CHAPTER 11

Sperm Activation

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11.1 INTRODUCTION

Fertilization is a sophisticated process for the production of offspring. Successful fertilization requires that sperm respond to various activation signals at the correct time and correct sites. The signals mediate sperm-egg interactions and induce initiation and activation of motility, chemotaxis and the acrosome reaction in sperm. The molecular mechanisms underlying the induction of these events have been reviewed in Ward and Kopf (1993). The sperm-egg interaction is induced at the egg-jelly coat surrounding the egg. Thus, the environment around the egg easily influences it. For example, although sperm-egg interactions are generally induced at the egg-coat, various species have made modifications with respect to the location of these interactions and the extracellular signals involved in them.

Fertilization of amphibians is completed under species-specific conditions (Duellman and Trueb 1994; Wake and Dickie 1998). In most anurans and the ancestral groups of urodeles, females spawn their eggs into water and sperm are ejaculated towards the egg. In those species that undergo external fertilization, sperm begin to move as a result of the decrease of osmolality when they are spawned into water (Hardy and Dent 1986a; Inoda and Morisawa 1987; Morisawa and Morisawa 1990). The change of osmolality is a general cue for the initiation of sperm motility in lower vertebrates living in an aquatic condition (Morisawa and Suzuki 1980; Takai and Morisawa 1995; Morisawa, *et al.* 1999). However, some fishes control sperm motility by other factors (Morisawa *et al.* 1983a, b; Tanimoto and Morisawa 1988; Morisawa *et al.* 1992; Yanagimachi *et al.* 1992; Tanimoto *et al.* 1994). Thus, the initiation of sperm motility is triggered in a species-specific manner. These extracellular

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signals activate the intracellular cascade mediated by cyclic nucleotides and/or Ca^{2+}. The details of this process are reviewed in Morisawa *et al.* (1999).

The sperm acrosome reaction is crucial for successful fertilization in amphibians, as well as in most other animals. This reaction, which is induced in the egg-coat (Picheral 1977; Yoshizaki and Katagiri 1982; Campanella *et al.* 1997; Nakai *et al.* 1999; Ueda *et al.* 2002), causes sperm to bind to the egg envelope (Nakai *et al.* 1999; Ueda *et al.* 2002) and to release the enzymes that dissolve the vitelline envelope (Elinson 1971; Iwao and Katagiri 1982; Cabada *et al.* 1989; Nakai *et al.* 1999), thereby allowing sperm to enter. Though the mechanism that induces the acrosome reaction is unknown in amphibians, some modifications may exist among species. The sperm acrosome reaction is induced by the ZP3 molecule at the zona pellucida in mice (Bleil and Wassarman 1980). In this case, the acrosome reaction causes sperm to release hydrolases from the acrosomal vesicle (Jones *et al.* 1988) and bind to the zona pellucida (Bleil *et al.* 1988; Tanii *et al.* 2001). However, in the musk shrew, *Suncus murinus*, the acrosome vesicle is already shed from the sperm head before the sperm reaches the zona pellucida (Bedford *et al.* 1997a,b). Thus the sperm acrosome reaction is induced in different sites among mammalian species, which raises a question about the role of the sperm acrosome reaction in mammalian species (Bedford 1998).

In this chapter, we focused on the extracellular signals involved in the control of sperm activation in amphibian fertilization, especially in the internal fertilization of urodeles, and we describe the responsiveness of sperm to these signals.

11.2 MATERIALS AND METHODS

11.2.1 Gametes

Mature eggs were surgically removed from the posterior oviduct of female Japanese newts, *Cynops pyrrhogaster*. This aglandular region of the pars convoluta (Greven 1998) is often referred to as the uterus or ovisac, and complete jelly coats cover the mature eggs in this area (Sever *et al.* 1996; Wake and Dickie 1998). To obtain dry sperm, vasa deferentia of males were isolated, and their contents were pushed out from the duct with fine forceps.

11.2.2 Jelly Extract

After adding an aliquot of saline to each mature egg and shaking the eggs at 4 °C for an hour, the eggs were centrifuged, and the supernatant was collected as the jelly extract (JE). Steinberg's salt solution (ST: 58.2 mM NaCl, 0.67 mM KCl, 0.83 mM $MgSO_4$, 0.67 mM Ca $(NO_3)_2$, 3 mM Hepes-NaOH; pH 8.5) and reconstructed salt solution (20 mM NaCl, 2.66 mM KCl, 0.39 mM $MgSO_4$, 5.06 mM Ca $(NO_3)_2$, 3 mM Hepes-NaOH; pH 8.5) were used in the experiment examining sperm motility. A modified Steinberg's salt solution (58.2 mM NaCl, 0.67 mM KCl, 0.83 mM $MgSO_4$, 6 mM Ca $(NO_3)_2$, 3 mM Hepes-NaOH; pH 8.5) was used for the experiment examining the induction of the acrosome reaction.

11.2.3 Cation Concentrations

Egg-jellies were collected from the mature eggs obtained in the uterus of *Cynops pyrrhogaster*. The egg-jellies were baked for 10h at 700 °C and subsequently suspended in distilled water of the estimated volume of the egg-jelly matrices. The cation concentrations were estimated by ICP luminescence analysis. The experiments were independently performed three times and the egg-jellies of 61-86 eggs were used in each experiment.

11.2.4 Sperm Motility

0ne µl of dry sperm was added to the 50 µl of jelly extract prepared with the Steinberg's salt solution or the reconstructed salt solution. One µl of the sperm suspension was then dropped onto a glass slide and mounted with a cover glass. Sperm were observed under a microscope at 1, 3, 5 and 10 min. Sperm in which the undulating membrane was in motion were considered to be moving sperm. Among them, those in which only a portion of the undulating membrane was moving were categorized as having a motility index of 1, and those in which the entire membrane showed visible wave movement were assigned a motility index of 2. In some sperm, the movement was so strong that only the afterimage of the undulating membrane was visible; these sperm were given a motility index of 3. Finally, sperm whose undulating membrane was moving so vigorously that it could not be seen were assigned a motility index of 4. Some of the sperm at 4 were folded in the middle part of the tail because of too vigorous motion and were categorized as 4'.

