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K^+-independent initiation of motility in chum salmon sperm treated with an organic alcohol, glycerol

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Summary

Sperm of salmonid fishes are quiescent in the presence of millimolar concentrations of extracellular K^+, but motility initiation occurs when sperm are suspended in K^+-free medium. In this study, glycerol ($CH_2OHCHOHCH_2OH$) treatment of intact sperm in the presence of K^+ induced the initiation of motility even though a large amount of K^+ was present. Another organic alcohol, erythritol ($CH_2OH(CHOH)_2CH_2OH$), had a similar effect, but ethylene glycol (CH_2OHCH_2OH) did not initiate sperm motility. Furthermore, this glycerol-treated sperm showed motility without subsequent addition of ATP and cAMP. CCCP, an uncoupler of the mitochondrial electron-transport chain involved in ATP synthesis, suppressed motility of glycerol-treated sperm, suggesting that ATP synthesis is required for dynein to slide microtubules in glycerol-treated sperm. The amount of intracellular cAMP ($[cAMP]_i$) in glycerol-treated sperm did not increase on motility activation, but a protein kinase A (PKA) inhibitor, H-89, inhibited glycerol-treated sperm motility. In addition, phosphorylation of protein associated with motility initiation also occurred in glycerol-treated sperm, suggesting that the glycerol treatment induces activation of PKA without an increase in $[cAMP]_i$. Taken together, it can be concluded that organic alcohol, glycerol and erythritol induce phosphorylation for motility initiation, bypassing the increase in $[cAMP]_i$ as a result of a decrease in extracellular K^+ concentration.

Key words: sperm motility, salmon, organic alcohol, phosphorylation, cAMP, eukaryotic flagella.

Introduction

Sperm motility is vital for the transport of the male genetic material to the egg. It is known that there are several sperm motility regulatory mechanisms in teleosts (Morisawa, 1994). In salmonid fish, a decrease in extracellular K^+ concentration triggers the initiation of flagellar motility. This mechanism is suited to the spawning ground of these fish, since freshwater contains very low amounts of K^+. This K^+-dependent regulatory mechanism has been well studied. The decrease in extracellular $[K^+]$ induces K^+ efflux thorough certain K^+ channels, leading to hyperpolarization of the plasma membrane and resulting in Ca^{2+} influx through dihidropiridine-sensitive Ca^{2+} channels (Kho et al., 2001). Subsequently, cAMP is produced (Morisawa and Okuno, 1982) and induces phosphorylation of axonemal proteins to initiate flagellar motility (Hayashi et al., 1987; Inaba et al., 1998). In the signal transduction pathway for motility initiation, a decrease in $[K^+]$ is the first signal.

Interestingly, addition of extracellular Ca^{2+} promotes initiation of motility, even in the presence of up to 10 mmol l^{-1} K^+ (Tanimoto and Morisawa, 1988). In addition, motility is suppressed by Ca^{2+} channel blockers (Tanimoto and Morisawa, 1988; Kho et al., 2001). These observations together suggest that the increase in intracellular $[Ca^{2+}]$, rather than efflux of K^+, plays a major role in the initiation of motility. On the other hand, Boitano and Omoto (1991) showed that the presence of divalent cations other than Ca^{2+} induces motility initiation, even in the presence of K^+, suggesting that the effect of Ca^{2+} is not mainly to initiate motility but a membrane potential that may be associated with motility initiation. The inconsistency of these results suggests that membrane hyperpolarization and Ca^{2+} influx may act independently in increasing cAMP production. Furthermore, activation of motility does not require the increase in cAMP in one specific condition. Demembranated sperm requires the addition of an appropriate concentration of cAMP and a low concentration of Ca^{2+} ($<10^{-8.5}$ mol l^{-1}) to reactivate motility, and high beat frequency can be achieved in the presence of 200 μmol l^{-1} Mg-ATP^{2-} (Okuno and Morisawa, 1989). In the presence of low concentrations of Mg-ATP^{2-} (<50 μmol l^{-1}), however, addition of cAMP is not required for reactivating demembranated sperm (M. Okuno, unpublished data).

Previous studies have given reliable results that signal transduction is required for motility initiation in salmonid fish sperm. However, signal transduction from the plasma membrane to the flagellar axoneme is still not fully understood. In other words, is the initiation of motility only regulated by a production of cAMP that is induced by the membrane hyperpolarization as a result of the decrease in extracellular K^+ and so on?

The aim of this study was to investigate a new membrane-permeabilized sperm model, which conserves membrane structure, rather than the triton-treated demembranated sperm model. The new model gives us clues about the mechanisms of signal transduction. In early cilia and flagella studies, the plasma membranes were permeabilized by treatment with 50% glycerol, and flagella motility was reactivated by an addition of exogenous ATP (Hoffman-Berling, 1955). We treated salmonid sperm with glycerol ($CH_2OHCHOHCH_2OH$) under slightly different conditions. The results were unexpected and surprising. The glycerol-treated sperm moved without addition of exogenous ATP and cAMP in the presence of a high concentration of K^+, suggesting that the glycerol-treated sperm conserve their plasma membrane and K^+ does not inhibit motility. Furthermore, treatment with another organic alcohol, erythritol ($CH_2OH(CHOH)_2CH_2OH$), also induced motility initiation in the presence of a large amount of K^+, although ethylene glycol (CH_2OHCH_2OH) did not.

