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Requirement of Ca^{2+} on activation of sperm motility in euryhaline tilapia *Oreochromis mossambicus*

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Summary

Euryhaline tilapia *Oreochromis mossambicus* acclimates to the external spawning environment by modulating its mechanism for regulating sperm motility. Adaptation of sperm was performed by acclimating fish in various environments. In this paper, regulatory mechanisms of freshwater-acclimated tilapia were studied in detail. Tilapia sperm motility was vigorous in hypotonic conditions and decreased with increasing osmolality. Sperm motility was reduced in hypotonic conditions when extracellular Ca^{2+} was chelated; however, extracellular Ca^{2+} was not a major factor for motility activation since sperm were motile even when extracellular Ca^{2+} levels were nominally depleted by EGTA. The Ca^{2+} indicator, fluo 3, showed that intracellular [Ca^{2+}] increased on motility activation independently of extracellular [Ca^{2+}], accompanied by swelling of the sperm neck region called the sleeve structure. Intracellular [Ca^{2+}] was not increased under hypertonic conditions, in which sperm were immotile, even on addition of extracellular Ca^{2+}. It is possible that Ca^{2+} is stored in the neck region. Demembranated sperm were reactivated in the presence of Ca^{2+}, but cAMP failed to reactivate the motility. Furthermore, we detected phosphorylation and dephosphorylation of three proteins at serine and threonine residues on motility activation. It is likely that hypotonic shock causes an increase in intracellular [Ca^{2+}] that activates motility activation *via* phosphorylation of some flagellar proteins.

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

Introduction

It is widely accepted that sperm motility in teleosts is regulated by, and suited to, the environment in which they reproduce. In many teleosts, which spawn in either freshwater or seawater, sperm motility is initiated by osmotic shock when sperm are ejaculated. These osmolality dependent regulatory systems are quite different in freshwater cyprinid teleosts, such as carp, gold fish and zebrafish, and marine teleosts, such as the pufferfish and flounder. Sperm of freshwater teleosts are quiescent at the osmolality of seminal plasma, referred to as isotonic conditions (approximately 300 mosmol kg^{-1}). They begin to move when suspended in hypotonic water (<300 mosmol kg^{-1}) and show high motility (as % motile sperm) in freshwater. By contrast, sperm of marine teleosts begin to move when released into hypertonic water (>300 mosmol kg^{-1}), and show the highest motility at approximately 1000 mosmol kg^{-1}, which is almost equivalent to seawater (Morisawa and Suzuki, 1980; Oda and Morisawa, 1993). It has therefore been assumed that the difference in osmolality between seminal plasma and the external water, corresponding to the spawning ground, is an important regulatory factor of sperm motility in many teleosts. If this is the case, the regulatory system of sperm motility seems to be reversed in freshwater and seawater teleosts, since the osmolality of seminal plasma is intermediate between that of freshwater and seawater.

The tilapia *Oreochromis mossambicus*, a euryhaline teleost, has the can acclimate to wide range of salinities, from freshwater to seawater, by a mechanism that includes chloride cells (Sakamoto et al., 1997; Balm et al., 1994; Borski et al., 1994). A unique feature of tilapia is that they can reproduce in both freshwater and seawater, even though there is a large osmotic difference between those two environments (Brock, 1954). Thus, it has been assumed that tilapia sperm can swim independently of osmolality, or modulate their regulatory mechanism to suit fertilization in either high or low salinity. It is possible that the motility regulatory system of tilapia sperm is quite different from those reported for other teleosts. We have already reported that tilapia sperm acclimated to seawater were motile in hypertonic water, i.e. seawater, in the presence of Ca^{2+}, whereas in tilapia acclimated to freshwater sperm could not swim in hypertonic conditions even in the presence of Ca^{2+} (Morita and Okuno, 1998). Linhart et al. (1999)

reported that during acclimation of the fish from freshwater to seawater, tilapia sperm adapt to conditions of high salinity by changing their motility from being independent of extracellular Ca^{2+} to Ca^{2+} dependent. It was also reported recently that sperm motility was physically suppressed by high viscous components in seminal plasma in the Nile tilapia *Oreochromis niloticus*, another species that cannot adapt to seawater. This result suggests that motility activation could be induced simply by mechanical release from viscosity immobilization (Mochida et al., 1999). These reports led us to question whether the motility regulatory mechanism of tilapia sperm differs from an osmotic shock related mechanism. In the present work, we examine the regulatory mechanism of sperm motility in tilapia acclimated to freshwater and demonstrate that sperm motility is regulated by osmolality, together with a limited role for extracellular Ca^{2+}. Furthermore, we demonstrate that on exposure to hypotonic conditions, various protein phosphorylations and dephosphorylations occur that are associated with the increase in intracellular Ca^{2+} levels.

Materials and methods

Chemicals

Biotinylated anti-phosphoserine (pS) and anti-phosphothreonine (pT) antibody, extravidin-conjugated horseradish peroxidase, E-64, Pepstatin A and phenylmethylsulphonyl fluoride (PMSF) were purchased from Sigma Chemical Co. (St Louis, MO, USA). The enhanced chemiluminescence (ECL) kit was from Amersham Pharmacia Biotech (Buckinghamshire, England), and Chaps and fluo-3 AM from Dojindo (Kumamoto, Japan). All other chemicals were obtained from Wako Chemical Co. (Osaka, Japan).

