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Characteristics of Sperm Motility Induced on the Egg-Jelly in the Internal Fertilization of the Newt, *Cynops pyrrhogaster*

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ABSTRACT—Most urodeles undergo internal fertilization and sperm are directly inseminated onto the surface of egg-jelly. Feature of sperm motility induced on the egg-jelly was examined in the newt, *Cynops pyrrhogaster*. When sperm were directly inseminated onto an egg-jelly, sperm motility was immediately induced on its surface. The egg-jelly of *C. pyrrhogaster* was composed of six sublayers that were added by turns in oviduct. When the eggs with various sets of the sublayers were obtained and sperm were inseminated onto the egg-jelly, the immediate activity for the initiation of sperm motility was observed only on the outermost sublayer. Similarly, the immediate initiation of sperm motility was induced in the sperm suspended in the extract of the egg-jelly (JE). The initiation of sperm motility was affected by the external pH, and the motility was activated in the moving sperm. A K^+-channel antagonist, charybdotoxin (CTX), or a Ca^{2+}-channel antagonist, gallopamil inhibited the initiation of sperm motility in a dose dependent manner. These results demonstrated the feature of the mechanism regulating sperm motility under stable surroundings in the internal fertilization of amphibians.

Key words: sperm motility, internal fertilization, newt, egg-jelly, ion channel

INTRODUCTION

Sperm motility is significant for achieving fertilization. It is initiated in the sperm ejaculated into the surroundings (Morisawa, 1994). The moving sperm reach an egg through the activation of motility and chemotactic attraction (Morisawa, 1994). Factors for regulating sperm motility are unique to each species and are suited to the environment in which the fertilization occurs (Morisawa, 1994). In the horseshoe crab, *Limulus polyphemus*, sperm are immotile when they are ejaculated. They begin to move by encountering a sperm motility-initiating factor exuded from the egg (Clapper and Brown, 1980). In herring, *Clupea pallasi*, two distinct factors are supposed to act for regulating sperm motility around the eggs (Yanagimachi *et al*., 1992; Morisawa *et al*., 1992). One of the factors is herring sperm-activating peptide (HSAP) that is a soluble peptide and localized in the outermost layer of chorion (Oda *et al*., 1995; Oda *et al*., 1998). The other is sperm motility-initiating factor (SMIF) that is a water-insoluble glycoprotein and localized around micropyle (Yanagimachi *et al*., 1992; Griffin *et al*., 1996). It was reported that nifedipine, a calcium ion channel antagonist, inhibited the effect of SMIF but did not affect that of HSAP (Vines *et al*., 2002). Thus, the two factors affect sperm to induce motility through the distinct signaling cascades.

The change of external osmolality triggers the initiation of sperm motility in many fishes (Morisawa, 1994; Darszon *et al*., 1999, 2001). In carp, sperm begin to move by virtue of a decrease in osmotic pressure. The initiation of sperm motility is inhibited by the treatment of sperm with the K^+-channel antagonist, suggesting that the K^+ efflux is a critical event (Krasznai *et al*., 1995). Some Ca^{2+}-channel antagonists also suppress or inhibit sperm motility, suggesting that the Ca^{2+}-channel participates in the initiation of sperm motility in carp (Krasznai *et al*., 2000).

In amphibians, reproduction occurs in distinct modes, such as external fertilization in many anurans (Katagiri, 1987; Wake and Dickie, 1998) and internal fertilization in most urodeles (Wake and Dickie, 1998; Onitake *et al*., 2000; Itoh *et al*., 2002). The low osmotic pressure in water has been thought to be the key factor for initiating sperm motility in amphibians because sperm have been observed to begin to move in low osmotic solutions (Hardy and Dent, 1986; Inoda and Morisawa, 1987). However, in most urodeles, sperm packed by spermatophore are presented to the female and quiescently stored in spermatheca (Tsutsui, 1931; Wake and Dickie, 1998). Fertilization occurs in the cloaca of the female with the sperm stored for a long duration (Tsutsui, 1931). Thus, it is difficult for the stored sperm

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to be induced by the decrease of osmolality at fertilization.

