Activation of Xenopus Eggs by Proteases: Possible Involvement of a Sperm Protease in Fertilization

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Egg activation in cross-fertilization between Xenopus eggs and Cynops sperm may be caused by a protease activity against Boc-Gly-Arg-Arg-MCA in the sperm acrosome. To determine the role of the sperm protease in fertilization, the protease was purified from Cynops sperm using several chromatographic techniques. We found that purified sperm protease readily hydrolyzes Boc-Gly-Arg-Arg-MCA and Z-Arg-Arg-MCA, that protease activity was inhibited by the trypsin inhibitors aprotinin and leupeptin, and that not only the purified protease, but also cathepsin B, induces activation in Xenopus eggs. We inseminated unfertilized Xenopus eggs with homologous sperm in the presence of various peptidyl MCA substrates or protease inhibitors and demonstrated that trypsin inhibitors or MCA substrates containing Arg-Arg-MCA reversibly inhibited fertilization of both fully jellied and denuded eggs. Sperm motility was not affected by the reagents. An extract obtained from Xenopus sperm showed hydrolytic activity against Boc-Gly-Arg-Arg-MCA, Z-Arg-Arg-MCA, and Arg-MCA. These results suggest that the tryptic protease in Xenopus sperm is involved in fertilization, most likely by participating in egg activation. © 1999 Academic Press

INTRODUCTION

In the fertilization of most animals, such as sea urchins, fish, frogs, and mammals, egg activation is accompanied by the release of Ca\(^{2+}\) ions into the egg cytoplasm (Miyazaki et al., 1993). It remains unclear, however, how a fertilizing sperm induces intracellular Ca\(^{2+}\) release, but several mechanisms are possible. The binding of sperm to a receptor in the egg plasma membrane may change the conformation of the receptor that initiates the cascades that cause the intracellular Ca\(^{2+}\) release (Jaffe, 1990). Or egg activation may be induced by a protein isolated from the sperm acrosome, as in the invertebrate Urechis (Gould and Stephano, 1987, 1991; Gould et al., 1996). In the starfish Asterina miniata, the application of proteases to eggs causes activation responses similar to those seen at fertilization (Carroll and Jaffe, 1995). In the frog Xenopus laevis, unfertilized eggs can be activated by a sperm extract of the newt Cynops pyrrhogaster and is accompanied by the propagative Ca\(^{2+}\) release across the entire egg surface (Iwao et al., 1994, 1995). The active component is probably a protease localized in the sperm acrosome (Iwao et al., 1994). We recently demonstrated that a peptide containing the RGD sequence induces the intracellular Ca\(^{2+}\) release that activates Xenopus eggs (Iwao and Fujimura, 1996). Investigation into cross-fertilization between (a) eggs of a voltage-insensitive species and sperm of a voltage-sensitive species (Iwao and Jaffe, 1989) and (b) eggs of voltage-sensitive species and sperm of a voltage-insensitive species (Jaffe et al., 1983; Iwao and Jaffe, 1989; Iwao et al., 1994) indicates that sperm contributes the voltage-sensitive component of egg activation. This suggests that either an extracellularly exposed protein that transduces a signal from the sperm at fertilization or the agent active in releasing the bound Ca\(^{2+}\) ions derives from the sperm cytosol. We note a variety of intriguing observations: In mammals, injection of the sperm extract into the unfertilized egg causes intracellular Ca\(^{2+}\) release and Ca\(^{2+}\) oscillation (Parrington et al., 1996). Similar Ca\(^{2+}\) release in ascidian eggs can be induced by human sperm extract (Wilding et al., 1997). It is proposed that an "oscellin" in the sperm head, which is homologous to Escherichia coli glucosamine-6-phosphate deaminase/iosmerase (GNPDA), causes calcium oscillation in hamster eggs (Parrington et al., 1996). Recombinant and purified GNPDA, however, failed to...
elevate intracellular \(Ca^{2+}\) when injected into mouse eggs (Wolosker et al., 1998). Thus, the nature of oscillin still remains unknown.

Xenopus sperm initiates hydrolysis of phosphatidylinositol 4,5-biphosphate (PI(PI)) to produce PI, at fertilization (Nuccitelli et al., 1993; Stith et al., 1994), which triggers a transient and propagative increase in the intracellular level of free \(Ca^{2+}\) ions (Busa and Nuccitelli, 1985; Nuccitelli et al., 1993). PI, receptors are localized in the cortical endoplasmic reticulum in Xenopus eggs (Kume et al., 1993). These observations suggest that the fertilizing sperm transmits a signal to produce PI, into egg cytoplasm at the time of binding between sperm and egg membranes.

Amphibian sperm have strong proteolytic activities in the acrosomal region (Elinson, 1974; Iwao and Katagiri, 1982; Iwao et al., 1994; Penn and Gledhill, 1972; Raisman and Cabada, 1977). The vitelline coat lysin of the toad Bufo japonicus can be purified and characterized as a tryptic protease (Yamasaki et al., 1988). Furthermore, fertilization in fully jellied or dejellied frog eggs which are surrounded with vitelline coats is inhibited by either soybean trypsin inhibitor (Cabada et al., 1978; Elinson, 1971; Takamune et al., 1986) or leupeptin (Takamune et al., 1986). Although these observations all suggest involvement of a tryptic protease during fertilization of amphibians, the detailed mechanisms have not yet been investigated.

To determine the molecular mechanism of egg activation in Xenopus fertilization, we tried two methods to induce egg activation: with highly purified Cynops sperm protease and with cathepsin B. We also attempted to inhibit fertilization using various substrates or protease inhibitors in the fertilization of denuded eggs, with the jelly-treated sperm and with cathepsin B. We also attempted to inhibit fertilization using various substrates or protease inhibitors in the fertilization of denuded eggs, the detailed mechanisms have not yet been investigated.

