The Journal of Experimental Biology 215, 630-641

doi:10.1242/jeb.063628

RESEARCH ARTICLE

Transient Ca^{2+} mobilization caused by osmotic shock initiates salmonid fish sperm motility

Gen L. Takei, Chinatsu Mukai and Makoto Okuno*

Department of Life Sciences, Graduate School of Arts and Sciences, The University of Tokyo Komaba 3-8-1, Meguro-ku, Tokyo, 153-8902 Japan

*Author for correspondence (cokuno@mail.ecc.u-tokyo.ac.jp)

Accepted 2 November 2011

SUMMARY

Salmonid fish sperm motility is known to be suppressed in millimolar concentrations of extracellular K^+, and dilution of K^+ upon spawning triggers cAMP-dependent signaling for motility initiation. In a previous study, however, we demonstrated that suspending sperm in a 10% glycerol solution and subsequent dilution into a low-osmotic solution induced motility independently of extracellular K^+ and cAMP. In the present study, we further investigated the glycerol-induced motility mechanism. We found that treatment with solutions consisting of organic or inorganic ions, as well as glycerol, induced sperm motility in an osmolarity-dependent manner. Elimination of intracellular Ca^{2+} by BAPTA-AM significantly inhibited glycerol-treated sperm motility, whereas removal of extracellular Ca^{2+} by EGTA did not. Monitoring intracellular Ca^{2+}, using fluo-4, revealed that intracellular Ca^{2+} increased when sperm were suspended in hypertonic solutions, and a subsequent dilution into a hypotonic solution led to a decrease in intracellular Ca^{2+} concomitant with motility initiation. In addition, upon dilution of sperm into a hypertonic glycerol solution prior to demembranation, the motility of demembranated sperm was reactivated in the absence of cAMP. The motility recovery suggests that completion of axonemal maturation occurred during exposure to a hypertonic environment. As a result, it is likely that glycerol treatment of sperm undergoing hypertonic shock causes mobilization of intracellular Ca^{2+} from the intracellular Ca^{2+} store and also causes maturation of axonemal proteins for motility initiation. The subsequent dilution into a hypotonic solution induces a decrease in intracellular Ca^{2+} and flagellar movement. This novel mechanism of sperm motility initiation seems to act in a salvaging manner for the well-known K^+-dependent pathway.

Key words: sperm motility, motility initiation, salmonid fish, osmotic shock, Ca^{2+}, calcium imaging.

INTRODUCTION

Sperm motility is indispensable for fertilization success. Fish sperm are known to be motile for very short periods, usually limited to minutes (Cosson, 2010). Thus, they must be kept immotile until they come into contact with female gametes. Therefore, initiation and activation of sperm motility must be precisely controlled in order to prevent exhaustion prior to ejaculation.

The sperm of teleosts are kept immotile in the testis. Upon spawning, their motility is triggered by exposure to an environment different from seminal plasma (Alavi and Cosson, 2006). Teleosts inhabit, and reproduce in, various environments. Therefore, initiation and activation of teleost sperm motility have adapted to these various environments. For example, sperm of freshwater teleosts, such as common carp and zebrafish, are immotile in an isotonic environment (~300 mosmol kg^{-1}). When these sperm were suspended into a hypotonic condition (<300 mosmol kg^{-1}, such as fresh water), they initiate motility in a Ca^{2+}-dependent and cAMP-independent manner (Krasznai et al., 2000; Takai and Morisawa, 1995; Morisawa and Suzuki, 1980). By contrast, sperm of marine teleosts, such as flounder and puffer fish, become motile in a Ca^{2+}-dependent and cAMP-independent manner (Oda and Morisawa, 1993; Takai and Morisawa, 1995) when released into hypertonic conditions (>300 mosmol kg^{-1}, such as sea water). Moreover, sperm of tilapia, a euryhalin teleost, activate their motility by a change in osmolarity between the seminal plasma and the external medium. Tilapia sperm, which have acclimated to fresh water, activate their motility by hypo-osmotic shock, whereas tilapia sperm, which have acclimated to seawater, activate motility by hyper-osmotic shock. The signaling pathway of tilapia sperm is Ca^{2+} dependent and cAMP independent (Morita et al., 2003). These facts suggest that osmotic shock and a Ca^{2+} change commonly play an important role in sperm motility activation in various teleosts.

By contrast, sperm motility of salmonid fishes is suppressed by the millimolar concentrations of extracellular K^+ in seminal plasma, and a decrease in extracellular K^+, upon spawning, triggers the signaling for initiation of sperm motility (Morisawa and Suzuki, 1980). The decrease in K^+ causes efflux of K^+ through certain K^+ channels and is followed by hyperpolarization of the plasma membrane (Tanimoto et al., 1994; Boitano and Omoto, 1991). Subsequently, the intracellular Ca^{2+} concentration increases (Cosson et al., 1989; Boitano and Omoto, 1992; Tanimoto et al., 1994), stimulating synthesis of cAMP (Morisawa and Ishida, 1987). The increase in cAMP leads to the initiation of sperm motility through cAMP-dependent protein phosphorylation of the axonemal components, including the 15-kDa protein and 22-kDa dynein light chain (Morisawa and Okuno, 1982; Morisawa and Hayashi, 1985; Hayashi et al., 1987; Inaba et al., 1998).

However, Morita and colleagues (Morita el al., 2005) reported that treatment of sperm with a 10% glycerol solution, before subsequent dilution into a low-osmotic solution, induced the

initiation of motility even though a high concentration of extracellular K^+ – for example, 100 mmol l^{-1} – was present. It was also reported that some axonemal components, including the 22-kDa dynein light chain, were phosphorylated in the glycerol-treated sperm in the absence of cAMP synthesis. The detected pattern of phosphorylation was different from that formed with accompanying cAMP synthesis. This result indicates the presence of a novel salvaging pathway for salmonid fish sperm motility initiation that bypasses K^+ efflux and the synthesis of cAMP. However, a detailed mechanism of motility initiation due to glycerol treatment remains unclear, and certain questions arise concerning which effect of glycerol treatment induces sperm motility and what is happening downstream from the glycerol treatment.

Although this glycerol-treated initiation of sperm motility is an artificial, yet specific, means of initiation, elucidation of this mechanism and comparison with conventional signaling pathways are expected to give us valuable information for understanding the signaling mechanism for salmonid fish sperm motility. Thus, in the present study, we investigated the motility initiation mechanism by glycerol treatment, specifically focusing on the triggering factor for initiation and the involvement of Ca^{2+}, using the fluorescent dye Fluo-4. Finally, we revealed that the motility of salmonid fish sperm could be initiated in an osmolarity- and Ca^{2+}-dependent manner similar to that of the sperm of other teleosts.

MATERIALS AND METHODS

Chemicals and solutions

Chelerythrine, calphostin C, H-89, H-85, BAPTA-AM, U 73122 and U 73343 were purchased from Merck KGaA (Darmstad, Germany). Dithiothreitol (DTT) was purchased from Sigma (St Louis, MO, USA). EGTA and Fluo-4 AM were purchased from Dojindo (Kumamoto, Japan). All other chemicals were reagent grade and were purchased from Wako Pure chemicals (Osaka, Japan).

Glycerol solution consisted of 0.5–2 mol l^{-1} glycerol (or other substances), 150 mmol l^{-1} KCl, 0.5 mmol l^{-1} DTT, 0.5 mmol l^{-1} EDTA and 20 mmol l^{-1} Hepes–NaOH, pH 8.0. Sodium test solution (STS) contained 100 mmol l^{-1} NaCl, 10 mmol l^{-1} Hepes–NaOH, pH 8.0, and potassium test solution (PTS) contained 100 mmol l^{-1} KCl and 10 mmol l^{-1} Hepes–NaOH, pH 8.0. Artificial seminal plasma (ASP) consisted of 130 mmol l^{-1} NaCl, 40 mmol l^{-1} KCl, 2.5 mmol l^{-1} $CaCl_2$, 1.5 mmol l^{-1} $MgCl_2$ and 10 mmol l^{-1} Hepes–NaOH, pH 8.3. The composition of ASP was based on the data of Kho and colleagues (Kho et al., 2001), with slight modification.

BAPTA-AM and inhibitors were dissolved in dimethylsulfoxide (DMSO), and the final concentration of DMSO in all experimental solutions was set below 0.5% (v/v). For control experiments, the same concentration of DMSO was added to each experimental solution.

Collection of sperm

In the present study, the sperm of the rainbow trout *Oncorhynchus mykiss* Walbaum 1792 and chum salmon *Oncorhynchus keta* (Walbaum 1792) were used as salmonid fish sperm. Chum salmon returning to the Ohtsuchi River in Iwate prefecture, Japan, from late November to December were kindly provided by the Ohtsuchi fisherman's union. Rainbow trout were kindly provided by the Oshino Branch of Yamanashi Prefectural Fisheries Experiment Station. Two strains of rainbow trout were used: a 'fall strain', which gave sperm from November to January and a 'summer strain' that matured from June to July. No significant difference in sperm behavior between the two strains was detected. Semen was collected by inserting a pipette directly into the sperm duct and stored on ice until use.

Activation of sperm motility

In the present study, sperm motility was activated by two methods as follows. Semen was diluted directly into 2000 volumes of potassium-free STS (intact sperm). Semen was diluted 100-fold with ice-cold hypertonic solution for 15 s; and then 2 µl of the sperm suspension was resuspended in 40 µl of PTS on a glass slide (glycerol-treated sperm). Sperm movements were recorded using a video recorder (HR-G11, Victor JVC, Kanagawa, Japan) and a CCD camera (WAT-902H Ultimate, Watec, Yamagata, Japan) mounted on a phase-contrast microscope (Optiphoto; Nikon, Tokyo, Japan). In order to elucidate which factor in the glycerol treatment is important for motility initiation, glycerol was substituted for other substances at various concentrations.

