrawn from the egg, and the bolu of injectate has been brought into contact with the cytoplasm by the recoiling yolk membrane.

buffers were effective enough to alter the behavior of the calcium wave, and by implication to alter cytoplasmic pH, in a graded fashion with buffer pH.

The actual buffered concentration of free calcium is likewise uncertain, since the calcium/EGTA buffer must contend not only with the egg's passive and active calcium buffering systems, but also with the unknown cytoplasmic magnesium concentration and with the change in pH imposed by the egg's cytoplasm. Fortunately, the free magnesium concentration is probably low enough (<10 mM, probably ~1 mM; see references 13, 22, 37) that the buffered calcium level will be only slightly affected by it. Further, because calcium buffers at or above threshold will elicit the calcium wave almost immediately, pH changes, magnesium binding and cytoplasmic calcium buffers' effects should be rather small at the time when the injected buffers exert their effect, so the buffered free calcium concentration should be close to the desired value.

RESULTS

A Simplifying Assumption

As noted in the introduction, medaka egg activation is accompanied by a transient increase in the cytoplasmic concentration of free calcium (35). This increase occurs first at the site of sperm entry (the animal pole) or artificial stimulation and propagates through the egg's cytoplasm as a narrow, ring-shaped zone of high free calcium (a "calcium wave") that closes upon itself at the antipode (19). The medaka egg cytoplasm contains many large (3–100-μm diameter) cortical vesicles which are virtually close-packed just beneath the plasma membrane. While propagating through the cytoplasm, the calcium wave apparently elicits fusion of these vesicles with the plasma membrane (19). This conjecture is consistent with the known relationship between calcium and secretory events in other sytems (40) and is supported by the findings that raising free calcium will cause vesicle fusion, and that no vesicle fusion occurs in regions which the calcium wave cannot enter (see below). Therefore, to simplify the presentation of the results, I have assumed that the progress of the calcium wave can be monitored by observing the corresponding wave of vesicle fusion.

Calcium Buffers

CALCIUM AND ACTIVATION: Calcium buffer injections were performed to determine whether an increase in cytoplasmic free calcium is necessary to activate the medaka egg, and to explore the relationship of free calcium, activation and pH. The calcium buffers were made with 20 mM EGTA and had free calcium levels spanning the physiological range. Because of possible pH effects on the threshold free calcium required to trigger calcium-stimulated calcium release (as suggested by the pH buffer injection experiments, which were performed first), and because the free calcium concentration of a calcium/EGTA buffering system is strongly pH-dependent (see Table I), the calcium buffers were also strongly pH-buffered with 40 mM MOPS or PIPES at pH 6.5, 7.0, or 7.5. Each buffer was tested in at least 20 eggs from at least three females, for a total of 496 eggs. About half of the eggs were fertilized while the buffer was still effective. The results of these injections are given in Table II.

The main findings were: (*a*) Increasing free calcium above some threshold level will elicit a wave of vesicle fusion and, therefore by implication, the calcium wave. At pH 7.0, the threshold free calcium required to elicit the calcium wave is

TABLE I
Total Calcium/EGTA Ratios Used To Make the Calcium Buffering Solutions

	$[Ca^{2+}]_{free}$, μM*							
	(0.01) 0.017	(0.03) 0.05	(0.1) 0.17	(0.3) 0.51	(1.0) 1.7	(3.0) 5.1	(10) 17	(30) 51
pH 6.5					0.30	0.57	0.81	0.93
pH 7.0			0.30	0.56	0.81	0.93	0.98	
pH 7.5	0.29	0.56	0.81	0.93	0.98			

* The values in parentheses were the desired free calcium concentrations. The values below these are corrected for hydrogen ion concentration, and are probably closer to the actual free calcium concentration of the buffers (see Materials and Methods).

TABLE II
Results of Injection of pH-buffered Calcium Buffers

	$[Ca^{2+}]_{free}$, μM							
	0.017	0.05	0.17	0.51	1.7	5.1	17	51
pH 6.5								
MOPS					– (20)	– (6) * (15)	+ (20) – (1)	
PIPES					– (21)	* (21)	+ (12) * (8)	+ (20)
pH 7.0								
MOPS			– (21)	– (26)	– (13) * (8)	+ (22)	+ (21)	
PIPES				– (20)	– (9) * (12)	+ (19) – (1)	+ (6)	
pH 7.5								
MOPS	–(20)	–(24)	– (19) + (2)	– (24) * (3)	+ (20)			
PIPES			– (21)	– (4) * (17)	+ (20)			

–, no activation; *, local activation; +, activation. The numbers in parentheses show the number of eggs for which a given result was obtained.