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**MATERIALS AND METHODS**

**Chemicals and Solutions**

Peptidyl-4-methylcoumaryl-7-amides (MCAs), benzylloxycarbonyl (Z)-Phe-Arg-MCA, Z-Arg-Arg-MCA, succinyl (Suc)-Gly-Pro-MCA, Suc-Ala-Pro-Ala-MCA, Suc-Leu-Val-Tyr-MCA, Suc-Ala-Ala-Pro-Phe-MCA, t-butyloxycarbonyl (Boc)-Gly-Arg-MCA, Boc-Val-Pro-Arg-MCA, Boc-Leu-Thr-Arg-MCA, Boc-Leu-Gly-Arg-MCA, and Arg-MCA, were obtained from the Peptide Institute. Leupeptin, aprotinin, bestatin, antipain, chymostatin, benzamidine, L-trans-epoxysuccinylleucylamido 4-guanidino butane (E-64), cathepsin B, and trypsin were purchased from Sigma Aldrich Japan. Ethylenediaminetetraacetic acid (EDTA), ethylene glycol bis-(β-aminoethyl ether)-N,N',N'-tetraacetic acid (EGTA), 4-(2-aminoethyl)benzenesulfonyl fluoride (HCl (p-ABSF)), N-ethylmaleimide (NEM), and CA-074 were obtained from Wako Pure Chemical Industries, Katayama Chemicals, Nakarai Tesque, and the Peptide Institute, respectively. The ionic compositions of solutions in mM were as follows: 100% De Boer's solution (DB), 110 NaCl, 1.3 KCl, 1.3 CaCl2, 5.7 Tris-Cl (pH 7.4), 100% Steinberg's solution (SB), 58.0 NaCl, 0.67 KCl, 0.34 CaCl2, 0.85 MgSO4, 4.6 Tris-Cl (pH 7.4).

**Gametes**

The clawed frogs, X. laevis, were purchased from Hamamatsu Seibutsu Kyouzai, Japan, and maintained in our laboratory. Sexually mature newts, C. pyrrhogaster, were collected near Yamaguchi, Japan. To obtain mature Xenopus eggs, females were injected with 375-500 IU of human chorionic gonadotropin (HCG; Tekoku Zoki, Tokyo) in the dorsal lymph sac and were kept at 18°C for 12-18 h. Mature eggs were obtained by squeezing females or by oviposition in 0.1 M NaCl. Egg jelly coats were removed by 3% cysteine-HCl (pH 8.5), 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 1 h. Xenopus sperm suspension for insemination was prepared by mincing a piece of testis in 50% SB.

**Artificial Insemination of Jellied and Denuded Eggs**

To determine the effect of MCA substrates or protease inhibitors on Xenopus fertilization, the sperm suspension was mixed with the reagent to be tested and incubated for 10 min at 20°C prior to insemination. Unfertilized eggs were incubated for 10 min in 50% SB containing the same reagent prior to insemination. A sufficient amount of 10% SB was added to the dish 60 min after insemination and the eggs were incubated at 20°C.

Insemination of denuded eggs was performed according to the method of Kline et al. (1991). Vitelline coats of dejellied eggs were removed with fine watchmaker's forceps in 33% DB. The dejellied eggs were placed in a depression (about 1 cm in diameter) in agar-coated dishes filled with 33% DB. The volume of solution in the depression was 200-300 μl. A jelly extract was prepared by shaking fully jellied eggs in 33% DB (about 8 ml of solution per 3 g of eggs) for 45 min and then adding 10% Ficoll. Egg jelly extract (150 μl) was combined with 15 μl of a sperm stock containing 1 testis + 100 μl 33% DB. To determine the effect of protease inhibitors or substrates on the fertilization of denuded eggs, the reagents to be tested were subsequently added to the mixture of sperm and egg jelly extract just before insemination. In some experiments, sperm were incubated with egg jelly extract in the absence of the reagents for 10 min and then the reagent to be tested was added prior to insemination.

Activation of Xenopus eggs can be detected in the live egg by movement of pigments, cortical contraction, and formation of fertilization coat. Fertilization was determined in live eggs by occurrence of first cleavage. Sections of eggs were examined in order to confirm breakdown of cortical granules and emission of the second polar body. Eggs were fixed in Smith's solution (0.5% K2Cr2O7, 3.6% formaldehyde, 2.5% acetic acid), for 24 h, or in Helly's solution (2.5% K2Cr2O7, 5% HgCl2, 1.8% formaldehyde), for 3 h, and then embedded in paraffin. The 15-μm-thick serial sections were stained with Feulgen's reagent and Fast green for observation of nuclei or by PAS reaction for observation of cortical granules.
Preparation of Sperm Extract

Cynops sperm extracts were prepared according to the method described previously (Iwao et al., 1994). Mature sperm of Cynops were collected from sperm ducts and suspended in 10% SB at a concentration of approximately 5 × 10^7 cells/ml. After rapid freezing at −80°C, the thawed suspension of sperm was centrifuged at 10,000g for 20 min at 2°C. The supernatant was again centrifuged at 100,000g for 1 h at 2°C. The supernatant was collected as the sperm extract and stored at −80°C.

To obtain mature Xenopus sperm, males were injected with 250 IU of HCG in the dorsal lymph sac and were kept at 18°C for 2–3 days. The sperm were obtained by mincing testes in 100% DB. After tissue debris was removed by decantation, the suspension was centrifuged at 700g for 10 min at 2°C. The sedimented sperm were suspended in 10% SB and frozen at −80°C. The thawed suspension was centrifuged at 10,000g for 20 min at 2°C. The supernatant was centrifuged at 200,000g for 60 min at 2°C. The supernatant was collected as the sperm extract and stored at −80°C until use.

