Localization of sperm intracellular Ca^{2+} keeps fertilizability in the newt vas deferens

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Abstract

Sperm intracellular Ca^{2+} is crucial for the induction of sperm-egg interaction, but little is known about the significance of Ca^{2+} maintenance prior to induction. In sperm of the newt *Cynops pyrrhogaster*, intracellular Ca^{2+} is localized to the midpiece during storage in the vas deferens, while extracellular Ca^{2+} is influxed in modified Steinberg's salt solution to promote a spontaneous acrosome reaction related to the decline of sperm quality. In the present study, sperm from the vas deferens were loaded with the Ca^{2+} indicator Fluo8H, and changes in Ca^{2+} localization in modified Steinberg's salt solution were examined. Calcium ions expanded from the cytoplasmic area of the midpiece to the entire tail in most sperm during a 1-h incubation and localized to the principal piece in some sperm within 24 h. Similar changes in Ca^{2+} localization were observed in reconstructed vas deferens solution that included ions and pH at equivalent levels to those in the vas deferens fluid. Sperm with Ca^{2+} localization in the entire tail or the principal piece weakened or lost responsiveness to sperm motility-initiating substances, which trigger sperm motility for fertilization, but responded to a trigger for acrosome reaction. The change in Ca^{2+} localization was delayed and transiently reversed by ethylene glycol tetraacetic acid or a mixture of Ca^{2+} channel blockers including Ni^{2+} and diltiazem. These results suggest that *C. pyrrhogaster* sperm localize intracellular Ca^{2+} to the midpiece through Ca^{2+} transport in the vas deferens to allow for responses to sperm motility-initiating substances.

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Introduction

Sperm intracellular Ca^{2+} plays a major role in the regulation of sperm function, such as capacitation, acrosome reaction and motility (Darszon *et al.* 2006). Ca^{2+} increase is crucial for the induction of each event and occurs through various species-dependent Ca^{2+} -permeable channels and transporters. However, how the intracellular Ca^{2+} is maintained prior to these inductions is generally ignored since it is simply quite low without a specific stimulus in most cell types.

In the internal fertilization of the newt *Cynops pyrrhogaster*, sperm undergo acrosome reaction and initiate motility on the egg jelly in response to acrosome reaction-inducing substance (ARIS) and sperm motility-initiating substance (SMIS), respectively (Watanabe *et al.* 2009, 2010, Yokoe *et al.* 2016). Ca^{2+} influx into sperm occurs in both events through distinct Ca^{2+} -permeable channels, such as the T-type voltage-dependent Ca^{2+} channel, transient receptor potential vanilloid 4 (TRPV4), and N-methyl D-Aspartate-type glutamate receptor (NMDAR) (Hiyoshi *et al.* 2007,

Watanabe *et al.* 2011, Takayama-Watanabe *et al.* 2015, Endo *et al.* 2019). CatSper channels, which are essential for the regulation of sperm motility in mammals and echinoderms (Ren *et al.* 2001, Seifert *et al.* 2015), are suggested to be absent in amphibians including *C. pyrrhogaster* (Cai & Clapham 2008, Watanabe & Takayama-Watanabe 2014). A recent report in *C. pyrrhogaster* found that some sperm stored in the vas deferens spontaneously underwent the acrosome reaction during incubation in modified Steinberg's salt solution (MST) when the Ca^{2+} concentration and pH were adjusted to those in the egg jelly (Kon *et al.* 2017). This phenomenon is influenced by extracellular Ca^{2+} and tends to occur after a longer storage period. Ectopic induction of the acrosome reaction severely decreased the fertilization efficiency (Takahashi *et al.* 2006), which suggests that the spontaneous acrosome reaction correlates with a decline in sperm quality for fertilization. Acrosome-reacted sperm are rarely seen in storage in the vas deferens of healthy males (Kon *et al.* 2017). Therefore, vas deferens secretions likely stabilize sperm intracellular Ca^{2+} before the onset

of fertilization. Intracellular Ca^{2+} is localized to the midpiece of *C. pyrrhogaster* sperm (Watanabe *et al.* 2011). This observation is based on sperm stored in the vas deferens for a short time period, though we recently found that Ca^{2+} was sometimes localized to the principal piece in male newts that were captured at the end of their reproductive season (Fig. 1A and B). This suggests that sperm actually maintain their intracellular Ca^{2+} to localize to the midpiece in the vas deferens in correlation with sperm storage. However, it is unknown why and how intracellular Ca^{2+} is localized in *C. pyrrhogaster* sperm.

In the present study, to show the significance of the localization of sperm intracellular Ca^{2+} for fertilization and the mechanism to maintain it in the vas deferens, we examined the changes in Ca^{2+} localization in sperm during incubation in MST using the Ca^{2+} indicator Fluo8H. The localization of intracellular Ca^{2+} time-dependently changed in MST from the midpiece to the principal piece. Similar changes also occurred in reconstructed vas deferens solution (RVDS), which includes ions and pH at equivalent levels to the vas deferens fluid. In egg jelly extract (JE) containing ARIS and SMIS, sperm with Ca^{2+} localization in the principal piece immediately underwent acrosome reaction but never initiated motility. On the other hand, chelation of extracellular Ca^{2+} or blockage of Ca^{2+} influx transiently delayed and reversed the change in Ca^{2+} localization. These results suggest that the localization of intracellular Ca^{2+} is maintained in the midpiece partly through Ca^{2+} transport in the vas deferens to allow sperm to respond to SMIS and initiate motility at fertilization.