11.2.5 Sperm Acrosome Reaction

0ne µl of dry sperm was added to 50 µl of the JE prepared with the modified Steinberg's salt solution. The sperm were fixed in 2.5% glutaraldehyde at 0.5, 1, 2, 5 or 15 min intervals, and observed by microscope. Sperm that had lost the acrosomal vesicle on the tip of the sperm head were regarded as acrosome-reacted sperm.

11.2.6 Control of Sperm Motility in the Internal Fertilization of Urodeles

Most urodeles undergo internal fertilization (Greven 1998; Sever 2002). In this process, females receive a spermatophore in which sperm are packed. Sperm in the spermatophore are transported to the spermathecae in the cloaca of females and stored until fertilization (Hardy and Dent 1986b). The duration of storage is sometimes more than a hundred days (Tsutui 1931; Sever 2002). Eggs pass through the oviduct, and sperm in the spermathecae are directly inseminated onto the surface of egg-jelly at the exit of the oviduct. The sperm then begin to move, marking the first step of fertilization, and pass into the egg under environmental conditions of the cloacal cavity. Chemoattraction of sperm, as proposed for the egg-jelly of the external fertilizing frog *Xenopus laevis* (Al-Anzi and Chandler 1998), is unknown in urodeles.

The mechanics of internal fertilization in urodeles suggest that the signal for the initiation of sperm motility is present in the egg-jelly. Amphibian egg-jelly is often composed of several sublayers (Good and Daniel 1943; Katagiri 1965; Humphries 1966; Freeman 1968; Shivars and James 1970; Gusseck and Hedrick 1971; Yurewicz *et al.* 1975; MacLaughlin and Humphries 1978; Carroll *et al.* 1992; Omata 1993; Wake and Dickie 1998, Onitake *et al.* 2000; Fig. 11.1). Each layer has unique carbohydrate components (Bonnell and Chandler 1996; Okimura *et al.* 2001) that accumulate during passage through different regions of the oviduct (Wake and Dickie 1998; Okimura *et al.* 2001). Here, we describe the responsiveness of sperm to the extracellular signals controlling sperm motility using *Cynops pyrrhogaster*.

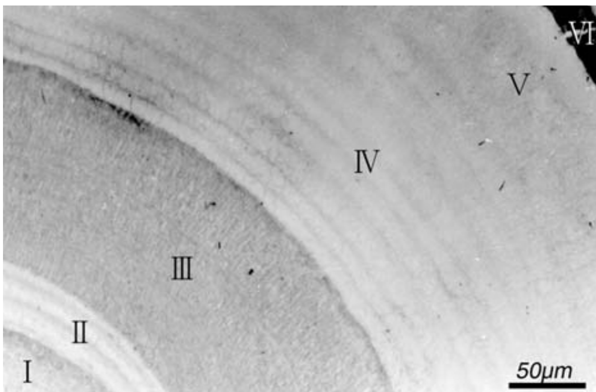

Fig. 11.1 A section of the egg-jelly of *Cynops pyrrhogaster*. A paraffin section of the egg-jelly was stained with hematoxylin and eosin. Six sublayers (I-VI) were identified by morphological features. The innermost and the third layers stained with hematoxylin. The second and fourth layers possess additional lamellae. The fifth layer has a smooth appearance. The outermost layer strongly stains with eosin. After Sasaki et al. 2002. Zygote 10: 1-9, Fig. 2.

11.3 RESULTS AND DISCUSSION

11.3.1 Initiation of Sperm Motility

Cynops pyrrhogaster undergoes the typical style of internal fertilization in urodeles. Its sperm are about 400 µm long (Fig. 11.2) and move forward in the egg-jelly by means of the undulating membrane on the tail region (Ukita *et al.* 1999; Itoh *et al.* 2002). Although only sperm stored in the cloaca of females contribute to fertilization in nature, most sperm in the efferent duct have already acquired the responsiveness to the signals of sperm-egg interaction (Ukita *et al.* 1999; Nakai *et al.* 1999; Sasaki *et al.* 2001). Indeed, they can contribute to successful fertilization when they are directly inseminated onto the surface of egg-jelly (Ukita *et al.* 1999). Thus, sperm of *C. pyrrhogaster* have acquired the capacity for fertilization in the efferent duct.

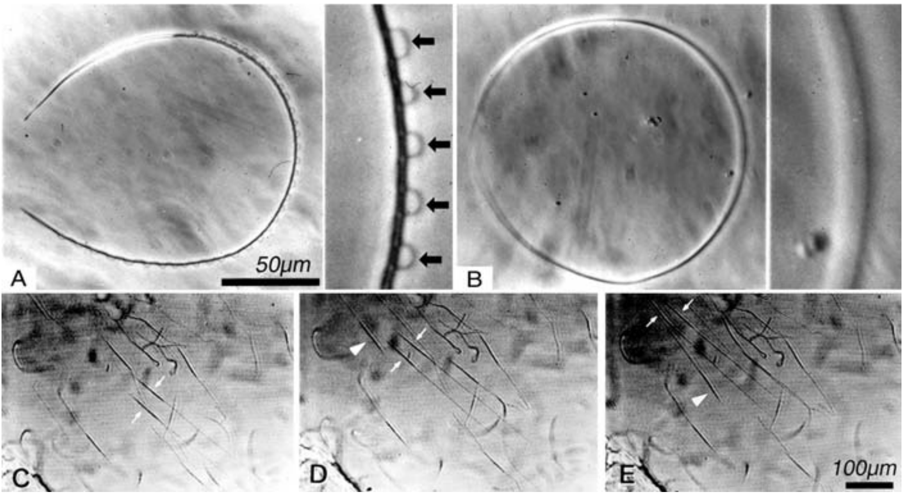

Fig. 11.2 Moving sperm of *Cynops pyrrhogaster*. **A**. Sperm were collected from the vas deferens and suspended in the Steinberg's salt solution. Left column shows the whole figure of a sperm with a head of about 100 μm and a tail region of about 300 μm. Right column shows a high magnification view of the tail region. The undulating membrane (arrows) is visible in the ceased sperm. **B**. A moving sperm in the egg-jelly extract. It moved so vigorously that the undulating membrane is not visible by light microscopy. **C**, **D**, **E**. Sperm showing the forward motility in the egg-jelly. (**D**) and (**E**) show the same view observed after 5 sec of (**C**) and (**D**). Arrows and arrowheads indicate the moving sperm in the egg-jelly. Sperm of *C. pyrrhogaster* move forward without bending. After Ukita et al. 1999. Zoological Science 16: 793-802, Figs. 4 and 5.

