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Role of Ca^{2+} and Calmodulin on the Initiation of Sperm Motility in Salmonid Fishes

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Abstract K^+ efflux through a certain type of K^+ channels causes the change of membrane potential and leads to cAMP synthesis in the transmembrane cell signaling for the initiation of sperm motility in the salmonid fishes. The addition of Ca^{2+} conferred motility to the trout sperm that were immobilized by external K^+ and other alkaline metals, Rb^+ and Cs^+, suggesting the participation of external Ca^{2+} in the initiation of sperm motility. L-type Ca^{2+} channel blockers such as nifedipine, nimodipine, and FS-2 inhibited the motility, but N-type Ca^{2+} channel blocker, w-conotoxin MvIIA, did not. On the other hand, the membrane hyperpolarization and cAMP synthesis were suppressed by Ca^{2+} channel blockers, nifedipine, and trifluoroperazine. Furthermore, these suppressions were relieved by the addition of K^+ ionophore, valinomycin. Inhibitors of calmodulin, such as W-7, trifluoperazine, and calmidazol-Cl, inhibited the sperm motility, membrane hyperpolarization, and cAMP synthesis. The results suggest that Ca^{2+} influx through Ca^{2+} channels that are sensitive to specific Ca^{2+} channel blockers and calmodulin participate in the changes of membrane potential, leading to synthesis of cAMP in the cell signaling for the initiation of trout sperm motility.

Key words: Sperm motility, Ca^{2+} ion channel, calmodulin

Cyclic AMP and Ca^{2+} are widely known to be the critical factors regulating motility and acrosome reaction in the sperm of vertebrate and invertebrate species [27, 46]. Mammalian sperm requires Ca^{2+} [33, 44] and cAMP [25, 33, 36] for the activation of sperm motility. Sperm activation peptides (SAPs), named as speract or resact and derived from the jelly layer covering the sea urchin egg, bind to a 77 kDa protein that is closely coupled to sperm guanylyl cyclase [39]. Transient activation of the enzyme increases cGMP and cAMP, which activate cyclic nucleotide-gated potassium channels in the sperm cell. Resulting transient hyperpolarization of the sperm plasma membrane [3, 11, 12, 15, 16, 48] triggers the activation of sperm motility [18, 40] and chemotaxis [45]. In the ascidians *Ciona intestinalis* and *C. savignyl*, Morisawa *et al.* [30] demonstrated that extracellular Ca^{2+} and intracellular cAMP are the second messengers prerequisite for the activation of motility of *Ciona* sperm. Cell signaling cascade for the activation of sperm motility in *Ciona* has been well established by Yoshida *et al.* [47], who found the sperm-activating and sperm-attracting factor (SAAF) derived from the unfertilized egg. The activation of sperm motility caused by SAAF requires the increase in intracellular Ca^{2+} via influx of extracellular Ca^{2+} through the Ca^{2+} channel [47], and participation of calmodulin [35] and calmodulin kinase II [34] in the SAAF-dependent activation of *Ciona* sperm motility has been suggested. On the other hand, the SAAF causes the increase in K^+ permeability of the plasma membrane, resulting in membrane hyperpolarization. The change of membrane potential activates adenylyl cyclase [22], and the increased cAMP triggers the final step of cell signaling; PKA-dependent phosphorylation of 26 kDa protein and 21 kDa dynein light chain [35]. However, it is still unknown whether the Ca^{2+} signaling is an independent process or is involved in membrane hyperpolarization-induced cAMP signaling in the salmonid fishes. Changes in environmental ionic and osmotic surrounding of sperm are factors belonging to a category other than egg-derived SAPs and SAAF as factors for the regulation of sperm motility [32]. Decrease in K^+ surrounding salmonid fish sperm causes K^+ efflux through certain K^+ channels, resulting in membrane hyperpolarization to lead to a cAMP synthesis. The subsequent cAMP-dependent phosphorylation of axonemal proteins with 15 kDa molecular mass [19] and dynein light chain [20] triggers the final step for the cAMP-dependent initiation of sperm motility [31]. Thus, the intracellular signal cascades

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such as cAMP-dependent phosphorylation of proteins have been clarified in the salmonid fishes, whereas the transmembrane cell signaling cascade triggered by decrease in external concentration of potassium ion still remains obscure.

Several studies have shown that the addition of millimolar concentration of divalent cations to the K^+-dependent immotile rainbow trout sperm induces sperm motility [4, 5, 13, 42], suggesting the participation of extracellular Ca^{2+} in the initiation of salmonid sperm motility. A Ca^{2+} channel blocker, verapamil [13, 42], inhibits sperm motility and increases intracellular Ca^{2+} concentration in the sperm cells during the initiation of trout sperm motility [6, 13, 42]. However, the characteristics of Ca^{2+} channels that participate in the initiation of trout sperm motility and the role of calmodulin have not yet been clarified. It is not clear whether the Ca^{2+} channel is independent of or involved in the membrane hyperpolarization-induced cAMP signaling.

Ca^{2+}-binding protein, calmodulin (CaM), is involved in the sperm activation in mammals as well as in other species through the control of axonemal function; i.e. activities of dynein ATPase and myosin light chain kinase [26]. During the regulation of flagellar motility in sea urchins, CaM is considered to be a Ca^{2+} sensor that detects the changes of intracellular Ca^{2+} concentration and the flagellar wave changes from symmetric to asymmetric and *vice versa* [9, 10]. Purified adenylyl cyclase has affinity to CaM in the sea urchin [7]. In the fowl sperm, one of CaM-dependent myosin light chain kinases is localized in the midpiece region and regulates the temperature-dependent activation of sperm motility [2]. In the dog sperm, reactivated sperm motility is reduced by CaM-dependent protein phosphatase, calcineurin [43]. These studies suggest that CaM, CaM-dependent kinase, and calcineurin play roles in the regulation of flagellar motility.

