Mechanism for Increase in Intracellular Concentration of Free Calcium in Fertilized Sea Urchin Egg

A Method for Estimating Intracellular Concentration of Free Calcium

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ABSTRACT Intracellular free calcium concentration in the sea urchin egg was calculated to increase from 0.1 mM in an unfertilized egg to 1 mM in a fertilized egg 10 min after fertilization, based on measurement of the dissociation constant between free calcium and sea urchin egg homogenate. The dissociation constant between free calcium (dialyzable calcium) and homogenate of sea urchin eggs was measured by means of dialysis equilibrium. The dissociation constant of the unfertilized egg was about \(10^{-4}\) M and that of the fertilized egg was about \(10^{-3}\) M in three species of sea urchin, *Hemicentrotus pulcherrimus*, *Anthocidaris crassispina*, and *Pseudocentrotus depressus*. An increase in the dissociation constant of the unfertilized egg homogenate was observed after the addition of calcium ion at a concentration above 0.3 mM, the dissociation constant becoming the same as that observed in the fertilized egg homogenate after the administration of CaCl\(_2\) at a concentration above 1 mM. Sodium ion also caused a decrease in the calcium-binding ability of the unfertilized egg homogenate. Therefore, penetration of calcium ion or sodium ion upon fertilization might induce an increase in the dissociation constant and then intracellular concentration of free calcium would increase at fertilization. Almost all calcium-binding ability of the egg homogenate was found in the microsomal fraction, and the substance which bound calcium was thought to be protein in nature, since trypsin could decrease the level of calcium-binding substance in the homogenate of the eggs.

INTRODUCTION

It has been demonstrated that the level of free calcium in the sea urchin egg increases upon fertilization (Mazia, 1937), and that the penetration of calcium
ion at fertilization, reported by Nakazawa et al. (1970), might cause an increase in intracellular concentration of calcium in the egg, which can be calculated to be equivalent to 0.4 mM. Since the increase in the free calcium concentration, which has been reported to be 0.5 mM (Mazia, 1937), seems to be very similar to that caused by calcium penetration, it may be assumed that the free calcium which increases in concentration at fertilization may be due to calcium ion which penetrates into the egg at fertilization. However, Mazia has also reported that a considerable amount of calcium in the egg could not be filtered by ultrafiltration (Mazia, 1937), suggesting that there might be a considerable level of large molecules or a fine structure either of which binds calcium in the eggs. If there were such molecules or fine structure which had an ability to bind with calcium, almost all the calcium ion which penetrates the egg at fertilization might be bound and the level of free calcium in the egg would probably scarcely increase. If, upon fertilization, the level of free calcium increases, then an increase in the dissociation constant of the large molecules or fine structure mentioned above and/or a decrease in the ability of calcium binding in those large molecules may also be observed.

In the present paper we estimated the dissociation constant between calcium and the egg homogenate and calculated the concentration of free calcium in the egg, the concentration depending on the dissociation constant measured. We found also that calcium ion in high concentrations caused an increase in the dissociation constant between egg homogenate and free calcium.

MATERIALS AND METHODS

Eggs of the sea urchin *Hemicentrotus pulcherrimus*, *Pseudocentrotus depressus*, and *Anthocidaris crassispina* were collected at Sagami Bay. The eggs and sperm were spawned by 0.5 M KCl injection into the body cavity. Insemination was carried out in filtered seawater. The eggs were twice washed with ice-cold 0.5 M NH₄Cl or 0.5 M KCl to remove calcium ion from the seawater and were collected by centrifugation at 2,000 rpm for 2 min at 4°C. When cytolysis or morphological change of the egg was observed, the eggs were discarded. The eggs thus collected were homogenized in an ice bath by a Teflon pestle homogenizer with 0.2 M KCl containing 10 mM Tris buffer at pH 7.2. 1 ml of the egg homogenate thus obtained was poured into a Visking tube and dialyzed with vigorous stirring against 1 liter of 0.2 M Tris solution buffered with 10 mM Tris-HCl at pH 7.2 containing an appropriate concentration of CaCl₂. After dialysis of the egg homogenate at 4°C for 24 h, the volume of the egg homogenate in the Visking tube was measured by a mescilinder and equal volumes of concentrated nitric acid were added, and then the mixture was heated for 30 min over boiling water. Sometimes the sample was digested with perchloric acid on a burner, giving a similar result. Calcium content of each sample was measured according to the method of Yanagisawa (1951). After 2 ml of 0.004% sodium-p-chlorophenol azo-1.8-dihydroxy-naphthalene-3.6-disulfate (Sunchromine Fast Blue MB) was poured into 0.5 ml of the
samples, 2.5 ml of 2 N NaOH was added. The concentration of calcium was determined photometrically at 620 nm on a Hitachi spectrophotometer (Hitachi Ltd., Tokyo, Japan, model 124). Calcium determination was also performed by the atomic absorbance method on an atomic absorbance photometer (Hitachi model 508), which gave almost the same results as the method mentioned above. The calcium content of the egg homogenate in the dialyzing bag was calculated so as to represent the calcium concentration in the original egg volume. In some cases, the egg homogenate was treated with phospholipase C, trypsin, and adenosine 3'5'-cyclic adenosine monophosphate (cAMP) for 15 min at 30°C before the dialysis. All of the experiments were repeated from three to five times, and each value represents the mean of closely agreeing numbers.