Like those of anuran amphibians (Inoda and Morisawa 1987), sperm of *Cynops pyrrhogaster* become motile as a result of a decrease in osmolality. When added to water or solutions at low osmolality, most sperm begin to move within 1 min (Ukita *et al.* 1999; unpublished data; Fig. 11.3). Street (1940) reported that fertilization of *C. pyrrhogaster* eggs was completed within 15 min when sperm were inseminated onto the surface of egg-jelly.

Monovalent cation solutions can induce sperm motility at pH 8.5, which is the estimated pH on the surface of egg-jelly of *Cynops pyrrhogaster* (Ukita *et al.* 1999; Fig. 11.4). When sperm were added to 60 mM Na^+ at pH 8.5 (Ukita *et al.* 1999), the percentage of moving sperm was gradually increased and was greater than 90% at 10 min. When added to 60 mM K^+ at pH 8.5, the percentage was immediately increased, reaching more than 90% in only 3 min. The activity for the initiation of sperm motility, therefore was stronger in the K^+ solution, suggesting that each monovalent cation acts on sperm via a different mechanism. Activity in both Na^+ and K^+ 60 mM solution probably is sufficient for achieving fertilization. However, the natural concentrations of both cations are so low (< 23.8 mM Na^+, <2.7 mM K^+; Table 11.1) in the egg-jelly of *C. pyrrhogaster* that they are probably insufficient to serve as the sole native factors for the initiation of sperm motility.

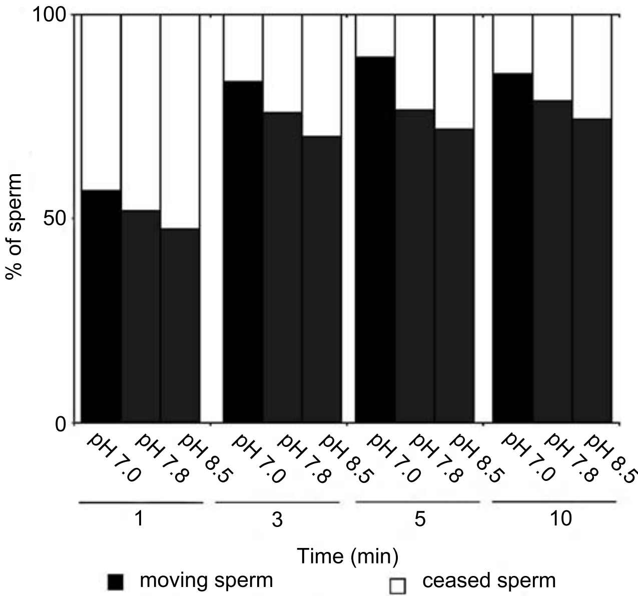

Fig. 11.3 Initiation of sperm motility in water. Sperm collected from the vas deferens were suspended in 10mM Tris-HCl at pH 7.0, 7.8 and 8.5. Sperm motility was observed according to the methods described in the text.

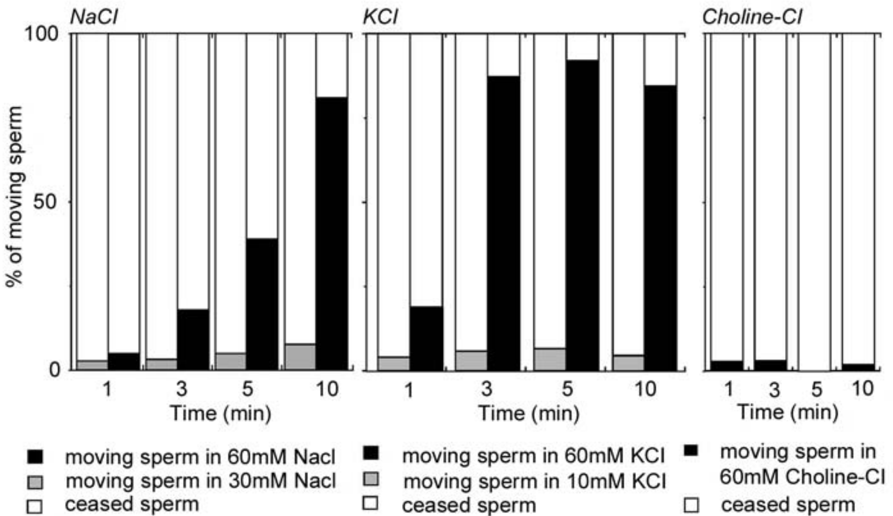

Fig. 11.4 Initiation of sperm motility in the monovalent-ion solution. Sperm collected from the vas deferens were suspended in various concentrations of NaCl or KCl solutions at pH 8.5. As the control, 60 mM chloin-Cl at pH 8.5 was used instead of the monovalent-ion solutions. Sperm motility was observed according to the methods described in the text.

Table 11.1 Cation concentrations in the egg-jelly of *Cynops pyrrhogaster*.