In this paper, we showed in the rainbow trout and steelhead trout that Ca^{2+} influx through the dihydropyridine-sensitive Ca^{2+} channel led to hyperpolarization of the plasma membrane and a subsequent synthesis of cAMP to initiate sperm motility. Effects of calmodulin inhibitors on sperm motility, membrane potential, and cAMP synthesis were examined.

MATERIALS AND METHODS

Chemicals and Solutions

W-5, W-7, KN-92, and KN-93 were purchased from Seikagakukogyo (Tokyo, Japan); 3,3'-dipropylthiadicarbocyanine iodide ($DiSC_3(5)$) from Molecular Probe (Leiden, The Netherlands); carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and valinomycin (Val) from Sigma Chemical Co. (St. Louis, MO, U.S.A.); Ca^{2+} channel blockers, nifedipine, nimodipine, taicatoxin, FS-2, ω-conotoxin MVIIA, PLTX-II, sFTX3.3, and FTX3.3 from Alomone Laboratories (Jerusalem, Israel); and cyclic AMP enzyme immunoassay (EIA) system (PRN 225) from BIOTRAK (Amersham Pharmacia Biotech., England). CCCP, Val, $DiSC_3(5)$, and nifedipine were dissolved in dimethylsulfoxide 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. Other reagents used were of analytical grade.

Collection of Sperm

Mature males of rainbow trout and steelhead trout, *Oncorhynchus mykiss*, were provided by Yamanashi Prefectural Fisheries Experiment Station Oshino Trout Hatchery, Japan, and kept in an indoor aquarium at 10°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 bovine serum albumin solution and dried to prevent sperm from sticking to the glass slide. A two-step dilution procedure for sperm motility activation was used, as reported by Cosson *et al.* [13], 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 $MgCl_2$, and 10 mM HEPES-NaOH at pH 7.8. Sperm motility was completely suppressed by 40 mM KCl in the ASP. The composition of the ASP was based on the ionic and osmotic composition of the seminal plasma of rainbow trout [28]. The prediluted sperm was placed on the glass slide, which was placed under 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 the addition of activation solution (AS) consisting of 150 mM NaCl and 10 mM HEPES-NaOH at pH 7.8. The second dilution step reduced 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, Tokyo, Japan).

Effects of Ca^{2+} Channel Blockers and Calmodulin Inhibitors on Sperm Motility

The effects of ion channel blockers on the initiation of sperm motility were investigated by diluting the semen in the ASP at the ratio of 1:10 and one volume of the sperm

suspension was resuspended in 100 volumes of AS containing various concentrations of Ca^{2+} channel blockers and calmodulin inhibitors.

Membrane Potential Measurement

The semen was diluted in ASP at a ratio of 1:10. After predilution, 20 µl of the sperm suspension were diluted in the 2 ml of AS containing 1 µM $DiSC_3(5)$ in a quartz cuvette in the presence or absence of Ca^{2+} channel blocker or calmodulin inhibitors. Mitochondrial potential was eliminated by 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.

Hyperpolarization and depolarization of the plasma membrane resulted in a decrease and an increase in fluorescence intensity, respectively, under experimental conditions.

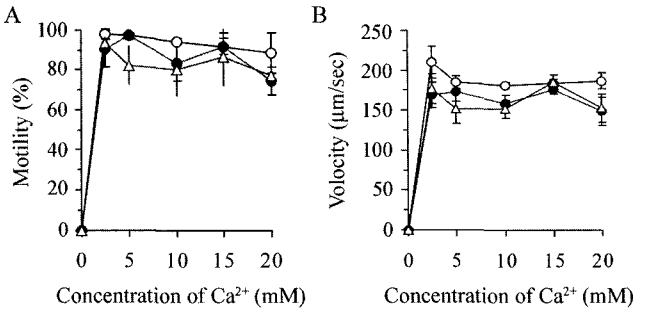

Fig. 1. Effect of Ca^{2+} on the motility of rainbow trout sperm immobilized by K^+, Rb^+, and Cs^{2+}.
Semen was diluted at the ratio of 1:100 in the solutions containing 10 mM KCl (open circle), 5 mM RbCl (filled circle), or 20 mM CsCl (open triangle), and various concentrations of $CaCl_2$ were then added. Percentage of motile sperm (**A**) and their velocity (**B**) were measured. Osmolality of the solutions was kept at constant 300 mOsmol/kg by replacing each alkaline metal ion for NaCl. Data represent means±SD (n=3).

Assay of cAMP

The semen was diluted in the ASP at the ratio of 1:10, and 3 µl of the sperm suspension were resuspended in 300 µl AS alone or AS containing KCl, ion channel blockers,

