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Acclimation of sperm motility apparatus in seawater-acclimated euryhaline tilapia *Oreochromis mossambicus*

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Summary

Euryhaline tilapia *Oreochromis mossambicus* can reproduce in freshwater and in seawater. Regulation of sperm motility appears to be modulated during acclimation of the fish from freshwater to seawater, being independent of extracellular Ca^{2+} in freshwater and dependent on extracellular Ca^{2+} in seawater. In the presence of extracellular Ca^{2+}, sperm of seawater-acclimated tilapia (SWT) showed motility even in a hypertonic environment, whereas sperm of freshwater-acclimated tilapia (FWT) were not motile. The Ca^{2+} indicator, fluo-3, revealed that intracellular Ca^{2+} concentration, $[Ca^{2+}]i$, of SWT sperm increased only in the presence of extracellular Ca^{2+} in hypotonic or hypertonic conditions. Since the increased $[Ca^{2+}]i$ in FWT sperm occurred under hypotonic conditions *via* intracellular Ca^{2+} stores, it is likely that tilapia modulate their source of increasing $[Ca^{2+}]i$ from intracellular stores (in FWT sperm) to extracellular stores (in SWT sperm). Experiments using demembranated sperm revealed that Ca^{2+} is necessary for activation of motility, suggesting that Ca^{2+} plays a key role in motility regulation in SWT sperm. We detected three phosphoproteins associated with the activation of sperm motility. Serine and threonine residues of two proteins of 15 kDa and 18 kDa became dephosphorylated in hypotonic conditions but remained phosphorylated in hypertonic conditions, suggesting that these protein phosphorylations were not only related to motility activation under hypertonic conditions but also resistant to osmotic pressure. The threonine residue(s) of a 41 kDa protein was also phosphorylated in dry sperm, even in FWT sperm in motility-feasible hypotonic conditions. It is likely that acclimation of the motility apparatus is associated with modulation of the flow of Ca^{2+} to increase $[Ca^{2+}]i$ and protein phosphorylation.

Key words: sperm motility, protein phosphorylation, Ca^{2+}, osmolality, tilapia, *Oreochromis mossambicus*.

Introduction

Habitats of teleosts range widely from freshwater to seawater. They have developed a mechanism for homeostasis of the body and reproductive system. Initiation/activation of teleost sperm motility is a critically important component of reproduction. Initiation/activation of motility is triggered by exposure to an environment different from that of seminal plasma, namely hypertonic seawater for sperm of marine teleosts such as puffer fish and hypotonic freshwater for freshwater teleosts such as carp (Morisawa and Suzuki, 1980; Morisawa, 1994). Since the osmotic shock needed for motility activation is opposite in seawater and freshwater, different mechanisms may be involved in the signal transduction that transmits an extracellular stimulus into an intracellular signal.

Euryhaline tilapia *Oreochromis mossambicus* can habituate and reproduce in both freshwater and seawater, so the question arises as to how the sperm of tilapia overcome the difference in osmolality to activate motility in both habitats. We previously reported that sperm of freshwater-acclimated tilapia (FWT) adjust to low osmotic pressure corresponding to freshwater (Morita et al., 2003). FWT sperm exhibit motility only in lower osmolality solutions (<500 mOsm kg^{-1}). Thus, the motility regulatory mechanism of FWT sperm suits a low osmolality environment such as freshwater. However, euryhaline tilapia also reproduce in seawater, where the osmolality is approximately 1000 mOsm kg^{-1}. In addition, physiological studies have shown that seawater-acclimated tilapia (SWT) sperm should suit a high osmolality (Morita and Okuma, 1998; Linhart et al., 2000). SWT must therefore modulate their motility apparatus to suit high osmolality.

In FWT sperm, an increase in intracellular Ca^{2+} ($[Ca^{2+}]i$) is associated with motility activation (Morita et al., 2003). In hypotonic conditions, both motility activation and increased $[Ca^{2+}]i$ occur even when extracellular Ca^{2+} is chelated. However, the increase in Ca^{2+} and motility activation do not occur in conditions more hypertonic than 500 mOsm kg^{-1}, even in the presence of extracellular Ca^{2+}. On the other hand,

SWT sperm must move in hypertonic conditions in order to reproduce in seawater of osmolality >500 mOsm kg^{-1}. If SWT sperm require increased $[Ca^{2+}]i$ for motility activation, the method of increasing $[Ca^{2+}]i$ must be modulated to supply Ca^{2+} in a hypertonic environment.

Protein phosphorylation is also involved in sperm motility activation in many animals. Protein phosphorylation in FWT sperm is observed only in motility-feasible hypotonic conditions associated with the increase in $[Ca^{2+}]i$ (Morita et al., 2003). Therefore, it is also important to investigate if the protein phosphorylation cascades exist in SWT sperm for motility activation in hypertonic conditions.

The aim of the present study was to further our understanding of the sperm motility regulatory mechanism in SWT. We report here that increased $[Ca^{2+}]i$ is also tightly linked with sperm motility activation; however, the flow of Ca^{2+} was modulated. It is likely that influx of extracellular Ca^{2+} also plays a significant role and that subsequent protein phosphorylation cascades are also modulated.

Materials and methods

Chemicals

Biotin-conjugated anti-phosphoserine and anti-phosphothreonine antibodies, extravidin-conjugated horseradish peroxidase, E-64, Pepstatin A and PMSF were purchased from Sigma Chemical Co (St Louis, MO, USA). The enhanced chemiluminescence (ECL) kit was from Amersham Pharmacia Biotech (Buckinghamshire, England). Protein marker and PVDF membrane were from Biorad (California, USA). Chaps and fluo-3 AM were from Dojindo (Kumamoto, Japan). All other chemicals were from Wako Chem. Co (Osaka, Japan).

Fish

All tilapia *Oreochromis mossambicus* L. (body mass 500–750 g) used in this study were collected using a casting net at a brackish water region of Aja River, south of Okinawa, Japan. These tilapia were acclimated to freshwater or seawater. For freshwater-acclimated tilapia (FWT), fishes were directly transferred to freshwater. For seawater-acclimated tilapia (SWT), fishes were maintained for 3 days in 30% seawater, then the salinity was increased up to 100% seawater within 5 days by gradual addition of 100% seawater. All FWT and SWT were acclimated in the ratio three males to one female in 1 ton freshwater tanks or in 1 ton running seawater tanks for at least 1 month before use.