Cation	*Concentration*
Na^+	23.83 ± 6.09
K^+	2.66 ± 1.07
Ca^{2+}	5.06 ± 0.40
Mg^{2+}	0.39 ± 0.70

The bivalent cation Ca^{2+} is also known to be deposited in egg-jelly and to be involved in sperm-egg interactions (Ishihara *et al.* 1984; Shimoda *et al.* 1994). The egg-jelly of *C. pyrrhogaster* contains about 5-6 mM Ca^{2+} (Ukita *et al.* 1999; Table 11.1). In *C. pyrrhogaster*, 6 mM of Ca^{2+} is sufficient to stimulate the initiation of sperm motility by the co-effect with each monovalent cation, unless Ca^{2+} itself induces the sperm motility (Fig. 11.5). Thus, Ca^{2+} in the egg-jelly acts as one of the major factors for controlling sperm motility in fertilization.

Although the monovalent cations present in the egg-jelly are insufficient for inducing the initiation of sperm motility in *Cynops pyrrhogaster*, egg-jelly plays a key role in the initiation of sperm motility in the internal fertilization of urodeles. Most sperm of *C. pyrrhogaster* began to move within 1 min after being suspended in the egg-jelly extract (Ukita *et al.* 1999; Fig. 11.6). This strong activity disappears by digestion with trypsin (Ukita *et al.* 1999), suggesting that egg-jelly contains a proteinacious substance for the induction of sperm motility. The sperm motility inducing substance (SMIS) has a molecular weight of about 50 kDa, and the inactive form has a molecular weight of over 500 kDa (Mizuno *et al.* 1999). The activation process is unique among the known systems for the initiation of sperm motility. The sperm head of *C. pyrrhogaster* exhibits a protease activity (Iwao *et al.* 1994), and this activity may be involved in the activation of the SMIS.

The SMIS is suggested to be the major factor involved in inducing sperm motility in the fertilization of *Cynops pyrrhogaster*. When the egg-jelly extract was prepared using saline containing the estimated concentrations of the cations in egg-jelly and adjusted to pH 8.5, most sperm began to move within 3 min (Fig. 11.7). When suspended only in saline, on the other hand, the percentage of moving sperm remained low even after 10 min. When sperm were placed on the surface of egg-jelly, most of them also began to move within 3 min and went into the jelly matrices. Our recent data suggest that the SMIS activity is localized in the sticky layer, the outermost layer of egg-jelly (Watanabe *et al.*, 2003). This localization is reasonable for the initiation of sperm motility in the internal fertilization of the newt.

The initiation of sperm motility is controlled by various factors. An increase or decrease of osmolality triggers the sperm motility in many fishes living in sea or fresh water when the sperm are ejaculated (Morisawa *et al.* 1999). In the Salmonidae, the dilution of K^+ around sperm triggers the initiation of their motility when they are ejaculated into water (Morisawa *et al.* 1983a; Tanimoto

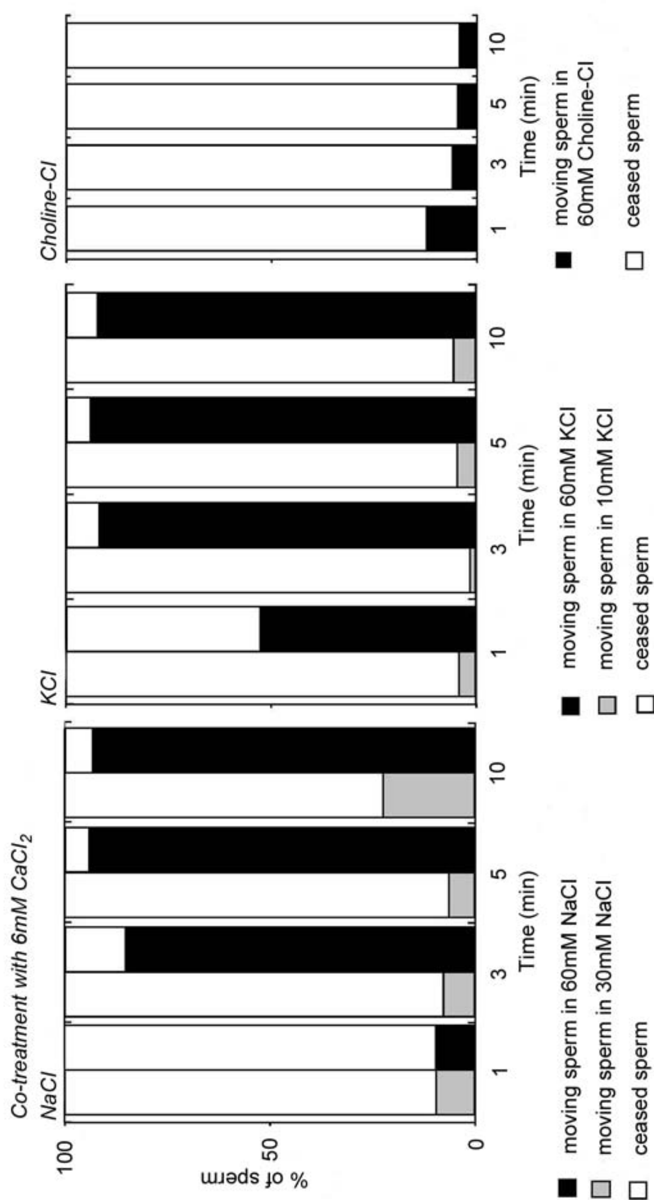

Fig. 11.5 Initiation of sperm motility in the monovalent-ion solutions with addition of calcium ion. Sperm collected from the vas deferens were suspended in various concentrations of NaCl or KCl solutions containing 6 mM $CaCl_2$ at pH 8.5. As the control, 60 mM chloin-Cl containing 6 mM $CaCl_2$ at pH 8.5 was used instead of the monovalent-ion solutions. Sperm motility was observed according to the methods described in the text.