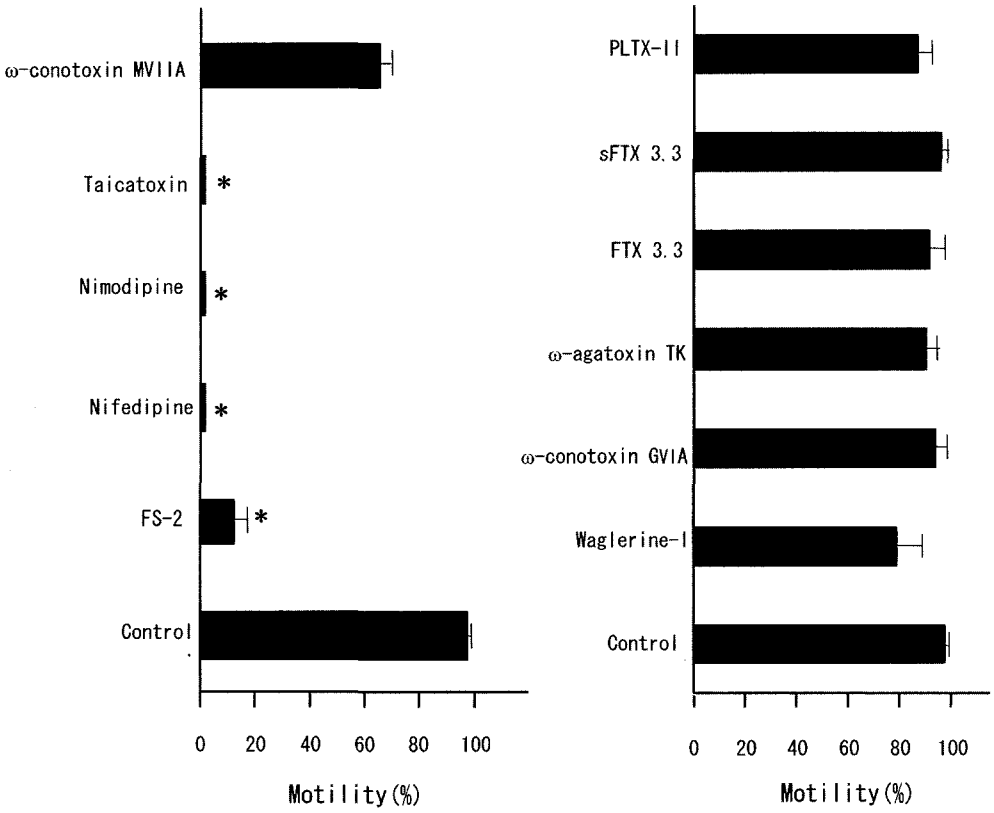

Fig. 2. Effects of Ca^{2+} channel blockers on the sperm motility in steelhead trout.
The semen was diluted at the ratio of 1:10 in ASP containing Ca^{2+} channel blockers, ω-conotoxin MVIIA (200 µM), taicatoxin (5 µM), nimodipine (20 µM), nifedipine (100 µM), FS-2 (20 µM), PLTX-II (500 µM), sFTX 3.3 (100 µM), FTX 3.3 (10 µM), ω-agatoxin TK (2 mM), ω-conotoxin GVIA (10 µM), or waglerine-I (60 µM). After incubation for 5 min, one volume of the sperm suspension was suspended in 100 volumes of AS containing the same concentration of blockers, and the percentage of motile sperm was measured. Data represent means±SD (n=3). Data marked with an asterisk show significant difference from the control ($p<0.05$).

calmodulin inhibitors, and/or valinomycin. After necessary incubation time, 180 µl of the suspension were mixed with 20 µl of kit buffer (lysis reagent) to stop cAMP synthesis and dissolve the cells. Then, 100 µl of the mixure were added to the well of the kit to quantify cAMP by the method described in the manual of cAMP EIA system (BIOTRAK). The cAMP level of each sample was calculated by measuring absorbance at 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 measurment from BIO-RAD (Bio-Rad Protein Assay, Bio-Rad, U.S.A.).

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

RESULTS

Effects of Ca^{2+} on Sperm Motility

We have recently reported that sperms of steelhead trout, rainbow trout, and masu salmon were completely immotile in medium containing K^+, Rb^+, or Cs^+ at over 10 mM, 5 mM, or 20 mM concentrations, respectively [23]. Over 80% of rainbow trout sperms became motile (Fig. 1A) with swimming velocity of around 180 µm/sec (Fig. 1B), when 2.5 mM Ca^{2+} was added to the sperms immotile in the presence of 10 mM K^+, 5 mM Rb^+, and 20 mM Cs^+.

Effects of Ca^{2+} Channel Blockers on Membrane Potential and cAMP Synthesis

In order to determine what types of Ca^{2+} channel were involved in the sperm motility, the effects of various Ca^{2+} channel blockers on the sperm motility of steelhead trout were examined (Fig. 2). FS-2 (20 µM) and taicatoxin (5 µM), which are considered as the blockers of L-type Ca^{2+} channel in the neuron, completely inhibited sperm motility of the steelhead trout. Dihydropyridine, nimodipine (20 µM), and nifedipine (100 µM), which are known as blockers of T-type Ca^{2+} channels in the sperm [24] and as blockers of L-type Ca^{2+} channel in other excitable cells, inhibited the sperm motility. The blockers of other types of

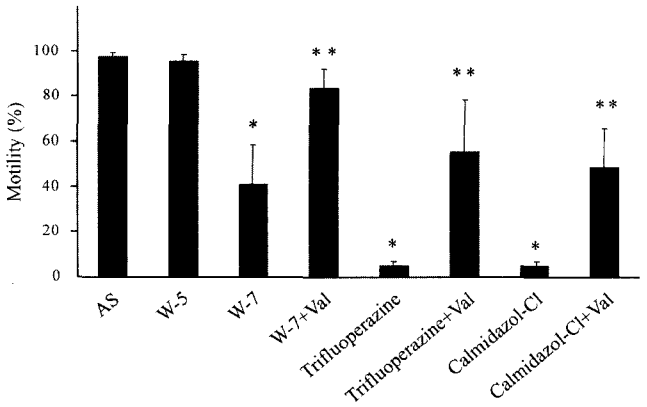

Fig. 3. Effects of calmodulin inhibitors, Ca^{2+}-channel blocker, and valinomycin on sperm motility in the rainbow trout.
Semen was diluted at the ratio of 1:10 in ASP containing 200 µM W-5, an inactive analogue of W-7, and calmodulin inhibitors, including 200 µM W-7, 200 µM trifluoroperazine, and 25 µM calmidazol-Cl. After incubation for 5 min, aliquots of the sperm suspensions were diluted in AS containing the same concentration of inhibitors or blockers, and percentage of motile sperm was measured. Valinomycin (Val) at 0.1 µM concentration was added to the sperm treated with W-7, trifluoroperazine, and calmidazol-Cl. Data represent means±SD (n=3). Data marked with an asterisk show significant difference from the control ($p<0.05$). Double asterisks show no significant difference from the control and W-5.

