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Transmembrane Cell Signaling for the Initiation of Trout Sperm Motility: Roles of Ion Channels and Membrane Hyperpolarization for Cyclic AMP Synthesis

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ABSTRACT—Motility of trout sperm is suppressed by extracellular K^+ at rest and initiated by the decrease in the extracellular K^+ concentration, and Ca^{2+} and cyclic AMP participate in the cell signaling for the initiation of sperm motility. We showed here that tetraethyl ammonium (TEA) and some blockers used as the voltage-dependent K^+ channel blockers in the neuron inhibited the motility in the sperm of the rainbow trout and the steelhead trout, *Oncorhynchus mykiss*. Other types of K^+ channel blockers, e.g., apamin, a blocker of small conductance Ca^{2+}-dependent K^+ channels, did not inhibit sperm motility. L-type Ca^{2+} channel blockers such as nifedipine, nimodipine, etc. also inhibited the motility, but other types of Ca^{2+} channel blockers did not. On the other hand, the gradual increase in external K^+ concentration caused both gradual decreases in the amplitude of hyperpolarization of the sperm plasma membrane and cyclic AMP synthesis. TEA and nifedipin suppressed both hyperpolarization and cyclic AMP synthesis, and these suppressions were relieved by addition of the K^+ ionophore, valinomycin. The calmodulin inhibitors W-7, trifluoroperazine and calmidazol-Cl inhibited the sperm motility, membrane hyperpolarization and cyclic AMP synthesis, although only at rather high concentrations. These results suggest that K^+ efflux through K^+ channels and Ca^{2+} influx through Ca^{2+} channels that are sensitive to specific channel blockers cause the changes of membrane potential and lead to a synthesis of cyclic AMP in the cell signaling for the initiation of trout sperm motility.

INTRODUCTION

Initiation and activation of sperm motility are prerequisite processes for the contact and fusion of male and female gametes at fertilization. The phenomena are under the regulation of cAMP and Ca^{2+} in vertebrates and invertebrates. Mammalian sperm requires Ca^{2+} and cyclic AMP (Tash and Means, 1983; Okamura *et al*., 1985) for the activation of sperm motility. Sperm activating peptides, SAPs, derived from the jelly layer of sea urchin egg (Suzuki, 1995) have been shown to increase cyclic GMP and cyclic AMP to cause transient hyperpolarization of sperm membrane potential and, in turn, regulation of sperm motility (Cook *et al*., 1994). Cell signaling for the initiation and activation of sperm motility has been well studied in the ascidians, *Ciona intestinalis* and *C. savignyi* and the Pacific herring, *Clupea pallasi* (Oda *et al*., 1998; Pillai *et al*., 1993). In *Ciona*, whose cell signaling for activation of sperm motility has been well established, the sperm-activating and -attracting factor, SAAF, released from unfertilized egg activates sperm motility (Yoshida *et al*., 1993). Upon the activation of sperm motility, SAAF increases the potassium permeability of the plasma membrane, and the K^+ efflux induces membrane hyperpolarization (Izumi *et al*., 1999). The hyperpolarization activates adenylyl cyclase to increase the intracellular cyclic AMP, resulting in phosphorylation of axonemal proteins including an outer arm dynein light chain and a 26kDa phosphoprotein that serves as an axoneme component (Nomura *et al*., 2000). On the other hand, the activation of sperm motility caused by SAAF requires an increase in intracellular Ca^{2+} via an influx of extracellular Ca^{2+} through Ca^{2+} channels (Yoshida *et al*., 1994), and participation of calmodulin in the SAAF-dependent activation of *Ciona* sperm motility has been suggested (Nomura *et al*., 2000). However, it is still unknown whether the Ca^{2+} signaling is an independent process or is involved in membrane hyperpolarization-

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induced cyclic AMP signaling in the salmonid fishes.

In addition to egg-derived SAPs and SAAF, changes in the environmental ionic and osmotic conditions surrounding sperm constitute a separate category of factors regulating sperm motility (Morisawa and Suzuki, 1980). For example, a decrease in K^+ concentration surrounding the sperm of salmonid fish causes a K^+ efflux (Tanimoto *et al.*, 1994), membrane hyperpolarization (Gatti *et al.*, 1990; Tanimoto *et al.*, 1988; Boitano and Omoto, 1991), an increase in intracellular Ca^{2+} (Cosson *et al.*, 1989) and cyclic AMP synthesis (Morisawa and Ishida, 1987). The subsequent cyclic AMP-dependent phosphorylation of axonemal proteins with a molecular mass of 15 kDa (Hayashi, *et al.*, 1987) and dynein light chain (Inaba *et al.*, 1998) triggers the final step for the cyclic AMP-dependent initiation of sperm motility (Morisawa and Okuno, 1982). Thus, the first trigger, the decrease in external K^+ concentration, and the intracellular signal cascades, such as cyclic AMP-dependent phosphorylation of proteins, have been clarified in the salmonid fishes. However, the characteristics of the transmembrane cell signaling cascade, i.e., the ion channels that participate in the initiation of trout sperm motility, remain to be clarified. Further, the relationship among K^+ channels, Ca^{2+} channels, membrane potential and cyclic AMP synthesis also remains to be clarified.

The results of the present study revealed that, in the trout, K^+ efflux through certain K^+ channels that are open at rest and Ca^{2+} influx through dihydropyridine-sensitive Ca^{2+} channels lead to hyperpolarization of the plasma membrane and subsequent synthesis of cyclic AMP to initiate sperm motility. The effects of calmodulin inhibitors on sperm motility, membrane potential and cyclic AMP synthesis were also investigated. However, the role of calmodulin on the transmembrane cell signaling for the initiation of sperm motility still remains to be determined.