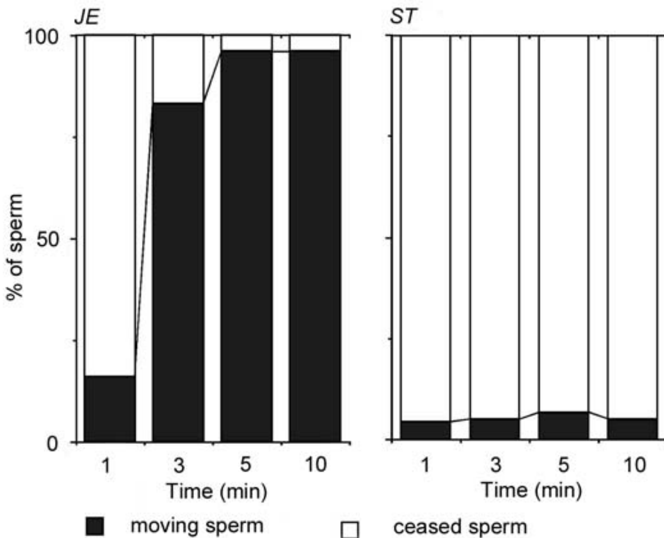

Fig. 11.6 Initiation of sperm motility in the jelly extract. The egg-jelly extract was prepared with the Steinberg's salt solution (pH 8.5) of the same volume as that of the egg-jelly. Sperm collected from the vas deferens were suspended in the egg-jelly extract. As the control, the Steinberg's salt solution was used instead of the egg-jelly extract. Sperm motility was observed according to the methods described in the text.

and Morisawa 1988). Additional substances derived from the egg are involved in the initiation of sperm motility in other species (Clapper and Brown 1980, 1982; Morisawa *et al.* 1992). These findings indicate that the factors that externally control the initiation of sperm motility are not conserved, probably because they vary according to the fertilization mode of each species. Although the motility of sperm of *C. pyrrhogaster* is induced by SMIS, changes in the osmotic environment or the concentration of monovalent cations can induce the motility independently of SMIS. This variation may reflect the ability of *C. pyrrhogaster* to adapt fertilization to different conditions.

11.3.2 Activation of Sperm Motility

The sperm of *Cynops pyrrhogaster* have a long tail. Although the tail is kept straight in moving sperm (Fig. 11.2), the undulating membrane creates a vigorous wave movement. When sperm were added to an egg-jelly extract prepared with the Steinberg's salt solution, the undulating membrane immediately began to move in most sperm (Figs. 11.6, 11.8).

The activation of sperm motility was also seen in the moving sperm of *Cynops pyrrhogaster* in 60 mM Na^+ at pH 8.5 (Figs. 11.4, 11.9). Under this condition, about 40% of sperm were moving within 5 min, and more than 50% of them showed a motility index of 1 or 2. At 10 min, 50% of them showed more vigorous motility. Sperm with a motility index of 4′ also appeared in this

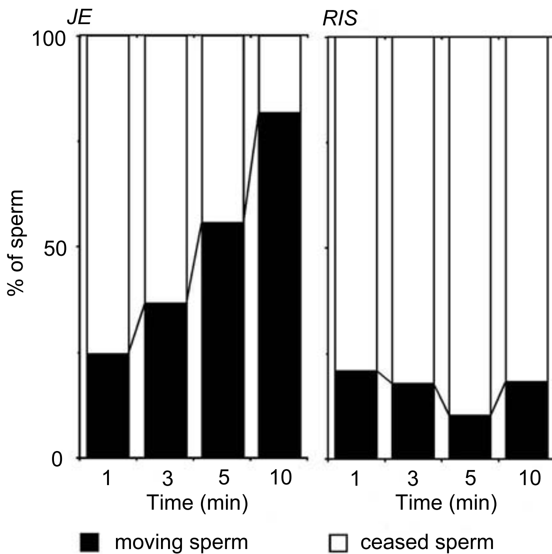

Fig. 11.7 Initiation of sperm motility in the jelly extract prepared with reconstructed ionic solution. The reconstructed ionic solution was prepared based on the estimated concentrations of cations in the egg-jelly of *Cynops pyrrhogaster* and adjusted at pH 8.5. The egg-jelly extract was prepared with the ionic solution of the same volume as that of egg-jelly. Sperm collected from vas deferens were suspended in the egg-jelly extract. As the control, the reconstructed ionic solution was used instead of the egg-jelly extract. Sperm motility was observed according to the methods described in the text.

period. In contrast, the motility of sperm was not as vigorous in 60 mM K^+ at pH 8.5 (Fig. 11.4, 11.9). At 3 min after adding the K^+ solution, more than 90% of sperm were already moving and more than 80% of sperm showed a motility index of 1 or 2. At 10 min, the percentage of moving sperm remained high but that of sperm with a motility index of 1 or 2 also remained high. This difference supports the hypothesis that those cations acts on sperm via a different mechanism to control their motility.

Interestingly, the percentage of sperm classified as having a motility of 1 or 2 also remained high for 10 min in water (Fig. 10). This suggests that the motility of sperm of *Cynops pyrrhogaster* can be controlled in two distinct ways. The first manner of controlling motility is through the decrease of osmolality or high concentration of K^+, and leads to induction of nonvigorous motility. The second way is mediation through the jelly extract or high concentration of Na^+, and leads to induction of vigorous motility.

The extracellular signals for the control of sperm motility are species-specific (Morisawa 1994; Morisawa *et al.* 1999). Although it is unknown why various signals for the control of sperm motility are used among species, the environment around sperm may be one of the mediators in the selection of a

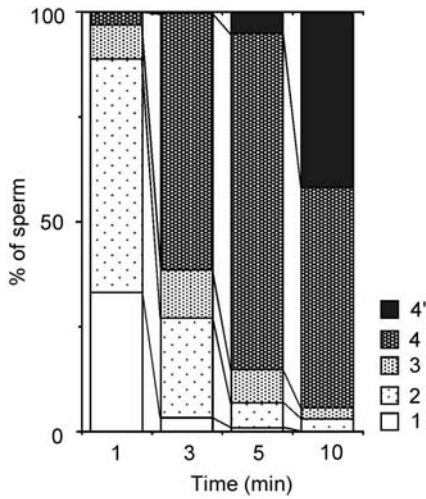

Fig. 11.8 Activation of sperm motility in the jelly extract. The egg-jelly extract was prepared with the Steinberg's salt solution (pH 8.5) of the same volume as that of egg-jelly. Sperm collected from the vas deferens were suspended in the egg-jelly extract. As the control, the Steinberg's salt solution was used instead of the egg-jelly extract. Activation of sperm motility was observed according to the method in the text. The larger motility index indicates more vigorous motion of the undulating membrane.

