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Activation of *Xenopus* Eggs by *Cynops* Sperm Extract is Dependent upon Both Extra- and Intra-Cellular Ca Activities

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ABSTRACT—When unfertilized *Xenopus* eggs were treated by *Cynops* sperm extract in 10% Steinberg's solution (SB), egg's membranes hyperpolarized to about −37 mV and then depolarized to elicit a positive-going potential amounting to about +34 mV. The eggs underwent cortical contraction and resumption of meiosis. Activation of eggs in various external solutions indicates that the hyperpolarization is due mainly to opening of Na channels, but the positive-going potential is due to Cl channels on the egg's plasma membranes. Since the activation was inhibited by $CdCl_2$, $CoCl_2$, or $NiCl_2$ as well as by amiloride, Ca influx through Ca channels is necessary for the activation by the sperm extract. A propagative intracellular Ca release was induced not only by *Cynops* sperm, but also by their sperm extract. Injection of BAPTA or heparin into the eggs completely inhibited activation, indicating that egg activation requires an intracellular Ca release dependent upon receptors for inositol 1,4,5-trisphosphate.

INTRODUCTION

The sperm induces egg activation to initiate embryonic development at fertilization. The mechanism how the initiation of egg activation is regulated remained unclear. In the invertebrate *Urechis*, the egg activation, including elicitation of a positive ferilization potential, can be induced by a protein isolated from the sperm acrosome [6–8]. In sea urchin eggs, a transmembrane glycoprotein on egg plasma membrane was characterized as a receptor for sperm-binding [5] and treatment with antiserum against a fragment of the receptor induces egg activation [4]. Recently, we have demonstrated that *Xenopus* eggs can be activated by the extract of *Cynops* sperm and its active component is a protease localized in the sperm acrosome [10].

Xenopus sperm triggers a transient, propagative increase in intracellular Ca^{2+} [1, 17] which is an indispensable signal for egg activation [2, 11]. In *Xenopus* eggs, the sperm initiates hydrolysis of phosphatidyl inositol 4,5-bisphosphate (PIP_2) to produce inositol 1,4,5-trisphosphate (IP_3), which is necessary for generation of the wave of Ca^{2+} at fertilization [17, 20]. The stimulation of exogenously introduced receptors can activate *Xenopus* eggs via coupling with G-protein [11], but it has not yet been determined the mechanism how the sperm triggers the production of IP_3 to generate the intracellular increase of Ca^{2+}. We have demonstrated that the voltage dependence is sensitive to extracellular Ca^{2+} activity in the activation of *Xenopus* egg by *Cynops* sperm or by their extract [10].

In order to understand the mechanism of egg activation that operates at the initial phase of amphibian fertilization, we attempted to clarify how the extract of *Cynops* sperm causes the egg activation in *Xenopus*, in particular, for role of both extra- and intra-cellular Ca^{2+} activities. We have demonstrated in this study that Ca influx through Ca channels on the egg plasma membrane induces a local increase in Ca^{2+} to cause the propagative Ca^{2+} wave in egg cytoplasm.

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MATERIALS AND METHODS

Chemicals and solutions

De Boer's solution (DB) whose final composition in mM was, 110 NaCl, 1.3 KCl, 1.3 $CaCl_2$, 5.7 Tris-HCl (pH 7.4); Steinberg's solution (SB), 58 NaCl, 0.67 KCl, 0.34 $Ca(NO_3)_2$, 0.85 $MgSO_4$, and 4.6 Tris-HCl (pH 7.4); 3×NKP solution, 120 NaCl, 7.5 KCl, 4 NaH_2PO_4, 6 Na_2HPO_4 (pH 7.2); Ca-free phosphate buffer (Ca-free PB), 1 EGTA, 50 Na_2HPO_4, 50 NaH_2PO_4, 5 KCl (pH 7.0); Ca-containing phosphate buffer (Ca-PB), 1 $CaCl_2$ 50 Na_2HPO_4, 50 NaH_2PO_4, 5 KCl (pH 7.0).

Gametes

Sexually mature newts, *Cynops pyrrhogaster*, were collected near Yamaguchi, Japan. The clawed frogs, *Xenopus laevis*, were purchased from dealers and maintained in our laboratory. To obtain mature *Xenopus* eggs, females were injected with 500 IU of human chorionic gonadotropin (HCG; Teikoku Zoki, Tokyo) in the dorsal lymph sac and were kept at 18°C for 12 hr. Ovulation in *Cynops* was induced by two injections of 100 IU each of HCG at intervals of 48 hr at 23°C. Mature eggs were obtained from the lowest portion of oviducts by squeezing females. Jelly coats of *Xenopus* eggs were removed by immersion in 2% cysteine-HCl (pH

7.8), which was followed by thorough washing with 100 mM NaCl and 50 mM Tris-HCl (pH 7.0). The dejellied eggs were kept in DB at 18°C and used within 2 hr. The sperm of *Cynops* were obtained from sperm ducts by squeezing males or by dissection. In *Xenopus*, the sperm were obtained by mincing a piece of testis.

Artificial insemination and microinjection

For insemination of fully jellied *Xenopus* eggs, some drops of the sperm suspension in 50% SB or in appropriate solutions were placed on a petri dish. Unfertilized eggs were then directly dropped after squeezing the females. About 15 min after insemination, sufficient amount of 10% SB was added and incubated at 18°C. To avoid artificial activation of *Xenopus* eggs at the time of insertion of a micropipet for microinjection, the dejellied eggs were placed in Ca-free PB containing 4% polyvinylpyrrolidinone (PVP) and injected with a glass micropipet with a tip diameter of 20–30 μm. The injected eggs were incubated in Ca-PB for several minutes, and then stored in 3×NKP containing 4% PVP until use.