Sperm collection

Fishes were anesthetized with 0.1% (v/v) 2-phenoxyethanol and testes dissected out from the abdomen. Sperm were collected by inserting a fine disposable transfer pipette (Iuchiseieido, Japan) into the sperm duct, taking great care not to contaminate the blood. Collected sperm were put onto a small Petri dish and stored on ice during the experiments, which lasted less than 7 h.

Assessment of sperm motility

Sperm motility was observed as described previously (Morita et al., 2003). Sperm were suspended in various solutions: electrolyte solution (0–500 mmol l^{-1} NaCl or KCl, 10 mmol l^{-1} Hepes-NaOH, pH 8.0), nonelectrolyte solution (0–900 mmol l^{-1} mannitol, 10 mmol l^{-1} Hepes-NaOH, pH 8.0), NaCl+Ca^{2+} solution (0–750 mmol l^{-1} NaCl, 0, 2 or 10 mmol l^{-1} $CaCl_2$, 10 mmol l^{-1} Hepes-NaOH, pH 8.0), Ca^{2+}-chelated NaCl solution (0–200 mmol l^{-1} NaCl, 5 mmol l^{-1} EGTA, 10 mmol l^{-1} Hepes-NaOH, pH 8.0), artificial seawater (ASW; 420 mmol l^{-1} NaCl, 9.0 mmol l^{-1} KCl, 10 mmol l^{-1} $CaCl_2$, 24.5 mmol l^{-1} $MgCl_2$, 25.5 mmol l^{-1} $MgSO_4$, 2.15 mmol l^{-1} $NaHCO_3$, 10 mmol l^{-1} Hepes-NaOH, pH 8.0). Velocities and beat frequency of sperm were determined from photographic records. Briefly, photographs (0.5 s exposure time) were taken using a Nikon camera system mounted on a microscope (Optiphoto; Nikon, Japan) with dark field condenser and 10× objective. The waveform was traced from video recordings of dark field images using 20× objective lens.

Measurements of $[Ca^{2+}]i$ using fluo-3 and confocal microscopy

One volume of sperm was diluted into nine volumes of Ca^{2+}-depleted artificial seminal plasma (CFASP) containing EGTA (FWT: 143 mmol l^{-1} NaCl, 50.7 mmol l^{-1} KCl, 0.18 mmol l^{-1} $MgSO_4$, 0.15 mmol l^{-1} glucose, 5 mmol l^{-1} EGTA, 10 mmol l^{-1} Hepes-NaOH, pH 8.0; SWT: 132.4 mmol l^{-1} NaCl, 52.5 mmol l^{-1} KCl, 1.14 mmol l^{-1} $MgSO_4$, 0.15 mmol l^{-1} glucose, 5 mmol l^{-1} EGTA, 10 mmol l^{-1} Hepes-NaOH, pH 8.0). The sperm concentration of this suspension was about $4–5\times10^{12}$ cells ml^{-1}. This sperm suspension was loaded with fluo-3 AM by incubating with 500 μmol l^{-1} fluo-3 AM (from a 20 mmol l^{-1} stock solution in anhydrous dimethylsulphoxide) on ice for 2 h. Then, the sperm suspension was centrifuged at 1500 ***g*** for 5 min at 4°C. The pelleted sperm were washed once with CFASP and resuspended into the same volume of CFASP. The sperm suspension was diluted to 19 volumes of various experimental solutions: (i) 50 mmol l^{-1} NaCl, (ii) 50 mmol l^{-1} NaCl + 5 mmol l^{-1} EGTA, (iii) 50 mmol l^{-1} NaCl + 5 mmol l^{-1} $CaCl_2$, (iv) 300 mmol l^{-1} NaCl and (v) 300 mmol l^{-1} NaCl + 10 mmol l^{-1} $CaCl_2$. Suspended sperm were then placed on the slide glass, covered with the coverslip, and sealed with nail varnish to prevent evaporation. These preparations were observed with a confocal microscope (40× objective lens) (Fluoview FV 500; Olympus, Japan).

Reactivation of the demembranated sperm

Demembranated tilapia sperm were reactivated in reactivation solutions to examine the effect of Ca^{2+} and osmolality. Slides and coverslips were coated with 1% (w/v) bovine serum albumin (BSA) to prevent sperm sticking to the glass surface. 1 volume of dry sperm was suspended into 10 volumes of the demembranation solution (175 mmol l^{-1} potassium acetate, 1 mmol l^{-1} dithiothreitol, 1 mmol l^{-1} EDTA, 0.04% w/v Triton X-100, 20 mmol l^{-1} Hepes-NaOH,

pH 8.0) for 30 s on ice. Then, 1 volume of the demembranated sperm suspension was mixed with 20 volumes of the reactivation solution (containing 75, 175, 350, 500 or 650 mmol l^{-1} potassium acetate, 1 mmol l^{-1} dithiothreitol, 0.5 mmol l^{-1} EDTA, 0.5 mmol l^{-1} EGTA, 220 μmol l^{-1} Mg-ATP^{2-}, 1 mmol l^{-1} free Mg^{2+}, 10^{-9}–10^{-2} mol l^{-1} free Ca^{2+}, 20 mmol l^{-1} Hepes-NaOH, pH 8.0).

Fractionation of sperm flagella and sleeve structure

Sperm were activated by suspending dry sperm, prepared by centrifuging at 8000 ***g*** for 10 min at 4°C to remove seminal plasma, into solutions containing (i) 50 mmol l^{-1} NaCl + 5 mmol l^{-1} $CaCl_2$ and (ii) 300 mmol l^{-1} NaCl + 10 mmol l^{-1} $CaCl_2$, and incubating for 1 min at room temperature. Movements of sperm in these suspensions were recorded as described above. The percentage of motile sperm was determined from video recordings. SWT sperm were motile when suspended in hypotonic (50 mmol l^{-1} NaCl + 5 mmol l^{-1} $CaCl_2$) and hypertonic (300 mmol l^{-1} NaCl + 10 mmol l^{-1} $CaCl_2$) solutions. Immotile and motile sperm suspensions were centrifuged at 15 000 ***g*** for 10 min at 4°C. Then 1 volume of flagella and sleeve structures in each sperm pellet were eluted with 7 volumes of urea solution (8 mol l^{-1} urea, 2 mol l^{-1} thiourea, 1% w/v Chaps, 1 mmol l^{-1} EDTA, 100 mmol l^{-1} dithiothreitol, 1 mmol l^{-1} phenylmethylsulphonyl fluoride (PMSF), 15 μmol l^{-1} E-64, 1.5 μmol l^{-1} Pepstatin A) to a concentration of 1.0×10^{13} cells ml^{-1}. Heads were pelleted by centrifugation at 15 000 ***g*** for 10 min at 4°C. SDS sample buffer was added to these flagella and sleeve suspensions, which were stored at –80°C before analysis by 1-D polyacrylamide gel electrophoresis (PAGE) and western blotting.