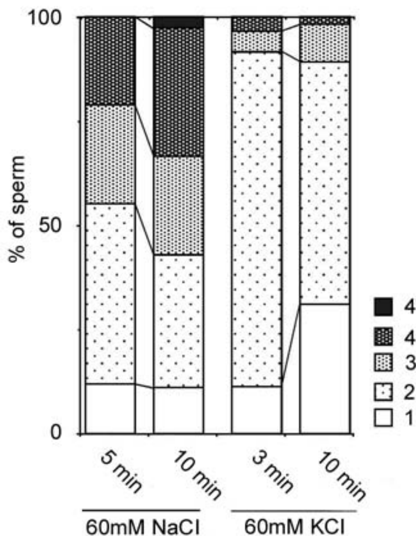

Fig. 11.9 Activation of sperm motility in the monovalent-ion solution. Sperm collected from the vas deferens were suspended in 60 mM NaCl or KCl at pH 8.5. Activation of sperm motility was observed according to the methods described in the text. A larger motility index indicates more vigorous motion of the undulating membrane.

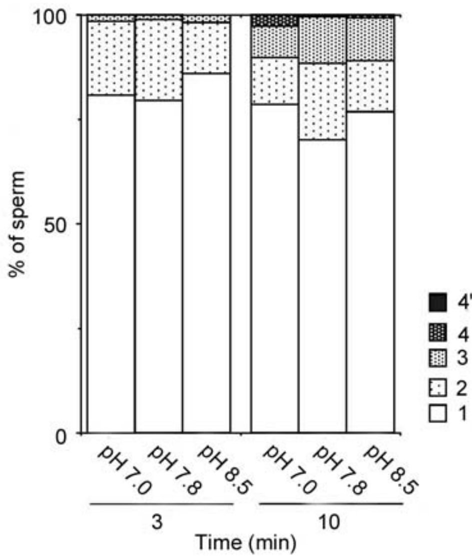

Fig. 11.10 Activation of sperm motility in water. Sperm collected from the vas deferens were suspended in 10mM Tris-HCl at pH 7.0, 7.8 and 8.5. Activation of sperm motility was observed according to the methods described in the text. A larger motility index indicates more vigorous motion of the undulating membrane.

suitable signal for the reproduction of each species. Sperm motility can be controlled artificially by a high concentration of monovalent cation through either of the above mechanisms in *C. pyrrhogaster*. This fact may indicate that the sperm response to motility-controlling extracellular signals is flexible, which would allow species-specific signaling in the fertilization process. Thus, it is an interesting question how these two distinct mechanisms control sperm motility; *i.e.*, whether each signal stimulates distinct cascades or whether each signal stimulates distinct steps of an identical cascade. Unfortunately, we have little information about the intracellular signaling cascades in newt sperm.

In urodeles, the intracellular signaling for controlling sperm motility remains to be clarified. A decrease of osmolality is the signal for the initiation of sperm motility in many freshwater fishes and in the anuran *Xenopus laevis* (Inoda and Morisawa 1987). The hypo-osmotic shock immediately induces membrane permeabilization and reorganization of lipid structure (Márián *et al.* 1993). The efflux of K^+ and the influx of Ca^{2+} occur through channels in the sperm (Krasznai *et al.* 1995; 2000), which leads to the initiation of motility. The increase of osmolality acts in a mirror image on the sperm of many marine fishes (Takai and Morisawa 1995; Morisawa, *et al.* 1999). In these cases, the influx of K^+ occurs through the potassium channel and the concentration of Ca^{2+} is increased, which leads to the initiation of sperm motility (Takai and Morisaw, 1995; Detweiler and Thomas 1998; Morisawa *et al.*, 1999). Thus, the

K^+ flow in the sperm of *C. pyrrhogaster* may trigger the osmolality-induced signaling for the initiation of sperm motility. The high concentration of external K^+ also affects the signaling cascade, which causes the same pattern of sperm motility. On the other hand, intracellular pH may also control the initiation of sperm motility (Morisawa *et al.* 1999). Hypo-osmotic shock induces fast alkalinization through the Na^+/H^+ exchanger in carp sperm (Márián *et al.* 1997). High pH in the carp sperm results in the initiation of sperm motility even in the presence of a low concentration of K^+ (Morisawa *et al.* 1999). A similar event may cause the initiation of sperm motility in *C. pyrrhogaster* induced by the high concentration of Na^+. In the case of sperm treated with the egg-jelly extract, Na^+ in the saline can contribute to the change of intracellular pH, although the concentration of Na^+ is not sufficient to initiate sperm motility.