In newt fertilization, sperm in the spermatheca are directly inseminated onto the egg-jelly. The amphibian egg-jelly is composed of several layers with a stable fibrous-network containing diffusible globular-molecules (Bonnell *et al*., 1996; Watanabe and Onitake, 2002). As in other species, it plays an important role in the sperm-egg interaction (Ishihara *et al*., 1984; Al-Anzi and Chandler, 1998; Ukita *et al*., 1999; Onitake *et al*., 2000; Sasaki *et al*., 2002). In the newt, *Cynops pyrrhogaster*, the egg-jelly is composed of six sublayers, each of which contains unique carbohydrate components (Okimura *et al*., 2001; Watanabe and Onitake, 2002). The sperm motility-inducing substance (SMIS) is contained in the egg-jelly. It may act as the key factor to initiate sperm motility, and some cations in the egg-jelly may be involved in controlling the motility (Ukita *et al*., 1999; Mizuno *et al*., 1999). However, the mechanism for regulating sperm motility in the egg-jelly is not clarified. In the present study, we estimated the involvement of the egg-jelly substances in controlling sperm motility under the physiological environment in the fertilization of *C. pyrrhogaster.*

MATERIALS AND METHODS

Gametes

Mature newts, *Cynops pyrrhogaster*, were collected in Yamagata Prefecture and stored at 4°C until they were used in experiments.

Ovulation was induced by several injections of the gonadotropin (HCG; Teikoku Zoki Inc., Tokyo, Japan) at a dose of 100 IU daily. Mature eggs were surgically obtained from the uterus, which was the most posterior portion of the oviduct. The eggs with every set of jelly sublayers were obtained from more anterior portions of the oviduct.

Dry sperm was obtained by pushing it out from *vas deferens* with forceps. Eggs and dry sperm were kept in the moist chamber until use.

Test solution

The reconstructed ion solution (RIS; 20.0 mM NaCl, 2.66 mM KCl, 0.39 mM $MgSO_4$, 5.06 mM $CaCl_2$, 40mM Choline-Cl, 10mM Tris-HCl; pH7.8 or pH8.5) was prepared according to the estimated concentrations of cations in the egg-jelly of *C. pyrrhogaster* (Ukita *et al*., 1999).

For the preparation of egg-jelly extract (JE), 20 μl of RIS was added to each egg and they were vigorously shaken at 4°C for 1 hr. The eggs were centrifuged at 16000×g at 4°C for 30 min and the supernatant was collected. It was stored at –35°C until use.

In some experiments, 60 mM NaCl or KCl was prepared with 10 mM Tris-HCl (pH8.5) as the monovalent cation solution.

Insemination assay

Egg-jelly with every set of sublayers was mechanically dissected from the eggs. Dry sperm was suspended in a 1000-fold volume of RIS. The egg-jelly was put on a glass slide and placed under a microscope. One-μl of the sperm suspension was directly inseminated onto it. Sperm were observed and recorded with a time-lapse videocassette recorder for 10 min at room temperature. Any sperm that moved more than 50 μm from the inseminated site during a period of 10 min was evaluated as a "motile sperm." Experiments were independently carried out at least twenty times, and more than one hundred sperm were observed under each condition. Then, the inseminated egg-jelly was embedded in O.C.T-compound (Miles Inc., U.S.A) and frozen at –80°C. Sections of 8 μm in thickness were put on a glass slide, and the number of sublayers was estimated in each egg-jelly.

Estimation of sperm motility

One-μl of dry sperm was suspended in 100 μl of test solution. One-μl of the sperm suspension was put on a glass slide, covered with a cover glass and placed under a microscope (IMT-2, Olympus, Tokyo). Sperm motility was observed and recorded with a time-lapse videocassette recorder (AG-6720, Panasonic, Tokyo) at 1, 3, 5, 7 and 10 min after suspending. Experiments were independently carried out at least six times, and more than 20 sperm were observed under each condition. The percentage of motile sperm to total sperm was calculated.

To estimate the activation of motility, the motilities of moving sperm were categorized into four groups. When a part of the undulating membrane was moving, it was categorized as motility index 1 (MI 1). The motility in which the whole undulating membrane was moving gently was categorized as MI 2. When the whole undulating membrane was moving so vigorously that the afterimage of the wave movement was visible, it was categorized as MI 3. Finally, the motility of sperm in which the undulating membrane was moving too vigorously to be visible was categorized as MI 4. Some of the sperm at MI 4 were seen to have a folded tail as a result of the vigorous motility. Those were categorized as MI 4'.

Treatment of sperm with the ion channel antagonist

A K^+-channel antagonist, charybdotoxin (LATOXAN, France) (Castle *et al*., 1989; Sugg *et al*., 1990) was prepared with the test solution at concentrations of 234nM, 23.4nM and 2.34 nM. A Ca^{2+}-channel antagonist, gallopamil (RBI, U.S.A.) (Frank, 1986; Krasznai *et al*., 2000) was prepared with the test solution at concentrations of 1mM, 0.1mM and 0.01mM. One-μl of dry sperm was pretreated with 10 μl of RIS containing the channel antagonist for 3 min at room temperature, and 90 μ of the JE containing the antagonist at the same concentration was added to it.

