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Membrane Hyperpolarization Increases cAMP to Induce the Initiation of Sperm Motility in Salmonid Fishes, Rainbow Trout and Masu Salmon

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Abstract Sperm motility of salmonid fishes is suppressed by external K^+ and initiated by decrease of K^+ concentration surrounding the sperm. It was shown that the decrease in external K^+ concentration induced not only the initiation of sperm motility, but also hyperpolarization of the plasma membrane and synthesis of cAMP in the sperm of rainbow trout, steelhead trout, and masu salmon. Inhibitors of K^+ channels, especially voltage-dependent K^+ channels, inhibited these three reactions, and the inhibitions were abolished by subsequent addition of a K^+ ionophore, valinomycin, suggesting that K^+ efflux through the K^+ channel contributes to rapid changes in the membrane potential of sperm and cAMP synthesis, thereby resulting in the initiation of sperm motility of salmonid fishes.

Key words: Sperm motility, K^+ channel, membrane potential

The mechanism underlying the initiation of sperm motility is attracting much attention. One of the most successful studies to elucidate the mechanism was performed with fish sperm [13, 24], especially salmonid fishes such as rainbow trout, chum salmon, masu salmon, and char [29, 30, 36]. Sperm motility of these salmonid fishes is completely suppressed in the medium containing K^+, suggesting that low motility of salmonid sperm is suppressed in the sperm duct by a high concentration of seminal K^+ surrounding the spermatozoa [24]. Motility is initiated under experimental conditions when the semen is diluted into K^+-free solution [2, 29, 30], suggesting that spermatozoa become motile when they are released into K^+-deficient fresh water at natural spawning.

The mechanism of the initiation of sperm motility was investigated mainly in rainbow trout. It has been reported that a decrease in K^+ immediately causes a significant and transient increase in intracellular cAMP [4, 27], which is a trigger for the phenomenon [28]. The second messenger causes phosphorylation of dynein light chain [23] and tyrosine residues of a protein with molecular mass of 15 kDa through activation of proton kinesis [20], which is indispensable for the initiation of sperm motility. Despite such well investigated intracellular signaling for the initiation of trout sperm motility, transmembrane cell signaling for generation of cAMP has been completely unknown in the rainbow trout as well as other salmonid species. In regard to the question of cell signaling, it has been suggested that the transport of K^+ and Ca^{2+} through ion channels at the plasma membrane of spermatozoa may participate in membrane hyperpolarization and the initiation of sperm motility [5, 6, 17, 31, 40], implicating the relationship between changes in membrane potential by the decrease in K^+ concentration surrounding the sperm and cAMP synthesis.

The effects of various ion channel blockers on sperm motility, potential changes of the sperm plasma membrane, and cAMP levels during the initiation of sperm motility in salmonid fishes were investigated in order to clarify the transmembrane cell signaling. The results clearly indicate that the membrane hyperpolarization through the opening of voltage-dependent K^+ channels increases cAMP synthesis to lead the initiation of sperm motility in salmonid fishes.

Materials and Methods

Chemicals

$DiSC_3(5)$, 3,3-dipropylthiadicarbocyanine iodide, was purchased from Molecular Probe (Leiden, Netherlands); carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), valinomycin (Val) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.); tetraethylammonium chloride (TEA) was from Nacalai tesque (Kyoto, Japan); K^+ channel modulators were from Alomone Laboratories (Jerusalem, Israel). CCCP, Val, and $DiSC_3(5)$ were dissolved in dimethylsulfoxide

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(DMSO). The concentration of a solvent was kept below 1% v/v in all experiments. Other reagents used were of analytical grade.

Collection of Sperm

Mature males of the rainbow trout *Oncorhynchus mykiss*, the steelhead trout *Oncorhynchus mykiss*, and the masu salmon *Oncorhynchus masou* were provided by Yamanashi Prefectural Fisheries Experiment Station, Oshino Trout Hatchery, and kept in an indoor aquarium at 20°C. The fish were not fed during experiments. Semen was collected directly by inserting a pipette into the sperm duct and stored on ice until use.

Assessment of Sperm Motility

A glass slide was coated with 1% bovine serum albumin and then dried to prevent sperm from sticking to the slide. A two-step dilution procedure for measuring sperm motility was used, as reported by Cosson *et al.* [15], to achieve synchronous induction of sperm motility. The sperms were diluted 10-fold in a physiological saline (ASP) consisting of 130 mM NaCl, 40 mM KCl, 2.5 mM $CaCl_2$, 1.5 mM $MgCl_2$, and 10 mM HEPES at pH 7.8. The composition of the ASP was based on the ionic and osmotic compositions of the seminal plasma of rainbow trout [25]. Sperm motility was completely suppressed in the ASP by 40 mM KCl. The prediluted sperm was placed on the glass slide, which was placed on the stage of a microscope (Nikon Optiphoto, Tokyo, Japan) with a dark field condenser lens without a cover glass. The sperm suspension was diluted 100-fold by addition of activation solution (AS) consisting of 150 mM NaCl and 10 mM HEPES at pH 7.8. The second dilution step reduced the K^+ concentration surrounding the sperm, thereby inducing motility of the sperm on the glass slide. The motility of the sperm was recorded on a camcorder (Hamamatsu C2400 SIT; Shizuoka, Japan) attached to the microscope. The percentage of motile sperm and their velocity were measured from the images of the tracks of sperm using a Cellsoft semen analyzer (Cellsoft series 3000, NAC incorporated, Tokyo, Japan).