Materials and methods

Gametes

The Committee for Animal Experiments of Yamagata University approved the experimental protocol (No. 29148). Newts were treated under the Guidelines for the Proper Conduct of Animal Experiments in Japan. Adult newts were captured in Yamagata prefecture, Japan in early spring or late autumn (i.e., at the beginning or the end of the reproductive season). Newts were maintained under hibernation conditions at 4°C. Female newts were injected three times daily with 300 IU human chorionic gonadotropin at room temperature to induce ovulation. Jellyed eggs were obtained from the most posterior region of the oviduct termed the uterus. Sperm were collected from the vas deferens fluid by squeezing the vas deferens of mature males. Gametes were stored in the moist chamber.

Chemicals

Nifedipine (a specific blocker of L-type voltage-dependent Ca^{2+} channels; Furukawa *et al.* 2009), NiCl_2 (a selective blocker of Cav3.2; Lee *et al.* 1999), RN1734 (a TRP channel blocker; Vincent *et al.* 2009), MK801 (a NMDAR blocker; Wong *et al.* 1986), diltiazem (a CNG channel blocker; Kolesnikov *et al.* 1990), and A23187 (a Ca^{2+} ionophore; Talbot *et al.* 1976) were used at final concentrations of 100 $\mu\text{mol/L}$ (Nifedipine, NiCl_2 , and RN1734), 500 $\mu\text{mol/L}$ (MK801), 50 $\mu\text{mol/L}$ (diltiazem), and 10 $\mu\text{mol/L}$ (A23187). These chemicals were all purchased from Sigma-Aldrich. Ethylene glycol tetraacetic acid (EGTA; Dojindo Lab., Tokyo, Japan) was used at a final concentration of 10 mmol/L in MST (58.2 mmol/L NaCl, 0.67 mmol/L KCl, 6 mmol/L $\text{Ca}(\text{NO}_3)_2$, 0.83 mmol/L MgSO_4 , 10 mmol/L Tris-HCl: pH8.5) or 2 mmol/L in vas deferens fluid.

Figure 1 Localization patterns of intracellular Ca^{2+} in *C. pyrrhogaster* sperm. (A and B) Typical patterns of Ca^{2+} localization in sperm obtained from the vas deferens of males that were captured in early spring (A) and late autumn (B). (C, D, E, F and G) Ca^{2+} localization in sperm incubated in MST. (C) Sperm with Ca^{2+} localization to the midpiece. (D, E and F) Sperm with Ca^{2+} localization to the entire tail (midpiece and principal piece). (D, E, and F) Sperm with higher Ca^{2+} levels in the midpiece than in the principal piece, equal Ca^{2+} levels between the midpiece and principal piece, and lower Ca^{2+} levels in the midpiece than in the principal piece, respectively. (G) Sperm with Ca^{2+} localization to the principal piece. Arrows indicate the border between the midpiece and the principal piece. Bars: 50 μm (A and B) and 25 μm (C, D, E, F and G).

Ca²⁺ imaging

Sperm were obtained from males that were maintained in hibernation in the laboratory within 2 months or for more than 6 months. A Ca²⁺ indicator, Fluo8H-AM (5 µmol/L) (AAT Bioquest Inc., Sunnyvale, CA, USA), was loaded into sperm in the vas deferens fluid at 17°C for 2 h. In some experiments, a fluorescent dye for the mitochondria, MitoRed (20 µmol/L) (Dojindo Lab., Tokyo, Japan), was coloaded. Sperm were incubated in MST, RVDS (20 mmol/L NaCl, 6 mmol/L Na₂SO₄, 1 mmol/L KCl, 0.1 mmol/L Ca(NO₃)₂, 0.06 mmol/L MgSO₄, 10 mmol/L HEPES–NaOH: pH6.9), or RVDS whose Ca²⁺ concentration and/or pH was raised to 6 mmol/L and/or 8.5 at room temperature for up to 24 h. The effect of a Ca²⁺ ionophore (A23187), EGTA or Ca²⁺-permeable channel blocker was examined by the addition of each chemical to a salt solution. The mixture of Ca²⁺-permeable channels was prepared by the addition of nifedipine, NiCl₂, RN1734, MK801, and diltiazem to MST. In some experiments, a blocker mixture without containing one of the blockers was prepared. Ca²⁺ localization was observed using a confocal laser scanning microscope (488 (Fluo8H) and 561 (MitoRed) nm for excitation) (C2; Nikon Co.) or a fluorescence microscope (ECRIPSE Ti2-E/B, Nikon Co.). Localization of intracellular Ca²⁺ was evaluated in more than 100 sperm. The criteria were as follows: localization to the midpiece and to the entire tail with higher levels in the midpiece, equal levels between the midpiece and the principal piece, and lower levels in the midpiece, and to the principal piece.