To estimate the sperm motility on the egg-jelly of the mature eggs, dry sperm was pretreated with 100 μl of RIS containing 1mM gallopamil for 3 min at room temperature. One-μl of the sperm suspension was directly inseminated onto the egg-jelly.

RESULTS

Motility of the sperm inseminated on the egg-jelly

To characterize the sperm motility on the surface of the egg-jelly, the sperm were directly inseminated onto the egg-jelly and the motility was observed in each sperm. Moving sperm were first seen after 5 sec on the egg-jelly (Fig.1), and 68.6% of the sperm began to move within 3 min (Fig. 1, Table 1). The rate of the moving sperm was gradually increased, and 84.5% of the sperm were motile at 10 min. When the sperm were directly inseminated on the surface of the J1, J3 or J4 sublayer, 4.1–13.9% of the sperm were moving within 3 min, and the rate of moving sperm was low until 10 min (Table 1). This result indicates that the activity for the immediate initiation of sperm motility was localized in the outermost sublayer of the egg-jelly. On the other hand, 25.1% of sperm were moving on the surface of J2 sublayer at 10 min (Table 1), suggesting that the weak activity for the initiation of sperm motility existed in the sublayer. However, unlike the surface of the outermost sublayer, the immediate

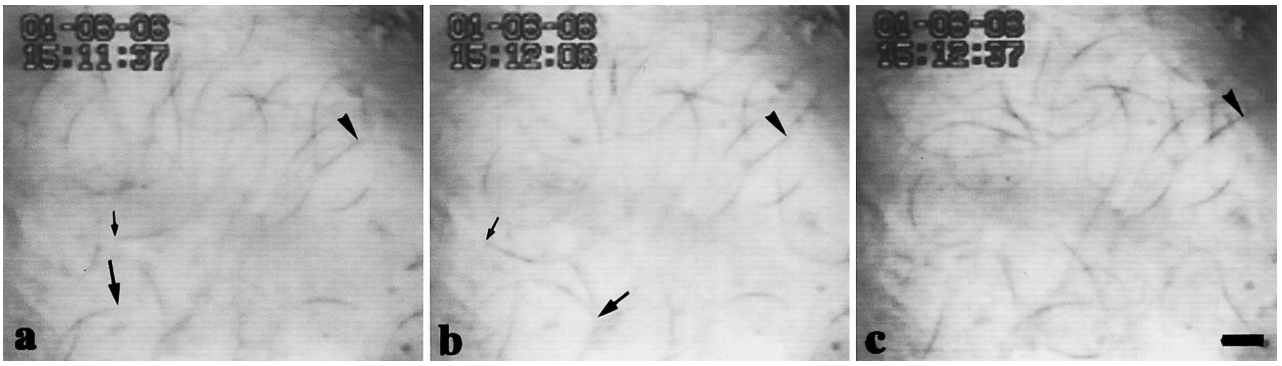

Fig. 1. Moving sperm on the egg-jelly of *C. pyrrhogaster*. One-μl of sperm suspension was directly inseminated onto the egg-jelly, and the sperm motility was recorded with a time-lapse videocassette recorder. (a) shows a view of the sperm on the surface of the outermost sublayer of the egg-jelly. (b) and (c) show the same field as (a) at 30 sec and 60 sec. Arrowheads, large arrows and small arrows indicate the tips of the distinct sperm moving on the egg-jelly. Bar: 100 μm.

Table 1. Sperm motility on the egg-jelly of *C. pyrrhogaster.*

Sublayer	Exp. (n)	No. of total sperm	Time (min)	No. of moving sperm (%±S. E.)
			0 – 3	157 (68.6±5.5)
Full	29	224	3 – 5	26 (11.1±3.2)
			5 – 10	11 (4.8±1.6)
			0 – 3	18 (13.9±4.2)
JO-J4	20	157	3 – 5	7 (3.8±2.0)
			5 – 10	7 (4.2±1.7)
			0 – 3	7 (4.1±2.5)
JO-J3	21	221	3 – 5	3 (1.7±1.4)
			5 – 10	5 (3.7±2.7)
			0 – 3	32 (11.6±3.6)
JO-J2	27	227	3 – 5	27 (11.1±3.2)
			5 – 10	8 (2.4±1.2)
			0 – 3	8 (5.8±2.6)
JO-J1	20	160	3 – 5	7 (4.2±2.2)
			5 – 10	7 (4.1±1.9)
			0 – 3	N. D.
JO	N. D.	N. D.	3 – 5	N. D.
			5 – 10	N. D.

