DEPHOSPHORYLATION OF A 30 KDA PROTEIN OF FOWL SPERMATOZOA BY THE ADDITION OF MYOSIN LIGHT CHAIN KINASE SUBSTRATE PEPTIDE INHIBITS THE FLAGELLAR MOTILITY

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Summary. Phosphorylation of demembranated fowl sperm proteins during incubation with [γ-^{32}P]ATP and various protein kinase substrate peptides at 30°C was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A marked difference in phosphorylation was observed in a 30 kDa protein. This protein was strongly phosphorylated after the addition of Kemptide, a cAMP-dependent protein kinase (PKA) substrate peptide; Syntide 2, a calmodulin-dependent protein kinase II substrate peptide; a protein kinase C (PKC) substrate peptide; as well as control samples but only slightly phosphorylated in the presence of a myosin light chain kinase (MLCK) substrate peptide. The motility of demembranated spermatozoa at 30°C remained high in control samples and following the addition of Kemptide, Syntide 2 and PKC substrate peptide, but decreased markedly following the addition of MLCK substrate peptide. These results suggest that the 30 kDa protein is identified as a substrate for MLCK or a MLCK-like protein in fowl spermatozoa and that phosphorylation-dephosphorylation of this protein is involved in the regulation of flagellar movement at 30°C.

Substantial evidence implicates a role for protein phosphorylation-dephosphorylation systems in the regulation of sperm movement, but details of the mechanisms remain unclear (reviewed in [1-8]). Fowl spermatozoa provide an excellent model for investigating this regulatory mechanism, since they display the unique phenomenon of reversible temperature-dependent immobilization: in simple salt solutions they become immotile at the avian body temperature of 40-41°C, but motility is restored by decreasing the temperature [9-14]. The axoneme itself appears to be directly involved in this regulatory system, since the motility of demembranated spermatozoa is, as with intact spermatozoa, negligible at 40°C and restored at 30°C [15,16]. However, the intracellular molecular mechanisms involved in the immobilization and restoration of motility have remained unsolved.

It is recognized that cAMP is indispensable for the initiation and activation of the motility of spermatozoa from a variety of species. Therefore, it seems likely that protein phosphorylation by cAMP-dependent protein kinase (PKA) is involved in the activation of sperm motility (reviewed in [1-8]). However, unlike that of other species, the motility of demembranated fowl spermatozoa is not restored by the addition of cAMP at 40°C [16,17].

In fowl spermatozoa, intracellular free Ca^{2+} seems to be essential for the maintenance of motility, since the motility of intact spermatozoa at 40°C can be restored by the addition of Ca^{2+} [12,15,18]. Furthermore, the motility of demembranated fowl spermatozoa is inhibited in Ca^{2+}

-free medium at 30°C [19], and the motility of intact spermatozoa loaded with an intracellular Ca^{2+} chelator, 1, 2-bis (2-aminophenoxy) ethane-*N, N, N', N'*-tetraacetic acid, tetraacetoxymethy ester (BAPTA/AM) is also negligible at 30°C, but can be instantly restored by the subsequent addition of Ca^{2+} [20]. However, even in the presence of Ca^{2+}, the addition of calmodulin antagonists such as *N*-(6-aminohexyl)-5-chloro-1-naphthalensulfonamide hydrochloride (W-7) and trifluoperazine inhibited sperm motility at 30°C and 40°C [20]. Therefore, it may be assumed that phosphorylation by Ca^{2+}/calmodulin-dependent protein kinases, rather than PKA, is likely to be a regulatory step in the maintenance of fowl sperm motility.

Recently, the involvement of axonemal myosin light chain kinase (MLCK), or a MLCK-like protein, has been proposed to be a candidate of Ca^{2+}/calmodulin dependent protein kinase, since the motility of both intact and demembranated fowl spermatozoa at 30°C decreased markedly following the addition of 1-(5-chlornaphthalene-1-sulfonyl)-1H-hexahydro-1, 4-diazepine hydrochloride (ML-9), a specific inhibitor of MLCK, but not following the addition of *N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (H-8), a specific inhibitor of PKA [21]. If MLCK or a MLCK-like protein affects the maintenance of fowl sperm motility, then the question of its target and precise mechanisms of action is raised. We report here that the 30 kDa protein is a substrate for MLCK, or a MLCK-like protein, and that dephosphorylation of this protein is associated with the inhibition of flagellar movement of fowl spermatozoa.

