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DIRECT EVIDENCE FOR IONIC MESSENGERS IN THE TWO PHASES OF METABOLIC DEREPRESSION AT FERTILIZATION IN THE SEA URCHIN EGG[1]

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ABSTRACT It has been proposed that the release of intracellular ionic calcium may be the universal factor promoting activation of egg metabolism at fertilization. If this hypothesis is valid, then fertilization and all parthenogenetic agents which evoke the entire program of activation would necessarily involve an intracellular rise in ionic calcium. Fertilization or parthenogenetic activation of Lytechinus pictus eggs was monitored after injection with the Ca-sensitive photoprotein aequorin. We estimated the peak calcium transient at 2.5-4.5 μM free calcium, 45-60 s after activation and lasting 2-3 min, assuming equal distribution throughout the cytoplasm. The calcium release at fertilization was shown to be from intracellular stores. The threshold for the discharge of the cortical vesicles was between 9-18 μM calcium, suggesting that the transient calcium release is confined to the inner subsurface of the egg. Parthenogenetic treatments, ionophore A23187, non-electrolyte 1M urea and hypertonic medium 1.6 x concentrated sea water, all acted to release calcium from intracellular stores. Sperm, ionophore and non-electrolyte release from the same calcium store which can be recharged after 40 min. Hypertonic medium releases from a different store. It has been proposed that the second ionic messenger is an increase in intracellular pH, which is responsible for the late steps in activation such as protein and DNA syntheses. For this hypothesis to be valid, treatment with weak bases, which only evoke late steps in activation, should not release intracellular calcium but should raise intracellular pH. Measurements with aequorin injected eggs showed some calcium entry from external solutions with NH_4Cl exposures but that it was not essential to weak base activation. Measurement with microelectrodes of intracellular pH in intact eggs did show a rise in pH with NH_4Cl and other weak bases as well as in normal fertilization.

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INTRODUCTION

The fertilization of the sea urchin egg leads to a sequence of changes at the egg surface and the interior cytoplasm, resulting in the activation of metabolism and cell division. The first change is at the membrane with an increase in Na^+ permeability, followed several minutes later by the development of new K^+ permeability (1). Within a few seconds of insemination the cortical vesicles start to fuse with the egg membrane protease is secreted and the egg surface is considerably modified (2,3,4,5,6). The first directly detectable intracellular change is a transient rise in ionic calcium (7). The intracellular change in calcium is followed by a rise in the pH of egg homogenates (8,9) and by a rise of intracellular pH as measured in intact eggs with microelectrodes reported here.

We have been working on the hypothesis that the early events of fertilization are causally linked to calcium release and that the resulting surface changes lead to the change in intracellular pH which in turn is responsible for the late events of activation such as protein and DNA syntheses. This hypothesis originated from the observation that exposure of sea urchin eggs to ammonia, which is known to raise intracellular pH, activated the late responses while bypassing completely all the early events such as cortical reaction and the increase in O_2 uptake (10,11). If this hypothesis is valid, then parthenogenetic agents which evoke the entire program of activation would necessarily involve an intracellular Ca^{2+} release, but ammonia or other weak bases would not. To examine this question we chose representatives of four widely different classes of parthenogenetic agents and treated eggs which had been previously microinjected with the Ca^{2+}-sensitive photoprotein aequorin. We utilized a divalent ionophore (A23187), a non-electrolyte (1 M urea), a weak base (10 mM NH_4Cl) and a hypertonic medium (1.6 X concentrated artificial sea water). The first agent, A23187, is known to cause a transient release of intracellular Ca^{2+} and its action is described in our previous paper (7), as well as in some experiments reported here.

In addition, by using more than one activating agent in sequential treatments, we explored the question to what extent these different agents act on the same Ca^{2+} store and how fast it could be recharged. Finally, we made direct measurements of intracellular pH to confirm that both fertilization and weak base activation raise intracellular pH throughout the cytoplasm of sea urchin eggs for extended periods.

MATERIALS AND METHODS

Handling of Gametes. Eggs and sperm of the sea urchin

Lytechinus pictus were obtained by the injection of 0.5 M KCl into the coelomic cavity. The jelly coats were removed from the eggs by treatment with acidified sea water pH 5 for one minute and repeated washing with natural sea water. Eggs were stuck down with polylysine treated plastic petri dishes filled with sea water maintained at 18 ± 0.5 °C.