The present study reveals another motility initiation mechanism, different from the K^+-dependent process, and shows that glycerol treatment alters the regulatory mechanism for inducing motility initiation in a K^+-rich solution.

Materials and methods

Chemicals

[γ-^{32}P]ATP (222 TBq mmol l^{-1}) was purchased from Amersham Pharmacia Biotech (Buckinghamshire, England), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and SQ-22536 were from Biomol Research Laboratory (Plymouth Meeting, PA, USA), cyclic AMP enzyme immunoassay (EIA) system (RPN 225) was from Biotrak (Amersham Pharmacia Biotech, Carlsbad, CA, USA). All other chemicals used were reagent grade from Wako Pure Chemicals (Osaka, Japan).

Collection of sperm

Chum salmon *Oncorhynchus keta* L. return to the Ohtsuchi River in Iwate prefecture, Japan, from the end of November to December. Fish were collected during this period with permission of Ohtsuchi fishermen's union. Semen was collected directly by inserting a pipette into the sperm duct and stored on ice until use.

Organic alcohol treatment of sperm

The semen was diluted 200-fold with ice-cold glycerol solution consisting of 10% (v/v) glycerol (1.3 mol l^{-1} glycerol), 1.3 mol l^{-1} ethylene glycol or 1.3 mol l^{-1} erythritol, 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). After 10 s incubation, 1 μl of the sperm suspension was suspended in 40 μl of experimental solution on a glass slide coated with 1% (w/v) BSA for observation. Sperm were completely immotile in the glycerol solution. The incubation time in the glycerol solution was critical for motility initiation; incubation for longer than 20 s failed to activate motility.

Observation of sperm motility

Sperm movements were recorded using a Video recorder (SLV-LF 1; Sony, Tokyo, Japan) and a CCD camera (CD-5C; RF system, Tokyo, Japan) mounted on a microscope (Optiphoto; Nikon, Tokyo, Japan) equipped with a dark field or phase contrast condenser. Sperm trajectories were taken with 0.5 s exposure on high sensitive film (SUPERIA 1600 or 800, Fuji film, Tokyo, Japan) with microscope camera (Nikon, UFX-II) mounted on the microscope equipped with dark field condenser. Then, velocity of sperm was calculated from the length of trajectories and the beat frequency from the number of waves of trajectories.

Effect of inhibitors

The semen was diluted tenfold in artificial seminal plasma (ASP) containing inhibitors as described in Kho et al. (2001). The composition of ASP was 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 7.8). ASP also contained 0.1% (v/v) DMSO, in which inhibitors were resuspended. After an appropriate incubation time (30 min), the sperm suspension was resuspended in 100 mmol l^{-1} NaCl and 10 mmol l^{-1} Hepes-NaOH (pH 8.0) in the ratio 1:100. For glycerol treatment, the sperm suspension was resuspended in glycerol solution for 10 s at a dilution ratio of 1:10. Then, 1 vol of the suspension was resuspended in 40 vol of the activation solution (100 mmol l^{-1} KCl and 10 mmol l^{-1} Hepes-NaOH, pH 8.0).

Cyclic AMP assay

The cyclic AMP assay was carried out according to the method described previously (Kho et al., 2001). In the intact sperm assay, semen was resuspended in ASP at a dilution ratio of 1:10. Then, 6 μl of the sperm suspension were transferred into 600 μl of 100 mmol l^{-1} NaCl or 100 mmol l^{-1} KCl solution. In the glycerol-treated sperm assay, semen was resuspended in glycerol solution at a dilution ratio of 1:25. Then, 10 μl of the sperm suspension were transferred into 400 μl of 100 mmol l^{-1} NaCl, 100 mmol l^{-1} KCl and 300 mmol l^{-1} KCl solutions. After the appropriate incubation time (15 s), assessment of cAMP production was carried out using cAMP EIA kit (Biotrak RPN 225; Amersham Pharmacia Biotech). Briefly, 360 μl of sperm suspension was mixed with 40 μl of Kit buffer (lysis reagent) to stop cAMP synthesis and dissolve the cells. 100 μl of the mixture was then used for cAMP measurement according to the manufacture's instructions. The amount of cAMP in each sample was calculated by measuring absorbance at 450 nm with a micro plate reader (Model 550; Bio-Rad, Richmond, CA, USA).

