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Intracellular Calcium Release and the Mechanisms of Parthenogenetic Activation of the Sea Urchin Egg

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Parthenogenetic activation of *Lytechinus pictus* eggs can be monitored after injection with the Ca-sensitive photoprotein aequorin to estimate calcium release during activation. Parthenogenetic treatments, including the nonelectrolyte urea, hypertonic sea water, and ionophore A23187, all acted to release Ca^{2+} from intracellular stores. Ionophore and urea solutions release Ca^{2+} from the same intracellular store as normal fertilization. This intracellular store can be reloaded after 40 min and discharged again. Hypertonic medium appears to release Ca^{2+} from a different intracellular store. Treatment with the weak base NH_4Cl did not release intracellular Ca^{2+} but did result in a momentary Ca^{2+} influx if Ca^{2+} was present in the external solution. Ca^{2+} influx was not required for ammonia activation.

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 (Steinhardt *et al.,* 1971). 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 (Millonig, 1969; Vacquier *et al.* 1972; Fodor *et al.,* 1975; Shapiro, 1975; Eddy and Shapiro, 1976). The first directly detectable intracellular change is a transient rise in ionic calcium (Steinhardt *et al.,* 1977). The intracellular change in calcium is followed by a rise in the pH of egg homogenates (Johnson *et al.,* 1976; Lopo and Vacquier, 1977) and by a rise in the intracellular pH as measured in intact eggs with microelectrodes (Shen and Steinhardt, 1978).

Steinhardt and Epel and their collaborators (Steinhardt and Epel, 1974; Epel *et al.,* 1974; Johnson *et al;* 1976) 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 synthesis and DNA synthesis. 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 of the early events such as the cortical reaction and the increase in O_2 uptake (Steinhardt and Mazia, 1973; Epel *et al.,* 1974). If this hypothesis is valid, then parthenogenetic agents which evoke the entire program of activation would necessarily involve an intracellular calcium 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 nonelectrolyte (1 *M*

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urea), a weak base (10 m*M* NH_4Cl), and a hypertonic medium (1.6× concentrated artificial sea water). The first agent, A23187, is known to cause a transient release of intracellular calcium, and its action is described in our previous paper (Steinhardt *et al.,* 1977) as well as in some experiments reported here.

In addition, by using more than one activating agent in sequential treatments, we explored the questions to what extent these different agents act on the same calcium store and how fast it could be recharged.

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 1 min and repeated washing with natural sea water. Eggs were stuck down on polylysine-treated plastic petri dishes filled with sea water maintained at 18 ± 0.5°C.

Sperm was stored "dry" at 4°C and delivered in a small capillary tube by a pulley system to the egg dish in the light-tight cage.

Experimental procedures. Eggs were microinjected with 7 pliters of a solution of 0.20 m*M* aequorin in 0.25 *M* K_2SO_4, 2 μ*M* K_2EDTA (ethylenediaminetetraacetic acid), and 10 m*M* phosphate buffer at pH 7.5. The methods for the preparation, storage, and microinjection of the aequorin solutions are described in detail elsewhere (Steinhardt *et al.,* 1977). The present results are based on about 70 observations of responses from over 1000 individually microinjected cells.

Detection of light emitted by aequorin. Light emitted by small groups of microinjected eggs (23–50 per dish) as a consequence of the reaction of Ca^{2+} and aequorin was collected and transmitted to a photomultiplier tube by a fiber-optics light guide (⅛-in. diameter, 1-ft long, 0.35 N.A.; American Optical UVLGM-3-12). We used an EMI 9789A photomultiplier tube, especially selected for low dark current and run at an anode voltage of 870 V. The anode current was proportional to the intensity of the light. One nanoampere corresponded to 4×10^{-5} parts of injected aequorin exhausted per second. For other details of our procedures, see our previous report (Steinhardt *et al.,* 1977).