Experimental Procedure-Aequorin. Eggs were microinjected with 7 pl of a solution of 0.2 mM aequorin in 0.25 M K_2SO_4, 2 uM K_2EDTA and 10 mM phosphate buffer at pH 7.5. The methods for preparation, storage and microinjection of the aequorin solutions and the photometric methods for monitoring responses are described in detail elsewhere (7).

Experimental Procedure- pH. We measured intracellular pH (pH_i) by means of the Thomas type pH sensitive microelectrodes with recessed tips (12). For our experiments, these electrodes had to be constructed with tip diameters of the outer insulating glass of ca. 0.5 μ and with distances of 5 μ or less to the inner pH sensitive surface. The pH microelectrodes used in these experiments gave a linear response of slope 56-59 mV/pH unit over the range pH 2-9 and had less than a 3 mV drift when calibrated before and after each experiment in 100 mM phosphate buffers (pH 6.6 and 7.6). The full response times of the pH microelectrodes used in this study were 30 s or less and were unaffected by the presence of high protein concentrations (50 mg/ml bovine serum albumin). In our experiments, two micro-electrodes penetrated the egg. Measurements of the egg membrane potential were made with conventional electrodes (1). The pH sensitive microelectrode, after penetration and healing of the cell membrane, recorded the membrane potential and a voltage proportional to pH. In order to determine that both microelectrodes were implanted in the egg with minimal membrane damage, a current pulse was passed through the conventional microelectrode periodically and the corresponding membrane potential deflection was monitored by the pH sensitive microelectrode. To obtain the intracellular pH, the membrane potential was subtracted from the pH microelectrode record.

Solutions. Natural sea water was adjusted to pH 8.1 (SW). Artificial sea water pH 8.1 (ASW) containing 460 mM NaCl, 55 mM $MgCl_2$, 11 mM $CaCl_2$, 10 mM KCl and 5 mM $NaHCO_3$. Zero calcium sea water (0-Ca EGTA SW) with 15 mM NaCl substituted for the $CaCl_2$ plus 2 mM EGTA (ethyleneglycol bis (β-aminoethyl ether) -N,N-tetraacetic acid). Urea solution (Urea) 1 M urea plus 0.1 mM $CaCl_2$ adjusted to pH 8.1 with NaOH. Zero calcium Urea solution (0-Ca Urea) 1 M urea with no calcium plus 2 mM EGTA was adjusted to pH 8.1. The divalent ionophore (obtained from R. Hamill, Eli Lilly Co., Indianapolis) A23187 was delivered dissolved in dimethyl sulfoxide by the same remote control system used for sperm with continuous stirring of the medium

bathing the eggs. Dimethyl sulfoxide (DMSO) alone had no effects at the concentrations used. The final concentrations in the dish were 40 μM A23187 and 0.8% DMSO. Hypertonic medium (1.6 x ASW) was 1.6 x concentrated ASW pH 8.1. Rapid changes of solution were accomplished by a perfusion system. The perfusion rate was 0.25 ml/s; the fluid in the petri dish containing the eggs was replaced every 10 s.

RESULTS

Fertilization and Ionophore. As reported in our previous paper, fertilization or ionophore treatment released intracellular Ca^{2+} in a transient which could be estimated to reach at least 15 μM in the subcortical cytoplasm (7). In the experiments reported here, the responses are normalized per injected egg as many of the parthenogenetic treatments used could not be scored for elevation of fertilization membranes.

TABLE 1
CHARACTERISTICS OF AEQUORIN RESPONSES OF EGGS TO VARIOUS TREATMENTS[a]

Treatment	Number of Experiments	Response Amplitude (nA)	Response Half-Width (sec)
Fertilization	9	0.084 ± 0.019	58 ± 6.3
A23187	2	0.119 ± 0.082	117 ± 7.5
0-Ca A23187	2	0.085 ± 0.042	78 ± 21
A23187 (pooled)	4	0.102 ± 0.039	98 ± 15
Urea	6	0.388 ± 0.135	33 ± 6.7
0-Ca Urea	3[b]	0.152 ± 0.016	16 ± 4
Urea (pooled)	9	0.309 ± 0.096	28 ± 5.3
NH_4Cl ASW	5	0.085 ± 0.046	20 ± 4.3
1.6 x ASW	4	0.063 ± 0.0107	18 ± 3
0-Ca 1.6 x ASW	1	0.21	30
1.6 x ASW (after fertilization)	3	0.099 ± 0.024	16 ± 1.7
1.6 x ASW (after A23187)	2	0.864 ± 0.637	20 ± 7

[a] Figures are mean response ± S.E. in nanoamps per injected egg.
[b] In 2 other experiments, a response occurred on rinsing out 0-Ca Urea.