Phosphorylation assay

In glycerol-treated sperm, extracellular ATP was completely unnecessary. However, [γ-^{32}P]ATP was added in glycerol solution, activation solutions and inactivation solution to investigate phosphorylation of proteins in glycerol-treated sperm. 20 μl of semen were resuspended and incubated for 1 min in the ice-cold 200 μl glycerol solution containing 4 MBq ml^{-1} [γ-^{32}P]ATP (222 TBq mmol^{-1}) and centrifuged at 10 000 ***g*** for 5 min at 4°C. The sperm pellet was diluted into 50 μl activation solution (100 mmol l^{-1} KCl or NaCl and 10 mmol l^{-1} Hepes-NaOH, pH 8.0) or inactivation solution (300 mmol l^{-1} KCl and 10 mmol l^{-1} Hepes-NaOH, pH 8.0). Both of them contained 8 MBq ml^{-1} [γ-^{32}P]ATP (222 TBq mmol^{-1}). After 5 min incubation at room temperature, the suspensions were centrifuged at 10 000 ***g*** for 5 min at 4°C. The supernatant was then decanted and the sperm pellet eluted for 1 min on ice with 30 μl of elution solution (8 mol l^{-1} urea and 10% 2-mercaptoethanol). Residuals were removed by centrifugation at 12 000 ***g*** for 5 min at 4°C. Then, 2× sample buffer [100 mmol l^{-1} Tris-HCl, pH 6.8, 4% (w/v) SDS, 12% (v/v) 2-mercaptoethanol, 20% (v/v) glycerol and 0.02% (w/v) Bromophenol Blue] was added to the supernatants and boiled for 2 min.

In the Triton model (demembranated sperm), sperm were treated with demembranation solution [0.4% (w/v) Triton X-100, 150 mmol l^{-1} potassium acetate, 1 mmol l^{-1} EDTA, 1 mmol l^{-1} DTT and 20 mmol l^{-1} Hepes-NaOH, pH 8.0] for 10 min on ice and centrifuged at 10 000 ***g*** for 1 min at 4°C. The demembranated sperm were resuspended with reactivation solution [150 mmol l^{-1} potassium acetate, 1 mmol l^{-1} EGTA, 1 mmol l^{-1} DTT, 2 mmol l^{-1} $MgCl_2$, 2 MBq ml^{-1} [γ-^{32}P]ATP (6000 Ci mmol) and 20 mmol l^{-1} Hepes-NaOH, pH 8.0] with or without 10 μmol l^{-1} cAMP. Phosphorylation reactions were carried out for 5 min at room temperature and suspensions were centrifuged at 10 000 ***g*** for 5 min at 4°C. The supernatant was then decanted, the demembranated sperm pellet eluted and sample prepared for SDS-PAGE as described above.

Samples were subjected to 12.5% SDS-PAGE (Laemmli, 1970), followed by staining with Coomassie Brilliant Blue. ^{32}P-labeled proteins were examined by exposing the gel to the X-ray film for 72 h at –80°C.

Statistical analysis

The data were subjected by two-way analysis of variance (ANOVA) followed by Fisher's PLSD for multiple-group comparisons. If there was a significant difference, the Bonferroni *post-hoc* test was conducted. Beat frequency and velocity data were subjected to analysis of covariance (ANCOVA; StatView J-5).

All experiments were carried out at room temperature (15–20°C), and the results are given as means ± S.D.

Results

Motility of glycerol-treated sperm

Sperm of salmonid fish are immotile in the presence of more than 10 mmol l^{-1} extracellular K^+. When suspended in K^+-free solution, motility is initiated (Fig. 1). By contrast, sperm incubated in glycerol ($CH_2OHCHOHCH_2OH$) solution for 10 s exhibited initiation of motility even in K^+-rich solutions, such as 100 mmol l^{-1} KCl buffered by Hepes, and K^+-free solution (100 mmol l^{-1} NaCl; Fig. 1). Motility initiation in K^+-rich solution required the presence of glycerol at less than 0.25% (v/v). Motility of glycerol-treated sperm, like intact sperm, lasted for about 20–40 s. Intact sperm did not move in the K^+-rich solutions.

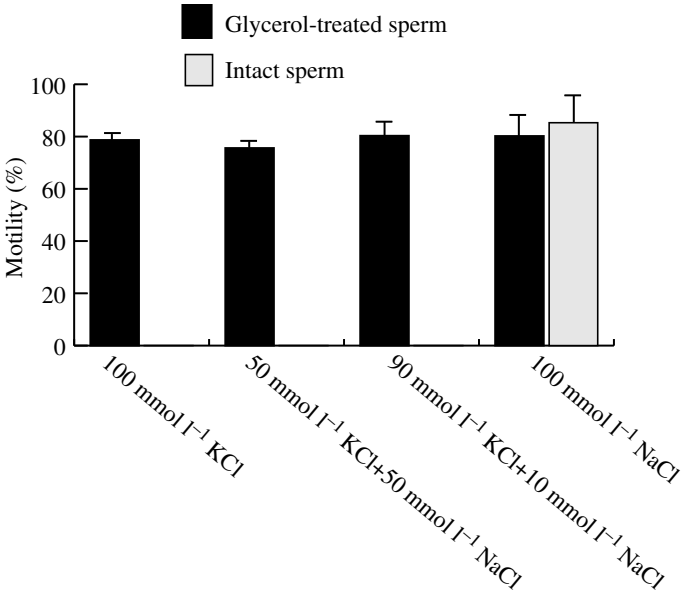

Fig. 1. Effect of ions on glycerol-treated sperm. Sperm were incubated in a glycerol solution for 10 s exactly. Then, 1 vol of this suspension was diluted in 40 vol of an activation solution (KCl 100 mmol l^{-1}, KCl 50 mmol l^{-1}+NaCl 50 mmol l^{-1}, KCl 10 mmol l^{-1}+NaCl 90 mmol l^{-1} or NaCl 100 mmol l^{-1}). Gray bar, percentage motility of intact sperm; black bars, glycerol-treated sperm. Intact sperm showed motility only with 100 mmol l^{-1} NaCl. Values are means ± S.D. (*N*=5).

