

## Soluble Sperm Extract Triggers Inositol 1,4,5-Trisphosphate-induced $\text{Ca}^{2+}$ Release in the Oocytes of the Sea Urchin, *Anthocidaris crassispina*

Masaki Osawa

*Sugashima Marine Biological Laboratory, Nagoya University, Toba, Mie 517, Japan*

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**ABSTRACT.** The substance in sperm which induces the activation of eggs at fertilization has yet to be elucidated. Osawa *et al.* reported that the soluble extract of sperm (spex) causes inositol 1,4,5-trisphosphate ( $\text{IP}_3$ )-induced  $\text{Ca}^{2+}$  release (IICR) in fertilized eggs of sea urchins when externally applied [OSAWA *et al.*, (1992). *Zool. Sci.* 9: 1206a]. This paper reports that spex also caused IICR in immature oocytes of *Anthocidaris crassispina* at their germinal vesicle stage and induced a transient increase in their intracellular  $\text{Ca}^{2+}$  concentration ( $\text{Ca}_i$ -transient). The peak value of this  $\text{Ca}_i$ -transient was  $160 \pm 16$  nM, a small increase by about 60 nM from the basal intracellular  $\text{Ca}^{2+}$  concentration,  $[\text{Ca}^{2+}]_i$ , of  $96 \pm 12$  nM. This reaction was completely blocked by heparin, an IICR inhibitor. Furthermore, the active factor in spex was resistant to pronase and did not show species specificity. It was found that  $\text{Ca}_i$ -transient can also be induced in oocytes by sperm. The peak value of the  $\text{Ca}_i$ -transient in this case was dependent on sperm concentration and was a maximum of approximately 250 nM, which was significantly lower than that at normal fertilization, i.e.,  $941 \pm 136$  nM. The  $\text{Ca}_i$ -transient induced by sperm in oocytes was not suppressed, though its time course was delayed, by heparin.

The mechanism by which sperm initiate the development of eggs at fertilization has yet to be explained clearly. The hypotheses which have been proposed to date to explain this mechanism can be classified into two groups. The first group assumes that egg activation occurs as sperm act on the egg surface (7, 11, 12, 17, 20), while the second group proposes that a substance in sperm is injected into eggs through membrane fusion between sperm and eggs (5, 16, 35, 39). Both groups suggest the existence of a substance, i.e., a first messenger of fertilization, in sperm which activates eggs. Substances assumed for the hypotheses of the first group include bindin-like acrosomal protein for *Urechis* (11, 12), bindin for sea urchins (7), and adenosine 5'-triphosphate (ATP) for *Xenopus* (20). Those for the hypotheses of the second group are, for example, protein of high molecular weight extracted from sperm for hamsters and sea urchins (5, 35), and inositol 1,4,5-trisphosphate ( $\text{IP}_3$ ) (16), cyclic guanosine 3',5'-monophosphate (cGMP) (39) of low molecular weight for sea urchins.

By whatever mechanism the fertilization of sea urchin eggs may occur, a transient increase in  $\text{Ca}^{2+}$  concentration ( $\text{Ca}_i$ -transient) takes place in these eggs, and

the development of the eggs is initiated. This  $\text{Ca}_i$ -transient is generated by the discharge of  $\text{Ca}^{2+}$  from the endoplasmic reticulum (2, 25, 37), and both the  $\text{IP}_3$  receptor and the ryanodine receptor may be involved in the  $\text{Ca}_i$ -transient (9, 10, 21, 32, 33). To understand the mechanism by which the sperm causes the  $\text{Ca}_i$ -transient at fertilization of an egg, Osawa *et al.* have attempted to find the substance in spex which induces the  $\text{Ca}_i$ -transient in eggs. No such substance has yet been identified. We have ascertained, however, that both sperm and spex induce the  $\text{Ca}_i$ -transient in fertilized eggs (28, 29, 30). We have also found that the  $\text{Ca}_i$ -transient caused in fertilized eggs by the external application of spex is induced by the activation of the  $\text{IP}_3$  receptor (29) through GTP-binding protein (31), which is regarded as one of the signal transduction pathways during normal fertilization (15, 19, 26, 27, 36, 38). This active factor in spex could be considered as having a role in egg activation.