In our previous paper (Steinhardt, *et al.,* 1977), we used *N*-ethylmaleimide to release intracellular calcium and exhaust the aequorin in eggs and concluded that all of the injected aequorin was available to react with the intracellular free calcium. It has since come to our attention that, under certain conditions, *N*-ethylmaleimide itself reacts with aequorin to emit light and inhibits the aequorin–calcium reaction (Shimomura *et al.,* 1974). These effects would invalidate our interpretations of our previous experiment. We have now performed control experiments, in which aequorin was injected into droplets of cytoplasm-like buffer (0.45 *M* KCl, 10 m*M* $MgCl_2$, 50 m*M* Hepes (pH 6.7), 10 m*M* K_2EGTA) containing 1 m*M* N-ethylmaleimide. No light was emitted for over 1 hr. The subsequent addition of excess $CaCl_2$ led to the same emission of light as that when no *N*-ethylmaleimide was present in the droplet. We conclude that, under the conditions of our experiments, *N*-ethylmaleimide–aequorin interactions are negligible, and we believe that these experiments still show that injected aequorin is fully available to react with intracellular free calcium.

Solutions. Solutions used included: natural sea water (SW) adjusted to pH 8.1; artificial sea water (ASW), pH 8.1, containing 460 m*M* NaCl, 55 m*M* $MgCl_2$, 11 m*M* $CaCl_2$, 10 m*M* KCl, and 5 m*M* $NaHCO_3$; zero-calcium sea water (0-Ca EGTA SW) with 15 m*M* NaCl substituted for the $CaCl_2$ plus 2 m*M* EGTA [ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid]; a urea solution (UREA) of 1 *M* urea plus 0.1 m*M* $CaCl_2$ adjusted to pH 8.1 with NaOH; a zero-calcium urea solution (0-Ca UREA)

of 1 *M* urea with no calcium plus 2 m*M* EGTA, also 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 alone had no effects at the concentrations used. The final concentrations in the dish were 40 μM A23187 and 0.8% dimethyl sulfoxide (DMSO). Hypertonic medium (1.6× ASW) was 1.6× concentrated artificial sea water, pH 8.1. Rapid changes of solution were accomplished by a perfusion system controlled from outside the light-tight cage. The perfusion rate was 0.25 ml/sec; the fluid in the petri dish containing the eggs was replaced every 10 sec.

Aqueous solutions of urea decompose slowly into ammonium cyanate (Marier and Rose, 1964). We were concerned that our urea solutions might contain sufficient ammonia to activate eggs. We measured the ammonium ion concentration in our urea solutions with ammonium ion-selective electrodes (kindly provided by Dr. F. Orme). The electrodes were calibrated in 1 *M* glucose plus various concentrations of ammonium, and we determined that urea does not affect the electrode response to ammonium. Our 1 *M* urea solutions contained ammonium at concentrations less than 100 μM, which is far less than the amount required for activation.

RESULTS

Nonelectrolyte Activation

In six experiments, treatment with the nonelectrolyte urea medium (UREA), as defined in Materials and Methods, resulted in light emission from aequorin-injected eggs (Fig. 1). The responses fell into three classes. In four experiments, light was emitted within a few minutes of washing in the UREA, without any emission on reentering SW (Fig. $1A_1$). In a fifth experiment, some light was emitted in the UREA wash and a small response also occurred upon the reentry to SW (Fig. $1A_2$). Finally, in the sixth experiment, the light response was small upon entry into UREA and large as the SW was washed back in (Fig. $1A_3$). Since we were limited to observing small groups of