between 1.7 and 5.1 μM, well below the estimated 30-μM peak calcium achieved in the calcium wave (19). Buffers which clamp free calcium below threshold prevent activation in the buffered region if the egg is fertilized while the buffer is still effective (for ~10 min after injection), and prevent activation altogether if the spreading buffer reaches the animal pole before the sperm does. An affected region was considered unactivated if no cortical vesicle fusion occurred, and if ooplasmic segregation (movement of most of the cytoplasm toward the animal pole, and of oil droplet inclusions toward the vegetal pole) failed or was incomplete, within the region. If no fusion occurred in the buffered region, then no fusion occurred in the area vegetal to the buffered region until the fusion wave from neighboring, unaffected regions of the cytoplasm invaded the lateral boundaries of this vegetal area, suggesting that the injected buffer blocked the passage of the calcium wave through the affected region. These observations satisfy the criteria outlined in the introduction.

(*b*) The threshold free calcium required to elicit a given effect (no activation, local activation, or activation) is a function of pH. For induction of the calcium wave, it is between 0.51 and 1.7 μM at pH 7.5; 1.7 and 5.1 μM at pH 7.0; and 5.1 and 17 μM at pH 6.5. Thus, for a 10-fold increase in hydrogen ion concentration, free calcium must also be raised about tenfold to elicit a given effect. Similar results were obtained in pilot experiments with solutions containing 40 mM EGTA, KCl and no pH buffer (16), suggesting that the observed pH dependence is not an artifact caused by alteration of buffer pH by cytoplasmic buffering systems.

Most of the eggs that were fertilized after the calcium buffers were no longer effective, or while they were still effective in the case of buffers near threshold, developed normally (93%; 147 of 158) as judged by visual comparison with uninjected controls, so the buffers were not acting as nonspecific poisons. On the other hand, 50% (40 of 80) of the eggs fertilized before a subthreshold buffer was overcome, and that therefore had a large unactivated region, developed abnormally. The same was true for ~17% (13 of 76) of the eggs injected with a suprathreshold buffer and fertilized before the micropyle closed and blocked sperm entry. In mild cases, abnormalities, such as distorted or missing structures, appeared only in the anterior regions of the embryo (heart, pericardium, eyes, head, etc.). In more severe cases, more posterior regions were included; in the most severe, the entire embryo was affected, and axiation sometimes failed to occur. These teratogenic effects are very similar to those produced in *Fundulus* by application of inhibitors of transcription and translation during fertilization (5, 51). Apparently, free calcium must achieve a high level throughout the entire cytoplasm, but must not remain high too long, to ensure normal activation and development.

LOCAL ACTIVATION: Local activation sometimes occurred when an egg was injected with a buffer having free calcium at or just below threshold. It was characterized by cortical vesicle fusion, depletion or inactivation of the calcium sensitive stores (determined by failure of the calcium wave to traverse the affected region when the egg was subsequently fertilized), and ooplasmic segregation (movement of cytoplasm to the animal boundary and migration of oil droplets to the vegetal boundary), which occurred only in the small region initially affected by the buffer. If the egg was fertilized while the buffer was still effective, activation was sometimes inhibited in a ring- or crescent-shaped area at the margin of the activated region. Local activation may have been an artifact due to pipette-induced calcium leakage into the cytoplasm, since the response was observed only during one set of experiments carried out in the summer of 1979, but not in similar experiments performed in winter/spring 1982 with a different set of pipettes made of different glass with a different puller. Other differences between the two sets of experiments, such as different populations of fish, or possible changes in the composition of the tap water used to culture the fish or the distilled water used to make the buffers, may also be responsible for the observed differences. That the response was real, however, was suggested by two observations. First, while a population of eggs may exhibit a mixed response to a near-threshold buffer (say, both local activation and no activation), most of the eggs from a particular female would show only one response, suggesting that small differences in calcium sensitivity may exist between batches of eggs from different females. Second, no correlation was apparent between local activation and the care exercised during injection or the shape, taper, and cleanliness of the pipette tip, suggesting that the response was not caused by pipette-induced calcium leakage.

pH Buffers

EFFECTS ON ACTIVATION AND DEVELOPMENT: pH buffer injection experiments were performed to test whether short-term changes of cytoplasmic pH would elicit any of the visible aspects of activation (such as cortical vesicle fusion or ooplasmic segregation) in unfertilized eggs or affect the development of fertilized eggs. Both MOPS (pKa = 7.2) and PIPES (pKa = 6.8) were used since by itself, neither could effectively cover the entire range of pH values tested (see Table III), and effects observed with one buffer could be checked with the other, within the range of overlap of buffer effectiveness. Each buffer, at each pH, was injected into at least nine eggs from at least three different females, for a total of 518 eggs (see Table III). Aside from artifactual mechanical activation, no visible aspects of activation were elicited by the buffers. Development was normal, as judged by visual comparison with KCl-injected and uninjected controls, whether the eggs were fertilized while the buffer was still effective (for ~8 min after injection, as judged by effects on the calcium transient-see below) or sometime afterward.

