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# PREVENTION OF THE CORTICAL REACTION IN FERTILIZED SEA URCHIN EGGS BY INJECTION OF CALCIUM-CHELATING LIGANDS

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## Summary

Eggs from the sea urchin, *Lytechinus pictus*, were injected with either EGTA or EDTA, and were subsequently fertilized. EGTA prevented cortical vesicle discharge and formation of the fertilization membrane. EDTA had either no effect, or sometimes retarded the elevation of the fertilization membrane, or reduced the percentage of eggs with elevated membranes. Theoretical considerations lead to estimates of the probable effects of EGTA and EDTA on the internally released calcium which triggers the cortical reaction. Whether or not cytoplasmic calcium buffers are considered, it is concluded: (1) that normally several times the threshold calcium concentration for the cortical reaction is released into a subsurface space; (2) that if a rapidly-equilibrating high-affinity buffer is present, it is locally saturated by the calcium released internally; (3) the injected EDTA reduces the subsurface free calcium concentration normally reached to approximately threshold for the cortical reaction, while injected EGTA reduces the calcium concentration to below this threshold; and (4) a rise in the internal ionic calcium concentration is a necessary step in the activation of the cortical reaction at fertilization.

## Introduction

In an earlier paper [1], we showed that a low level of calcium is sufficient to discharge the cortical vesicles in isolated egg cortices. We also demonstrated a correlation between the release of calcium from an internal store and the discharge of cortical vesicles, when eggs were fertilized [1] or activated parthenogenetically [2]. To complete the proof that calcium plays an essential

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Abbreviation: EGTA, ethyleneglycol-bis(β-aminoethylether)-*N*,*N*′-tetraacetic acid.

role in this early event in activation, it would be necessary to show that a specific interference with a rise in intracellular free calcium blocks the cortical reaction in eggs at fertilization.

In this report, we describe the results of experiments designed to prevent the intracellular free calcium concentration from rising sufficiently to activate egg cells, and we present calculations of the probable changes in intracellular calcium responsible for the cortical reaction.

## Materials and Methods

Dejellied *Lytechinus pictus* eggs were affixed to a plastic Petri dish coated with poly-(DL-lysine) HBr. Eggs were filled with solutions drawn by suction into specially cleaned micropipettes with bevelled tips having a $0.5 \times 1.5$ $\mu$m opening. Eggs were penetrated with a micropipette held in a micromanipulator and attached to a pulsed pressure system. The pressure pulse was adjusted (usually 30 ms at 2 bar) to inject approx. 2 pl, or about 0.25% of an egg volume. The responses of unfertilised eggs to sperm added to the dish were observed either in a stereomicroscope or an inverted compound microscope, with bright-field transmitted illumination at a magnification of 40—81×. Details of these methods have been published elsewhere [1].

## Results

In two experiments, we injected a field of unfertilised eggs with 0.1 M $K_2$EGTA, pH 6.7, in 0.4 M KCl. The final intracellular concentration of EGTA was about 0.25 mM. The percentages of eggs which appeared undamaged in these two experiments were 55% and 29%. On adding sperm, none of the injected healthy-looking eggs (32 total) was activated.

This result might be due to a nonspecific toxic effect of EGTA. To check for this possibility, we injected a field of 26 unfertilised eggs with 0.1 M $K_2$EDTA, pH 6.7, in 0.4 M KCl. Half of these eggs appeared uninjured. Under these conditions, the excess intracellular magnesium will drastically reduce the buffering effect of EDTA on the level of intracellular calcium. When we fertilized these eggs, about 65% of the healthy-looking eggs elevated their fertilization membranes. This is about the usual success rate for fertilization of injected healthy-looking eggs [1]. However, we did notice that the elevation of fertilization membranes appeared to occur more slowly in some of the EDTA-injected eggs than in uninjected eggs.