It is known that when an immature oocyte of starfish (1) and that of hamster (8) are inseminated, the  $[\text{Ca}^{2+}]_i$  increases in both of them. In the case of an oocyte of sea urchin, a transient depolarization and sperm entry occur by insemination (4, 6, 23). Investigation was conducted to find whether or not the  $\text{Ca}_i$ -transient is induced in the oocyte not only by sperm but also by spex. The signal transduction was also investigated by injecting heparin, an  $\text{IP}_3$  receptor inhibitor (34), into the oocyte.

Abbreviations used: ASW, artificial sea water;  $\text{Ca}_i$ -transient, a transient increase in intracellular  $\text{Ca}^{2+}$  concentration;  $[\text{Ca}^{2+}]_i$ , intracellular  $\text{Ca}^{2+}$  concentration; EPPS, N-2-hydroxyethylpiperazine-N'-3-propanesulfonic acid; IICR, inositol 1,4,5-trisphosphate induced  $\text{Ca}^{2+}$  release;  $\text{IP}_3$ , inositol 1,4,5-trisphosphate; spex, sperm extract.

## MATERIALS AND METHODS

**Preparation of gametes.** *Anthocidaris crassispina* was used for experiments. Eggs and sperm were obtained by injecting 0.5 M KCl into the coelomic cavity of sea urchins. Oocytes having germinal vesicle were present among eggs at a ratio of several percent to the eggs when the above injection was made into female sea urchins in the early period of their reproductive season. The oocytes in their meiotic prophase used for experiments were those which had their diameter greater than 90% of the diameter of the eggs and the germinal vesicle diameter at 40–50% of that of the oocytes themselves.

Both eggs and oocytes were washed for removing their jelly layer two times with Ca-Mg-free artificial sea water (450 mM NaCl, 10 mM KCl, 2 mM EDTA, 10 mM EPPS-Tris; pH 8.2) and again two times with artificial sea water (ASW) (430 mM NaCl, 10 mM KCl, 10 mM CaCl<sub>2</sub>, 20 mM MgCl<sub>2</sub>, 30 mM MgSO<sub>4</sub>, 2 mM NaHCO<sub>3</sub>, 10 mM EPPS-Tris; pH 8.2). These eggs and oocytes were then attached to cover glasses coated with poly-L-lysine (mol. wt. 1,000–4,000; Sigma Chem. Co., St. Louis, MO, U.S.A.). Experiments were conducted at 25 ± 1°C.

**Microinjection and [Ca<sup>2+</sup>]<sub>i</sub> measurement.** A modified Hiramoto method (14,) was used for microinjecting into cells a Ca<sup>2+</sup> sensitive fluorescent dye, Indo-1 (Dojindo Laboratory, Kumamoto), diluted to 10 mM with an injection buffer (100 mM potassium aspartate, 10 mM HEPES; pH 7.0). Heparin was dissolved in the above solution to a concentration of 40 mg/ml. The volume injected was always adjusted to about 1% of the volume of the egg based on the fluorescent intensity of Indo-1. Intracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) was measured by the use of a Nikon Diaphot inverted microscope using a 20 × objective fitted with various accessories described later. A 100-W mercury short-arc lamp was used as an excitation light source, and an excitation interference filter (355 nm) and a dichroic mirror (380 nm) were set on a filter cassette for epifluorescence (Nikon, Tokyo). The emission light was divided into two by a half prism (HPC-15; Chuo Precision Industrial Co., Ltd., Tokyo). One light was let to pass through an emission interference filter of 405 nm and the other through that of 485 nm, and each light was measured by an photomultiplier (R647-01; Hamamatsu Photonics K.K., Hamamatsu) connected to an I-V converter. Measured values were recorded on a recorder (3057; Yokogawa Electric Co., Tokyo), and the [Ca<sup>2+</sup>]<sub>i</sub> was calculated based on the ratio of fluorescence emissions at 405 nm and 485 nm to excitation at 355 nm. This calculation was made on the basis of the assumption that the dissociation constant of Indo-1 for Ca<sup>2+</sup> in the cell is 250 nM (13). All experimental data are expressed in mean ± SD with the number of recordings.