Insemination assay

Insemination was performed according to [Takahashi et al. \(2013\)](#). Sperm were obtained from the vas deferens of males that were maintained under hibernation within 2 months or for more than 6 months in the laboratory ([Kon et al. 2017](#)). Sperm were suspended in a 1000-fold volume of MST, and 1 µL of the suspension (approximately 600 sperm) was placed on the surface of the jelly layer of a mature egg. In some experiments, sperm were preincubated in MST for 1 h. The inseminated egg was set in a moist chamber for 10–15 min and then immersed in distilled water. The egg was observed with binoculars 1 day after insemination. Fertilization was evaluated by the presence of a cleaving embryo.

Egg jelly extract

Egg jelly extract was prepared according to [Ukita et al. \(1999\)](#). Briefly, eggs were shaken in MST for 1 h. The supernatant was collected and centrifuged at 20,000 g at 4°C for 30 min. The supernatant was collected as the JE and stored at –25°C until experimental use.

Acrosome reaction

Fluo8H-loaded sperm were incubated in RVDS at room temperature for 1 or 24 h and then incubated in JE for 5 min. The absence of an acrosome was examined in live Fluo8H-loaded sperm simultaneously by clarifying the localization

of intracellular Ca²⁺ using merged fluorescent images and differential interference contrast images under a confocal laser scanning microscope (C2; Nikon Co.). The acrosome reaction was evaluated in more than 50 sperm with Ca²⁺ localized in the midpiece, the principal piece, or the entire tail.

Sperm motility

Fluo8H-loaded sperm were incubated in RVDS at room temperature for 1 or 24 h and then incubated in JE for 5 min. Sperm motility was observed simultaneously with identifying the localization of intracellular Ca²⁺ using merged fluorescent images and differential interference contrast images under a confocal laser scanning microscope (C2; Nikon Co.). The motility of sperm with Ca²⁺ localized in the midpiece, the principal piece, or the entire tail was evaluated in more than 50 sperm. The criteria were as follows: motile-sperm moved forward; undulating (partial) and (whole)-sperm did not move forward, but the undulating membrane, which provides thrust for motility, was undulating in part of the tail or in the entire tail, respectively; and quiescent-sperm neither moved forward nor undulated the undulating membrane.

Statistical analysis

Significant differences were evaluated using Welch's *t*-test ($P < 0.01$ or $P < 0.05$).

Results

Changes in the localization of sperm intracellular Ca²⁺ in MST

The calcium concentration (6 mmol/L) and pH (8.5) of MST used in the present study were adjusted to those in the egg jelly, which makes the sperm promote spontaneous acrosome reactions ([Ukita et al. 1999](#), [Kon et al. 2017](#)). Sperm were obtained from the vas deferens of males that had shown reproductive behaviors in the field and were loaded with Fluo8H. When those sperm were incubated in MST, intracellular Ca²⁺ was localized to the midpiece in 77 ± 4.2% of the sperm at 5 min ([Figs 1 and 2A](#)), similarly to what was previously reported in [Watanabe et al. \(2011\)](#). Some sperm showed Ca²⁺ uniformly in the midpiece and principal piece or in the principal piece alone. Ca²⁺ distribution in part of the principal piece or midpiece in addition to the entire midpiece or principal piece, respectively, and abundantly in the midpiece or principal piece were also observed. Those distribution patterns were regarded as Ca²⁺ localization to the entire tail, with higher levels in the midpiece or principal piece. By observing the image under high magnification, intracellular Ca²⁺ detected by Fluo8H in the midpiece and principal piece was detected in the cytoplasm but not in the mitochondria and their localizing area ([Fig. 3](#)). At 1 h, 70 ± 16% of the sperm showed Ca²⁺ uniformly localized in the entire tail (i.e., the midpiece and principal piece) ([Fig. 2B](#)). Ca²⁺ localization to the

Figure 2 Changes in the localization of sperm intracellular Ca^{2+} in saline. Fluo8H-loaded sperm were incubated in MST (A and B) or RVDS (C and D) for up to 24 h. The localization of intracellular Ca^{2+} was observed using a fluorescence microscope. More than 100 sperm were observed in each experiment and divided into 5 groups according to the Ca^{2+} localization patterns as follows. Intracellular Ca^{2+} was localized to the midpiece (M); the entire tail with higher levels in the midpiece (M>P), equal levels between the midpiece and the principal piece (M=P), or lower levels in the midpiece (M<P); and the principal piece (P). (A and C) 5-min incubation and (B and D) 1–24-h incubation. All data are presented from three independent experiments.

midpiece was observed in only $8.6 \pm 6.1\%$ of sperm. At 24 h, $54 \pm 22\%$ and $23 \pm 17\%$ of the sperm showed Ca^{2+} localization uniformly in the entire tail and the principal piece, respectively. Intracellular Ca^{2+} was not localized in the midpiece in any sperm.