Purification of Cynops Sperm Protease

All chromatographies were performed with a FPLC system (Pharmacia Biotechnologies) at 4°C. The sperm extract from up to 4 × 10^9 Cynops sperm was applied to a 0.2 ml/min to a 5 × 20-cm column containing soybean agglutinin (SBA)-agarose (Vectastain) equilibrated in 10% SB. After washing through equilibration buffer, bound glycoproteins were eluted with 10% SB containing 1 M NaCl. Fractions containing greater than 50% of the activity of the peak tube were pooled. After the buffer was changed to 10% SB with a PD-10 column (Pharmacia Biotechnologies), the activity peak pool from the SBA-affinity chromatography was applied at 1 ml/min to a 2.5 × 12.5-cm column containing aprotinin-agarose (Sigma Aldrich Japan) equilibrated in 10% SB. Bound proteins were eluted with 10% SB containing 1 M NaCl and fractions containing greater than 50% of the activity of the peak tube were pooled. After the buffer was changed, the activity peak pool from the aprotinin-affinity chromatography was applied to an ion-exchange column (Mono Q; Pharmacia Biotechnologies) equilibrated in 10% SB. Bound proteins were eluted with a linear 5.8–500 mM gradient of NaCl. To determine the molecular weight, the peak activity eluted at approximately 350 mM was applied to a gel-filtration column (Superose 12; Pharmacia) equilibrated with 10% SB containing 350 mM NaCl.

Assay for Proteolytic Activities

To determine the protease activities of the sperm extract, amidolytic activities against various peptidyl MCA substrates were assayed. MCA substrates were dissolved in dimethyl sulfoxide at 10 mM and stored at −20°C. Before use each solution was diluted with 50 mM Heps–NaOH (pH 7.4) to a final concentration of 10 μM. The extract or cathepsin B (20 μl) was added to 1 ml of the substrate solution, and the initial velocity of AMC formation at 20°C was measured by a spectrofluorophotometer (excitation at 380 nm and emission at 460 nm; RF-500C; Shimadzu Corp.). In some cases, the extract (20 μl) was added to the MCA substrate solution (50 μM, 20 μl). After incubation for 30–60 min at 25°C, 0.1 M sodium chloroacetate was added to the mixture and formation of AMC was measured fluorometrically. To determine the effect of protease inhibitors, each inhibitor (10 μl) was added to the extract (10 μl) and preincubated for 30 min at 25°C. The amidolytic activities against the MCA substrates were measured as described above.

Electrophoresis

In SDS-polyacrylamide gel electrophoresis (SDS-PAGE), gels were stained using Coomassie brilliant blue or using silver stain II kit (Wako Pure Chemical Industries) for proteins. To measure protease activity after electrophoresis, samples containing active proteases were separated on a 7.5% SDS-polyacrylamide gel and following electrophoresis the gels were incubated for 1 h in 10 mM Tris–HCl (pH 8.0) containing 0.1% Triton X-100 to remove SDS. The gels were cut into 2-mm fragments, and each fragment was incubated in 150 μl of 50 mM Hepes–NaOH (pH 7.4) at 4°C for 12 h with constant rocking. The eluted protease activity was measured as described above.

Electrical Recordings and Measurement of [Ca^{2+}]

To record 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 chart recorder (WR-3701; Graphitec), and a DAT data recorder (RD-125T; TEAC). All the experiments were carried out at 18–23°C.

Measurement of intracellular Ca^{2+} in Xenopus eggs was performed with aequorin as described previously (Iwao and Fujimura, 1996).

RESULTS

Purification and Characterization of Cynops Sperm Protease

Xenopus eggs can be activated with an extract obtained from Cynops sperm, and this activation can be inhibited by substrates containing Arg-Arg-MCA at the carboxyl terminus or by protease inhibitors (Iwao et al., 1994, 1995). Because of the close correlation between egg activation and the Boc-Gly-Arg-MCA amidolysis exhibited by the sperm extract, we initially attempted to purify and characterize amidolytic activity against Boc-Gly-Arg-MCA from the sperm extract using several chromatographic techniques. The initial crude sperm extract obtained from 4 × 10^7 sperm contained the hydrolytic activity of 532 mM AMC/min against Boc-Gly-Arg-MCA (see Table 1; purification is illustrated in Fig. 1). Since this enzyme is precipitated by SBA (Iwao et al., 1994), it binds to an SBA-conjugated agarose-affinity column and was eluted by 1 M NaCl (Fig. 1A). The peak fractions were pooled and applied to an aprotinin-conjugated agarose-affinity column, and the bound enzyme was eluted by 1 M NaCl (Fig. 1B). Further purification by a Mono-Q ion-exchange column produced a fraction that was highly enriched in amidase (Fig. 1C; purification is summarized in Table 1).
The molecular weight of the purified enzyme was estimated at approximately 380 kDa by gel filtration with a Superose 12 column (Fig. 1D). The molecular weight of the enzyme under nonreducing conditions was also determined by 3.5% SDS–PAGE (data not shown). The purified enzyme migrated approximately to the 380-kDa position upon SDS–PAGE under nonreducing conditions (Fig. 2A, lane 1), but 100- and 65-kDa bands both appeared in SDS–PAGE under reducing conditions (Fig. 2A, lane 2). When the 380-kDa fraction that had been obtained by SDS–PAGE under non-