Preparation of sperm extract

Sperm extracts were prepared according to the method described previously [10]. The sperm of *Cynops* were collected from sperm ducts and suspended in 10% SB at a concentration of about 5×10^6 cells/ml. After rapid freezing at −80°C, the thawed suspension of sperm was centrifuged at 10,000×g for 20 min at 2°C. The supernatant was again centrifuged at 100,000×g for 1 hr at 2°C. The supernatant was collected as the sperm extract and stored at −80°C. One unit of the activity was for convenience defined as the amount of activity in 1 μl of the sperm extract. In some cases, the extract was desalted by a PD-10 column ("Pharmacia"), and then concentrated by lyophilization.

Electrical recordings

To record a fertilization potential or an activation potential, one electrode (10–20 MΩ with 3 M KCl) was inserted into the animal hemisphere of an egg, by increasing the capacitance compensation to produce oscillation. Recordings were made with a microelectrode amplifier (MEZ-7101 or MEZ-8301; Nihonkohden), a digital storage-oscilloscope (DS-6612; Iwatsu), a voltage clamp amplifier (CEZ-1100; Nihonkohden), and a chart recorder (WR-3701; Graphtec). Currents were measured with a converter between ground and an Ag-AgCl reference electrode, which was connected to the bath via an agar bridge. All the experiments were carried out at 18–23°C.

Activation of *Xenopus* eggs can be detected in the live egg by movement of pigments, cortical contraction, and formation of fertilization coat. Sections of *Xenopus* eggs were examined in order to confirm breakdown of cortical granules, as well as emission of the second polar body. Eggs were fixed in Smith's solution, and embedded in paraffin. The 10-μm-thick serial sections were stained with Feulgen's reaction and fast green for observation of nuclei or by the PAS reaction for observation of cortical granules.

Measurements for intracellular Ca ions

Measurements of activities of intracellular Ca^{2+} in *Xenopus* eggs were performed by the methods described previously [13]. The dejellied *Xenopus* eggs were placed in 100% SB, and their vitelline coats were removed by fine watchmarker's forceps. Aequorin used in the present study was a gift from Dr. Osamu Shimomura (MBL, Woods Hole). Aequorin was dissolved at a concentration of 10 mg/ml in water containing 100 μM EGTA and 10 mM Pipes at pH 7.0. The denuded eggs were immersed in 0.05 M phosphate buffer (pH 7.0) for 10 min, and then 25–50 nl of the aequorin solution was injected. Final concentration of aequorin and EGTA was approximately 0.2–0.4 mg/ml and 2–4 μM, respectively. About 10 min after injection, the eggs were transferred to 100% SB. An image of the luminescence emitted from aequorin in the presence of Ca^{2+} was obtained by a two-dimensional photon-counting system (ARGUS-100/VIM, Hamamatu Photonics). Technical details were reported previously [22, 23].

RESULTS

Ion channels which opened at activation of Xenopus eggs by Cynops sperm extract

When unfertilized *Xenopus* eggs were treated by *Cynops* sperm extract (1 unit/μl; 20 μl) in 10% SB ($[Na^+]_o$=5.8 mM, $[K^+]_o$=0.067 mM, $[Cl]_o$=6.3 mM), the egg underwent hyperpolarization to about −37 mV 2–3 min after treatment (Fig. 1A; Table 1). The egg membrane then deporlarized to reach a positive potential of about +38 mV 3–4 min after appearance of hyperpolarization. The egg underwent cortical contraction 2–3 min after eliciting the positive potential.

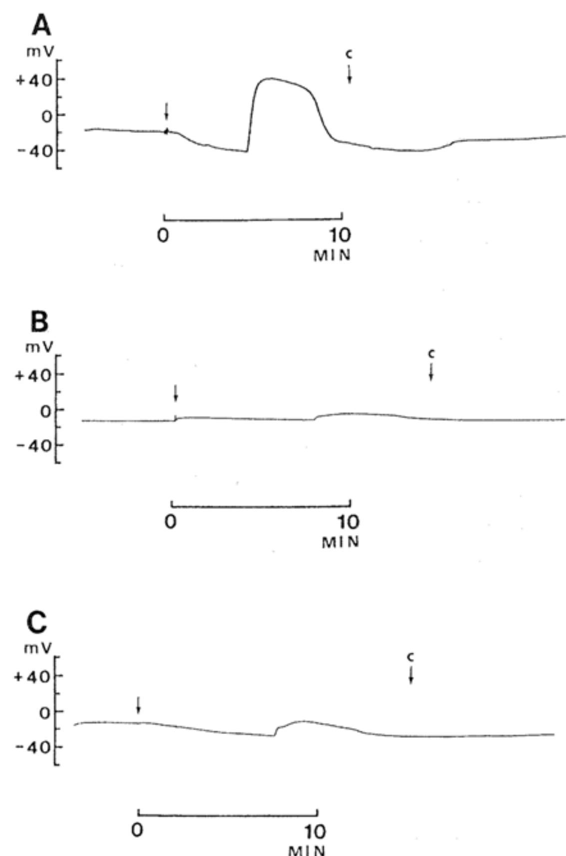


FIG. 1. Electrical responses of dejellied *Xenopus* eggs to an extract of *Cynops* sperm. The change in potential in 10% SB (A), in 10% SB containing 52.2 mM NaCl (B), or in 10% SB containing 56.9 mM choline-Cl (C), showing a decrease of amount of both hyperpolarization and a positive-going potential in NaCl, but only of a positive-going potential in choline-Cl. Arrows indicate timing of extract treatment. c, Beginning of cortical contraction.