Changes in the localization of sperm intracellular Ca^{2+} in RVDS

Reconstructed vas deferens solution is saline with pH and ion concentrations that are adjusted to those of the vas deferens fluid (Takayama-Watanabe *et al.* 2015). Notably, it contains 0.1 mmol/L Ca^{2+} at pH6.9, which are lower than those levels for MST. When Fluo8H-loaded sperm were incubated in RVDS, fluorescence from the Fluo8H was weaker than that in MST (Fig. 4). The fluorescence was at a similar strength to that in MST when the pH and Ca^{2+} concentration in RVDS were raised to those in MST. Additionally, the different pH between RVDS and MST hardly influenced the fluorescence intensity from the sperm when sperm intracellular Ca^{2+} was saturated by treatment with A23187 (Fig. 5). Thus, the weak fluorescence in RVDS was due to the relatively low pH and Ca^{2+} concentration surrounding sperm. Localization of Ca^{2+} to the midpiece was observed in $76 \pm 9.6\%$ of the sperm in RVDS at 5 min (Fig. 2C). This localization was still observed in $40 \pm 7.4\%$ of sperm at 1 h (Fig. 2D).

Figure 3 Distribution of intracellular Ca^{2+} detected by Fluo8H in the midpiece. (A) Schematic drawing of a cross section of the urodele sperm midpiece. Bold lines indicate the approximate positions observed in (B, C, D and E). (B and C) Merged, longitudinal images of the midpiece and the principal piece of sperm in which a Ca^{2+} indicator (Fluo8H) and a fluorescent dye for mitochondrion (MitoRed) were loaded. (D and E) Isolated images of (C) showing the distributions of Fluo8H-bound Ca^{2+} (D) and MitoRed-bound mitochondria (E). Green: Fluo8H, Red: MitoRed. Bars: 5 μm .

However, sperm with Ca^{2+} localization to the midpiece decreased to $0.75 \pm 0.92\%$ at 4 hours, and $81 \pm 8.1\%$ of the sperm showed Ca^{2+} localization uniformly in the entire tail. At 24 h, $58 \pm 5.1\%$ and $15 \pm 7.9\%$ of the sperm showed Ca^{2+} localization uniformly in the entire tail and to the principal piece, respectively. These results indicate that a specific factor other than ions and pH is needed to maintain the localization of sperm intracellular Ca^{2+} in the vas deferens.

Figure 4 Effects of extracellular Ca²⁺ and pH on sperm intracellular Ca²⁺ levels. Fluo8H-loaded sperm were incubated for 5 min in MST (A), RVDS (B), RVDS in which the pH was raised to that in MST (C), RVDS in which the Ca²⁺ concentration was raised to that in MST (D), and RVDS in which both the pH and Ca²⁺ concentration were raised to those in MST (E). Each photograph was obtained under the same laser intensity and capturing conditions on a confocal laser scanning microscope.

Significance of the localization of sperm intracellular Ca²⁺ to the midpiece

It has been reported that sperm are not newly produced in newts during hibernation because of the cessation of spermatogenesis (Yazawa *et al.* 2003, Watanabe & Takayama-Watanabe 2014, Yokoe *et al.* 2014). The localization of sperm intracellular Ca²⁺ was examined in male newts that were maintained under hibernation for more than 6 months in the laboratory. Calcium localization uniformly in the entire tail was mostly observed in the sperm after 5 min incubation in MST (Table 1). However, fertilization was achieved at a high percentage when an aliquot of suspension that included approximately 600 sperm were used for insemination of an egg by natural fertilization (Takahashi *et al.* 2006) (Table 2). Because the sperm population stored for more than 6 months in the vas deferens included sperm with every pattern of intracellular Ca²⁺ localization (Table 1), we could not deny the possibility that eggs were fertilized with sperm that were in the population and had Ca²⁺ localization to the midpiece. Additionally, Ca²⁺ localization to the midpiece was recovered by treatment of the sperm with JE (Fig. 6), suggesting that a specific substance in the egg jelly promotes Ca²⁺ localization to the midpiece in the sperm and contributes to the success of fertilization in the insemination assay. Next, the responsiveness to extracellular triggers that act during fertilization was examined in sperm with different intracellular Ca²⁺ localization. When Fluo8H-loaded sperm were incubated in RVDS for 1 or 24 h and then suspended in JE containing ARIS and SMIS, most sperm underwent an acrosome reaction regardless of the intracellular Ca²⁺ localization (Fig. 7). In contrast, forward motility was decreased in sperm with Ca²⁺ localization uniformly in the entire tail (Fig. 8). Sperm with Ca²⁺ localization to the principal piece never showed motility. These results clearly indicate that localization of intracellular Ca²⁺ to the midpiece is needed for the initiation of sperm motility in response to SMIS. The fertilization rate decreased when eggs were inseminated with sperm that had been preincubated in MST for 1 h (Table 3), which may support the significance of Ca²⁺ in the midpiece for sperm fertilizability though an event other than the change of Ca²⁺ localization, such as spontaneous acrosome reaction should influence the fertilizability of preincubated sperm.