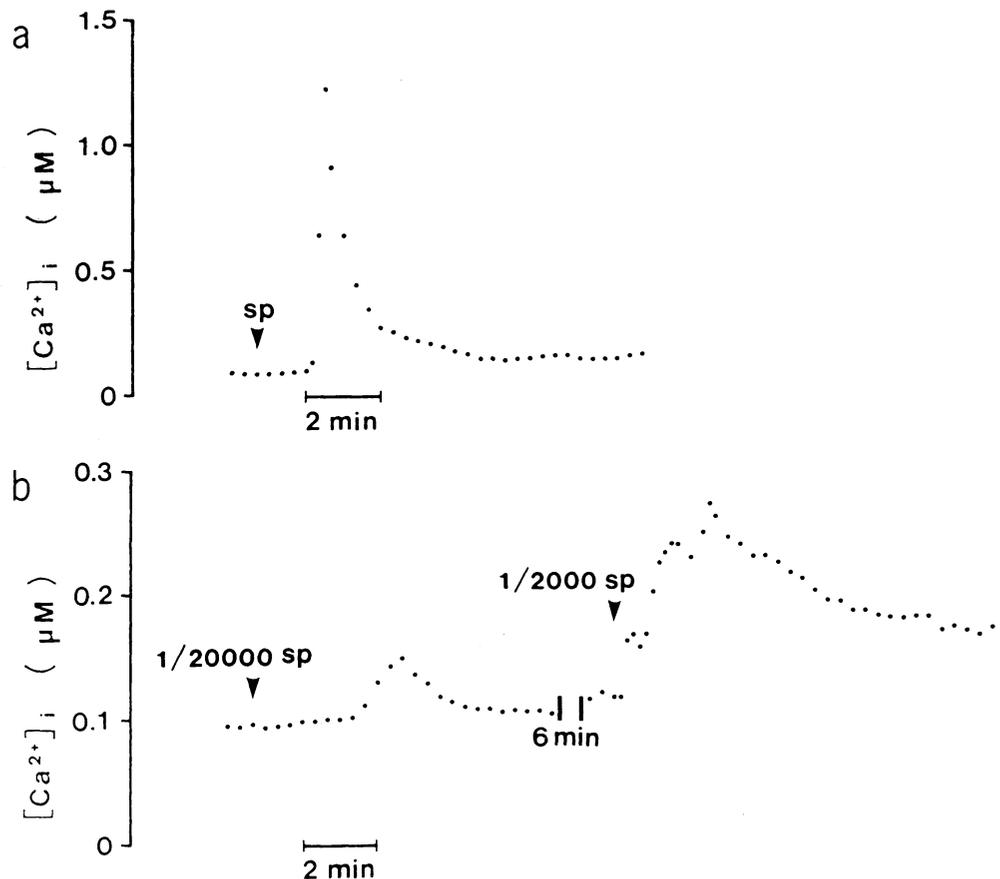
**Preparation of spex.** Sperm was frozen at -80°C, thawed at room temperature, and centrifuged at 8,000 × g for 60 minutes at 4°C for the preparation of spex. The supernatant was adjusted to pH 8.2 using Tris, and was called "crude spex." For further purification, the crude extract whose pH was not

adjusted was incubated at 100°C for 10 minutes, and was centrifuged at 8,000 × g for 30 minutes at 4°C. The supernatant was passed through an ultrafilter with a fractionating molecular weight of 5000 (Ultrafree UFP1 LCC, Millipore Co., Bedford, MA, U.S.A.), and the filtrated solution was collected. The spex of *Hemicentrotus pulcherrimus* was also prepared by the same method. The solution posited as the 'spex' of concentration 1 was diluted 10 times and 100 times with ASW, and was adjusted to pH 8.2 using Tris. A sample of this spex was added in an amount equal to the volume of ASW in the chamber where oocytes were present, and changes in the [Ca<sup>2+</sup>]<sub>i</sub> were observed.

**Pronase (actinase) treatment.** The pronase treatment of spex was conducted by dissolving pronase (actinase) (Kaken Pharmaceutical Co., Ltd.) to 250 µg/ml in spex diluted 10 times with ASW and incubating the solution for 3 hours at 37°C. To denature pronase, the solution was incubated for 1 hour at 100°C, and the activity of the spex was examined. The same operation was performed using a sample with ASW instead of spex as a control, and the sample was confirmed not to have any activity to oocytes.