Non-electrolyte Activation. Treatments with the non-electrolyte urea medium (Urea) resulted in light emission from aequorin-injected eggs (Table 1). The light emission resulting from the Urea treatment did not require calcium in the external solution. We removed Ca^{2+} by prewashing the injected eggs

with 0-Ca EGTA SW, which we have shown previously to not cause a response by itself (7). We followed these prewashes with treatment with 0-Ca Urea (also with EGTA) and then back to 0-Ca EGTA SW. In 8 experiments of this type without external calcium, the eggs exhibited a Ca^{2+} transient release similar to that observed when Ca^{2+} was present (Table 1). We concluded that non-electrolyte treatment releases Ca^{2+} from an intracellular store.

Weak Base Activation. In five experiments, treatment with 10 mM NH_4Cl in SW pH 8.1 resulted in a brief light emission just as the ammonia-containing solution comes into contact with the eggs (Table 1). However, this response was not due to an internal release of Ca^{2+}. After previously prewashing in 0-Ca EGTA SW, eggs were exposed to 10 mM NH_4Cl in 0-Ca EGTA SW and no light emission was ever observed. We therefore concluded that ammonia treatment briefly permits Ca^{2+} entry from the external solution during the initial contact, but that ammonia does not release Ca^{2+} from an intracellular store.

Does ammonia treatment activate by the transient Ca^{2+} entry or by another pathway such as pH_i elevation (8,10)? We tested this question by prewashing eggs either in 0-Ca EGTA SW or in ASW and resuspending them at an egg concentration of 1% in four different lots in these four solutions: ASW, ASW plus 10 mM NH_4Cl, 0-Ca EGTA SW, and 0-Ca EGTA SW plus 10 mM NH_4Cl, all at pH 8.1. They were then incubated in their respective media in thin layers on the bottom of a beaker at 18°C. Aliquots were removed at 80 and 100 min and fixed in 3:1 ethanol:acetic acid. They were stained with 2% Orcein in 45% acetic acid and examined for condensed chromosomes and the breakdown of the nuclear envelope. Eggs in NH_4Cl in both ASW and 0-Ca EGTA SW showed condensed chromosomes while the controls in just ASW and 0-Ca EGTA SW had intact nuclear envelopes and no condensed chromosomes. We concluded that while exposure to ammonia-containing solutions can allow some Ca^{2+} to enter the egg from the outside, ammonia activation does not involve an intracellular release of Ca^{2+} and does not require Ca^{2+}.

Hypertonic Medium. The Loeb method for inducing cell division after activation by parthenogenetic agents involves a secondary treatment with hypertonicity (13,14). After a 30 min pretreatment with 10 mM NH_4Cl SW, aequorin-injected eggs were washed in normal SW and then exposed to 1.6 x ASW. The light emitted showed a roughly similar intensity and duration to those observed in activation (Table 1). Eggs pretreated as above with 10 mM NH_4Cl, washed in 0-Ca EGTA SW, and then exposed to 1.6 x ASW without Ca^{2+} plus 2 mM EGTA also demonstrated a similar Ca^{2+} transient (Table 1). Therefore, we concluded that hypertonic treatment also releases from an intracellular store.

The Calcium Store. Do all parthenogenetic agents which release calcium do so from the same intracellular store? To answer this question, we used double treatments in combination of sperm, non-electrolyte, ionophore or hypertonic medium. In the first of these experiments, we fertilized a field of aequorin-injected eggs and submitted them to a series of washes with Urea at sequential time points. Eggs which had just responded to fertilization could not respond to Urea, but after 40 min a full calcium transient could be elicited. In nine similar experiments in which Urea treatments were repeated or the ionophore A23187 was followed by Urea or vice versa, or sperm was followed by ionophore, the second treatment evoked little or no additional Ca^{2+} release if it closely followed the first (fig. 1). Therefore, we believe non-electrolytes, ionophore and normal fertilization activate by releasing Ca^{2+} from the same intracellular store, which can be recharged in 40 min and released again.