Delay in the change in sperm intracellular Ca²⁺ by suppression of Ca²⁺ influx

To estimate the contribution of extracellular Ca²⁺ to the changes in Ca²⁺ localization, Fluo8H-loaded sperm were observed in the vas deferens fluid. Fluorescence from Fluo8H was observed in the tail of many sperm, although the amount of Fluo8H incorporated was highly varied among the sperm (Fig. 9). When EGTA was

Figure 5 Effect of extracellular pH on the fluorescent intensity from Fluo8H-loaded sperm. Fluo8H-loaded sperm were incubated for 5 min in A23187 (10 μ mol/L) containing RVDS whose Ca^{2+} concentration was raised to 6 mmol/L (A) or Ca^{2+} concentration and pH were raised to 6 mmol/L and 8.5, respectively (B). Sperm were incubated in RVDS as a control (C). Each photograph was obtained under the same laser intensity and capturing conditions on a confocal laser scanning microscope.

added to the vas deferens fluid, the fluorescence was severely decreased within 5 min. This result suggests that Ca^{2+} influx into sperm maintains the intracellular Ca^{2+} concentration in the vas deferens. However, it was difficult to precisely determine the localization of Ca^{2+} in those sperm because of the high sperm concentration in the vas deferens fluid. Then, sperm were incubated in MST containing EGTA. EGTA also weakened the fluorescence from Fluo8H in each sperm, suggesting that the intracellular Ca^{2+} level was decreased. Intracellular Ca^{2+} was still localized to the midpiece in $58 \pm 24\%$ and $41 \pm 17\%$ of the sperm at 1 and 2 h, respectively (Fig. 10A). However, sperm with Ca^{2+} localization to the midpiece decreased to $7.5 \pm 4.0\%$ at 4 h, and those with Ca^{2+} localization uniformly in the entire tail increased to $84 \pm 6.4\%$. At 24 h, $57 \pm 12\%$ and $20 \pm 7.8\%$ of the sperm showed Ca^{2+} localization uniformly in the entire tail and to the principal piece, respectively. Next, the participation of Ca^{2+} -permeable channels in the changes in the localization of intracellular Ca^{2+} was pharmacologically examined. Ni^{2+} , RN1734, nifedipine, MK801, or diltiazem alone had no effect (Fig. 10B), but a mixture of all the blockers delayed the change in the localization of intracellular Ca^{2+} (Fig. 10C). The blocker mixture did not weaken the fluorescence. These results suggest that multiple types of Ca^{2+} -permeable channels participate in the initial change in the localization of intracellular Ca^{2+} in the sperm.

Reversibility of the change of sperm intracellular Ca^{2+} localization

To examine the reversibility of the changes in sperm Ca^{2+} localization, sperm were incubated in MST for 1 h before EGTA was added to the MST. One hour after EGTA addition, $28 \pm 2.1\%$ of the sperm showed Ca^{2+} localization to the midpiece in contrast to the $2.6 \pm 2.0\%$ of sperm without the addition of EGTA (Fig. 11A).

This localization was also seen in $45 \pm 13\%$ of sperm at 2 h, but it decreased to $2.5 \pm 3.6\%$ at 4 hours. Intracellular Ca^{2+} was uniformly localized in the entire tail in $78 \pm 12\%$ of the sperm at 4 h. At 24 h, $60 \pm 3.9\%$ and $18 \pm 6.5\%$ of the sperm showed Ca^{2+} localization uniformly in the entire tail and to the principal piece, respectively. These results suggest that the intracellular Ca^{2+} that once appeared in the principal piece when the sperm were in MST was transiently decreased by the depletion of extracellular Ca^{2+} . However, the mixture of Ca^{2+} channel blockers did not reverse the change in Ca^{2+} localization (Fig. 11B). This suggests that an additional channel or transporter should be blocked to reverse the change.

Characterization of Ca^{2+} -permeable channels that change Ca^{2+} localization

To further characterize the participation of Ca^{2+} -permeable channels in the changes in Ca^{2+} localization, we selected a sperm population that mostly showed strong Ca^{2+} distribution to the midpiece (Fig. 12). The change in Ca^{2+} localization was also delayed in those sperm by the Ca^{2+} channel blocker mixture (Fig. 12A and C). When diltiazem was removed from the blocker mixture, the change in the Ca^{2+} localization was promoted within 5 min (Fig. 12A). After 1 h, the suppression of the change in Ca^{2+} localization completely disappeared by the removal of Ni^{2+} (Fig. 12C). Finally, the change in the Ca^{2+} localization was suppressed by the copresence of diltiazem and Ni^{2+} (Fig. 12D). Although diltiazem blocks L-type voltage-dependent Ca^{2+} channels (Kraus *et al.* 1998), nifedipine had no effect. Nickel ion also blocks other voltage-dependent Ca^{2+} channels (Adachi-Akahane & Nagao 2000). However, only L-type and T-type voltage-dependent Ca^{2+} channels are suggested to be present in the *C. pyrrhogaster* sperm (Watanabe & Takayama-Watanabe 2014). Thus, the results of the

Table 1 Percentages of sperm with different localization patterns of intracellular Ca^{2+} in correlation to the storage duration in the vas deferens.