Materials and Methods

Chemicals. Protein kinase substrate peptides, Leu-Arg-Arg-Ala-Ser-Leu-Gly (Kemptide), Pro-Leu-Ala-Arg-Thr-Leu-Ser-Val-Ala-Gly-Leu-Pro-Gly-Lys-Lys (Syntide 2) and pGlu-Lys-Arg-Pro-Ser-Gln-Arg-Ser-Lys-Tyr-Leu were obtained from Sigma Chemical Co. (St Louis, MO), and Lys-Lys-Arg-Pro-Gln-Arg-Ala-Thr-Ser-Asn-Val-Phe-Ser-NH_2 was from Peninsula Laboratories, Inc. (Belmont, CA). Calyculin A was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Adenosine 5'-triphosphate (ATP), dithiothreitol, potassium glutamate, *N*-Tris-[hydroxymethyl]-methyl-2-aminoethane-sulphonic acid (TES) and Triton X-100 were purchased from Sigma Chemical Co. (St Louis, MO). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) molecular weight standards were from Life Technologies, Inc. (Gaithersburg, MD). [γ-^{32}P]ATP was purchased from Du Pont-New England Nuclear (Boston, MA). Other chemicals were of regent grade from Nacalai Tesque, Inc. (Kyoto, Japan).

Preparation of spermatozoa. Ejaculated spermatozoa from commercial White Leghorn roosters were diluted approximately 10-fold in 150 mM NaCl with 20 mM TES at pH 7.4 and centrifuged at 700 x *g* for 13 min at room temperature (20-25°C). The washed spermatozoa were reconstituted in the same buffer to give a final concentration of approximately 1 x 10^9 cells/ml. Samples of 3-4 ml were poured into 30 ml Erlenmeyer flasks with a screw cap.

Phosphorylation of endogenous proteins and electrophoresis. Phosphorylation reaction and electrophoresis on polyacrylamide gels of demembranated sperm proteins were carried out according to the methods described previously [22], but with some modifications. Briefly, demembranated spermatozoa were incubated for 2.5 min at 30°C with extraction-reactivation medium, containing 0.1% Triton X-100, 200 mM sucrose, 25 mM potassium glutamate, 1 mM $MgSO_4$, 1 mM dithiothreitol and 20 mM Tris-HCl buffer (pH 7.9), 0.1 mM ATP and approximately 7000 cpm/pmol [γ-^{32}P]ATP. To examine the effects of protein kinases on phosphorylation, various protein kinase substrate peptides were added to the medium. At the end of the incubation, sodium pyrophosphate and unlabeled ATP, at final concentrations of 15 mM and 10 mM, respectively, were added to stop the reaction, and the samples were placed on ice. Each sample was centrifuged, and Laemmli [23] sample buffer was added to the pellets and boiling for 5 min.

Samples containing protein from approximately 2.7 x 10^6 spermatozoa were loaded on to 10% or 15% SDS-polyacrylamide slab gels, and subjected to electrophoresis. Autoradiography was performed at -80°C for 1-2 days exposure to X-ray film with an intensifying screen (Lightning plus, Du Pont, Wilmington, DE).

Analysis of demembranated sperm motility. Demembranation and reactivation of spermatozoa were performed according to the method described previously [16]. The extraction medium consisted of 0.1% Triton X-100, 200 mM sucrose, 25 mM potassium glutamate, 1 mM $MgSO_4$, 1 mM dithiothreitol and 20 mM Tris-HCl buffer (pH 7.9). The reactivation medium consisted of 0.5 mM ATP, 200 mM sucrose, 25 mM potassium glutamate, 1.5 mM $MgSO_4$, 1 mM dithiothreitol and 20 mM Tris-HCl buffer (pH 7.9). The reactivation medium, containing demembranated spermatozoa was incubated in a water bath for 5 min at 30°C or 40°C. The suspension of demembranated spermatozoa was then placed into a microscope slide chamber (Sekisui Chemical Co., Ltd., UR-157 type, Tokyo, Japan) on a thermostatically-controlled warm plate, and the motility of spermatozoa was recorded by videomicroscopy (magnification on the black and white monitor was approximately x 600) at 30°C or 40°C [24]. Measurements were made on a total of 200-300 spermatozoa, distributed uniformly among the three or more fields, to determine the percentage motility.

Statistical analysis. Percentage motility was transformed using arc sine transformation. The results were analyzed by Duncan's multiple-range tests [25].