## RESULTS

For quantitative comparison between the Ca<sub>i</sub>-transient induced at normal fertilization and that by insemination of oocytes, a Ca<sup>2+</sup> sensitive fluorescent dye, Indo-1 (Fig. 1a and 1b) was employed. There was little difference in basal [Ca<sup>2+</sup>]<sub>i</sub> between the eggs and the oocytes (Table I), and the value of basal [Ca<sup>2+</sup>]<sub>i</sub> was nearly constant all the time. After insemination, a Ca<sub>i</sub>-transient was induced in the oocytes (Fig. 1b), and the peak value of the Ca<sub>i</sub>-transient was low as compared to that of the Ca<sub>i</sub>-transient at normal fertilization (941 ± 136 nM, n = 5). Peak values of the Ca<sub>i</sub>-transient induced by insemination with the sperm prepared by diluting 'dry sperm' 20,000 times and 2,000 times with ASW were 161 ± 47 nM (n = 7) and 249 ± 87 nM (n = 10), respectively. This means that the peak value of the Ca<sub>i</sub>-transient induced when an oocyte was inseminated was dependent on the number of the spermatozoa which interacted with the oocyte. The peak value, however, did not rise above the level reached with the sperm diluted 2,000 times even when sperm of higher concentration, i.e., the sperm diluted 200 times, was used (Table I). In addition, the time length from the start of the change in [Ca<sup>2+</sup>]<sub>i</sub> to the arrival at a peak value was 34.3 ± 6.5 sec (n = 5) at normal fertilization and 62.3 ± 16.2 sec (n = 8) when the sperm diluted 2,000 times was applied to oocytes; in other words, the latter took 1.8 times or more longer than the former. This quantitative difference may be attributed to insufficient reaction by oocytes to sperm. Considering that there could be a phenomenon similar to the interaction occurring at fertilization, an experiment was carried out to see if spex can induce the



**Fig. 1.** Comparison between Ca<sub>i</sub>-transient induced by insemination of oocyte and that which occurs at normal fertilization (a) indicates a Ca<sub>i</sub>-transient at normal fertilization triggered by insemination at (sp). (b) represents a Ca<sub>i</sub>-transient induced in oocytes by insemination with sperm prepared by diluting 'dry sperm' 20,000 times and 2,000 times, and applied at (1/20,000 sp) and (1/2,000 sp), respectively. The sperm seen under a microscope along the circumferential edge of an oocyte numbered 1–10 when diluted 20,000 times and 20–50 when diluted 2,000 times. No fertilization membrane was formed on oocytes, and large cytoplasmic cones protruding from oocytes were often observed. (22, 23, 24).

same interaction as sperm does in oocytes (Fig. 2a). By external application to oocytes, a spex diluted 200 times induced no interaction while that diluted 20 times induced a Ca<sub>i</sub>-transient whose peak value was  $160 \pm 16$  nM (n=9) (Table I). The time length from the start of the Ca<sub>i</sub>-transient to the arrival at the peak value was  $39.4 \pm 18.8$  sec (n=7), which was shorter than that at the time of insemination. This seems partly due to the stimulation of an oocyte by sperm which started at one point where the oocyte and sperm had interaction, while that by the spex started from all over the surface of the oocyte. When the spex was externally applied to unfertilized eggs, no change was induced in [Ca<sup>2+</sup>]<sub>i</sub> whether the spex was 'normal' or crude (Fig. 2b). The factor in spex which induced the Ca<sub>i</sub>-transient in fertilized eggs was pronase-insensitive and had no species specificity (28, 30). Investigation was also conducted to find whether the factor which acts on oocytes has the same characteristics as those of the factor in spex which

acts on fertilized eggs (Fig. 3). It became clear that spex did not lose its activity by the pronase treatment (Fig. 3a). This would suggest that the active factor in spex was not peptide. In addition, it was also confirmed that a similar Ca<sub>i</sub>-transient was induced when the spex of *Hemicentrotus pulcherrimus* diluted 20 times was applied to oocytes of *Anthocardaris crassispina*. The active factor in spex showed no species specificity, at least, in this combination. It was, therefore, ascertained that these activities of spex to the oocytes are the same as those of spex to the fertilized eggs.