MATERIALS AND METHODS

Chemicals

$DiSC_3(5)$, 3, 3'-dipropylthiadicarbocyanine iodide, was purchased from Molecular Probe (Leiden, Netherlands); carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and valinomycin were from Sigma Chemical Co. (St. Louis, MO); W-5, W-7, KN-92 and KN-93 were from Seikagakukogyo (Tokyo, Japan); tetraethylammonium chloride (TEA) from Nacalai Tesque (Kyoto, Japan); the K^+ channel blockers, α-dendrotoxin, β-dendrotoxin, γ-dendrotoxin, δ-dendrotoxin, dendrotoxin-I, apamin and mast cell-degranulating peptide (MCD-peptide), and the Ca^{2+} channel blockers, nifedipine, nimodipine, taicatoxin, FS-2, ω-conotoxin MVIIA, PLTX-II, sFTX3.3 and FTX3.3 were from Alomone Laboratories (Jerusalem, Israel); and the cyclic AMP enzyme immunoassay (EIA) system (PRN 225) was from BIOTRAK (Amersham Pharmacia Biotech, Buckinghamshire, England). CCCP, valinomycin, $DiSC_3(5)$ and nifedipin were dissolved in DMSO at 0.2, 0.1 and 0.1% v/v, respectively. Nimodipine was dissolved in 1% ethanol. These concentrations had no deteriorating effect on sperm motility, and the media used in the control experiment contained the same concentration of the solvents. All other reagents used were of analytical grade.

Collection of sperm

Mature males of the rainbow trout and the steelhead trout, which is the sea-run rainbow trout, *Oncorhynchus mykiss* were provided by the Yamanashi Prefectural Fisheries Experiment Station of the Oshino Trout Hatchery (Fujiyoshida, Japan) and kept in an indoor aquarium at 10°C. The fish were not fed during the 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 glass. A two-step dilution procedure for measuring sperm motility was used, as reported by Cosson *et al.* (1989), to achieve synchronous induction of sperm motility. The semen was diluted 10-fold in physiological saline (ASP) consisting of 130 mM NaCl, 40 mM KCl, 2.5 mM $CaCl_2$, 1.5 mM $MgCl_2$ and10 mM HEPES-NaOH at pH 7.8. The composition of the ASP was based on the ionic and osmotic composition of the seminal plasma of the rainbow trout (Morisawa, 1985). Sperm motility was completely suppressed in the ASP by the 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 and 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-NaOH 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 video camera (Hamamatsu C2400 SIT; Hamamatsu, Japan) attached to the microscope. The percentage of motile sperm and their velocity were measured from images of the tracks of sperm using a Cellsoft semen analyzer (Cellsoft series 3000; NAC Incorporated, Tokyo, Japan).

Effects of ion channel blockers and calmodulin inhibitors on sperm motility

The effects of ion channel blockers and calmodulin inhibitors on the initiation of sperm motility were investigated by diluting the sperm in the ASP containing various concentrations of ion channel blockers and calmodulin inhibitors, and then suspending the sperm in AS containing each blocker at the same concentration.

Membrane potential measurement

The semen was diluted in the ASP at a dilution ratio of 1:10. After predilution, 20 μl of the sperm suspension was diluted in 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 subsequent addition of 1 μM CCCP, followed by addition of 1 μM valinomycin to make the plasma membrane maximally permeable to K^+. Finally, KCl was 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 emission wavelength of 670 nm with continuous stirring at 18°C. A single loading of 1 μM $DiSC_3(5)$ to the 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 the experimental conditions.

Assay of cyclic AMP

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

tion was measured using a commercial reagent for protein measurement from BIO-RAD (Bio-Rad Protein Assay; Bio-Rad Laboratories, Hercules, CA).