In the experiment on the effect of K^+ concentration on sperm motility, KCl was substituted for NaCl on an equimolar basis in AS. Effects of alkaline metals on sperm motility were investigated by suspending the prediluted sperm in AS in which KCl, RbCl, CsCl, or LiCl was substituted for NaCl on an equimolar basis.

The effect of ion channel blockers on the initiation of sperm motility was investigated by diluting the sperm in the ASP at the ratio 1:10 containing various concentrations of ion channel blockers, and then one volume of the sperm suspension was resuspended in 100 volumes of AS containing each blocker at the same concentration. Viability of the sperm was checked by diluting the prediluted sperm in AS without inhibitors. In the experiments on the effect of TEA and valinomycin, sperms were preincubated in the ASP without TEA.

For measuring the time course of sperm motility, the semen was diluted 1,000-fold in AS without predilution, and their motility and velocity were measured at 5, 10, 15, 20, 25, and 30 sec after dilution.

Measurement of Membrane Potential

The semen was diluted in ASP at a ratio of 1:10. After predilution, 20 µl of the sperm suspension was diluted in the 2 ml of AS containing 1 µM $DiSC_3(5)$ in a quartz cuvette in the presence or absence of an inhibitor. Mitochondrial potential was eliminated by a subsequent addition of 1 µM CCCP. Then, 1 µM valinomycin was added to make the plasma membrane maximally permeable to K^+, and KCl was subsequently added. During the procedures, $DiSC_3(5)$ fluorescence was monitored by a fluorescence spectrophotometer (Hitachi 650-10S; Hitachi, Tokyo, Japan) at an excitation wavelength of 620 nm and an emission wavelength of 670 nm with continuous stirring at 18°C. One µM $DiSC_3(5)$ loading alone to trout sperm did not significantly change the motility. Hyperpolarization and depolarization of the plasma membrane resulted in a decrease and an increase in fluorescence intensity, respectively, under experimental conditions.

Assay of cAMP

The semen was diluted in the ASP at a ratio of 1:10, and 3 µM sperm suspension was suspended in 300 µM AS alone or AS containing KCl, ion channel blockers, and/or valinomycin. After incubation for a necessary period of time, 180 µl of the suspension was mixed with 20 µl of kit buffer (lysis reagent) to stop cAMP synthesis and dissolve the cells. Then, 100 µl of the mixture was added to the well of the kit to quantify cAMP by the method described in the manual of the cyclic AMP EIA system (BIOTRAK RPN 225; Amersham Pharmacia Biotech, England). The cAMP level of each sample was calculated by measuring absorbance of 450 nm with a microplate reader (Model 550; Bio-Rad, Richmond, CA, U.S.A.).

The protein concentration was measured by the method of Bradford [8] using a commercial reagent for protein measurement from BIO-RAD (Bio-Rad Protein Assay; Bio-Rad Laboratories, Hercules, CA, U.S.A.).

A two-paired student's t-test was used for the statistical analysis. Data were expressed as the means±SD.

Results

Sperm Motility

Just after dilution of the ASP-prediluted sperm into AS, more than 95% of the rainbow trout sperm became motile

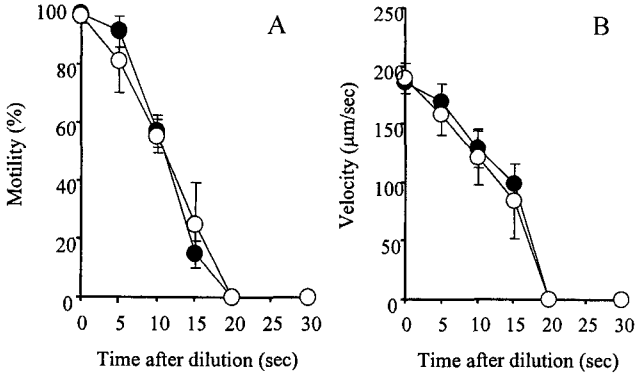

Fig. 1. Profile of short-term sperm motility in rainbow trout and masu salmon.
One volume of the semen was directly suspended in 1,000 volumes of AS, and percentage of motile sperm (**A**) and their velocity (**B**) were measured in the rainbow trout (filled circle) and the masu salmon (open circle). The data are expressed as mean±SD (n=5).

(Fig. 1A). The sperm initially swam straightforwardly, and the percentage of motile sperm declined within 15 sec. The swimming pattern became circular, and the swimming halted by 25 sec after the dilution. The sperm initiated motility with an average velocity of 180 μm/sec (Fig. 1B), and the velocity sharply declined with passage of time by 20 sec after the dilution, in good agreement with results obtained previously [14]. The motility parameters had no significant difference between rainbow trout and masu salmon (Figs. 1A and 1B).

Effects of alkaline metals on the two parameters of sperm motility, percentage of motile sperm and velocity, were investigated in rainbow trout (Fig. 2A) and masu salmon (Fig. 2B). Sperm motility in the rainbow trout was not affected in the presence of KCl at concentrations up to 2.5 mM, and the percentage of the motile sperm declined to 25% at 5 mM K^+ and was completely suppressed at KCl concentrations over 10 mM. Velocity of the sperm underlying motility also became half of that at 5 mM. When the medium contained RbCl, the number of motile sperm and the velocity of the motile sperm decreased with an increase in Rb^+ concentration over 2.5 mM, and the motility ceased at 5 mM. In the medium containing CsCl, the number of motile spermatozoa and the velocity of the motile spermatozoa decreased with an increase in Cs^+ concentration over 2.5 mM, and motility ceased at 20 mM. In contrast, LiCl and NaCl did not inhibit sperm motility at 0–40 mM concentration. Almost all spermatozoa exhibited vigorous and progressive movement in these media. Effect of the alkaline metals on the motile pattern of sperm in the masu salmon was similar to those in the rainbow trout. The observed ionic selectivity ($Rb^+ > K^+ > Cs^+ \gg Li^+ > Na^+$) suggests that the plasma membrane of spermatozoa in the salmonid fishes has selective permeability to K^+ over Na^+.