An experiment was conducted to investigate the transduction pathway through which spex induces the Ca<sub>i</sub>-transient in oocytes. Because it was known that the activating effect of spex on fertilized eggs is inhibited by heparin (29), heparin was previously injected into oocytes to see whether or not the activating effect of spex on oocytes would be inhibited by it. The application of spex to heparin-loaded oocytes did not generate any

**Table I.** PEAK VALUES OF  $Ca_i$ -TRANSIENT INDUCED IN EGG AND OOCYTE BY SPERM AND SPEX, AND BASAL  $[Ca^{2+}]_i$  OF EGG AND OOCYTE.

Number of $Ca_i$ -transients / Number of experiments	Peak $[Ca^{2+}]_i$ (nM) <sup>a)</sup>	Condition <sup>b)</sup>
5/5	941 ± 136	Egg-Sperm (fertilization)
6/7	161 ± 47	Oocyte-1/20,000 sperm
8/10	249 ± 87	Oocyte-1/2,000 sperm
5/5	248 ± 30	Oocyte-1/200 sperm
0/5	—	Oocyte-1/200 spex
8/9	160 ± 16	Oocyte-1/20 spex
9/10	252 ± 95	Heparin-loaded oocyte <sup>c)</sup> -1/2,000 sperm
0/7	—	Heparin-loaded oocyte <sup>c)</sup> -1/20 spex

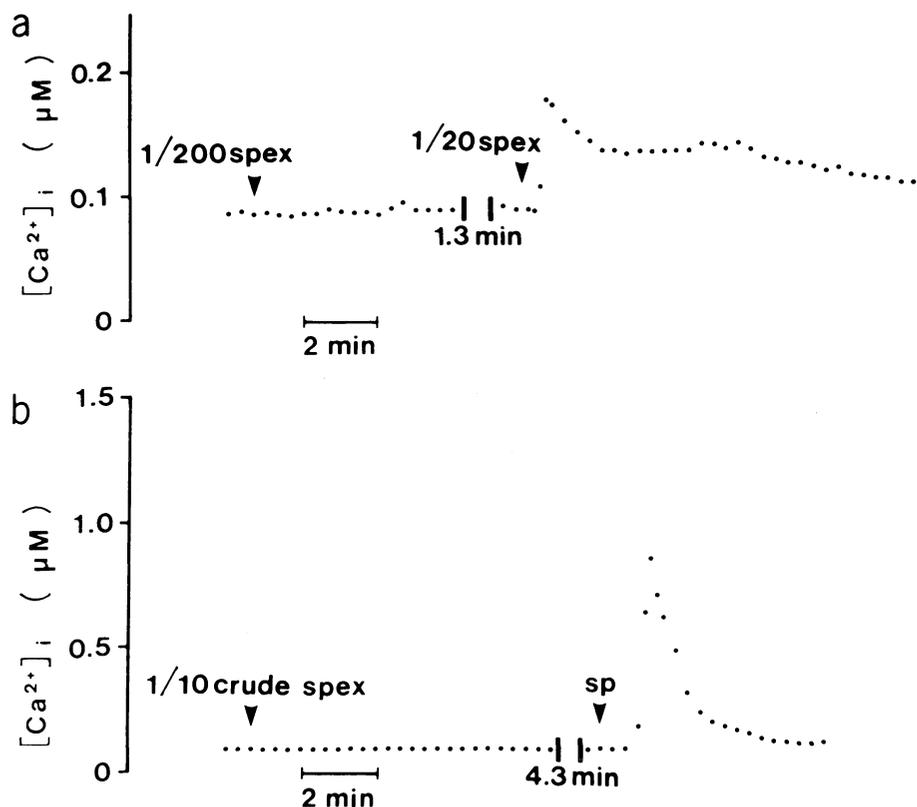
Number of experiments	Basal $[Ca^{2+}]_i$	Cells measured
5	86 ± 8	Egg
29	96 ± 12	Oocyte

(Notes)