One-μl of sperm suspension was directly inseminated onto the egg-jelly with the various sets of sublayers. Experiments were independently performed at least 20 times, and more than 150 sperm were observed under each condition. Rapid initiation of sperm motility was observed only in the egg-jelly with the full sublayer. Values are means±S.E.

initiation of sperm motility was not observed on the J2 sublayer.

Initiation of sperm motility in the egg-jelly extract (JE)

When the JE was prepared with the reconstructed ion solution (RIS) at the pH 7.8 and 1 μl of dry sperm was added to it, 14.7% of the sperm were moving at 1 min (Fig. 2). The rate of moving sperm was gradually increased, and 54.9% of the sperm were moving at 10 min. When dry sperm was added to the JE at the pH 8.5, 17.3% of the sperm were moving at 1 min (Fig. 2). The rate of moving sperm was immediately increased and 90.8% of the sperm was moving at 10 min. In the control, 7.9% or 5.5% of the sperm was moving in the RISs at the pH 7.8 or 8.5 at 1 min (Fig. 2). The rate of moving sperm remained low for 10 min. These results indicate that sperm motility was immediately induced in the JE as previously reported (Ukita *et al.*, 1999), and that the activity in the JE depends on the external pH.

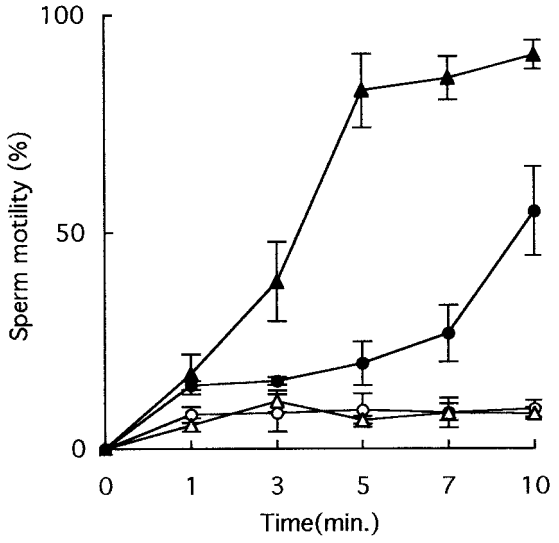

Fig. 2. Initiation of sperm motility of *C. pyrrhogaster* in the JE. One-μl of dry sperm was added to the test solution, and sperm motility was recorded with a time-lapse videocassette recorder. The rate of moving sperm was estimated at 1, 3, 5, 7 and 10 min. Solid circle and solid triangle indicate sperm motility in the JEs at pH 7.8 and 8.5. Open circle and open triangle indicate RISs at pH 7.8 and 8.5, as controls.

Effect of charybdotoxin in the initiation of sperm motility

To investigate the participation of the K^+-channel in the initiation of sperm motility induced by the JE, the sperm

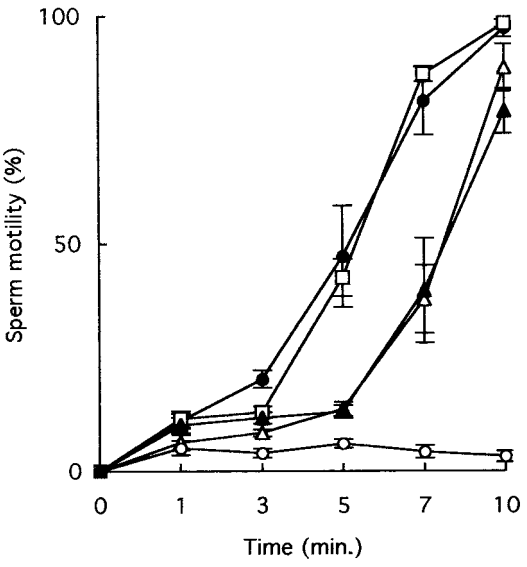

Fig. 3. Effect of charybdotoxin (CTX) on the initiation of sperm motility of *C. pyrrhogaster*. One-μl of dry sperm was pretreated with 10 μl of RIS containing CTX for 3 min at room temperature, and 90 μl of the test solution containing CTX was added to it. The pH of the test solution was adjusted to 8.5, which was the estimated pH on the surface of the egg-jelly. Sperm motility was recorded with a time-lapse videocassette recorder, and the rate of moving sperm was estimated at 1, 3, 5, 7 and 10 min. Sperm motility in the JE containing CTX at 2.34 nM (open square), 23.4 nM (open triangle) and 234 nM (solid triangle) were examined. As a control, JE (solid circle) and RIS (open circle) not containing CTX were examined. The immediate initiation of sperm motility was significantly inhibited. However, most sperm were moving at 10 min.