Effect of a Ca^{2+} chelator

The effect of extracellular Ca^{2+} on motility activation was examined using the Ca^{2+} chelator EGTA. 1 mmol l^{-1} EGTA was added to the STS in intact sperm or PTS in glycerol-treated sperm to remove extracellular Ca^{2+}. The resultant concentrations of free Ca^{2+} in those solutions were calculated to be less than 10^{-10} mol l^{-1}, including Ca^{2+} contamination from semen. The concentration of free Ca^{2+} was calculated by the algorithms published by Goldstein (Goldstein, 1979).

To investigate the effect of intracellular Ca^{2+} on motility activation, intracellular Ca^{2+} was eliminated by using BAPTA-AM. To load BAPTA into sperm cells, semen was diluted twofold in ASP containing 200 or 1000 µmol l^{-1} of BAPTA-AM (final concentration 100 and 500 µmol l^{-1}, respectively) and incubated for 30 min at room temperature. After incubation, the sperm suspension was treated by the two methods of activation previously described.

Ca^{2+} imaging

Intracellular Ca^{2+} was monitored by a Ca^{2+}-fluorescent dye, Fluo-4 (excitation 495 nm; emission 518 nm). Semen was suspended in the same volume of ASP, containing 150 µmol l^{-1} of Fluo-4 AM (final concentration 75 µmol l^{-1}) and 0.6% (v/v) Powerload (final concentration 0.3%) and incubated at 10°C for 2 h for dye loading. After dye loading, semen was placed at a window of the perfusion chamber. The perfusion chamber was made by longitudinally putting two strips of plastic tape at 10 mm intervals on glass slides and then fixing a coverslip to the strips with additional plastic tapes. ASP without Fluo-4 was applied to one side the chamber window and absorbed by filter paper at the other window. Additionally, various experimental solutions (glycerol solution, STS, PTS) were perfused using the same method. Fluorescent images emitted from sperm were captured using fluorescence microscopy (BX51, Olympus, Tokyo, Japan) and an Olympus filter set (excitation filter, BP470–490; dichromatic mirror, DM 505; emission filter, BA 510–550) and recorded digitally using an EMCCD camera (Luca S, Andor Technology, Belfast, Northern Ireland). Recorded images were analyzed with Andor Solis software (Andor Technology, Belfast, Northern Ireland). The fluorescence intensity (FI) of the sperm head was determined by selecting a square region surrounding the sperm head and then calculating the mean FI value of this region using Andor Solis. Calculated values were multiplied by the area of the square and the background FI value was subtracted, which was determined in the same manner as that for the sperm head. The FI value of the tail region was determined similarly, except for the size of the square, using the center of the sperm tail.

Demembranation and reactivation of sperm

Reactivation of sperm flagellar movement was performed based on the methods of Morisawa and Okuno (Morisawa and Okuno, 1982)

and Cosson and colleagues (Cosson et al., 1995), with slight modifications. Briefly, dry sperm was diluted in ASP (or STS, glycerol solution in other experiments) by a factor of 100. After the appropriate incubation time, 5 µl of the sperm suspension was mixed with 200 µl of demembranating solution, containing 150 mmol l^{-1} K-acetate, 0.5 mmol l^{-1} EDTA, 20 mmol l^{-1} Hepes–KOH (pH 8.2), 2 mmol l^{-1} DTT and 0.04% Triton-X100. After a 30 s incubation on ice, 2.5 µl of the demembranated sperm suspension was again diluted with 50 µl of reactivation solution, containing 150 mmol l^{-1} K-acetate, 1 mmol l^{-1} EGTA, 20 mmol l^{-1} Hepes–KOH (pH 8.2), 2 mmol l^{-1} DTT and 0.2 mmol l^{-1} ATP, with or without 10 µmol l^{-1} cAMP. After gentle mixing, an aliquot of reactivated sperm suspension was placed onto glass slides, covered with a coverslip and observed using a phase-contrast microscope.

Effect of inhibitors

The semen was diluted twofold in ASP containing various concentrations of inhibitors, as illustrated in the figures, and incubated for an appropriate time (30–60 min). After incubation, the sperm motility was examined by the two methods described previously using solutions containing the same concentrations of inhibitors. The effects of inhibitors on sperm motility were expressed in terms of the percentage of motile spermatozoa. The incubation time and temperature were noted during individual experiments. U 73122 [an inhibitor of phospholipase C (PLC)], H-89 [an inhibitor of protein kinase A (PKA)] and chelerythrine and calphostin C [both inhibitors of protein kinase C (PKC)] were used in this study.

RESULTS

Effect of osmolarity of glycerol solution on motility activation

Morita and colleagues (Morita et al., 2005) reported that, when chum salmon sperm were treated once with 1.3 mol l^{-1} glycerol or an organic alcohol solution followed by dilution with a hypotonic solution, motility initiation was observed even in motility-inhibiting PTS. In rainbow trout sperm, treatment with 1.3 mol l^{-1} glycerol and an organic alcohol, such as erythritol, also provided high sperm motility in PTS (data not shown). To elucidate the motility-triggering factor of treatment with glycerol, we first substituted several compounds for glycerol and looked at the effect of osmolarity on rainbow trout sperm motility. Fig. 1 shows the result of using glycerol, NaCl and KCl. No sperm motility was observed when sperm were treated with a 0.5 osmol l^{-1} glycerol solution. Treatment with a glycerol solution of more than 0.75 osmol l^{-1} caused sperm motility, and they exhibited the highest motility at 1.25 osmol l^{-1}. Treatment with a glycerol solution of more than 1.5 osmol l^{-1} caused a gradual decrease in sperm motility. Similar results were obtained with electrolyte solutions (NaCl or KCl). However, motility was considerably lower in high-osmotic (>1.25 osmol l^{-1}) electrolyte solutions owing to sperm aggregation.

To investigate the effect of the chemical properties of solutes, such as molecular weight and charge, on sperm motility initiation, we

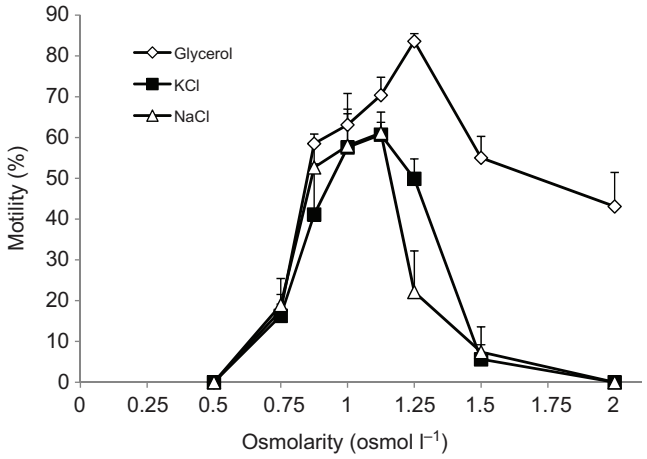

Fig. 1. Effect of osmolarity on initiation of sperm motility. Rainbow trout sperm were treated with hypertonic solutions of various osmolarities for 15 s and subsequently diluted into a potassium solution to activate the motility. The percentage of motile sperm was determined in each condition. Osmolarity was adjusted with glycerol, potassium chloride or sodium chloride. Bars represent the s.e.m. *N*=4.

treated chum salmon sperm with various compounds, including organic alcohol (glycerol, ethylene glycol, polyethylene glycol 200), sugar (sucrose), organic ion (choline chloride) and inorganic ions (NaCl, KCl and sodium acetate). The results are shown in Table 1. As previously shown by Morita and colleagues (Morita et al., 2005) and Fig. 1, organic alcohol and inorganic ions, with the exception of ethylene glycol, induced sperm motility. In addition, treatment with organic ions, such as choline chloride, and sugars, such as sucrose, also induced motility. Furthermore, osmolarity greater than 0.75 osmol l^{-1} was required for motility initiation in all solutes. These results indicate that treatment with a hyper-osmotic solution and the subsequent dilution into hypo-osmotic solution induces salmonid fish sperm motility, independent of solute molecular weight or charge.

The percentage of motility-initiated sperm reached a plateau at 1–1.125 osmol l^{-1} of a glycerol–NaCl–KCl solution. Therefore, hypertonic solutions of 1.125 osmol l^{-1} were used in the following experiments.

Effect of a Ca^{2+} chelator on sperm motility activation

The effects of Ca^{2+} on sperm motility initiation were examined. To test the effect of Ca^{2+} on motility activation of glycerol-treated and intact sperm, extracellular and intracellular Ca^{2+} ions were removed using EGTA and BAPTA-AM, respectively.

In both intact and glycerol-treated sperm from rainbow trout, the elimination of extracellular Ca^{2+} by 1 mmol l^{-1} EGTA had no effect on sperm motility initiation (Fig. 2A), although the swimming paths of intact sperm were altered considerably. This effect occurred at a

Table 1. Activation of chum salmon sperm motility by various chemicals

	Sucrose	PEG 200	Choline chloride	Glycerol	Sodium acetate	KCl	NaCl	Ethylene glycol
Molecular weight	342.3	200	139.6	92.1	82.03	74.55	58.44	62.07
Molarity (mol l^{-1})	0.75	0.75	0.38	0.75	0.38	0.38	0.38	–
Osmolarity (osmol l^{-1})	0.75	0.75	0.75	0.75	0.75	0.75	0.75	–
Motility (>50%)	○	○	○	○	○	○	○	×

Glycerol in glycerol solution was substituted by various chemicals and whether treatment with these solutions was able to initiate chum salmon sperm motility was examined. The osmolarity and molarity required for motility activation and molecular weight of each chemical are also shown. Sperm that exhibited a level greater than 50% were defined as 'motile' and are represented by circles, while low motility of less than 50% is represented by crosses.