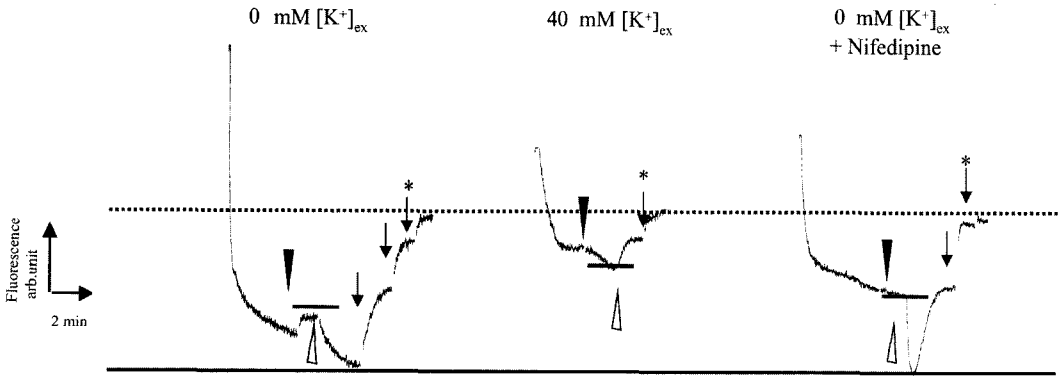

Fig. 4. Effect of Ca^{2+} channel blocker on membrane potential of rainbow trout sperm.
The semen was diluted at the ratio of 1:10 in the ASP containing external 40 mM KCl, 40 mM $[K^+]_{ex}$, containing 100 µM nifedipine and incubated for 5 min. The sperm suspension was then diluted in AS (0 mM KCl) containing 1 µM $DiSC_3(5)$ in the presence of the blockers. After the fluorescence level, namely the membrane potential, became stable, 1 µM 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 KCl (arrows) depolarized the plasma membrane. The final levels of the membrane potential 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 the addition of valinomycin. The difference of membrane potential of sperm, before (solid bars) and after the addition of valinomycin (solid line in the bottom), became larger concomitantly with the increase in the presence of nifedipine. $[K^+]_{ex}$ at 40 mM concentration (40 mM $[K^+]_{ex}$) inhibits membrane hyperpolarization (middle).

Ca^{2+} channel than L- and T-types, such as ω-conotoxin MvIIA, PLX-II, and sFTX, showed slight inhibition at the concentration of 100–500 μM. Trifluoperazine that can block Ca^{2+} channel as well as calmodulin has been reported to inhibit the acrosome reaction in sea urchin sperm and the membrane potential of mouse sperm [14]. This blocker inhibited trout sperm motility, and the inhibition was relieved by the addition of 0.1 μM valinomycin (Fig. 3). In addition, nifedipine inhibited the membrane hyperpolarization of the rainbow trout sperm at the concentration of 100 μM (Fig. 4). These results suggest that Ca^{2+} influx via dihydropyridine-sensitive Ca^{2+} channel contributes to membrane hyperpolarization and the initiation of sperm motility. Trifluoperazine (100 μM), a Ca^{2+} channel blocker, inhibited cAMP synthesis in the sperm of steelhead trout (Fig. 5), and subsequent addition of valinomycin slightly increased the cAMP.

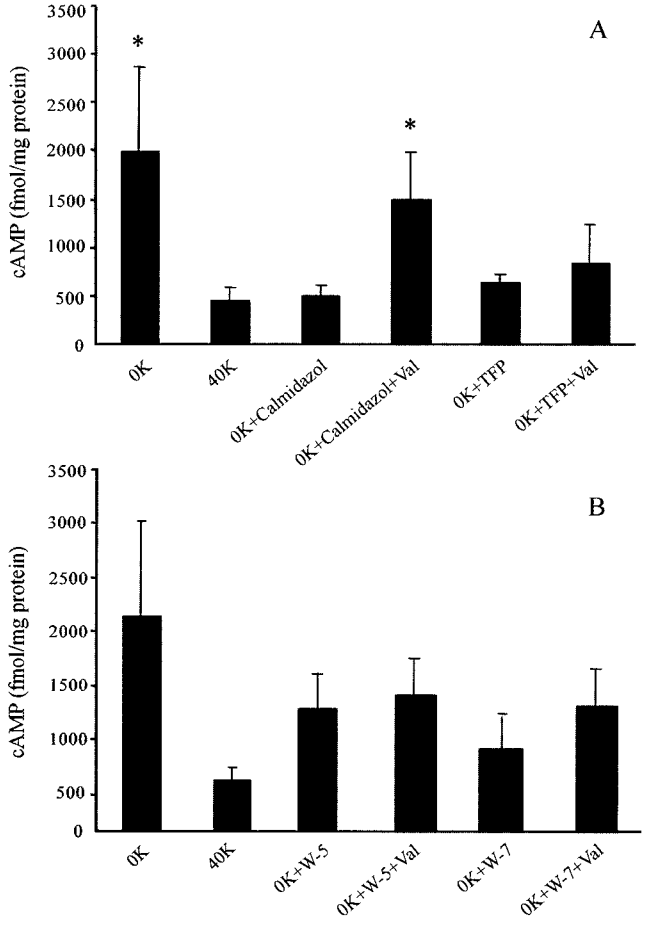

Fig. 5. Effects of Ca^{2+} channel blockers, calmodulin inhibitor, and valinomycin on cyclic AMP synthesis in steelhead trout sperm.