Additions of ATP and cAMP were not required to initiate the motility of glycerol-treated sperm, whereas the demembranated sperm required ATP and cAMP to be reactivated. This observation suggests that the properties of the glycerol-treated sperm were quite different from both the intact sperm and demembranated sperm, since fluctuations in extracellular [K^+] are not closely related to the initiation of motility.

Other organic alcohols, ethylene glycol (CH_2OHCH_2OH) and erythritol ($CH_2OH(CHOH)_2CH_2OH$) were also tested (Fig. 2A). Ethylene glycol treatment did not induce motility initiation in 100 mmol l^{-1} KCl solution (Fig. 2B), whereas erythritol treatment induced motility initiation in KCl solution (Fig. 2B).

Demembranated sperm showed motility only with $10^{-8.5}$ mol l^{-1} Ca^{2+} supplemented with appropriate concentrations of Mg-ATP^{2-} (Okuno and Morisawa, 1989). By contrast, intact sperm motility is enhanced when a millimolar concentration of Ca^{2+} is added even in the presence of extracellular K^+ (Tanimoto and Morisawa, 1988). In glycerol-

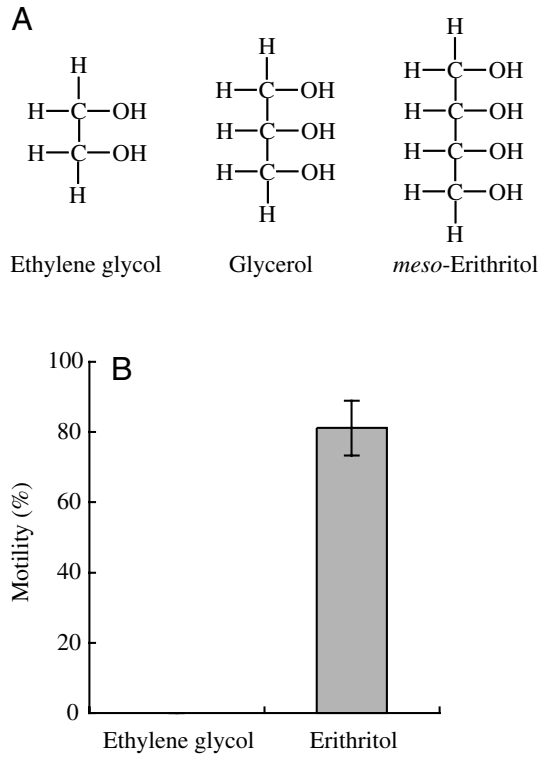

Fig. 2. Effect of organic alcohol on sperm motility. (A) Chemical formulae of three organic alcohols. (B) Effects of ethylene glycol and erythritol on sperm motility. Sperm were first incubated with one of these alcohol solutions (1.3 mol l^{-1}) and then diluted into 100 mmol l^{-1} KCl solution. Values are means ± S.D. (N=5).

treated sperm, addition of Ca^{2+} had no apparent effect on motility initiation (Fig. 3). Furthermore, when Ca^{2+} concentrations in glycerol solutions were changed from 10^{-5} to 10^{-9} mol l^{-1}, there was no effect on motility initiation (data not shown).

Sperm velocity increased significantly with beat frequency (ANCOVA, $F_{1,80}$=282, P<0.0001, Fig. 4A). The slopes for glycerol-treated and intact sperm were significantly different ($F_{1,80}$=77.4, P<0.0001). Dark field microscopy revealed that the trajectory pitch of glycerol-treated sperm was smaller than that of intact ones (Fig. 4B,C), indicating that swimming velocity achieved with one stroke of the glycerol-treated sperm was lower than that of intact sperm.

Effect of inhibitors

When an uncoupler of mitochondrial electron transport chain, CCCP, was applied in ASP, motility initiation was inhibited both in glycerol-treated and intact sperm (Fig. 5A). Therefore, inhibition of mitochondrial ATP synthesis suppressed motility in glycerol-treated sperm. It is likely that ATP is synthesized and maintained in glycerol-treated sperm as well as in intact sperm. Even when ATP was added to the solution for glycerol-treated sperm, they failed to maintain motility for more than 30 s.

An adenylyl cyclase inhibitor, SQ22536, did not

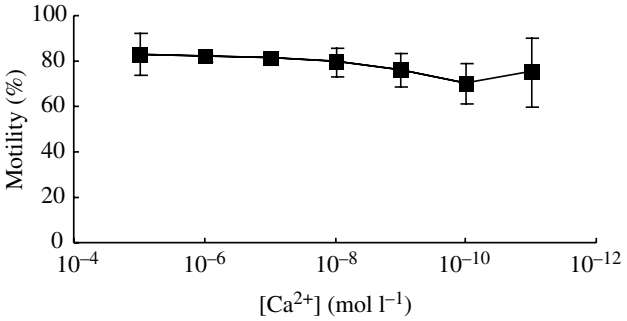

Fig. 3. Effect of extracellular Ca^{2+} concentration on glycerol-treated sperm. Glycerol-treated sperm were suspended in 100 mmol l^{-1} NaCl solutions containing various concentration of Ca^{2+}. Values are means ± S.D. (N=5).

significantly inhibit glycerol-treated sperm motility compare to the intact sperm (P<0.05; Fig. 5B). On the other hand, a PKC inhibitor, H-89, inhibited glycerol-treated sperm (P<0.05; Fig. 5C).