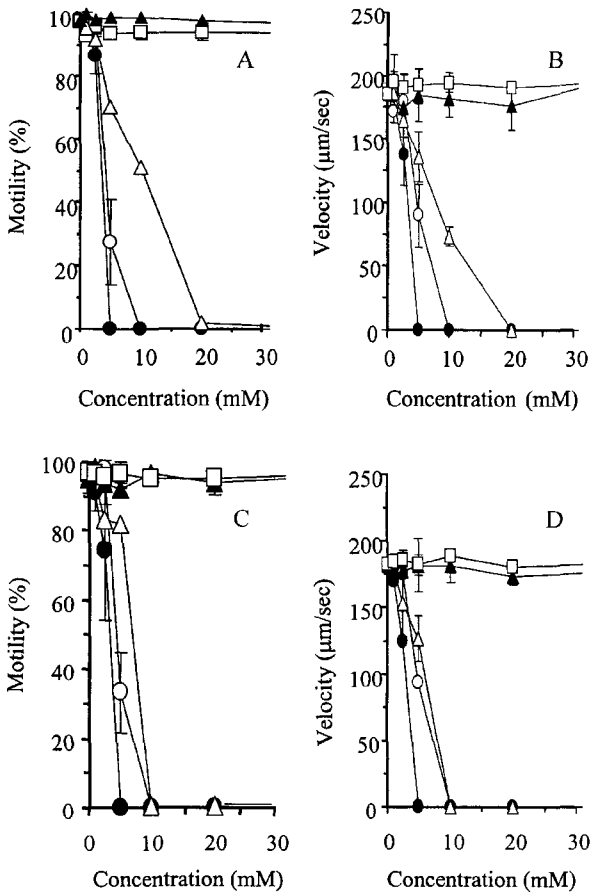

Fig. 2. Effects of alkaline metals on the sperm motility in rainbow trout and masu salmon.
The semen was diluted in ASP at the ratio of 1:10. One volume of the sperm suspension was suspended in 100 volumes of AS containing various concentrations of KCl (open circle), RbCl (filled circle), CsCl (open triangle), LiCl (filled triangle) and NaCl (open square), and percentage of motile sperm (left) and their velocity (right) in the rainbow trout (**A**, **B**) and the masu salmon (**C**, **D**) were measured. The data are expressed as mean±SD (n=5).

In order to elucidate the role of the K^+ channel in the initiation of sperm motility, sperm motility in the presence of K^+ channel blockers was examined in rainbow trout and masu salmon. As shown in Fig. 3, TEA, which is generally known to inhibit the efflux of K^+ through voltage-dependent K^+ channels in the plasma membrane [38], suppressed the motility of sperm by 50% in masu salmon at around 25 mM. This value was higher than that previously obtained in rainbow trout (9.0 mM) [40], which is comparable to the dose for inhibition of the acrosome reaction in sea urchin sperm [34, 35]. Sperm of the steelhead trout exhibited full motility in AS, and the motility was inhibited in the presence of K^+. Sperm motility of the steelhead trout was also blocked in the AS containing 25 mM TEA (Fig. 5). Although the inhibition required higher concentration of the inhibitor, sperm motility was reversibly recovered, when the concentration of TEA was decreased by addition

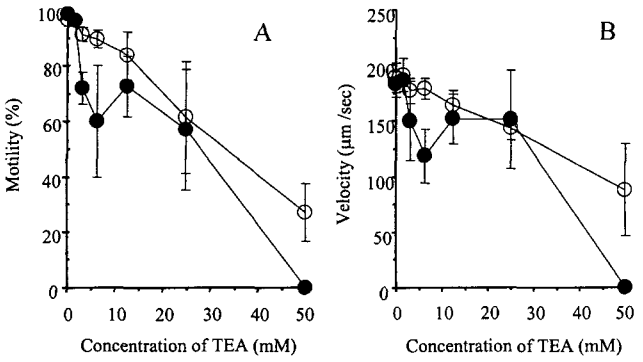

Fig. 3. Effect of TEA on the sperm motility in rainbow trout and masu salmon.
The semen was diluted in ASP without TEA at the ratio of 1:10. One volume of the sperm suspension was suspended in 100 volumes of AS containing various concentrations of TEA, and percentage of motile sperm (**A**) and their velocity (**B**) were measured in the rainbow trout (filled circle) and the masu salmon (open circle). The data are expressed as mean±SD (n=5).