An equal volume of SDS-PAGE sample buffer was added to the urea-extracted flagella and sleeve. The samples (equivalent to 1.5×10^{11} cells) were subjected to SDS-PAGE in tricine buffer, according to the method of Schagger and Jagow (1987).

Western blotting was performed according to Towbin et al. (1979), with a little modification. Gels obtained from tricine-SDS-PAGE were placed on polyvinylidene difluoride membranes (PVDF; Biorad, USA) and electrically transferred. The membranes were blocked by incubation with 5% (w/v) bovine serum albumin in TTBS (137 mmol l^{-1} NaCl, 0.1% w/v Tween 20, 20 mmol l^{-1} Tris-HCl, pH 7.4) overnight at 4°C for detection of phosphoproteins. The membranes were washed three times for 10 min with TTBS followed by incubation for 2 h at room temperature with anti-phosphoserine antibody (diluted 1:20 000) and anti-phosphothreonine antibody (diluted 1:20 000). Then, the membranes were washed and incubated with horseradish peroxidase-conjugated extravidin (diluted 1:25 000) in TTBS. The membranes were again washed three times and subjected to an enhanced-chemiluminescence (ECL) reaction, performed according to the manufacturer's instructions (Amersham Pharmacia Biotech). The resulting membranes were exposed to X-ray film for 5–30 s.

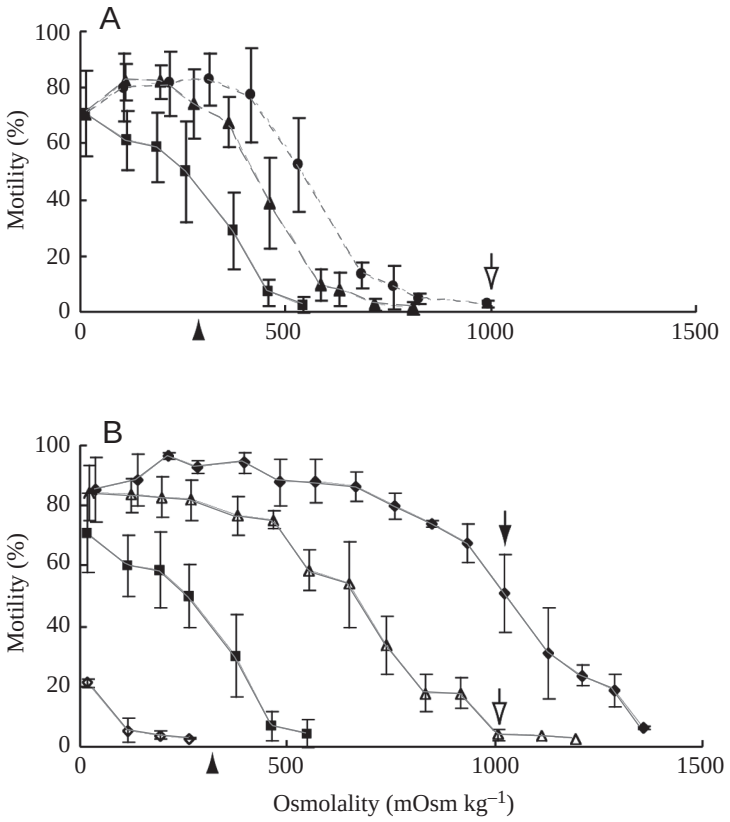

Fig. 1. Effects of osmolality and extracellular Ca^{2+} on motility in sperm of seawater-acclimated tilapia *Oreochromis mossambicus*. Sperm were suspended in different concentrations of electrolytes (NaCl and KCl) and nonelectrolyte (mannitol) containing 10 mmol l^{-1} Hepes-NaOH (pH 8.0). The percentage of motile sperm was measured from video recordings. (A) Motility in NaCl, KCl and mannitol solutions. Filled squares, NaCl; filled triangles, KCl; filled circles, mannitol. (B) The effect of Ca^{2+} on motility. Filled squares, NaCl alone; open triangle, NaCl solutions containing 2 mmol l^{-1} $CaCl_2$; filled diamond, NaCl solutions containing 10 mmol l^{-1} $CaCl_2$; open diamond, NaCl solutions containing 5 mmol l^{-1} EGTA. Filled arrowheads indicate the osmotic pressure of seminal plasma, i.e. isotonic osmolality. Open arrows and filled arrows indicate the osmotic pressure of seawater. Values are means ± S.D.; *N*=150 sperm from 5 fish for each point.

Table 1. *Sperm motility of freshwater-acclimated tilapia (FWT) and seawater-acclimated tilapia (SWT) in Hepes buffer and artificial seawater*

Motility (%)	FWT	SWT
10 mmol l^{-1} Hepes buffer	85.2±7.9	71.1±15.2
Artificial seawater (ASW)	0	60.2±27.2

Values are means ± S.D. (*N*=5).

Statistical analysis

Group comparisons were performed using a one-way or two-

way analysis of variance (ANOVA) followed by the least-significant test, where quality of variance criteria were met. Otherwise Mann–Whitney and Kruskal–Wallis procedures were used for the swimming velocity and beat frequency results.