Change in intracellular content of cyclic AMP on the process of motility initiation

Intracellular cyclic AMP (cAMP) concentration ($[cAMP]_i$) was increased in intact sperm when they were exposed to K^+-free solution (100 mmol l^{-1} NaCl), but not to K^+-rich solution (100 mmol l^{-1} KCl; Fig. 6). Glycerol-treated sperm showed vigorous motility even in K^+-rich solutions. $[cAMP]_i$ in glycerol-treated sperm, however, was not increased in K^+-rich solution even though 90% of sperm showed motility, and no increase in $[cAMP]_i$ was observed with 300 mmol l^{-1} KCl, although sperm did not move because of the high osmolality (Fig. 6). It is unlikely that $[cAMP]_i$ is important in the motility initiation process in glycerol-treated sperm. In other words, $[cAMP]_i$ increases in the initiation process of intact sperm motility.

Protein phosphorylation in glycerol-treated sperm

In salmonids, protein phosphorylation occurs during the initiation of sperm motility. Many sperm and axonemal proteins are phosphorylated in a cAMP-dependent manner. Previous studies clearly showed that a 15 kDa protein (Morisawa and Hayashi, 1985) and 21 kDa dynein light chain (LC2), 29 kDa protein (Inaba et al., 1998, 1999), and PKA regulatory subunit (Inaba, 2002) were phosphorylated in a cAMP-dependent manner. In glycerol-treated sperm, however, $[cAMP]_i$ was not increased (Fig. 6). Then, we examined incorporation of ^{32}P in the glycerol-treated sperm and demembranated sperm as a control.

In demembranated sperm, a 18 kDa protein corresponding to the 15 kDa protein described in Morisawa and Hayashi (1985), dynein light chain, 29 kDa protein, PKA regulatory subunit, 80, 125 and 300 kDa proteins were phosphorylated in a cAMP-dependent manner (Fig. 7A). Glycerol-treated sperm presented a very low incorporation of ^{32}P, suggesting that activity of protein kinase is very low. Among the phosphoproteins the PKA regulatory subunit and 300 kDa