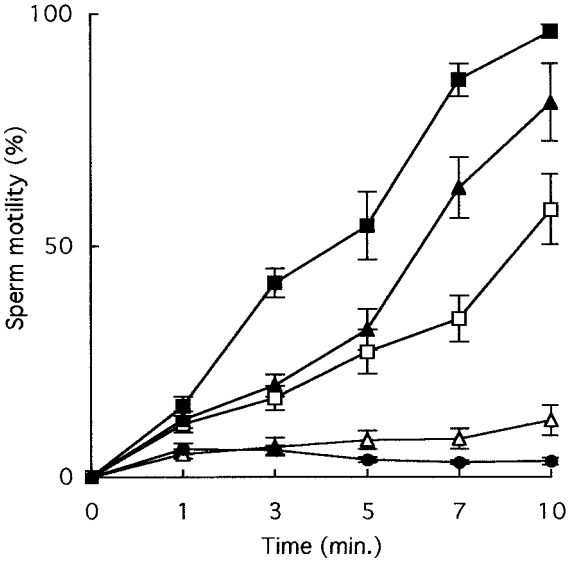

Fig. 4. Effect of gallopamil in the initiation of the sperm motility of *C. pyrrhogaster*. One-μl of dry sperm was pretreated with 10 μl of RIS containing gallopamil for 3 min at room temperature, and 90 μl of test solution containing gallopamil was added to it. The pH of the test solution was adjusted to 8.5, which was the estimated pH on the surface of the egg-jelly. Sperm motility was recorded with a time-lapse videocassette recorder, and the rate of moving sperm was estimated at 1, 3, 5, 7 and 10 min. Sperm motility in the JE containing gallopamil at 1 (open triangle), 0.1 (open square) and 0.01 mM (solid triangle) were examined. As controls, JE (solid square) or RIS (solid circle) without containing gallopamil was examined.

were treated with charybdotoxin (CTX), and the motility was observed.

In the JE containing 2.34nM CTX, 11.5% of the sperm were moving at 1 min, and the rate of moving sperm was gradually increased until 87.2% of the sperm were moving at 7 min (Fig. 3). Whereas, in the JE containing 23.4 nM or 234 nM CTX, 6.3% or 10.2% of the sperm were moving at 1 min, and the rate of moving sperm remained low for 7 min (Fig. 3). In the controls, the rate of moving sperm remained low in RIS for 10 min and it was immediately increased to 81.2% in the JE at 7 min. These results indicate that CTX inhibited the initiation of sperm motility induced by the JE in a dose-dependent manner. However, the rate of moving sperm was increased to 88.6-98.3% at 10 min in each concentration of CTX (Fig. 3).

Effect of gallopamil in the initiation of sperm motility

To investigate the participation of the Ca^{2+}-channel in the initiation of sperm motility induced by the JE, the sperm were treated with gallopamil, and the motility was observed.

In the JE containing 0.01 mM gallopamil, 7.0% of the sperm were moving at 1 min, and the rate of moving sperm was increased to 89.2% at 10 min (Fig. 4). Whereas, in the JE containing 1 mM gallopamil, the rate of moving sperm remained low, and 3.3% of the sperm were moving at 10 min (Fig. 4).

Whereas, when sperm treated with 1 mM gallopamil were directly inseminated onto the egg-jelly, only 7.1% of the sperm were motile within 3 min (Table 2). The rate of moving sperm was less than 10% within 10 min.

Table 2. Motility of gallopamil-treated sperm on the egg-jelly of *Cynops pyrrhogaster*

Pretreatment	Exp. (n)	No. of total sperm	Time (min)	No. of moving sperm (%±S. E.)
1mM gallopamil in RIS	20	158	0 – 3	10 (7.1±2.8)
			3 – 5	2 (1.3±0.9)
			5 – 10	1 (0.5±0.5)
RIS	20	161	0 – 3	119 (68.6±7.2)
			3 – 5	18 (12.2±4.4)
			5 – 10	6 (4.1±2.0)

One-μl of sperm suspension containing 1 mM gallopamil was directly inseminated onto each egg-jelly of the mature egg. Experiments were independently performed 20 times, and more than 150 sperm were observed under each condition. The initiation of motility was severely inhibited in the gallopamil-treated sperm. Values are means±S.E.