TABLE III
*Results of pH Buffer Injections**

	pH									
	6.1	6.3	6.5	6.7	6.9	7.1	7.3	7.5	7.7	7.9
MOPS	19/0	19/0	9/0	18/0	18/0	42/4	26/3	64/25	37/14	38/13
PIPES	18/0	18/0	27/1	18/0	18/1	18/0	18/1	34/3	26/5	34/15

* Number of eggs injected/number of eggs that activated.

Pipette-induced activation was observed in some experiments upon KCl control injection (5 of 43 eggs injected) or buffer injection, especially at higher pH values (Table III). This activation was probably due to calcium leaking into the egg through a transient wound left by the exiting pipette, and not to the injected KCl or buffer solutions, for the following reasons: (*a*) the proportion of activating eggs varied greatly from run to run with a given buffer (for example, from a low of 0 out of 6 to a high of 5 out of 7 with pH-7.5 MOPS), in contrast to the highly reproducible results obtained with the calcium buffers; (*b*) the proportion of activating eggs was in part a function of the care exercised during injection and of the shape, taper, and cleanliness of the pipette tip; (*c*) in general, activation would not occur until the pipette tip was completely withdrawn from the egg; (*d*) passage of the injected solutions through a Chelex column to remove traces of calcium did not prevent activation; and (*e*) it is unlikely that every solution injected was marginally capable of activating the eggs. A higher sensitivity to a calcium leak at higher pH, manifesting as an increased proportion of artifactually activated eggs, would be consistent with the observed pH dependence of the threshold free calcium concentration required to elicit the calcium wave.

EFFECTS ON THE CALCIUM TRANSIENT: When eggs were fertilized while an injected pH buffer was still effective, the velocity of propagation of the calcium wave, as monitored by cortical vesicle fusion, was altered during passage through the region of cytoplasm affected by the buffer. If buffer pH was 6.9 or lower, the wave was slowed relative to neighboring, unbuffered regions (see Fig. 2, *a–d*). The effect was just noticeable at pH 6.9. The lower the pH, the more pronounced was the effect until, at pH 6.1, the wave was barely capable of entering the buffered region. Conversely, if the buffer pH was 7.3 or above, the calcium wave was accelerated in the buffered region, and wave velocity increased with increasing pH (see Fig. 2, *e–g*). The slowing or acceleration of the wave became less pronounced as the buffer was diluted by diffusion and/or overcome by the egg's active buffering systems, and could not be detected by ~8–10 min after injection. These results suggest that the threshold free calcium required to trigger calcium-stimulated calcium release may be pH dependent.

Buffers of pH 6.9 and below were slowing the wave, rather than simply preventing vesicle fusion in the affected region while the calcium wave propagated through it normally. This was apparent because fusion did not occur at the vegetal boundary of the affected region until the delayed wave of fusion reached it. In fact, when buffer pH was sufficiently low (and wave delay correspondingly great), fusion in the area vegetal to the buffered region first occurred when the fusion wave from neighboring, unaffected regions of the cytoplasm invaded the lateral boundaries of this vegetal area. These observations suggest that the calcium wave itself was greatly slowed within the buffered region.

Control injections of KCl, however, did cause a slight delay of fusion of cortical vesicles in the buffered region, since a normal fusion wave did reach the vegetal boundary while some vesicles were still unfused in the buffered region. This effect was superimposed upon the effects due to the pH buffers, and was also pH-dependent: it was most pronounced at the lowest pH and was almost unnoticeable at the highest. Oddly, while injected KCl slowed fusion of the cortical vesicles, it accelerated incorporation of the fused vesicles into the plasma membrane. This effect was also observed with the buffers and was pH-dependent, being more pronounced at high pH than at low.