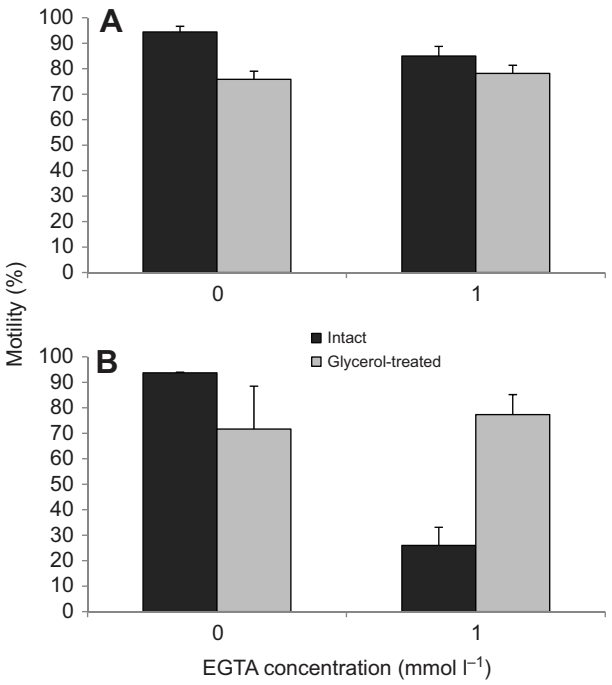

Fig. 2. Effect of extracellular calcium ions on sperm activation. The effect of extracellular calcium ions on rainbow trout (A) and chum salmon (B) sperm activation was evaluated by chelating calcium ions in the media with 1 mmol l^{-1} EGTA. Motility activation was significantly inhibited by 1 mmol l^{-1} EGTA in intact sperm of chum salmon (P<0.01). No influence on motility rate was observed in intact sperm of rainbow trout and glycerol-treated sperm of both species. Statistical tests were performed using Student's t-tests. The calculated concentration of calcium ions in the media was $<10^{-10}$ mol l^{-1}. The concentration of free Ca^{2+} was calculated by the algorithms published by Goldstein (Goldstein, 1979). Values are means ± s.e.m. N=4.

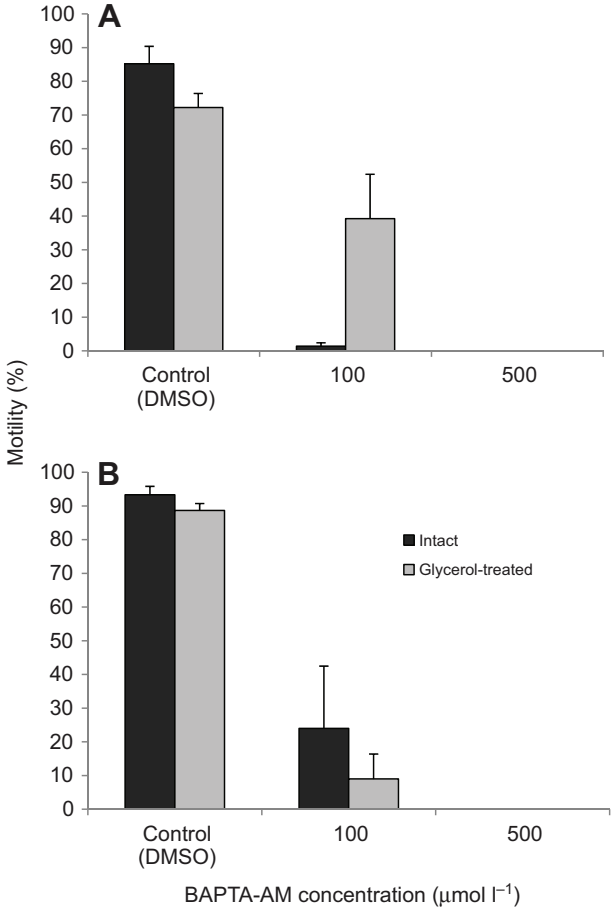

Fig. 3. Effect of intracellular calcium ions on sperm activation. The effect of intracellular calcium ions on rainbow trout (A) and chum salmon (B) sperm activation was evaluated by chelating intracellular calcium ions using 100 or 500 μmol l^{-1} BAPTA-AM. Semen was diluted with ASP containing each concentration of BAPTA-AM and incubated for 30 min at room temperature. A significant decrease (P<0.01) of motility was observed in both intact sperm and glycerol-treated sperm of both species. Statistical tests were performed using Student's t-tests. Values are means ± s.e.m. N=4–5.

Ca^{2+} concentration calculated to be $<10^{-10}$ mol l^{-1}. By contrast, when sperm were preloaded with BAPTA-AM to eliminate intracellular Ca^{2+}, the motility of both intact and glycerol-treated sperm was significantly inhibited, in a dose-dependent manner (Fig. 3A).

In the case of chum salmon sperm, motility initiation of intact sperm was significantly inhibited by 1 mmol l^{-1} EGTA, unlike rainbow trout sperm, whereas glycerol-treated chum salmon sperm were not inhibited, similar to rainbow trout sperm (Fig. 2B). As in the case of rainbow trout sperm, BAPTA-loaded chum salmon sperm motility was severely inhibited in both intact and glycerol-treated sperm (Fig. 3B). These results indicate that Ca^{2+} plays an important role in both intact and glycerol-treated sperm; intact sperm are more dependent on extracellular Ca^{2+}, contrary to glycerol-treated sperm, which showed no dependency on extracellular Ca^{2+}.

Change in intracellular Ca^{2+} concentration during motility activation

In glycerol-treated sperm, high motility was observed even when extracellular Ca^{2+} had been depleted with EGTA. However, when sperm were loaded with BAPTA-AM, glycerol-treated sperm showed decreased motility. This result suggests that mobilization of Ca^{2+} from intracellular Ca^{2+} stores might be involved in the regulatory mechanism of glycerol-treated sperm. In order to examine the involvement of Ca^{2+} during motility activation, intracellular Ca^{2+} was monitored with the fluorescent dye Fluo-4, using a perfusion technique. Fig. 4 shows the change in the level of intracellular Ca^{2+} in rainbow trout sperm during motility activation.

When sperm were perfused with a hyper-osmotic glycerol solution, a slight decrease in FI was observed following the onset of perfusion, and a subsequent rapid increase of FI, mainly in the head-to-neck region, was also observed (Fig. 4A,B,F,G,K). The increased FI was followed by a gradual decrease over time (Fig. 4B,G). By contrast, PTS perfusion following glycerol solution perfusion (glycerol treatment) caused a rapid decrease in FI in the head region (Fig. 4A,F). The FI at the center of the tail region did not change in glycerol-treated sperm (Fig. 4K). Perfusion with other hypertonic solutions, which consisted of either ionic or nonionic substances, also caused an increase in FI, whereas perfusion of hypotonic STS, following hypertonic solution perfusion, caused a decrease in FI, as shown in Fig. 4A,F (data not shown). These results demonstrate that hypertonic treatment causes a rapid increase of intracellular Ca^{2+}, and the subsequent hypotonic shock causes a rapid decrease in intracellular Ca^{2+}.

It was very hard to determine the absolute value of the intracellular Ca^{2+} concentration owing to individual variance and the technical difficulty of these experiments. However, the maximum value of intracellular Ca^{2+} in the head region during perfusion with a glycerol solution was assumed to be approximately 10^{-6} mol l^{-1}, as

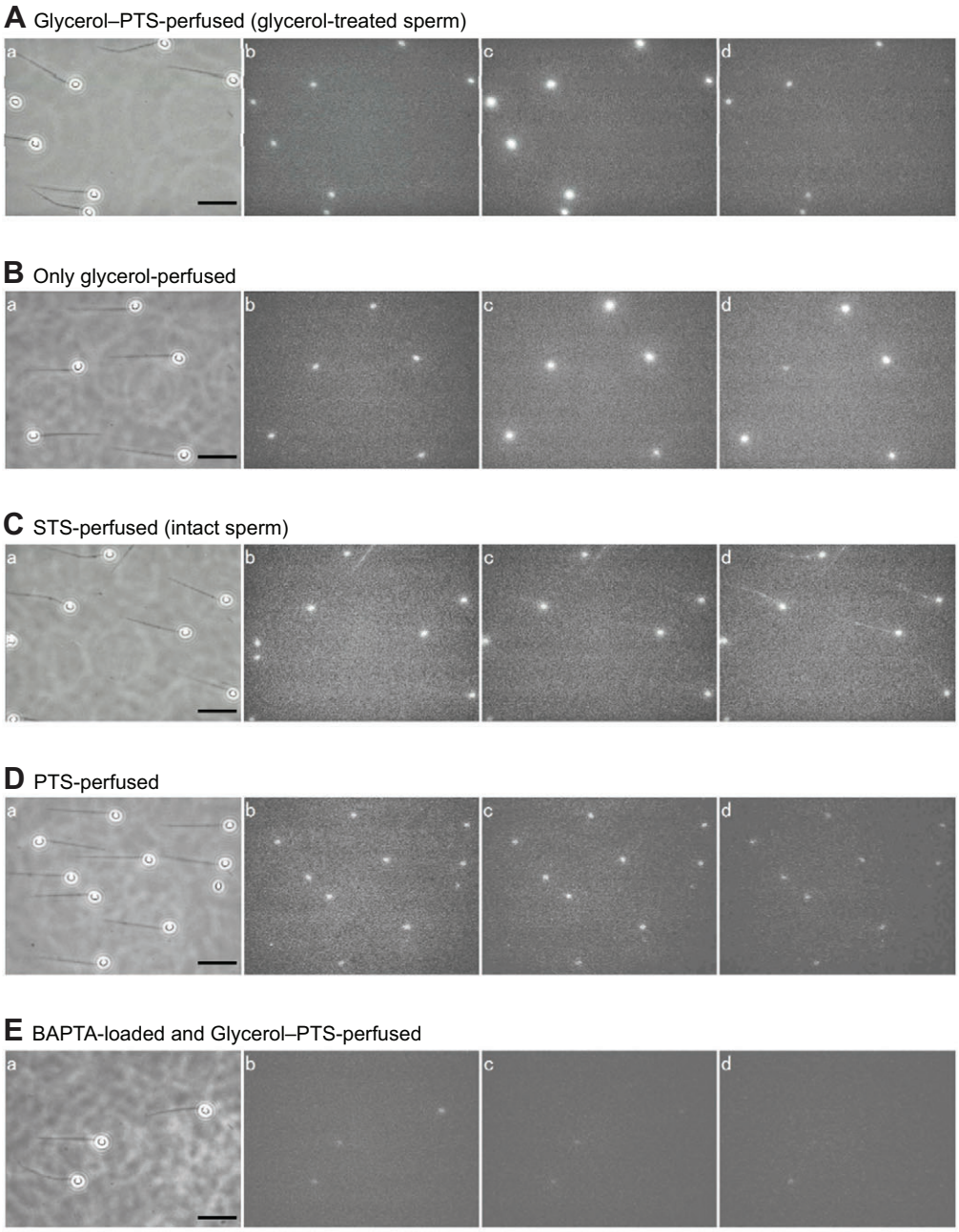

Fig. 4. For legend see facing page.