Fish

All tilapia *Oreochromis mossambicus* (body mass 500–750 g) used in this study were collected with a casting net at a blackish water region of Aja River, in the southern part of Okinawa, Japan. These tilapia were acclimated to the various conditions in groups of 3 males to 1 female in 1 ton freshwater tanks for at least 1 month before use.

Sperm collection

Fish were anesthetized with an appropriate amount of 2-phenoxyethanol, and the 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 transferred to a small Petri dish and stored on ice.

Measurement of sperm motility

In this study, NaCl and KCl were used as electrolytes and mannitol as a nonelectrolyte. All solutions contained 10 mmol l^{-1} Hepes-NaOH buffer, pH 8.0. Approximately 0.05 μl of semen were immediately diluted into 45 μl of solution on a glass slide with fine glass capillary tube, and covered with a coverslip. Sperm movements were recorded using a video recorder (SLV-LF1; Sony, Japan) and a CCD camera (cs 226; Olympus, Japan) mounted on a phase contrast microscope (Optiphoto, Nikon). Percentage motility was counted from the video recordings. Sperm were counted as motile if they either exhibited progressive movement or spontaneous flagellar beating if the sperm head was attached to the glass slide.

Measurement of components and osmolality of seminal plasma

Dry sperm were transferred to 1.5 ml Eppendorf tubes, and centrifuged for 3 min at 8000 ***g*** (4°C). The supernatants were used as seminal plasma. Osmolality of the seminal plasma and experimental solutions was measured by vapor-pressure osmometer (VPO5506; Wesco, USA).

Electrolyte components in seminal plasma were measured after dilution to an appropriate concentration in millipore-filtered water. 10 ml of the diluted seminal plasma solution were subjected to polarized zeeman atomic absorption spectrophotometer (Z-6100; Hitachi, Japan) to measure Na^+, K^+, Mg^{2+} and Ca^{2+} concentrations.

Measurements of intracellular [Ca^{2+}] with fluo-3 and confocal microscope

Sperm was diluted 1:9 (v/v) with Ca^{2+}-depleted artificial seminal plasma (CFASP) containing EGTA (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 and 10 mmol l^{-1} Hepes-NaOH, pH 8.0). The sperm concentration of this suspension was approximately 4–5×10^{12} cells ml^{-1}. This sperm suspension was loaded with fluo-3 AM by incubation with 500 μmol l^{-1} fluo-3 AM [from a 20 mmol l^{-1} stock solution in anhydrous dimethyl sulphoxide (DMSO)] on ice for 2 h, followed by centrifugion at 1500 ***g*** for 5 min at 4°C. The pelleted sperm were washed once with CFASP and resuspended in the same volume of CFASP before dilution 1:19 (v/v) into 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} $CaCl_2$, 300 mmol l^{-1} NaCl and (iv) 300 mmol l^{-1} NaCl + 5 mmol l^{-1} $CaCl_2$. The diluted sperm were then placed on glass slides, covered with a coverslip, and sealed with nail varnish to prevent evaporation. The 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 to examine the effect of [Ca^{2+}] and osmolality. Glass slides and coverslips were coated with 1% (w/v) bovine serum albumin (BSA) to prevent sperm sticking to the glass surface. Demembranation and reactivation were carried out according to the method described previously (Okuno and Morisawa, 1989). Dry sperm was suspended 1:10 (v/v) in the demembranation solution [175 mmol l^{-1} potassium acetate, 1 mmol l^{-1} dithiothreithol (DTT), 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 the

demembranated sperm was mixed 1:20 (v/v) with the reactivation solution (175 mmol l^{-1} potassium acetate, 1 mmol l^{-1} DTT, 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 the dry sperm in various solutions: (i) 50 mmol l^{-1} NaCl + 5 mmol l^{-1} EGTA, (ii) 50 mmol l^{-1} NaCl + 5 mmol l^{-1} $CaCl_2$ and (iii) 300 mmol l^{-1} NaCl, and incubated for 1 min at room temperature. Movements of sperm in these suspensions were recorded by a video recorder (SLV-LF1; Sony) and a CCD camera (cs 226; Olympus) mounted on a microscope (Optiphot; Nikon, Japan) equipped with a dark-field or phase-contrast condenser. The percentage of motile sperm was counted from the video recordings. Immotile sperm samples were prepared by suspending them into 300 mmol l^{-1} NaCl solution (activation solution iii). Sperm were motile when suspended in 50 mmol l^{-1} NaCl + 5 mmol l^{-1} EGTA (activation solution i) or 50 mmol l^{-1} NaCl + 5 mmol l^{-1} $CaCl_2$ (activation solution ii). The immotile and motile sperm suspensions were centrifuged at 15 000 ***g*** for 10 min at 4°C. Sperm pellets were resuspended to a concentration of 1.0×10^{13} cell ml^{-1} in urea solution (8 mmol l^{-1} urea, 2 mmol l^{-1} thiourea, 1% (w/v) Chaps, 1 mmol l^{-1} PMSF, 15 μmol l^{-1} E-64, 1.5 μmol l^{-1} Pepstatin A and 100 mmol l^{-1} DTT). Heads were pelleted by centrifugation at 15 000 ***g*** for 10 min at 4°C. SDS-sample buffer was added to the supernatant containing flagella and sleeve structures for 1-D polyacrylamide gel electrophoresis (PAGE). Suspensions were stored at –80°C until used.