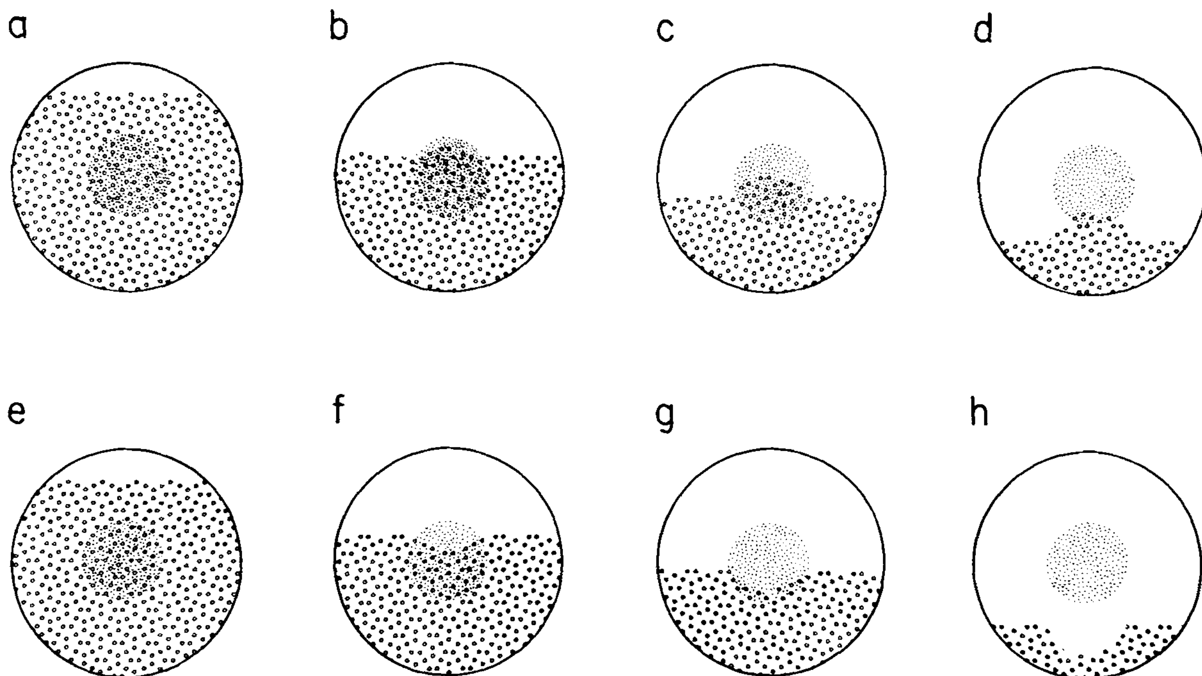

FIGURE 2 Schematic results of pH buffer injections. Face views of eggs that have been injected with a pH buffer midway between the animal and vegetal poles, and subsequently fertilized. (*a–d*) Results of injection of a buffer of pH <7.1; ~6.5 for the example shown. Note that the vesicle fusion wave is progressively slowed, relative to unbuffered regions, as it passes through the buffered region. (*e–h*) Result of injection with a buffer with pH >7.1; ~7.7 for this example. The wave is progressively accelerated as it passes through the buffered region.

CO_2 and NH_4Cl Exposure

CO_2 and NH_4Cl were added to the bathing Ringer's to test the effect of long-term changes in cytoplasmic pH on egg activation and development. Exposure of various cell types to CO_2-containing media causes a large, prolonged decrease in cytoplasmic pH due to the intracellular formation of carbonic acid by CO_2, which readily permeates the plasma membrane (38); subsequent removal of CO_2 causes a prolonged pH increase. Conversely, exposure to a weak penetrating base such as the free NH_3 in a solution containing NH_4Cl causes a large, prolonged increase in cytoplasmic pH, while subsequent removal causes a decrease (38). The ionic permeability of the medaka egg plasma membrane is so low (33) that NH_4^+ flux across this membrane should be negligible.

Several regimens were used to test the effect of CO_2 and NH_4Cl on unfertilized and fertilized eggs. When possible, each run of a given experimental regimen was performed with eggs from a single female. The minimum temporal resolution in the CO_2 and NH_4Cl experiments was limited to ~5 min by the time required to observe and judge the stage of development of each egg, and by the natural spread in the timing of developmental events in eggs from a single female. This is sufficient resolution to observe anomalies in the timing of ooplasmic segregation (most of which normally occurs before first division) and the first three divisions; in these experiments, first division occurred within 1 to 1.5 h after fertilization, and subsequent divisions 30–45 min apart, depending upon the ambient temperature. Ooplasmic segregation refers to the movement of the egg's cytoplasm to the animal pole, and the simultaneous migration of oil droplet cytoplasmic inclusions to the (opposite) vegetal pole.

I should point out that the significance of these experiments is limited by the fact that I did not directly monitor the effects of the treatments, if any, on cytoplasmic pH. In addition, the number of eggs studied in these tedious experiments was relatively small. The small number of eggs per regimen, however, does not necessarily make the results unreliable, because medaka egg development is remarkably reproducible: of more than 700 uninjected control eggs which I observed throughout the course of the experiments reported in this paper, only 33 exhibited aberrant development relative to their siblings.