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

RESULTS

Effects of K^+- and Ca^{2+}-channel blockers on sperm motility

Just after dilution of the ASP-pre-diluted sperm into AS,

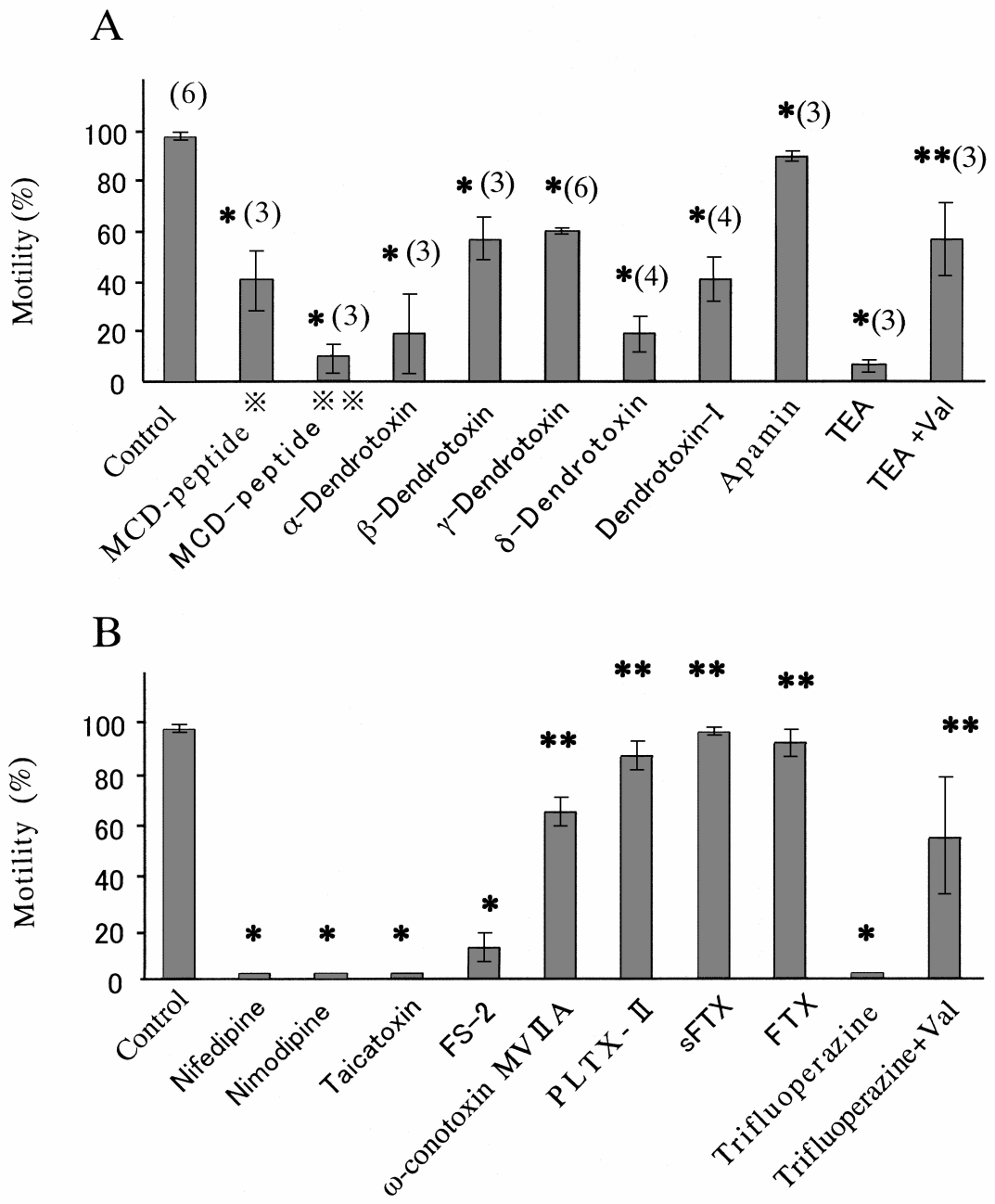

Fig. 1. Effects of blockers of K^+- and Ca^{2+}-channels on sperm motility. Sperm of the rainbow trout were diluted in ASP containing blockers of the voltage-dependent K^+ channel (A): α-dendrotoxin (5 μM), β-dendrotoxin (1 μM), γ-dendrotoxin (1 μM), δ-dendrotoxin (1 μM), dendrotoxin-I (1 μM), apamin (10 μM), MCD-peptide (5※ and 10 μM※※), TEA (25 mM); or blockers of the Ca^{2+} channel (B): nifedipine (100 μM), nimodipine (20 μM), taicatoxin (5 μM), FS-2 (20 μM), ω-conotoxin MVIIA (200 μM), PLTX-II (0.5 μM), sFTX3.3 (100 μM), FTX3.3 (10 μM) and trifluoroperazine (200 μM). After incubation for 5 minutes, aliquots of the sperm suspensions were diluted in AS containing the same concentration of blockers, and the percentage of motile sperm was measured. Valinomycin (Val) at a concentration of 0.1 μM was added to the immotile sperm in the presence of TEA or trifluoroperazine. Note that the sperm exhibited full motility in AS (control), but that some blockers known as blockers of the voltage-dependent K^+ channels and blockers of L-/T-type Ca^{2+} channels inhibited sperm motility. Inhibition of motility by TEA or trifluoroperazine was relieved by addition of valinomycin. The data are expressed as the means±SD. Numbers of experiments are indicated in the parentheses in (A), and all experiments were performed in triplicate in (B). Data marked with asterisks were significantly different from the control data ($p<0.05$). TEA+Val and trifluoroperazine+Val were significantly different from +TEA ($p<0.05$). Double asterisks indicate an absence of significant difference.

more than 95% of the sperm of the rainbow trout and steelhead trout began to move straight with an average velocity of 180 μm/sec. The percentage of motile sperm declined within 15 sec, and the swimming pattern became circular and halted by 25 sec (data not shown). The results were almost the same as those obtained previously in chum salmon (Morisawa and Suzuki, 1980), masu salmon, char (Morisawa *et al.*, 1983) and rainbow trout (Morisawa *et al.*, 1983; Cosson *et al.*, 1991). Blockers effective for the voltage-dependent K^+ channels that are mainly used in the neurons, β-dendrotoxin, δ-dendrotoxin, γ-dendrotoxin and dendrotoxin-I at the concentration of 1 μM and α-dendrotoxin at the concentration of 5 μM, effectively inhibited the sperm motility of rainbow trout (Fig. 1A). The blocker concentrations effective for inhibiting the motility of live sperm cells in the present study were higher than those used in the investigation of ion fluxes through the isolated sperm plasma membrane (Galindo *et al.*, 2000). MCD-peptide at a concentration of 5 μM, which has been shown to inhibit the motility of *Ciona* sperm (Izumi *et al.*, 1999), also inhibited trout sperm motility in the present work (Fig. 1A). Blockers of other types of K^+ channels did not inhibit the sperm motility (data not shown). For example, apamin—a blocker of the small conductance Ca^{2+} -dependent K^+ channel—and iberiotoxin, paxilline, penitrem and verruculogen-blockers of the large conductance Ca^{2+} -activated K^+ channel-did not inhibit sperm motility at concentrations up to 200 μM (Fig. 1A). TEA, which is generally known to inhibit the efflux of K^+ through the voltage-dependent K^+ channels (Stanfield, 1983), suppressed 50% of sperm motility at concentrations lower than 25 mM in the rainbow trout (Fig. 1). The experimental media was kept at a constant osmolality of 300 mOsm/kg by replacing TEA for NaCl in AS. Sperm motility, which was blocked by 25–200 mM TEA, was reversibly recovered when the concentration of TEA was decreased by addition of AS, indicating that TEA is a useful tool for investigating transmembrane cell signaling in the trout sperm. Furthermore, sperm motility that was completely inhibited by 25 mM TEA was rescued when the plasma membrane was made maximally permeable