Gel electrophoresis and western blotting analysis

Flagella and sleeves (equivalent to approximately $2–3\times10^{11}$ cells) extracted by urea were subjected to tricine-buffered SDS-PAGE (Schagger and Jagow, 1987) in 10% polyacrylamide gels containing 0.1% SDS.

Western blotting was performed according to the method of Towbin et al. (1979), with a little modification. After electrophoresis the tricine SDS-PAGE gels were placed on polyvinylidene difluoride (PVDF) membranes (Biorad, USA) and electrically transferred. The membranes were blocked by incubation with 2% (w/v) BSA in TTBS (137 mmol l^{-1} NaCl, 0.1% (w/v) Tween 20 and 20 mmol l^{-1} Tris-HCl, pH 7.4) overnight at 4°C. The membranes were washed three times with TTBS followed by incubation for 2 h at room temperature with anti-pS antibody (1:20,000 dilution) or anti-pT antibody (1:20,000 dilution) as the primary antibodies. Then the membranes were washed and incubated with extravidine-conjugated with horseradish peroxidase (1:25,000 dilution) in TTBS for 1 h at room temperature. The membranes were again washed three times and subjected to the enhanced chemiluminescence (ECL) reaction, carried out according to the manufacturer's protocol. The membranes were exposed to X-ray film for 5–30 s.

Results

Effect of osmolality and extracellular Ca^{2+} on sperm motility

Sperm of the freshwater-acclimated tilapia in freshwater exhibited vigorous motility for 30 min or more. Sperm motility was observed after suspension in solutions containing various concentrations of electrolytes (NaCl and KCl) and a nonelectrolyte (mannitol). Changes in motility with osmolality are shown in Fig. 1A. Osmolalities of all solutions were measured using a vapor-pressure osmometer (VPO5506; Wesco), and shown in the

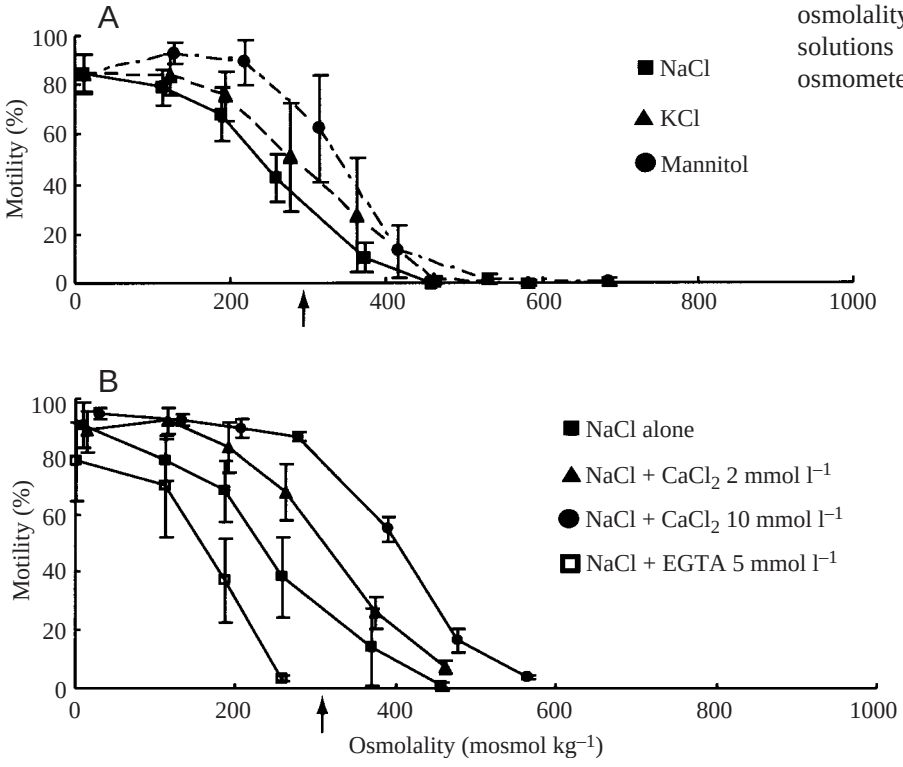

Fig. 1. Effects of osmolality and extracellular [Ca^{2+}] on motility in sperm of tilapia *Oreochromis mossambicus*. Sperm were suspended in solutions of 10 mmol l^{-1} Hepes-NaOH, pH 8.0, containing different concentrations of electrolytes and a nonelectrolyte (mannitol) to give the required osmolality.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

Table 1. *Components and osmotic pressure of seminal plasma in freshwater-acclimated tilapia sperm*

[Electrolyte] (mmol l^{-1})				
Na^+	K^+	Mg^{2+}	Ca^{2+}	Osmolality (mosmol kg^{-1})
142.3±21.3	50.7±7.0	0.18±0.17	2.0±0.77	299.0±24.0

Values are means ± S.D. (N=5).

figure rather than [electrolyte]. Sperm motility was high in hypotonic conditions (0–200 mosmol kg^{-1}) and decreased as osmolality increased over 200 mosmol kg^{-1}. Similar results were obtained in solutions containing both electrolytes (NaCl and KCl) and the nonelectrolyte (mannitol), so osmolality is probably a major factor in regulating sperm motility. The arrows in Fig. 1 indicate the osmotic pressure of seminal plasma (Table 1), which is referred to as the isotonic condition. Approximately 20–50% sperm were motile at this isotonic condition.