Trypsin and phospholipase C were purchased commercially from Sigma Chemical Co., St. Louis, Mo. cAMP was also obtained commercially from Boehringer Mannheim Co., West Germany.

RESULTS

Level of Total and Nondialyzable Calcium in the Sea Urchin Eggs

As shown in Table I, the level of calcium in the egg did not change upon fertilization. The increase in the level of total calcium at fertilization, which should be caused by the penetration of calcium into the eggs (Nakazawa et al., 1970), could not be detected, because the amount of calcium which penetrates into eggs at fertilization is very small compared with the intracellular concentration of total calcium in the egg. As also shown in Table I, part of the calcium in the eggs could not be dialyzed against calcium-free medium. The concentration of nondialyzable calcium was 9.0 mM in the unfertilized eggs of *H. pulcherrimus*, 11.1 mM in *A. crassispina*, and 14.2 mM in *P. depressus*.

| TABLE I | CONCENTRATION OF TOTAL CALCIUM AND NONDIALYZABLE CALCIUM IN SEA URCHIN EGG |
|----------|---------------------------------|
|          | Concentration of calcium         |
|          | *H. pulcherrimus* | *A. crassispina* | *P. depressus* |
| Total calcium |                        |                      |                |
| Unfertilized egg | 26.0±1.2 | 34.8±2.3 | 24.2±2.6 |
| Fertilized egg | 25.7±2.8 | 34.4±2.5 | 24.7±3.1 |
| 10 min | 26.6±2.9 | 34.2±3.0 | 23.9±3.7 |
| 20 min | 26.6±2.9 | 34.2±3.0 | 23.9±3.7 |
| Nondialyzable calcium |                        |                      |                |
| Unfertilized egg | 9.0±0.2 | 11.1±1.2 | 14.2±2.0 |
| Fertilized egg | 9.1±0.3 | 11.5±1.6 | 14.0±3.1 |
| 10 min | 8.9±0.7 | 10.6±1.9 | 14.3±0.5 |
No change in the level of nondialyzable calcium was observed upon fertiliza-
tion (Table I). For convenience, nondialyzable calcium was called "fixed"
calcium. The difference in the total and fixed calcium was very high and did
not change at fertilization (Table I). If intracellular free calcium concentra-
tion increases upon fertilization as reported by Mazia (1937), it is possible
that dialyzable calcium may be bound reversibly with large molecules or with
a fine structure in the eggs; change in the dissociation constant between
calcium and such molecules and structures and/or change in the level of
calcium at the calcium-binding site ("calcium radicals") of the large mole-
cules or fine structure might regulate the intracellular concentration of free
calcium in the eggs.

Calculation of the Dissociation Constant between Calcium and Calcium Radicals

Fig. 1a and b shows the relationship between free calcium concentration and
bound calcium concentration which was found in the homogenate of the eggs
after 24-h dialysis against each concentration of calcium (free calcium), re-
spectively. In this experiment, free calcium means that contained in the outer
medium of the dialyzing bag. We call "bound" calcium the difference be-
tween the concentration of fixed calcium and the calcium which was found in the
homogenate inside of the dialyzing bag, after dialysis against specific concen-
tration of CaCl₂ solution (free calcium). The concentration of bound calcium
of the unfertilized egg homogenate at a concentration of free calcium below
0.5 mM was higher than that of the fertilized egg. If the concentration of free
calcium was higher than 1 mM, bound calcium concentration of the un-
fertilized egg homogenate was almost the same as that of the fertilized egg.
The same profile of the relation between free and bound calcium concentra-
tion was observed when eggs were washed with 0.5 M KCl or 0.5 M NH₄Cl
solution, before homogenization with 0.2 M KCl. Except for the method of
preparing homogenate, previously described, we could not obtain reproduc-
ible results.