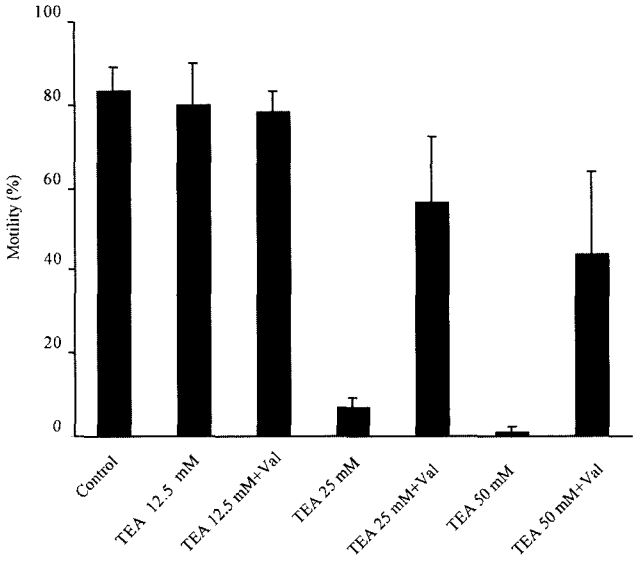

Fig. 5. Effects of TEA and valinomycin on the sperm motility in steelhead trout.
The semen was diluted in ASP without TEA at the ratio of 1:10. One volume of the sperm suspension was diluted in 100 volumes of AS containing 0 mM (control), 12.5 mM, 25 mM, and 50 mM TEA. Valinomycin (Val) at the concentration of 100 nM was added to TEA+Val. The data are expressed as mean±SD (n=5).

of AS or when K^+ permeability to the plasma membrane was increased by addition of valinomycin (Fig. 5), indicating that TEA could be a useful tool for investigating transmembrane cell signaling. Ba^{2+}, a well-known potent blocker of K^+ current through K^+ channels in the plasma membrane in various cells [16], inhibited the initiation of sperm motility in rainbow trout and masu salmon. The half-maximal concentration of Ba^{2+} necessary to inhibit the motility was 0.15 mM (data not shown).

Among inhibitors of voltage-dependent K^+ channel, 1 μM dendrotoxin-I, 1 μM β-dendrotoxin, 5 μM α-dendrotoxin, or 10 μM MCD-peptide effectively inhibited sperm motility of rainbow trout (Fig. 4). However, δ-dendrotoxin did not inhibit sperm motility of rainbow trout. Nonvoltage-dependent K^+ channel inhibitors, such as dendrotoxin-K, agitoxin, stichodactylatoxin, tityustoxin, and margatoxin did not inhibit sperm motility of rainbow trout.

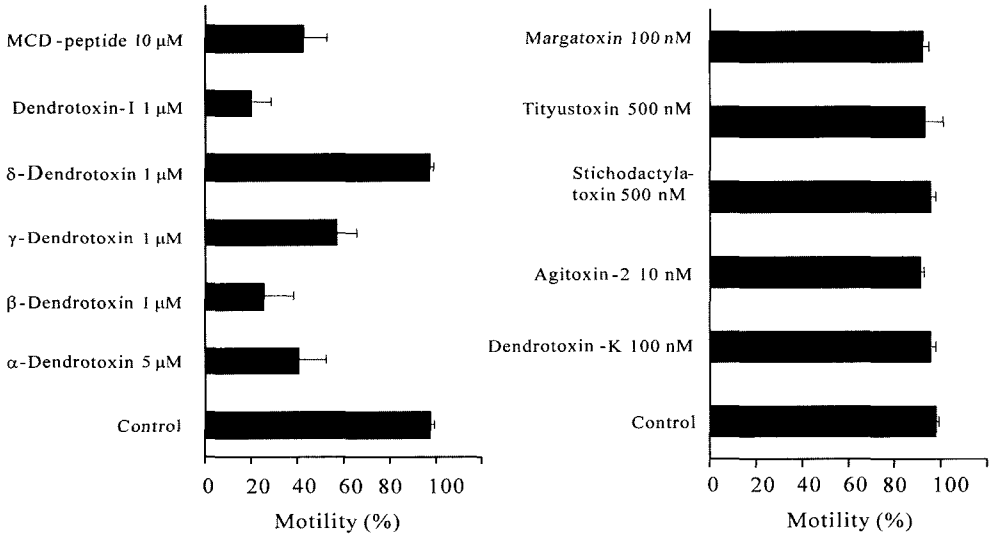

Fig. 4. Effects of K^+ channel blockers on sperm motility in the rainbow trout.
One volume of the semen was diluted in 10 volumes of ASP containing blockers of K^+ channel. After incubation for 5 min, aliquots of the sperm suspensions were diluted in AS containing the same concentration of blockers, and percentage of motile sperm was measured. Note that sperm exhibit full motility in AS (control), but some organic toxins known as the blockers of the voltage-dependent K^+ channels inhibited sperm motility (left). Other toxins, margatoxin, tityustoxin, stichodactylatoxin, agitoxin, dendrotoxin-K, in the right column did not. The data are expressed as mean±SD (n=5).

The inhibition of sperm motility by K^+ channel inhibitor was recovered when K^+ selectivity of the plasma membrane was cancelled by K^+ ionophore. As shown in Fig. 5, TEA completely suppressed the sperm motility of steelhead trout, but a subsequent addition of 100 nM valinomycin, a K^+-selective ionophore, recommenced motility.