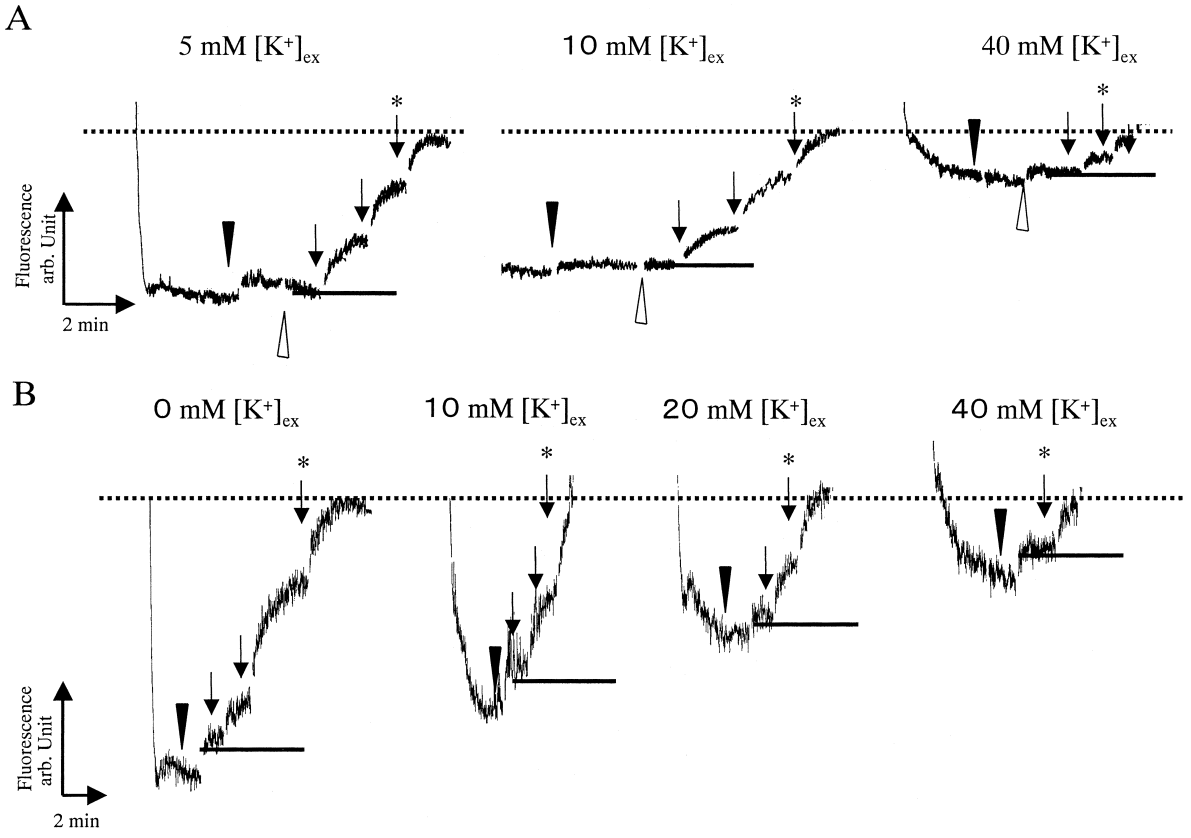

Fig. 2. Membrane potential of the sperm in the presence of various concentrations of external K^+, ($[K^+]_{ex}$). The sperm of rainbow trout diluted in ASP containing 40 mM $[K^+]_{ex}$ were diluted in AS containing the fluorescent dye $DiSC_3(5)$ and 0, 10, 20 or 40 mM $[K^+]_{ex}$. After the fluorescence level became stable, CCCP was added (filled arrowhead in A) to cancel mitochondrial membrane potential. After the fluorescence became stable, which represents the membrane potential of the sperm at each K^+ concentration (solid bar), 0.1 μM valinomycin was added (open arrowhead) in A and then KCl was added (arrow) to the final K^+ concentration of $[K^+]_{ex}$ (arrow with asterisk). In B, valinomycin was not added, and KCl was directly added. After the fluorescence level, namely the membrane potential, became stable in the presence of external 75 (A) or 50 mM $[K^+]_{ex}$, the levels of 0, 10, 20 or 40 mM $[K^+]_{ex}$ were aligned (dotted line). Note that the membrane potential of the sperm (solid bar) in 40 mM $[K^+]_{ex}$ may be the membrane potential of the sperm in the seminal plasma containing 40 mM $[K^+]_{ex}$ (Morisawa, 1985), and the potential may become negative concomitantly with the decrease in K^+ concentration from 40 mM to 0 mM $[K^+]_{ex}$. Typical patterns of three experiments (A) and four experiments (B) are shown.

to K^+ by a K^+-selective ionophore, valinomycin, at a concentration of 0.1 μM. These results suggest that K^+ permeability that is sensitive to TEA and some other specific K^+ channel blockers is essential for the initiation of trout sperm motility.

FS-2 (20 μM) and taicatoxin (5 μM), which are considered to be blockers of L-type Ca^{2+} channels in the neuron, completely inhibited the sperm motility of the rainbow trout (Fig. 1B). Dihydropyridine, nimodipine (20 μM) and nifedipine (100 μM), which are known as blockers of T-type Ca^{2+} channels in sperm (Lievano *et al*., 1996) and as blockers of L-type Ca^{2+} channels in other excitable cells, inhibited sperm motility. Blockers of Ca^{2+} channels other than the L- and T- types Ca^{2+} channels such as ω-conotoxin MVIIA, PLTX-II, and FTX at the concentration of 0.5–200 μM, showed only slight inhibition. Trifluoroperazine, which can block Ca^{2+} channels as effectively as calmodulin, has been reported to inhibit the acrosome reaction in sea urchin sperm and the membrane potential of mouse sperm (Espinosa and Darszon, 1995). This blocker inhibited trout sperm motility, and this inhibition was relieved by addition of 0.1 μM valinomycin (Fig. 1B).

Effects of extracellular K^+ concentration and K^+ channel blocker on membrane potential

K^+ at a concentration of 40 mM in the seminal plasma (Morisawa, 1985) completely suppresses sperm motility in the rainbow trout (Morisawa *et al*., 1983). A decrease in K^+ concentration in the area surrounding the sperm causes an increase in sperm motility (Morisawa and Morisawa, 1986). In order to determine whether the initiation of sperm motility by the decrease in K^+ concentration in the area surrounding the sperm correlates with K^+-dependent changes in membrane potential, we directly monitored membrane potential using the fluorescent probe, $DisC_3(5)$. Hyperpolarization of the membrane decreased $DisC_3(5)$ fluorescence, while depolarization increased its fluorescence. As shown in Fig. 2, when rainbow trout sperm suspended in the ASP were suspended in AS containing $DisC_3(5)$, the sperm became motile, and the dye fluorescence level became stable. After the membrane potential in AS became stable, the potential from mitochondria was cancelled by addition of CCCP, but this caused only a small increase in fluorescence (Fig. 2A), suggesting that