The dissociation constant between calcium and the radicals, which were
supposed to bind with calcium, can be calculated, based on the relation be-
tween bound and free calcium estimated in the fertilized and unfertilized egg
homogenate. The dissociation constant formula is

\[
\frac{[\text{free Ca]} \cdot [\text{free radicals}]}{[\text{Ca-bound radicals}]} = \text{dissociation constant.}
\]

For convenience we will use the following abbreviations: free calcium con-
centration \((F)\); concentration of radicals bound with calcium (equivalent to
bound calcium concentration) \((B)\); total radical concentration \((R)\); free ra-
dical concentration \((r)\); dissociation constant between calcium and radicals
Figure 1. Relationship between free calcium and bound calcium concentration in the homogenate of the sea urchin egg, *H. pulcherrimus* (a) and *A. crassispina* (b). Open circles, fertilized egg homogenate; solid circles, unfertilized egg homogenate. Eggs of *H. pulcherrimus* and *A. crassispina* were washed with 0.5 M NH₄Cl or 0.5 M KCl solution, respectively. Dialysis was performed at 4°C for 24 h. Free calcium concentration is that of outer medium of dialyzing bag, which contains the egg homogenate. Volume of both bound and nondialyzable calcium was that of the difference between calcium concentration bound in dialyzing bag and free calcium concentration. Bound calcium concentration in the eggs is based on the number of eggs contained in the egg homogenate. The volume of 2 × 10⁶ eggs is about 1 ml, since the diameter of an egg is 100 μm.
which bind with calcium \((K)\). Thus,

\[
\frac{F \cdot r}{B} = K,
\]

and since \(r = R - B\), it follows that

\[
\frac{F(R - B)}{B} = K,
\]

or,

\[
\frac{R}{B} - 1 = \frac{K}{F}.
\]

Therefore, the relationship between reciprocals of the bound calcium concentration and the free calcium concentration is a linear function. Figs. 2 and 3 show the relation of reciprocals of the concentration in bound and that in free calcium, demonstrating linear relationships between them and showing the dissociation constant and the concentration of calcium-binding radicals in the egg homogenate. In the unfertilized egg homogenate, the dissociation constant was lower than that of the fertilized egg homogenate at a calcium concentration below 0.1 mM, but the dissociation constant was the same value.

![Figure 2. Relation of double reciprocal plots of free and bound calcium concentration in H. pulcherrimus. 1/F and 1/B indicate reciprocals of free and bound calcium concentration, respectively. Open circles, fertilized egg homogenate; solid circles, unfertilized egg homogenate. These were calculated from the values shown in Fig. 1.](image-url)
Figure 3. Relation of reciprocal of free and that of bound calcium concentration in *A. crassispina*. Open circles, fertilized egg homogenate; solid circles, unfertilized egg homogenate.

### Table II

Dissociation Constant and Concentration of Calcium Radicals in the Eggs

|                  | *H. pulcherimus* | *A. crassispina* | *P. depressus* |
|------------------|------------------|-----------------|---------------|
| Concentration of calcium radical, mM | 29.1             | 30.8            | 15.4          |
| Dissociation constant |
| Unfertilized egg, M | $1.03 \times 10^{-4}$ | $0.92 \times 10^{-4}$ | $1.41 \times 10^{-4}$ |
| Fertilized egg (10 min after fertilization), M | $1.10 \times 10^{-3}$ | $0.40 \times 10^{-3}$ | $1.00 \times 10^{-3}$|

as that found in the fertilized egg homogenate, when the concentration of free calcium was higher than 1 mM, in the three species of common Japanese sea urchin (Table II). As also shown in Table II, however, no difference in the concentration of calcium radicals between fertilized and unfertilized eggs was found in any concentration of free calcium. When the eggs were washed with 0.5 M NaCl or sucrose solution in a concentration between 0.6 M and 1 M, the linear relation between the double reciprocal of bound and free calcium was not observed. It was also difficult to obtain the linear relation of double reciprocal plots when the eggs were homogenized with sucrose or NaCl solution.