Effects of K^+ and K^+ Channel Blocker on Membrane Potential

The sperm of salmonid fishes were activated by a decrease in external K^+ (Fig. 1). In order to determine if K^+ contributes to membrane potential in rainbow trout sperm, membrane potential was directly monitored using the fluorescent probe, $DiSC_3(5)$. The positively charged $DiSC_3(5)$ is redistributed across the lipid bilayer of the plasma membrane, driven by the membrane potential, and hyperpolarization of the membrane decreases $DiSC_3(5)$ fluorescence, while depolarization increases its fluorescence. As shown in Fig. 6, when the sperm of rainbow trout suspended in ASP were resuspended in AS containing $DiSC_3(5)$, sperm became motile, and fluorescence of the dye reached the stable level. After the membrane potential in AS became stable, potential from mitochondria was cancelled by the addition of CCCP, causing a slight increase in fluorescence

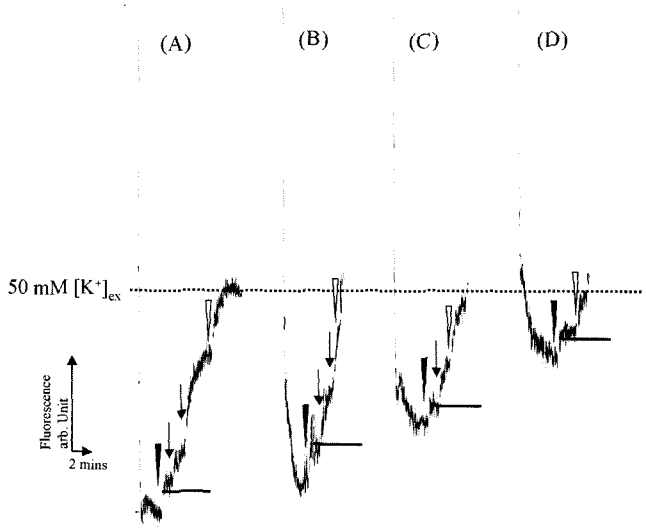

Fig. 6. Membrane potential of rainbow trout sperm in the presence of various concentrations of external K^+ ($[K^+]_{ex}$).
The sperm of rainbow trout diluted in ASP containing 40 mM $[K^+]_{ex}$ were diluted in AS containing the fluorescence dye $DiSC_3(5)$ and 0 (**A**), 10 (**B**), 20 (**C**), or 40 mM (**D**) $[K^+]_{ex}$. After the fluorescence level became stable, CCCP was added (filled arrowhead) to cancel mitochondrial membrane potential. After the fluorescence became stable, which represents the membrane potential of the sperm at each K^+ concentration (solid bar), KCl was sequentially added (arrow) to a final K^+ concentration of 50 mM (open arrowhead). Note that the fluorescence level, namely membrane potential, was the same in the presence of 50 mM $[K^+]_{ex}$ (dotted line), and membrane potential of sperm (solid line) became negative concomitant with decrease in K^+ concentration (from D to A).

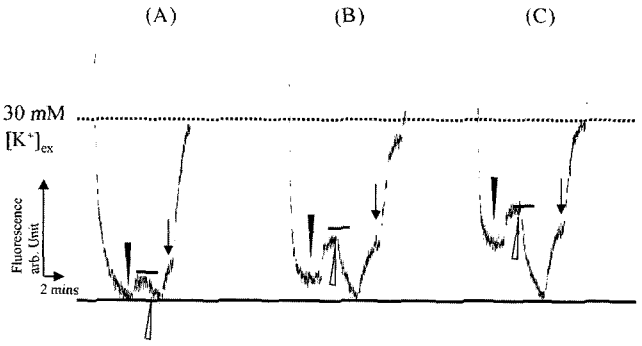

Fig. 7. Effect of TEA on membrane potential of rainbow trout sperm.
The sperm suspended in ASP containing 40 mM KCl were diluted in AS containing 0 (**A**), 25 (**B**), or 50 mM (**C**) TEA. After the fluorescence level became stable, CCCP was added (filled arrowhead) to cancel mitochondrial membrane potential, exhibiting sperm membrane potential (solid bar). After the membrane potential became stable, valinomycin was added (open arrowhead) to eleminate K^+ selectivity of the plasma membrane. Subsequent addition of 30 mM KCl (arrow) depolarized the plasma membrane to reach the same level. Note that the membrane potential reached the same level (solid line in the bottom) after addition of valinomycin. The difference of membrane potential of sperm before (solid bars) and after addition of valinomycin (solid line in the bottom) became larger concomitant with the increase in TEA concentration.

(Fig. 6A), suggesting that the mitochondrial potential is negligible in the sperm cells. An increase in K^+ concentration causes a decrease in the intensity of fluorescence, and the intensity decreased in a K^+-concentration-dependent manner (Figs. 6B, 6C, and 6D), showing stepwise depolarization of the plasma membrane. Subsequent additions of KCl induced depolarization of the sperm membrane, and the membrane potential reached the same level as in the presence of 50 mM KCl.

TEA, a K^+ channel blocker [40], inhibited both sperm motility (Fig. 5) and hyperpolarization of the sperm plasma membrane at 25 and 50 mM concentrations (Fig. 7). The subsequent addition of valinomycin caused the hyperpolarization, and the level of membrane potential in both the absence (Fig. 7A) and presence (Figs. 7B and 7C) of TEA reached the same level. The subsequent addition of KCl depolarized the plasma membrane, and the membrane potential in the presence of 30 mM KCl reached the same level. The difference between the levels after the addition of CCCP and valinomycin increased 6 and 10 times in the presence of 25 mM (Fig. 7B) and 50 mM (Fig. 7C) TEA, respectively, when they were compared with that in the absence of TEA (Fig. 7A). These results suggest that TEA inhibits not only sperm motility, but also membrane hyperpolarization.