Sperm retained motility even when suspended in 150 mmol l^{-1} KCl solution, which was an almost isotonic condition, suggesting that the system of motility regulation in this fish is very different from that of salmonid fish, where decreased [K^+] causes increased sperm motility (Morisawa and Suzuki, 1980).

Addition of Ca^{2+} to the solutions caused increased sperm motility at the osmolalities less than 500 mosmol kg^{-1}, as shown in Fig. 1B. However, sperm motility ceased above 600 mosmol kg^{-1}. Removal of extracellular Ca^{2+} by EGTA reduced motility, and sperm were almost quiescent at the isotonic condition. However, even in the presence of EGTA, motility was almost equivalent to that observed in the presence of Ca^{2+} at osmolalities less than 100 mosmol kg^{-1}. Similar results were observed in solutions containing KCl and mannitol (data not shown). Seminal plasma contains approximately 2 mmol l^{-1} Ca^{2+} (Table 1), so it was assumed that Ca^{2+} contaminated from seminal plasma was responsible for the increased motility in the experiments shown in Fig. 1A. It was also likely that in hypotonic conditions sperm did not require extracellular Ca^{2+} for motility. Mg^{2+} had no effect on sperm motility (data not shown).

Measurement of intracellular Ca^{2+}

In freshwater, tilapia sperm exhibited high motility even when the extracellular [Ca^{2+}] was depleted with EGTA. However, sperm motility in medium of lower osmolality than isotonic medium was improved by the addition of Ca^{2+}. Ca^{2+} might therefore be involved in the regulatory mechanism of flagellar motility in tilapia.

In order to examine this possibility, the intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ was measured using fluorescent dye. Fig. 2 shows the increase in $[Ca^{2+}]_i$ after motility activation. Sperm diluted in solution containing 50 mmol l^{-1} NaCl + 5 mmol l^{-1} $CaCl_2$ (Fig. 2A) or 5 mmol l^{-1} EGTA (Fig. 2B) exhibited high motility for approximately 30 min and then stopped. Sperm did not move in the solution containing high NaCl (300 mmol l^{-1}) regardless of the presence of Ca^{2+} (Fig. 2C,D). Fluorescence micrographs taken after the flagella motility stopped revealed increased fluorescence in sleeve structures of sperm that had exhibited motility. Even when extracellular Ca^{2+} was chelated with EGTA, $[Ca^{2+}]$i were increased (Fig. 2B), suggesting that the increased intracellular Ca^{2+} was supplied from intracellular Ca^{2+} stores. No fluorescence was observed in sperm that were immotile under hypertonic conditions.

The sleeve structures were expanded in solutions containing 50 mmol l^{-1} NaCl (Fig. 2A,B; low osmotic condition), and contained Ca^{2+}, as indicated by fluo-3 localization, suggesting that this Ca^{2+} was supplied from Ca^{2+} stores within the sleeve structures. On the other hand, the sleeve structure was not expanded nor was the $[Ca^{2+}]_i$ increased in solutions containing 300 mmol l^{-1} NaCl with or without 5 mmol l^{-1} $CaCl_2$ (Fig. 2C,D). It is likely that tilapia sperm do not have the capability to increase $[Ca^{2+}]_i$ in a high osmotic environment. In addition, since the sleeve structure is shrunk in the isotonic and hypertonic conditions, supplementation of intracellular Ca^{2+} stores is stimulated by hypotonic conditions, associated with swelling of the sleeve structure, and high osmotic pressures suppress the function of the Ca^{2+} store in sleeve structures (Fig. 2E).

Ca^{2+} requirement for reactivation of the demembranated sperm

Soluble components of the cytoplasm, including ions, soluble proteins, nucleotides etc, diffuse away from the axoneme when sperm are demembranated. It is then possible to examine the effect of intracellular factors, such as Ca^{2+}, directly by changing the composition of the solution. It is very difficult to control the $[Ca^{2+}]_i$ because the cell membrane is impermeable to Ca^{2+}. We used the demembranated sperm model to apply Ca^{2+} directly to the flagellar axoneme. As shown in Fig. 3A, demembranated sperm were not reactivated in the presence of ATP only. An appropriate concentration of Ca^{2+} (10^{-4} mol l^{-1}) in the reactivating solution was necessary for motility to occur. Addition of either 10 μmol l^{-1} cAMP or 10 μmol l^{-1} cGMP failed to reactivate the sperm movement.

More detailed experiments using the demembranated sperm were performed to investigate the effect of Ca^{2+} on reactivation. Motility was very low at 10^{-9} mol l^{-1} Ca^{2+}, began to increase at 10^{-7} mol l^{-1} and was maximal at 10^{-4} mol l^{-1} Ca^{2+}. Reactivated sperm motility was suppressed at Ca^{2+} levels of 10^{-2} mol l^{-1} or higher (Fig. 3B). It seems that increased $[Ca^{2+}]_i$ rather than increased cAMP or cGMP levels was necessary to activate sperm motility. We found no