11.3.4 The Possible Role of Ca^{2+} in the Activation of Sperm Motility

When 6 mM Ca^{2+} was added to the monovalent cation solutions, sperm motility was immediately activated in both cases (Fig. 11.11). In the co-treatment of Na^+ with Ca^{2+}, more than 50% of sperm showed a motility index of 4 within 3 min and more than 90% of sperm showed an index of 4 within 10 min. In the case of K^+, sperm motility was activated in a pattern similar to that for Na^+. Because K^+ itself has little effect on increasing sperm motility (Fig. 11.9), this result indicates that Ca^{2+} has a strong ability to activate the motility in moving sperm. In many species, the increase of intracellular Ca^{2+} is a major event in the initiation of sperm motility. The Ca^{2+} is provided by the influx through the calcium channel and/or the release from the intracellular stock. In the case of *Cynops pyrrhogaster*, the activation of sperm motility is strengthened by the influx of Ca^{2+} (Fig. 11.11), although it is also induced by Na^+ without the external Ca^{2+} (Fig. 11.9). However, the findings suggest that both the extracellular and the intracellular stock of Ca^{2+} can contribute to the activation of sperm motility. The effect of Ca^{2+} on sperm activation has been described well in many animals (Ward and Kopf 1993). In mice, one of the Ca^{2+} channels, CatSper, is specifically localized in the sperm plasma membrane (Ren *et al.* 2001). The knockout mouse of the *CatSper* gene is sterile because the motility does not become vigorous in the moving sperm. In these sperm, the influx of Ca^{2+} is not observed by cyclic AMP, which mediates the activation of sperm motility in mice. A similar mechanism may act in the urodele sperm to specifically cause the activation of sperm motility.

11.3.5 Induction of Sperm Acrosome Reaction

Sperm acrosome reaction is reported to be induced at or near the vitelline envelope in *Bufo bufo japonica* (Yoshizaki and Katagiri 1982). This observation is supported by the reports that a glycoprotein in the egg-jelly maintains the acrosomal vesicle of the sperm of *Bufo arenarum* (Arranz and Cabada 2000), and the components of the vitelline envelope can induce sperm acrosome

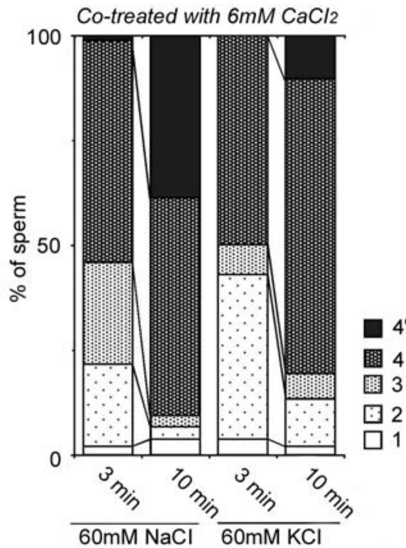

Fig. 11.11 Activation of sperm motility in the monovalent-ion solution with calcium ion. Sperm collected from vas deferens were suspended in 60mM NaCl or KCl containing 6 mM $CaCl_2$ at pH 8.5. Activation of sperm motility was observed according to the methods described in the text. A larger motility index indicates more vigorous motion of the undulating membrane.

reaction (Barisone *et al.* 2002). Ueda *et al.* (2002) suggested induction of the acrosome reaction is founded in the oviductal extract from the pars recta, the anteriormost portion of oviduct in *Xenopus laevis*. Induction depends on external Ca^{2+}, and binding to the vitelline envelope is seen in the acrosome-reacted sperm. The substance to induce the sperm acrosome reaction is supposed to be more than 10 kDa in molecular weight. However, the molecular basis of the induction of sperm acrosome reaction is still unknown.

Sperm of urodeles have large acrosomal vesicles (Picheral 1977; Onitake *et al.* 2000). The sperm acrosome reaction is considered to be induced in egg-jelly unlike the anurans, because sperm lacking an acrosomal vesicle are observed in egg-jelly (Picheral 1977), and most sperm lose the acrosomal vesicle in passing through the egg-jelly (Sasaki *et al.* 2002). The reaction is also induced in sperm treated with the egg-jelly extract (JE) (Takai and Onitake 1990), and these sperm show binding activity to the vitelline envelope of the eggs (Nakai *et al.* 1999). Thus, the sperm acrosome reaction acts not only for digesting but also for binding to the vitelline envelope in the fertilization process of urodeles.

When sperm of *Cynops pyrrhogaster* were treated with the JE, the acrosome reaction occurred in most of them within 1 min (Sasaki *et al.* 2002; Fig. 11.12). The activity for the induction of the sperm acrosome reaction has been detected in a fraction of more than 500 kDa of the JE (Onitake *et al.* 2000; Sasaki

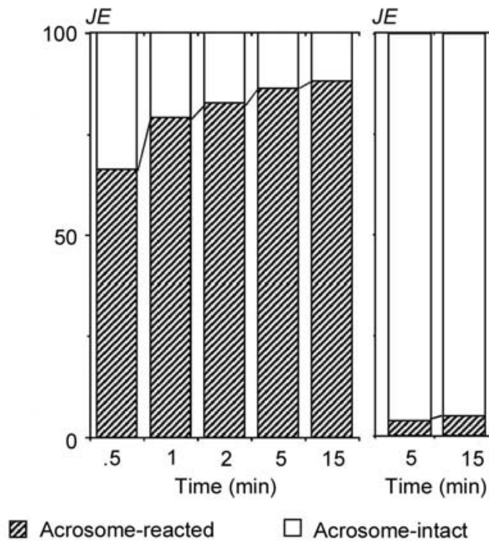

Fig. 11.12 Acrosome reaction in the sperm treated with the jelly extract. The egg-jelly extract was prepared with the Steinberg's salt solution (pH 8.5) of the same volume as that of egg-jelly. As the control, the Steinberg's salt solution was used instead of the egg-jelly extract. Acrosome-reacted sperm were estimated according to the methods described in the text.

et al. 2002). In this fraction, the high molecular weight components that make up the jelly structure are detected by SDS-PAGE. The results suggest that one of the components is responsible for the induction of the sperm acrosome reaction in *C. pyrrhogaster*. It has also been reported that an acrosome reaction-inducing substance of macromolecules exists in the egg-jelly of starfish (Uno and Hoshi 1978; Ikadai and Hoshi 1981; Hoshi *et al.* 1999). The saccharide chain containing the repeating pentasaccharide units of the molecule is crucial for activity. In *C. pyrrhogaster*, the induction of the acrosome reaction is inhibited by some lectins, suggesting that carbohydrate components are involved in the activity (Sasaki *et al.* 2002).