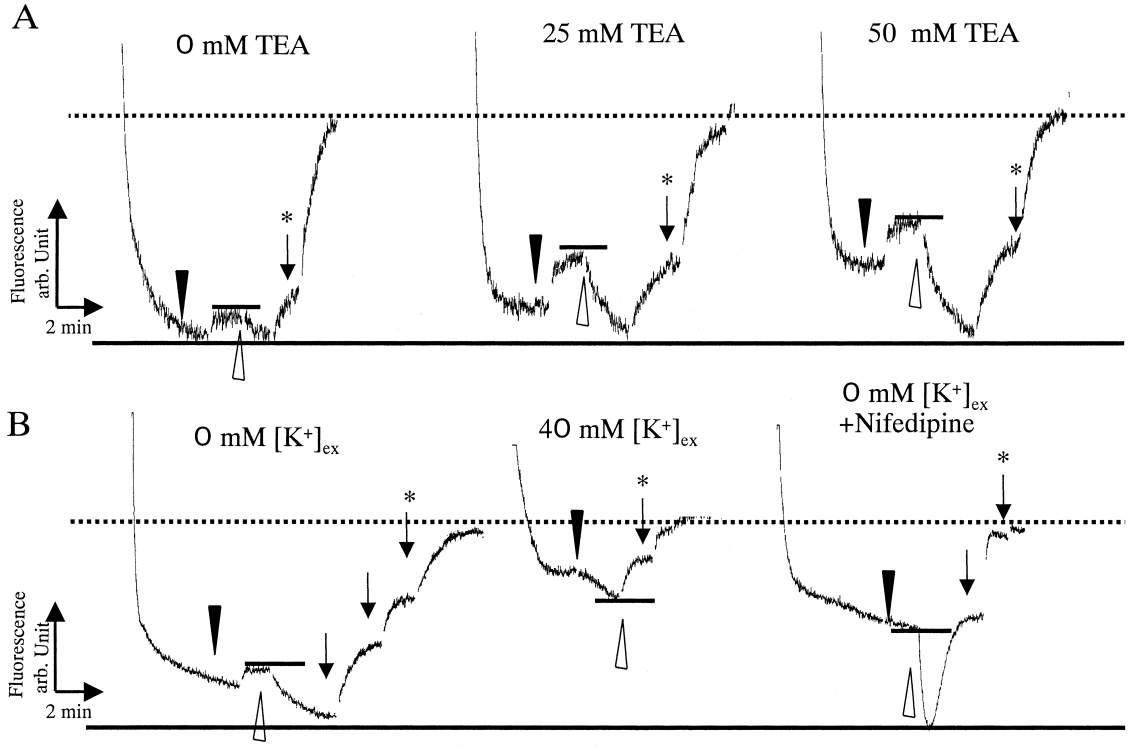

Fig. 3. Effects of a K^+- or Ca^{2+}- channel blocker on membrane potential of the sperm. The sperm of the rainbow trout diluted in the ASP containing external 40 mM KCl, 40 mM $[K^+]_{ex}$, were diluted in AS without KCl containing 0 mM, 25 mM or 50 mM TEA (A) or 100 μM nifedipine (B). After the fluorescence level, namely the membrane potential, became stable, CCCP was added (filled arrowhead). The stable level reached shows the sperm membrane potential (solid bar). Valinomycin at the concentration of 0.1 μM was then added (open arrowhead) to make the plasma membrane maximally permeable to K^+. Subsequent addition of 30 mM KCl (arrow) depolarized the plasma membrane. The final levels after addition of 30 mM $[K^+]_{ex}$ (arrow with asterisk) were aligned (dotted line). 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 concomitantly with the increase in TEA concentration or presence of nifedipine. These findings suggest that a K^+ channel blocker, TEA, and Ca^{2+} channel blocker, nifedipine, inhibited the hyperpolarization of the plasma membrane. $[K^+]_{ex}$ at the concentration of 40 mM inhibited membrane hyperpolarization (middle in B).

the mitochondrial potential was negligible in the sperm cells. Therefore, the stable fluorescence level in the presence (Fig. 2A) or absence (Fig. 2B) of added CCCP is considered to represent the membrane potential of the sperm at each K^+ concentration. The stepwise increase in external K^+ concentration (arrows in Fig. 2) caused a stepwise decrease in the intensity of fluorescence, i.e., a stepwise depolarization of the plasma membrane both in the presence (Fig. 2A) and absence (Fig. 2B) of valinomycin. After the fluorescence intensity at the same final external concentration of K^+ (75 or 50 mM) following stepwise addition of K^+ was aligned (dotted line in Fig. 2), the levels of membrane potential in each experiment were compared. Consequently, the fluorescence intensity, which is comparable to the resting membrane potential at each K^+ concentration (solid bars in Fig. 2) decreased with an increase in external K^+ concentration. This suggests that the sperm plasma membrane behaves as a potassium electrode whose potential is defined by the Nernst equation, and that the gradual decrease in K^+ concentration surrounding the sperm caused a gradual hyperpolarization of the plasma membrane. The decrease in fluorescence intensity was not due to quenching causing by KCl, since the fluorescence of AS containing $DisC_3(5)$ did not change upon addition of 40 mM KCl (data not shown).

The degree of membrane hyperpolarization in the rainbow trout sperm decreased with increasing concentration of TEA, a K^+ channel blocker (solid bars in Fig. 3A). Subsequent addition of valinomycin caused the hyperpolarization, and the membrane potentials (solid line at the bottom of Fig. 3A) were identical in the presence or absence of TEA. The difference between the fluorescence level after addition of CCCP and that after addition of valinomycin was 3- or 4-fold larger in the presence of 25 mM and 50 mM TEA, respectively, than in the absence of TEA (Fig. 3A). These results suggest that TEA inhibited not only the sperm motility (Fig. 1A) but also the membrane hyperpolarization (Fig. 3A).

Nifedipine, a blocker of L-type Ca^{2+} channels in general and of T-type Ca^{2+} channels in sperm cells (Lievano *et al.*, 1996) completely inhibited the hyperpolarization of the sperm plasma membrane (Fig. 3B) as well as the sperm motility (Fig. 1B) in rainbow trout. These results suggest that Ca^{2+} influx via the dihydropyridine-sensitive Ca^{2+} channel contributes to the membrane hyperpolarization and the initiation of sperm motility. Trifluoroperazine, which can inhibit Ca^{2+} channels (Espinosa and Darszon, 1995), suppressed the membrane hyperpolarization (data not shown).