To obtain more information on this point, we injected a further 38 eggs with $K_2$EDTA as before; 22 of these eggs appeared undamaged. We followed the timecourse of the cortical reaction in these eggs by photographing them in ordinary bright-field optics at 16×, taking one photograph roughly every 20 s after insemination. Of these eggs, 9 discharged their cortical vesicles, as judged by fertilization membrane elevation, within 8 min. Four of these eggs reacted at the normal rapid rate to sperm, beginning to elevate their membranes about 1 min after insemination. However, 4 other eggs elevated their membranes only gradually, beginning about 1.5 min (2 eggs), 2.25 min (1 egg) or 3 min (1 egg) after insemination. The ninth egg was slightly out of focus, and we could not

determine exactly when its cortical vesicles discharged. In summary, about half the fertilized eggs injected before fertilization with EDTA elevated their membranes more slowly than usual.

We were worried that the delay in membrane elevation in some eggs might be a consequence of latent damage resulting from our microinjection procedure. To test for this, we injected (in the same dish) an additional 51 eggs with 0.5 M KCl. On adding sperm, 26 of the 37 apparently undamaged eggs discharged their cortical vesicles. All of these eggs responded within the first 1 min 10 s after insemination. Thus the injection procedure did not retard membrane elevation in fertilized eggs. Moreover, it left 70% of the healthy-looking eggs fertilizable, which is somewhat higher than the 41% of healthy-looking EDTA-injected eggs from the same animal which were fertilizable.

## Theoretical considerations

### *EGTA and EDTA acting alone*

In our first attempt to understand these results, we supposed that EGTA and EDTA acted alone intracellularly in regulating the peak free calcium concentration attained following fertilization. We showed previously [1] that the cortical vesicles require about 15 $\mu$M calcium to fuse rapidly with the plasma membrane, and that the threshold requirement for calcium is fairly sharp. Thus, 9 $\mu$M calcium failed to discharge the cortical vesicles in less than 15 min. We also concluded that the internal free calcium concentration reaches this high level or higher in only a small portion of the egg cytoplasm, possibly only a few percent of the cell volume lying just beneath (perhaps within 1 $\mu$m of) the plasma membrane.

Suppose that, normally 15 $\mu$M total calcium ($[Ca_T]$) is released into this critical volume. What would be the effect of a total ligand concentration ($[L_T]$) of 0.25 mM EGTA? The reaction governing the resulting free calcium concentration is

$$[Ca^{2+}] + [L^{2-}] \rightleftharpoons [CaL] \qquad K_L = 6.1 \cdot 10^5\ M^{-1} \tag{1}$$

where L here represents the EGTA ligand, and $K_L$ is the apparent association constant of calcium with EGTA at pH 6.7 [1]. Then we have

$$[CaL] = [Ca^{2+}][L^{2-}]K_L \tag{2}$$

$$[Ca_T] = [Ca^{2+}] + [CaL] \tag{3}$$

$$[L_T] = [L^{2-}] + [CaL] \tag{4}$$

from which

$$[Ca^{2+}]^2 + ([L_T] - [Ca_T] + 1/K_L)[Ca^{2+}] - [Ca_T]/K_L = 0 \tag{5}$$

the only positive root of which is $[Ca^{2+}] = 0.1238$ $\mu$M. This accounts clearly for the block to activation by EGTA.

What would we expect to be the effect of EDTA injections? With EDTA, we must consider the effect of the normal level of intracellular free magnesium concentration, which has been estimated from egg cell homogenates to be about 10 mM in *Lytechinus* [3]. The result of a divalent cation reacting with

the ligand competitively with calcium is to reduce the effective binding constant of the ligand for calcium, so that

$$K_L = K_a\left(\frac{1}{1 + [Mg^{2+}]K_{Mg}}\right) \tag{6}$$

where $K_a$ is the apparent binding constant of the ligand with calcium in the absence of magnesium, $K_{Mg}$ is the apparent binding constant of the ligand for magnesium, and $K_L$ is the effective binding constant of the ligand with calcium in the presence of excess magnesium $[Mg^{2+}]$ (the derivation is similar to that for $[H^+]$ in Portzehl et al. [4]). A magnesium concentration of 10 mM reduces $K_a$ of EDTA from $1.0648 \cdot 10^7\ M^{-1}$ at pH 6.7 to a $K_L$ of $1.0182 \cdot 10^4\ M^{-1}$. (By contrast, this magnesium concentration reduces the apparent binding constant of EGTA with calcium by only 0.1%, so its effect on EGTA-calcium chelation can be neglected.) Using this value for $K_L$, we calculated that the intracellular calcium concentration in unfertilized eggs injected with EDTA would be reduced from 15 $\mu$M to 4.3 $\mu$M.