derived from the similar value obtained when sperm were perfused with a Ca^{2+} ionophore (data not shown). When sperm were preloaded with BAPTA, almost no change of FI during perfusion was observed, suggesting the removal of intracellular Ca^{2+} by BAPTA (Fig. 4E,J).

Intact sperm exhibited a different pattern of FI. Perfusion of STS, using intact sperm, also caused a rapid increase of FI, following a slight decrease of FI at the beginning of perfusion. However, in contrast to glycerol-treated sperm, the fluorescence of intact sperm was observed mainly in the tail region, and fluorescence of the head region was less bright (Fig. 4C,H,L). In fact, the maximum FI value of intact sperm in the head region was only 1.5-fold higher when compared with the value before perfusion, whereas glycerol-treated sperm showed more than a fourfold increase (Fig. 5A). In addition, the time required for the onset of the increase in FI of the head in intact sperm was significantly shorter than that of glycerol-perfused sperm (Fig. 5B). Moreover, the fluorescence emission pattern was quite different between intact and glycerol-perfused sperm. In glycerol-perfused sperm, emission started from the specific region of the head around the basal body and mid-piece (Fig. 6A). By contrast, no such specific region was observed in intact sperm (Fig. 6B). These results show that the change in intracellular Ca^{2+} during motility activation is different between intact and glycerol-treated sperm. There was no significant change when sperm were perfused with PTS (Fig. 4D,I).

Motility of demembranated sperm

Demembranated and reactivated trout sperm motility experiments were performed to examine whether glycerol treatment induces axonemal maturation (Fig. 7). As reported previously by Morisawa

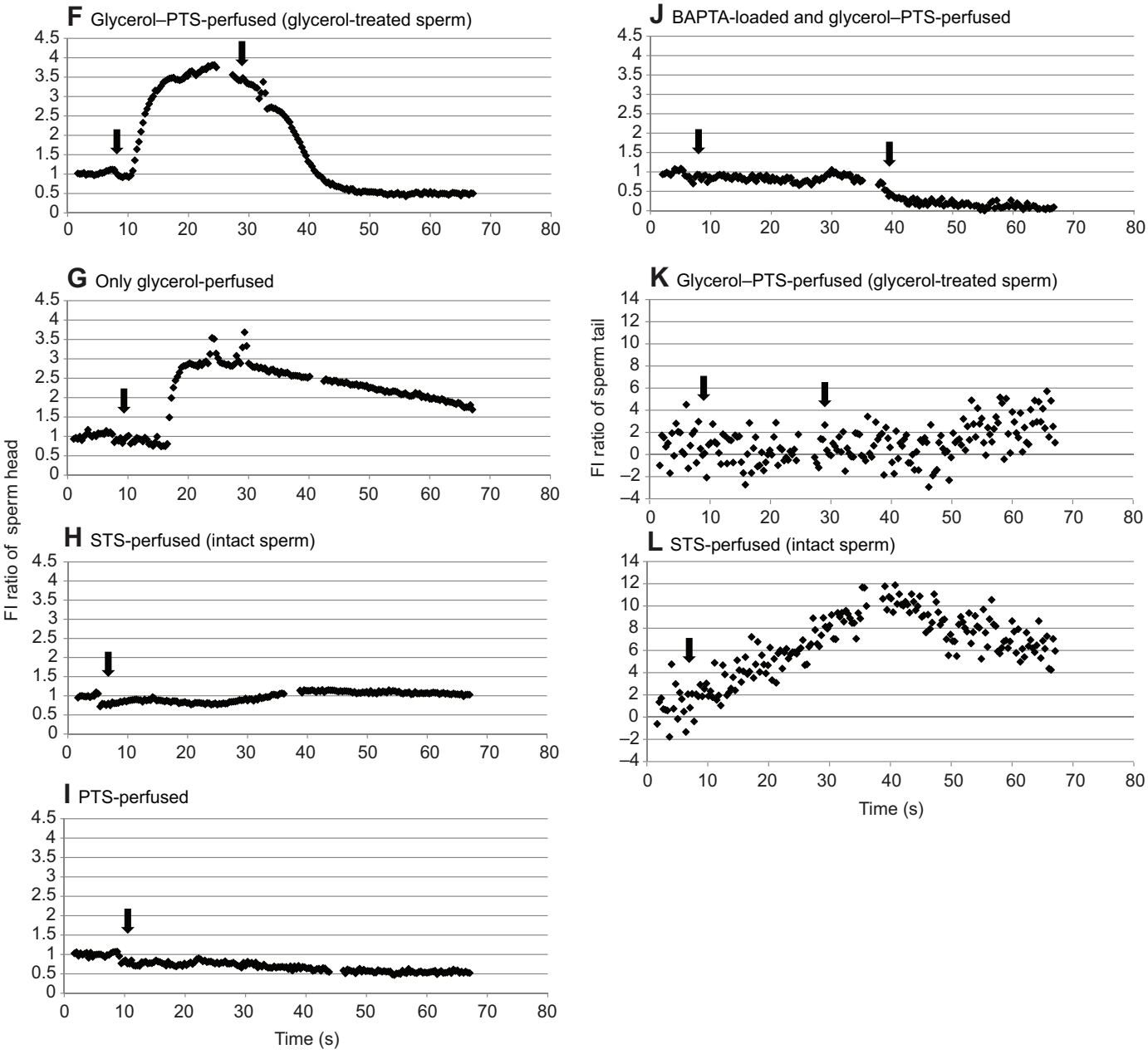

Fig. 4. Change in intracellular Ca^{2+} concentration of rainbow trout spermatozoa indicated by Fluo-4. Rainbow trout sperm were incubated with Fluo-4 AM in ASP for 2 h, and loaded sperm were applied to a perfusion chamber. Then, various experimental solutions were perfused, and fluorescence was observed by fluorescence microscopy. (A–E) Fluorescence images of sperm during perfusion. (F–J) Typical Fl change of sperm head during perfusion. (K,L) Typical Fl change of sperm tail during perfusion. (A,F,K) Glycerol solution and subsequently PTS were perfused (glycerol-treated sperm). Fluorescence increased primarily in the head region after glycerol solution perfusion and rapidly decreased after PTS perfusion. (B,G) When only a glycerol solution was perfused, the fluorescence increased, as shown in glycerol-treated sperm, but there was not a rapid decrease. (C,H,L) Sperm perfused with STS (intact sperm) showed an increase of fluorescence primarily in the tail region. (D,I) When PTS was perfused, sperm did not show an increase at all. (E,J) Sperm preloaded with 500 μmol l^{-1} BAPTA-AM were perfused with a glycerol solution and subsequently PTS. Almost no change in fluorescence was observed in these sperm. In A–E, panel a: phase-contrast images, panel b: sperm fluorescence images before perfusion, panel c: 8 s after perfusion and panel d: 8 s after a second perfusion (or 30 s after first perfusion without second perfusion) are shown sequentially from left to right. Arrows in the graph indicate the onset of perfusion. Scale bars, 20 μm.

and Okuno (Morisawa and Okuno, 1982) and Cosson and colleagues (Cosson et al., 1995), demembranated trout sperm were almost completely immotile in the reactivation solution that contained 200 μmol l^{-1} ATP without cAMP. Supplementation of cAMP to the reactivation solution caused reactivated motility in more than 70% of sperm. By contrast, when trout sperm were diluted in STS, in which sperm exhibited motility, for 30 s before demembranation, flagellar movement occurred without cAMP, as reported by Morisawa and Okuno (Morisawa and Okuno, 1982). This result indicates that reduction of external K^+ induces maturation of the sperm motility apparatus, including protein phosphorylation for motility initiation. Similarly, when sperm were diluted in a glycerol