Role of cAMP on the Activation of Sperm Motility

The sperm diluted in potassium-free AS were motile (Fig. 1), and the concentration of intracellular cAMP was 2,340 fmol/mg protein (Fig. 8), whereas the concentration

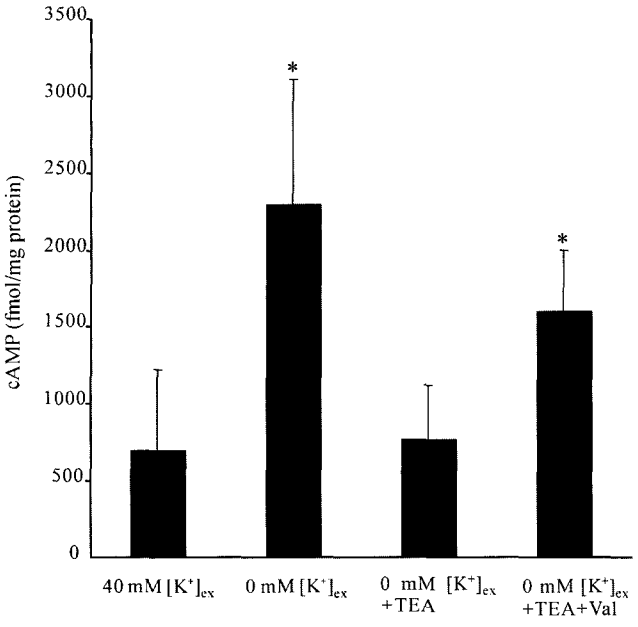

Fig. 8. Effects of TEA and valinomycin on the cAMP synthesis. The cAMP levels of valinomycin or TEA-treated sperm were measured at 5 sec as described in Materials and Methods. The data are expressed as mean±SD (n=5). An asterisk indicates a significant difference after paired t-test ($p<0.05$) from the data in the presence of 40 mM $[K^+]_{ex}$.

of cAMP in the presence of 40 mM K^+ was 690 fmol/mg protein. The sperm diluted in the AS containing TEA became immotile and the cAMP level was almost the same as that of 40 mM K^+. When valinomycin was added to the TEA-dependent immotile sperm, the sperm became motile (Fig. 5), and the cAMP was increased up to 1,588 fmol/mg protein after 5 sec (Fig. 8). MCD-peptide, which inhibited sperm motility (Fig. 4), also strongly inhibited the cAMP, and the subsequent addition of valinomycin increased the cAMP, although the valinomycin treatment could not recover the motility (data not shown).

DISCUSSION

In salmonid fishes, it is well established that the high concentration of K^+ (around 40 mM) contained in the seminal plasma [24] enforces the sperm quiescent in the sperm duct. The decrease in K^+ surrounding sperm at spawning in fresh water confers mobility to the sperm [29, 30]. However, duration of sperm motility in salmonid fishes is very short (20–25 sec). The detailed studies by Cosson *et al.* [14, 15] in rainbow trout as well as this present study in the same species and masu salmon (Fig. 1) showed that about half of the sperm suspended in K^+-free medium became immotile within 10 sec after suspension, and almost all sperm became quiescent at 25–30 sec. Rb^+ and Cs^+ as well as K^+ inhibited motility, but Na^+ and Li^+ did not inhibit sperm motility of both rainbow trout and masu salmon with the ionic selectivity sequence of $Rb^+ > K^+ > Cs^+ \gg Li^+ > Na^+$ (Fig. 2). This ionic selectivity is similar to that reported for the speract-induced hyperpolarization in intact sea urchin sperm [1], suggesting participation of K^+ channels on the regulation of sperm motility in salmonid fishes as well as that of sperm motility of acrosome reaction in sea urchin.

The sperm motility of rainbow trout was suppressed in the presence of TEA and Ba^{2+} at half the maximal concentrations of 9.0 mM and 0.036 mM, respectively [40], whose concentration is comparable to that of other functions occurring with sperm and oocyte. Furthermore, voltage-dependent K^+ channel blockers, such as α-dendrotoxin, β-dendrotoxin, dendrotoxin-I, and MCD-peptide, also effectively inhibited the motility of sperm in rainbow trout (Fig. 4). The K^+ selectivity on blockage of the initiation of sperm motility by all K^+ channel blockers examined suggests that voltage-dependent K^+ current produced from the inside to the outside of sperm cells plays an important role in the initiation of sperm motility in salmonid fishes.

Ion channel-dependent K^+ permeability contributes mainly to the resting potential of many kinds of cells [22]. It has also been reported that the changes of membrane potential and of K^+ permeability regulate sperm functions. With regard to the acrosome reaction, the membrane potential of sea urchin sperm in seawater is almost unresponsive to the addition of KCl in seawater, indicating low resting K^+ permeability of the sperm [1, 3]. The speract, a decapeptide released from the egg jelly layer of sea urchin, increases K^+ permeability by increasing cGMP [11], thus inducing hyperpolarization of the sperm membrane [1, 32]. The membrane hyperpolarization followed by the subsequent membrane depolarization [18] is necessary for the elevation of cAMP [3, 12] and subsequent Ca^{2+} influx [33], respectively, resulting in the acrome reaction. Concerning sperm motility in rainbow trout, measurement of membrane potential using lipophilic tracers showed a K^+ concentration-dependent change in membrane potential on a time scale of minutes [17], although trout sperm was irreversibly activated in less than one sec and ceased to move within 20 sec (Fig. 1). Therefore, relevance of the time scales of the membrane potential change in relation to the activation of sperm motility is unclear. Boitano and Omoto [5, 6] further showed that membrane hyperpolarization of trout sperm induced with both Cs^+ and valinomycin in K^+-supplemented medium could initiate motility. In the present study, the decrease in extracellular K^+ concentration induced a gradual increase in sperm motility (Fig. 2) and hyperpolarization of plasma membrane (Fig. 6) in the trout sperm. The experimental condition, in which the K^+ concentration decreased from 40 mM to 0 mM, is similar to the environmental changes in K^+ concentration at natural