Results and Discussion

No marked difference in the phosphorylation status of demembranated sperm proteins was obvious between control samples and those with added Leu-Arg-Arg-Ala-Ser-Leu-Gly (Kemptide), a PKA substrate peptide, or pGlu-Lys-Arg-Pro-Ser-Gln-Arg-Ser-Lys-Tyr-Leu, a PKC substrate peptide. In contrast, dephosphorylation of approximately 80 kDa and 105 kDa proteins was observed after the addition of Pro-Leu-Ala-Arg-Thr-Leu-Ser-Val-Ala-Gly-Leu-Pro-Gly-Lys-Lys (Syntide 2), a calmodulin-dependent protein kinase II substrate peptide, or Lys-Lys-Arg-Pro-Gln-Arg-Ala-Thr-Ser-Asn-Val-Phe-Ser-NH_2, a MLCK substrate peptide. In addition, a 30 kDa protein was slightly phosphorylated following MLCK substrate peptide treatment, whereas this protein was strongly phosphorylated in the presence of Kemptide, Syntide 2 or PKC substrate peptide as well as control (Fig. 1a, b, denoted by arrow).

In the presence of ATP, the percentage motility of demembranated spermatozoa without substrate peptides was high at 30°C, but negligible at 40°C. At 30°C, the addition of Kemptide, Syntide 2 or PKC substrate peptide, did not appreciably affect the motility of demembranated spermatozoa. In contrast, inhibition of motility of demembranated spermatozoa was observed following the addition of MLCK substrate peptide. At 40°C, no stimulation or inhibition of motility of demembranated spermatozoa was observed following the addition of any substrate peptides (Table 1). The presence of calyculin A, a inhibitor of protein phosphatase type 1 and type 2, permitted reactivation of demembranated spermatozoa at 40°C, and this effect was maintained even in the presence of Kemptide, Syntide 2 and PKC substrate peptide. However, no stimulation of motility was observed following the addition of calyculin A and MLCK substrate peptide at both 30°C and 40°C (Table 2). Demembranated spermatozoa, stimulated by calyculin A at 40°C, lost their motility in a dose-dependent manner following exposure to MLCK substrate peptide (Fig. 2).

There is substantial evidence that PKA is involved in the activation of sperm motility (reviewed in [1-8]). For example, phosphorylation of a 15 kDa axonemal protein by PKA is essential for the initiation of trout sperm motility [26]; a 55 kDa protein which is phosphorylated in a cAMP-dependent manner is apparently related to the motility state of bovine spermatozoa [27]; axokinin, a soluble 56 kDa phosphoprotein, seems to play a key role in mediating the cAMP response in dog spermatozoa [28,29]; and the type II regulatory subunit of PKA has been identified as the major cAMP-dependent phosphoprotein in bovine spermatozoa [30,31]; and a heat-stable protein inhibitor of PKA, purified from rabbit skeletal muscle or rat testis, inhibits reactivation of demembranated mammalian and sea urchin spermatozoa [28,32]. Moreover, it has been reported that reactivation of the motility of demembranated dog spermatozoa produced by ATP alone is also inhibited by H-8, a specific inhibitor of PKA, with half-maximal inhibition of motility is achieved with 50 μM drug [29].

However, the presence of even 1 mM H-8 did not inhibit the motility of either intact or demembranated fowl spermatozoa at 30°C [21]. Furthermore, the motility of demembranated fowl spermatozoa was not restored by the addition of cAMP at 40°C [16,17], and was maintained even in the presence of phosphodiesterase at 30°C [16]. In the study reported here, PKA substrate peptide was also ineffective as a inhibitor of the motility of demembranated spermatozoa at 30°C. Therefore, it seems likely that the protein phosphorylation of axonemal protein(s) by PKA may not be involved in the maintenance of fowl sperm motility. Neither did the addition of PKC substrate peptide affect the motility and phosphorylation state of demembranated spermatozoa, even though the activation of this enzyme may contribute to a decrease in the flagellar movement of fowl spermatozoa [33]. This may be due to loss of PKC by the demembranation process, since it has been suggested that endogenous PKC is present in the cytoplasmic matrix or the membrane of fowl spermatozoa [33].

The present results showed that the 80 kDa and 105 kDa proteins, but not the 30 kDa protein, were dephosphorylated by the addition of Syntide 2, one of calmodulin-dependent protein kinases substrate peptide, but this peptide did not inhibit sperm motility. In contrast, the addition of a MLCK substrate peptide, another kind of calmodulin-dependent protein kinase substrate peptide, decreased the phosphorylation levels of the 30 kDa protein in addition to 80 kDa and 105 kDa proteins, and inhibited the motility of demembranated spermatozoa at 30°C. Thus, it may be possible that phosphorylation of the 30 kDa protein by endogenous MLCK or a MLCK-like protein might be necessary for the activation of fowl sperm motility at 30°C.