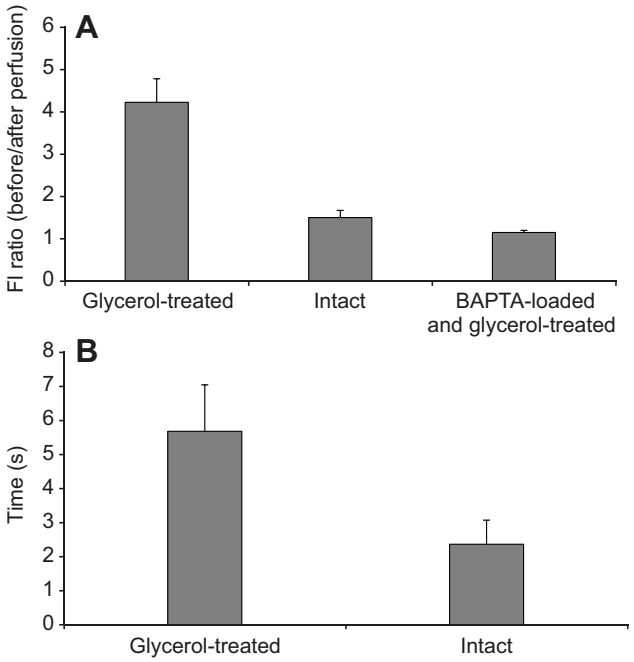

Fig. 5. Characterization of the FI change of trout sperm during perfusion. Maximum FI values of sperm head after perfusion (A) and the time required to emit after onset of perfusion (B) were determined. (A) Maximum FI values after perfusion were expressed as the ratio of the raw value of the maximum FI to the mean FI value of pre-perfusion. The fluorescence of glycerol-treated sperm heads increased by fourfold, whereas glycerol-treated BAPTA-loaded sperm showed no increase of FI. The fluorescence of intact sperm heads increased, although significantly less than that of glycerol-treated sperm. (B) The time-point of slight decrease in FI was set to be the beginning of perfusion, and the time that passed from the beginning of perfusion to the onset of an increase in fluorescence was measured in each sperm head region. The time required to emit was significantly longer in glycerol-treated sperm than in intact sperm (P<0.05). Statistical tests were performed using Student's t-tests. Values are means ± s.e.m. N=4–8.

solution for 15 s before demembranation, >50% of the sperm were reactivated when cAMP was not supplemented in the reactivation solution. Demembranation of sperm after glycerol treatment also resulted in reactivated motility in the absence of cAMP; however, the percentage of motile sperm was slightly lower than the preceding three preparations. These results indicate that hypertonic shock induces maturation of axonemal components, similar to the effects of external K^+ reduction.

Effect of inhibitors

When U 73122, an inhibitor of PLC, was applied to experimental solutions, motility activation of both intact and glycerol-treated sperm was significantly inhibited at 20 μmol l^{-1} and was completely inhibited at 50 μmol l^{-1} (Fig. 8). By contrast, the same concentrations of U 73343, an inactive analog of U 73122, did not inhibit sperm motility (Fig. 8).

Fig. 9 shows the effect of H-89, a potent inhibitor of PKA. In rainbow trout, H-89 significantly inhibited activation of intact sperm motility in a dose-dependent manner. Only 12% of intact sperm were activated in the presence of 30 μmol l^{-1} H-89. In contrast, 50% of glycerol-treated sperm activated motility at the same concentrations of H-89. H-85, an inactive analog of H-89, did not significantly inhibit either intact or glycerol-treated sperm motility at a concentration of 50 μmol l^{-1} or less. Chum salmon sperm exhibited results similar to those of the rainbow trout sperm (data not shown).

The effect of the PKC inhibitors chelerythrine and calphostin C on both intact and glycerol-treated sperm were examined (Fig. 10). Applying 15 μmol l^{-1} chelerythrine significantly inhibited glycerol-treated sperm motility in both the rainbow trout and chum salmon (Fig. 10A,B). By contrast, the same concentrations of chelerythrine showed weaker motility inhibition effects on intact sperm (Fig. 10A,B). Similar results were obtained with calphostin C. Calphostin C inhibited glycerol-treated sperm motility in a dose-dependent manner, whereas calphostin C did not affect intact sperm motility at these concentrations (Fig. 10C,D).

DISCUSSION

Osmolarity shock induces salmonid fish sperm motility

It has been known that activation of salmonid fish sperm motility is triggered by a decrease of extracellular K^+ ions (Morisawa and Suzuki, 1980). However, our previous study demonstrated that, when salmonid fish sperm were treated with a 10% glycerol solution and subsequently diluted into the low-osmotic solution, sperm initiate motility even in the presence of a high concentration of extracellular K^+ (Morita et al., 2005). It was also reported that not only glycerol treatment but also treatment with erythritol, an organic alcohol, induced initiation of sperm motility contrary to ethylene glycol treatment, which did not induce sperm motility initiation. Based on these results, we previously concluded that the chemical properties and molecular weight of organic alcohol might be important factors for initiation of motility. In the present study, however, we found that treatment of sperm with compounds other than organic alcohol (NaCl, KCl, etc.) also induced sperm motility, and motility initiation was dependent on the osmolarity of the solution (Fig. 1 and Table 1). These results indicate that motility initiation by glycerol is dependent on the osmotic shock caused by glycerol treatment, but less dependent on the molecular weight and electric charge of the substances.

Hypertonic-shock-induced Ca^{2+} increase and its relation to motility activation

The effects of intracellular Ca^{2+} increase in rainbow trout sperm motility have been revealed by many authors, but their source and function are still obscure (Tanimoto and Morisawa, 1988; Cosson et al., 1989; Boitano and Omoto, 1992). Several reports suggested that the increase in intracellular Ca^{2+}, by the entry of external Ca^{2+}, is important for the initiation of sperm motility (Tanimoto and Morisawa, 1988; Cosson et al., 1989). In addition, Kho and colleagues (Kho et al., 2001) reported that the influx of Ca^{2+}, through dihydropyridine-sensitive T- and/or L-type Ca^{2+} channels, caused the changes in membrane potential, resulting in the activation of adenylyl cyclase to produce cAMP. Boitano and Omoto (Boitano and Omoto, 1992) reported that, in addition to activation of motility, Ca^{2+} release from intracellular stores also regulates the swimming pattern in trout sperm in a manner similar to that of sea urchin sperm (Brokaw, 1979; Okuno and Brokaw, 1981). By contrast, Okuno and Morisawa (Okuno and Morisawa, 1989) have shown an inhibitory effect of relatively low Ca^{2+} concentrations on the reactivation of the demembranated trout sperm.

In the present study, we demonstrated that intracellular Ca^{2+} increased upon motility activation, especially in the tail region (Fig. 4C,H,L) and the motility of intact sperm was inhibited by the elimination of intracellular Ca^{2+} by BAPTA-AM (Fig. 3). These results are consistent with previous studies that discussed the effects

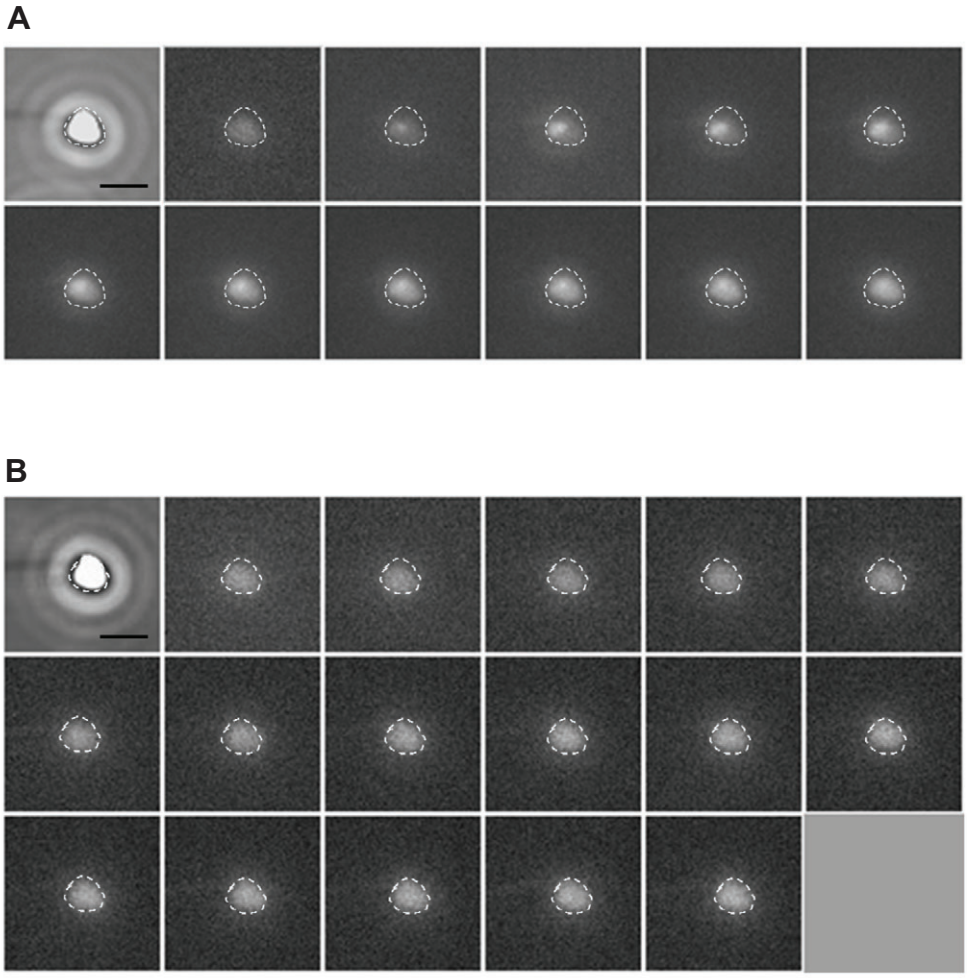

Fig. 6. Change in fluorescence of sperm heads during glycerol solution perfusion. Magnified images of sperm heads during perfusion with glycerol solution (A) or STS (B) are sequentially displayed at intervals of 0.3 s (broken line indicates boundary of sperm head). The starting point of fluorescence was observed in the basal region of sperm heads in glycerol-perfused sperm. Scale bars, 3 μm.

of Ca^{2+} on trout sperm motility (Tanimoto et al., 1994; Cosson et al., 1989; Boitano and Omoto, 1992). When extracellular Ca^{2+} was eliminated by EGTA, the motility of chum salmon intact sperm was inhibited (Fig. 2B). By contrast, in rainbow trout, EGTA did not severely affect the percentage of intact sperm that initiated motility (Fig. 2A). However, their swimming path was altered from straight to circular (data not shown). Because we frequently found that sperm near the end of the breeding season or poor-quality sperm generally show such a circular swimming path, it is assumed that the intact sperm of rainbow trout were also impaired by EGTA. These results indicate that, in intact sperm of both species, the Ca^{2+} increase is induced more or less by the entry of external Ca^{2+} and that sperm of chum salmon are more dependent on extracellular Ca^{2+} than the rainbow trout. This difference might come from the difference in the habitats of these species – rainbow trout inhabit inland waters, whereas chum salmon migrate.

