Motility-Associated and Cyclic AMP-dependent Protein Phosphorylation in the Sperm of the Chum Salmon, *Oncorhynchus keta*

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ABSTRACT

Cyclic AMP-dependent cell signaling pathway has been considered to play an essential role in the initiation of sperm motility in salmonid fishes. We showed here that inhibitors of cyclic AMP-dependent protein kinase blocked the motility of the sperm of chum salmon whose plasma membrane was removed with TritonX-100. Seven proteins, which were phosphorylated by cAMP concomitantly with motility initiation of the demembranated sperm, were identified. Among them two proteins were identified as 21 kDa light chain of the outer arm dynein and 48 kDa regulatory subunit of protein kinase-A (PKA) from their molecular masses. Five proteins had the molecular masses of 28 kDa, 33 kDa, 41 kDa, 63 kDa and 83 kDa. Phosphorylation of these proteins as well as the motility of the demembranated sperm were significantly inhibited by the PKA inhibitors. When the demembranated sperm were prepared after pre-activation, all of these 7 proteins had less labeled ³²P. These results suggest that cAMP-dependent phosphorylation of multiple proteins through activation of PKA is involved in the initiation of sperm motility in the salmonid fish.

Cyclic AMP is one of the essential second messengers for the regulation of sperm motility in starfish (11), sea urchin (11, 3), tunicate (3), salmonid fishes (19, 20) and mammals (6, 15, 27, 30). It has been reported that the increase in cAMP concentration in sperm causes activation of cAMP-dependent protein kinase (A-kinase, PKA) (29). The kinase phosphorylates several flagellar proteins, including the regulatory subunit of PKA (6, 9, 25, 29) and the light chain of the outer arm dynein (1, 9, 10, 28). Sperm of salmonid fishes whose motility is triggered by a decrease in the extracellular potassium ion concentration at spawning (18) have provided a novel

model for understanding the molecular mechanism of the regulation of sperm motility. The decrease in K⁺ surrounding sperm activates adenylyl cyclase, resulting in an increase in the intracellular cAMP concentration (22). The synthesized cyclic AMP phosphorylates 22 kDa dynein light chain (10), the 48 kDa regulatory subunit of PKA (9) and 15 kDa protein (5, 12, 21). The 15 kDa protein is phosphorylated at tyrosine residues in a cAMP-dependent manner (5), possibility through activation of tyrosine kinase by PKA and is localized at the base of the flagellum, a part that is close to the centrioles of sperm (13). On the other hand, the dynein light chain and the PKA regulatory subunit have been isolated from the axoneme of sperm flagella (9, 10) and considered to be located along the axoneme of the sperm flagellum. These suggest that two cascade processes in regard to cAMP-dependent protein phosphorylation are involved in the initiation of sperm motility in salmonid fishes.

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Five new proteins whose phosphorylation was inhibited by PKA inhibitors and associated with sperm motility were identified. Furthermore, ^{32}P -labeling did not occur in the five proteins isolated from the demembranated sperm whose motility had been initiated in K^+ -free medium before the demembration, suggesting that multiple cAMP-dependent protein phosphorylation, at least in the five proteins, is associated with the initiation of sperm motility in the salmonid fish.

MATERIALS AND METHODS

Chemicals. H-89 (*N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide) and H-85 (*N*-[2-(*N*-formyl-*p*-chlorocinnamylamino)ethyl]-5-isoquinolinesulfonamide) were purchased from Seikagaku Kogyo Co. (Tokyo, Japan). PKAI (synthetic peptide inhibitor of PKA) was from Calbiochem Co. (Schwalbach, Germany). $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was from Dupont/NEN Life Science Products (Boston, MA, U.S.A.). Molecular markers of SDS-PAGE were from Bio-Rad (Hercules, CA). Cyclic AMP, ATP and Triton X-100 were from Wako Pure Chemical Co. (Osaka, Japan). All other reagents were purchased from Nacalai tesque (Tokyo, Japan).

H89 and H85 were dissolved in dimethyl sulfoxide (DMSO) at a final concentration of DMSO of less than 1%. The concentrations had no effect on sperm motility (8).