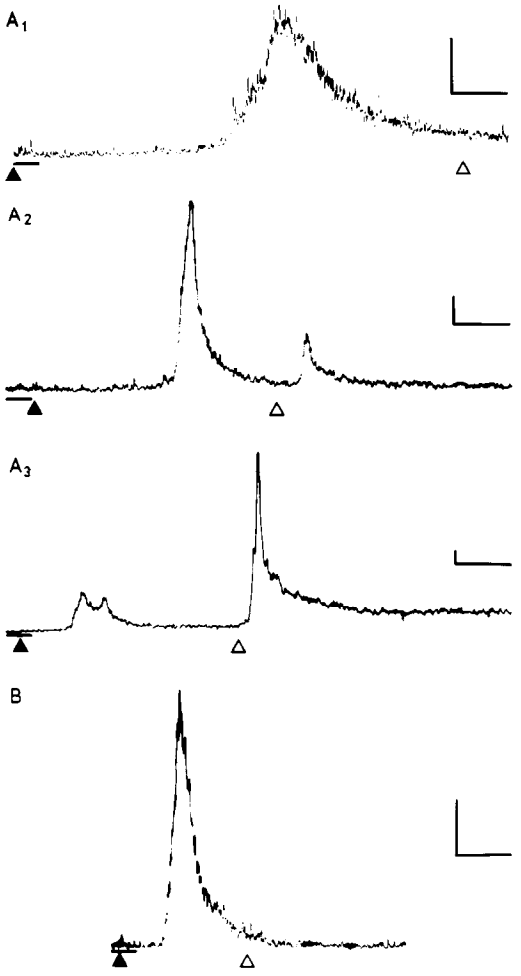

FIG. 1. (A) Light emitted by eggs filled with aequorin and exposed to UREA solution (solid triangles) followed by a sea water rinse (open triangles). The numbers of healthy-looking eggs injected with aequorin were 18 in A_1, 27 in A_2, and 21 in A_3. (B) Thirty-three successfully injected eggs were washed with 0-Ca EGTA SW and exposed to 0-Ca UREA (solid triangle) followed by a 0-Ca EGTA SW rinse (open triangle). In this and all subsequent figures, the vertical bar represents 1 nA of photomultiplier anode current, the horizontal bar represents 1 min, and the short horizontal line on the left is the zero light level. All experiments were conducted at 18°C, and the anode current was smoothed with a time constant of 400 msec. The composition of solutions is given in Materials and Methods.

injected eggs, these differences may reflect the variability of individual eggs in the response to nonelectrolyte. Once a group of eggs had been treated with UREA and SW washes and therefore had emitted light, indicating a transient Ca^{2+} release, that group of eggs required more than 30 min to recover its ability to respond to UREA (see the following section on the calcium store).

The light emission resulting from the UREA and SW treatment did not require calcium in the external solutions. We removed calcium by prewashing the injected eggs with 0-Ca EGTA SW, which we had shown previously not to cause a response by itself (Steinhardt *et al.,* 1977). We followed these prewashes with a treatment with 0-Ca UREA (also with EGTA) and then returned to 0-Ca EGTA SW. In five experiments of this type without external calcium, the eggs exhibited a Ca^{2+} transient release similar to that observed when calcium was present (Fig. 1B). In two of these experiments, the only emission occurred upon the return to 0-Ca EGTA SW, while in one experiment, the responses to UREA and the return to SW were similar. Based on the results obtained in the absence of external calcium, we concluded that nonelectrolyte treatment releases Ca^{2+} from an intracellular store.

We were interested in comparing the responses to UREA to those evoked by fertilization and by A23187 activations (Steinhardt *et al.,* 1977). Unfortunately, quantitative comparisons are particularly difficult because there is a great deal of variability in the form and magnitude of the responses. In most cases, the responses had a rapid rise, a rounded peak, and a slow roughly exponential decay. The variability in the amplitude of the responses was due in part to a variable percentage of eggs surviving the injection procedure. We attempted to correct for this by dividing the peak response magnitudes by the number of injected eggs that appeared healthy, but since we had previously found (Steinhardt *et al.,* 1977) that only 30–70% of the injected healthy-looking eggs are fertilizable, this correction is far from perfect. Other factors, such as imperfect synchrony in egg responses, also contribute to the variability. In the case of urea responses, the problem is confounded by the fact that responses may occur only to the UREA treatment or the SW rinse, or to both. Furthermore, urea responses were sometimes complex, consisting of two or more peaks or declining in two phases—one fast and one slow. We decided to use the half-width of a response as a rough index of its duration, but the limitations of this measure must be appreciated in attempting to compare responses whose time courses are not identical.