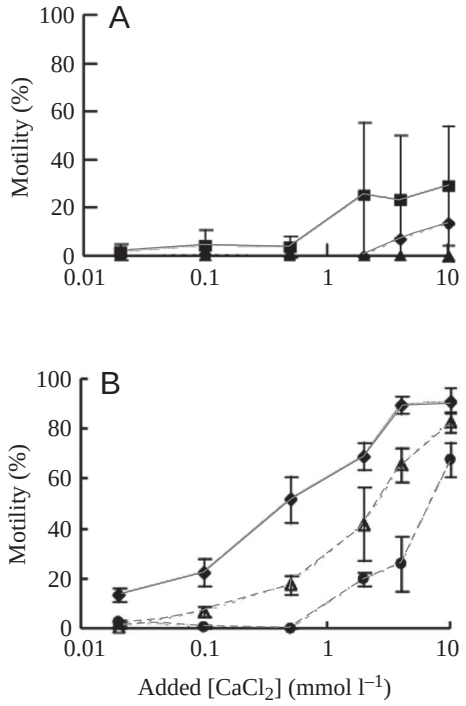

Fig. 2. Effect of Ca^{2+} concentration on sperm motility in hypertonic conditions. (A) FWT and (B) SWT sperm. Squares, hypertonic solution containing 200 mmol l^{-1} NaCl; diamonds, 250 mmol l^{-1} NaCl (500 mOsm kg^{-1}); triangles, 350 mmol l^{-1} NaCl (700 mOsm kg^{-1}); circles, 500 mOsm kg^{-1} (1000 mOsm kg^{-1}). Values are means ± S.D.; *N*=150 sperm from 5 fish for each for each point.

Results

Comparative observation of motility in FWT and SWT sperm

In this study, as reported by Brock (1954), SWT reproduced in the tank containing running seawater. We therefore first investigated the effect of osmolality on SWT sperm motility using electrolytes (NaCl, KCl) and a nonelectrolyte (mannitol). When SWT sperm were suspended in various concentrations of NaCl buffered with 10 mmol l^{-1} Hepes, they exhibited motility at low osmolality, as shown Fig. 1A. Motility decreased to <50% at isotonic conditions and to almost zero at 500 mOsm kg^{-1}. In the solution of osmolality equivalent to seawater (1000 mOsm kg^{-1}) SWT sperm did not show motility (open arrow in Fig. 1A). By contrast, approx. 60% of SWT sperm showed motility in artificial seawater (not shown), suggesting that components in artificial seawater and seawater, such as divalent cations Ca^{2+} or Mg^{2+}, cause increased motility under conditions of high osmolality. When Ca^{2+} was added to NaCl solutions, SWT sperm motility was increased in a dose-dependent manner (Fig. 1B). Approximately 50% were motile in the presence of 10 mmol l^{-1} $CaCl_2$ at 1000 mOsm kg^{-1} (filled arrow in Fig. 1B). As in NaCl solutions, motility decreased when osmolality was raised by KCl or mannitol and could be restored by addition of Ca^{2+} (data not shown). Addition of Mg^{2+} had no effect on SWT sperm motility.

To further examine the effect of Ca^{2+}, EGTA was added to remove Ca^{2+} completely, since considerable amounts of Ca^{2+} are derived from seminal plasma, which contains approx. 2 mmol l^{-1} Ca^{2+} in the experimental conditions shown in Fig. 1A. Under these conditions, sperm motility was drastically suppressed (Fig. 1B). The response of SWT sperm to changes in osmolality was different from that of FWT sperm. The motility of FWT sperm (Fig. 2A) in 200 mmol l^{-1} NaCl solution (approximately 400 mOsm kg^{-1}) increased from 0% to approximately 20% on addition of Ca^{2+} (≥2 mmol l^{-1}). However, no motility was observed at 350 mmol l^{-1} NaCl even in the presence of 10 mmol l^{-1} Ca^{2+}. In contrast, the effect of Ca^{2+}on SWT sperm was more marked, as shown in Fig. 2B, and rose dose-dependently in the presence of extracellular Ca^{2+}. More than 90% of

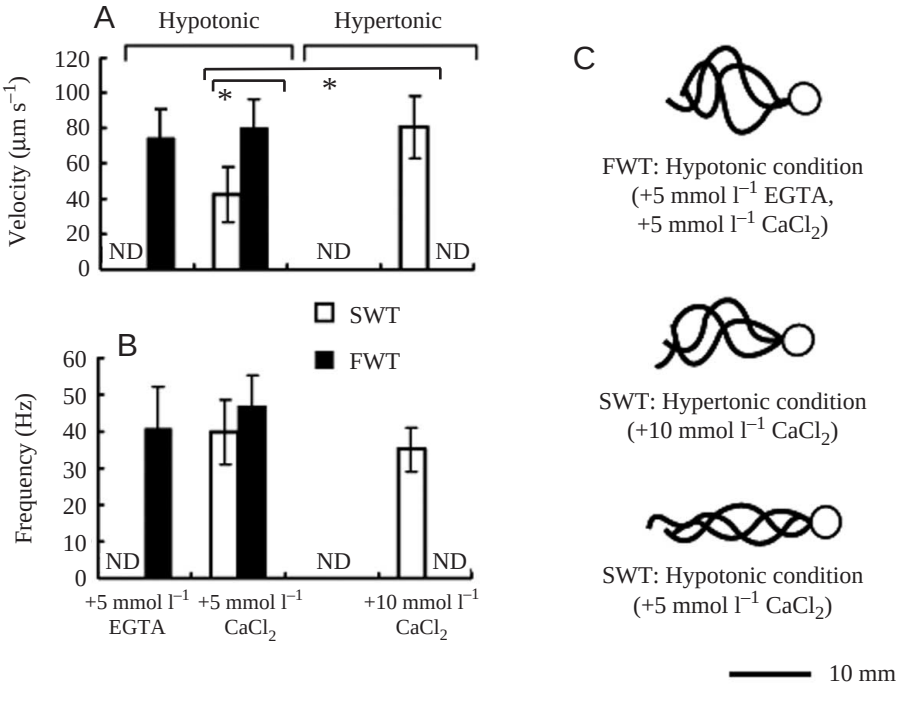

Fig. 3. Swimming velocity, beat frequency and wave form of FWT and SWT sperm. (A) Swimming velocity, (B) beat frequency and (C) waveform of FWT and SWT sperm in hypotonic (50 mmol l^{-1} NaCl) and hypertonic (300 mmol l^{-1} NaCl) conditions with or without Ca^{2+}. Values are means ± S.D. (*N*=4). ND, not determined. *The velocity of FWT and SWT sperm in the hypotonic condition+Ca^{2+} (A) were significantly different, and of SWT sperm in hypotonic+Ca^{2+} and hypertonic solutions (*P*<0.01).