A. Cyclic AMP levels were measured 5 seconds after suspending the sperm in the media containing 0 mM $[K^+]_{ex}$ (0 K), 40 mM $[K^+]_{ex}$ (40 K), 25 μM calmidazol-Cl (0 K+calmidazol), calmidazol-Cl+0.1 μM valinomycin (0 K+calmidazol+Val), 100 μM trifluoroperazine (0 K+TFP), or trifluoroperazine+0.1 μM valinomycin (0 K+TFP+Val). **B**. Cyclic AMP levels were measured 5 sec after suspending the sperm in the media containing 0 mM $[K^+]_{ex}$, 40 mM $[K^+]_{ex}$, 200 μM W-5 (0 K+W-5), W-5+ 0.1 μM valinomycin (0 K+W-5+Val), 200 μM W-7 (0 K+W-7), or W-7+ 0.1 μM valinomycin (0 K+W-7+Val). The control (0 K) had no K^+, calmodulin inhibitors, Ca^{2+} channel blocker, and valinomycin. Cyclic AMP level in the sperm whose motility was completely inhibited by calmidazol and trifluoroperazin was the same as that in the immotile sperm in the presence of 40 mM $[K^+]_{ex}$. Subsequent addition of valinomycin initiated sperm motility and increased cAMP. The data represent means±SD (n=3). Data marked with an asterisk show significant difference after paired *t*-test ($p<0.05$) from that in the presence of 40 mM $[K^+]_{ex}$.

Effects of Inhibitors of Calmodulin, Calmodulin Kinase II, and Valinomycin on Sperm Motility, Membrane Potential, and cAMP Synthesis

Almost all sperm became motile, when the sperm of rainbow trout or steelhead trout were 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. 6). The 100 μM concentration of W-7 was required for 50% inhibition of sperm motility. Half maximal inhibition concentration of another calmodulin inhibitor, calmidazol-Cl, was 12.5 μM. The sperm, whose motility were inhibited in the presence of 25 μM calmidazol-Cl or 200 μM W-7, became motile when valinomycin was

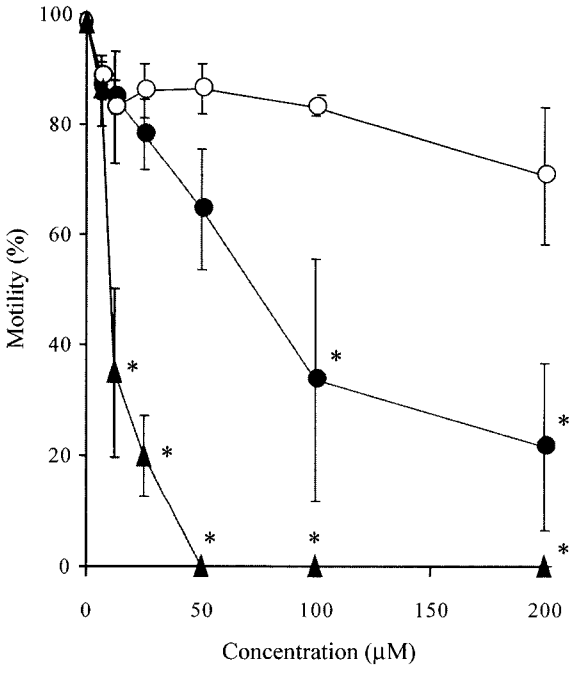

Fig. 6. Effects of calmodulin inhibitors on sperm motility in the rainbow trout.

The semen was diluted at the ratio of 1:10 in ASP containing various concentrations of calmodulin inhibitors, W-5 (open circle), W-7 (filled circle), and calmidazol-Cl (filled triangle). After incubation for 5 min, one volume of the sperm suspension was suspended in 100 volumes of AS containing the same concentration of inhibitors. Data represent means±SD (n=3). Data marked with an asterisk show significant difference from the control and W-5 ($p<0.05$).

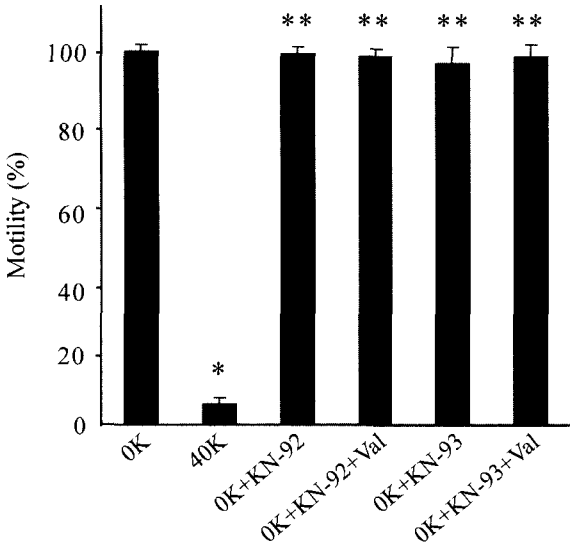

Fig. 7. Effects of calmodulin kinase II inhibitors and valinomycin on sperm motility in the steelhead trout.
Semen was diluted at the ratio of 1:10 in ASP containing 50 μM KN-92 (inactive analogue of KN-93) and 50 μM KN-93 (calmodulin kinase II inhibitor). After incubation, aliquots of the sperm suspension were diluted in AS containing the same concentration of inhibitors, and the percentage of motile sperm was measured. Valinomycin (Val) at the concentration of 0.1 μM was added to the sperm treated with calmodulin kinase II inhibitors. Data represent means±SD (n=3). Data marked with an asterisk show significant difference from the control ($p<0.05$). Double asterisks show no significant difference from the control (0 K).

added (Fig. 3). Sperm motility in steelhead trout was unaffected by the inhibitor of calmodulin kinase II, KN-93 and its inactive analogue, KN-92, at the concentration up to 50 μM (Fig. 7).