In glycerol-treated sperm, intracellular Ca^{2+} increased when sperm were exposed to a hypertonic solution (Fig. 4A,F), and the removal of intracellular Ca^{2+} by BAPTA-AM inhibited both sperm motility and the increase of intracellular Ca^{2+} (Fig. 3, Fig. 4E,J). These results indicate that intracellular Ca^{2+} is a prerequisite for the initiation of salmonid fish sperm motility in glycerol-treated sperm, as well as in intact sperm. By contrast, elimination of extracellular Ca^{2+} by EGTA did not influence glycerol-treated sperm motility (Fig. 2). In addition, U73122, an inhibitor of a PLC, inhibited initiation of glycerol-treated sperm motility (Fig. 8). Therefore, unlike intact sperm, it is likely that the mobilization of Ca^{2+} is from the intracellular Ca^{2+} store by means of the PLC–inositol (1,4,5)-trisphosphate (IP_3) pathways, not through the influx from the extracellular medium, and this plays an important role in the motility initiation of glycerol-treated sperm. This idea is supported by the observation that the intracellular Ca^{2+} concentration increased when sperm were suspended in a hypertonic glycerol solution where most of the extracellular Ca^{2+} had been removed by 0.5 mmol l^{-1} EDTA (Fig. 4A,F). The difference in time required for the onset of the increase in intracellular Ca^{2+} between intact and glycerol-perfused sperm might be reflective of the difference in Ca^{2+} mobilization (Fig. 5B). Collectively, the data indicate that hypertonic-shock-induced mobilization of Ca^{2+} from intracellular Ca^{2+} stores by means of the PLC–IP_3 pathway has an important role in glycerol-treated sperm motility initiation. Interestingly, this result coincides with a previous report of puffer fish sperm in which the release of Ca^{2+} from an intracellular store, in response to hypertonic shock, has a key role in motility initiation (Oda and Morisawa, 1993). This idea is very suggestive of evolutionary adaptation in response to reproductive background.

In glycerol-treated sperm, the Ca^{2+} increase occurred from a specific region of the head close to the basal body and mid-piece (Fig. 6A), whereas such a specific point of Ca^{2+} burst could be identified neither in intact sperm (Fig. 6B) nor in sperm treated with A23187, a Ca^{2+} ionophore (data not shown). These results suggest the existence of a Ca^{2+} store near the basal body that releases Ca^{2+} in response to hypertonic shock. Ho and Suarez (Ho and Suarez, 2001) reported that an IP_3 receptor-gated Ca^{2+} store exists at a

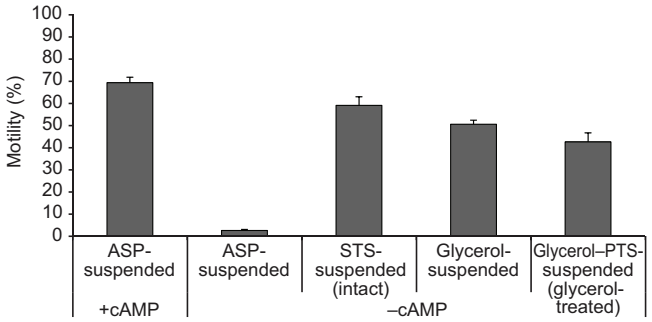

Fig. 7. Motility of demembranated sperm after glycerol treatment. Demembranated sperm were reactivated in the presence of 200 μmol l^{-1} ATP. 10 μmol l^{-1} cAMP was required for trout sperm flagellar movements under these conditions. When sperm were diluted in STS for 30 s before demembranation, sperm reactivated their motility when cAMP was not supplemented to the reactivation solution ('intact'). Likewise, when sperm were suspended in a glycerol solution for 15 s before demembranation, sperm reactivated their motility when cAMP did not supplement the reactivation solution ('glycerol-suspended'). Demembranation of sperm after glycerol treatment also caused reactivated sperm motility in the absence of cAMP ('glycerol-treated'). Values are means ± s.e.m. *N*=4.

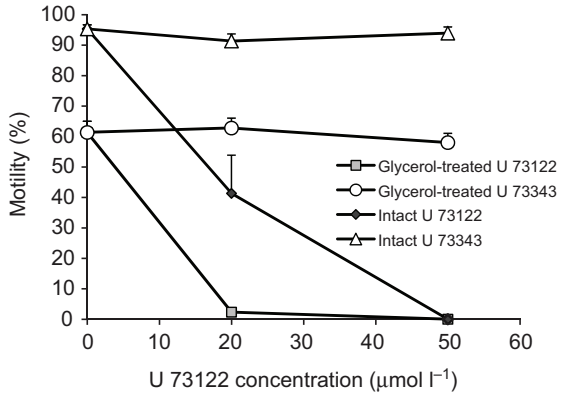

Fig. 8. Effect of the PLC inhibitor U 73122 on initiation of sperm motility in rainbow trout. Semen was diluted with ASP containing U 73122 or U 73343, an inactive analog of U 73122, for 1 h at room temperature. Motility was determined from video recordings. U 73122 significantly (P<0.01) inhibited sperm motility of intact and glycerol-treated sperm, whereas U 73343 did not inhibit sperm motility. Statistical tests were performed using Student's *t*-tests. Values are means ± s.e.m. *N*=4.

redundant nuclear envelope in the neck region of bovine sperm. A similar membrane structure was observed between the basal body and mitochondria of trout sperm with an osmiophilic mass (Billard, 1983). It is plausible that this structure is the IP_3 receptor-gated Ca^{2+} store in trout sperm.

Decrease of intracellular Ca^{2+} caused by hypotonic shock triggers motility initiation

When sperm were suspended and maintained in a hypertonic solution, intracellular Ca^{2+} increased in response to hypertonic shock and was maintained at a high level (Fig. 4B,G). Although intracellular Ca^{2+} increased under this condition, the sperm did not initiate motility. When sperm were diluted into a hypotonic solution following hypertonic exposure, they initiated motility concomitantly with a decrease in increased intracellular Ca^{2+} (Fig. 4A,F). These results suggest that not only the increase in Ca^{2+} by hyper-osmotic shock but also the subsequent decrease of intracellular Ca^{2+} are required for motility initiation. It is reported that relatively low concentrations of Ca^{2+} inhibit the motility of demembranated trout sperm (Okuno and Morisawa, 1989). In addition, Su and Vacquier (Su and Vacquier, 2002) reported that the intracellular Ca^{2+} of swimming sea urchin sperm is maintained at a low level by a K^+-dependent Na^+/Ca^{2+} exchanger, and blockage of this exchanger inhibited sperm motility. In conclusion, we speculate as follows: the increase of Ca^{2+} induced by hypertonic shock leads to the acquisition of the capacity for motility to the motile apparatus; however, the sperm cannot then move at that moment as hypertonic shock increased intracellular Ca^{2+} to an inhibitory level that is much higher than that of intact sperm (Fig. 5A). Then, the subsequent hypo-osmosis-induced removal of Ca^{2+} triggers the motility initiation. In intact sperm, the Ca^{2+} increase appears to be low enough to avoid an inhibitory effect on the axoneme. The mechanism by which hypotonic shock induces a decrease in intracellular Ca^{2+} is still unclear. It is known that mechano-sensitive cation channels are involved in the regulation of the motility of marine and freshwater fish sperm (Krasznai et al., 2003). Therefore, the opening of a mechano-sensitive channel by hypotonic shock might contribute to the decrease of Ca^{2+}. It is also probable that some Ca^{2+} pumps are activated in response to hypotonic shock to remove intracellular Ca^{2+}. Additional experiments are needed in order to elucidate the mechanism by which hypotonic shock induces a decrease in intracellular Ca^{2+}.

Diversity of Ca^{2+} bursts in sperm during motility initiation

In intact sperm, the magnitude of increase in intracellular Ca^{2+} was less than in glycerol-treated sperm (Fig. 4F,H, Fig. 5A). An increase of Ca^{2+} was mainly observed in the tail region of intact sperm, whereas a major increase of Ca^{2+} was observed in the head region of glycerol-treated sperm (Fig. 4A,C,F,H,K,L). These results lead to speculation that the increase of Ca^{2+} necessary for flagellar activation is as minimal as that observed in intact sperm (Fig. 4, Fig. 5A) and that the burst of Ca^{2+} in the head of glycerol-treated sperm might cause the increase of Ca^{2+} in the flagellum, which is sufficient for motility activation.