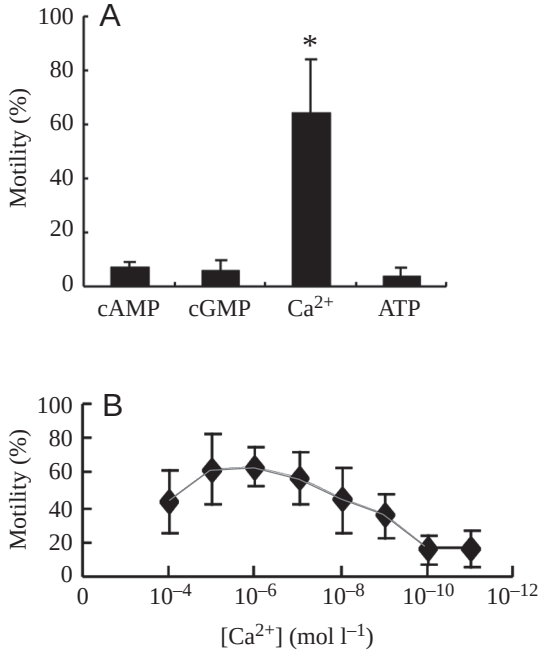

Fig. 4. Effect of (A) cAMP (10 μmol l^{-1}), cGMP (10 μmol l^{-1}), Ca^{2+} (10^{-4} mol l^{-1} free Ca^{2+}) and reactivation solution (B) various concentrations of Ca^{2+} on reactivation of sperm motility in demembranated sperm. The demembranated sperm were suspended in reactivation solution containing 220 μmol l^{-1} Mg-ATP^{2-}, 175 mmol l^{-1} potassium acetate, 1 mmol l^{-1} free Mg^{2+}, 1 mmol l^{-1} DTT, 0.5 mmol l^{-1} EGTA, 0.5 mmol l^{-1} EDTA, 20 mmol l^{-1} Hepes-NaOH (pH 8.0). Values are means ± S.D. (*N*=5 in A; *N*=7 in B). *Significantly different (*P*<0.01).

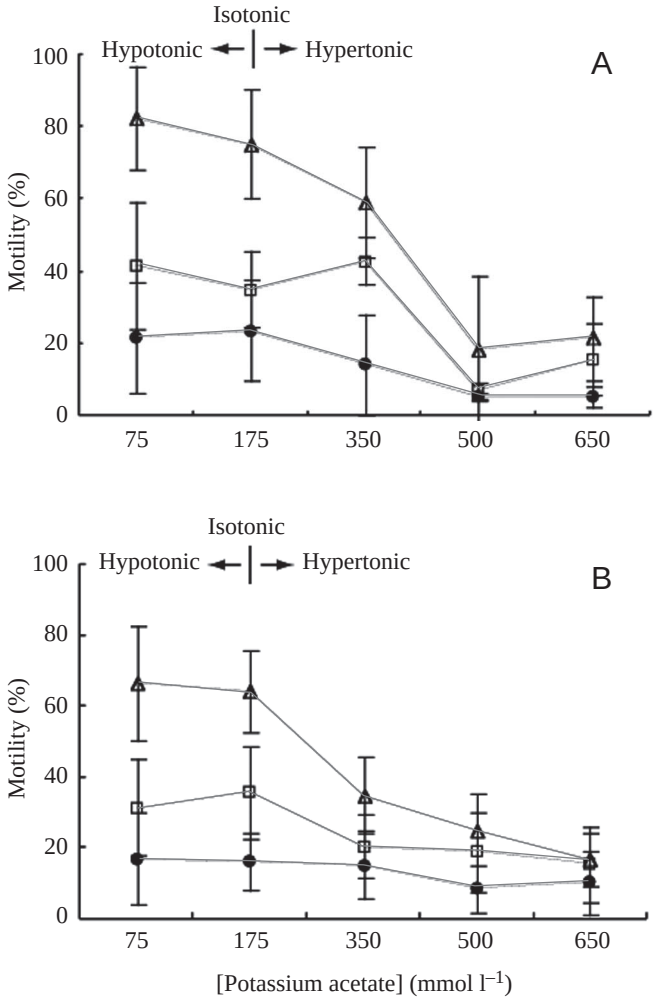

Fig. 5. Effect of osmolality and Ca^{2+} on motility in (A) FWT and (B) SWT demembranated sperm. The demembranated sperm were suspended in reactivation solutions containing 220 μmol l^{-1} Mg-ATP^{2-}, 75–650 mmol l^{-1} potassium acetate, 1 mmol l^{-1} free Mg^{2+}, 1 mmol l^{-1} DTT, 0.5 mmol l^{-1} EGTA, 0.5 mmol l^{-1} EDTA, 20 mmol l^{-1} Hepes-NaOH (pH 8.0). Open triangles, 10^{-4} mol l^{-1} Ca^{2+}; open squares, 10^{-7} mol l^{-1} Ca^{2+}; filled circles, 10^{-8} mol l^{-1} Ca^{2+}. Values are means ± S.D. (*N*=7).

SWT sperm were motile in the presence of 5 mmol l^{-1} Ca^{2+} at 250 mmol l^{-1} NaCl. The higher the osmolality, the larger the extracellular [Ca^{2+}] necessary to activate motility. It is likely that SWT sperm require extracellular Ca^{2+} to acquire motility in the hypertonic environment.

We then assessed sperm motility by determining swimming velocity, flagellar beat frequency and waveform, as shown in Fig. 3. The swimming velocities of FWT and SWT sperm were different in the hypotonic Ca^{2+}-containing condition (50 mmol l^{-1} NaCl + 5 mmol l^{-1} $CaCl_2$). In this hypotonic solution, the swimming velocity of FWT sperm was greater that that of SWT sperm (Fig. 3A.). By contrast, SWT sperm in hypertonic Ca^{2+}-containing solution (300 mmol l^{-1} NaCl + 10 mmol l^{-1} $CaCl_2$) swam as fast as FWT sperm in hypotonic solution (Fig. 3A). In this Ca^{2+}-containing hypertonic solution, SWT sperm showed vigorous motility, and the swimming velocity of SWT sperm was faster than that of sperm in the Ca^{2+}-containing hypotonic condition (Fig. 3A).

On the other hand, the beat frequency of FWT and SWT in Ca^{2+}-containing hypertonic and hypotonic conditions was the same (Fig. 3B). Hence, the pitch of one stroke by SWT sperm in the hypertonic condition was larger than that of sperm in the hypotonic condition. As shown in Fig. 3C, the waveform of SWT sperm in the hypertonic condition was different from that in the hypotonic condition but similar to the waveform of FWT sperm in the hypotonic condition. Therefore, it is plausible that FWT and SWT sperm are suited to hypotonic or hypertonic conditions, corresponding to their habitat, suggesting that the mechanism for regulating the sperm motility is modulated during acclimation of these fish.