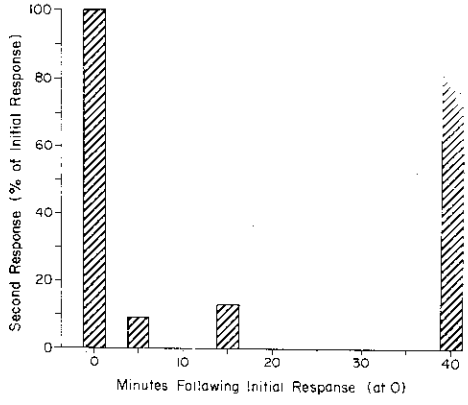

FIGURE 1. Pooled responses from 9 experiments in which aequorin-filled eggs were initially fertilized or exposed to Urea or to ionophore, and subsequently exposed one or more times to Urea or ionophore. The responses to subsequent treatments were expressed as a percentage of the initial response, and the average responses for 4 treatments each at intervals of about 5, 15 or 40 min after the previous treatment are shown.

Experiments with hypertonic SW gave quite different results. When eggs are first activated by sperm or by ionophore, a subsequent exposure to 1.6 x ASW evoked a typical response even if given at the short intervals when a second treatment

with ionophore or non-electrolyte would have resulted in little or no response from the nearly empty and refractory intracellular store (Table 1). Taken together, our results suggest that hypertonic SW releases Ca^{2+} from some other intracellular source than the internal store released by sperm, ionophore and non-electrolyte.

Intracellular pH (pH_i). Unfertilized eggs of L. pictus were placed in SW, penetrated with both microelectrodes and inseminated by the addition of dilute suspensions of sperm. Unfertilized eggs maintained a steady intracellular pH of 6.84 $\pm$ 0.02 (n=44). Fig. 2 shows a typical response to fertilization.

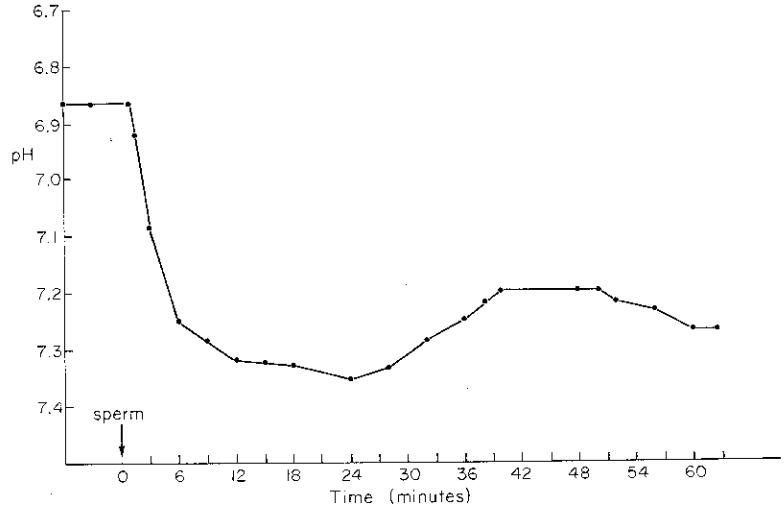

Figure 2. Continuous recording of intracellular pH during fertilization.

The rapid increase in pH_i closely parallelled the kinetics of the H^+ efflux recorded with extracellular electrodes under the same conditions. Generally within 6-8 min the intracellular pH of fertilized eggs stabilized at their higher new values of 7.27 $\pm$ 0.03 (n=15) and remained close to those values throughout the 60 min recording periods used in these experiments.

We also examined the intracellular pH changes during ammonia activation. Unfertilized eggs were exposed to different concentrations of NH_4Cl and in some cases to procaine (fig. 3). The higher concentrations of NH_4Cl acted more quickly in raising pH_i as expected. However, there were two unexpected features to these results. First, the H^+ generation was fin-

ished well before the change in pH_i. Second, given enough time, the different NH_4Cl concentrations achieved the same extent of an increase in pH (0.72 ± 0.01, n=8) before leveling off.