The following regimens were used to test the effect of Ringer's containing 10 mM NH_4Cl, pH 7.3, on the eggs: (*a*) Eggs were exposed to NH_4Cl-Ringer's for periods of 60, 30, 10, or 5 min immediately before or after fertilization in standard Ringer's (20 eggs total). (*b*) Eggs were exposed to NH_4Cl-Ringer's for 10-min periods beginning 40, 30, or 20 min before, or 10, 20, 30, 40, 50, or 70 min after fertilization (25 eggs total). (*c*) Eggs were exposed to NH_4Cl-Ringer's for various periods before fertilization in NH_4Cl-Ringer's, and kept in this medium for various periods after fertilization; if the times in NH_4Cl-Ringer's before (b) and after (a) fertilization are designated by b/a, the regimens used were 5/5, 5/10, 5/30, 5/55, 10/5, 10/10, 10/30, 10/50, 30/5, 30/10, and 30/30 (30 eggs total). (*d*) Finally, eggs were either fertilized and immediately placed in NH_4Cl-Ringer's (three eggs), or placed in NH_4Cl-Ringer's and immediately fertilized (three eggs), and kept in this medium until hatching. Altogether, 81 experimental and 33 control eggs were used.

Placement of unfertilized eggs in NH_4Cl-Ringer's and subsequent replacement in normal Ringer's (see regimens *a* and *b* above) elicited no visible signs of activation. After fertilization, the eggs developed normally, with the same timing of events through third division as for untreated control eggs. Eggs fertilized during (regimens *c* and *d*) or before (regimens *a*, *b*, and *d*) exposure to NH_4Cl-Ringer's also developed normally as compared with controls. Thus, alterations of cytoplasmic pH (and other metabolic changes) caused by exposure to and removal from NH_4Cl-containing medium have no developmentally significant effects.

The effect of 5% CO_2-equilibrated Ringer's, pH 7.3, was determined with the following regimens: (*a*) Eggs were exposed to CO_2-Ringer's for periods of 60, 30, 10, and 5 min immediately before or after fertilization in standard Ringer's (16 eggs total). (*b*) Eggs were exposed to CO_2-Ringer's for 30-min periods beginning 75, 60, or 45 min before (nine eggs), or 10, 30, or 50 min after fertilization (21 eggs). (*c*) Eggs were exposed to CO_2-Ringer for 10-min periods beginning 10, 30, 50, or 70 min after fertilization (24 eggs total). (*d*) Eggs were exposed to CO_2-Ringer's for various periods before and after fertilization in this medium; using the notation of the NH_4Cl regimen *c* above, the regimens were 30/10, 30/30, and 10/30 (eight eggs total). (*e*) Finally, three eggs were exposed to CO_2-Ringer's for 4 h, beginning 30 min after fertilization. Altogether, 81 experimental and 32 control eggs were used.

Exposure to CO_2-Ringer's and subsequent replacement in normal Ringer's (see regimens *a* and *b*) neither activated the eggs nor interfered with development after fertilization. However, some effects on the timing of ooplasmic segregation and/or the first three divisions were observed with certain regimens involving exposure to CO_2-Ringer's after fertilization. Marginal delays (≤5 min) of one or more of the first three divisions were observed in eggs exposed to CO_2-Ringer's for 30-min periods immediately after fertilization (regimen *a* and 10/30, 30/30 of regimen *d*) and for 10-min periods beginning 10 and 30 min after fertilization (regimen *c*). Eggs exposed to CO_2-Ringer's for 60 min after fertilization (regimen *a*) were slightly behind controls in cytoplasmic segregation, and far behind in oil droplet segregation, at the end of the exposure period. The first division occurred 20 min later, the second ~10 min later, and the third ~8 min later than in controls. The effects of exposure to CO_2-Ringer's for 30-min periods beginning 10, 30, and 50 min after fertilization (regimen *b*; I shall designate them 10–40, 30–60, and 50–80, respectively) were similar. At the end of the exposure period, ooplasmic segregation in the 10–40 eggs appeared to have progressed about as far as in control eggs. Cytoplasmic but not oil droplet segregation then began to lag behind the controls, with the result that the first and subsequent divisions occurred ~6–8 min later than in the controls. Oil droplet segregation in the 30–60 and 50–80 eggs was apparently greatly slowed during exposure to CO_2-Ringer's, but cytoplasmic segregation progressed normally. First division occurred at the same time as in controls, sometimes while the 50–80 eggs were still in CO_2-Ringer's. The eggs then gradually fell behind the controls, until by third division they were marginally behind. Eggs exposed to CO_2-Ringer's for 4 h (regimen *e*) were roughly one division behind the controls (4 vs. 5) at the end of the exposure period. Cytoplasmic and oil droplet segregation had gone to completion while these eggs were in CO_2, so oil droplet segregation is only slowed and not arrested by CO_2 treatment.

The effects of exposure to CO_2 thus had three visible manifestations: a delay in the onset of one or more of the first three divisions, a slight slowing of cytoplasmic segregation, and a great slowing of oil droplet segregation. Cytoplasmic segregation and the timing of first division seemed to be most

sensitive between 10 and 30 min after fertilization (compare the 10–40 results with the 30–60 results). Effects on oil droplet segregation were more pronounced at later times, after the rate of oil droplet segregation normally increases (41). However, the longer the period of exposure to CO_2, the further the eggs fell behind the controls.