The change in the dissociation constant after fertilization at a concentration
Calcium Concentration in Fertilized Sea Urchin Egg

FIGURE 4. Change in the dissociation constant between free calcium and homogenate of the egg *H. pulcherrimus* after fertilization. Fertilized eggs were washed twice with 0.5 M KCl and collected by hand-driven centrifuge after fertilization and homogenized with 0.2 M KCl solution containing Tris-buffer at pH 7.2 in ice bath. The dissociation constant was measured by the method described before.

The concentration of calcium below 0.1 mM was also measured by the same method as mentioned above. As shown in Fig. 4, the dissociation constant was found to increase for 10 min after fertilization; thereafter, the dissociation constant remained at the 10-min value. This value was also found in unfertilized egg homogenate at a concentration higher than 1 mM. The value of the dissociation constant in the homogenate of fertilized egg 5 min after fertilization also increased to the same value as that at 10 min after fertilization, if free calcium concentration was higher than 1 mM. On the other hand, the concentration of calcium radicals was found to be unchanged before and after fertilization. Even 5 min after fertilization, when the dissociation constant was supposed to be increasing, the concentration of calcium radicals kept the same level as that found in fertilized and unfertilized eggs. Since no change in the level of calcium radicals could be observed in any case, it is probable that the concentration at the site of calcium binding in the eggs was not changed, and the character of the calcium-binding radicals was changed at fertilization.

**Effect of Calcium Ion and Sodium Ion on the Dissociation Constant of Calcium Radicals**

Since the concentration of the radical which binds with calcium was found to be unchanged in the unfertilized egg homogenate even at high calcium concentration, which could cause an increase in the dissociation constant, it may be assumed that concentration of the calcium radical in unfertilized egg homogenate would not be changed if the free calcium concentration was between 0.2 mM and 1 mM. At this concentration of free calcium, values of the reciprocals of bound calcium found in the homogenate diverged greatly.
from the values in the double-reciprocal plots of free and bound calcium in the fertilized and unfertilized eggs (Fig. 2). This divergence may have been due to the change in the dissociation constant of the unfertilized egg homogenate, which would be caused by the calcium ion being above 0.2 mM. Then, when the dissociation constant of the unfertilized egg homogenate was calculated, it was supposed that the value of the calcium radical concentration was unchanged in the egg homogenate. As shown in Fig. 5, the dissociation constant between calcium and calcium radicals observed in the unfertilized egg

![Figure 5](image)

**Figure 5.** Effect of calcium ion concentration on the dissociation constant between calcium and the homogenate of the egg, *H. pulcherrimus*. Open circles, fertilized egg homogenate; solid circles, unfertilized egg homogenate. The dissociation constant was calculated by the assumption that the concentration of calcium radical would not be changed with calcium concentration.

![Figure 6](image)

**Figure 6.** Effect of sodium ion and lithium ion on the calcium-binding capacity of unfertilized egg homogenate of *H. pulcherrimus*. Eggs were washed with 0.5 M KCl. The homogenate of unfertilized egg was preincubated with NaCl and LiCl, respectively at 30°C for 20 min. Dialysis was performed against 0.2 mM CaCl₂ solution containing NaCl and LiCl, respectively. Dotted line shows bound calcium found in fertilized egg homogenate as a percentage of that found in unfertilized egg.
homogenate increased, depending on calcium ion concentration, while no change in the dissociation constant of the fertilized egg homogenate was observed.

The effect of some other metal ions on the alteration of the dissociation constant was also examined. MgCl₂ did not change the dissociation constant as far as examined. Addition of NaCl to the homogenate caused a decrease in the calcium-binding capacity of unfertilized egg homogenate (Fig. 6), although no change in the capacity of calcium binding was observed by treatment with LiCl. cAMP, at a concentration between $10^{-8}$ M and $10^{-4}$ M, also failed to cause any change in the binding ability of the radicals.

**Nature and Localization of Calcium Radical in the Egg**

The homogenate was treated with trypsin and phospholipase C to determine the nature of the unknown substance which might contain calcium radicals. Although treatment with phospholipase C (5 U/ml) had very little effect on the binding of calcium with the egg homogenate, administration of trypsin to the homogenate caused a decrease in the bound calcium. As shown in Fig. 7,

![Figure 7](image)