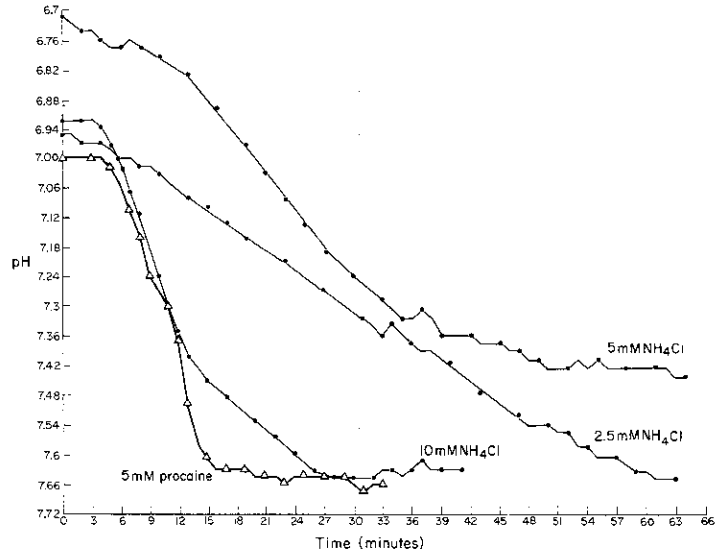

FIGURE 3. Continuous recording of pH_i during activation by NH_4Cl at 2.5, 5 and 10 mM (0) and procaine at 5 mM ( ). Twice the experimental concentration of NH_4Cl or procaine was dissolved in SW and the pH was adjusted to 8.1 with NaOH prior to the experiment. At t=0, equal volume of NH_4Cl or procaine was added to the experimental sea water bath.

Apparently, the intact egg can regulate its intracellular pH and responds only slowly to a weak base stimulus. The pH (7.65 ± 0.05, n=8) reached in NH_4Cl or procaine is higher than the pH reached in fertilized eggs and may represent a point at which the intracellular buffers are very effective in resisting further pH increase.

Further evidence of more complex regulation of pH_i was observed when a NH_4Cl stimulus is washed out. With a 50-fold dilution of a 10 mM NH_4Cl stimulus, there is only a partial recovery of lower pH values and then the pH_i continues to drift slowly upward. With a 500-fold dilution of the 10 mM NH_4Cl stimulus, the pH_i again only partially recovers (~30%) and then remains steady at this level well above the control values for unstimulated eggs.

DISCUSSION

The metabolic derepression at fertilization or parthenogenetic activation of the sea urchin egg is hypothesized to occur in two main phases (10,11). In the first phase, intracellular Ca^{2+} release mediates a number of changes, most likely by means of the discharge of the cortical vesicles (7,15). In the second phase, a rise in intracellular pH is postulated to be the principal mediator in the activation of protein and DNA syntheses (8,10,11). Thus, in this broad outline of the program of activation, there is an essential role for two ionic messengers. If this hypothesis is valid, one must be able to demonstrate directly the postulated changes in Ca^{2+} and pH. Furthermore, it is also essential to show how the first ionic messenger, Ca^{2+}, leads to the second ionic message, the rise in intracellular pH. Finally it is necessary to demonstrate directly under *in vitro* controlled conditions how these ions act at physiological concentrations to affect specific reactions.

In the experiments reported here and those previously (7) fertilization, divalent ionophore A23187 and non-electrolyte, all result in a transient release of intracellular Ca^{2+}. Furthermore, we have recently shown that when the intracellular rise in Ca^{2+} is prevented by injections of EGTA, activation by sperm is prevented (16). In contrast to the other forms of activation, ammonia activation proceeds without an intracellular Ca^{2+} elevation.

Fertilization, divalent ionophore and non-electrolyte treatment all can lead to discharge of the cortical vesicles (15,17), but ammonia treatment does not (10). We suggest that parthenogenetic agents which release intracellular Ca^{2+} activate the eggs by initiating the cortical reaction or another surface change, which in turn, results in a signal from the changed egg surface going into the interior cytoplasm. We are not inclined to believe that this signal from the surface is primarily Ca^{2+} since our previous experiments (7) indicated that the transient Ca^{2+} rise is largely confined to the inner subsurface of the egg. The fact that ammonia activation need not be accompanied by a rise in intracellular Ca^{2+} is consistent with the postulated roles for Ca^{2+} and pH.

If pH is the signal from the surface to the interior, then both normal fertilization and weak base activation with substances such as NH_4Cl should show a rise in pH_i. Our direct measurements with intracellular electrodes do demonstrate that the intracellular pH is elevated by these treatments for considerable periods, suggesting that this increase in pH is a leading candidate in further investigations of the mechanisms of derepression of protein synthesis. Preliminary results in-

dicate rapid changes in the rate of incorporation of amino acids into new protein can be accomplished with intracellular pH changes of 0.1 pH units (Grainger, Winkler, Shen and Steinhardt, manuscript in preparation).

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