Finally, although the timing of early events may in some cases have been slightly altered, all eggs exposed to CO_2-Ringer's before, during and after fertilization developed into normal embryos.

DISCUSSION

Calcium and the Calcium Transient

As noted previously (19), if a transient increase in cytoplasmic free calcium is necessary for egg activation, then (*a*) such an increase in free calcium must accompany activation, (*b*) raising free calcium to a level equal to or below that reached during activation must activate the egg; and (*c*) preventing a rise in free calcium must prevent activation. Criterion *a* is certainly fulfilled in the medaka egg, as well as in echinoderm (44), frog (briefly mentioned in reference 48; and W. J. Wassermann, personal communication), and mouse (7) eggs. Are the other criteria met in the medaka egg as well?

The local activation phenomenon suggests that at least some aspects of medaka egg activation can be elicited by raising free calcium to between 0.51 and 1.7 μM at pH 7.0. Higher levels of free calcium elicit the calcium wave, which is certainly accompanied by full activation. The "null point" of the pH buffer injections, that is, the pH which had no noticeable affect on the rate of propagation of the calcium wave, was about 7.1. This must, then, be the pH at threshold free calcium during the normal passage of the wave. The threshold free calcium required to elicit the wave at pH 7.0 is between 1.7 and 5.1 μM; at pH 7.5, it is between 0.51 and 1.7 μM. At pH 7.1, it would be $\sim$2 μM. Both this and the local activation threshold are well below the estimated 30 μM free calcium achieved in the calcium wave (19). These results are as close as one can come to satisfying criterion *b*) in the medaka egg or any other system exhibiting calcium-stimulated calcium release, without somehow disabling the release mechanism.

Finally, preventing a rise in free calcium can prevent the calcium wave from entering the buffered region, blocking activation there, and can block initiation altogether, thus preventing activation. These observations satisfy criterion *c*).

Thus, an increase in free calcium is both necessary and sufficient to elicit the calcium wave, and is necessary to activate the egg. Abnormal development occurred in eggs in which activation was inhibited locally, or which were activated with a suprathreshold buffer before fertilization. Apparently, to ensure normal activation, free calcium must achieve a high level throughout the entire cytoplasm, but must not remain high too long.

While the above criteria have also been fulfilled in sea urchin eggs (23, 57), all three have not been demonstrated in the same species. Solutions containing high concentrations of $CaCl_2$ (200–500 mM) have been found to activate frog (6, 24) and mouse (14) eggs upon injection, but the resulting initial cytoplasmic free calcium was undoubtedly very high and may have caused activation as a secondary effect, so criterion *b*) was not fulfilled. The results I present here are thus the first complete demonstration that all three criteria are fulfilled for the egg of a single species.

I should note here that the existence of a relatively low threshold free calcium level that can trigger the calcium wave, and the observation that the wave can be blocked by preventing a rise in free calcium, provide further strong support that the calcium transient is initiated and propagated by calcium-stimulated calcium release.

pH and the Calcium Transient

The results of the pH buffer injections clearly show that the rate of propagation of the calcium wave through the egg's cytoplasm is a function of cytoplasmic pH: below pH 7.1 the wave is slowed, while above pH 7.1 it is accelerated. Further, calcium buffer injections show that the threshold free calcium required to elicit the calcium wave is a function of cytoplasmic pH; for a tenfold increase in hydrogen ion concentration, free calcium must be raised tenfold to elicit the calcium wave. To my knowledge, this is the first report of such a dependence.

In earlier papers (19, 35), my colleagues and I presented evidence that the calcium transient is locally elicited, and propagates through the cytoplasm, by a mechanism of calcium-stimulated calcium release: a local increase in free calcium triggers release of calcium from local stores, greatly raising local free calcium. The released calcium then diffuses to neighboring regions, raising free calcium and triggering release there, etc. Increased activity of calcium sinks then restores free calcium to resting levels behind the zone of release. One can postulate three simple mechanisms by which cytoplasmic pH could affect the rate of propagation: by altering resting free calcium; by changing the efficiency of release or uptake by the calcium sources or sinks; and by altering the threshold concentration of free calcium required to trigger calcium-stimulated calcium release. For example, less calcium must diffuse ahead of the wave, and therefore less time is required, to trigger release if resting free calcium is elevated by increased pH, so the wave may be accelerated under these conditions. However, resting free calcium is already two orders of magnitude lower than the level achieved in the calcium wave. A decrease in pH which lowered the resting free calcium by even another order of magnitude would have little effect on the rate of wave propagation. Further, the observed effect is in the "wrong" direction. For example, lowering pH—thus raising hydrogen ion concentration—should raise free calcium through calcium/hydrogen exchange or other mechanisms (9, 30, 36, 39) and thus increase wave velocity. In fact, the opposite is observed. Finally, a pH effect is observed even in the presence of a calcium buffer, which should swamp any effect of pH on resting free calcium level. Therefore, this mechanism is unlikely. Similarly, an effect of cytoplasmic pH on the efficiency of release or uptake by the calcium sources or sinks should be swamped by the calcium buffer. This leaves the third mechanism: the threshold concentration of free calcium required to trigger calcium-stimulated calcium release might be a function of pH. For example, if higher pH—thus lower hydrogen ion concentration—lowers this threshold, then less calcium must diffuse ahead to trigger release, and the wave will propagate more quickly.