It has been proposed that the activation of protein phosphatase type 1 (PP1), present in the fowl sperm axoneme, may be involved in the inhibition of fowl sperm motility at 40°C, since, in addition to calyculin A and okadaic acid, inhibitors 1 and 2, specific inhibitors of PP1 [34], also stimulated the motility of demembranated spermatozoa at 40°C [35]. In the present study, demembranated fowl spermatozoa, stimulated by the addition of calyculin A at 40°C, lost their motility in the presence of MLCK substrate peptide in a dose dependent manner, but not in the presence of Kemptide, Syntide 2 or PKC substrate peptide. These results suggest that the phosphorylation-dephosphorylation of axonemal protein(s) by at least MLCK, or a MLCK-like protein, and PP1 may be involved in the regulation of fowl sperm motility. Additional studies

are therefore necessary to elucidate the target of the action of PP1 in axoneme: so far we have been unable to obtain evidence that the 30 kDa protein is dephosphorylated by PP1.

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Table 1. Effects of protein kinase substrate peptides on the motility of demembranated fowl spermatozoa at 30°C and 40°C

Substance	Motility (%)	
	30°C	40°C
None (control)	79.0±4.1[a]	2.0±1.3[a]
Leu-Arg-Arg-Ala-Ser-Leu-Gly (Kemptide)	77.8±6.4[a]	0.9±0.9[a]
Pro-Leu-Ala-Arg-Thr-Leu-Ser-Val-Ala -Gly-Leu-Pro-Gly-Lys-Lys (Syntide 2)	70.9±7.6[a]	0.3±0.3[a]
Lys-Lys-Arg-Pro-Gln-Arg-Ala-Thr-Ser -Asn-Val-Phe-Ser-NH_2 (MLCK substrate)	1.9±1.9[b]	0.0±0.0[a]
pGlu-Lys-Arg-Pro-Ser-Gln-Arg-Ser -Lys-Tyr-Leu (PKC substrate)	65.2±8.3[a]	0.6±0.6[a]

Each peptide added in the reactivation medium was 0.5 mM. Each value represents the mean (±S.E.M.) of five samples of spermatozoa. Within columns, values with different superscripts differ significantly ($P<0.01$) from each other.

Table 2. Effects of calyculin A and protein kinase substrate peptides on the motility of demembranated fowl spermatozoa at 30℃ and 40℃

Substance		Motility (%)	
		30°C	40℃
None (control)		81.1±3.9[a]	2.8±1.6[a]
Calyculin A		85.7±2.3[a]	74.4±3.9[b]
Calyculin A	+ Kemptide	85.7±4.9[a]	74.8±5.1[b]
	+ Syntide 2	83.6±4.4[a]	64.3±6.2[bc]
	+ MLCK substrate	2.9±1.3[b]	0.0±0.0[a]
	+ PKC substrate	77.7±4.6[a]	49.5±2.7[c]

Calyculin A and each peptide added in the reactivation medium were 100 nM and 0.5 mM, respectively. Each value represents the mean (±S.E.M.) of five samples of spermatozoa. Within columns, values with different superscripts differ significantly ($P<0.01$) from each other.

Legends for figures

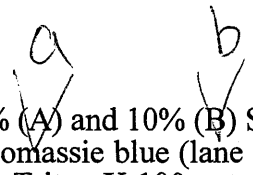

Fig. 1. 15% (A) and 10% (B) SDS-PAGE profile of demembranated fowl sperm proteins stained with the Coomassie blue (lane 6) and corresponding autoradiography of phosphorylated proteins (lanes 1-5). Triton X-100-extracted and reactivated samples were incubated for 2.5 min at 30°C. Approximately 7000 cpm/pmol [γ-^{32}P]ATP and 0.1 mM ATP were added in the medium. Samples containing protein from approximately 2.7 x 10^6 spermatozoa were loaded. Lane 1, no treatment (control); lane 2, presence of 0.5 mM MLCK substrate peptide; lane 3, presence of 0.5 mM Kemptide; lane 4, presence of 0.5 mM Syntide 2; lane 5, presence of 0.5 mM PKC substrate peptide.

Fig. 2. The time course of motility of demembranated fowl spermatozoa at 40°C following addition of 100 nM calyculin A and various concentrations of MLCK substrate peptide (■; no addition, ○; 0.05 mM, ●; 0.1 mM, □; 0.5 mM). Each point represents the mean (±S.E.M.) of five samples of spermatozoa. $^*P<0.01$ compared with value of control (no addition) at each period.