This is well below the calcium threshold for discharging isolated cortices, yet the eggs filled with EDTA were not prevented from discharging their cortical vesicles. One way out of this difficulty is to suppose that normally the subsurface intracellular free calcium concentration rises above the threshold for cortical vesicle discharge. How much calcium must be released into this space to account for our results?

We begin by noting that some of the eggs injected with EDTA elevated their membranes abnormally slowly, some elevated at the normal rapid rate, and sometimes a lower than usual percentage of eggs elevated following fertilization. We take this to mean that EDTA reduced the intracellular free calcium concentration at the subsurface to approximately the threshold for normal cortical vesicle discharge, i.e., $[Ca^{2+}]$ is approx. 15 $\mu$M. For this to occur in the presence of 0.25 mM EDTA ($[L_T]$), with $K_L = 1.0182 \cdot 10^4\ M^{-1}$, Eqns. 1—4 yield

$$[Ca_T] = [Ca^{2+}]\left(1 + \frac{K_L[L_T]}{1 + [Ca^{2+}]K_L}\right), \tag{7}$$

which gives $[Ca_T] = 48\ \mu M$. Thus we conclude that three times as much calcium must be released into the subsurface space as is necessary for cortical vesicle discharge, in order for eggs injected with EDTA to still be able to elevate their fertilization membranes. This would then be the level of free calcium normally attained in uninjected eggs.

Can we still account for the block of activation by EGTA? Solving Eqn. 5 with a $[Ca_T]$ of 48 $\mu$M, $[L_T] = 0.25$ mM and $K_L = 6.1 \cdot 10^5\ M^{-1}$, we obtain $[Ca^{2+}] = 0.38\ \mu M$, which is still well below threshold for cortical vesicle discharge.

*EGTA and EDTA competing with a cytoplasmic calcium buffer*

In the above discussion, we suppose that injected EGTA or EDTA act alone in regulating the internal free calcium concentration, and that normally all the calcium released into a critical submembrane space is available to cause cortical vesicles to discharge. These assumptions seem simplistic and unrealistic, since

there are obviously very powerful calcium binding agents inside eggs. The total calcium content of sea urchin eggs is about 7 mM [3], while the intracellular free calcium concentration is definitely less than 1 $\mu$M, probably about 0.1 $\mu$M or even less [1,5].

Simply regarding eggs as containing a single cytoplasmic calcium buffer leads to trouble. A buffer capable of holding a $[Ca_T]$ of 7 mM to a $[Ca^{2+}]$ of 0.1 $\mu$M would require a release of 1 M of calcium to reach a $[Ca^{2+}]$ of 15 $\mu$M. Alternatively, this buffer could saturate at $[Ca_T]$ levels of tens of millimoles. In any case, the injection of 0.25 mM EGTA would be without effect.

Nakamura and Yasumasu [6] report finding a calcium-binding buffer in sea urchin egg homogenates with a binding constant $K_X = 1 \cdot 10^4\ M^{-1}$. In order to maintain $[Ca^{2+}]$ at 0.1 $\mu$M, over 7 M of this buffer is required if $[Ca_T] = 7$ mM, and 0.17 M of calcium would have to be released to achieve a $[Ca^{2+}]$ of 15 $\mu$M. Evidently, some other system is responsible for maintaining calcium at very low concentrations. Brinley et al. [7] describe a cytoplasmic buffer in squid giant axon that reduces $[Ca^{2+}]$ to 1/1667 of $[Ca_T]$. This buffer would require a $[Ca_T]$ of 25 mM to be released for $[Ca^{2+}]$ to reach 15 $\mu$M. This seems unreasonably high. Moreover, the $[Ca^{2+}]$ would be unaffected by 0.25 mM EDTA or EGTA. Apparently, this mitochondrial system does not control the transient $[Ca^{2+}]$ level in the submembrane space. These objections apply to mitochondrial calcium buffering in general [8]. We believe it is primarily responsible for chelating calcium after it diffuses away from the subsurface region. Clearly, a second-stage high-affinity rapidly-equilibrating buffer is required to control the calcium concentration at the free levels normally occurring.