Activation of sperm motility in the moving sperm

Motilities of the moving sperm were observed in the JE or the monovalent cation solution. They were classified

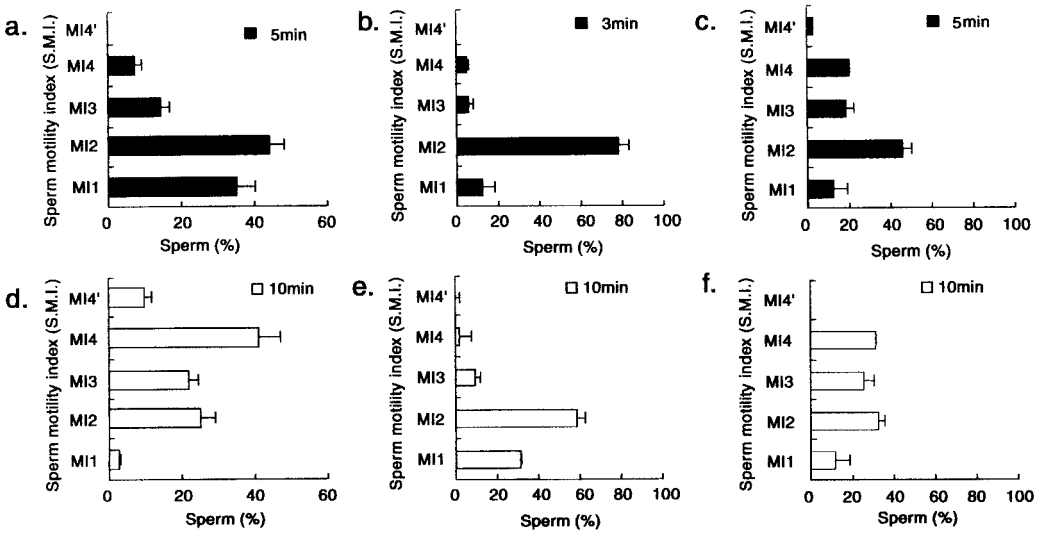

Fig. 5. Activation of motility in moving sperm of *C. pyrrhogaster*. The change of motility of the moving sperm was estimated in test solutions. (a) and (d) indicate sperm motility in the JE at 5 min (a) and 10 min (d). (b) and (e) indicate sperm motility in the 60 mM KCl at 3 min (b) and 10 min (e). (c) and (f) indicate sperm motility in the 60 mM NaCl at 5 min (c) and (f). The motility index (MI) 1 indicates the sperm in which a part of the undulating membrane was moving. MI 2, 3 and 4 indicate the sperm in which the whole undulating membrane was moving weakly, moderately and fully, respectively. MI 4' indicates the sperm whose tails were folded by the too-vigorous movement of the undulating membrane.

according to the motility indices (MIs): that were MI 1 indicating sperm that a part of the undulating membrane was moving, MI 2 indicating those that the whole undulating membrane was moving gently, MI 3 indicating those the whole undulating membrane was moving so vigorously that the afterimage of the wave movement was visible, and MI 4 indicating those that the undulating membrane was moving too vigorously to be visible. The sperm that have a folded tail as a result of the vigorous motility were categorized as MI 4'.

When sperm were suspended in the JE at pH8.5, the motility in the moving sperm was first weak. At 5min, 53.4% of the sperm were moving and many of the moving sperm were at MI 1 or 2 (Fig. 5a). Vigorously moving sperm categorized as MI 4' were not seen. At 10 min, the rate of moving sperm at MI 1 or MI 2 decreased. In contrast, many of the moving sperm was at MI 3 or MI 4, and the moving sperm at MI 4' were seen in 9.5% of them (Fig. 5d).

It is known that a high concentration of K^+ or Na^+ could trigger the initiation of motility in *Cynops* sperm (Ukita *et al*., 1999). When sperm were suspended in the 60 mM KCl at pH8.5, 87.2% of the sperm were moving at 3 min and many of the moving sperm were at MI 1 or MI 2 (Fig. 5b). Vigorously moving sperm categorized as MI 4' were not seen. At 10 min, 84.4% of the total sperm were moving, and many of the moving sperm were at MI 1 or MI 2 (Fig. 5e). The moving sperm at MI 3 or MI 4 were few even at 10 min. Vigorously moving sperm categorized as MI 4' were not seen.