How might pH alter this threshold? Since free calcium must be increased in direct proportion to hydrogen ion to elicit a response, perhaps the calcium binding site for calcium-stimulated calcium release can also bind one proton, and proton binding competitively inhibits calcium binding to this site. The binding of calcium to calmodulin, a small protein which apparently mediates calcium regulation of many aspects of

cellular metabolism, exhibits a pH dependence similar to that of medaka egg activation: between pH 6.5 and 7.5, free calcium must be decreased in direct proportion to hydrogen ion to maintain a constant concentration of calcium-calmodulin complex (45; see also reference 3). This suggests that calmodulin might mediate at least some aspects of medaka egg activation. However, in preliminary experiments, injected anticalmodulin antibody failed to block activation (my unpublished results), which may argue against mediation of calcium release by calmodulin. In any case, the calcium (or calmodulin) target must be either an integral membrane protein or associated with one, so a mechanism similar to one proposed for the pH sensitivity of calcium release in muscle (see below) may apply here as well (20): the surface charge density of biological membranes is very sensitive to bulk hydrogen ion concentration over the physiological range. The concentration of calcium and other cations is higher in the Stern layer (diffuse double layer) near the membrane than in bulk solution (because the net surface charge is negative) and is a function of the surface charge density, and, therefore, of bulk pH. Lowering pH, thus raising bulk hydrogen ion concentration, will decrease surface charge density, and thus also decrease free calcium near the membrane. Bulk free calcium must then be increased to raise free calcium near the membrane sufficiently to activate the receptor. Conversely, lowering bulk hydrogen ion will decrease the bulk free calcium required to activate the receptor.

A pH-dependent calcium sensitivity could also explain the apparent derepression of sea urchin egg metabolism by the pH increase: lowering hydrogen ion concentration may allow the affected systems to "see" and be stimulated by resting levels of free calcium.

Cardiac muscle sarcomplasmic reticulum apparently also exhibits pH-dependent calcium-stimulated calcium release (12). As in the medaka egg, raising hydrogen ion concentration increases the free calcium required to trigger release, and vice versa. Sarcoplasmic reticulum is simply modified endoplasmic reticulum. My colleagues and I earlier suggested that the calcium-stimulated source in medaka eggs may be a modified endoplasmic reticulum (19; see also reference 26). If this organelle is indeed the calcium-stimulated source in the medaka egg, a pH dependence of the calcium threshold might be expected. Further, a recent ultrastructural study of *Xenopus* eggs has revealed the presence of membrane junctions between the plasma membrane and the cortical endoplasmic reticulum (15). Similar junctions between the plasma membrane and the sarcoplasmic reticulum in muscle are thought to be involved in the triggering of calcium release from the sarcoplasmic reticulum. Finally, studies of the calcium source in sea urchin eggs indicate that ATP is required to keep the source loaded and sensitive (1, 2). Since the endoplasmic reticulum has been shown to sequester calcium in an ATP-dependent fashion (46, 47), this evidence also supports the idea that this organelle is the calcium-stimulated source.

pH and Activation of Development

The results of injection of pH buffers demonstrate that short-term, local changes in the pH of the cytoplasm of the medaka egg do not have any developmentally significant effects. Similarly, long-term, global changes in cytoplasmic pH—and any other effect upon egg metabolism—caused by exposure to NH_4Cl neither elicit visible signs of activation nor alter the course and timing of developmental events in fertilized eggs. It is not clear whether the effects of CO_2 on the timing of division and rate of ooplasmic segregation are due to alteration of cytoplasmic pH or to some other effect on egg metabolism. If the events leading to or the mechanisms underlying division and segregation were pH-sensitive, exposure to NH_4Cl should also have had an effect on these events, but none was observed. Further, a CO_2-induced pH change should not have lasted so long (due to metabolic compensation by the cell; see reference 38) as to exert a retarding influence on the egg over a period of 4 h, or even 1 h. In any case, eggs developed into normal embryos whether or not they were affected by a particular CO_2 treatment regimen. Therefore, either CO_2 had an equivalent effect on all developmentally significant aspects of metabolism, or these aspects are independent of pH changes, whether naturally occurring or artificially imposed.