The membrane potential changed slightly in the presence of 50 μM W-5. On the other hand, 50 μM W-7, trifluoperazine (TFP), and calmidazol-Cl strongly inhibited membrane hyperpolarization (Fig. 8). However, subsequent addition of valinomycin caused only a little membrane hyperpolarization. The level of membrane potential could not reach the same level as that of control or in the presence of W-5 (c.f. solid line in the bottom of Fig. 8A). In addition, stepwise addition of KCl could not cause the stepwise depolarization of the plasma membrane (data not shown). These results implicate that calmodulin inhibitors may not directly affect calmodulin. The inhibitors of calmodulin kinase II, KN-93 and KN-92, had no effects on the membrane potential of the rainbow trout sperm (Fig. 9).

Calmidazol-Cl as well as trifluroperazine also decreased cAMP 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 cAMP (Fig. 5A). Effect of W-7 and valinomycin on cAMP synthesis was not prominent (Fig. 5B), indicating that the reagent may not specifically inhibit calmodulin. However, these results at least suggest that membrane hyperpolarization is the prerequisite process for the cAMP synthesis. Inhibitors of calmodulin kinase II, KN-93 and KN-92, had no effects on the cAMP synthesis (Fig. 10).

DISCUSSION

In the cell signaling for the activation of sperm motility in the salmonid fish, it is well established that high concentration of K^+ in the seminal plasma inhibits sperm motility. The decrease of K^+ surrounding the sperm immediately causes significant and transient increase in intracellular cAMP [29], which is a trigger for the intracellular cell-signaling cascade of the initiation of sperm motility [31]. Furthermore, K^+ efflux through a certain kind of K^+ channel causes hyperpolarization of the plasma membrane of sperm and cAMP synthesis, resulting in the initiation of sperm motility in several species of the salmonid fish [23].

The inhibition of sperm motility by the external K^+ [4, 42] was shown by the addition of several mM Ca^{2+} in rainbow trout (Fig. 1). Removal of external Ca^{2+} by EDTA or EGTA prevented the initiation of sperm motility in these species [4, 13]. Moreover, the initiation of motility in rainbow trout sperm was associated with a six-fold increase in intracellular free Ca^{2+}, which was prevented by the presence of K^+ in the external medium [13]. These observations suggest that intracellular Ca^{2+}, which is necessary for the initiation of sperm motility, may derive from an influx of external Ca^{2+} in the salmonid fish. However, the role of Ca^{2+} in the changes of membrane potential and cAMP synthesis remains unknown. In the present study, Ca^{2+} influx through a Ca^{2+} channel(s) having sensitivity to nifedipine, nimodipine, trifluoroperazine, FS-2, and taicatoxin (Figs. 2 and 3), which are generally regarded as blockers of the L-type Ca^{2+} channel, may be involved in the initiation of trout sperm motility. The role of T-type Ca^{2+} channel has been suggested in the sperm activation by egg-derived factors, SAPs, in sea urchins [17], and the existence of dihydropyridine-sensitive T-type Ca^{2+} channel has been reported in the mouse spermatocyte [37]. 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 salmonid sperm motility. Furthermore, the dihydropyridine-sensitive Ca^{2+} channel blocker, nifedipine, shifted the membrane potential to the depolarizing direction (Fig. 4). Thus, it is suggested that not only the K^+ channel but also the Ca^{2+} channel may be involved in membrane hyperpolarization.

Sequential processes, including activation of adenylyl cyclase, cAMP synthesis and its transient degradation by the activation of phosphodiesterase, occur within a short period of time by the decrease in external K^+ at the initiation of trout sperm motility [29]. We have recently shown that blockers of K^+ channel such as TEA suppressed cAMP synthesis [23]. The suppression of cAMP synthesis