Effects of channel blockers and valinomycin on cyclic AMP synthesis

The cyclic AMP concentration was 698 fmol/mg protein in the immotile sperm of rainbow trout in the presence of 40 mM K^+ (40 mM $[K]_{ex}$ in Fig. 4). The concentration increased significantly to 2291 fmol/mg protein (0 mM $[K]_{ex}$ in Fig. 4) when sperm motility was initiated in AS without K^+. When sperm were immobilized by 25 mM TEA, the cyclic AMP level became 774 fmol/mg protein, almost the same as that in the presence of 40 mM K^+ (+TEA in Fig. 4). Addition of valinomycin to the sperm immobilized by TEA recovered sperm motility (Fig. 1A), and the cyclic AMP level increased to about 1588 fmol/mg protein (TEA+Val in Fig. 4), which was significantly different from that in the immotile sperm under the influence of 40 mM K^+ (40 mM $[K]_{ex}$ in Fig. 4). The cyclic AMP concentration in the immotile sperm of steelhead trout (465 fmol/mg protein) increased to 1990 fmol/mg protein in the presence of 0 mM $[K]_{ex}$, and to 545 fmol/mg protein in the presence of 200 mM TEA. Valinomycin relieved the cyclic AMP concentration up to 1071 fmol/mg protein (n=3). MCD-peptide, a blocker of the voltage-dependent K^+ channel, inhibited the sperm motility of the rainbow trout (Fig. 1A). MCD-peptide strongly decreased the cyclic AMP, and subsequent addition of valinomycin recovered the cyclic AMP (data not shown). A Ca^{2+} channel blocker, trifluoroperazine, at a concentration of 200 μM inhibited cyclic AMP synthesis in the sperm of steelhead

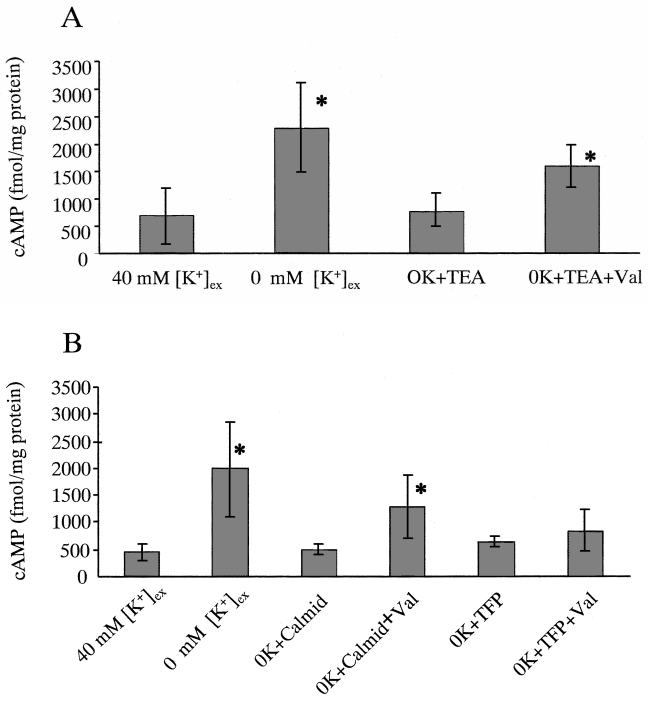

Fig. 4. Effects of the K^+ channel blocker, Ca^{2+} channel blocker and calmodulin inhibitor on cAMP synthesis. Cyclic AMP levels of sperm were measured 5 seconds after suspending the sperm of rainbow trout in the media containing 40 mM K^+ (40mM $[K^+]_{ex}$), 0 mM K^+ (0 mM $[K^+]_{ex}$), 25 mM TEA (0K+TEA) or TEA and 0.1 μM valinomycin (0K+TEA+Val) in A. In B, cyclic AMP levels of steelhead sperm were measured in the media containing 40 mM K^+ (40 mM$[K^+]_{ex}$), 0 mM K^+ (0 mM$[K^+]_{ex}$), 25 μM calmidazol (0K+Calmid), calmidazol + 0.1 μM valinomycin (0K+Calmid+Val), 100 μM trifluoroperazine (0K+TFP) or trifluoroperazine + 0.1 μM valinomycin (0K+TFP+Val). The control (0 mM$[K^+]_{ex}$) had no K^+ channel blocker, calmoduline inhibitors or valinomycin. Cyclic AMP level in the sperm whose motility was completely inhibited by TEA, calmidazol and trifluoroperazine was the same as that in the immotile sperm in the presence of 40 mM$[K^+]_{ex}$. Subsequent addition of valinomycin caused the initiation of sperm motility and increase in cAMP. The data are indicated as the means±SD (n=3). An asterisk indicates a significant difference after paired t-test ($p<0.05$) from the data in the presence of 40 mM$[K^+]_{ex}$.

trout (Fig. 4B). Subsequent addition of valinomycin caused a slight increase in cyclic AMP.

Effects of inhibitors of calmodulin and valinomycin on sperm motility, membrane potential and cyclic AMP synthesis

Almost all rainbow trout or steelhead trout sperm became motile when suspended in AS. More than 80% of the rainbow trout sperm were motile in AS containing 0-100 μM W-5, an inactive analogue of W-7 (Fig. 5). W-7 at a concentration of 100 μM was required for 50% inhibition of sperm motility (Fig. 5). The half- maximal concentration required for inhibition by another calmodulin inhibitor, calmidazol-Cl, was 12.5 μM. Sperm whose motility was inhibited in the presence of 25 μM calmidazol-Cl or 200 μM W-7 became motile when valinomycin was added (data not shown). Sperm motility in steelhead trout was unaffected by the inhibitor of calmodulin kinase II, KN-93, or by its inactive analogue, KN-92, at concentrations up to 50 μM (data not shown).