**Figure 7.** Effect of trypsin treatment on the double reciprocal relation between free and bound calcium in the homogenate of unfertilized egg, *H. pulcherrimus.* Open circles, fertilized egg homogenate; solid circles, unfertilized egg homogenate; solid triangles, unfertilized egg homogenate treated with 5% trypsin solution; open triangles, unfertilized egg homogenate treated with 10% trypsin solution at 30°C for 20 min. B means the concentration of bound calcium in moles and F means the concentration of free calcium in moles. Eggs were washed with 0.5 M KCl.
the decrease in the bound calcium was not caused by the change in the dissociation constant, but the calcium radicals diminished. Treatment of the egg homogenate with 5% trypsin for 10 min at 30°C caused a 57.8% decrease in the concentration of the radical and treatment with 10% trypsin caused a 64.3% decrease, whereas no change in the dissociation constant was observed for either. These facts suggest that the large molecules having the radicals might be protein in nature.

As shown in Table III, almost all the nondialyzable calcium (fixed calcium) was found in a fraction supposed to contain mitochondria, and nearly 60% of the calcium radicals were found in the microsomal fraction. Therefore, the substance having calcium radicals may be expected to be located in the membrane structure of the egg. The Ca-binding substance in the microsome fraction was extracted by 24-h treatment with 0.6 M KCl at 4°C. This substance,

| Precipitate obtained with centrifugation | Nondialyzable calcium | Calcium radical |
|----------------------------------------|-----------------------|-----------------|
| At 1,000 g for 10 min                   | 24.9                  | 20.4            |
| At 8,000 g for 10 min                   | 51.6                  | 10.9            |
| At 105,000 g for 2 h                    | 9.4                   | 56.2            |

Eggs were homogenized with 0.5 M sucrose in ice bath and centrifuged on a Hitachi ultracentrifuge (model 55 P. 2).

Figure 8. Sephadex G-200 column chromatographs of partially purified calcium-binding substance. The column was 1.8 x 80 cm and the swelled gel height, 75 cm. The flow rate was 20 ml/h. Elution was performed with 0.6 M KCl, and 4.0-ml fractions were collected. Calcium-binding substance was associated primarily with second peak.
thus extracted, was soluble in pure water. Column chromatography with Sephadex G-200 was done, and the Ca-binding substance was found at the second peak shown in Fig. 8. Using the elution pattern from the column chromatograph, we roughly estimated the molecular weight to be about 35,000. Calcium-binding ability of partially purified substance could not be found after Pronase treatment. The molecular weight and amino acid composition of the calcium-binding substance will be published elsewhere.

Free Calcium Concentration in the Egg

A test, based on total calcium level (Table I), dissociation constant, and intracellular calcium radical concentration in the egg (Table II), was performed to calculate the free calcium concentration. As the content of total calcium and fixed calcium (Table I) and the concentration of calcium radicals (Table II) did not change before and after fertilization, the change in the content of free calcium at fertilization might almost depend on the change of the dissociation constant between the calcium-binding substance and free calcium. The amount of free calcium calculated in the egg is shown in Fig. 9.

![Figure 9. Change in free calcium concentration in the egg of sea urchin H. pulcherrimus after fertilization. The content of free calcium was calculated by the formula for the dissociation constant using the values of the dissociation constant as shown in Fig. 4, the concentration of calcium radical, and total bound calcium concentration in the egg.](image)

The free calcium concentration in the unfertilized egg of *H. pulcherrimus* was about 0.1 mM, then increased to 1 mM within 10 min after insemination, and thereafter, changed no further. In the other two species of Japanese sea urchin, *P. depressus* and *A. crassispina*, the concentration of free calcium in fertilized and unfertilized eggs were almost the same as those in the *H. pulcherrimus* egg, as shown in Table IV.
TABLE IV

CONCENTRATION OF FREE CALCIUM IN FERTILIZED AND UNFERTILIZED EGG

|                     | H. pulcherrimus | A. crassispina | P. depressus |
|---------------------|-----------------|----------------|--------------|
| Unfertilized egg, mM | 0.14            | 0.27           | 0.24         |
| Fertilized egg (10 min after fertilization), mM | 1.24            | 1.05           | 1.29         |

DISCUSSION

Mazia (1937) determined free calcium concentration in the egg by means of ultrafiltration and demonstrated an increase in the level of free calcium upon fertilization. Calculation of the free calcium in the fertilized egg, based on the dissociation constant between calcium and the calcium-binding substance in the present paper, shows almost the same values of free calcium concentration as those determined by Mazia, although the free calcium concentration in the unfertilized egg as determined by Mazia (1937) was somewhat higher than that of the present paper. This difference might be due to species difference or to the inevitable dilution of free calcium in the homogenate during homogenization of the eggs, which might induce an increase in free calcium depending on dissociation of bound calcium in the egg. However, in the present paper an increase in free calcium concentration in the egg at fertilization agrees with Mazia's report. This increase might depend on an increase in the dissociation constant between calcium and the radical, which might be located in the microsomal fraction, because among several factors which might determine the free calcium concentration in the egg, only the dissociation constant of a calcium-binding substance was found to increase at fertilization.