Motility measurement of demembranated sperm. Mature males of the chum salmon, *Oncorhynchus keta* were captured from late October to early December in Otsuchi Bay and Otsuchi river, Iwate prefecture, Japan. Semen was obtained by inserting a Pasteur pipette into the sperm duct. Preparation of demembranated sperm was based on the method described previously (19). One volume of semen was suspended in 20 volumes of extraction medium containing 0.15 M KCl, 2 mM MgCl_2 , 0.5 mM EDTA, 2 mM Tris-HCl (pH8.0), 0.04% Triton X-100 and 0.5 mM DTT and incubated on ice for 1 min to solubilize the plasma membrane of the sperm. One volume of the suspension containing the demembranated sperm was re-suspended in 25 volumes of reactivation medium containing 0.15 M KCl, 2 mM MgCl_2 , 1 mM EGTA, 20 mM Tris-HCl (pH8.0), 0.1 mM cAMP, 2% polyethylene glycol (molecular mass of 20,000) and 0.5 mM DTT with or without inhibitors. The motility of the demembranated sperm was then reactivated by adding 0.5 mM ATP to the reactivation medium. Sperm motility was recorded on a videotape via a CCD camera (Hama-

matsu Photonics, Hamamatsu, Japan) attached to a microscope (Nikon, Tokyo, Japan) with phase contrast illumination and analyzed by counting motile and immotile sperm. The percentage of sperm motility was normalized by taking the percentage of motile sperm (the number of motile sperm/all sperm) in the absence of inhibitors as 100%.

Phosphorylation assay and solubilization of flagellar protein. Phosphorylation assay was performed as described previously (21) with some modifications. Semen was centrifuged at $2,500\times g$ for 5 min at 4°C to collect sperm. Sperm were suspended in 15 volumes of extraction medium used for preparing the demembranated sperm.

After incubation on ice for 1 min, the suspension was centrifuged at $2,500\times g$ for 5 min at 4°C . The precipitate containing the demembranated sperm was re-suspended in 3 volumes of phosphorylation assay solution containing 0.15 M KCl, 2 mM MgCl_2 , 1 mM EGTA, 20 mM Tris-HCl (pH8.0), and 0.5 mM DTT. The protein concentration in the sperm was measured by Bradford's method (2) and adjusted to 3.8 mg/mL in the solution. Cyclic AMP (0.1 mM) and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (0.5 mM, 3 GBq/mmol) were added. PKA inhibitors were added before the addition of ATP. After incubation at room temperature for 1 min, phosphorylation of proteins was terminated by adding 7 M urea and flagellar proteins were solubilized by continuous incubation for 15 min on ice. After centrifugation at $10,000\times g$ for 15 min at 4°C , the supernatant was collected and analyzed by SDS-PAGE using 10% polyacrylamide gel.

After sperm were demembranated, re-suspended sperm in reactivation medium were homogenized by POLYTORON AG® (KINEMATICA, Luzern, Switzerland) for 10 min on ice. The homogenate was centrifuged at $2,000\times g$ for 1 min at 4°C , and this supernatant used as 'flagellar fractions'. The flagellar fractions were assayed phosphorylation with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and the phosphorylated flagella were collected by centrifugation at $8,000\times g$ for 10 min at 4°C . The collected flagella were suspended in 20 volumes of the solution containing 0.6 M NaCl, 2 mM MgCl_2 , 1 mM EGTA, 20 mM Tris-HCl (pH8.0) and 0.5 mM DTT, and incubated for 30 min on ice. After incubation, the suspension was centrifuged at $5,000\times g$ for 30 min at 4°C , and the supernatant were analyzed by SDS-PAGE using 15% polyacrylamide gel.

SDS-PAGE and autoradiography. Sample buffer containing SDS at a final concentration of 10% SDS, 15% 2-mercapthoethanol, 40% glycerol,

0.004% bromophenol blue, 312.5 mM Tris-HCl (pH6.8) was added to the supernatants, and the samples were boiled for 2 min at 100°C and subjected to SDS-PAGE. SDS-PAGE was carried out according to the method of Laemmli (14). The separating gel used was 10% (w/v) polyacrylamide containing 0.1% (w/v) SDS. After electrophoresis, the gel was stained with Coomassie Brilliant Blue, dried and exposed to Kodak X-Omat film (Eastman-Kodak, NY, USA.) backed with an intensifying filter (Toshiba, Tokyo, Japan) for 48 h at -80°C. The degree of phosphorylation was quantified by measuring the intensity of bands on the film using NIH Image ver. 1.61. Background density was subtracted from the density of each peak that represented each band on the film and was used as the demonstrated quantity of phosphorylation of the band.