When sperm were suspended in the 60 mM NaCl at pH8.5, the motility in the moving sperm was first weak. At 5 min, 38.5% of the sperm were moving. Many of the moving sperm were at MI 1 or MI 2 (Fig. 5c). No sperm moving at MI 4' were observed. At 10 min, 80.7% of the sperm were moving, and more than 50% of the moving sperm were at MI 3 or MI 4 (Fig. 5f). The moving sperm at MI 4' were seen in 2.6% of them.

DISCUSSION

Sperm motility is controlled in the species-specific manner according to the fertilization environment (Morisawa, 1994). In amphibians whose fertilization occurs in water, the initiation of motility is induced by the decrease of osmolality around sperm (Inoda and Morisawa, 1987). On the other hand, in most urodeles, fertilization is achieved in the cloaca of female (Tsutsui, 1931; Greven, 1998). Sperm are quiescently stored in spermatheca for a long duration and directly inseminated onto an egg at fertilization. We have reported that the egg-jelly extract (JE) contained sperm motility-inducing substance (SMIS) in the newt, *Cynops pyrrhogaster* (Ukita *et al*., 1999).

The SMIS acts as a key factor in the initiation of sperm motility in the JE (Ukita *et al*, 1999). When sperm were added to the JE, most of them began to move within 3 min (Ukita *et al*., 1999; Fig. 2). This immediate initiation is the major feature in the JE-induced sperm motility. In the present study, similar motility was observed in the sperm inseminated onto the egg-jelly (Fig. 1, Table 1), suggesting that SMIS also acts as the key factor for the initiation of sperm motility in the internal fertilization of *C. pyrrhogaster*. The SMIS is a proteinacious factor (Ukita *et al.,* 1999; Onitake *et al*., 2000). The active- and the inactive- forms of SMIS may exist in the egg-jelly (Mizuno *et al*, 1999), suggesting that the inactive form of SMIS is activated at fertilization.

The activity for the immediate initiation of sperm motility was localized in the outermost sublayer of the egg-jelly

(Table 1). The layered composition is widely observed in amphibian egg-jelly (reviewed in Greven 2002) and contributes to the successful fertilization (Omata 1993; Itoh *et al.,* 2002; Sasaki *et al.,* 2002; Watanabe and Onitake 2002). It has been reported that amphibian egg-jelly contained some factors involving in sperm chemotaxis and acrosome reaction (Ishihara *et al.,* 1984; Al-Anzi and Chandler, 1996; Sasaki *et al.,* 2002). In *C. pyrrhogaster*, the activity for the induction of acrosome reaction is also localized in the outermost sublayer. These indicate that amphibian egg-jelly is functionally organized extracellular matrices for achieving the fertilization.

The localization of the activity for the immediate initiation of sperm motility suggests that SMIS specifically acts in species that undergo internal fertilization. Components of each sublayer of the egg-jelly are fundamentally secreted in the specific region of oviduct (Okimura *et al*., 1999; Greven, 2002) and those of the outermost sublayer are secreted in *uterus*. It is reported that the secretion from the *uterus* changed to fit with the mode of viviparous mode in European salamanders (Greven, 1998). It is needed to investigate the secretion in *uterus* in order to clarify the uniqueness of the SMIS in amphibian fertilization.

The weak activity for the initiation of sperm motility was observed in the J2 sublayer (Table 2). It did not cause the immediate initiation of sperm motility, suggesting that the factor inducing sperm motility is distinct between in the J2 sublayer and in the outermost sublayer. It was reported that the J2 sublayers contained a unique conbination of carbohydrate components in the egg-jelly (Okimura *et al*., 2001). Whereas, sperm of *C. pyrrhogaster* began to move by the effect of high pH or monovalent cation at a high concentration (Ukita *et al*., 1999). Thus, the local environment in the J2 sublayer may cause sperm to begin to move. Although this activity does not contribute to the initiation of sperm motility at fertilization, it may have an role in cotrolling sperm motility in the egg-jelly.

The pH in the JE affected the activity to induce sperm motility (Fig. 2). It was known that the external pH strongly affects the initiation of sperm motility in some species (Morisawa *et al*., 1999). In contrast, motility can be induced independently of external pH in urodele sperm when they are suspended in low osmotic solution (Hardy and Dent, 1986). Although the decrease of osmolality does not occur at fertilization in newt, the sperm appear to initiate the motility through the other signaling cascade. The decrease of osmolality is the major cue in lower vertebrates living in freshwater (Morisawa and Suzuki, 1980). Thus, the initiation of sperm motility by the egg-jelly substance is thought to be an additional function that corresponds to the mode of internal fertilization. It is probable that the pH independent signaling cascade might function before the mode of internal fertilization is established in urodele species. Further study is needed to clarify this hypothesis.