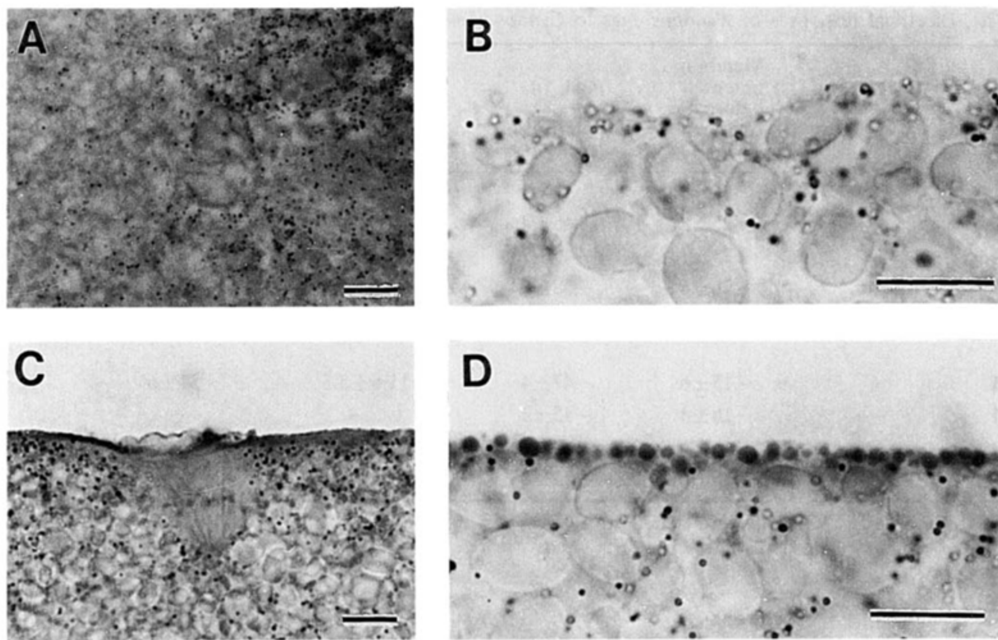


FIG. 2. A section of the animal hemisphere of a *Xenopus* egg treated with an extract of *Cynops* sperm in 10% SB (A) or in 10% SB containing 0.5 μM $CdCl_2$ (C), showing an egg pronucleus in the egg treated in 10% SB, but an egg nucleus at the second meiotic metaphase in the egg treated in $CdCl_2$. A section of the vegetal hemisphere of *Xenopus* egg treated with an extract of *Cynops* sperm in 10% SB (B) or in 10% SB containing 0.5 μM $CdCl_2$ (D), showing loss of cortical granules after treatment in 10% SB, but cortical granules beneath the egg cortex after treatment in $CdCl_2$. The eggs were fixed about 60 min after treatment with the extract. Bars, 10 μm.

TABLE 1. Electrical responses of *Xenopus* eggs to *Cynops* sperm extract under various conditions of external ions

External solutions**	Membrane potential before treatment (mV)	Peak of hyper-polarization (mV)	Interval between start of treatment and positive potential (min)	Peak of positive potential (mV)	Duration of positive potential (min)
10% SB	−22±8*	−37±7	3.3±1.0	34±5	3.5±0.5
NaCl 52.2 mM	−8±2	−12±2	3.6±1.6	−4±3	—
Choline-Cl 56.9 mM	−12±2	−23±3	6.6±1.4	−11±3	—
KCl 0.67 mM	−17±5	−35±3	3.6±0.6	30±9	2.8±0.6
6.7 mM	−22±5	−28±2	3.4±0.7	24±1	1.4±1.9

* Mean±SD (n=7–10). ** Ions were added to 10% SB.

The egg activation was confirmed by resumption of meiosis to form egg pronuclei (Fig. 2A) and by discharge of cortical granules (Fig. 2B). These potential changes were quite similar to those induced by *Cynops* sperm [9]. When the eggs were treated in 10% SB containing 52.2 mM NaCl to increase $[Na^+]_o$ to 10-fold ($[Na^+]_o$=5.8 mM, $[K^+]_o$=0.067 mM, $[Cl^-]_o$=58.5 mM), not only the amount of hyperpolarization, but the amount of positive potential (depolarization) decreased (Fig. 1B; Table 1). All the eggs underwent activation to show cortical contraction. In 10% SB containing 56.9 mM choline chloride to increase $[Cl]_o$ to 10-fold ($[Na^+]_o$]=5.8 mM, $[K^+]_o$]=0.067 mM, $[Cl^-]_o$=63.2 mM), the amount of depolarization was reduced to −11 mV, but the hyperpolarization phase was almost unaffected (Fig. 1C; Table 1). Furthermore, an increase of $[K^+]_o$ up to 6.7 mM did not affect the potential changes by the sperm extract at all (Table 1). These results indicate that the hyperpolarization is due to opening of Na channels, but the succeeding depolarization (the positive potential) is due to opening of Cl channels on egg plasma membrane, which is well consistent with the results obtained at cross-fertilization of *Xenopus* eggs by *Cynops* sperm [9].