RESULTS

Effects of inhibitors of protein kinase-A on the motility of demembranated sperm

Motility of the demembranated sperm of the rainbow trout (19) and chum salmon (20), which were demembranated by Triton X-100, is not reactivated only in the presence of ATP. The addition of cAMP causes the initiation of motility, suggesting that a cAMP dependent regulatory mechanism controls the motility initiation in salmonid sperm. To elucidate the role of cAMP-dependent protein kinase-A

(PKA) in the initiation of sperm motility, we examined the effects of several inhibitors of PKA on the reactivation of the demembranated sperm. An inhibitor, which is specific for PKA, H-89, very efficiently inhibited the reactivation of the demembranated sperm (Fig. 1). Nevertheless, H-85, which is a non-inhibitory structural analogue of H-89, slightly inhibited the reactivation of the demembranated sperm, and the inhibitory effect was far less than that of H-89. PKAI, which is a synthetic peptide and very specific inhibitor of PKA, inhibited the reactivation of the demembranated sperm at a concentration of 20 nM.

Solubilization of flagellar proteins by urea

Sample buffer for SDS-PAGE has been used to dissolve flagellar proteins (5, 24). In the present study, the addition of sample buffer to sperm suspension made the solution viscous due to the solubilized DNA from sperm heads with SDS. However, when 7 M urea was used, viscosity derived from DNA was not found, and the sperm flagellum was completely dissolved, and sperm heads remained intact (Fig. 2). Sperm heads were quickly removed by centrifugation, and flagellar proteins were recovered in the supernatant. This simple and efficient method was used for the subsequent phosphorylation studies.

Fig. 1 Effects of PKA inhibitors on the motility of demembranated sperm. Demembranated sperm were reactivated by 0.5 mM ATP and 0.1 mM cAMP in the presence or absence of PKA inhibitors: H-85 (60 μ M), H-89 (60 μ M) and PKAI (20 nM). Relative motility represents the percentage of motile sperm relative to that in the absence of inhibitors (control). Values are expressed as the mean $\pm$ SD. Single asterisk represents significant inhibition as compared with the control ($P < 0.001$, $n = 5$). Double asterisks represent significant inhibition as compared with H-85. Note that the demembranated sperm was not reactivated in the absence of both cAMP and PKA inhibitors (-cAMP).

Fig. 2 Solubilization of axonemal proteins from the demembrated sperm by urea solution. Images seen through phase contrast microscope show that axoneme of the demembrated sperm (arrow) existed before incubation with 7 M urea solution (left), and the axoneme was solubilized after urea incubation (right). Heads (arrowheads) were intact both before and after incubation. Bar represents 5 μ m.

Detection of cAMP- dependent phosphoproteins associated with sperm motility

To elucidate the role of PKA-dependent protein phosphorylation on the initiation of sperm motility, we tried to detect phosphoproteins which are associated with reactivation of the demembrated sperm. Sperm plasma membrane was removed with Triton X-100 and the demembrated sperm were incubated in the phosphorylation assay solution (see Materials and Methods) containing [γ - 32 P]ATP. As shown in Fig. 3, (lane a), only faint 32 P_i incorporations were detected in several proteins in the absence of cAMP. But phosphorylation was detected in 10 proteins with molecular masses of 21 kDa, 23 kDa, 28 kDa, 33 kDa, 36 kDa, 41 kDa, 48 kDa,

Fig. 3 Phosphorylation of flagellar proteins at the cAMP-dependent initiation of sperm motility. Demembrated sperm were immotile in the absence of cAMP (see Fig. 1), and 21 kDa, 23 kDa, 41 kDa, 48 kDa, 63 kDa and 70 kDa proteins were slightly phosphorylated in the absence of cAMP (lane a). When motility of the demembrated sperm was reactivated in the presence of 0.1 mM cAMP (lane b), 10 proteins with molecular masses of 21 kDa, 23 kDa, 28 kDa, 33 kDa, 36 kDa, 41 kDa, 48 kDa, 63 kDa, 70 kDa and 83 kDa (arrows with black and white heads), were phosphorylated. In lane c, when intact sperm were allowed to be motile for 2 min and then the sperm were demembrated and reactivated by 0.1 mM cAMP, 32 P_i incorporation were reduced in the proteins (arrows with black heads, see Table 1). The numbers on the right show molecular mass markers.

63 kDa, 70 kDa and 83 kDa in the presence of cAMP (Fig. 3, lane b). To examine whether the phosphorylation of these 10 proteins is really coupled with the initiation of sperm motility, we investigated phosphorylation of the demembrated sperm after *in vivo* activation as follows: motility of intact sperm was initiated *in vivo* at first by diluting one

volume of semen to 40 volumes of K⁺-free medium containing 100 mM NaCl, 5 mM CaCl₂ and 5 mM NaHCO₃. After the motility was completed the plasma membrane of the sperm was solubilized in the demembranated medium containing [γ -³²P]ATP in the presence of cAMP. Incorporation of ³²P was significantly reduced in the seven proteins with molecular masses of 21 kDa, 28 kDa, 33 kDa, 41 kDa, 48 kDa, 63 kDa and 83 kDa (Fig. 3, lane c and Table 1). This reduction of ³²P incorporation may be due to that these proteins were already phosphorylated upon *in vivo* initiation of sperm motility. Three other proteins with molecular masses of 23 kDa, 36 kDa and 70 kDa had similar ³²P-incorporation to that observed in lane b. These suggest that among phosphorylations, 7 proteins other than 23, 36 and 70 kDa are coupled with the initiation of salmonid sperm motility.