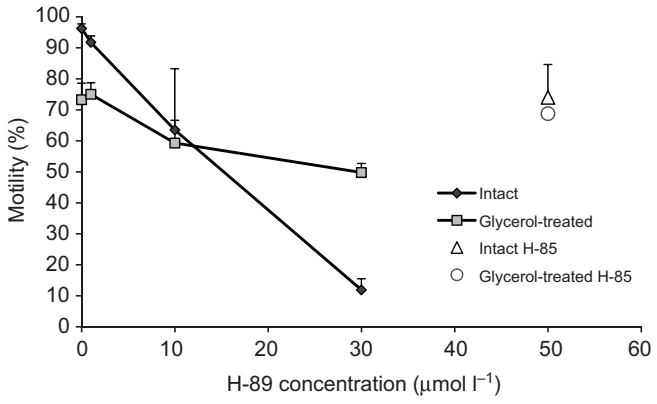

Fig. 9. Effect H-89 on initiation of sperm motility in rainbow trout. Semen was diluted and incubated with ASP containing H-89, a PKA inhibitor, for 30 min at room temperature. Motility was determined from video recordings. H-89 inhibited the motility of intact sperm in a dose-dependent manner (P<0.01 at 30 μmol l^{-1}). Glycerol-treated sperm motility was also inhibited, but rather less than for intact sperm. H-85, an inactive analog of H-89, did not inhibit sperm motility significantly. Statistical tests were performed using Student's *t*-tests. Values are means ± s.e.m. *N*=4.

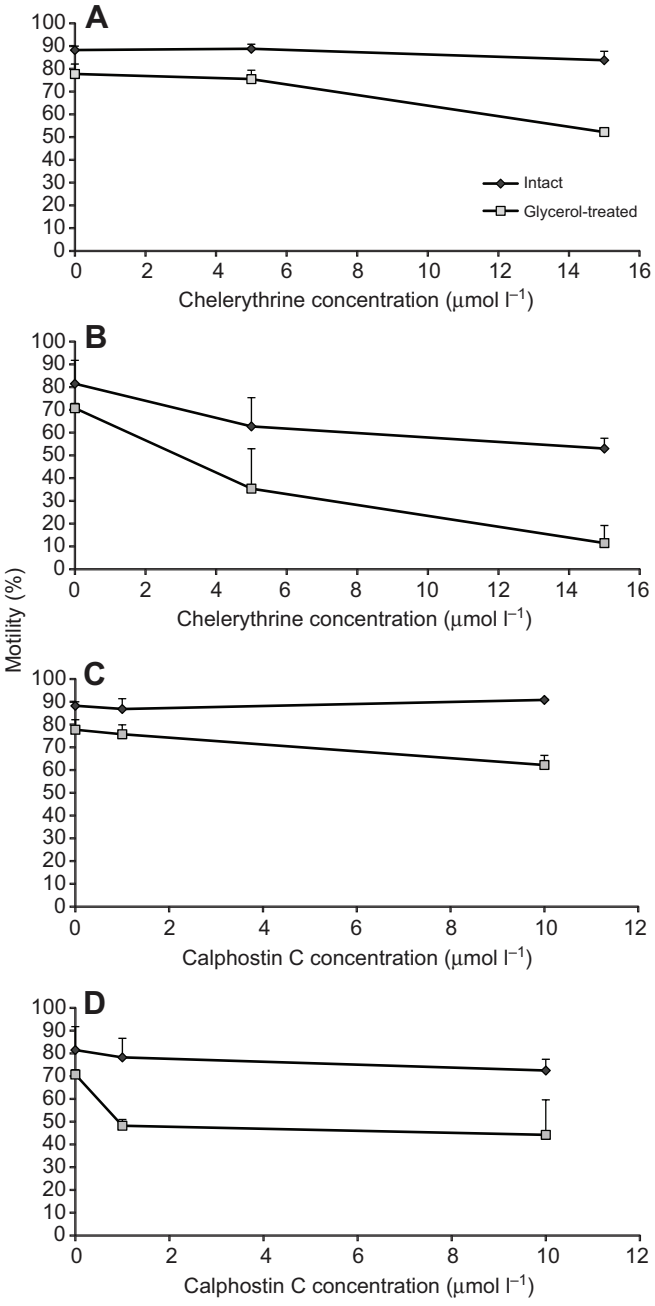

Fig. 10. Effect of PKC inhibitors on initiation of sperm motility in salmonid fishes. Semen was diluted and incubated with ASP containing (A,B) chelerythrine or (C,D) calphostin C, an inhibitor of PKC, for 30 min at room temperature. Motility was determined from video recordings. Glycerol-treated sperm motility of both rainbow trout and chum salmon was significantly inhibited by chelerythrine (P<0.01 at 15 μmol l^{-1}) and by calphostin C (P<0.05 at 10 μmol l^{-1}). Chelerythrine and calphostin C were not so effective in intact sperm as in glycerol-treated sperm. Statistical tests were performed using Student's t-tests. Values are means ± s.e.m. N=4.

Alternatively, an increase in Ca^{2+} might be necessary only at the base of the flagellum for motility initiation.

Protein phosphorylation and maturation in relation to motility

cAMP-dependent phosphorylation of intraflagellar proteins has been demonstrated to be indispensable for activation of sperm motility in many species (Brandt and Hoskins, 1980; Tash and Means, 1982; Ishiguro et al., 1982). In salmonid fish sperm, the involvement of cAMP in motility initiation was first reported by Morisawa and Okuno (Morisawa and Okuno, 1982). They demonstrated that cAMP-induced maturation of the non-motile axonemal apparatus is required for demembranated trout sperm motility (see also Fig. 7). In addition, they demonstrated that flagellar movement occurred without cAMP when trout sperm were diluted in potassium-free medium for 30 s prior to demembranation, suggesting that reduction of external K^+ induces maturation of the sperm motile apparatus (Morisawa and Okuno, 1982) (Fig. 7). The following studies have demonstrated that maturation induced by cAMP includes the phosphorylation of axonemal proteins, such as the 15-kDa protein and 22-kDa dynein light chain (Morisawa and Hayashi, 1985; Hayashi et al., 1987; Inaba et al., 1998). Our previous study reported that the 22-kDa dynein light chain and other axonemal proteins, except for the 15- and 29-kDa proteins, were phosphorylated in glycerol-treated salmonid fish sperm without the synthesis of cAMP. This result indicates that cAMP-independent maturation must occur in glycerol-treated sperm. In some species of sperm, Ca^{2+}-dependent phosphorylation is demonstrated to be important in motility regulation (Morita et al., 2006; White et al., 2007). As the increase of intracellular Ca^{2+} was achieved by hypertonic shock in glycerol-treated sperm, we consider that axonemal maturation might have occurred during exposure to a hypertonic environment. Indeed, the present study demonstrated that dilution of sperm into a glycerol solution, as well as STS, induced maturation of the sperm axoneme. A subsequent hypotonic shock was not necessary to induce maturation of the axoneme (Fig. 7). This result supports the idea, mentioned above, that phosphorylation of axonemal proteins is completed during hypertonic dilution and that subsequent hypotonic shock might function as a trigger. Notably, increasing intracellular Ca^{2+} from the intracellular store in response to hypertonic shock induces the maturation of the axoneme necessary for flagellar movement through Ca^{2+}-dependent phosphorylation of axonemal proteins, and the subsequent removal of Ca^{2+} by hypotonic shock specifically induces sperm motility by unlocking the Ca^{2+}-inhibitory effect.

The results discussed above raise a new question: what is the key signaling element for phosphorylation of axonemal proteins in glycerol-treated sperm? It has been revealed that the catalytic subunit of PKA, which phosphorylates proteins in a cAMP-dependent manner, is present in trout sperm (Itoh et al., 2003), suggesting that this signaling molecule might play an important role in intact sperm. In our previous study, however, cAMP was not synthesized upon motility initiation of glycerol-treated sperm. Then we proposed that PKA is activated without the synthesis of cAMP. In the present study, H-89, a potent inhibitor of PKA, inhibited the motility of intact sperm in a dose-dependent manner (Fig. 9). This result is consistent with previous reports that cAMP-dependent phosphorylation of axonemal proteins by PKA is implicated in the motility of intact sperm. In glycerol-treated sperm, H-89 inhibited motility at 30 μmol l^{-1}, which was weaker than what was required for inhibiting intact sperm (Fig. 9). This result suggests that the contribution of PKA to the motility of glycerol-treated sperm is less than that to intact sperm. Alternatively, the inhibition of the motility of glycerol-treated sperm by H-89 was caused by nonspecific inhibition because it is reported that H-89 at concentrations of approximately 30 μmol l^{-1} acts on other kinases, as well, *in vitro* (Chijiwa et al., 1990).

Next, we tested the involvement of Ca^{2+}-dependent phosphorylation in sperm motility initiation, such as calmodulin kinases and PKC, using inhibitors as these signaling elements are

reported to be involved in the regulation of motility in some species (Morita et al., 2006; White et al., 2007). However, W-7, which inhibits calmodulin, did not inhibit the motility of glycerol-treated sperm (data not shown). By contrast, PKC inhibitors (chelerythrine and calphostin C) inhibited the motility of glycerol-treated sperm at a concentration that did not inhibit intact sperm motility, although inhibition was not so severe in rainbow trout sperm ($P<0.05$; Fig. 10). These results suggest that PKC, which does not affect intact sperm initiation, might function at least partially on phosphorylation of axonemal proteins in glycerol-treated sperm. The result that U 73122 inhibited glycerol-treated sperm motility supports this idea, as PKC is known to phosphorylate proteins in a diacylglycerol-dependent manner (Fig. 8). Further investigations are needed to elucidate the key signaling molecule in glycerol-treated sperm.

Conclusions

The present study proposes a novel mechanism for sperm motility initiation that acts as a salvaging pathway for the well-known K^+-dependent pathway through phosphorylation of the same axonemal proteins, including the 22-kDa dynein light chain. This means that salmonid fish sperm possess diverse mechanisms for the initiation of motility. One mechanism revealed in the present study shares features common to those of other teleosts that initiate motility in a osmolarity- and Ca^{2+}-dependent manner. Further investigations on the mechanism of glycerol-treated sperm motility and actual function of this novel signaling pathway under physiological condition are needed and might lead to the generation of further valuable information.