Demembranated sperm of FWT and SWT sperm

The present study revealed that demembranated SWT sperm, like demembranated FWT sperm, required Ca^{2+} but not cAMP or cGMP to reactivate the motility (Fig. 4). It is likely that increased [Ca^{2+}]i is necessary to activate both FWT and

SWT sperm motility, even though motility-feasible conditions were different for FWT and SWT sperm.

The effect of $[K^+]$ on motility reactivation in both FWT and SWT sperm was studied. Takai and Morisawa (1995) reported that $[K^+]$ is important in marine and freshwater teleosts. In marine teleosts, an increase in $[K^+]$ is necessary to reactivate the demembranated sperm. In freshwater teleosts such as zebra fish, on the other hand, a decrease in $[K^+]$ is necessary. We examined the effect of $[K^+]$ in demembranated FWT and SWT sperm. Both FWT and SWT demembranated sperm exhibited a high ratio of reactivation in hypotonic and isotonic $[K^+]$ solutions (Fig. 5A,B) in the presence of 0.1 mmol l^{-1} Ca^{2+}. In hypertonic solutions, motility was decreased gradually, suggesting that $[K^+]$ did not affect the activation cascades in tilapia sperm in the isotonic and hypotonic range of solutions tested.

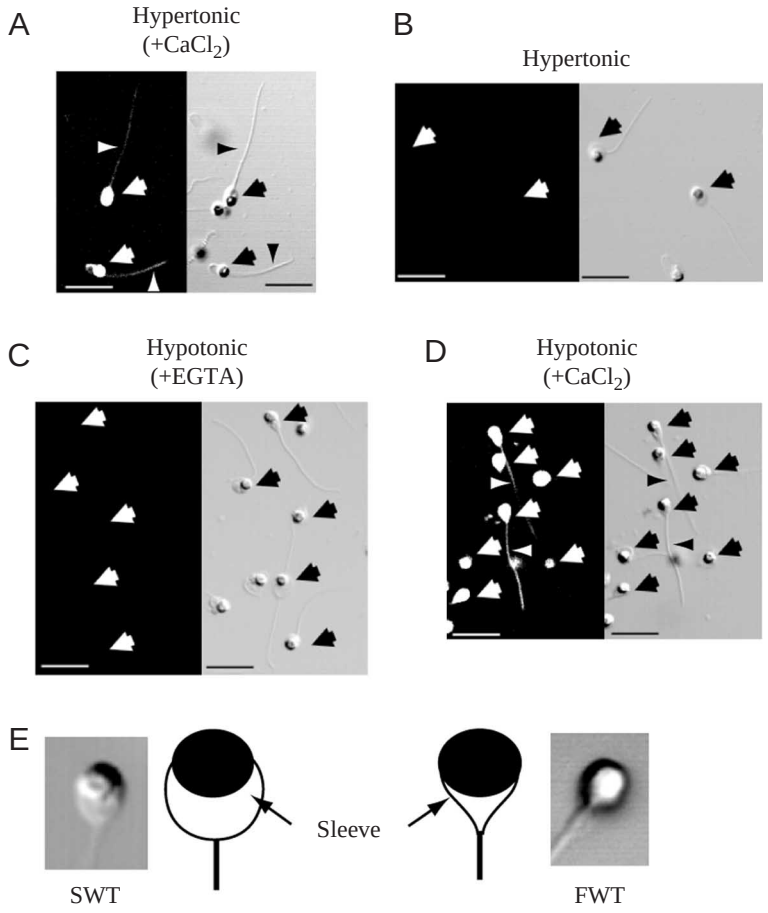

Fig. 6. Changes in $[Ca^{2+}]$i in SWT sperm subjected to hypotonic or hypertonic conditions, indicated by fluo-3 AM. Sperm were incubated with 500 μmol l^{-1} fluo-3 AM in artificial seminal plasma (ASP) for 2 h. Approximately 90% of sperm moved after dilution into Ca^{2+}-containing hypotonic or hypertonic conditions: (A) 50 mmol l^{-1} NaCl + 5 mmol l^{-1} $CaCl_2$ or (D) 300 mmol l^{-1} NaCl + 10 mmol l^{-1} $CaCl_2$. Motility was suppressed in Ca^{2+}-free hypotonic or hypertonic solutions: (C) 50 mmol l^{-1} NaCl + 5 mmol l^{-1} EGTA or (B) 300 mmol l^{-1} NaCl. Arrows, head and sleeve regions; arrowheads, flagella. (E) In hypertonic conditions the sleeve structure of SWT was expanded but that of FWT was shrunken. Bars, 10 μm. For details of microscopy, see Materials and methods.

Increase in intracellular $[Ca^{2+}]$

SWT sperm showed motility in hypotonic and hypertonic conditions in the presence of extracellular $[Ca^{2+}]$ (Figs 1B, 2B). In SWT sperm, increased $[Ca^{2+}]$i was observed in motility-feasible hypotonic and hypertonic conditions with Ca^{2+} present (Fig. 6A,D). As shown in our previous study (Morita et al., 2003), in hypertonic conditions, $[Ca^{2+}]$i in FWT sperm did not increase even on addition of extracellular Ca^{2+}. By contrast, in SWT sperm, $[Ca^{2+}]$i only increased when extracellular Ca^{2+} was added, suggesting that the increased $[Ca^{2+}]$i is extracellular $[Ca^{2+}]$-dependent. The increase in $[Ca^{2+}]$i of FWT and SWT sperm occurred in each different motility-feasible condition, suggesting that ways of mobilising Ca^{2+} also differ between FWT and SWT sperm. It is possible that the mechanism of raising $[Ca^{2+}]$i in FWT sperm is driven hypo-osmotically and that of SWT sperm depends on Ca^{2+} influx from extracellular regions.

Increased $[Ca^{2+}]$i was mainly observed in the head area, including the sleeve structure (arrows in Fig. 6A,D). A weak fluorescence signal was also observed in flagella (arrowheads in Fig. 6A,D). As shown in our previous study, swelling of the sleeve structure in FWT sperm occurred in hypotonic conditions corresponding to the motility practicable condition in which the increase in $[Ca^{2+}]$i was observed (Morita et al., 2003). The sleeve structure in SWT sperm was swollen in both hypotonic and hypertonic conditions independently of $[Ca^{2+}]$, as shown in Fig. 6E.