Phosphoproteins extracted by high salt solution

In order to examine the nature of cAMP dependent phosphoproteins extracted by 7 M urea solution, we tried to extract these phosphoproteins by high salt (0.6 M NaCl) by which dynein light chain and PKA subunit could be extracted from sperm flagella (9). As a result, 21 kDa and 48 kDa proteins could be extracted from sperm flagella by the high salt solution (Fig. 4), suggesting that 21 kDa and 48 kDa protein extracted by the urea solution would be correspondent to the component of outer arm dynein and PKA. 28 kDa, 33 kDa and 41 kDa proteins could not be extracted (Fig. 4). It is not clear whether 2 phosphoproteins in the molecular masses between 66 kDa to 99 kDa (Fig. 4) were 63 kDa and 83 kDa phosphoproteins which were showed in Fig. 3, since we used 15% polyacrylamide gel for SDS-PAGE.

Effect of PKA inhibitors on the phosphorylation of the seven phosphoproteins

H-89 and PKAI inhibited the phosphorylation of the seven proteins (Fig. 5), concomitantly with inhibition of the motility of the demembranated sperm (Fig. 1). H-89 did not significantly inhibit phosphorylation of the seven proteins (Fig. 5) or reactivation of the demembranated sperm (Fig. 1). The inhibition of phosphorylation by H-89 was efficient in 33 kDa and 63 kDa proteins. Inhibition of phosphorylation by PKAI was more efficient in 28 kDa protein than that in other proteins (Table 2). Inhibition of phosphorylation of 21 kDa protein by H-89 and PKAI was not prominent when compared with that of 33 kDa, 63 kDa and 28 kDa proteins but the inhibition of 21 kDa protein by the inhibitors was clear when compared with the control (Table 2). These results suggest that PKA-dependent phosphorylation of these seven proteins, especially that of 28 kDa, 33 kDa and 63 kDa, plays a key role in the initiation of sperm motility in the salmonid fish.

DISCUSSION

The contribution of phosphoproteins and protein phosphorylations to sperm motility has been studied in many animal species (1, 3-6, 10, 21, 23-25, 27-30) but details of the roles of these phosphoproteins in cell signaling underlying the regulatory mechanism of sperm motility have been unclear. It has been suggested that cAMP-dependent phosphorylation of 15kDa protein (5, 21), dynein light chain of the outer arm (10) and regulatory subunit of protein kinase-A (9) is coupled with sperm motility in the sperm of salmonid fishes.

SDS solution, which has been widely used for solubilization of protein from cells, could not be

Table 1 *Quantitative Analysis of Cyclic AMP-dependent Phosphorylation of the Ten Proteins*

	Relative densitometry (%) ^a									
	21kDa	23kDa	28kDa	33kDa	36kDa	41kDa	48kDa	63kDa	70kDa	83kDa
- cAMP	30.3 ^b	27.6	25.8	14.6	48.6	24.2	23.6	19.8	44.3	21.2
+ cAMP	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
pre-activat. ^c	57.8	99.6	58.2	52.8	99.0	36.4	50.2	49.4	97.9	55.8

^aBand intensity of the ten proteins in Fig. 3, value relative to that in the presence of cAMP.

^bThe mean for at least four different experiments.

^cWhen intact sperm were allowed to be motile for 2 min and then the sperm were demembranated and reactivated in the presence of cAMP and [γ -³²P] ATP.

Fig. 4 Cyclic AMP-dependent phosphorylation of 0.6 M NaCl extracted proteins. The high salt 0.6 M NaCl could solubilize phosphorylated 21 kDa protein (arrow) and 48 kDa protein (arrowhead) from sperm flagella. Molecular masses of phosphoproteins indicated with white arrowheads could not be identified in 15% polyacrylamide gel. The numbers on the right show molecular mass markers.