The egg-jelly of *Cynops pyrrhogaster* is composed of six sublayers (Onitake *et al.* 2000; Okimura *et al.* 2001; Fig. 11.1). The activity for the induction of the acrosome reaction is localized in the outermost sublayer (Sasaki *et al.* 2002), suggesting that the acrosome reaction-inducing substance is localized in the sublayer. At fertilization, sperm inseminated onto the surface of egg-jelly are induced to undergo the acrosome reaction and then enter inner portion of the egg-jelly, unlike *Bufo* and *Xenopus*. The acrosome reaction is also thought to be induced in the outer layer of egg-jelly in the primitive frog, *Discoglossus pictus* (Campanella *et al.* 1997). The time at which the acrosome reaction is induced in the fertilization process may be important for achieving a successful fertilization.

The induction of the acrosome reaction depends on the external Ca^{2+} (Takai and Onitake 1990; Onitake *et al.* 2000) and is inhibited by a Ca^{2+} channel blocker (unpublished data). An influx of Ca^{2+} has been shown upon induction of the acrosome reaction in many animals (Ward and Kopf 1993). About 6 mM Ca^{2+} deposited in the egg-jelly of *C. pyrrhogaster* is sufficient to induce the acrosome reaction in sperm (Nakai *et al.* 1999; Ukita *et al.* 1999), suggesting that the egg-jelly also acts as a source of Ca^{2+} to ensure the sperm acrosome reaction.

11.3.7 Responsiveness of Urodele Sperm to the External Signals for Sperm Activation

The sperm of urodeles are rendered quiescent by the effects of polysaccharides in the sperm cap of the spermatophore (Duellman and Trueb 1994). The spermatophore is lodged in the cloaca of female, and sperm are released during degeneration of the cap matrix. Sperm then migrate along the dorsal wall of the cloaca and are stored in the spermathecae (Hardy and Dent 1986b). Sperm remain quiescent in the spermathecae during the storage period (Hardy and Dent 1986a; Duellman and Trueb 1994). In the internal fertilization of urodeles, hundreds of sperm are inseminated onto the surface of egg-jelly. They pass through the egg-coat, where they are affected by several factors that mediate the sperm-egg interaction. Finally, some sperm nuclei enter an egg, because urodele eggs are characterized by physiological polyspermy. The number of sperm nuclei in a spawned egg is less than 15 in many species (Elinson 1986). Thus, the number of sperm that can pass through the egg-coat and contribute to the fertilization is limited. Although it is unknown which sperm participate in the fertilization, the timing of the sperm-egg interaction may be one of the factors involved in excluding the excess sperm at fertilization. Urodele eggs can be used to estimate the varieties of responsiveness of sperm to the external signals for the interaction, because the environment around these eggs is stable at fertilization. Indeed, the eggs can be fertilized by sperm placed directly onto the egg-jelly without immersion in solution (Ukita *et al.* 1999).

The egg-jelly contains six kinds of detectable cations, Na^{+}, K^{+}, Ca^{2+}, Mg^{2+}, Ba^{2+} and St^{2+} (Ukita *et al.* 1999). The first four cations are quantitatively major. When sperm from the vas deferens were added to an ionic solution reconstructed according to the estimated concentrations of those cations and the estimated pH in the egg-jelly (RIS) (Ukita *et al.* 1999), most sperm were actually quiescent (Fig. 11.7). However, when they were added to the egg-jelly extract (JE) prepared with the RIS, the percentage of moving sperm gradually increased and, at 10 min, most of the sperm were moving. Sperm began to move immediately in the JE prepared with Steinberg's salt solution (ST) (Fig. 11.6), suggesting that each sperm has a variety of responses to the external signals for the initiation of sperm motility in the egg-jelly. A variety of responses to those for the activation of sperm motility are also seen (Fig. 11.13). The strength of the waving of the undulating membrane varied at 10 min after sperm were added in the JE-RIS. Such a range of responses to the initiation and

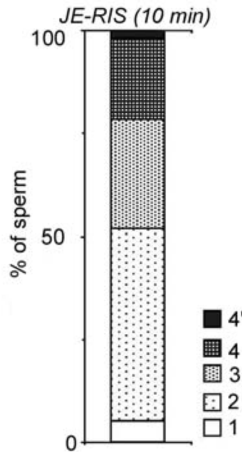

Fig. 11.13 Activation of sperm motility in the jelly extract prepared with reconstructed ionic solution. The reconstructed ionic solution was prepared based on the estimated concentrations of cations in egg-jelly of *Cynops pyrrhogaster* and adjusted at pH 8.5. The egg-jelly extract was prepared with the ionic solution of the same volume as that of egg-jelly. Sperm collected from the vas deferens were suspended in the egg-jelly extract. Activation of sperm motility was observed according to the methods described in the text. A larger motility index indicates more vigorous motion of the undulating membrane.

activation of sperm motility is critical to the fertilization of *Cynops pyrrhogaster*, because fertilization is completed within 15 min after insemination (Ukita *et al.* 1999). On the other hand, the acrosome reaction was induced within 1 min after sperm were added in the JE-RIS (unpublished data). Thus, sperm of *C. pyrrhogaster* are homogeneous in their responsiveness to the external signals for the induction of the acrosome reaction in egg-jelly, whereas they are heterogeneous in their responsiveness to the signals controlling sperm motility. This suggests that the responsiveness to the signals controlling sperm motility in egg-jelly influences the chance for each sperm to contribute to fertilization in nature. Although the molecular basis of the heterogeneous responsiveness in sperm is unknown, it may be caused by a quantitative difference in the ion channels because the motility of most sperm is immediately initiated/activated in the JE-ST (Figs. 11.6, 11.8). In the internal fertilization of urodeles, sperm are quiescently stored in the spermathecae (Hardy and Dent 1986a). After storage, which may last several months or more (Sever 2002), surviving sperm are expelled from the spermathecae onto the egg-jelly surface. The heterogeneous responsiveness of sperm is proposed to be the major factor in the selection of superior sperm to contribute to fertilization.

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