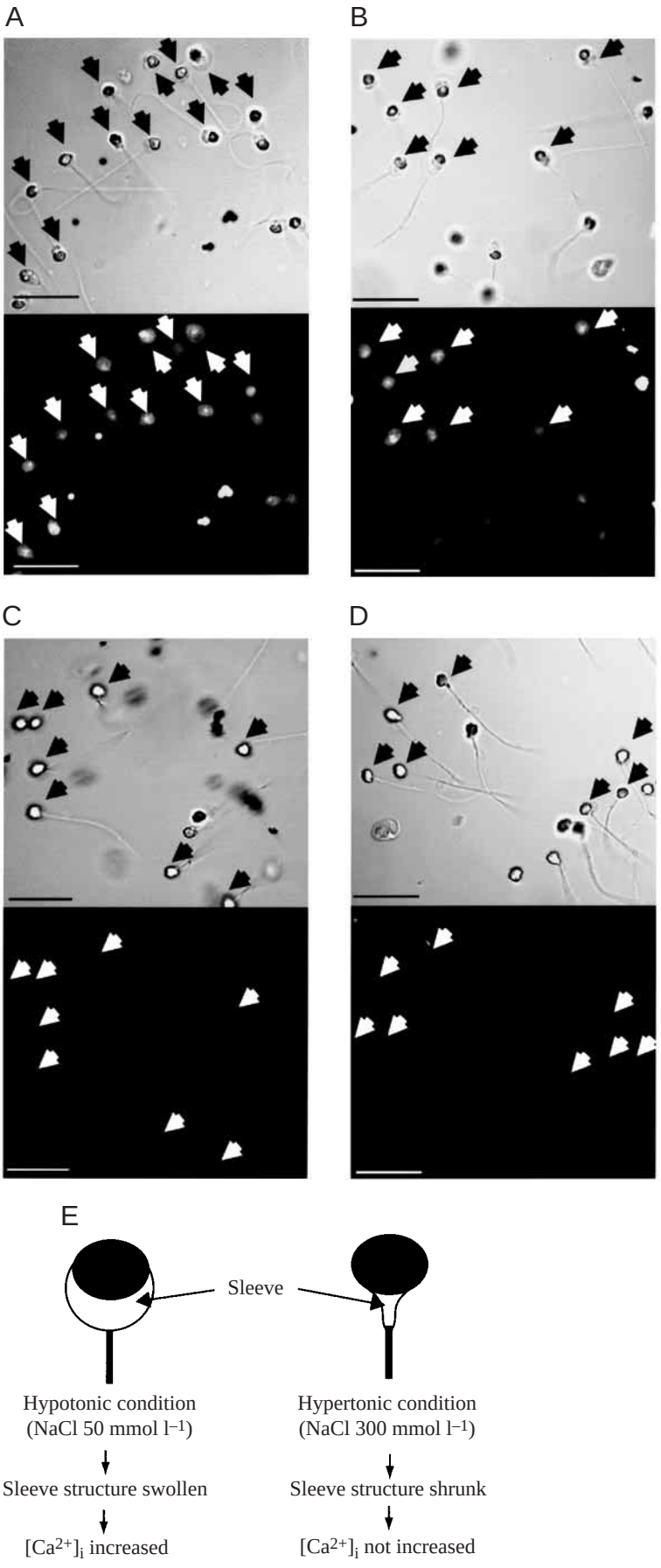

Fig. 2. Change of $[Ca^{2+}]_i$ in hypotonic or hypertonic condition indicated by fluo-3 AM. Sperm were incubated with 500 μmol l^{-1} fluo-3 AM in ASP (artificial seminal plasma) for 2 h. (A,B) Approximately 90% of sperm showed movement after dilution in hypotonic conditions: NaCl 50 mmol l^{-1} + $CaCl_2$ 5 mmol l^{-1} (A) or NaCl 50 mmol l^{-1} + EGTA 5 mmol l^{-1} (B). Confocal micrographs were taken approximately 30 min after the onset of activation, when almost all sperm had stopped moving. (C,D) In hypertonic conditions (300 mmol l^{-1} NaCl), sperm did not move even in the presence of Ca^{2+} (D) and $[Ca^{2+}]_i$ was not increased, as indicated by the absence of fluorescence. Upper panels, phase contrast micrographs; lower photos, fluorescence micrographs. (E) Diagram of sleeve structure expanded in hypotonic conditions (as in A and B) but was shrunk in hypertonic conditions (as in C and D). Bars, 20 μm.

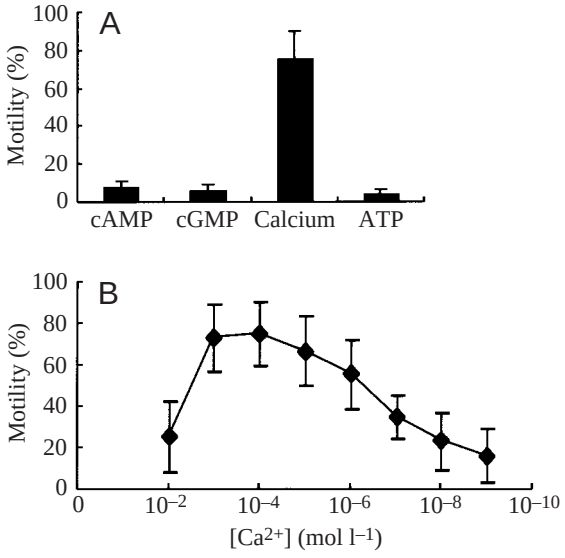

Fig. 3. Effect of (A) cAMP, cGMP and Ca^{2+}, and (B) various concentrations of Ca^{2+} on demembranated sperm motility. Demembranated sperm were suspended in reactivation solutions containing 220 μmol l^{-1} Mg-ATP^{2+}, 175 mmol l^{-1} potassium acetate, 1 mmol l^{-1} free Mg^{2+}, 1 mmol l^{-1} DTT, 0.5 mmol l^{-1} EGTA, 0.5 mmol l^{-1} EDTA, 20 mmol l^{-1} Hepes-NaOH (pH 8.0). (A) Solutions contained 10 μmol l^{-1} cAMP, 10 μmol l^{-1} cGMP, 10^{-4} mol l^{-1} free Ca^{2+} or reactivation solution only (ATP). (B) Effect of $[Ca^{2+}]$ on reactivation of sperm motility. Values are means ± S.D.; *N*=5 (A); *N*=7 (B).