spawning. Furthermore, the membrane hyperpolarization was suppressed by K^+ channel inhibitor (Fig. 7). Therefore, it is possible that K^+ efflux through the K^+ channel is suppressed by a high seminal K^+ concentration at rest of trout sperm in the male reproductive tract. Upon spawning of the sperm in fresh water, a decrease in the environmental K^+ concentration may cause spontaneous efflux of K^+ through the channel to lead to the membrane hyperpolarization.

The cooperative role of membrane hyperpolarization and cAMP synthesis in the fast forward swimming of *Paramecium* was suggested by Bonini *et al.* [7]. It was also found that adenylate cyclase of *Paramecium* cilia has the property of voltage-dependent K^+ channel [37], suggesting that the hyperpolarization of the plasma membrane by K^+ efflux could directly synthesize cAMP through the activation of adenylate cyclase. It was previously demonstrated that the decrease in K^+ might induce the synthesis of intracellular cAMP via Ca^{2+}-dependent activation of adenylate cyclase [27], and resulting cAMP-dependent phosphorylation of axonemal protein triggers the final step of the initiation of sperm motility [25]. Although changes in extracellular K^+ and cAMP are known to influence sperm motility, their relationship between the ion and second messenger has been unknown. In the present study, suppression of sperm motility (Fig. 5), membrane hyperpolarization (Fig. 7), and cAMP synthesis (Fig. 8) by K^+ channel blocker were restored by subsequent addition of a K^+ ionophore, valinomycin. These results clearly indicate that K^+ efflux through the K^+ channel contributes to rapid changes in the membrane potential and synthesis of cAMP, which is a second messenger, triggering the following cell signaling, and cAMP-dependent phosphorylation of proteins [23] for the initiation of sperm motility.

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References

1. Babcock, D. F., M. Mosma, D. E. Battaglia, and A. Darszon. 1992. Early persistent activation of sperm K^+ channels by the egg peptide speract. *Proc. Natl. Acad. Sci. USA* **89:** 6001-6005.
2. Baynes, S. M., A. P. Scott, and A. P. Dawson. 1981. Rainbow trout, *Salmo gairdneri* Richardson, spermatozoa: Effects of cations and pH on motility. *J. Fish Biol.* **19:** 259-267.
3. Beltran, C., O. Zapata, and A. Darszon. 1996. Membrane potential regulates sea urchin sperm adenylylcyclase. *Biochemistry* **35:** 7591-7598.
4. Benau, D. and C. Terner. 1980. Initiation, prolongation and reactivation of the motility of salmonid spermatozoa. *Gamet. Res.* **3:** 247-257.
5. Boitano, S. and C. K. Omoto. 1991. Membrane hyperpolarization activates trout sperm without an increase in intracellular pH. *J. Cell Sci.* **98:** 343-349.
6. Boitano, S. and C. K. Omoto. 1992. Trout sperm swimming patterns and role of intracellular Ca^{2+}. *Cell Motil. Cytoskel.* **21:** 74-82.
7. Bonini, N. M., M. C. Gustin, and D. L. Nelson. 1986. Regulation of ciliary motility by membrane potential in *Paramecium*: A role for cyclic AMP. *Cell Motil. Cytoskel.* **6:** 256-272.
8. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248-254.
9. Cabrini, G. and A. S. Verkman. 1986. Localization of cyanine dye binding to brush-border membranes by quenching of n-(9-anthroyloxy) fatty acid probes. *Biochim. Biophys. Acta* **862:** 285-293.
10. Christen, R., J. L. Gatti, and R. Billard. 1987. Trout sperm motility: The transient movement of trout sperm is related to changes in the concentration of ATP following the activation of the flagellar movement. *Eur. J. Biochem.* **166:** 667-671.
11. Cook, S. P. and D. F. Babcock. 1993. Activation of Ca^{2+} permeability by cAMP is coordinated through the pHi increase induced by speract. *J. Biol. Chem.* **268:** 22408-22413.
12. Cook, S. P. and D. F. Babcock. 1993. Selective modulation by cGMP of the K^+ channel activated by speract. *J. Biol. Chem.* **268:** 22402-22407.
13. Cosson, J., R. Billard, C. Cibert, C. Dreanno, and M. Suquet. 1999. Ionic factors regulating the motility of fish sperm, pp. 161-186. *In* C. Gagnon (ed.), *The Male Gamete: From Basic Science to Clinical Applications.* Cache River Press.
14. Cosson, M. P., R. Billard, and R. Billard. 1991. Synchronous triggering of trout sperm is followed by an invariable set sequence of movement parameters whatever the incubation medium. *Cell Motil. Cytoskel.* **20:** 55-68.
15. Cosson, M. P., R. Billard, and L. Letellier. 1989. Rise of internal Ca^{2+} accompanies the initiation of trout sperm motility. *Cell Motil. Cytoskel.* **14:** 424-434.
16. Eaton, D. C. and M. S. Brodwick. 1980. Effects of barium on the potassium conductance of squid axon. *J. Gen. Physiol.* **75:** 727-750.
17. Gatti, J. L., R. Billard, and R. Christen. 1990. Ionic regulation of the plasma membrane potential of rainbow trout, *Salmo gaiirdneri*, spermatozoa: Role in the initiation of sperm motility. *J. Cell. Physiol.* **143:** 546-554.