	M	M > P	M = P	M < P	P
Exp. 1*	69 ± 4.4	14 ± 6.8	23 ± 2.4	0.70 ± 0.90	0
Exp. 2 [‡]	13 ± 9.7	4.7 ± 3.7	54 ± 1.6	15 ± 7.5	17 ± 8.9

Sperm were stored in the vas deferens within 2 months (*) and more than 6 months ([‡]) before experiments.

Table 2 Fertilization rate in inseminating sperm after the storage in the vas deferens.

Exp.	Eggs (n)	Fertilized n (%)
1-1*	17	10 (59)
1-2*	12	9 (75)
1-3*	26	20 (77)
Total	55	39 (71)
2-1 [#]	16	4 (25)
2-2 [#]	21	16 (76)
2-3 [#]	34	28 (82)
Total	71	48 (68)

Sperm were stored in the vas deferens for less than 2 months (*) and 6 months (#) before experiments.

present study suggest that T-type voltage-dependent Ca²⁺ channels and CNG channels are major participants in changing the Ca²⁺ localization from the midpiece to the principal piece. However, reversing the change in Ca²⁺ localization was not observed by Ca²⁺ channel blocker mixture alone (Fig. 12B and D). This result supports that

Figure 6 Changes in Ca²⁺ localization in the egg jelly extract. Fluo8H-loaded sperm were incubated in MST (black column) or JE (white column) for 5 min. The localization of intracellular Ca²⁺ was observed using a fluorescence microscope. More than 100 sperm were observed in each experiment and divided into 5 groups according to the Ca²⁺ localization patterns as follows. Intracellular Ca²⁺ was localized to the midpiece (M); the entire tail with higher levels in the midpiece (M>P), equal levels between the midpiece and the principal piece (M=P), or lower levels in the midpiece (M<P); and the principal piece (P). Data were obtained using sperm populations in which the initial intracellular Ca²⁺ distribution was mostly observed in the midpiece (A) or the principal piece (B). Asterisks indicate significant differences ($P<0.01$ ** and 0.05 *).

Figure 7 ARIS-inducing acrosome reaction in sperm with different intracellular Ca²⁺ localization. Fluo8H-loaded sperm were incubated in RVDS for 1 or 24 h. Egg jelly extract was added to an aliquot of sperm suspension and incubated for 5 minutes. The acrosome reaction was evaluated in more than 100 sperm under a confocal laser microscope, and the localization of intracellular Ca²⁺ was assessed. The percentage of acrosome reaction was calculated in sperm with Ca²⁺ localization to the midpiece (M), the entire tail with equal levels between the midpiece and the principal piece (M=P), or the principal piece (P). All data are presented from three independent experiments. Asterisks indicate significant differences (** $P<0.01$).

an additional channel or transporter is needed to reverse the change.

Discussion

Sperm storage allows fertilization to be achieved independently of the timing of spermatogenesis and ensures the success of a specific species-dependent reproductive strategy (Neubauer & Wolfner 1999). In *C. pyrrhogaster*, sperm are stored in the vas deferens for months during hibernation. The long storage results in a decline in sperm quality, which is characterized by the increasing potential of spontaneous acrosome reaction observed by 1–24-h incubation in MST (Kon et al. 2017). In the present study, localization of intracellular Ca²⁺ changed from the midpiece to the principal piece during the 1–24-h incubation in MST (Fig. 2). The change in Ca²⁺ localization also occurred in RVDS but was rarely seen in sperm stored in the vas deferens for a relatively short time period (Table 1). These results suggest that Ca²⁺ localization in sperm is maintained in the midpiece in the vas deferens. In the internal fertilization of *C. pyrrhogaster*, sperm stored in the sperm reservoir of females are inseminated on the jelly layer of ovulated eggs, responding to ARIS and SMIS for the induction of acrosome reaction and the initiation of motility, and penetrating the egg jelly. It takes 5–10 min for inseminated sperm to initiate motility and enter the

Figure 8 SMIS-inducing motility in sperm with different intracellular Ca^{2+} localization. Fluo8H-loaded sperm were incubated in MST for 1 (A and B) or 24 h (C and D). Egg jelly extract was added to an aliquot of sperm suspension and incubated for 5 min. Sperm motility was observed in more than 50 sperm under a confocal laser microscope, and the localization of intracellular Ca^{2+} was assessed. (A) Intracellular Ca^{2+} was localized to the midpiece (M). (B and C) Intracellular Ca^{2+} was localized to the entire tail and its level was equal between the midpiece and the principal piece (M=P). (D) Intracellular Ca^{2+} was localized to the principal piece (P). Motile: sperm was moving forward. Undulating (partial) and (whole): sperm did not move forward, but the undulating membrane that provides thrust for motility was undulating in part of the tail and in the entire tail, respectively. Quiescent: sperm neither moved forward nor showed undulation of the undulating membrane. All data are presented from three independent experiments. Asterisks indicate significant differences ($P < 0.01^{**}$ and 0.05^{*}).