Table 1 compares responses to insemination, exposure to ionophore in the presence or absence of calcium, and exposure to urea in the presence or absence of calcium. Note that the peak amplitudes are expressed in nanoamperes per injected healthy-looking egg. Since roughly 50% of such eggs were fertilizable, a value of 0.08 nA/injected egg corresponds roughly to 0.16 nA/fertilized egg, which is similar to the result reported in our earlier paper (Steinhardt *et al.,* 1977). Since we had no visual indication of what proportion of urea-treated eggs was activated (the fertilization membrane dissolves in urea), we could not use the more appropriate measure of nanoamperes per activated egg to compare response amplitudes.

The results indicate that all five treatments elicit roughly similar responses. It is possible that slightly smaller responses are obtained in 0-Ca media, but these differences are not significant for either A23187 or UREA (two-sided t tests, $P > 0.05$). Perhaps soaking eggs in 0-Ca SW depletes the intracellular calcium store to some extent. It appears that the UREA responses are somewhat larger than those elicited by sperm, but this difference is also not significant (two-sided t test, $P > 0.05$). The duration and latency of responses are unaffected by external calcium, so these data may be pooled for the four ionophore and

nine urea experiments. The results now suggest a briefer response to UREA than to sperm ($P < 0.01$) and a longer ($P < 0.05$) but earlier ($P < 0.01$) response to A23187 than to sperm. These apparent differences in duration may be artifacts, especially in the case of UREA responses, where slight differences in response shape have a large influence on half-width. Since additional experiments (see below) suggest that all of these treatments release calcium from the same intracellular store, we do not regard these differences between UREA, A23187, and fertilization responses as particularly meaningful.

Weak Base Activation

In five experiments, treatment with 10 m*M* NH_4Cl in SW (pH 8.1) resulted in a brief light emission as the ammonia-containing solution came into contact with the eggs (Fig. 2A and Table 1). The responses to ammonia were briefer than the responses to sperm and ionophore (two-sided *t* test, $P < 0.01$). Since the ammonia responses were uniform in shape, we are confident of the statistical significance of this difference in duration. However, ammonia responses are not due to an internal release of Ca^{2+}. After prewashing in 0-Ca EGTA SW, eggs were exposed to 10 m*M* NH_4Cl in 0-Ca EGTA SW in two experiments, and no light emission was observed at all (Fig. 2B). We therefore concluded that ammonia treatment briefly permits calcium entry from the external solution during the initial contact, but that ammonia does not act directly to release Ca^{2+} from an intracellular store.

Does ammonia treatment activate by the transient calcium entry or by another pathway such as intracellular pH elevation

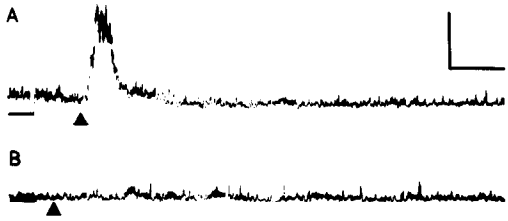

FIG. 2. Responses of eggs to exposure to 10 m*M* NH_4Cl in SW. (A) Eighteen eggs were successfully injected with aequorin, and the ammonia was introduced (triangle) in SW. (B) Twenty-five injected eggs appeared healthy, and the ammonia was introduced (triangle) in 0-Ca EGTA SW.

TABLE 1

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

Treatment	Number of experiments	Response amplitude[b] (nA/egg)	Response half-width (sec)	Response latency (sec)
Fertilization	9	0.084 ± 0.019	58 ± 6.3	79 ± 8.6
A23187	2	0.119 ± 0.082	117 ± 7.5	28 ± 2.5
0-Ca A23187	2	0.085 ± 0.042	78 ± 21	22 ± 3.5
A23187 pooled	4	0.102 ± 0.039	98 ± 15	24 ± 2.5
UREA	6	0.388 ± 0.135	33 ± 6.7	101 ± 25
0-Ca UREA	3[c]	0.152 ± 0.016	16 ± 4	152 ± 51
UREA pooled	9	0.309 ± 0.096	28 ± 5.3	118 ± 24
NH_4Cl ASW	5	0.085 ± 0.046	20 ± 4.3	35 ± 12
1.6× ASW	4	0.063 ± 0.017	18 ± 3.0	46 ± 9.4
0-Ca 1.6× ASW	1	0.21	30	72
1.6× ASW after fertilization	3	0.099 ± 0.024	16 ± 1.7	41 ± 21
1.6× ASW after A23187	2	0.864 ± 0.637	20 ± 7	23 ± 0
Fertilization after 1.6× ASW	1	<0.002	>100	~70
A23187 after 1.6× ASW	2	0.007 ± 0.003	>350	~20
UREA after NH_4Cl ASW	1	0.139	91	235
Fertilization after NH_4Cl ASW	1	0.035	60	55
1.6× ASW after NH_4Cl ASW	1	0.050	23	65