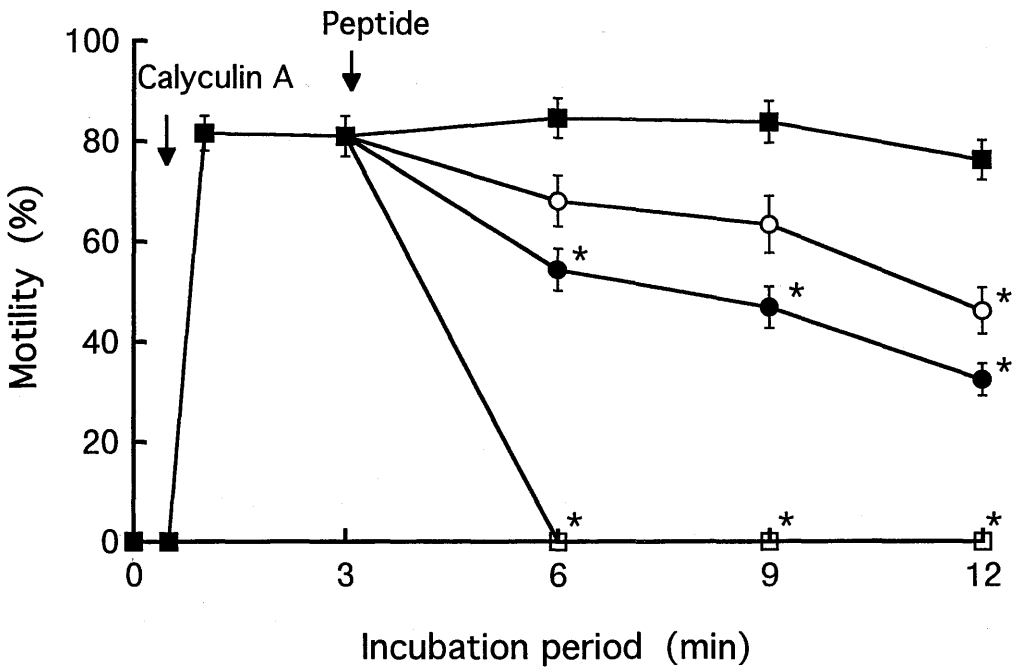

Fig. 2

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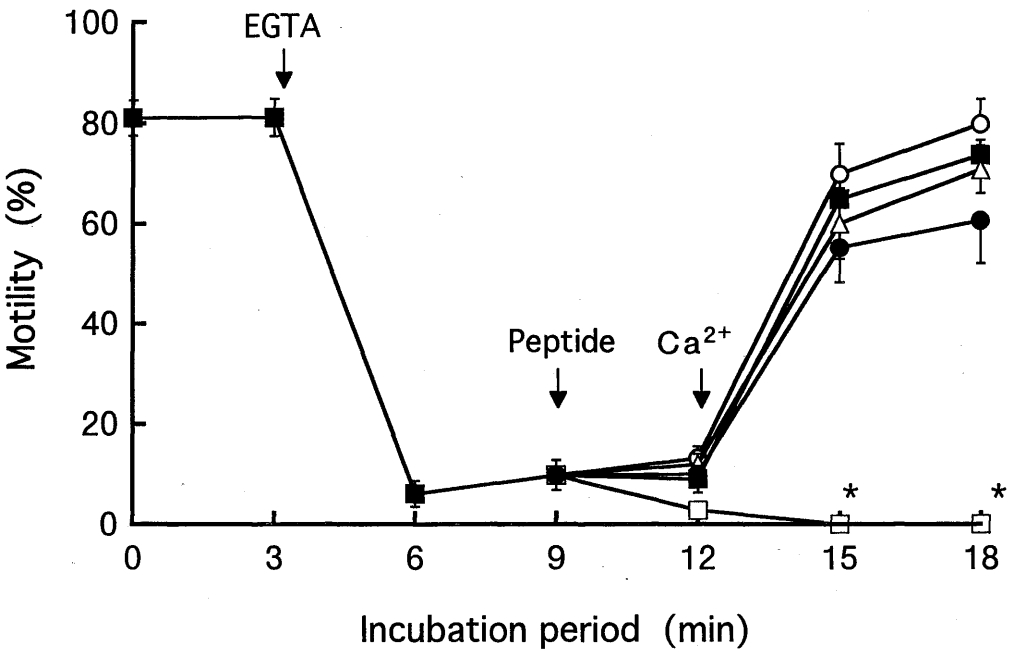

Fig. 3.

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