jelly layer (Watanabe *et al.* 2010, Takahashi *et al.* 2013). Although Ca^{2+} remained localized to the midpiece in many sperm in saline for at least 5 min (Fig. 2), the activity recovering Ca^{2+} localization to the midpiece was also present in the egg jelly (Fig. 6). These results suggest the significance of maintaining intracellular Ca^{2+} localization prior to the onset of fertilization in *C. pyrrhogaster*. Actually, sperm that did not maintain the localization of intracellular Ca^{2+} in the midpiece completely lost responsiveness to SMIS (Fig. 8). SMIS is a key ligand protein for achieving internal fertilization

Table 3 Fertilization rates in the insemination of the sperm after the incubation in MST.

Exp.	Unincubated		1 h in MST	
	Fertilized (n.) / total (n.)	%	Fertilized (n.) / total (n.)	%
1	7/11	64	0/11	0
2	6/9	67	0/13	0
3	4/7	57	0/11	0
4	5/13	38	0/12	0
5	8/8	100	5/8	63
	Mean	69	Mean	13*

* $P < 0.01$ by Student's *t*-test.

Figure 9 Decrease in intracellular Ca^{2+} in sperm by chelating Ca^{2+} in the vas deferens fluid. EGTA was added to the vas deferens fluid including Fluo8H-loaded sperm, and the fluorescence from the Ca^{2+} indicator was observed after 5 min using a fluorescence microscope. (A) and (B) present the sperm in the vas deferens fluid without (A) or with (B) EGTA. The fluorescence in (A) and (B) was optimized for the sperm with strong fluorescence, although fluorescence was observed in all sperm. Bars: 25 μm .

in *C. pyrrhogaster* (Watanabe *et al.* 2010, Takayama-Watanabe *et al.* 2014). Its receptor is suggested to be localized to the midpiece (Yokoe *et al.* 2016, Sato *et al.* 2017), and the SMIS-induced initiation of sperm motility is delayed by the decrease in intracellular Ca^{2+} (Watanabe *et al.* 2011). Thus, maintaining the role of intracellular Ca^{2+} in the midpiece allows sperm to efficiently initiate motility during the fertilization process.

Although the localization of intracellular Ca^{2+} did not influence the induction of the acrosome reaction by ARIS (Fig. 7), the change in Ca^{2+} localization showed some similarities to the spontaneous acrosome reaction in occurring in MST (Fig. 2), the quickness of the reaction in MST compared to RVDS (Fig. 2), and the dependency on extracellular Ca^{2+} (Fig. 10) (Kon *et al.* 2017). T-type voltage-dependent Ca^{2+} channels and CNG channels were involved in the changes in Ca^{2+} localization (Fig. 12), and the gating of these channels may lead to spontaneous acrosome reaction. Actually, Ni^{2+} and diltiazem independently inhibited the spontaneous acrosome reaction while nifedipine did not (Kon *et al.* 2020). However, the spontaneous acrosome reaction occurs only in a part of the sperm after 1- and 24-h incubation in MST (Kon *et al.* 2017), while most sperm changed their localization of intracellular Ca^{2+} within 1 h (Fig. 2). This suggests that the change in Ca^{2+} localization occurs through normal responses of the Ca^{2+} -permeable channels to a change in extracellular conditions, which leads to the spontaneous acrosome reaction in some sperm that exhibit a decrease in quality for fertilization (Kon *et al.* 2017). Therefore, the maintenance of Ca^{2+} localization additionally suppresses the spontaneous acrosome reaction, which decreases the fertilization efficiency (Takahashi *et al.* 2006).

In the present study, Ca^{2+} visualized by Fluo8H was not in the mitochondria but in the cytoplasm (Fig. 3). That Ca^{2+} appears to be largely influxed because a low Ca^{2+} concentration in the RVDS (Fig. 4) or chelating of extracellular Ca^{2+} remarkably decreased intracellular Ca^{2+} . Efflux of Ca^{2+} is also significant for promoting Ca^{2+} localization to the midpiece since reversal can be caused

Figure 10 Involvement of Ca²⁺ influx in the changes in the localization of sperm intracellular Ca²⁺. Fluo8H-loaded sperm were incubated for up to 24 h in MST containing EGTA (A), a Ca²⁺ channel blocker (B), or the mixture of Ca²⁺ channel blockers (C). Localization of intracellular Ca²⁺ was observed using a fluorescence microscope. More than 100 sperm were observed in each experiment and divided into 5 groups according to the Ca²⁺ localization patterns as follows. Intracellular Ca²⁺ was localized to the midpiece (M); the entire tail with higher levels in the midpiece (M>P), equal levels between the midpiece and the principal piece (M=P), or lower levels in the midpiece (M<P); and the principal piece (P). All data are presented from three independent experiments.

by the depletion of extracellular Ca²⁺. The mitochondria may also contribute to Ca²⁺ localization, which should be examined in future studies.