Such a buffer has been described by Baker and Schlaepfer [9] and Baker [10] in squid axon, and by Kendrick et al. [11] in rat brain synaptosomes. The squid axon cytoplasmic buffer had a binding constant $[K_X]$ of $2\text{—}3 \cdot 10^7\ M^{-1}$, and a capacity ($[X_T]$) of 35—50 $\mu$M [7]. A somewhat weaker buffer, with larger capacity, has been reported in skeletal muscle [12].

Supposing such a buffer ($[X^{2-}]$) were present in sea urchin egg cytoplasm, how would it interact with EDTA and EGTA in controlling the free subsurface calcium concentration? We need to consider the following reactions:

$$[Ca^{2+}] + [L^{2-}] \rightleftharpoons [CaL] \qquad K_L \tag{8}$$

$$[Ca^{2+}] + [X^{2-}] \rightleftharpoons [CaX] \qquad K_X \tag{9}$$

which lead to these equations:

$$[CaL] = [Ca^{2+}][L^{2-}]K_L \tag{10}$$

$$[CaX] = [Ca^{2+}][X^{2-}]K_X \tag{11}$$

$$[L_T] = [L^{2-}] + [CaL] \tag{12}$$

$$[X_T] = [X^{2-}] + [CaX] \tag{13}$$

$$[Ca_T] = [Ca^{2+}] + [CaL] + [CaX]; \tag{14}$$

from which we have:

$$[Ca_T] = [Ca^{2+}]\left(1 + \frac{K_L[L_T]}{1 + [Ca^{2+}]K_L} + \frac{K_X[X_T]}{1 + [Ca^{2+}]K_X}\right) \tag{15}$$

Assuming as before that $[Ca^{2+}]$ reaches 15 $\mu$M in fertilized eggs injected with 0.25 mM EDTA, we calculate that $[Ca_T] = 89$ $\mu$M of calcium is released into the subsurface space, if $[X_T] = 42.5$ $\mu$M of a cytoplasmic buffer with $K_X = 2.5 \cdot 10^6\ M^{-1}$ is present, as in squid axon. Then in the absence of EDTA, the free subsurface calcium concentration can be calculated from Eqn. 5 to reach 47 $\mu$M. Thus normally the subsurface free calcium reaches about three times the threshold concentration for cortical vesicle discharge, and about six times this amount is released into the subsurface space from a calcium store during activation. The main effect of a high-affinity buffer is to require that enough calcium be released locally to saturate it, in order to achieve high levels of free internal calcium concentration.

What would we expect 0.25 mM EGTA to do to this released calcium, with a cytoplasmic buffer present? We now need to solve Eqns. 10—14 for $[Ca^{2+}]$. The result is

$$[Ca^{2+}]^3 + p[Ca^{2+}]^2 + q[Ca^{2+}] + r = 0 \tag{16}$$

where

$$p = \frac{1}{K_L} + \frac{1}{K_X} + [L_T] + [X_T] - [Ca_T]$$

$$q = \frac{[L_T]}{K_X} + \frac{[X_T]}{K_L} - \frac{K_L + K_X}{K_L K_X}[Ca_T] + 1$$

and

$$r = -\frac{[Ca_T]}{K_L K_X}.$$

With $K_L = 6.1 \cdot 10^5\ M^{-1}$, $[L_T] = 0.25$ mM, $K_X = 2.5 \cdot 10^6\ M^{-1}$, $[X_T] = 42.5$ $\mu$M and $[Ca_T] = 89$ $\mu$M, we calculate $[Ca^{2+}] = 0.56$ $\mu$M, again well below threshold for cortical vesicle discharge.

## Discussion

The experiments described in this report were motivated by a desire to examine the idea that calcium ions are an essential intracellular messenger involed in triggering at least one early event in activation — cortical vesicle discharge and consequent fertilization membrane formation. The evidence for this hypothesis has been accumulating for decades. In 1939, Moser [13] reported that when eggs were exposed to mechanical or chemical irritants, in the presence of calcium, a cortical reaction was elicited. Steinhardt and Epel [3] and Chambers et al. [14] found that eggs could be parthenogenetically activated by the divalent cation ionophore A23187. Vacquier [15] and Steinhardt et al. [1] showed that calcium was effective in discharging isolated cortices. Thus the evidence seems clear that calcium is sufficient to trigger the cortical reaction in cortices.