Inhibition of egg activation by the sperm extract in the presence of Ca channel blockers

Since activation of *Xenopus* eggs by *Cynops* sperm extract did not occur in very low concentration of extracellular Ca^{2+}; $[Ca^{2+}]_o$=1.5 μM [10], it is worth to determine

Table 2. Electrical responses of *Xenopus* eggs to *Cynops* sperm extract under various conditions of external ions

External solutions**	Egg activation	Membrane potential before treatment (mV)	Peak of hyper-polarization (mV)	Interval between start of treatment and positive potential (min)	Peak of positive potential (mV)	Duration of positive potential (min)
$CdCl_2$						
0.1 μM	+	-25 ± 8*	-45 ± 4	5.7 ± 4.8	34 ± 10	4.4 ± 1.3
0.5 μM	+	-23 ± 13	-45 ± 7	5.3 ± 0.3	39 ± 6	12.5 ± 6.3
	—	-26 ± 6	-38 ± 6	5.1 ± 3.1	-20 ± 9	—
1.0 μM	—	-20 ± 3	-41 ± 6	—	—	—
$CoCl_2$						
0.1 mM	+	-15 ± 6	-47 ± 4	11.6 ± 5.3	31 ± 6	3.8 ± 0.2
1.0 mM	—	-16 ± 4	-45 ± 3	—	—	—
$NiCl_2$						
5.0 mM	—	-18 ± 2	-33 ± 4	—	—	—

* Mean$\pm$SD (n=7–10). ** Ions were added to 10% SB.

whether or not entry of extracellular Ca^{2+} through Ca channels is necessary for activation by the sperm extract. Unfertilized *Xenopus* eggs were treated by the sperm extract in various concentrations of $CdCl_2$, $CoCl_2$, or $NiCl_2$ in 10% SB (Table 2). Egg activation was not affected in 0.1 μM $CdCl_2$ at all. In 75% of eggs treated by the sperm extract in 0.5 μM $CdCl_2$, small positive-going potentials were elicited (Fig. 3A; Table 2), but no cortical contraction was observed. Their egg nuclei stayed at the second meiotic metaphase (Fig. 2C) and their cortical granules remained intact (Fig. 2D). Finally, no positive-going potential was detected in the eggs treated in 1 μM $CdCl_2$ (Fig. 3B). The hyperpolarization was, however, not affected at all. The egg activation was also inhibited either by 1 mM $CoCl_2$ or by 5 mM $NiCl_2$ (Table 2).

Unfertilized *Xenopus* eggs were treated by the sperm extract in various concentrations of amiloride which inhibits Ca channels as well as Na/Ca exchanger. The eggs elicited the positive-going potential and underwent cortical contraction in 0.5–1.0 mM amiloride, but appearance of positive-going potential was delayed. In 2 mM amiloride, the egg activation was completely inhibited, so that neither a positive-going potential nor cortical contraction was induced (Fig. 3C). When unfertilized eggs were treated by the sperm extract in various concentrations of verapamil; a Ca channel blocker, the egg activation was not inhibited, but the amount of positive-going potential significantly decreased when treated in 1 mM verapamil (Fig. 3D). These results suggest that an influx of Ca^{2+} through Ca channels on egg plasma

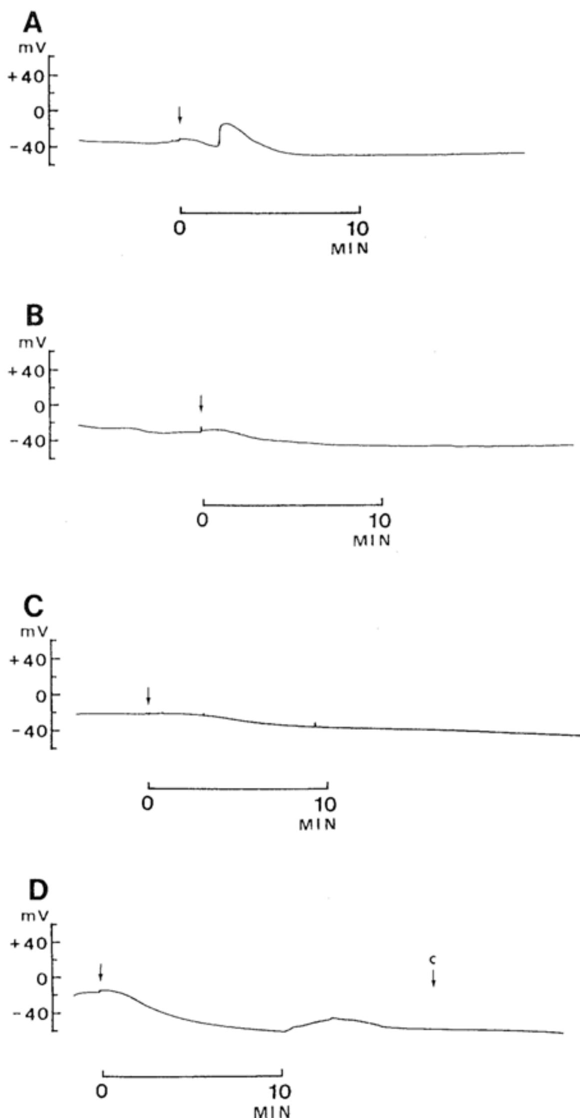


Fig. 3. Electrical responses of dejellied *Xenopus* eggs to an extract of *Cynops* sperm. The change in potential in 10% SB containing 0.5 μM (A) or 1 μM (B) $CdCl_2$, in 10% SB containing 2 mM amiloride (C), and in 10% SB containing 1 mM verapamil (D), showing the inhibition of eliciting a positive-going potential in 1.0 μM $CdCl_2$, 2 mM amiloride, but a decrease of the amount of positive-potential in 1 mM verapamil. Arrows indicate timing of extract treatment. c, Beginning of cortical contraction.

membrane is involved in activation of *Xenopus* eggs by *Cynops* sperm extrat.