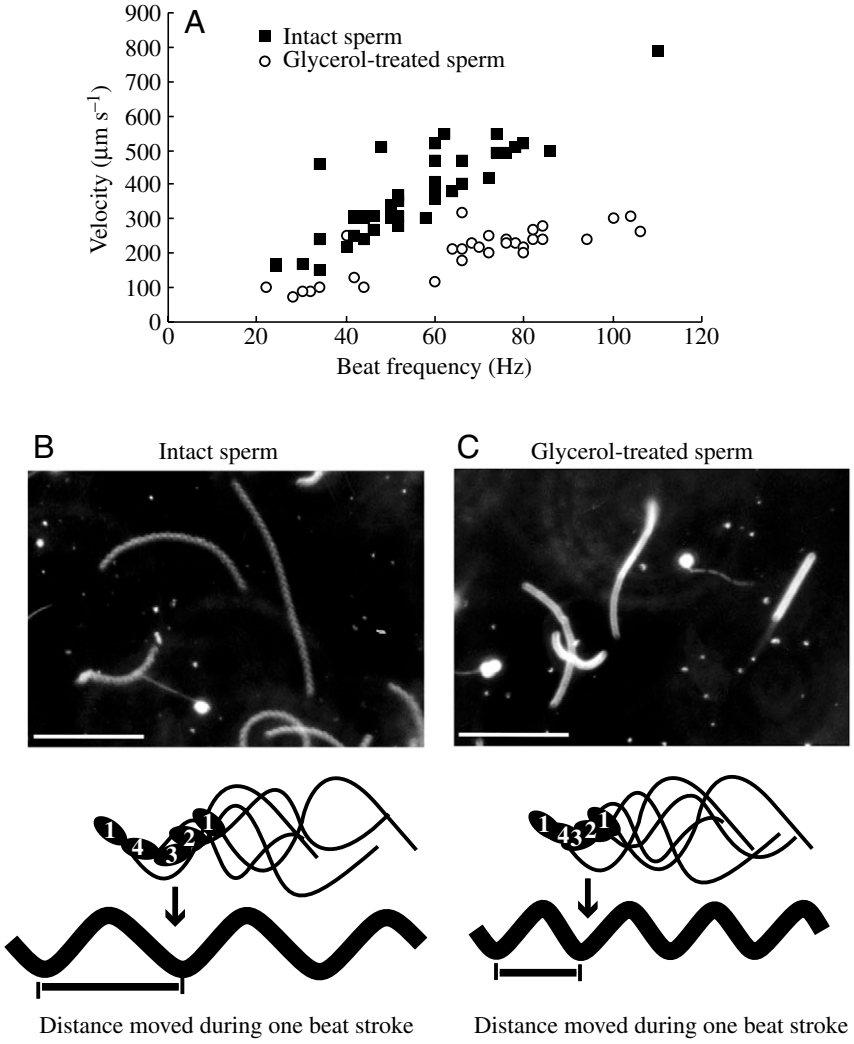

Fig. 4. Relationship between swimming velocity and beat frequency of glycerol-treated and intact sperm. Photographs were taken from 5 s after dilution to the end of sperm motility. Velocity and beat frequency of intact and glycerol-treated sperm were calculated from photographs. (A) Relationship between swimming velocity and beat frequency of glycerol-treated (open circles) and intact sperm (filled squares). The regressions are y=7.0x–11.1 for intact sperm (r^2=0.8, P<0.0001, N=49) and y=2.2x+22.6 for glycerol-treated sperm (r^2=0.6, P<0.0001, N=35). (B,C) Trajectories taken at 0.5 s exposures for intact (B) and glycerol-treated (C) sperm. Schema of the trajectory are represented below each photograph. Bars, 100 μm (N=3).

protein were only weakly phosphorylated (Fig. 7B; lane, glycerol treatment). Most proteins were phosphorylated when sperm were diluted in activating solutions. Dynein light chain, 125 and 300 kDa proteins were phosphorylated in 100 mmol l^{-1} NaCl and KCl solutions (Fig. 7B). These proteins were also phosphorylated in the demembranated sperm in a cAMP-dependent manner (Fig. 7A). A 70 kDa protein was phosphorylated in 100 mmol l^{-1} NaCl and KCl solutions. This protein was phosphorylated only in the glycerol-treated sperm, and not the demembranated sperm (Fig. 7A). The 18 and 29 kDa proteins in the glycerol-treated sperm were not as strongly phosphorylated as those of the demembranated sperm (Fig. 7A,B).

Discussions

It is known that the decrease in extracellular [K^+] causes the initiation of flagellar motility in salmonid fish sperm (Morisawa and Suzuki, 1980). The decrease in extracellular [K^+] is assumed to induce hyperpolarization of the plasma membrane potential, leading to an increase in intracellular [Ca^{2+}] followed by cAMP production (Tanimoto and Morisawa, 1988; Kho et al., 2001). However, glycerol-treated sperm showed motility even in the presence of 100 mmol l^{-1} KCl (Fig. 1), suggesting that the initiation of motility is independent of the decrease in extracellular [K^+]. In addition, it is likely that glycerol-treated sperm retained their plasma membrane as a cell barrier since the addition of cAMP and Mg-ATP^{2-} was not required for initiating the sperm flagellar motility. Moreover, the inhibition of ATP synthesis in mitochondria by CCCP suppressed the motility of glycerol-treated sperm (Fig. 5A), suggesting that the metabolic pathway for ATP synthesis is not affected in glycerol-treated sperm.

Glycerol-treated sperm motility was initiated even when a large amount of extracellular K^+ was present (Fig. 1). Not only glycerol but also erythritol (Fig. 2A) induced motility initiation in the presence of a large amount of K^+ (Fig. 2B), but ethylene glycol treatment did not induce motility activation (Fig. 2B). The molecular size of alcohol is important for induction of motility initiation, and must be equal to or larger than glycerol.

In intact sperm, the decrease in extracellular [K^+] is identified as a trigger for the initiation of flagellar motility. K^+ efflux *via* K^+ channels induces hyperpolarization of the plasma membrane (Tanimoto and Morisawa, 1988; Kho et

al., 2001), followed by Ca^{2+} influx *via* the dihydropyridine-sensitive Ca^{2+} channel (Kho et al., 2001). Kho et al. (2001) then postulated that the membrane hyperpolarization activates adenylyl cyclase for the production of cAMP. It is known that activation of adenylyl cyclase is associated with membrane hyperpolarization in ascidian (Izumi et al., 1999) and sea urchin sperm (Beltran et al., 1996). However, in the present study, hyperpolarization did not occur with a high concentration of extracellular K^+ in glycerol and erythritol-treated sperm. Moreover, cAMP production was not observed in the K^+-rich condition. Motility of glycerol-treated sperm was not strongly inhibited by an adenylyl cyclase inhibitor, SQ22536 (Fig. 5B). Furthermore, the present study shows that an increase in $[cAMP]_i$ did not occur in glycerol-treated sperm (Fig. 6). By contrast, $[cAMP]_i$ of intact sperm increased in the motility-permissive 100 mmol l^{-1} NaCl solution (Fig. 6). Therefore, in glycerol-treated sperm, it is likely that initiation of flagellar motility is not associated with the cAMP synthesis pathway, which is induced by hyperpolarization as a result of K^+ efflux and Ca^{2+} influx in intact sperm. It is possible that the motility regulatory mechanism of glycerol-treated sperm is different from that in intact sperm.