Sperm intracellular Ca²⁺ was decreased by EGTA even in the vas deferens fluid (Fig. 9), suggesting that sperm promote Ca²⁺ transport through the plasma membrane in the vas deferens. In mammals, the sperm membrane lipid content is changed to influence membrane fluidity

in the epididymis (Gervasi & Visconti 2017). This phenomenon may be essential for the suppression of the acrosome reaction until sperm reach the oviduct. In aves, high concentrations of lactic acid cause cytoplasmic acidification, making sperm quiescent in the sperm storage tubules before the activation of motility (Matsuzaki *et al.* 2015). Sperm storage in the vas deferens of *C. pyrrhogaster* is unique in that sperm maintain their fertilizability by the ion transport through the plasma membrane. The mechanism for maintaining fertilizability appears to consist of two steps. One step is functional for several hours in MST by the modulation of Ca²⁺ transport through multiple types of channels/transporters (Figs 10, 11 and 12). In this step, the distribution of intracellular Ca²⁺ changes between the midpiece and the principal piece. Results of the present study revealed that blockage of Ca²⁺-permeable channels, including T-type voltage-dependent Ca²⁺ channels and CNG channels, was effective for the transient maintenance of Ca²⁺ localization in MST (Fig. 12). Participation of CNG channels may be reasonable because the cAMP levels in *C. pyrrhogaster* sperm are increased by a 5-min incubation in MST (Kon *et al.*

Figure 11 Reversibility of the changes in the distribution of intracellular Ca²⁺ in sperm. Fluo8H-loaded sperm were incubated in MST for 1 h, and EGTA (A) or the mixture of Ca²⁺ channel blockers (B) was added to the MST. The sperm were further incubated for up to 24 h. Localization of intracellular Ca²⁺ was observed using a fluorescence microscope. More than 100 sperm were observed in each experiment and divided into 5 groups according to the localization patterns of Ca²⁺ as follows. Intracellular Ca²⁺ was localized to the midpiece (M); the entire tail with higher levels in the midpiece (M>P), equal levels between the midpiece and the principal piece (M=P), or lower levels in the midpiece (M<P); and the principal piece (P). The time after the addition of EGTA or the mixture of Ca²⁺ channel blockers is expressed in the graph. All data are presented from three independent experiments.

Figure 12 Characterization of Ca^{2+} -permeable channels in sperm participating in the change in Ca^{2+} localization. Fluo8H-loaded sperm were incubated in MST containing the mixture of Ca^{2+} channel blockers without including one of the blockers (A and C), EGTA (B and D), or Ni^{2+} and diltiazem (B and D) for 5 min (A and B) and 1 h (C and D). Localization of intracellular Ca^{2+} was observed using a fluorescence microscope. More than 100 sperm were observed in each experiment and divided into 5 groups according to the Ca^{2+} localization patterns as follows. Intracellular Ca^{2+} was localized to the midpiece (M); the entire tail with higher levels in the midpiece (M>P), equal levels between the midpiece and the principal piece (M=P), or lower levels in the midpiece (M<P); and the principal piece (P). All data are presented from three independent experiments.

2017). The increase in cAMP can promote the gating of T-type voltage-dependent Ca^{2+} channels (Kim *et al.* 2006). Additionally, an unknown channel/transporter is required for the reversal in Ca^{2+} localization (Figs 11 and 12). We have reported that cysteine-rich secretory protein 2 (CRISP2) was expressed in the vas deferens of *C. pyrrhogaster* (Kon *et al.* 2017) as well as in the oviduct that secretes egg jelly components (Watanabe & Takayama-Watanabe 2014). CRISP family proteins possess an ion channel regulatory domain and potentially modulate Ca^{2+} influx through multiple types of Ca^{2+} channels (Roberts *et al.* 2007, Burnett *et al.* 2008). In mammalian sperm, another member of the Crisp family proteins, *Crisp1*, affects hyperactivated motility by blocking the CatSper channel (Ernesto *et al.* 2015). *Crisp2* is a candidate for the modulation of Ca^{2+} influx into the *C. pyrrhogaster* sperm to maintain intracellular Ca^{2+} localization in the vas deferens as well as in the egg jelly. The other step may be fundamentally needed for the modulation of Ca^{2+} transport in the vas deferens, as Ca^{2+} localization finally changed to the principal piece even in the absence of extracellular Ca^{2+} (Fig. 10). A mechanism other than the blockage of Ca^{2+} transport appears to work in this step.

The maintenance of sperm Ca^{2+} localization in the vas deferens of *C. pyrrhogaster* is a novel phenomenon in sperm storage sites. Surprisingly, it can sustain sperm fertilizability for months. Further study focused on the identification of molecules involved in this process will reveal the mechanism to maintain sperm quality for fertilization and may be applicable to fertilizing sperm of other vertebrates as related to sperm storage.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

N M and N S performed experiments and analyzed data. E T-W and A W conceived the study and wrote the paper.

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