### Table 1

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (mM AMC/min)</th>
<th>Specific activity (mM AMC/min/mg)</th>
<th>Recovery (%)</th>
<th>Purification (fold)</th>
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<tr>
<td>Sperm extract</td>
<td>8.8</td>
<td>532.4</td>
<td>60.5</td>
<td>(100)</td>
<td>(1.0)</td>
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<tr>
<td>SBA-agarose</td>
<td>0.99</td>
<td>270.8</td>
<td>272.3</td>
<td>50.9</td>
<td>4.5</td>
</tr>
<tr>
<td>Aprotinin-agarose</td>
<td>0.068</td>
<td>43.4</td>
<td>638.2</td>
<td>8.2</td>
<td>10.5</td>
</tr>
<tr>
<td>Mono-Q</td>
<td>0.02</td>
<td>26.4</td>
<td>1691.7</td>
<td>5.0</td>
<td>28.0</td>
</tr>
</tbody>
</table>

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**FIG. 1.** Purification of the sperm protease as described under Materials and Methods. Affinity chromatography of a sperm extract on a SBA-conjugated agarose column (A), affinity chromatography of an aprotinin-conjugated agarose column (B), a Mono-Q anion-exchange column (C), and a Superose 12 gel filtration column (D). Peak activity fractions (horizontal bar) were pooled for the subsequent chromatography. Open square, OD 280 nm; closed square, hydrolytic activity against Boc-Gly-Arg-Arg-MCA. In SBA chromatography, the sperm extract was loaded twice to the same column.
reducing conditions was then reelectrophoresed under reducing conditions, both 100- and 65-kDa bands appeared (Fig. 2B). To determine which band demonstrated enzymatic activity, we subjected the unheated, purified enzyme to SDS-PAGE under reducing conditions (Fig. 3A). Activity was recovered in fractions 17 and 18, which contained the 100-kDa bands of protein (Fig. 3B). These results indicate that the protease is found in a high-molecular-weight complex, but that the 100-kDa protein shows protease (amidase) activity against Boc-Gly-Arg-Arg-MCA.

The substrate specificity of the purified enzyme is summarized in Fig. 4. Amidase preferentially hydrolyzes Boc-Gly-Arg-Arg-MCA and Z-Arg-Arg-MCA (Fig. 4A). A relatively high hydrolytic activity was observed against Boc-Leu-Thr-Arg-MCA, Boc-Phe-Ser-Arg-MCA, Z-Phe-Arg-MCA, Boc-Leu-Gly-Arg-MCA, and Boc-Val-Leu-Lys-MCA. The substrate specificity of the enzyme is quite similar to that of cathepsin B (Fig. 4B). Purified enzyme hydrolyzes Boc-Gly-Arg-Arg-MCA maximally at pH 9.0. The activity recorded exceeded 70% of maximal from pH 7.0 to 9.5, and there is a sharp decline in activity below pH 6.0. NaCl did not affect the enzymatic activity up to 1 M. The enzyme was slightly stimulated in very low concentration of Ca$^{2+}$ (Fig. 5) and was highly inhibited by any of the inhibitors for trypsin, aprotinin, leupeptin, antipain, or p-ABSF, and also by chymostatin, an inhibitor for chymotrypsin (Fig. 5), but was not affected by either E-64 or NEM, inhibitors of thiol proteases. Enzymatic activity increased approximately two-fold in the presence of cysteine or the SH-reagents, 2-ME and DTT. CA-074, a specific inhibitor for cathepsin B, did not affect the sperm protease. These results indicate that the sperm protease with amidolytic activity against a peptidyl bond at the carboxyl terminus of double arginine residues (X-Arg-Arg-MCA) is a unique serine protease that
has both trypsic and chymotryptic properties, but which are different from those of cathepsin B.

**Activation of Xenopus Eggs with Purified Cynops Sperm Protease and Cathepsin B**

To determine whether the sperm protease can activate unfertilized Xenopus eggs, we treated dejellied eggs with the purified enzyme (1.5 mM AMC/min/ml against Boc-Gly-Arg-Arg-MCA, 20 µl in 10% SB). All eggs (10/10) demonstrated a positive-going activation potential (Fig. 6A) and underwent cortical contraction. Resumption of meiosis (formation of egg pronuclei) and cortical granule breakdown was confirmed in cytological sections of all eggs. When we treated dejellied Xenopus eggs with cathepsin B (5 U/ml, 4.5 mM AMC/min/ml against Boc-Gly-Arg-Arg-MCA, 20 µl) in 10–100% SB (pH 7.4), all (10/10) demonstrated a positive-going activation potential (Fig. 6B); all underwent cortical contraction, cortical granule breakdown, and formation of egg pronuclei. Cathepsin B (0.5 U/ml) induced activation in 40% (4/10) of the treated eggs, but activation did not occur unless the concentration was more than 0.05 U/ml. The height and duration of positive-going activation potentials induced by the purified enzyme or cathepsin B were similar to those observed in fertilized eggs (Table 2). While hyperpolarization before eliciting positive-going potential was observed at activation by crude sperm extract (Table 2; Iwao et al., 1994), no hyperpolarization before eliciting positive-going potential was detected at activation by the purified enzyme, by cathepsin B, or by Xenopus sperm. Hyperpolarization seems to be caused by a different component in the crude extract from the sperm protease. In contrast, the treatment with trypsin (0.1 mg/ml) did not affect membrane potential (Fig. 6C) and the egg did not undergo activation. Furthermore, cathepsin B (5 U/ml) induced an increase in intracellular Ca^{2+} level (Fig. 6D), whose pattern

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**FIG. 4.** Relative rates of hydrolytic activity against various peptidyl-MCA substrates by the purified sperm protease (A, 30 nM AMC/min against Boc-Gly-Arg-Arg-MCA) and by cathepsin B (B, 90 nM AMC/min against Boc-Gly-Arg-Arg-MCA). Concentration of substrates, 10 µM.
is similar to that observed in fertilized Xenopus eggs (Iwao and Fujimura, 1996). These results indicate that Xenopus eggs can be activated by the sperm protease that preferentially hydrolyzes X-Arg-Arg-MCA accompanied by intracellular Ca\(^{2+}\) release.