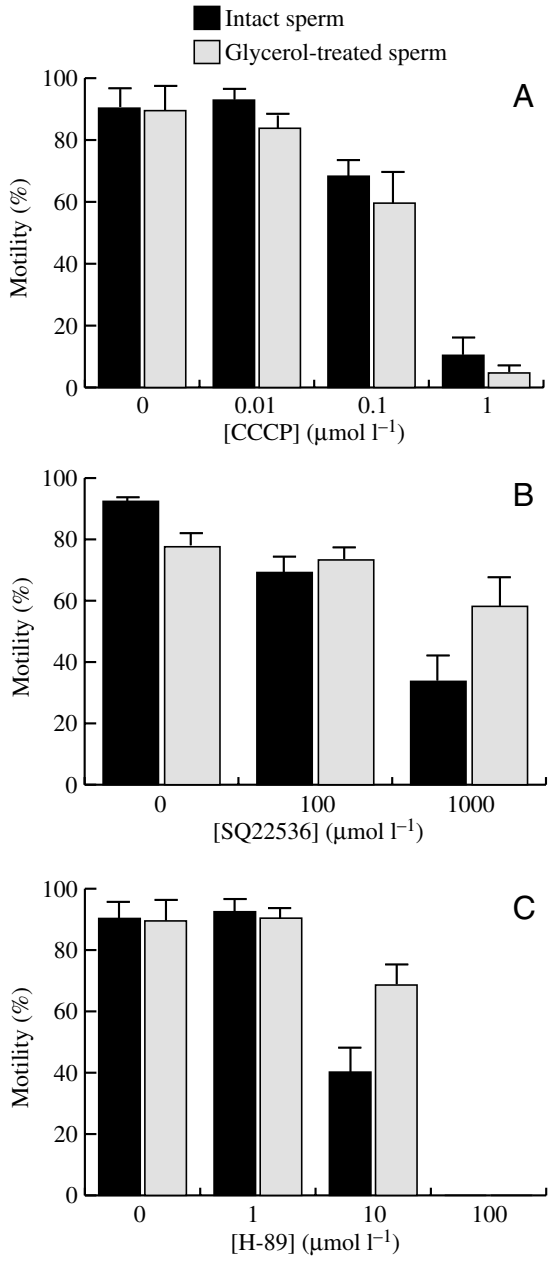

Fig. 5. Effect of inhibitors on glycerol-treated sperm. Sperm were incubated for 30 min in ASP containing (A) CCCP, an uncoupler of the mitochondrial electron transport chain for ATP synthesis; (B) SQ22536, an adenylyl cyclase inhibitor; (C) H-89, a PKA inhibitor. Gray bars, percentage motility of intact sperm; black bars, glycerol-treated sperm. Motility was determined from video recordings taken 5 s after motility initiation. Values are means ± S.D. (N=3).

As described above, cAMP production was not necessary for motility initiation in glycerol-treated sperm. However, a PKA inhibitor, H-89, weakly inhibited motility initiation of glycerol-treated sperm (Fig. 5C). PKA exists as an inactive tetramer, consisting of two regulatory and two catalytic subunits, at low cAMP concentrations (Robinson, 1970). Binding of cAMP to PKA regulatory subunits causes the release of PKA catalytic subunits, inducing PKA activation and resulting in protein phosphorylation (Taylor et al., 1990). Therefore, activation of PKA is tightly related to the increase in cAMP. The present study shows that the cAMP concentration increased on motility activation without K^+, as shown in Fig. 6, in agreement with previous studies that demonstrated an increase in cAMP followed by protein phosphorylation (Morisawa and Hayashi, 1985; Hayashi et al., 1987; Inaba et al., 1989). This means that protein phosphorylation could not occur unless cAMP was produced. However, the present experiments show that dynein light chain and PKA regulatory subunit of the glycerol-treated sperm were phosphorylated in both 100 mmol l^{-1} NaCl and KCl solutions (Fig. 7B). These proteins are known to be phosphorylated in a cAMP-dependent manner (Fig. 7A; Inaba, 2002; Inaba et al., 1998). Therefore, it is plausible that PKA is activated without the synthesis of cAMP. It is possible that glycerol- or erythritol treatment can release the regulatory subunits from an inactive PKA tetramer in the absence of cAMP. Then, the following question arises. Does organic alcohol itself affect the PKA state? If this is the case, proteins under the regulation of PKA could be phosphorylated during the glycerol treatment. However, ^{32}P-uptake in sperm was very low in the glycerol solution, except that the PKA regulatory subunit was weakly phosphorylated (Fig. 7B). Large amounts of ^{32}P-uptake occurred after sperm were treated with 100 or 300 mmol l^{-1} KCl. It is speculated that the high osmolality of glycerol solution (about 1300 mOsm kg^{-1}) inhibited the kinase activity in the presence of glycerol. Then, kinase activity is likely to rise as osmolality decreases.

As shown in Figs 6 and 7B, synthesis of cAMP is not required to induce protein phosphorylation of glycerol-treated sperm, although the 18 and 29 kDa proteins were not as strongly phosphorylated as those in demembranated sperm