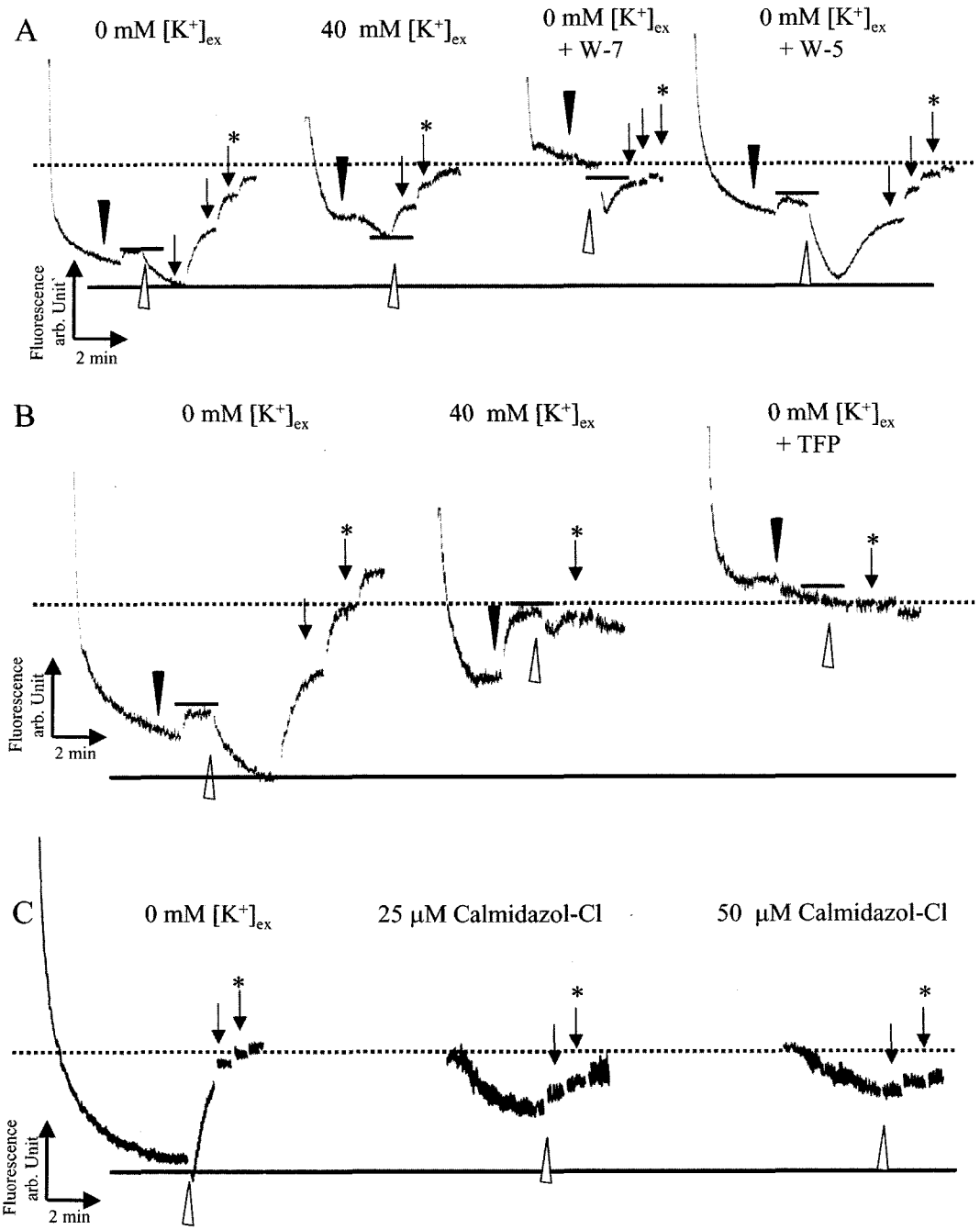

Fig. 8. Effects of calmodulin inhibitors on the membrane potential of steelhead trout sperm.
The semen was diluted in ASP containing external 40 mM KCl, 40 mM $[K^+]_{ex}$, at a dilution ratio of 1:10 in the presence of calmodulin inhibitors, 50 μM W-5, 50 μM W-7, 50 μM trifluoroperazine, and 50 μM calmidazol-Cl. One volume of the sperm suspension was then diluted in 100 volumes of AS (0 mM KCl) containing 1 μM $DiSC_3(5)$ and each calmodulin inhibitor. 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 added (open arrowhead) to make the plasma membrane maximally permeable to K^+. Then, KCl (arrows) was added. The final levels after addition of 30 mM $[K^+]_{ex}$ (arrow with asterisk) were aligned (dotted line). Note that the calmodulin inhibitors inhibited the membrane hyperpolarization. However, the membrane potential did not reach the same level (solid line in the bottom) after the addition of valinomycin, and addition of KCl showed only a slight membrane depolarization.

was relieved concomitantly with the relief of sperm motility and membrane hyperpolarization from the suppression by adding valinomycin to make the plasma membrane maximally permeable to K^+ and hyperpolarize the plasma membrane [23]. In this study, we showed that Ca^{2+} channel blockers, nifedipine [24] and trifluoroperazine [14], suppressed

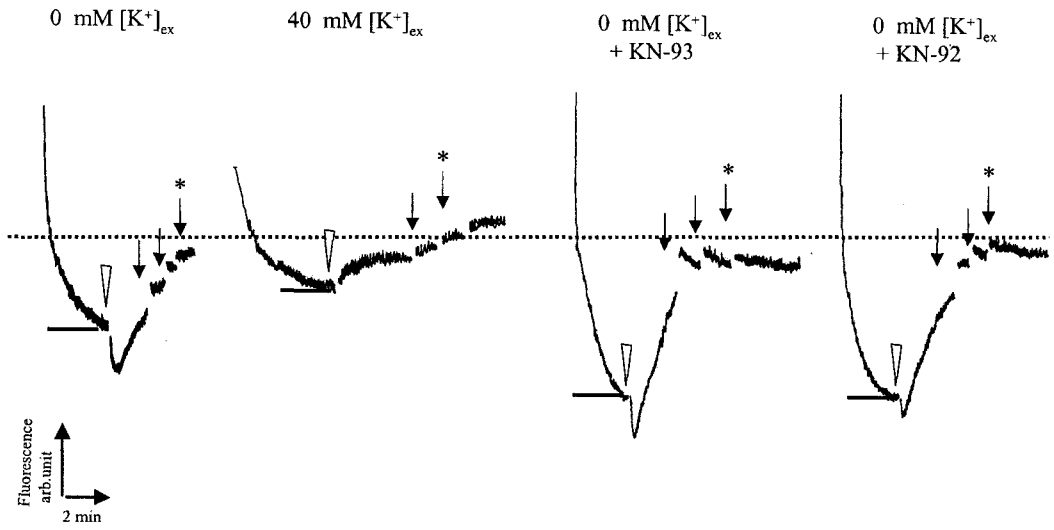

Fig. 9. Effects of calmodulin kinase II inhibitors on the membrane potential of rainbow trout sperm.
The semen was diluted in ASP containing 40 mM $[K^+]_{ex}$ in the presence of calmodulin kinase II inhibitor (50 μM KN-93) and inactive analogue of KN-93 (50 μM KN-92) at a dilution ratio of 1:10. The sperm suspension was then diluted in AS (0 mM KCl) containing 1 μM $DiSC_3(5)$ and calmodulin inhibitor. After the fluorescence level became stable, the stable level reached shows the sperm membrane potential (solid bar). Valinomycin at the concentration of 0.1 μM was added (open arrowhead) to make the plasma membrane maximally permeable to K^+. Then, KCl (arrows) was added. The final levels after the addition of 30 mM $[K^+]_{ex}$ (arrow with asterisk) were aligned (dotted line). Note that the calmodulin kinase inhibitor did not inhibit the membrane hyperpolarization, although membrane hyperpolarization was suppressed by 40 mM $[K^+]_{ex}$.