The membrane potential was slightly changed in the presence of W-5. On the other hand, W-7 (50 μM) and calmidazol (25 μM) strongly inhibited membrane hyperpolarization. However, subsequent addition of valinomycin caused only slight membrane hyperpolarization. The level of the membrane potential did not reach that seen in the controls or in the presence of W-5 (cf. solid line in the bottom of Fig. 3). In addition, stepwise addition of KCl did not cause a stepwise depolarization of the plasma membrane (data not shown). These results suggest that the calmodulin inhibitor may achieve its effect on sperm motility via some mechanism other than a direct effect on calmodulin.

Calmidazol-Cl also decreased cyclic AMP from a high level in 0 mM K^+ to a low level comparable to that in 40 mM K^+. Addition of valinomycin recovered the level of cyclic AMP (Fig. 4B). These results may suggest that these reagents do not specifically inhibit calmodulin. At the same time, however, they make clear that membrane hyperpolarization is prerequisite to cAMP synthesis.

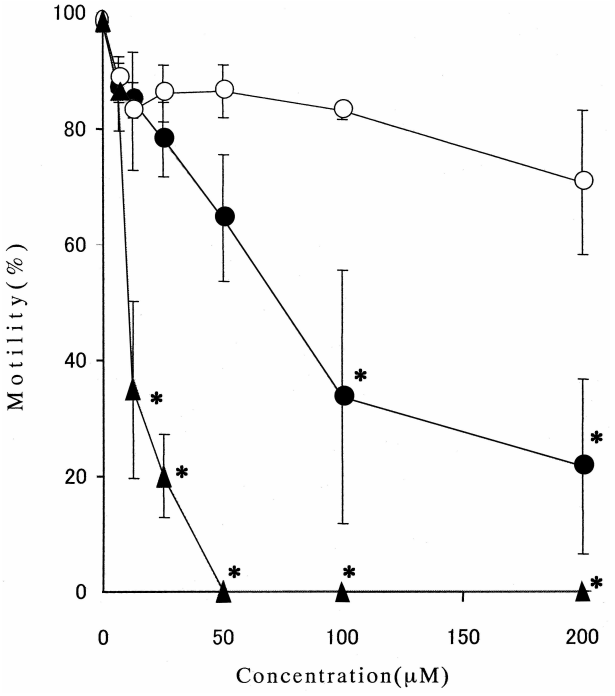

Fig. 5. Effects of calmodulin inhibitors on sperm motility. Sperm of rainbow trout diluted in ASP containing various concentrations of the calmodulin inhibitors, W-5 (open circle), W-7 (closed circle) and calmidazol-Cl (closed triangle), were suspended in AS containing the same concentration of the inhibitors, and the percentage of motile sperm was measured. The data are expressed as the means±SD=(n=3). An asterisk indicates a significant difference from the control or +W-5 ($p<0.05$).

DISCUSSION

Previous studies have shown that the high concentration of K^+ contained in the seminal plasma of salmonid fishes, around 40−80 mM (Morisawa, 1985), makes the sperm quiescent in the sperm duct. The decrease in K^+ surrounding the sperm at spawning in fresh water confers motility to the sperm (Morisawa and Suzuki, 1980; Morisawa *et al.*, 1983). The inhibition of sperm motility by high external K^+ concentration is relieved by the subsequent addition of several mM Ca^{2+} (Baynes *et al.*, 1981; Tanimoto and Morisawa, 1988), suggesting that external Ca^{2+} plays a role in the initiation of sperm motility (Boitano and Omoto, 1992). Furthermore, removal of external Ca^{2+} by EDTA or EGTA has been shown to prevent the initiation of sperm motility in rainbow trout (Baynes *et al.*, 1981; Cosson *et al.*, 1989). An actual increase in intracellular Ca^{2+} concentration concomitant with the initiation of sperm motility in K^+-free medium has been reported (Cosson *et al.*, 1989; Tanimoto *et al.*, 1994). These findings suggest that the influx of external Ca^{2+} through the plasma membrane constitutes an additional transmembrane process distinct from that of K^+ efflux (Tanimoto *et al.*, 1994), and that the Ca^{2+} entering the sperm cytoplasm participates in the activation of certain Ca^{2+}-specific proteins or enzymes.

We have shown here that several blockers known to block voltage-dependent K^+ channels in neurons and TEA, which is also known as a blocker of the voltage-dependent K^+ channels, suppressed the initiation of sperm motility in K^+- free medium. However, other types of K^+ channel blockers, such as apamin, a Ca^{2+}-dependent K^+ channel blocker, did not (Fig. 1A). These findings suggest that the efflux of K^+ (Tanimoto *et al.*, 1994) through a certain type of K^+ channels that are sensitive to TEA, dendrotoxins and MCD-peptide are involved in the initiation of trout sperm motility. Furthermore, the present study demonstrated a gradual decrease in electrical potential of the sperm plasma membrane, i.e., membrane hyperpolarization, which occurred concomitantly with a gradual decrease in external K^+ concentration (Fig. 2). These findings reflect the environmental ionic changes surrounding the sperm at natural spawning in K^+-deficient fresh water. The hyperpolarization is suggested to be caused by K^+ efflux (Tanimoto *et al.*, 1994) through the TEA sensitive K^+ channel (Fig. 3A). These results suggest that a kind of voltage dependent K^+

channel participates in the initiation of trout sperm motility. On the other hand, the concomitant occurrence of gradual decreases in the amplitude of hyperpolarization of the sperm plasma membrane and gradual decreases in environmental K^+ indicates the possible involvement of a TEA-sensitive K^+ channel that is open at rest. It seems possible that the K^+ efflux through the K^+ channel is suppressed by a seminal high K^+ concentration at rest of trout sperm motility in the male reproductive tract. At spawning of the sperm in freshwater, a decrease in the environmental K^+ concentration may cause a spontaneous efflux of K^+ through the channel, thereby leading to membrane hyperpolarization.