It has also been demonstrated that upon an influx of calcium ion into the egg at fertilization (Nakazawa et al., 1970), the increase in the free calcium concentration in the egg can be calculated to be almost the same as that of calcium ion which penetrated into the egg. Therefore, the calcium ion penetration might be a possible reason for the increase in the level of free calcium at fertilization. However, supposing a level of calcium-binding capacity in the egg high enough to bind the calcium ion which penetrates the egg, and also assuming no change in the dissociation constant of the calcium-binding substance from that of the unfertilized egg, almost all calcium penetrating the egg upon fertilization might be bound with the calcium-binding substance. If so, no increase in the calcium level might be observed, if the dissociation constant were not changed upon fertilization. Influx of calcium into the egg upon fertilization (Nakazawa et al. 1970) might have another role in the increase in free calcium content. The penetration might have some effects on the change in the dissociation constant of calcium-binding substance in the unfertilized egg, because the dissociation constant of the substance in the
unfertilized egg was found to be increased by calcium ion at a concentration higher than 0.2 mM, and reached the value of that in the fertilized egg at a concentration higher than 0.5 mM. Because the concentration of calcium which penetrates into the egg upon fertilization is calculated to be 0.4 mM (Nakazawa et al., 1970) and may easily be imagined to be higher than 0.4 mM, influx of calcium at fertilization could stimulate enough of an increase in the dissociation constant of the substance which is supposed to be located in the microsome or possibly in the membrane of the egg. If influx of calcium were to occur at a local part of the egg, the dissociation constant of the calcium-binding substance might be increased locally by calcium penetration, and calcium should be released at that part of the egg, which might cause an increase in the dissociation constant of the substance located nearby. Consequently, the increase in the dissociation constant might spread throughout the egg, and finally, the free calcium concentration would become that of the fertilized egg.

The dissociation constant of the calcium-binding substance in the fertilized egg was found to increase and reach its maximum within 10 min after fertilization, and, according to Nakazawa et al. (1970), $^{40}$Ca influx has also been reported to reach a maximum during this period. This coincidence also suggests that the influx of calcium ion into the egg observed at fertilization might cause a change in the dissociation constant of the calcium-binding substance, which could increase the free calcium concentration in the egg. There may also be another possibility for the induction of an increase in the dissociation constant of the calcium-binding substance. If sodium ion were to penetrate into eggs at fertilization, it should cause a decrease in the capacity of calcium binding of the substance. Therefore, the penetration of sodium ion may also be a reason for the increase in the free calcium level of the egg at fertilization.

Increase in the free calcium concentration after fertilization, might play an important role in the activation of several biochemical systems which have been reported to be stimulated upon fertilization. Calcium ion has been reported to activate many enzymes, for instance, phosphorylase (Ozawa et al., 1967), phosphodiesterase (Kakiuchi and Yamazaki, 1970), adenyl cyclase (Bradham et al., 1970), ATPase (Singer and Meister, 1945), and others. In the sea urchin egg, glycolysis has been reported to be activated (Yasumasu et al., 1973) and phosphorylase, one of glycogenolytic key enzymes, has been demonstrated to be stimulated upon fertilization (Shoger et al., 1973 and Yasumasu et al., 1973). Activation of adenyl cyclase at fertilization of the sea urchin egg was also reported by Castaneda and Tyler (1968). These enzymes might be activated by the increase in free calcium on fertilization. Epel (1969) and also Nakazawa et al. (1970) have suggested that one of several possible reasons for the increase in O$_2$ uptake at fertilization might be the increase in free calcium concentration in the egg. This increase, induced by the change in
the dissociation constant between the calcium-binding substance and calcium, might be an important trigger to activate several biochemical systems at fertilization. Since the calcium-binding substance, which is assumed to be a protein, was found mainly in the microsome, calcium level regulation might be one of the functions of the membrane of the sea urchin egg; the regulatory mechanism could be similar, for the cell function, to that of "relaxing factor" in muscle (Ebashi, 1961).

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