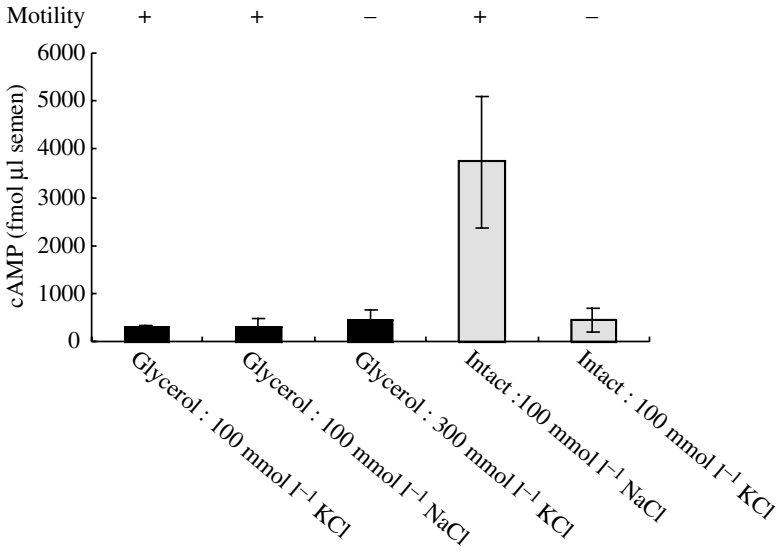

Fig. 6. Cyclic AMP synthesis in glycerol-treated and intact sperm. Sperm cAMP levels were measured 15 s after suspending intact and glycerol-treated sperm into various solutions. Glycerol-treated sperm were diluted in 100 mmol l^{-1} KCl, 100 mmol l^{-1} NaCl and 300 mmol l^{-1} KCl solutions. Intact sperm were diluted in 100 mmol l^{-1} NaCl and KCl solutions. Gray bars, percentage motility of intact sperm; black bars, glycerol-treated sperm. Values are means ± S.D. (N=3).

(Fig. 7A,B). In *Paramecium* cilia, phosphorylation of a 29 kDa protein regulates the sliding velocity of microtubules and swimming speed (Hamasaki et al., 1991). The swimming speed of glycerol-treated sperm at the same beat frequency was significantly lower than that of non-treated intact sperm (Fig. 4A). It is possible that phosphorylation of the 29 kDa protein regulates the swimming speed of salmonid fish sperm by changing the wave form *via* the sliding velocity of microtubules. But further studies are necessary to elucidate the mechanisms.

Glycerol- and erythritol-treated sperm exhibited surprising features, not only in sperm flagellar motility but also in protein phosphorylation, which is believed to be important in cell signaling. Further studies could provide more interesting and valuable information.

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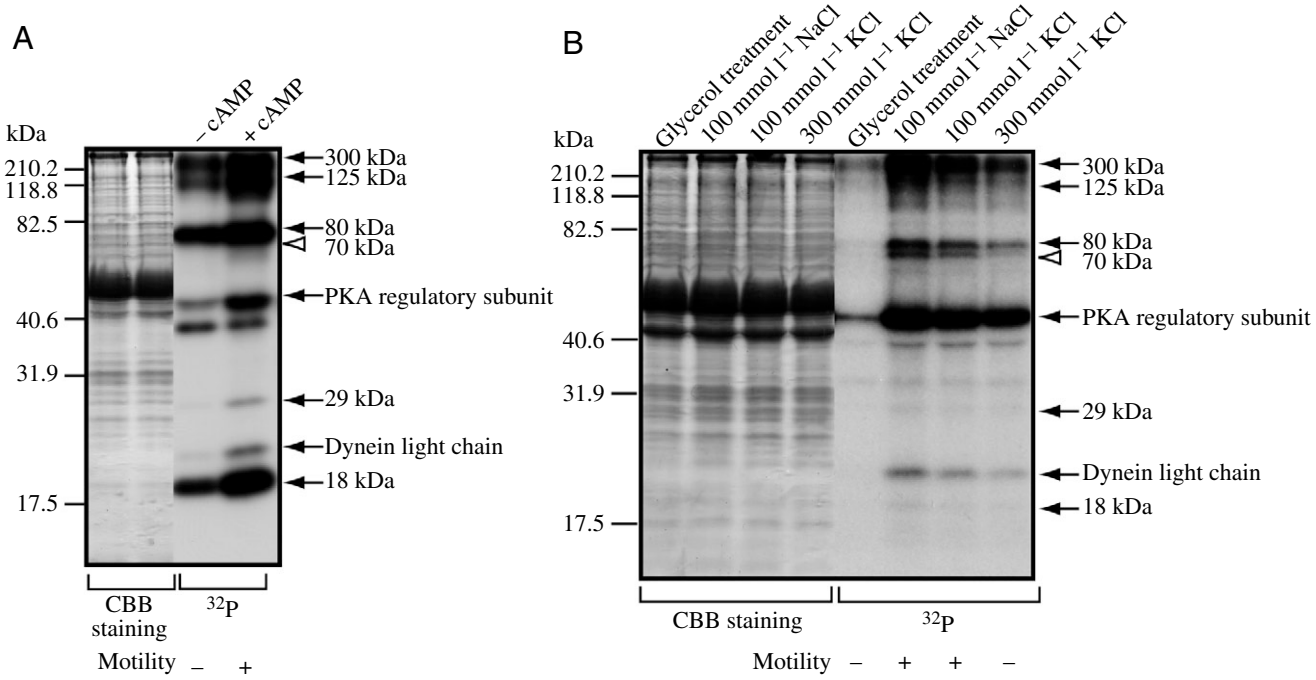

Fig. 7. Detection of phosphorylation in (A) demembranated and (B) glycerol-treated sperm treated with various solutions. ^{32}P-labelled proteins were separated by a 12.5% separating gel. Demembranated sperm were reactivated in the presence of cAMP. Glycerol-treated sperm showed motility in the 100 mmol l^{-1} NaCl and KCl solutions. Bars on the left side show molecular mass standards.

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