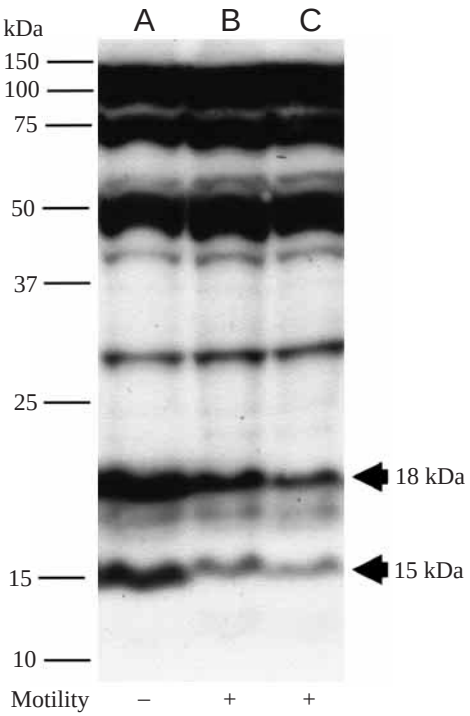

Fig. 4. Motility-dependent dephosphorylation at serine residues in hypotonic conditions. Sperm were diluted in either 300 mmol l^{-1} NaCl solution (lane A), 50 mmol l^{-1} NaCl + 5 mmol l^{-1} $CaCl_2$ (lane B), or 50 mmol l^{-1} NaCl + 5 mmol l^{-1} EGTA (lane C). Sperm were motile in the hypotonic solutions (containing 50 mmol l^{-1} NaCl; B,C) and immotile in the hypertonic solution (300 mmol l^{-1} NaCl; A). Sperm were collected and subjected to western blotting with anti-phosphoserine antibody. Numbers on the left indicate molecular mass markers. Motility is shown below the lanes.

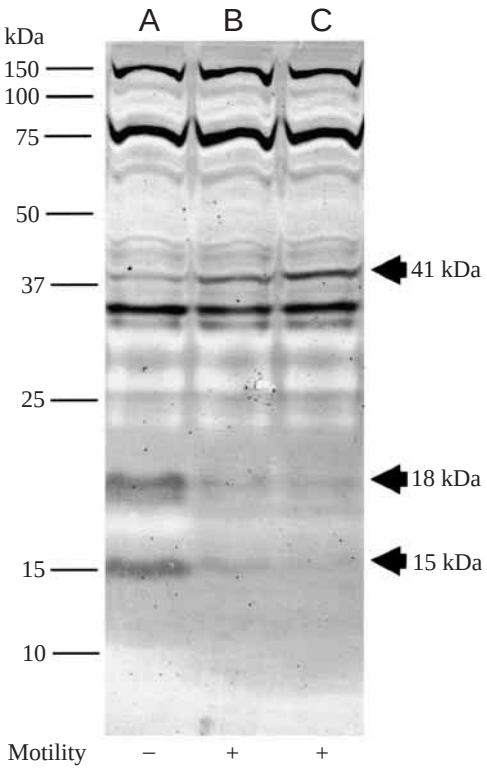

Fig. 5. Motility-dependent phosphorylation or dephosphorylation at threonine residues in hypotonic conditions. Sperm were diluted either in 300 mmol l^{-1} NaCl solution (lane A) or 50 mmol l^{-1} NaCl + 5 mmol l^{-1} $CaCl_2$ (lane B) or 50 mmol l^{-1} NaCl + 5 mmol l^{-1} EGTA (lane C). Sperm were motile in the hypotonic solutions (containing 50 mmol l^{-1} NaCl; B,C) and immotile in hypertonic solution (300 mmol l^{-1} NaCl; A). Sperm were collected and subjected to western blotting with anti-phosphothreonine antibody. Numbers on the left indicate molecular mass markers. Motility is shown below the lanes.

difference in motility when Ca^{2+} was either present or absent in the demembranation solution.

Protein phosphorylation during activation of sperm motility

Tilapia sperm exhibited vigorous motility under conditions of low osmotic pressure associated with increased $[Ca^{2+}]_i$. It is known that many kinds of kinases are activated by Ca^{2+}, including protein kinase C. Therefore, we examined the effect of protein phosphorylation on the activation of motility introduced by transferring sperm from a hypertonic (300 mmol l^{-1} NaCl) to a hypotonic (50 mmol l^{-1} NaCl) medium. The effect of Ca^{2+} in the hypotonic solution was also examined. Western blotting using phosphoserine (pS) and phosphothreonine (pT) antibodies were carried out on PVDF membranes transferred from tricine SDS–PAGE. We found no evidence of tyrosine phosphorylation using phosphotyrosine (pY) antibody in this study.