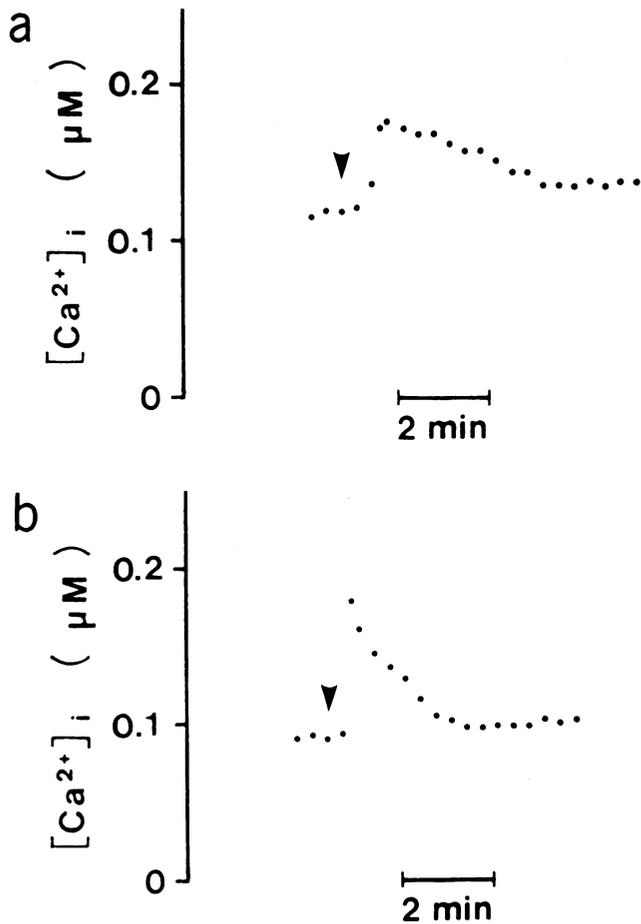
<sup>a)</sup> Peak  $[Ca^{2+}]_i$  is expressed in Mean ± S.D. Basal  $[Ca^{2+}]_i$  is used where no  $Ca_i$ -transient occurred.

<sup>b)</sup> Sperm diluted 200, 2,000, and 20,000 times from dry sperm were applied to oocytes, while spex diluted 20 times and that diluted 200 times were applied to oocytes.

<sup>c)</sup> Oocytes were loaded with heparin to its final concentration of 400  $\mu\text{g/ml}$ .



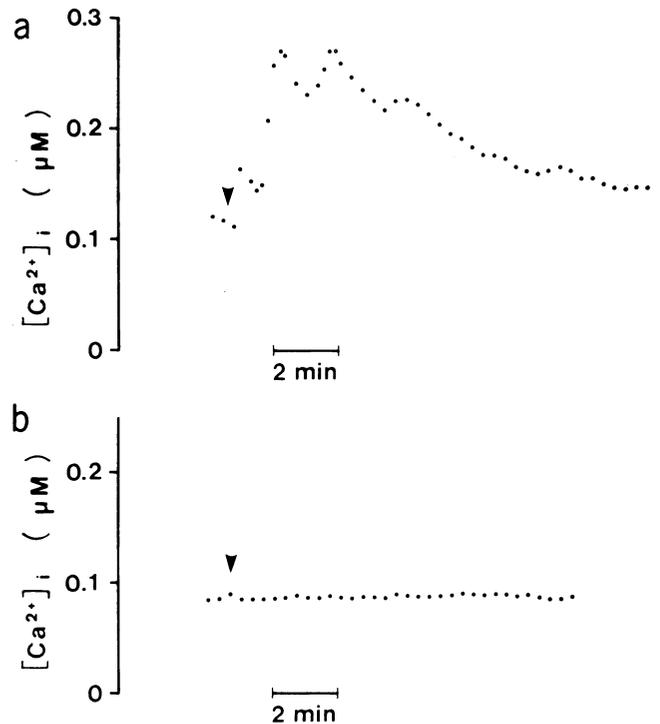
**Fig. 2.**  $[Ca^{2+}]_i$  when the spex was applied to oocytes and the 'crude spex' to eggs (a) indicates  $[Ca^{2+}]_i$  when a spex diluted 200 times and a spex diluted 20 times were applied to oocytes at (1/200 spex) and (1/20 spex), respectively. (b) indicates  $[Ca^{2+}]_i$  when 'crude spex' diluted 10 times was applied to an egg at (1/10 crude spex). The egg was later inseminated at (sp).