Propagative intracellular Ca^{2+} release during activation of Xenopus eggs by Cynops sperm extract

To determine whether or not activation of unfertilized *Xenopus* eggs is induced when the sperm extract is applied to a small area, about 2 μl of cocentrated sperm extract (10 units/μl) was applied to about 0.02 mm^2 of egg surface with a small glass pipet. The eggs elicited a potential change which is quite similar to that induced by a large amount of extract (1 unit/μl; 20 μl) (Fig. 5A). All the eggs underwent activation, so that cortical contraction began at the site of extract treatment, suggesting propagation of activation stimuli, for example intracellular Ca release induced by the extract treatment.

To monitor potential changes concomitant with intracellular Ca^{2+} activities during cross-fertilization or egg activation, unfertilized *Xenopus* egg was injected with aequorin. The egg was inseminated with about 10 μl of *Cynops* sperm suspension (about 10^5 sperm/ml) in 50% SB (Fig.4A). The luminescence for intracellular Ca^{2+} release was initiated at the site of an animal hemisphere and spread over the whole hemisphere. The luminescence was finally progressed to the vegetal hemisphere about 3 min after appearance of luminescence. The egg underwent a hyperpolarization followed by a positive-going potential (Fig. 4C). The luminescence was observed just after eliciting the positive-going potential. The amount of potential changes was small due to high $[Na^+]_o$ and $[Cl^-]_o$ in 50% SB. Thus, the pattern of propagative intracellular Ca release induced by *Cynops* sperm is quite similar to that induced by pricking [13].

When aequorin-injected *Xenopus* egg was locally treated by a small amount (1–5 μl) of concentrated sperm extract (10 units/μl) during recording the egg membrane potential in 50% SB (Fig. 4B), the egg underwent a hyperpolarization followed by a positive-going potential (Fig. 4D). The weak luminescence for intracellular Ca^{2+} release was detected at a site of an animal hemisphere after the peak of positive-going potential and spread over the whole egg surface. These results demonstrate that not only the sperm but also their extract induced the propagative intracellular Ca^{2+} release and that Ca^{2+} release occurs at eliciting a positive-going potential, but not during a hyperpolarization phase.

IP_3-receptor dependent intracellular Ca^{2+} release is necessary for activation by the sperm extract

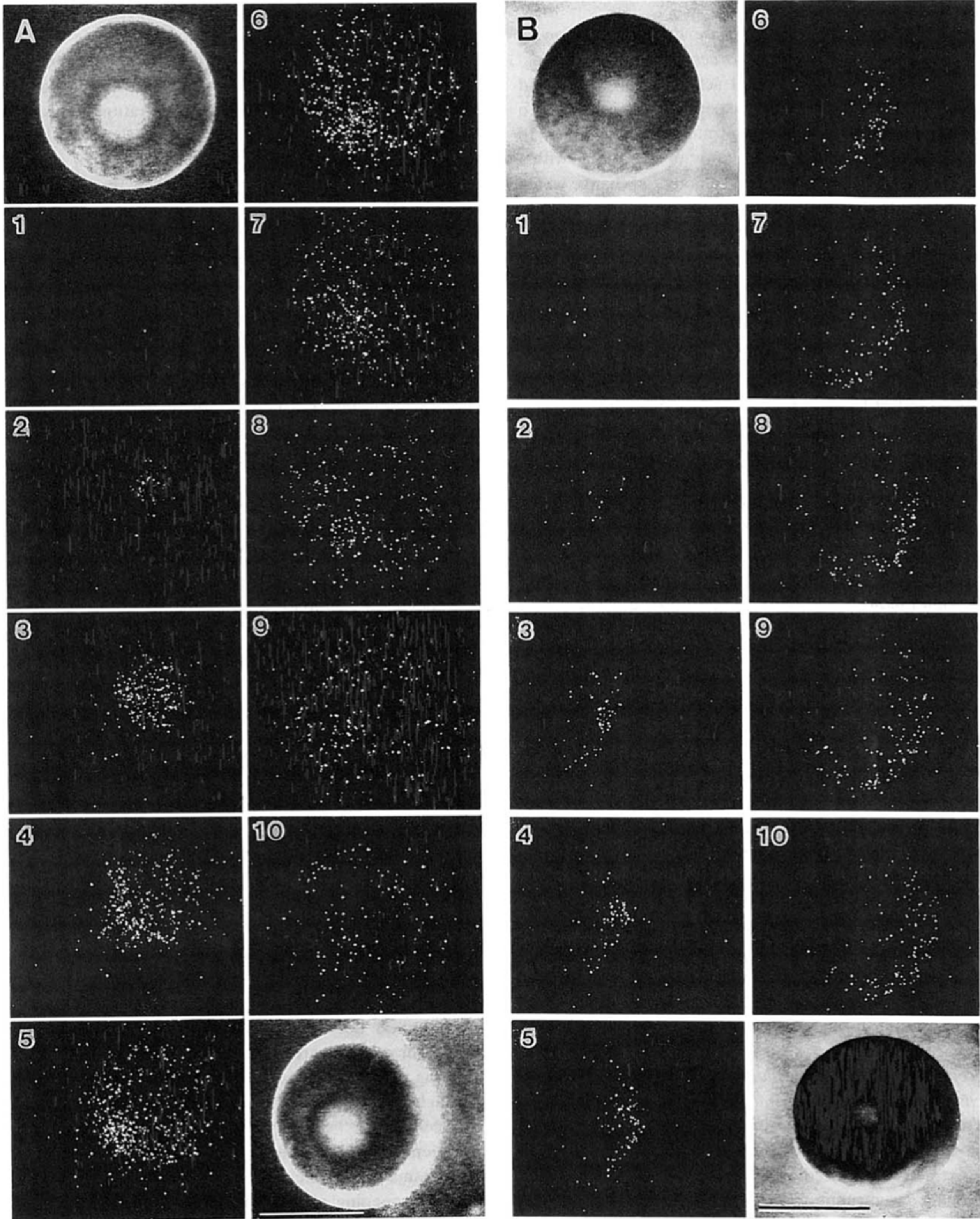
To determine whether or not the intracellular Ca^{2+} release is necessary for activation of *Xenopus* eggs by the sperm extract, unfertilized eggs were injected with BAPTA whose final concentration in cytoplasm was 2.5 mM. About 20 min after injection, the eggs were treated by the sperm extract (Fig. 5B; Table 3). The eggs underwent hyperpolarization from about −8 mV to about −38 mV, but neither a positive-going potential nor cortical contraction was observed. Egg activation was completely inhibited by BAPTA-injection because their cortical granules remained intact and their egg nuclei stayed at the second meiotic metaphase. The injection of distilled water did not affect egg activation at all.