Fig. 5 Effects of PKA inhibitors on the phosphorylation of proteins. Demembrated sperm were reactivated with 0.1mM cAMP and [γ - 32 P]ATP in the absence (lane a) and presence of PKA inhibitors; 60 μ M H-85 (lane b), 60 μ M H-89 (lane c) and 20 nM PKAI (lane d). Proteins were separated by SDS-PAGE and subjected to autoradiography. Phosphorylation of seven proteins that were identified as proteins phosphorylated in association with sperm motility (see Fig. 3) was inhibited by PKAI. The numbers on the right show molecular mass markers

Table 2 *Quantitative Analysis of the Effect of PKA Inhibitors on Phosphorylation of the Seven Proteins*

Inhibitor	Concentration	Relative densitometry (%) ^a						
		21kDa	28kDa	33kDa	41kDa	48kDa	63kDa	83kDa
—	—	100.0 ^b	100.0	100.0	100.0	100.0	100.0	100.0
H-85	60 μ M	67.8	53.0	55.1	73.9	70.2	68.3	63.7
H-89	60 μ M	43.7	27.2	22.7	46.4	47.2	30.4	36.7
PKAI	20nM	52.5	36.2	44.6	48.2	53.1	52.0	45.8

^aBand intensity of the seven proteins in Fig. 4, value relative to that in the absence of inhibitors.

^bThe mean for at least three different experiments.

used in salmonid sperm, since the addition of SDS to sperm suspension results in high viscosity of the solution which makes it difficult to analyze flagellar proteins by SDS-PAGE. In the present study, urea was able to certainly and quickly solubilize flagellar proteins without disruption of the sperm head (see Fig. 2). In addition, the 15 kDa protein (5, 21) whose phosphorylation could not be detected in the axoneme isolated by the conventional homogenization method (10), and therefore considered to be localized at the base of the flagellum (13), could be detected by the urea method (data not shown). This suggests that the procedure could solubilize the entire flagellum including its basal part. By this method we have detected seven proteins phosphorylated in association with the cAMP-dependent reactivation of the demembranated sperm (Fig. 3, Table 1). Among them, 21 kDa and 48 kDa proteins were considered to be the light chain of the outer arm dynein and the regulatory subunit of proteins kinase-A (9), respectively, judging from the molecular masses and prominent phosphorylation pattern (see Fig. 3). Five other phosphoproteins (28 kDa, 33 kDa, 41 kDa, 63 kDa and 83 kDa) was first found and described in detail in the present study. The phosphorylation of the five proteins as well as 21 kDa and 48 kDa proteins and the reactivation of demembranated sperm were inhibited by the inhibitors of PKA (see Fig. 1, 5 and Table 2). These results suggest that all of the cAMP-dependent phosphorylation of the sperm proteins is involved in the cell signaling underlying the initiation of sperm motility in the salmonid fish.

Several studies suggest that PKA is localized near the outer arm dynein and is involved in the phosphorylation of 21 kDa dynein light chain-related Tctex2 (t-complex testis expressed 2)-related (9, 10). Tctex2 is coded in the t-complex gene which participates in transmission ratio distortion and male infertility in mice (16). It is therefore possible that the 21 kDa light chain of the outer arm dynein and the 48 kDa regulatory subunit of PKA, which are phosphorylated in association with cAMP-dependent initiation of sperm motility, are closely distributed with each other in the sperm flagellum in the salmonid fish. On the other hand, PKA is present in several subfractions of flagellar axoneme, even in subfractions from the outer-arm-depleted axoneme in sea urchin sperm (1), suggesting that PKA-dependent phosphorylation occurs in an axonemal structure other than outer arm dynein. Several subunits which are composed of radial spoke, a structural component of axoneme, are known as phosphoproteins (26). PKA-

dependent phosphorylation was reported in the intermediate chain of inner arm dynein from the flagellum of *Chlamydomonas* (17, 26). In ascidian *Ciona*, a protein from axoneme with a molecular mass of 26 kDa, is phosphorylated in association with the activation of sperm motility (4). The 26 kDa protein is not extracted by a high salt solution and considered to be a component of the structure of axoneme other than outer arm dynein (24), which can be easily solubilized from sperm axoneme by high salt solution (7). The dynein light chain (Fig. 4. See also ref. 9) and regulatory subunit of PKA (Fig. 4) could be solubilized by the high salt solution from the axoneme of sperm of the chum salmon but at least 3 of the five phosphoproteins detected in this study could not be solubilized by the high salt solution, and only urea solution could solubilize them. From the evidence accumulated in past and present studies, it is possible that high salt soluble 21 kDa and 48 kDa proteins and urea soluble five phosphoproteins, which might be unknown components of an axonemal structure, form the multiple phosphoprotein networks. Further study on the localization of these proteins in the axoneme may lead us to a clearer understanding of the PKA-dependent pathway for the initiation of sperm motility.

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