**Fig. 3.** Effect of pronase treatment on the activity of the spex and species specificity of the effect of the spex to oocytes (a) shows a record obtained when a spex, treated with pronase and diluted 20 times, was applied. (b) indicates a record obtained when a spex, prepared from *Hemicentrotus pulcherrimus* and diluted 20 times, was applied to oocytes of *Anthocidaris crassispina*. Each spex was applied to the chamber at a respective point indicated by an arrowhead.

Ca<sub>i</sub>-transient (Fig. 4b), which therefore would suggest that spex triggers IP<sub>3</sub>-induced Ca<sup>2+</sup> release (IICR) in oocytes in the same way as it does in fertilized eggs (29, 31).

There was no significant difference between the peak value ( $252 \pm 95$  nM,  $n=10$ ) in heparin-loaded oocytes reached by the addition of sperm diluted 2,000 times and that which was attained in heavily inseminated oocytes into which no heparin had previously been injected (Fig. 1b, Fig. 4a, Table I). The time length, however, from the start of  $[Ca^{2+}]_i$  to the arrival at the peak value in heparin-loaded eggs was  $108.2 \pm 46.8$  sec ( $n=7$ ), which is 1.7 times longer than that in heparin-unloaded eggs. This delay of the time course caused by heparin at insemination was also observed at normal fertilization (3, 33, 39).



**Fig. 4.** Effect of heparin on Ca<sub>i</sub>-transient in oocytes induced by sperm and spex (a) represents a record obtained when sperm prepared by diluting 'dry sperm' 2,000 times was applied to the oocyte into which 400  $\mu g/ml$  of heparin had previously been injected. (b) shows a record obtained when spex diluted 20 times was applied to the oocyte loaded with 400  $\mu g/ml$  of heparin. Each of the sperm and the spex was applied to the chamber at a respective point indicated by an arrowhead.

When the oocytes in which the Ca<sub>i</sub>-transient had once been induced by insemination were reinseminated, the Ca<sub>i</sub>-transient was induced again (Fig. 1b). Investigation was made to see whether the same could occur by the application of spex (Fig. 5). In the experiment, after the first Ca<sub>i</sub>-transient induced by the application of spex diluted 20 times and subsequent perfusion, another Ca<sub>i</sub>-transient occurred when spex diluted 20 times was applied again. No desensitization by the spex of the first application was observed in the experiment conducted according to the time course shown in Fig. 5.

## DISCUSSION

*Ca<sub>i</sub>-transient induced by sperm.* When an oocyte is inseminated, membrane fusion between the oocyte and multiple spermatozoa is possible because, unlike normal fertilization, the polyspermy block (18) does not act (4). It was not possible to ascertain whether the Ca<sub>i</sub>-transient induced in oocytes at insemination was by a single spermatozoon or by multiple spermatozoa. The peak value of the Ca<sub>i</sub>-transient, on the other hand, varied ac-