Protein phosphorylation in SWT sperm during motility activation

Activation of SWT sperm was dependent on extracellular $[Ca^{2+}]$. Protein phosphorylation and dephosphorylation occur in FWT sperm during activation of motility (Morita et al., 2003). Therefore, we examined protein phosphorylation in relation to the $[Ca^{2+}]$ increase and motility activation. In hypotonic Ca^{2+}-containing conditions (50 mmol l^{-1} NaCl + 5 mmol l^{-1} $CaCl_2$), serine and threonine residues of 15 kDa and 18 kDa proteins were dephosphorylated (Fig. 7A,B, lanes b) compared to those of the dry sperm (Fig. 7A,B, lanes a). These dephosphorylations did not occur in the absence of extracellular Ca^{2+} even in hypotonic conditions (data not shown). By contrast, in hypertonic conditions, serine and threonine residues of 15 kDa and 18 kDa proteins were phosphorylated (Fig. 7A,B, lanes c). As previously shown (Morita et al., 2003), threonine residue(s) of a 41 kDa protein in FWT sperm were also phosphorylated in motility-feasible hypotonic conditions. This 41 kDa protein was also phosphorylated in threonine residues in dry sperm (Fig. 7B; lane a) and was retained under motile conditions (Fig. 7B, lanes b,c) in SWT sperm.

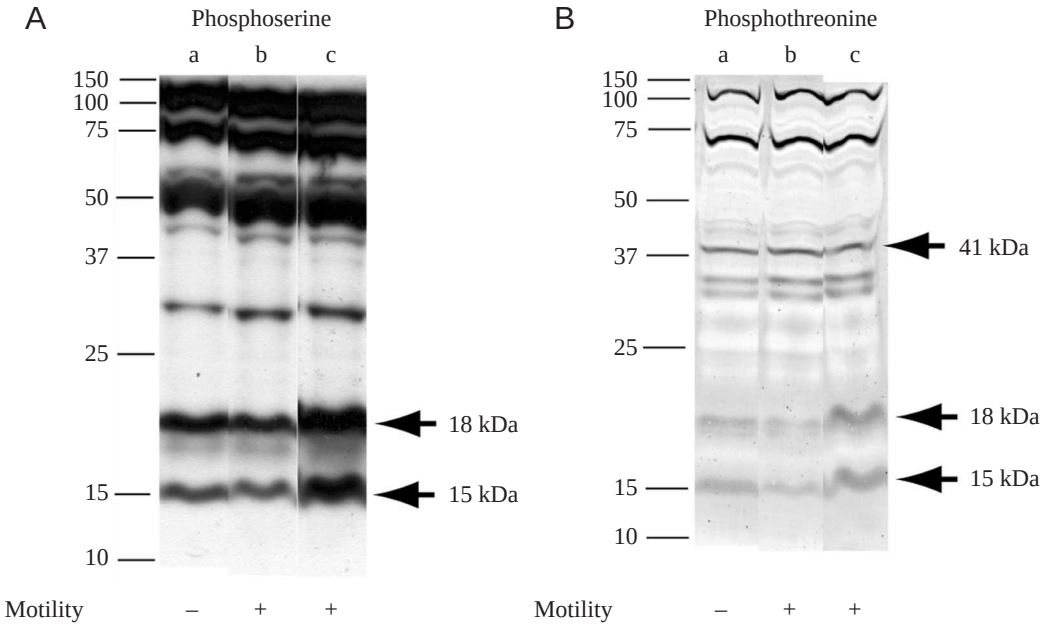

Fig. 7. Protein phosphorylation and dephosphorylation at serine (A) and threonine (B) residues in motility-feasible conditions. Sperm were diluted into either 50 mmol l^{-1} NaCl + 5 mmol l^{-1} $CaCl_2$ (lane b) or 300 mmol l^{-1} NaCl + 10 mmol l^{-1} $CaCl_2$ (lane c). Protein of dry sperm (lane a) was eluted after removal of seminal plasma by centrifugation. Sperm were motile in both the hypotonic solutions (50 mmol l^{-1} NaCl) and the hypertonic solution (300 mmol l^{-1} NaCl). Sperm were then collected and subjected to western blotting with (A) anti-phosphoserine antibody or (B) phosphothreonine antibody. Numbers on the left indicate molecular mass (kDa) obtained from molecular markers. Motility is shown below the lanes.

Discussion

In the present study, we have demonstrated that Ca^{2+} plays a significant role in motility activation in sperm of tilapia acclimated to seawater (SWT) as well as to freshwater (FWT). (1) Intact sperm exhibited vigorous motility from hypotonic up to hypertonic conditions equivalent to seawater, in the presence of Ca^{2+} (Figs 1–3). (2) Motility was seriously suppressed when extracellular [Ca^{2+}] was depleted by EGTA (Fig. 1). (3) [Ca^{2+}]i increased in motile conditions (Fig. 6). (4) Ca^{2+} was required to reactivate demembranated sperm (Figs 4, 5).

In sperm of puffer fish (Oda and Morisawa, 1993), lancelet (Tanaka et al., 2002), ascidians (Nomura et al., 2000; Yoshida et al., 1994, 2003), carp (Krasznai et al., 2000) and salmonidae (Boitano and Omoto, 1992; Tanimoto and Morisawa, 1998; Kho et al., 2001), increased [Ca^{2+}]i is known to play an important role in activation of the sperm motility. Demembranated sperm is assumed to simulate a regulatory mechanism of motility in intact sperm. In the presence of $>10^{-4}$ mol l^{-1} Ca^{2+}, demembranated FWT sperm were reactivated (Morita et al., 2003). In the present study we have shown that demembranated sperm of SWT is also required Ca^{2+} for reactivation (Fig. 4), suggesting that Ca^{2+} activates a second messenger for motility activation. In salmonid fishes, increased [Ca^{2+}]i was also observed on motility initiation/activation (Cosson et al., 1989). These results suggest that Ca^{2+} is tightly coupled to the initial phase of motility regulation. However, a series of experiments have demonstrated that Ca^{2+} sometimes works as an inhibitor. [Ca^{2+}] of more than $10^{-8.5}$ mol l^{-1} inhibits reactivation of demembranated salmonid sperm (Okuno and Morisawa, 1989). In demembranated sea urchin sperm, sperm flagella become quiescent and ‘cane’-shaped in high [Ca^{2+}] (10^{-4} mol l^{-1}; Gibbons and Gibbons, 1980; Okuno and Brokaw, 1981). In these cases, Ca^{2+} seems to suppress some wave parameters directly. Therefore, it is likely that Ca^{2+} has a biphasic role. Ca^{2+} in tilapia works predominantly in motility initiation/activation.