**Inhibition of Fertilization in Jellied Eggs by MCA Substrates and Protease Inhibitors**

To determine the role of proteases in the homologous fertilization of Xenopus, we investigated the effects of various peptidyl MCA substrates for proteases. When fully jellied Xenopus eggs were inseminated with Xenopus sperm in 50% SB, more than 95% of the eggs underwent cortical contraction approximately 20 min after insemination and cleaved about 80 min after insemination at 20°C (Figs. 7A and 7B, 0 mM). When unfertilized eggs were inseminated in 1 mM Boc-Gly-Arg-Arg-MCA, cortical contraction was not observed and cleavage did not occur during incubation with the MCA substrate (Fig. 7A and Table 3). Cortical granules in the egg cortex remained intact, and egg nuclei were arrested at the second meiotic metaphase (data not shown). However, of the eggs that had been inseminated in 1 mM Boc-Gly-Arg-Arg-MCA and washed with 10% SB 60 min after insemination, more than 50% underwent cortical contraction within 20 min after washing and cleaved normally approximately 90 min after washing (Fig. 7A). Fifty to one hundred percent of the eggs cleaved after washing the substrate, dependent on experiments. Egg activation after washing was confirmed by the breakdown of cortical granules and by the formation of egg pronuclei, and numerous sperm were observed on the vitelline envelope after washing (data not shown). These results indicate that Boc-Gly-Arg-Arg-MCA reversibly inhibits fertilization of jelled Xenopus eggs. When unfertilized eggs were inseminated in various MCA substrates (Table 3), fertilization was completely inhibited by the addition of 1 mM Boc-Gly-Arg-Arg-MCA or by 1 mM Z-Arg-Arg-MCA. Light microscopy did not reveal any effect of MCA substrates on sperm motility. By contrast, when eggs were inseminated in 1 mM Suc-Gly-Pro-MCA, 78% cleaved normally. Suc-Ala-Pro-Ala-MCA and Arg-MCA did not affect fertilization. These results indicate that a protease that hydrolyzes X-Arg-Arg-MCA is involved in the fertilization of Xenopus.

When unfertilized eggs were inseminated in 8 \(\mu\)M aprotinin, cortical contraction was not observed and cleavage did not occur during incubation with the inhibitor (Fig. 7B and Table 4). The presence of cortical granules and egg nuclei at the second meiotic metaphase was confirmed in the egg cortex by cytological sections; however, when the eggs that had been inseminated in 8 \(\mu\)M aprotinin were washed with 50% SB 60 min after insemination, almost all cleaved normally approximately 90 min after washing (Fig. 7B), indicating that the inhibition of fertilization by the protease inhibitor is reversible. When unfertilized eggs were inseminated in several inhibitors for serine proteases, in particular for trypsin, 0.2 mM leupeptin, 1 mM antipain, and 5 mM p-ABSF completely inhibited fertilization (Table 4). Neither chymostatin, E-64, CA-074, nor bestatin (an inhibitor for aminopeptidases) affected fertilization. These results indicate that a trypsinic serine protease is involved in Xenopus fertilization.

**Inhibition of Fertilization of Denuded Eggs by MCA Substrates and Protease Inhibitors**

When unfertilized, jellied eggs were inseminated in 1 mM Boc-Gly-Arg-Arg-MCA, 1 mM Z-Arg-Arg-MCA, 8 \(\mu\)M aprotinin, or 0.2 mM leupeptin, many sperm were observed either in jelly layers or on the outer surface of vitelline coats, but none was observed in perivitelline spaces. This indicates that the substrates and the inhibitors prevent
FIG. 6. Positive-going activation potentials of dejellied Xenopus eggs induced by the treatment with the purified sperm protease (A, 1.5 mM AMC/min/ml against Boc-Gly-Arg-Arg-MCA, 20 μl) and cathepsin B (B, 5 U/ml, 4.5 mM AMC/min/ml against Boc-Gly-Arg-Arg-MCA, 20 μl). No change in membrane potential of a dejellied egg occurred from the treatment with trypsin (C, 0.1 mg/ml, 1.6 mM AMC/min/ml against Boc-Gly-Arg-Arg-MCA, 20 μl). There was an increase in intracellular free Ca²⁺ level of an aequorin-injected egg in response to cathepsin B (D, 5 U/ml, 4.5 mM AMC/min/ml against Boc-Gly-Arg-Arg-MCA, 20 μl). Arrows, time of treatment.
sperm from passing through the vitelline coats. To determine whether fertilization is blocked by inhibiting sperm from passing through the vitelline coat, eggs whose vitelline coat had been removed were inseminated in a jelly extract containing various MCA substrates (Table 3) or inhibitors (Table 4). Fertilization of denuded eggs was inhibited by 0.1 mM Boc-Gly-Arg-Arg-MCA or 1 mM Z-Arg-Arg-MCA in most of the eggs. Intact cortical granules and egg nuclei that had arrested at the second meiotic metaphase were confirmed in the egg cortex. By contrast, when denuded eggs were inseminated in the presence of 1 mM Suc-Ala-Pro-Ala-MCA, 1 mM Suc-Gly-Pro-MCA, and 1 mM Arg-MCA, 46–78% of the eggs cleaved normally and on time. This suggests that a protease that hydrolyzes X-Arg-Arg-MCA is necessary for fertilization of denuded eggs. Fertilization was completely inhibited when denuded eggs were inseminated in the presence of 8 μM aprotinin, 0.2 mM leupeptin, 1 mM antipain, or 1 mM chymostatin, but not in the presence of either E-64 or bestatin, suggesting that a protease that has both trypsinic and chymotryptic properties is necessary for fertilization in denuded eggs. In addition, when denuded eggs were inseminated with the sperm that had been incubated in egg jelly extract for 10 min, 75% of the eggs were fertilized (n = 20) and cortical contraction was observed 10–15 min after insemination, but fertilization was completely inhibited in the presence of 1 mM Boc-Gly-Arg-Arg-MCA (0%, n = 20) or 8 μM aprotinin (0%, n = 20). Since cortical contraction in fertilization of denuded eggs was detected about 10 min after insemination with sperm in egg jelly extract, and the earliest fertilization responses, intracellular Ca²⁺ increase and elicitation of fertilization potential, occur 3–5 min before detection of cortical contraction (Iwao et al., 1997), the period of pretreatment of sperm with jelly extract appears to be