To know a role of inositol 1,4,5-trisphosphate (IP_3) receptors in egg activation process, unfertilized eggs were injected with heparin in various concentrations (Figs. 5C and D; Table 3). The injection of heparin whose final concentration in egg cytoplasm was 75 μM did not affect egg activation by the sperm extract. When the eggs were injected with higher concentration of heparin (150 μM), the amount of hyperpolarization was not changed, but that of a positive-going potential was decreased (Fig. 5C). The injection of heparin whose final concentration was 300 μM completely inhibited egg activation by the sperm extract, so that a hyperpolarization was detected (Fig. 5D). The egg nuclei at the second meiotic metaphase and intact cortical granules were confirmed by cytological sections. Furthermore, unfertilized eggs injected with 0.5 μM thapsigargin, an inhibitor for Ca-ATPase, underwent activation by the treatment of a small amount (2 μl) of sperm extract (Table 3). These results suggest that the Ca^{2+} release dependent upon IP_3 receptors in intracellular Ca^{2+} store is necessary for egg activation by the sperm extract, in particular eliciting the positive-going potential and undergoing cortical reactions, but the preceding hyperpolarization is independent of intracellular Ca^{2+} activity.

DISCUSSION

We showed in this study that the hyperpolarization is due to the opening of Na channels, and the succeeding depolarization (a positive-going potential) is due to the opening of Cl channels on the egg plasma membrane during the activation of *Xenopus* eggs by *Cynops* sperm extract. Properties of these potential changes are quite similar to those in cross-fertilization of *Xenopus* eggs with *Cynops* sperm [9]. These potential changes are probably caused by a sperm protease localized in an acrosomal region [10]. When a protease activity in the sperm extract was abolished by protease inhibitors or by competitive substrates, not only the positive-going potential, but also the hyperpolarization was inhibited [10]. In contrast, we observed in this study that inhibition of Ca channels does not affect the hyperpolarization phase. Furthermore, an increase in intracellular Ca^{2+} occurs after eliciting the positive-going potential. Thus, Ca^{2+} influx through Ca channels seems to be necessary for elevation of intracellular Ca^{2+} level, rather than for the initial interactions between the sperm protease and their putative receptors on egg plasma membrane.

The opening of Cl channels to produce a fertilization potential is dependent upon intracellular Ca^{2+} activity at normal fertilization in *Xenopus* [11, 12], but the opening of Na channels has not yet been determined. However, it has been recently reported that an ATP-activated Na^+ channels is involved in sperm-induced fertilization [15]. Furth-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ermore, treatment of *Xenopus* oocytes with trypsin induces Ca^{2+}-activated Cl^- currents [3]. These results suggest that a sperm protease of *Xenopus* which is similar to that of *Cynops* sperm is involved in activation of normal fertilization in *Xenopus*.

The propagative intracellular Ca^{2+} release induced by the sperm extract is quite similar to that induced by *Xenopus* sperm [17] or by pricking [13]. The requirement of an intracellular Ca release dependent upon inositol 1,4,5-trisphosphate (IP_3) receptors is also well consistent with the results obtained in normal fertilization of *Xenopus* eggs [17]. Injection of IP_3 into unfertilized eggs induces egg activation, whereas injection of heparin inhibits egg activation by sperm [20]. IP_3 mass increases after fertilization of *Xenopus* eggs preinjected with BAPTA or heparin [20]. Furthermore, IP_3 receptors are localized in cortical endoplasmic reticulum in *Xenopus* eggs [14]. These suggest that the sperm extract induces the propagative Ca release by producing IP_3. The opening of Cl channels is probably caused by increase in intracellular Ca^{2+}. However, the opening of Na channels seems to be independent of an increase in intracellular Ca^{2+}, because the hyperpolarization was induced even after inhibition of intracellular Ca^{2+} activities by BAPTA or by heparin.

We found in this study that Ca influx through egg plasma membrane is indispensable for egg activation. Although amiloride is also known as an inhibitor for Na/Ca exchanger which causes Ca influx in low $[Na^+]_o$ condition [18], $CdCl_2$ inhibits Ca channels in less than 100 μM, but does not affect Na/Ca exchanger in cardiac muscles [19]. These results suggest that the opening of Ca channels rather than stimulation of Na/Ca exchanger causes Ca entry during egg activation.