[a] Figures are means ± SE.

[b] Peak amplitude divided by the number of injected healthy-looking eggs.

[c] In two other experiments, a response occurred on rinsing out 0-Ca UREA.

(Steinhardt and Mazia, 1973; Johnson *et al.*, 1976)? We tested this question by prewashing eggs either in 0-Ca EGTA SW or in artificial sea water (ASW) and then resuspending them at an egg concentration of 1% in four different lots in these four solutions: ASW, ASW plus 10 m*M* NH_4Cl, 0-Ca EGTA SW, and 0-Ca EGTA SW plus 10 m*M* 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 ethanol–acetic acid (3:1). They were stained with 2% Orcein in 45% acetic acid and examined for condensed chromosomes and 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 (Loeb, 1913; Brandriff *et al.*, 1975). In four experiments, after a 30-min pretreatment with 10 m*M* NH_4Cl SW (pH 8.1), aequorin-injected eggs were washed in normal SW and then exposed to 1.6× concentrated artificial SW. The light emitted showed a roughly similar intensity but a somewhat briefer duration than those observed in activation (Fig. 3A and Table 1). In another experiment, eggs pretreated as above with 10 m*M* NH_4Cl, washed in 0-Ca EGTA SW, and then exposed to 1.6× ASW without calcium plus 2 m*M* EGTA demonstrated a similar calcium transient (Fig. 3B and Table 1). Therefore, we concluded that hypertonic treatment releases from an intracellular store.

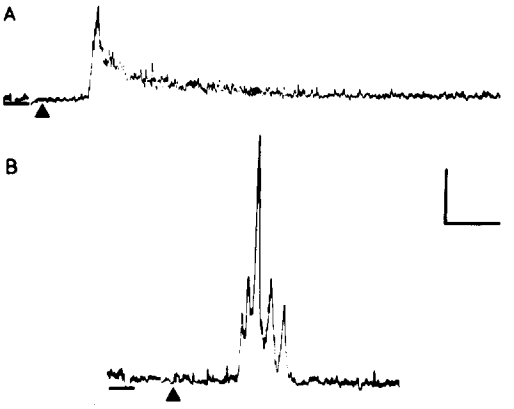

FIG. 3. Responses of eggs pretreated with ammonia and exposed to hypertonic sea water. (A) Twenty-one eggs were successfully injected with aequorin and washed in 1.6× ASW (triangle) after a 30-min exposure to ammonia. (B) Twenty eggs were filled successfully with aequorin, kept in ammonia for 30 min, washed in 0-Ca EGTA SW, and exposed to 0-Ca EGTA 1.6× ASW.

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 of combinations of sperm, nonelectrolyte, 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 (Fig. 4). This result suggests that fertilization and UREA release calcium from the same intracellular store. In this dish, 42 of the 50 injected eggs showed no visible damage, and 34 were successfully fertilized as scored by the elevation of the fertilization membrane. Since the UREA treatment at 5 min after fertilization did not give a significant increase in light emission, we concluded that the same eggs contributed to the response to sperm and to the response after 40 min to UREA. In several similar experiments in which UREA treatments were repeated, ionophore A23187 was followed by UREA or vice versa, or sperm was followed by iono-