A few years ago, the pH of homogenates of activated sea urchin eggs was observed to be more alkaline, by a few tenths of a unit, than that of homogenates of unactivated eggs (29, 31). Subsequently, direct and more reliable observations made with an intracellular pH microelectrode showed that the pH of the cytoplasm of an activated urchin egg increased by ~0.4 U over the unfertilized value, beginning ~1–2 min after activation and leveling off at the higher pH ~6–8 min after activation (42, 43). This change has been confirmed by dimethyloxazolidinedione (27) and ^{31}P-nuclear magnetic resonance (54) methods, and a similar increase has been observed in frog eggs using pH microelectrodes and ^{31}P-nuclear magnetic resonance (34, 49, 50). Experiments using weak penetrating acids and bases to alter sea urchin egg cytoplasmic pH suggested that the observed normal alkalinization was necessary for the expression of certain aspects of activation, the best studied being an increased rate of protein synthesis (21). The correlation between the time course and magnitude of the induced pH changes and changes in protein synthesis rate was not especially good, and the effects were only partially reversible, suggesting that at least part of the effect of these weak acid and base treatments on protein synthesis rates was due to some mechanism other than the induced pH change. Later studies involving natural and induced calcium transients, in addition to induced pH changes, showed that a prior calcium transient is necessary for a normal and complete change in protein synthesis (55). This finding was confirmed in a recent study using urchin egg homogenate dialyzed against pH and calcium buffers (51, 52); this study also provided the best evidence to date that the rate of protein synthesis in urchin eggs is, at least in part, a function of pH. However, a recent study in the egg of another echinoderm, a starfish, has shown that upon activation, a change in protein synthesis rate occurs without a change in cytoplasmic pH, yet treating starfish eggs with weak acids and bases will alter protein synthesis rates (28). This is additional evidence that such treatments may artifactually alter metabolism by means other than changing pH (27), and a demonstration that natural regulation of protein synthesis rates by pH is not universal, even within the echinoderms.

Local Activation

Local activation, which involved activation of only the small region of cytoplasm affected by the buffer, may be related to the similar results obtained by Chambers and Hinckley (4), and Epel (11) in sea urchin eggs locally exposed

to small doses of ionophore A23187. The suggestion has been made that the sea urchin results are caused by a localized ionophore-induced increase in cytoplasmic free calcium which is large enough to locally trigger cortical granule fusion and other aspects of activation, but not of sufficient magnitude to elicit the large, propagated increase characteristic of calcium-stimulated calcium release (25). Similarly, in the calcium buffer injection experiments reported here, the buffered level of free calcium may have been high enough to slowly release calcium from, and thereby deplete, the calcium-sensitive stores, to cause fusion of the cortical vesicles, and to activate the segregation machinery and other (but not necessarily all) aspects of activation. As the buffer spread away from the injection site, the egg's calcium regulating systems would have brought the buffered calcium level below that required for local activation. Alternatively, the buffered calcium level could have been just high enough to trigger calcium-stimulated calcium release, but as the buffer spread away from the injection site, the buffered calcium level was brought below threshold. Since the calcium wave travels more slowly than the diffusion-limited buffer front (19), the wave was soon damped. Some years ago, Yamamoto (56) found that, under certain conditions, a small stimulus could elicit a calcium wave which would die out before it had spread throughout the entire cytoplasm. If the "intensity" of the calcium wave is indeed a function of stimulus size, then a near threshold calcium buffer might have elicited a low intensity wave which quickly died out, producing local activation. Finally, the buffered calcium level may have been just below threshold, but calcium entering through a transient pipette-induced wound in the plasma membrane locally raised calcium above threshold; the elicited wave would then have been damped by the surrounding subthreshold buffer. While this last explanation is unlikely, (see Local Activation, in Results), it cannot be ruled out. Unfortunately, simply lowering external calcium may not be a good test of this last hypothesis, since wound closure is inhibited by low external calcium (my unpublished results).

SUMMARY

Short-term local and longer term global changes in the pH of the medaka egg's cytoplasm neither activated the eggs nor prevented normal development, although CO_2 exposure slowed certain aspects of early development. This suggests that even if a change in cytoplasmic pH normally occurs as a result of activation, it is not necessary for normal development. Injected pH buffers did, however, alter the rate of propagation of the calcium wave through the cytoplasm, suggesting that the threshold free calcium concentration for calcium-stimulated calcium release might be pH-dependent. This conjecture was supported by calcium buffer injection experiments, which demonstrated that if hydrogen ion concentration is raised tenfold, free calcium must also be raised tenfold to elicit the calcium wave. The calcium buffer experiments also demonstrated that an increase in cytoplasmic free calcium is necessary and sufficient to elicit the calcium wave; that the calcium wave is necessary for activation; and that a normal calcium wave is necessary to ensure normal development.

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