18. González-Martínez, M. T., A. Guerrero, E. Morales, L. D. Torre, and A. Darszon. 1992. A depolarization can trigger Ca^{2+} uptake and the acrosome reaction when preceded by a hyperpolarization in *L. pictus* sea urchin sperm. *Dev. Biol.* **150:** 193-202.
19. Hagiwara, S., S. Miyazaki, W. Moody, and J. Patlak. 1978. Blocking effects of barium and hydrogen ions on the potassium current during anomalous rectification in the starfish egg. *J. Physiol.* **279:** 167-185.
20. Hayashi, H., K. Yamamoto, H. Yonekawa, and M. Morisawa. 1987. Involvement of tyrosine kinase in the initiation of flagellar movement in rainbow trout spermatozoa. *J. Biol. Chem.* **262:** 16692-16698.
21. Hodgkin, A. L. 1951. The ionic basis of electrical activity in nerve and muscle. *Biol. Rev.* **26:** 339-409.
22. Hodgkin, A. L. and P. Horowicz. 1960. Potassium contractures in single muscle fibers. *J. Physiol.* **153:** 386-403.
23. Inaba, K., S. Morisawa, and M. Morisawa. 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.
24. Morisawa, M. 1985. Initiation mechanism of sperm motility at spawning in teleosts. *Zool. Sci.* **2:** 605-615.
25. Morisawa, M. and H. Hayashi. 1985. Phosphorylation of a 15 K axonemal protein is the trigger initiating trout sperm motility. *Biomed. Res.* **6:** 181-184.
26. Morisawa, M., T. Hirano, and K. Suzuki. 1979. Changes in blood and seminal plasma composition of the mature salmon, *Oncorhynchus keta*, during adaptation to freshwater. *Comp. Biochem. Physiol.* **64A:** 325-329.
27. Morisawa, M. and K. Ishida. 1987. Short-term changes in levels of cyclic AMP, adenylatecyclase, and phosphodiesterase during the initiation of sperm motility in rainbow trout. *J. Exp. Zool.* **242:** 199-204.
28. Morisawa, M. and M. Okuno. 1982. Cyclic AMP induces maturation of trout sperm axoneme to initiate motility. *Nature* **295:** 703-704.
29. Morisawa, M. and K. Suzuki. 1980. Osmolarity and potassium ion: Their roles in initiation of sperm motility in teleosts. *Science* **210:** 1145-1147.
30. Morisawa, M., K. Suzuki, and S. Morisawa. 1983. Effects of potassium and osmolality on spermatozoan motility of salmonid fishes. *J. Exp. Biol.* **107:** 105-113.
31. Nam, D. H., S. K. Lim, M. H. Chung, E. S. Lee, Y. S. Sohn, and D. Y. Ryu. 2001. Membrane transporter genes in cephabacin biosynthetic gene cluster of *Lysobacter lactamgenus*. *J. Miocrobiol. Biotechnol.* **11:** 153-159.
32. Reynaud, E., L. D. L. Torre, O. Zapata, A. Lievano, and A. Darszon. 1993. Ionic bases of the membrane potential and intracellular pH changes induced by speract in swollen sea urchin sperm. *FEBS Lett.* **329:** 210-214.
33. Ro, J. Y., J. Y. Kim, J. H. Ha, and C. H. Lee. 2002. Ga12 and Ga13 subunits modulate Ca^{2+}-induced histamine release in human umbilical cord blood-derived mast cells. *J. Microbiol. Biotechnol.* **12:** 476-483.
34. Schackmann, R. W., E. M. Eddy, and B. M. Shapiro. 1978. The acrosome reaction of *Strongylocentrotus purpuratus* sperm. *Develop. Biol.* **65:** 483-495.
35. Schackmann, R. W. and B. M. Shapiro. 1981. A partial sequence of ionic changes associated with the acrosome reaction of *Strongylocentrotus purpuratus*. *Develop. Biol.* **81:** 145-154.
36. Schlenk, W. and H. Kahmann. 1938. Die chemische Zusammensetzumg des Spermaliquors und ihre physiologische Bedeutung. Untersuchung am Forellensperma. *Biochem. Z.* **295:** 283-301.
37. Schultz, J. E., S. Klumpp, R. Benz, W. J. C. Schürhoff-Goeters, and A. Schmid. 1992. Regulation of adenylyl cyclase from *Paramecium* by an intrinsic potassium conductance. *Science* **255:** 600-603.
38. Stanfield, P. R. 1983. Tetraethylammonium ions and the potassium permeability of excitable cells. *Rev. Physiol. Biochem. Pharmacol.* **97:** 1-67.
39. Tanimoto, S., Y. Kudo, T. Nakazawa, and M. Morisawa. 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.
40. Tanimoto, S. and M. Morisawa. 1988. Roles of potassium and calcium channels in the initiation of sperm motility in rainbow trout. *Dev. Growth Diff.* **30:** 117-124.