sperm motility (Figs. 2 and 3), membrane hyperpolarization (Fig. 4 and 8), and cAMP synthesis (Fig. 5). The suppression of cAMP synthesis by the Ca^{2+} channel blockers as well as that by K^+ channel blocker [23] was also relieved by the addition of valinomycin (Fig. 5). The results suggest that K^+ efflux and Ca^{2+} influx through certain K^+ and Ca^{2+} channels, respectively, cause the membrane hyperpolarization, thus leading to the synthesis of cAMP that is a trigger for the intracellular cell signaling for the initiation of sperm motility in the salmonid fish.

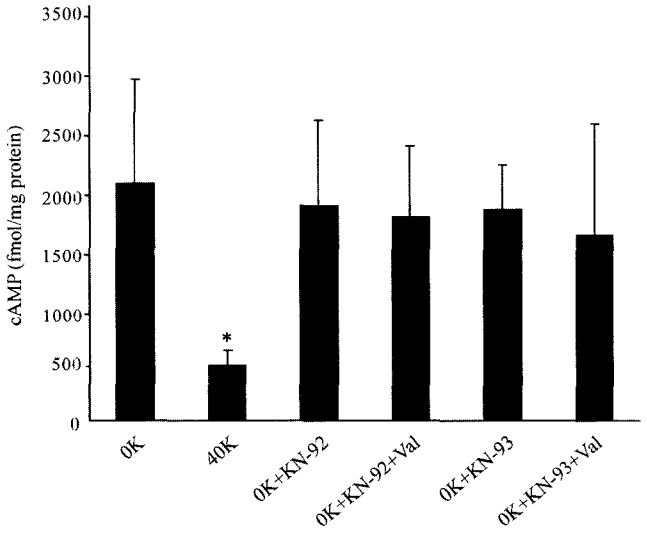

Fig. 10. Effects of calmodulin kinase II inhibitors on cAMP synthesis in steelhead trout sperm.
Cyclic AMP levels were measured 5 sec after suspending the sperm in the media containing 0 mM $[K^+]_{ex}$ (0 K), 40 mM $[K^+]_{ex}$ (40 K), 50 μM KN-92 (0 K+KN-92), KN-92+0.1 μM valinomycin (0 K+KN-92+Val), 50 μM KN-93 (0 K+KN-93), or KN-93+0.1 μM valinomycin (0 K+KN-93+Val). The control (0 K) had no K^+ and calmodulin kinase II inhibitors. The data represent means±SD (n=3). An asterisk shows a significant difference after paired *t*-test ($p<0.05$) from the control (0 K).

Calmodulin inhibitor, W-7, completely inhibits motility of sea urchin sperm, but does not inhibit motility of sea urchin sperm whose plasma membrane was removed by a detergent [21]. Furthermore, a high concentration of W-7 inhibits motility of live sperm, but does not inhibit motility of the demembraned sperm in fowl [1]. These results, therefore, indicate that calmodulin works at the plasma membrane: The calmodulin is present in trout sperm cells [41]. In the present study, W-7 at high concentration, trifluroperazin, and calmidazol suppressed sperm motility (Figs. 3 and 6), membrane hyperpolarization (Fig. 8), and cAMP synthesis (Fig. 5) in live sperm of trout. However, it is possible that the effect of the calmodulin inhibitors may not be specific, but rather are general deteriorating inhibitors on the plasma membrane, since hyperpolarization and depolarization by the addition of valinomycin and KCl, respectively, were not complete; i.e., the level of the membrane potential did not reach the same level as that of the control in the presence of W-5, an inactive analogue of calmodulin inhibitor. Moreover, as shown in Fig. 8, stepwise addition of KCl did not induce the stepwise depolarization of the plasma membrane. Thus, the role of calmodulin in the transmembrane cell signaling in the trout sperm motility still remains obscure. On the other hand,

calmodulin, which binds to the axoneme of sea urchin sperm flagellum, is considered to work as a mediator of the effect of Ca^{2+} on the asymmetry of flagellar bending [10]. In our preliminary experiments, a calmodulin inhibitor W-7 at the concentration up to 200 mM did not inhibit the motility of sperms whose plasma membrane were removed by a detergent, Triton X-100. Previous report by Okuno and Morisawa [37] showed that motility of the demembranated trout sperm starts only in the presence of Ca^{2+} lower than 10^{-8} M concentration. Since calmodulin is active at 10^{-6}–10^{-5} M Ca^{2+} concentration, it is highly possible that calmodulin is not active during the events occurring in the axoneme, a motile apparatus of sperm flagellum, during the initiation of trout sperm motility. Adenylyl cyclase has an affinity for calmodulin in sea urchin sperm [7], and the cAMP degrading enzyme, phosphodiesterase, is regulated by calmodulin [44]. The role of calmodulin in the regulation of cAMP production and degeneration, which occurs shortly after cAMP synthesis [29], should be further elucidated.

In conclusion, we attempted to elucidate the transmembrane cell signaling underlying the initiation of trout sperm motility during natural spawning as follows. A decrease in the environmental K^+ concentration surrounding the spawned sperm causes K^+ efflux and Ca^{2+} influx through the specific K^+ and dihydropyridine-sensitive L-/T-type Ca^{2+} channel, respectively, thereby leading to membrane hyperpolarization and Ca^{2+} influx. The membrane hyperpolarization induces synthesis of cAMP, which triggers a further process of cell signaling, i.e., cAMP-dependent protein phosphorylation, to initiate sperm motility in salmonid fishes.

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