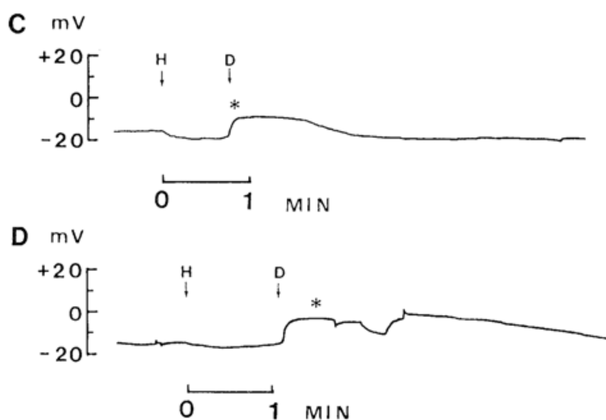

FIG. 4. A free calcium wave propagating along the surface of denuded *Xenopus* eggs. The unfertilized eggs were treated by *Cynops* sperm (A) concomitant with recording potential changes in 50% SB (C). The unfertilized eggs were treated by their extract (B) concomitant with recording potential changes in 50% SB (D). Successive photographs are 15 sec apart. Aequorin luminescence accumulated for 5 sec was shown in each photograph. The recordings showed the luminescence from 3.5 min after insemination in (A) and 1.8 min after extract treatment in (B). First frame in each series is the egg shortly before the insemination or the treatment, respectively. Last frame in each series is the egg showing cortical contraction after passage of the calcium wave. H, Beginning of hyperpolarization. D, Beginning of depolarization. Asterisk, Beginning of Ca wave. Bar, 1 mm.

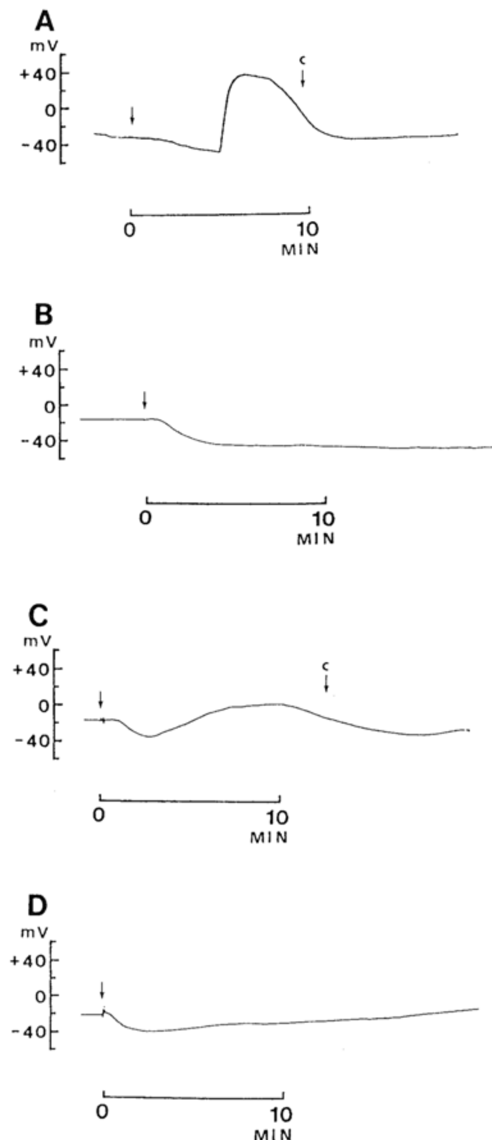

FIG. 5. Electrical responses of dejellied *Xenopus* eggs to an extract of *Cynops* sperm in 10% SB. The eggs was treated with a small amount (2 μl) of concentrated extract (10 units/μl) on a small area of an animal hemisphere (A), or with a large amount (20 μl) of the extract (1 unit/μl) on the whole surface of an animal hemisphere (B-D). The eggs had been injected with 2.5 mM BAPTA (B), or with heparin (C, 150 μM; D, 300 μM), showing the inhibition of eliciting a positive-going potential in BAPTA-injected eggs and heparin (300 μM)-injected eggs. Arrows indicate timing of extract treatment. c, Beginning of cortical contraction.

Although the mechanism how the stimulation by sperm entry induces the intracellular Ca^{2+} release is still controversial between species, there are several possibilities [16]: (1) a sperm agonist binds to a receptor molecule on egg plasma membrane to produce IP_3, which results in Ca^{2+} release from

TABLE 3. Electrical responses of *Xenopus* eggs which had been injected with BAPTA or heparin, to *Cynops* sperm extract in 10% SB

Injected with	Egg activation	Membrane potential before treatment (mV)	Peak of hyper-polarization (mV)	Interval between start of treatment and positive potential (min)	Peak of positive potential (mV)	Duration of positive potential (min)
BAPTA						
2.5 mM	—	−8±2*	−38±2	—	—	—
Heparin						
75 μM	+	−19±12	−40±9	7.1±5.4	27±8	5.7±0.4
150 μM	—	−22±6	−42±3	3.0±0.4	−14±12	—
300 μM	—	−16±2	−41±1	—	—	—
Tapsigargin						
0.1 μM	+	−21±7	−29±7	5.8±3.0	22±12	2.6±0.8
0.5 μM	+	−18±10	−29±8	7.5±1.4	37±6	5.2±1.1

* Mean±SD (n=7–10).