In this paper we sought to show that an unimpeded calcium release is necessary for eliciting the cortical reaction. We found that unfertilized eggs injected with EGTA were prevented from elevating fertilization membranes at fertiliza-

tion, while unfertilized eggs injected with EDTA were only slightly retarded in the rate and probability of occurrence of the cortical reaction. We conclude that EGTA acted specifically to reduce the subsurface free calcium concentration to below the threshold for cortical vesicle discharge, and that calcium is an essential link in the chain of events leading from fertilization to the cortical reaction.

In the second part of this paper, we present theoretical calculations of the probable levels of calcium released into the submembrane space, the likely levels of free calcium concentration normally attained, and some possible influences of cytoplasmic calcium buffers. First, we found that it is very unlikely that exactly enough calcium is normally released to trigger the cortical reaction, because in that case even the EDTA-injected eggs would have failed to react. If no cytoplasmic buffer operates rapidly on the subsurface calcium, then about 48 $\mu$M calcium must be released for the free subsurface calcium to drop to near the cortical reaction threshold of about 15 $\mu$M, as it appears to do in EDTA-injected eggs.

We went on to consider some effects of cytoplasmic buffers. Two classes of calcium-binding cytoplasmic buffer have been identified: slowly-equilibrating, large-capacity, low-affinity buffers usually attributed to mitochondria [7,8] and rapidly-equilibrating, low-capacity, high-affinity buffers usually attributed to proteins [9–12]. We have shown that if a mitochondrial buffer were regulating calcium in the subsurface space, a huge amount of total calcium would have to be released into this space, and then the additional presence of 0.25 mM EGTA would be without effect. On the other hand, if a small-capacity high-affinity buffer regulates calcium in the subsurface space, then enough calcium must normally be released to saturate this buffer, and still raise the free calcium concentration to well above the cortical reaction threshold. Then EDTA will reduce the free calcium to near threshold, and EGTA will reduce it to well below threshold. Our calculations assuming no cytoplasmic buffer, or a low-capacity high-affinity buffer like that found in squid axon, are summarized in Table I.

It must be emphasized that these values can be treated only as approximate, qualitative estimates of what is really happening to intracellular calcium. We do not know the characteristics of the various egg cytoplasmic calcium buffers, and have based our calculations on what are merely convenient and reasonable

TABLE I

CALCULATIONS OF INTRACELLULAR SUBSURFACE CALCIUM CONCENTRATIONS

$[Ca^{2+}]$ is the subsurface calcium concentration normally reached in uninjected eggs. $[Ca_T]$ is the total calcium concentration released into the subsurface space. $[Ca^{2+}]_{EDTA}$ is the subsurface free calcium concentration in eggs injected with EDTA. $[Ca^{2+}]_{EGTA}$ is the subsurface free calcium concentration in eggs injected with EGTA.

| | $[Ca^{2+}]$ ($\mu$M) | $[Ca_T]$ ($\mu$M) | $[Ca^{2+}]_{EDTA}$ ($\mu$M) | $[Ca^{2+}]_{EGTA}$ ($\mu$M) |
|---|---|---|---|---|
| Assuming no intracellular calcium buffer | 48 | 48 | 15 | 0.38 |
| Assuming a high-affinity, low-capacity calcium buffer | 47 | 89 | 15 | 0.56 |

assumptions, derived from measurements on other types of cells. We do not know precisely the value of the calcium threshold for cortical vesicle discharge, nor the exact volume (hence concentration) of injected solution. If anything, our preliminary calculations suggest the need for more data.

Nevertheless, certain qualitative conclusions emerge from the results and calculations. (1) The only type of cytoplasmic buffer likely to influence the subsurface calcium concentration is a high-affinity low-capacity buffer, which then must be saturated by the local calcium release; (2) whether or not such a buffer operates, several times the threshold concentration of free calcium for the cortical reaction must be attained normally after fertilization; (3) EDTA reduces this to near threshold, while EGTA reduces it to well below threshold for cortical vesicle discharge; (4) free internal calcium ions are necessary to activate the cortical reaction at fertilization.

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