Western blotting using anti-phosphoserine antibodies revealed that two proteins were dephosphorylated when sperm shifted from the immotile to the motile phase (Fig. 4). The changes in phosphorylation patterns differed in the presence and the absence of extracellular Ca^{2+} (Fig. 4B,C). Two protein bands of 18 kDa and 15 kDa were dephosphorylated after dilution into both hypotonic solutions (50 mmol l^{-1} NaCl + 5 mmol l^{-1} EGTA and 50 mmol l^{-1} NaCl + 5 mmol l^{-1} $CaCl_2$). Therefore, protein dephosphorylation accompanied the motility change whether or not extracellular Ca^{2+} was present.

In order to investigate further the effect of protein phosphorylation on motility activation, phosphorylation of threonine residue(s) was investigated. Changes in phosphorylation were detected in three proteins after motility activation was attained in hypotonic conditions (Fig. 5). The 41 kDa protein was phosphorylated and the 18 kDa and 15 kDa proteins were both dephosphorylated in 50 mmol l^{-1} NaCl solution with or without Ca^{2+}. Protein phosphorylation and dephosphorylation of threonine residue(s) thus occurred in conditions where sperm motility was activated.

Discussion

It has been reported that sperm from seawater-acclimated tilapia, *Tilapia mossambicus*, became immotile on depletion of extracellular Ca^{2+} (Morita and Okuno, 1998; Linhart et al., 1999). Sperm of the freshwater-acclimated tilapia should

acclimate to low osmotic pressure and lower extracellular $[Ca^{2+}]$ in freshwater for fertilization. In the present study, we focused on the regulatory mechanism of sperm motility in freshwater-acclimated tilapia to reveal the osmo-regulatory mechanism for sperm motility in tilapia, a euryhaline teleost. This study also furthers our general understanding of motility regulatory mechanisms in freshwater teleosts such as carp.

Increased $[Ca^{2+}]_i$ on initiation of motility was reported in the marine teleost, pufferfish (Oda and Morisawa, 1993) and salmonid fish (Cosson et al., 1989; Boitano and Omoto, 1992). Increased $[Ca^{2+}]_i$ seems to play an important role in the initiation process of sperm motility. In tilapia sperm, in hypotonic conditions, extracellular Ca^{2+} is not necessary to initiate motility (Fig. 1), although extracellular Ca^{2+} increases motility in certain extent. By contrast, sperm from seawater-reared tilapia require Ca^{2+} for motility (Linhart et al., 1999; Morita and Okuno, 1998).

Osmotic shock induces initiation of motility in sperm of teleosts fertilizing in external spawning grounds (Morisawa and Suzuki, 1980). However, the environment controlling the regulatory mechanism seems to be different, since osmosis between outside and inside the cell is reversed. Hypotonic shock triggers initiation of sperm motility in freshwater teleosts such as cyprinids. By contrast, hyper-osmolality triggers sperm motility in seawater teleosts such as pufferfish. *Tilapia mossambicus* can spawn from freshwater to seawater, as their sperm adapt to the external spawning ground by changing their sensitivity to extracellular $[Ca^{2+}]$ (Morita and Okuno, 1998; Linhart et al., 1999). However, the process by which the motility regulatory mechanism adapts remains obscure. In the present study, we examined the motility of sperm from freshwater-acclimated tilapia, and demonstrate that $[Ca^{2+}]_i$ plays a significant role in motility activation, although sperm can move without extracellular Ca^{2+}.

It is likely that $[Ca^{2+}]_i$ is necessary for motility activation, for the following reasons. Firstly, confocal microscopy using fluo-3 loaded sperm revealed that the $[Ca^{2+}]_i$ was increased independently of the extracellular $[Ca^{2+}]$ when sperm were suspended into a hypotonic solution (Fig. 2A,B). Increase in $[Ca^{2+}]_i$ occurred even when external Ca^{2+} was chelated (Fig. 2B). Therefore, it could be assumed that hypotonic shock triggered the supplementation of Ca^{2+} in the cytoplasm from some intracellular Ca^{2+} store, not from the outside the cell. Secondly, demembranated sperm were reactivated only in the presence of Ca^{2+} concentrations greater than 10^{-6} mol l^{-1}. cAMP and cGMP failed to reactivate the motility.

How is $[Ca^{2+}]_i$ increased in tilapia sperm? In salmonid fish sperm, decreased extracellular $[K^+]$ on ejaculation of sperm into freshwater is thought to induce hyperpolarization of the plasma membrane followed by a transient influx of Ca^{2+} *via* Ca^{2+} channels (Tanimoto and Morisawa, 1988). Thus, extracellular Ca^{2+} is necessary for sperm motility in this species. By contrast, extracellular Ca^{2+} is not necessary for motility of tilapia sperm, and high concentrations of KCl, approximately 100 mmol l^{-1}, had no effect on motility activation in the present study. Therefore, Ca^{2+} supplementation in tilapia must occur intracellularly. Since hypotonic shock induces swelling of the sleeve structure together with increased $[Ca^{2+}]$ in this area (Fig. 2A,B), sleeve structure and some other organelle may be candidates for supplying the Ca^{2+}, and regulated by hypotonic shock.