In *C. pyrrhogaster*, six kinds of cations are detected in the egg-jelly (Ukita *et al.,* 1999). Among them, K^+, Na^+ and Ca^{2+} may involve in the initiation of sperm motility because each cation affects the sperm motility under the experimental conditions (Ukita *et al.,* 1999). In the present study, a K^+-channel antagonist, CTX, inhibited the initiation of sperm motility in the JE at an early time (Fig. 3). This result suggests that a CTX-sensitive K^+-channel involves in the immediate initiation of sperm motility by the SMIS. In sea urchin, *Strongylocentrotus purpuratus*, a sperm-activating peptide, speract, is released from the egg-jelly and causes K^+ efflux through cGMP-dependent K^+ channels in the sperm (Galindo *et al.,* 2000; Darszon *et al*., 2001). A K^+-channel also involves in the initiation of sperm motility in fresh and sea water fishes (Tanimoto *et al*., 1988; Morisawa, 1994; Krasznai *et al.,* 1995; 2000; Darszon *et al.,* 1999; 2001).

On the other hand, most sperm were moving in the JE in spite of the existence of CTX at 10min (Fig. 3). It is unknown what induces the sperm motility in the later time. It is reported that several factors that can independently induce sperm motility are contained in the egg-jelly (Ukita *et al.,* 1999). Some of them may trigger the signaling cascade that acts independently of the CTX-sensitive K^+-channel. However, it does not act on the egg-jelly because the immediate initiation of sperm motility is induced through the other cascade.

A Ca^{2+}-channel antagonist, gallopamil, inhibited the initiation of sperm motility in the JE (Fig. 4) and on the egg-jelly (Table 2). These results indicate that a gallopamil-sensitive Ca^{2+}-channel plays a crucial role in the initiation of sperm motility by the SMIS. It is known that Ca^{2+} is sustained in the amphibian egg-jelly, which assures the induction of sperm acrosome reaction (Ishihara *et al*., 1984). The sustained Ca^{2+} is significant to assure the initiation of sperm motility as well as the induction of acrosome reaction in the internal fertilization of *C. pyrrhogaster*. In sea urchin, it is suggested that the Ca^{2+} influx occurred through Na^+/H^+ exchanger by speract (Schackman and Chock, 1986; Morisawa, 1994). This activation of the Na^+/H^+ exchanger leads to the intracellular alkalization, which causes the elevation of cAMP, and finally Ca^{2+} influx occurs in the sperm through the Ca^{2+}-channel (Morisawa, 1994). A similar mechanism may cause the initiation of sperm motility in *C. pyrrhogaster*.

In the present study, the motility of moving sperm was activated in the JE (Fig. 5a and 5d). It is suggested that the activation of sperm motility in moving sperm occurs in the egg-jelly at fertilization of *C. pyrrhogaster*. In the K^+ solution, though most sperm immediately began to move, their motility was remained weak (Fig. 5b and 5e). Whereas, in the Na^+ solution, many sperm made vigorous after they began to move (Fig. 5c and 5f). These results suggest that an external environment can control the activation of motility in the moving sperm. It was reported that about 20 mM of Na^+ was contained in the egg-jelly of *C. pyrrhogaster* (Ukita *et al.,* 1999). Although this concentration of Na^+ is not sufficient to induce sperm motility, Na^+ may involve in the activation of sperm motility in egg-jelly at fertilization. It is reported that

the influx of Na^+ into the sperm raises intracellular pH (Darszon *et al*., 2001). Similar event may involve in the activation of motility in the moving sperm of *C. pyrrhogaster*. In ascidians, sperm are activated by the sperm-activating and -attracting factor through a Ca^{2+} -channel (Yoshida *et al.,* 1994; Morisawa *et al.,* 1999). Recently, it is suggested in mice that motility is activated in the moving sperm through the specific Ca^{2+} -channel (Ren *et al.,* 2001). However, the mechanism to activate sperm motility is not known well. To understand the mechanism in the sperm of *C. pyrrhogaster*, it is needed to purify the SMIS and investigate the signaling cascade in details.

Sperm motility is controlled in a species-specific manner and various cues act to induce sperm motility according to the surroundings at fertilization. In amphibians, the mechanism to induce sperm motility has been studied in species that undergo external fertilization. The result of this study demonstrated some features of the mechanism for controlling sperm motility in the internal fertilization of urodeles. From further study about the molecular mechanism in newt sperm, it is expected to understand how the distinct cues can induce the sperm motility in amphibian species.

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