Ca^{2+} influx through Ca^{2+} channel(s) with sensitivity to nifedipine, nimodipine, trifluoroperazine, FS-2 and taicatoxin (Fig. 1B), which are generally regarded as the L-type Ca^{2+} channels, may be involved in the initiation of trout sperm motility, although types of Ca^{2+} channels other than L- and T-type may not be involved. T-type Ca^{2+} channels have been suggested to play a role in the sperm activation by egg-derived factors, SAPs, in sea urchins (Gonzalez-Martines *et al.*, 1987). The existence of dihydropyridine sensitive T-type Ca^{2+} channels has been reported in mouse spermatocytes (Santi *et al.*, 1996). Therefore, it seems possible that dihydropyridine sensitive T-and/or L-type Ca^{2+} channels participate in the cell signaling cascade of the initiation of trout sperm motility. Furthermore, the dihydropyridine, nifedipine, shifted the membrane potential in the depolarizing direction in the present series (Fig. 3B). These results suggest that not only K^+ channels but also Ca^{2+} channels participate in the occurrence of membrane hyperpolarization and initiation of sperm motility.

The role of cyclic AMP in the initiation of trout sperm motility has been clearly established. Sequential processes-i.e., the activation of adenylyl cyclase, cyclic AMP synthesis and its transient degradation by the activation of phosphodiesterase-occur within a short period after exposure of trout sperm to the K^+ deficient environment (Morisawa and Ishida, 1987). The synthesized cyclic AMP triggers the axonemal system for the initiation of sliding and bending of the flagellar axoneme (Morisawa and Okuno, 1982) through the phosphorylation of a protein with a molecular mass of 15 kDa (Hayashi *et al.*, 1987) and a dynein light chain (Inaba *et al.*, 1998). It has been demonstrated that the 26S proteasome plays a role in the initiation of sperm motility in the chum salmon and rainbow trout (Ohkawa *et al.*, 1997). In the present study, blockers of K^+ channels and Ca^{2+} channels and/or calmodulin suppressed cyclic AMP synthesis (Fig. 4). The suppression of cyclic AMP synthesis and, concomitantly, the decreases in sperm motility and membrane hyperpolarization induced by these blockers were relieved by making the plasma membrane maximally permeable to K^+ through the addition of valinomycin, which hyperpolarized the plasma membrane (Fig. 3A). Furthermore, nifedipine, a T-type Ca^{2+} channel blocker in sperm cells (Liveano *et al.*, 1996) and trifluoroperazine, a Ca^{2+} channel blocker in sperm cells (Espinosa and Darszon, 1995) suppressed sperm motility (Fig. 1B), membrane hyperpolarization (Fig. 3B), and cyclic AMP synthesis (Fig. 4B). These suppressions were also relived by addition of valinomycin. These results suggest that K^+ efflux and Ca^{2+} influx through certain K^+ and Ca^{2+} channels induce membrane hyperpolarization, thereby leading to synthesis of cAMP, which in turn triggers the intracellular cell signaling for the initiation of sperm motility in the trout. However, it is still unknown whether the cAMP synthesis is caused by the direct activation of adenylyl cyclase by the membrane hyperpolarization. This idea has previously been demonstrated in the regulation of motility of flagella in ascidian sperm (Izumi *et al.*, 1999) and *Paramecium* cilia with voltage-dependent K^+ channels (Schultz *et al.*, 1992) and acrosome reaction in sea urchin sperm (Beltran *et al.*, 1996).

The calmodulin inhibitor, W-7, at a concentration of 50 μM completely inhibits the motility of sea urchin sperm but does not inhibit the motility of sea urchin sperm whose plasma membrane has been removed by a detergent (Iwasa *et al.*, 1987). And a high concentration of W-7 inhibits the motility of live sperm but does not inhibit that of demembranated sperm in the fowl (Ashizawa *et al.*, 1994). These findings indicate that calmodulin works at the level of the plasma membrane. Calmodulin is known to be present in trout sperm cells (Tanimoto, personal communication). In the present study, a high concentration of W-7 suppressed sperm motility (Fig. 5), membrane hyperpolarization and cyclic AMP synthesis (data not shown) in live sperm of the rainbow trout. Calmidazol also inhibited sperm motility (Fig. 5) and cyclic AMP synthesis (Fig. 4). However, it seems possible that the effect of the calmodulin inhibitors may not be specific to calmodulin, but rather may be a general deteriorating effect on the plasma membrane, since the hyperpolarization and depolarization induced by addition of valinomycin and KCl, respectively, were not complete. That is, the level of the membrane potential did not reach that in the controls in the presence of W-5, an inactive analogue of calmodulin inhibitor, and stepwise addition of KCl did not cause stepwise depolarization of the plasma membranes, as shown in Fig. 3. Thus, the role of calmodulin in the transmembrane cell signaling in trout sperm motility still remains obscure. On the other hand, calmodulin, which binds to the axoneme of sea urchin sperm flagellum, is considered to mediate the effect of Ca^{2+} on the asymmetry of flagellar bending (Brokaw and Nagayama, 1985). In our preliminary experiments, a calmodulin inhibitor, W-7, at concentrations up to 200 μM did not inhibit the motility of sperm whose plasma membrane had been removed by a detergent, Triton X-100. A previous report by Okuno and Morisawa (1989) showed that motility of the demembranated trout sperm, starts only in the presence of Ca^{2+} at a concentration lower than 10^{-8} M. Since calmodulin is active at the Ca^{2+} concentration of 10^{-6}–10^{-5} M, it seems possible that calmodulin does not play a role in the events occurring in the axoneme, a motile apparatus of sperm flagellum, during the initiation of trout sperm motility. Adenylyl cyclase has an affinity to calmodulin in sea urchin sperm (Bookbinder *et al.*, 1990), and the cyclic AMP degrading enzyme, phosphodiesterase, is regulated by calmodulin (Tash and Means, 1983). The role of calmodulin in the regu-

lation of cyclic AMP production and degeneration, which occurs shortly after cyclic AMP synthesis (Morisawa and Ishida, 1987), should be elucidated.

The present study, along with those previous studies described above, suggests the following explanation of the transmembrane cell signaling underlying the initiation of trout sperm motility at the natural spawning. A decrease in the environmental K^+ concentration surrounding the spawned sperm causes a K^+ efflux and Ca^{2+} influx through the specific K^+ channel and dihydropyridine-sensitive L-/T-type Ca^{2+} channel, respectively, thereby leading to the membrane hyperpolarization and Ca^{2+} influx. The membrane hyperpolarization synthesizes cyclic AMP, which triggers the further process of cell signaling, i.e., cyclic AMP-dependent protein phosphorylations, to initiate sperm motility in salmonid fishes.

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