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Membrane potential at treatment (mV)</th>
<th>Peak of hyperpolarization (mV)</th>
<th>Peak of positive potential (mV)</th>
<th>Duration of positive potential (min)</th>
<th>No. of eggs</th>
</tr>
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<tr>
<td>Insemination with Xenopus sperm</td>
<td>−9 ± 3</td>
<td>−11 ± 4</td>
<td>+37 ± 4</td>
<td>7.0 ± 0.4</td>
<td>10</td>
</tr>
<tr>
<td>Crude Cynops sperm extract</td>
<td>−12 ± 7</td>
<td>−44 ± 5</td>
<td>+32 ± 4</td>
<td>3.5 ± 0.9</td>
<td>10</td>
</tr>
<tr>
<td>Purified Cynops sperm protease (30 nM/min)</td>
<td>−12 ± 4</td>
<td>−12 ± 5</td>
<td>+35 ± 4</td>
<td>7.5 ± 0.6</td>
<td>10</td>
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<tr>
<td>Cathepsin B (90 nM/min)</td>
<td>−10 ± 3</td>
<td>−11 ± 5</td>
<td>+30 ± 5</td>
<td>7.9 ± 0.4</td>
<td>10</td>
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<tr>
<td>Trypsin (32 nM/min)</td>
<td>−9 ± 2</td>
<td>−10 ± 2</td>
<td>−9 ± 3</td>
<td>—</td>
<td>10</td>
</tr>
</tbody>
</table>

Note. In 10% Steinberg’s solution (mean ± SD).

*Activity against Boc-Gly-Arg-Arg-MCA, see Materials and Methods for details.

![FIG. 7.](Image) (A) The rates of first cleavage in jellied Xenopus eggs inseminated with homologous sperm in Boc-Gly-Arg-Arg-MCA. The inseminated eggs were washed with fresh 50% SB 60 min after insemination (arrow). Closed circles, 0 mM; open circles, 0.01 mM; closed squares, 0.1 mM; open squares, 1 mM. (B) The rates of first cleavage in jellied Xenopus eggs inseminated with homologous sperm in aprotinin. The inseminated eggs were washed with fresh 50% SB 60 min after insemination (arrow). Closed circles, 0 M; open circles, 0.08 μM; closed squares, 0.8 μM; open squares, 8 μM.
sufficient to activate the sperm. Adding jelly extract to sperm presumably induces the acrosome reaction, although this has not yet been conclusively demonstrated for X. laevis. These results indicate that the inhibitors also specifically target a protease that is required for egg activation. However, since fertilization of denuded eggs with the sperm which had been treated with egg jelly extract was difficult in the absence of egg jelly extract, further investigation is necessary to clarify the role of egg jelly extract in the fertilization of denuded eggs.

### Proteolytic Activities in Xenopus Sperm

Having demonstrated that egg activation is induced by proteases and that a trypsin protease is involved in Xenopus fertilization, we next used various MCA substrates and inhibitors to further investigate the role of proteases in egg activation.

#### TABLE 3

Inhibition of Fertilization by Various MCA Substrates

<table>
<thead>
<tr>
<th>Treatment</th>
<th>mM</th>
<th>% cleavage</th>
<th>No. of eggs used</th>
<th>% cleavage</th>
<th>No. of eggs used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boc-Gly-Arg-Arg-MCA</td>
<td>1</td>
<td>0</td>
<td>44</td>
<td>4</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>100</td>
<td>25</td>
<td>11</td>
<td>45</td>
</tr>
<tr>
<td>Z-Arg-Arg-MCA</td>
<td>1</td>
<td>0</td>
<td>24</td>
<td>0</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>93</td>
<td>29</td>
<td>43</td>
<td>44</td>
</tr>
<tr>
<td>Boc-Phe-Ser-Arg-MCA</td>
<td>1</td>
<td>100</td>
<td>46</td>
<td>0</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>100</td>
<td>55</td>
<td>65</td>
<td>43</td>
</tr>
<tr>
<td>Suc-Ala-Pro-Ala-MCA</td>
<td>1</td>
<td>100</td>
<td>30</td>
<td>52</td>
<td>48</td>
</tr>
<tr>
<td>Suc-Gly-Pro-MCA</td>
<td>1</td>
<td>78</td>
<td>32</td>
<td>79</td>
<td>61</td>
</tr>
<tr>
<td>Arg-MCA</td>
<td>1</td>
<td>100</td>
<td>27</td>
<td>46</td>
<td>46</td>
</tr>
<tr>
<td>None</td>
<td>1</td>
<td>100</td>
<td>50</td>
<td>78</td>
<td>50</td>
</tr>
</tbody>
</table>

* Number of cleaved eggs was counted at 90 min after insemination.