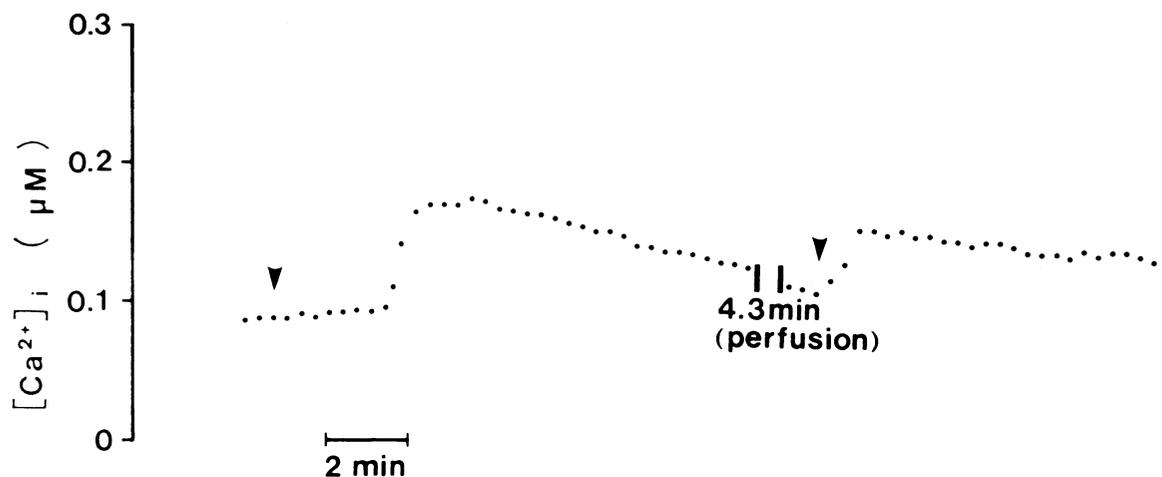


Fig. 5. Repeatability of the activity of spex to oocytes

Spex diluted 20 times was applied to the chamber at a point indicated by an arrowhead. After  $\text{Ca}_i$ -transient subsided, perfusion with ASW was performed for 258 seconds, and then spex diluted 20 times was applied again to the chamber at a point indicated by an arrowhead.

cording to the sperm concentration (Fig. 1, Table I), which, at least, indicates that multiple spermatozoa were involved in the induction of the  $\text{Ca}_i$ -transient. This is also in agreement with the result of experiments conducted using starfish oocytes (1).

Because heparin did not suppress the  $\text{Ca}_i$ -transient induced by sperm (Fig. 4a), it has been suggested concerning the signal transduction pathway through which sperm activate oocytes that the  $\text{Ca}_i$ -transient can be induced in oocytes through a pathway similar to that activated at normal fertilization (3, 9, 10, 32, 33), i.e., without activating the  $\text{IP}_3$  receptor. The time course, however, of the  $\text{Ca}_i$ -transient induced in heparin-loaded oocytes at insemination was delayed. This coincides with the delay of the time course of the  $\text{Ca}_i$ -transient caused in heparin-loaded eggs at insemination. The involvement, therefore, of the  $\text{IP}_3$  receptor in the  $\text{Ca}_i$ -transient induced in oocytes by sperm cannot be denied. In the case of normal fertilization, the involvement of the ryanodine receptor in addition to that of the  $\text{IP}_3$  receptor has been reported (9, 10, 21, 32, 33). Hence, the question yet to be answered will be which pathway sperm activate first in a direct way at fertilization: the pathway to the  $\text{IP}_3$  receptor or that to the ryanodine receptor?

**Activity of spex.** Osawa *et al.* already reported that the active factor in spex induces the  $\text{Ca}_i$ -transient in fertilized eggs (28, 29, 30). The question, however, is whether or not an active factor which acts on oocytes is the same active factor as this. The active factor in spex which induces the  $\text{Ca}_i$ -transient in oocytes is resistant to pronase, though no test has yet been performed on oocytes of other sea urchin species. This active factor has no species specificity (Fig. 3), and induces the  $\text{Ca}_i$ -transient by the IICR (Fig. 4b). These characteristics are the

same as those of the active factor which induces the  $\text{Ca}_i$ -transient in fertilized eggs (28, 29, 30). Although it is highly possible that these active factors are identical, purification is necessary for determining their molecular structure. The peak value of the  $\text{Ca}_i$ -transient induced by spex in fertilized eggs is several hundreds nM (29, 31), while that of the  $\text{Ca}_i$ -transient induced in oocytes is considerably lower (Table I). This result can be considered as reasonable in light of the report that the development of the  $\text{IP}_3$  receptor in immature oocytes of hamsters and starfish are low (1, 8). The biological significance of the existence of this receptor for spex in oocytes and fertilized eggs is not yet clear.

**Can the active factor in spex be the first messenger of fertilization?** In order for the active factor in spex to be the first messenger of fertilization, the active factor must activate unfertilized eggs. However, when we applied spex to unfertilized eggs, no changes were induced in  $[\text{Ca}^{2+}]_i$  (Fig. 2). The question, therefore, is why the unfertilized egg which chronologically comes between the oocyte and the fertilized egg is not activated by spex. It may be due either to the fact that the unfertilized egg has no receptor for the active factor or to the fact that the receptor in the unfertilized egg is somehow blocked. In order to assume that the active factor in spex is the first messenger of fertilization, the latter case should be true, and further that sperm should first remove the block so that the active factor can activate unfertilized eggs.

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