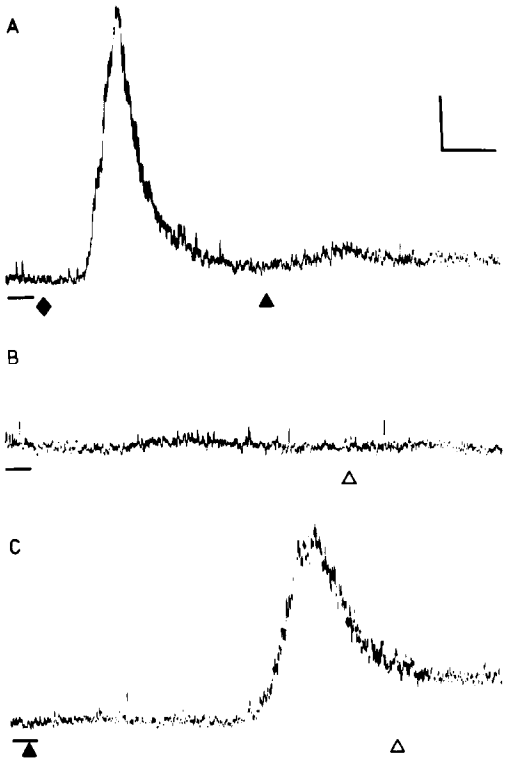

FIG. 4. Responses of one field of 42 healthy-looking eggs injected with aequorin to insemination and exposure to UREA solution. (A) Sperm was added (diamond), and UREA was introduced 5 min later (solid triangle): 34 eggs were fertilized by the sperm. (B) UREA was washed out (open triangle) with SW after 10.5 min. (C) UREA was reintroduced 40 min after the first exposure to UREA (solid triangle) and then washed out (open triangle).

phore, the second treatment evoked little or no additional calcium release if it closely followed the first (see Fig. 7). Therefore, we believe that nonelectrolyte, ionophore, and normal fertilization activate by releasing calcium from the same intracellular store.

In five experiments with hypertonic SW, we obtained quite different results. When eggs were first activated by sperm or by ionophore, a subsequent exposure to 1.6× ASW evoked a typical (or even exaggerated) response, even if given at the short intervals when a second treatment with ionophore or nonelectrolyte would have resulted in little or no response from the nearly empty and refractory intracellular store (Fig. 5A and Table 1). Taken together, our results suggest that hypertonic SW releases calcium from some intracellular source other than the internal store released by sperm, ionophore, and nonelectrolyte.

Treatment of eggs with hypertonic SW followed by fertilization or exposure to ionophore gave an even more curious result (Fig. 5B and Table 1). The transient calcium release caused by hypertonic SW was followed by a prolonged and greatly diminished release of intracellular Ca^{2+}, lasting several minutes. This correlated with a very slow release of cortical vesicles and a slow elevation of the fertilization membranes observed visually in parallel experiments done under identical conditions. Apparently, the hypertonic treatment can interfere with normal calcium release, altering both its amplitude and its time course.

Our experiments with ammonia demonstrated that treatment with weak bases can allow external Ca^{2+} to enter the egg transiently, but that such treatment does not release the intracellular Ca^{2+} store responsible for triggering the cortical reaction. Therefore, we would not expect a pretreatment with ammonia to occlude a subse-

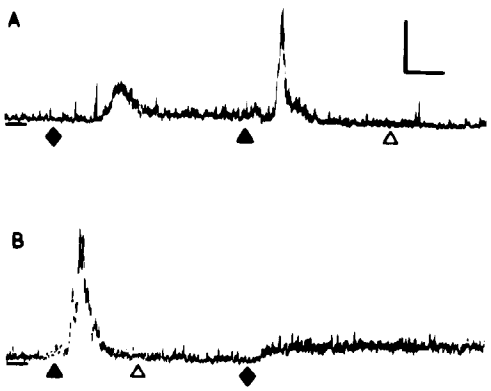

FIG. 5. Interactions between hypertonic sea water responses and those elicited by sperm or ionophore. (A) When 25 successfully injected eggs were inseminated (diamond), 18 elevated fertilization membranes. They were then exposed to 1.6× ASW (solid triangle) and finally rinsed in SW (open triangle). (B) Twenty-two healthy-looking eggs filled with aequorin were exposed to 1.6× ASW (solid triangle), rinsed in SW (open triangle), then exposed to A23187 (diamond).