#### TABLE 4

Inhibition of Fertilization by Various Protease Inhibitors

<table>
<thead>
<tr>
<th>Treatment</th>
<th>mM</th>
<th>% cleavage</th>
<th>No. of eggs used</th>
<th>% cleavage</th>
<th>No. of eggs used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aprotinin</td>
<td>0.008</td>
<td>5</td>
<td>40</td>
<td>0</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>0.0008</td>
<td>94</td>
<td>35</td>
<td>7</td>
<td>42</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>2</td>
<td>0</td>
<td>83</td>
<td>0</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>5</td>
<td>86</td>
<td>0</td>
<td>34</td>
</tr>
<tr>
<td>Antipain</td>
<td>1</td>
<td>0</td>
<td>26</td>
<td>0</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>94</td>
<td>34</td>
<td>22</td>
<td>49</td>
</tr>
<tr>
<td>p-ABSF</td>
<td>5</td>
<td>0</td>
<td>41</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>100</td>
<td>41</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Chymostatin 1</td>
<td>100</td>
<td>20</td>
<td>24</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>E-64</td>
<td>1</td>
<td>100</td>
<td>50</td>
<td>2</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>100</td>
<td>54</td>
<td>79</td>
<td>48</td>
</tr>
<tr>
<td>CA-074</td>
<td>1</td>
<td>100</td>
<td>50</td>
<td>56</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>100</td>
<td>50</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Bestatin</td>
<td>1</td>
<td>100</td>
<td>25</td>
<td>52</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>100</td>
<td>27</td>
<td>74</td>
<td>42</td>
</tr>
<tr>
<td>None</td>
<td>1</td>
<td>100</td>
<td>50</td>
<td>76</td>
<td>50</td>
</tr>
</tbody>
</table>

* Number of cleaved eggs was counted at 90 min after insemination.
strates (Fig. 8) to determine what kinds of hydrolytic activities exist in the Xenopus sperm extract. The sperm extract exhibited relatively higher activity against Boc-Gly-Arg-Arg-MCA, Z-Arg-Arg-MCA, or Arg-MCA. The activity against Arg-MCA was 423% of that against Boc-Gly-Arg-Arg-MCA (170 nM AMC/min/ml). Xenopus extract obtained from 5\times 10^7 sperm had a hydrolytic activity of 0.85 mM AMC/min against Boc-Gly-Arg-Arg-MCA.

**FIG. 8.** Relative rates of hydrolytic activity against various peptidyl-MCA substrates by Xenopus sperm extract which had 0.85 mM AMC/min/ml against Boc-Gly-Arg-Arg-MCA.

DISCUSSION

**Activation of Xenopus Eggs by Cynops Sperm Protease**

In the present study, we used highly purified protease from the sperm of the newt, C. pyrrhogaster. This protease was recovered in a high-molecular-weight fraction (380 kDa), which consisted of a 100- and a 65-kDa protein. The 100-kDa protein contains the protease activity, and its protease complex is evidently a dimeric or trimeric form; however, the 65-kDa protein might be the result of self-degradation. Using various peptidyl-MCA substrates and protease inhibitors, we clarified several biochemical properties. First, it is a trypsin-like protease that hydrolyzes X-Arg-Arg-MCA. Because the sperm protease is also inhibited by chymostatin, an inhibitor of chymotrypsin, it would appear to possess a chymotrypsin-like active center. However, its substrate specificity is quite different from that of chymotrypsin, because it does not hydrolyze Suc-Ala-Ala-Pro-Phe-MCA, a substrate for chymotrypsin. Second, the sperm protease activity is increased by SH reagents; separating the subunits may expose the active domain. Third, the sperm protease preferentially hydrolyzes peptidyl-MCA substrates for cathepsin B, a kind of thiol protease in the lysosome. Furthermore, although the sperm protease and cathepsin B both activate Xenopus eggs, as inhibitors for thiol proteases (E-64 and NEM) do not affect protease activity, this cannot be a thiol protease. CA-074, a specific inhibitor for cathepsin B, does not inhibit the sperm protease, thus, although the protease has substrate specificity similar to that of cathepsin B, its catalytic site is different.

The existence of trypsin-like proteases in sperm has been reported for several species: The ascidian, Halocynthia roretzi, has two trypsin-like proteases, acrosin and spermosin. The spermosin is localized in the sperm head, and the antibody against it inhibits fertilization (Sawada and Someno, 1996). Mammalian sperm have acrosin, a trypsin-like protease in the acrosome (Polakoski and McRorrie, 1973). Acrosin (proacrosin) is associated with the high-molecular-weight acrosomal matrix which is dissociated into lower subunits on reduction (Hardy et al., 1991). In the amphibian B. japonicus, a vitelline coat lysin in the sperm is a trypsin protease (Iwao and Katagiri, 1982; Yamasaki et al., 1988). This lysin is similar to the purified Cynops sperm protease, because the Bufo sperm protease has hydrolytic activity against Boc-Gln-Arg-MCA; a significant difference, however, is that the Bufo vitelline coatlysin has no hydrolytic activity against Z-Arg-Arg-MCA. The sperm of other amphibians, such as Rana pipiens (Elionson, 1971), Bufo arenarum (Cabada et al., 1978), and Leptodactylus chaquensis (Raisman and Cabada, 1977), have trypsin-like proteases that probably function as a vitelline coat lysin. Compared with these proteases, the Cynops sperm protease is unique in that it has trypsinic and chymotryptic properties.
We have demonstrated that the purified Cynops sperm protease activates Xenopus eggs. How does this occur? It is unlikely that the protease causes nonspecific damage to the plasma membrane that induces extracellular Ca$^{2+}$ influx into the egg cytoplasm because the eggs were not activated by other proteases, such as trypsin or chymotrypsin (data not shown), yet were activated by cathepsin B. It appears that the protease could cleave substrates on the extracellular aspect of the egg plasma membrane, but it should not enter the cytoplasm. A Xenopus egg is activated by RGD-sequence-containing peptides, probably by their interaction with receptor molecules on the plasma membrane (Iwao and Fujimura, 1996). Thus, the Xenopus egg appears to be activated by the binding of a sperm agonist (as a ligand) to a receptor on the egg membrane. Several hypotheses exist as to how the sperm protease activates the Xenopus eggs. First, the sperm protease itself may have an agonistic peptide sequence that acts as the ligand for the egg receptor to induce signal transduction for egg activation. Second, the egg receptor may be hydrolyzed by the sperm protease, exposing an agonistic peptide in the receptor; for example, a protease-activated receptor that initiates a G-protein-coupled cascade is activated by cleaving itself with thrombin, a tryptic endoprotease (Vu et al., 1991). Since stimulation of exogenously introduced receptors can activate Xenopus eggs via coupling with G proteins (Kline et al., 1988) that are insensitive to pertussis toxin (Kline et al., 1991), the sperm protease may stimulate hydrolysis of PI$\,\!_2$ to produce IP$\,\!_3$ through G proteins to cause intracellular Ca$^{2+}$ increase at fertilization. Third, the sperm protease may induce interaction of the receptor with lateral integrins, thus triggering the signaling pattern of signal transduction that is known to occur in the interaction between urokinase and its receptor (Andreasen et al., 1997). This third hypothesis is supported by the finding that the RGD-sequence-containing peptide that is a ligand of integrins (Hynes, 1992) causes egg activation accompanied by intracellular Ca$^{2+}$ release (Iwao and Fujimura, 1996). It has been recently found that peptides of sperm metalloprotease/disintegrins inhibit Xenopus fertilization (Shilling et al., 1997) and their local application to unfertilized eggs causes egg activation (Shilling et al., 1998).