In salmonid fish sperm, cAMP plays an important role as a second messenger. Both adenylate cyclase activity and the cAMP concentration increase on motility initiation in intact sperm (Morisawa and Ishida, 1987), and cAMP is necessary for reactivating the demembranated sperm (Morisawa and Okuno, 1982). It has been suggested that a cAMP dependent phosphorylation cascade is the main cause of motility activation (Hayashi et al., 1987; Jin et al., 1994). In tilapia, however, cAMP failed to activate the motility of demembranated sperm, which was activated by Ca^{2+}. In cyprinid sperm, cAMP is not required and only increased $[Ca^{2+}]$ is necessary to reactivate demembranated sperm (Krasznai et al., 2000). Thus, an increase in $[Ca^{2+}]_i$ could be sufficient to cause activation of motility. Increased $[Ca^{2+}]_i$ was obtained from extracellular Ca^{2+} *via* Ca^{2+} channels in Cyprinid sperm; however, the present study suggested that extracellular Ca^{2+} influx was not the major source of increased $[Ca^{2+}]_i$ in sperm of tilapia. Ca^{2+} stores are assumed to be involved in sperm of tilapia, operating only in hypotonic conditions to increase $[Ca^{2+}]_i$ in sleeve structures in the neck region of sperm (Fig. 2A,B,E). the sleeve was only expanded in hypotonic solutions, whereas in hypertonic conditions this structure was shrunk; this cell swelling is probably linked with the Ca^{2+} releasing mechanism in hypotonic conditions.

Osmotic regulation of $[Ca^{2+}]_i$ have also been reported in cyprinids, where Ca^{2+} influx for motility initiation is triggered by membrane hyperpolalization induced by the decreasing ion concentration resulting from the decrease in osmolality (Krasznai et al., 2000). In salmonid fish, a transient increase in $[Ca^{2+}]_i$ occurs by the release of Ca^{2+} from intracellular stores (Boitano and Omoto, 1992). In the marine teleost, pufferfish, it is reported that increased $[Ca^{2+}]_i$ initiates sperm motility in hypertonic conditions (Oda and Morisawa, 1993). Changes in osmotic pressure triggered an increase in $[Ca^{2+}]_i$, initiating motility in both freshwater and marine teleosts. It is supposed that osmolality-regulated changes in cell membranes trigger intracellular Ca^{2+} releasing pathways *via* some unknown mechanism.

Electron microscopic analysis (Don and Avtalion, 1993) showed that large amounts of cytoplasm surrounded by plasma membrane similar to endoplasmic reticulum (ER) exist in sleeve structures, and this ER-like structure could be a Ca^{2+} store operated by cell swelling in hypotonic conditions. It is well known that inositol triphosphate and ryanodine receptors attached to the ER, a Ca^{2+} store, and mobilize Ca^{2+} from this store into cytoplasm (Berridge, 1993). Hypotonic shock could then induce mobilization of Ca^{2+} *via* the inositol triphosphate and ryanodine receptors. Osmolality regulation of these two receptors has not, however, yet been reported, and it is possible that the increase in $[Ca^{2+}]_i$ is the result of rupture of ER-like structures in the sleeve caused by cell swelling. Low osmolality has an inhibitory effect on sperm motility in

cyprinid fish, however, and tip of flagellum of carp sperm became folded and swollen within 90–120 s of initiation of motility in freshwater, resulting in serious damage to sperm function (Perchec et al., 1996). Sperm of tilapia must therefore have a means of overcoming potential damage in conditions of low osmolality.

Protein phosphorylation occurs during initiation and activation of sperm motility in salmonid fish, echinoderms and mammals (Morisawa, 1994; Bracho et al., 1998; Nomura et al., 2000). The present study shows that serine and threonine residues of various proteins are phosphorylated and dephosphorylated in solutions that support motility. Since phosphorylation and dephosphorylation occur in many proteins, our criterion for detecting motility-associated phosphophorylation was that since the motility activation occurred within several seconds, any phosphorylation or dephosphorylation events associated with motility activation must occur within a time equal to or quicker than that of the motility activation. In the present study, we therefore prepared protein samples from sperm for analysis 1 min after the activation.

Phosphorylation at threonine residues occurred in a 41 kDa protein independently of extracellular [Ca^{2+}] (Fig. 5) and dephosphorylation at serine and threonine residues occurred in 18 kDa and 15 kDa proteins (Figs 4 and 5). It is likely that these proteins phosphorylation and dephosphorylation of these proteins was probably linked with increased [Ca^{2+}]$_i$ (Fig. 2), and possibly with the Ca^{2+} uptake mechanisms. Whether Ca^{2+} uptake also involved activation of kinases such as protein kinase C remains for to be investigated. It is likely that the phosphorylation/dephosphorylation of these proteins in hypotonic solutions is associated with initiation of motility and flagellar activation *via* dynein activation.

18 kDa and 15 kDa proteins are similar in molecular mass to the 15 kDa protein that plays an important role in initiation process of sperm motility in salmonids (Morisawa and Hayashi, 1986; Hayashi et al., 1987; Jin et al., 1994), although phosphorylation in the latter protein occurred at tyrosine residues. A 15 kDa protein was phosphorylated during initiation of motility of chum salmon sperm, in contrast to the dephosphorylation of 15 kDa and 18 kDa proteins seen in tilapia sperm under hypo-osmotic conditions. Therefore, it is possible that these proteins are not the same as the 15 kDa proteins in chum salmon sperm, where increased [Ca^{2+}] is required to stimulate cAMP synthesis needed to initiate sperm motility (Jin et al., 2000), *via* protein phosphorylation cascades. It is thus likely that not only protein phosphorylation but also protein dephosphorylation play important roles in the sperm motility activation process under hypotonic conditions in sperm of freshwater-acclimated tilapia.

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