quent Ca^{2+} release by another type of activating agent. To test this, we injected 23 eggs with aequorin and exposed them to 10 m*M* NH_4Cl SW (pH 8.1) for 20 min. A normal brief calcium transient was observed as the NH_4Cl first came into contact with the eggs. Following this treatment, the eggs were washed free of NH_4Cl with SW and then exposed to UREA. A normal Ca^{2+} release was observed (Fig. 6 and Table 1). Another experiment was performed in which exposure to ammonia was followed after 3 min by insemination (see Table 1). Again a brief response to ammonia (25-sec half-width, 0.027 nA/egg) was followed by a fertilization response (60-sec half-width, 0.035 nA/egg) whose amplitude fell in the normal range. In a further experiment (Table 1), an ammonia pretreatment led to a response (0.025 nA/egg, 32-sec half-width) which was followed by a typical response to hypertonic sea water treatment (0.050 nA/egg, 23-sec half-width). All of these results stand in contrast to our other experiments with successive treatments with sperm, nonelectrolyte, and ionophore, in which the second response was much smaller than the first. The results confirm that ammonia failed to release either the calcium store released by hypertonic sea water or the store released by sperm, urea, and ionophore. Furthermore, the Ca^{2+} influx triggered by ammonia does not itself release the stores.

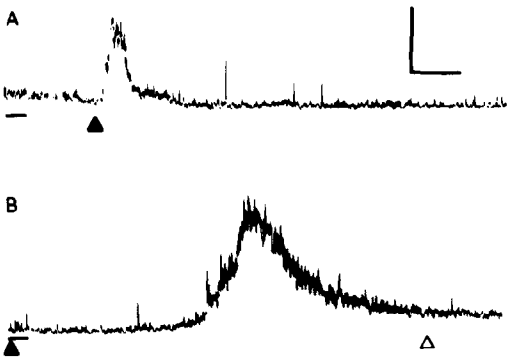

FIG. 6. Interactions between responses to ammonia and UREA solution in 18 successfully injected eggs. (A) Exposure to 10 m*M* NH_4Cl in SW (solid triangle). (B) The ammonia was washed out after 20 min, and UREA solution was introduced (solid triangle), then washed out with SW (open triangle).

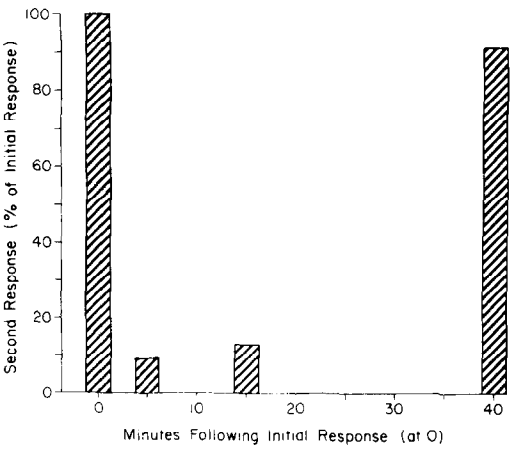

FIG. 7. Pooled responses from nine experiments in which aequorin-filled eggs were initially fertilized or exposed to UREA or to ionophore and subsequently exposed one or more times to UREA or ionophore. The response amplitudes to subsequent treatments are expressed as a percentage of the initial response, and the average responses for four treatments, each at intervals of about 5, 15, or 40 min after the previous treatment, are shown. For UREA treatments, responses to UREA and to subsequent SW rinse were combined to form a composite UREA response.

We wished to examine the question of how long it takes to recharge the intracellular store from which Ca^{2+} is released by sperm, urea, and ionophore. Since we found no clear differences in responses to these three agents, we pooled the data from nine experiments in which fertilization or exposure to urea or ionophore was followed at one more intervals by exposure to UREA or A23187 solutions. Figure 7 shows these pooled data and indicates that it takes over 40 min to fully recharge the intracellular calcium store.