**Involvement of Sperm Protease in Xenopus Fertilization**

We have demonstrated that fertilization of Xenopus is inhibited by substrates that contain Arg-Arg-MCA at a carboxyl terminus. This inhibition requires a higher substrate concentration in fully jellied eggs than in denuded eggs; similar results were obtained with protease inhibitors. Fertilization of fully jellied eggs was inhibited by several inhibitors of trypsin, but not by chymostatin, which did, in fact, inhibit fertilization of denuded eggs. The lower sensitivity found in fully jellied eggs may be due to the lower accessibility of the reagents in the jelly layers. Thus, Xenopus fertilization must involve a protease(s) that has both trypsic and chymotryptic properties. This conclusion is supported by the observations that Xenopus sperm extract contains higher amidolytic activity against Gly-Arg-Arg-MCA and that its activity is inhibited by trypsin inhibitors and a chymostatin inhibitor. Assuming localization of the protease in the sperm leads to several possible factors for inhibiting fertilization. Reagents may inhibit: (1) acrosome reaction in sperm, (2) an activity of vitelline coat lyso preventing sperm from passing through the vitelline coat, and/or (3) an interaction between sperm and egg membranes. We did not observe entry of sperm into perivitelline space in the jellied eggs in the presence of the substrates and inhibitors, suggesting that the acrosome reaction may be inhibited. Xenopus sperm has an acrosome cap at the anterior end of the head (Yoshizaki, 1987), but its acrosome reaction remains unclear. Because the sperm of the toad (Bufo bufo japonicus) undergoes acrosome reaction on the surface of the vitelline coat, and because PRE-induced acrosome reaction was inhibited by soybean trypsin inhibitor (Yoshizaki and Katagiri, 1982), a similar protease may be involved in the acrosome reaction of Xenopus sperm.

It is also possible that the activity of vitelline coat lysis is inhibited, resulting in the failure of sperm to pass through the vitelline coat. Bufo sperm has discernable serine protease activity in the acrosome region (Iwao and Katagiri, 1982), and the vitelline coat lysis has a hydrolytic activity against Boc-Gln-Arg-Arg-MCA (Yamasaki et al., 1988). Fertilization of denuded eggs whose vitelline coats have been removed is also inhibited by those same substrates or inhibitors, suggesting that inhibition of fertilization is not caused by inhibition of the vitelline coat lysis.

Finally, despite contact between sperm and egg membranes, membrane fusion and egg activation may be inhibited by substrates or inhibitors. We prefer this possibility because: (1) Cynops sperm fertilize Xenopus eggs and/or cause egg activation (Iwao, 1985), (2) activation by Cynops sperm extract is inhibited by either Boc-Gly-Arg-Arg-MCA or trypsin inhibitors (Iwao et al., 1994), and (3) the Cynops sperm protease that hydrolyzes X-Arg-Arg-MCA can induce egg activation. The substrate specificities of the sperm protease and its responses to inhibitors are both quite similar to those observed in homologous fertilization in Xenopus. Furthermore, since fertilization with the sperm that had been treated with egg jelly extract prior to insemination was blocked by Boc-Gly-Arg-Arg-MCA or aprotinin, a protease seems to be involved in the process of sperm-egg binding/fusion, rather than in the process of interaction between sperm and egg jelly, for example, the acrosome reaction. However, since we know nothing about the induction of the acrosome reaction in Xenopus sperm, further investigation is necessary to clarify the role of sperm protease in...
the sperm acrosome reaction. Fertilization-like responses can also be induced in starfish by some proteases, such as trypsin, chymotrypsin or Pronase (Carroll and Jaffe, 1995). Thus, Xenopus sperm may have a similar protease that cleaves a receptor molecule on egg plasma membrane during normal fertilization.

ACKNOWLEDGMENTS

Cordial thanks from the authors are due to Dr. Chiaki Katagiri for his reading the manuscript and valuable criticisms. This work was supported by the Grant-in-Aid for Scientific Research (C) from The Ministry of Education, Science, Sports, and Culture to Y.I.

REFERENCES


Received for publication June 17, 1998
Revised December 7, 1998
Accepted December 21, 1998