ACKNOWLEDGEMENTS

We thank the Oshino Branch of Yamanashi Prefectural Fisheries Experiment Station for supplying rainbow trout. We also thank the Ohtsuchi Kaiyo center of the Atmosphere and Ocean Research Institute, The University of Tokyo and Ohtsuchi fisherman's union for provision of facilities and materials, notably chum salmon. We wish for a quick recovery from the enormous damage caused by the huge 2011 Tohoku earthquake and tsunami.

FUNDING

A part of this research was supported by the Sasakawa Scientific Research Grant to G.L.T., and by Japan Society for the Promotion of Science (JSPS) Grants-in-Aid for Scientific Research (Wakate B) to C.M. and JSPS Grants-in-Aid for Scientific Research [17049012] to M.O. We thank Jacquelyn L. Nelson (Cornell University) for proof-reading of the manuscript.

LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
ASP	artificial seminal plasma
ATP	adenosine triphosphate
BAPTA	*O,O′*-bis(2-aminophenyl)ethyleneglycol-*N,N,N′,N′*-tetraacetic acid
cAMP	3′-5′-cyclic adenosine monophosphate
DMSO	dimethylsulfoxide
DTT	dithiothreitol
EGTA	ethylene glycol tetraacetic acid
Fluo-4	1-[2-amino-5-(2,7-difluoro-6-hydroxy-3-oxo-9-xanthenyl)phenoxy]-2-(2-amino-5-methylphenoxy)ethane-*N,N,N′,N′*-tetraacetic acid, pentaacetoxymethyl ester
H-85	*N*-[2-(*N*-formyl-*p*-chlorocinnamylamino)ethyl]-5-isoquinolinesulfonamide
H-89	*N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PTS	potassium test solution
STS	sodium test solution
U 73122	1-[6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione
U 73343	1-[6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-2,5-pyrrolidinedione

REFERENCES

Alavi, S. M. H. and Cosson, J. (2006). Sperm motility in fishes. (II) Effects of ions and osmolality: a review. *Cell. Biol. Int.* **30**, 1-14.
Billard, R. (1983). Ultrastructure of trout spermatozoa: changes after dilution and deep-freezing. *Cell. Tissue Res.* **228**, 205-218.
Boitano, S. and Omoto, C. K. (1991). Membrane hyperpolarization activates trout sperm without an increase in intracellular pH. *J. Cell Sci.* **98**, 343-349.
Boitano, S. and Omoto, C. K. (1992). Trout swimming patterns and role of intracellular Ca^{2+} *Cell. Motil. Cytoskel.* **21**, 74-82.
Brandt, H. and Hoskins, D. D. (1980). A cAMP-dependent phosphorylated motility protein in bovine epididymal sperm. *J. Biol. Chem.* **255**, 982-987.
Brokaw, C. J. (1979). Calcium-induced asymmetrical beating of triton-demembranated sea urchin sperm flagella. *J. Cell Biol.* **82**, 401-411.
Chijiwa, T., Mishima, A., Hagiwara, M., Sano, M., Hayashi, K., Inoue, T., Naito, K., Toshioka, T. and Hidaka, H. (1990). Inhibition of forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized selective inhibitor of cyclic AMP-dependent protein kinase, N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesufonamide (H-89), of PC12D phenochromocytoma cells. *J. Biol. Chem.* **265**, 5267-5272.
Cosson, J. (2010). Frenetic activation of fish spermatozoa flagella entails short-term motility, portending their precocious decadence. *J. Fish Biol.* **76**, 240-279.
Cosson, M. P., Billard, R. and Letellier, L. (1989). Rise of internal Ca^{2+} accompanies the initiation of trout sperm motility. *Cell. Motil. Cytoskel.* **14**, 424-434.
Cosson, M. P., Cosson, J., André, F. and Billard, R. (1995). cAMP/ATP relationship in the activation of trout sperm motility: Their interaction in membrane-deprived models and in live spermatozoa. *Cell. Motil. Cytoskel.* **31**, 159-176.
Goldstein, D. A. (1979). Calculation of the concentrations of free cations and cation-ligand complexes in solutions containing multiple divalent cations and ligands. *Biophys. J.* **26**, 235-242.
Hayashi, H., Yamamoto, K., Konekawa, H. and Morisawa, M. (1987). Involvement of tyrosine protein kinase in the initiation of flagellar movement in rainbow trout spermatozoa. *J. Biol. Chem.* **262**, 16692-16698.
Ho, H. C. and Suarez, S. S. (2001). An inositol **1**, 4, 5-triphosphate receptor-gated intracellular Ca^{2+} store is involved in regulating sperm hyperactivated motility. *Biol. Reprod.* **65**, 1606-1615.
Inaba, K., Morisawa, S. and Morisawa, M. (1998). Proteasomes regulate the motility of salmonid fish sperm through modulation of cAMP-dependent phosphorylation of an outer arm dynein light chain. *J. Cell Sci.* **111**, 1105-1115.
Ishiguro, K., Murofushi, H. and Sakai, H. (1982). Evidence that cAMP-dependent protein kinase and a protein factor are involved in reactivation of triton X-100 models of sea urchin and starfish spermatozoa. *J. Cell Biol.* **92**, 777-782.
Itoh, A., Inaba, K., Ohtake, H., Fujinoki, M. and Morisawa, M. (2003). Characterization of a cAMP-dependent protein kinase catalytic subunit from rainbow trout spermatozoa. *Biochem. Biophys. Res. Commun.* **305**, 855-861.
Kho, K. H., Tanimoto, S., Inaba, K., Oka, Y. and Morisawa, M. (2001). Transmembrane cell signaling for the initiation of trout sperm motility: roles of ion channels and membrane hyperpolarization for cyclic AMP synthesis. *Zool. Sci.* **18**, 919-928.
Krasznai, Z., Márián, T., Izumi, H., Damjanovich, S., Balkay, L. and Morisawa, M. (2000). Membrane hyperpolarization removes inactivation of Ca^{2+} channels, leading to Ca^{2+} influx and subsequent initiation of sperm motility in the common carp. *Proc. Natl. Acad. Sci. USA* **97**, 2052-2057.
Krasznai, Z., Morisawa, M., Krasznai, Z. T., Morisawa, S., Inaba, K., Bazsáné, Z. K., Rubovszky, B., Bodnár, B., Borsos, A. and Márián, T. (2003). Gadolinium, a mechano-sensitive channel blocker, inhibits osmosis-initiated motility of sea- and freshwater fish sperm, but does not affect human or ascidian sperm motility. *Cell. Motil. Cytoskel.* **55**, 232-243.
Morisawa, M. and Hayashi, H. (1985). Phosphorylation of a 15 kD axonemal protein is the trigger initiating trout sperm motility. *Biomed. Res.* **6**, 181-184.
Morisawa, M. and Ishida, K. (1987). Short-term changes in levels of cyclic AMP, adenylate cyclase, and phosphodiesterase during the initiation of sperm motility in rainbow trout. *J. Exp. Zool.* **242**, 199-204.
Morisawa, M. and Okuno, M. (1982). Cyclic AMP induces maturation of trout sperm axoneme to initiate sperm motility. *Nature* **295**, 703-704.
Morisawa, M. and Suzuki, K. (1980). Osmolality and potassium ion: their roles in initiation of sperm motility in teleosts. *Science* **210**, 1145-1147.
Morita, M., Takemura, A. and Okuno, M. (2003). Requirement of Ca^{2+} on activation of sperm motility in euryhaline tilapia *Oreochromis mossambicus*. *J. Exp. Biol.* **206**, 913-921.
Morita, M., Fujinoki, M. and Okuno, M. (2005). K^+-independent motility initiation in chum salmon sperm treated with an organic alcohol, glycerol. *J. Exp. Biol.* **208**, 4549-4556.
Morita, M., Takemura, A., Nakajima, A. and Okuno, M. (2006). Microtubule sliding movement in tilapia sperm flagella axoneme is regulated by Ca^{2+}/Calmodulin-dependent protein phosphorylation. *Cell. Motil. Cytoskel.* **63**, 459-470.
Oda, S. and Morisawa, M. (1993). Rises of Ca^{2+} and pH mediate the initiation of sperm motility by the hyperosmolality in marine teleosts. *Cell. Motil. Cytoskel.* **25**, 171-178.
Okuno, M. and Brokaw, C. J. (1981). Effects of Triton-extraction conditions on beat symmetry of sea urchin sperm flagella. *Cell. Motil.* **1**, 363-370.

Okuno, M. and Morisawa, M. (1989). Effect of Ca^{2+} on motility of rainbow trout sperm flagella demembranated with triton X-100. *Cell. Motil. Cytoskel.* **14**, 194-200.
Su, Y. H. and Vacquier, V. D. (2002). A flagellar K^+-dependent Na^+/Ca^{2+} exchanger keeps Ca^{2+} low in sea urchin spermatozoa. *Proc. Natl. Acad. Sci. USA* **99**, 6743-6748.
Takai, H. and Morisawa, M. (1995). Change in intracellular K^+ concentration caused by external osmolality change regulates sperm motility of marine and freshwater teleosts. *J. Cell Sci.* **108**, 1175-1181.
Tanimoto, S. and Morisawa, M. (1988). Roles for potassium and calcium channels to the initiation of sperm motility in rainbow trout. *Dev. Growth Differ.* **30**, 117-124.
Tanimoto, S., Kudo, Y., Nakazawa, T. and Morisawa, M. (1994). Implication that potassium flux and increase in intracellular calcium are necessary for the initiation of sperm motility in salmonid fishes. *Mol. Reprod. Dev.* **39**, 409-414.
Tash, J. S. and Means, A. R. (1982). Regulation of protein phosphorylation and motility of sperm by cyclic adenosine monophosphate and calcium. *Biol. Reprod.* **26**, 745-763.
White, D., Lamirande, E. D. and Gagnon, C. (2007). Protein kinase C is an important signaling mediator associated with motility of intact sea urchin spermatozoa. *J. Exp. Biol.* **210**, 4053-4064.