Ca^{2+} store in egg cytoplasm, (2) a sperm agonist induces Ca influx through egg plasma membrane to produce a local increase in intracellular Ca^{2+} level, which causes the propagative Ca^{2+} release, and (3) a substance from sperm is introduced through the connection between the sperm and the egg after their membrane fusion. Our results obtained here seems to support the possibility that the sperm opens Ca channels to increase Ca influx during egg activation. How does local increase in intracellular Ca^{2+} cause the Ca wave? The local increase in Ca^{2+} may stimulate phospholipase C to produce IP_3 (IP_3-induced Ca release, IICR), because heparin which is known as an inhibitor for IP_3 receptors inhibited egg activation by the sperm extract. Alternatively, the local increase in Ca^{2+} may stimulate directly Ca store to release Ca^{2+} (Ca-induced Ca-release, CICR). In this case, Ca may stimulate IP_3 receptors to induce Ca^{2+} release [17], rather than causes an overload of Ca stores to undergo Ca^{2+} release through Ca-ATPase, because thapsigargin which is known as an inhibitor for Ca-ATPase [21] did not inhibit egg activation by the sperm extract.

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REFERENCES

1 Busa WB, Nuccitelli R (1985) An elevated free cytosolic Ca^{2+} wave follows fertilization in eggs of the frog, *Xenopus laevis*. J Cell Biol 100: 1325–1329

2 Cross NL (1981) Initiation of the activation potential by an increase in intracellular calcium in eggs of the frog, *Rana pipiens*. Dev Biol 85: 380–384

3 Durieux ME, Salafranca MN, Lynch, KR (1994) Trypsin induces Ca^{2+}-activated currents in *X. laevis* oocytes. FEBS Letters 337: 235–238

4 Foltz KR, Lennarz, WJ (1992) Identification of the sea urchin egg receptor for sperm using an antiserum raised against a gragment of its extracellular domain. J Cell Biol 116: 647–658

5 Foltz KR, Partin JS, Jennarz, WJ (1993) Sea urchin egg receptor for sperm: sequence similarity of binding domain and hsp70. Science 259: 1421–1425

6 Gould M, Stephano JL (1987) Electrical responses of eggs to acrosomal protein similar to those induced by sperm. Science 235: 1654–1656

7 Gould M, Stephano JL (1991) Peptides from sperm acrosomal protein that initiate egg development. Dev Biol 146: 509–518

8 Gould M, Stephano JL, Holland LZ (1986) Isolation of protein from *Urechis* sperm acrosomal granules that binds sperm to eggs and initiates development. Dev Biol 117: 306–318

9 Iwao Y (1985) The membrane potential changes of amphibian eggs during species- and cross-fertilization. Dev Biol 111: 26–34

10 Iwao Y, Miki A, Kobayashi M, Onitake K (1994) Activation of *Xanopus* eggs by an extract of *Cynops* sperm. Dev Growth Differ 36: 469–479

11 Kline D (1988) Calcium-dependent events at fertilization of the frog egg: injection of a calcium buffer blocks ion channel opening, exocytosis, and formation of pronuclei. Dev. Biol 126: 346–361

12 Kline D, Nuccitelli R (1985) The wave of activation current in the *Xenopus* egg. Dev Biol 111: 471–487

13 Kubota HY, Yoshimoto Y, Yoneda M, Hiramoto Y (1987) Free calcium wave upon activation in *Xenopus* eggs. Dev Biol 119: 129–136

14 Kume S, Muto A, Aruga J, Nakagakwa T, Michikawa T, Furuichi T, Nakade S, Okano H, Mikoshiba K (1993) The *Xenopus* IP_3 receptor: Structure, function, and localization in oocytes and eggs. Cell 73: 555–570

15 Kupitz Y, Atlas D (1993) A putative ATP-activated Na^+ channel involved in sperm induced fertilization. Science 261: 484–486

16 Miyazaki S, Nakada K, Shirakawa H. (1993) Signal transduction of gamate interaction and intracellular calcium release mechanism at fertilization of mammalian eggs. In "Biology of The Germ Line in Animal and Man" Ed by H Mohri, M Takahashi, C Tachi, Japan Scientific Society Press, Tokyo, pp 125–143

17 Nuccitelli R, Yim, DL, Smart T (1993) The sperm-induced Ca^{2+} wave following fertilization of the *Xenopus* egg requires the production of Ins(1,4,5)P_3. Dev Biol 158: 200–212.

18 Philipson KD, Nicoll DA (1993) Molecular and kinetic aspects of sodium-calcium exchanger, In "Molecular Biology of Receptors and Transporters: Pumps, Transporters, and Channels Vol. 137C" Ed by M Friedlander, M Muechler, Academic Press, San Diego, pp 199–227

19 Sham JSK, Cleemann L, Morad M (1992) Gating of the cardiac Ca^{2+} release channel: The role of Na^+ current and Na^+-Ca^{2+} exchange. Sinence 255: 850–853

20 Stith BJ, Espinoza R, Roberts D, Smart T (1994) Sperm increase inositol 1,4,5-trisphosphate mass in *Xenopus laevis* eggs preinjected with calcium buffers or heparin. Dev Biol 165: 206–215

21 Thastrup O, Dawson AP, Scharff O, Foder B, Cullen PJ, Drøbak BK, Bjerrum PJ, Christensen SB, Hanley MR (1989) Thapsigargin, a novel molecular probe for studying intracellular calcium release and storage. Agents and Action 27: 17–23

22 Yoshimoto Y, Hiramoto Y (1991) Observation of intracellular Ca^{2+} with aequorin luminescence. Int Rev Cytol 129: 45–73

23 Yoshimoto Y, Iwamatsu T, Hirano K, Hiramoto Y (1989) The wave pattern of free calcium release upon fertilization in medaka and sand dollar eggs. Dev Growth Differ 28: 583–59