As shown above, increased [Ca^{2+}]i is necessary for activation of both demembranated FWT and SWT sperm motility. The levels of Ca^{2+} in habitats of FWT and SWT are quite different. Freshwater does not contain large amounts of Ca^{2+}, but seawater does, although both FWT and SWT sperm require increased [Ca^{2+}]i as a second messenger for motility activation. FWT sperm have difficulties in getting sufficiently high levels of extracellular Ca^{2+} for activation in freshwater, because there are not high enough amounts of Ca^{2+} present. By contrast, it would be easier for SWT sperm to utilize extracellular Ca^{2+} in seawater for motility activation. Therefore, it is reasonable to suggest that one major component in acclimation of motility regulatory mechanisms in tilapia is modulation of the flow of Ca^{2+} supply. The Ca^{2+} indicator, fluo 3, revealed that levels of [Ca^{2+}]i increased in SWT sperm in the presence of extracellular Ca^{2+} even at high osmolality (Fig. 6A,D). Increase in [Ca^{2+}]i in SWT sperm, however, did not occur in the absence of extracellular Ca^{2+} (Fig. 6B,D), suggesting that the Ca^{2+} influx is required to raise the intracellular Ca^{2+} level. We previously reported that FWT sperm do not exhibit motility at high osmotic pressure even in

the presence of extracellular Ca^{2+}, if the increase in $[Ca^{2+}]i$ does not also occur (Morita et al., 2003). It is plausible that FWT sperm have Ca^{2+} stores that can be released by osmotic shock to increase $[Ca^{2+}]i$, whereas SWT sperm do not. However, it is possible that SWT sperm may also have Ca^{2+} stores controlled by a ryanodine receptor, which is activated by attaching Ca^{2+} (Berridge, 1993). Thus, in SWT sperm, it is possible that the increase in $[Ca^{2+}]i$ occurs *via* Ca^{2+}-induced Ca^{2+} release. Preliminary experiments show that the ryanodine receptor inhibitor did inhibit SWT sperm motility in hypotonic conditions (M. Morita and M. Okuno, unpublished data).

SWT sperm also showed swelling of the sleeve structure in hypertonic conditions, whereas the sleeve of FWT sperm was shrunk (Fig. 6E). Volume regulation of the sleeve structure might possibly act as an important candidate to increase $[Ca^{2+}]i$ or another function of motility in conditions of high osmolality. It is reported that cell swelling induces increased $[Ca^{2+}]i$ in *Necturus* erythrocytes (Light et al., 2003). Therefore, it is likely that volume regulation of the sleeve structure is important for the motility regulatory mechanism by increasing $[Ca^{2+}]i$ even if the Ca^{2+} was influxed from the extracellular region.

The ionic environment is also an important factor responsible for motility activation. High concentrations of electrolytes, such as KCl and NaCl, are necessary for activating the motility of demembranated sperm of marine teleosts such as puffer fish. On the other hand, a decrease in electrolytes is required for motility activation of freshwater teleosts such as zebra fish sperm (Takai and Morisawa, 1995), where reactivation of the demembranated sperm failed to occur when the electrolytes were substituted for mannitol, suggesting that the presence of appropriate concentrations of ions is necessary for attaining motility. In comparison with puffer fish and zebra fish, the properties of demembranated tilapia sperm appeared similar to those of freshwater teleosts, even when acclimated to seawater. Furthermore, it is likely that the regulatory mechanism of tilapia sperm motility is not controlled by $[K^+]$, since the demembranated tilapia sperm were reactivated in isotonic K^+ solution (Fig. 5A,B) and the decrease and increase in $[K^+]$ had no effect on the reactivation ratios of either FWT or SWT demembranated sperm (Fig. 5A,B).

In sperm of salmonid fishes, it has been accepted that motility initiation is induced by phosphorylation of a 15 kDa protein *via* the increase in cAMP (Morisawa and Hayashi, 1985; Hayashi et al., 1987). We failed to reactivate demembranated tilapia sperm with cAMP as shown in Fig. 4. Furthermore, we added both cAMP and the catalytic subunit of A-kinase to eliminate the possibility that the latter was depleted on demembranation. Again, we failed to reactivate the demembranated sperm (data not shown). Thus, we conclude that a cAMP-dependent system is not involved in tilapia sperm motility activation. We previously reported that protein phosphorylation occurred during activation of FWT sperm motility accompanied by an increase in $[Ca^{2+}]$ (Morita et al., 2003). In the present study, protein phosphorylation of serine and threonine residues of various proteins was closely related to the activation of SWT sperm motility (Fig. 7). Taken together with our previous results, it is apparent that a protein phosphorylation cascade is involved in motility activation mechanism in tilapia sperm although the protein phosphorylation cascades of FWT and SWT sperm seem to be different.

Considering the relationship between motility activation and protein phosphorylation, three phosphoproteins, of 15 kDa, 18 kDa and 41 kDa, were detected. The 15 kDa and 18 kDa proteins were dephosphorylated in motility-feasible hypotonic conditions in both FWT and SWT sperm. By contrast, the 15 kDa and 18 kDa proteins were strongly phosphorylated in hypertonic conditions (Fig. 7). A threonine residue(s) of a 41 kDa protein in FWT sperm was phosphorylated in the motility-feasible hypotonic condition (Morita et al., 2003). The 41 kDa protein in SWT sperm was also phosphorylated in the dry sperm condition (Fig. 7, lane a), suggesting that the protein phosphorylation cascades related to motility activation are different in FWT and SWT sperm. It is possible that phosphorylation of the 41 kDa protein in the dry sperm condition is required for motility in the hypertonic condition. It is also likely that modulation of protein phosphorylation cascades is related to acclimation of the motility regulatory mechanism in FWT and SWT sperm. It is therefore suggested that acclimation of sperm is caused by modulation of spermatogenesis, involving modulation of protein phosphorylation cascades and mechanism of Ca^{2+} supply. However, it is still not certain what kinds of signal transduction are related to motility activation with respect to the supply of Ca^{2+} and protein phosphorylation.

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