DISCUSSION

The metabolic derepression at fertilization or parthenogenetic activation of the sea urchin egg is hypothesized to occur in two main phases (Steinhardt and Mazia, 1973; Epel *et al.*, 1974). In the first phase, intracellular Ca^{2+} release mediates a number of changes, most prominently the discharge of the cortical vesicles (Steinhardt

and Epel, 1974; Steinhardt *et al.*, 1977). In the second phase, a rise in intracellular pH is posulated to be the principal mediator in the activation of protein and DNA synthesis (Steinhardt and Mazia, 1973; Epel *et al.*, 1974, Johnson *et al.*, 1976). 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 message, 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 metabolic reactions.

At this point, we do not want to be the ones to insist that this hypothesis is proven. However, we would like to indicate, briefly, evidence derived from sea urchin eggs which bears directly on this hypothesis and to place these experiments in that context.

In the experiments reported here and those reported previously (Steinhardt *et al.*, 1977), fertilization, divalent ionophore A23187, and nonelectrolyte 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 (Zucker and Steinhardt, 1978). In contrast to the other forms of activation, ammonia activation proceeds without an intracellular Ca^{2+} elevation.

Fertilization, divalent ionophore, and nonelectrolyte treatment all can lead to discharge of the cortical vesicles (Motomura, 1934; Steinhardt and Epel, 1974), but ammonia treatment does not (Steinhardt and Mazia, 1973). We suggest that parthenogenetic agents other than ammonia activate eggs by releasing intracellular calcium, which then triggers the early surface events in activation, such as cortical vesicle discharge, and initiates a reaction resulting 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 (Steinhardt *et al.*, 1977) 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 and if the rise in intracellular pH could be blocked, activation of protein and DNA synthesis should be halted. The observations by Chambers (1976) that low levels of Na^+ were essential to development after the cortical reaction led Johnson *et al.* (1976) to the elegant demonstration that Na^+ uptake and H^+ release were coupled and necessary to the continuation of development. At the same time, Johnson and his co-workers demonstrated a rise in the pH of egg homogenates which was linked to the requirement for Na^+. Here was the possible link from the early to the late events in the program of activation. It remained for Shen and Steinhardt (1978) to show by direct measurements with intracellular microelectrodes that intracellular pH did, in fact, rise in both normal fertilization and ammonia activation. At this moment, pH studies have not yet been extended to the wide range of parthenogenetic conditions reported on here.

Thus the requirement that direct measurements show the postulated rise in Ca^{2+} and pH has been met, and part of the link between these two ionic messengers has been uncovered.

Both ionophore and ammonia activation require a secondary treatment with hypertonic sea water to result in cell division and subsequent development (Loeb, 1913; Brandriff *et al.*, 1975). We have shown here that hypertonicity results in an intracellular release of Ca^{2+}, but probably from a different source than that released by other activating agents. Although hypertonic sea water alone may activate sea urchin eggs at

high levels of tonicity, exposure to 1.6× ASW fails to activate *Lytechinus* eggs (Brandriff *et al.*, 1975). We are uncertain of the significance of the intracellular Ca^{2+} released by hypertonic sea water, except to point out that complete parthenogenetic development cannot be separated from intracellular Ca^{2+} release.

We have very limited information on the nature of the Ca^{2+} store. Our results suggest that the widely different activators, sperm, ionophore A23187, and nonelectrolytes, all release from the same intracellular store. On the contrary, neither hypertonic sea water, ammonia, nor the calcium influx which can be triggered by ammonia evokes a calcium release from this store. Neither does the transient calcium influx which occurs when unfertilized eggs are exposed to a high-calcium medium (Steinhardt *et al.*, 1977). We can also report that repeated releases are possible, but that a considerable period of time (over 40 min) is required to fully recharge the store. Finally, under our experimental conditions, we never saw a partial release; once Ca^{2+} started to come out of the intracellular store, it continued until it was empty. Normally the discharge of calcium from this intracellular store is an all-or-none process. We note, however, that it is possible under special experimental conditions to get a partial cortical reaction (Allen, 1954; Sugiyama, 1956).

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