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Regulation of Fowl Sperm Flagellar Motility by Protein Phosphatase Type 1 and Its Relationship with Dephosphorylation of Axonemal and/or Accessory Cytoskeletal Proteins

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The motility of demembranated fowl spermatozoa was vigorous at 30°C in the presence of ATP, but decreased markedly following the addition of recombinant protein phosphatase type 1 (PP-1) supplemented with Mn^{2+}. This inhibition was not restored by the addition of cAMP, within the range 1-1000 μM, but instantly restored by the addition of 50 ng/ml trypsin. Phosphorylation of demembranated fowl sperm proteins during incubation with [γ-^{32}P]ATP at 30°C was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A marked difference in phosphorylation status was observed in approximately 116, 86, 79, 50 and 29-kDa proteins. These proteins were dephosphorylated in the presence of PP-1 and Mn^{2+} compared with those in control samples. These results suggest that PP-1-mediated dephosphorylation of some of these proteins of the axoneme and/or accessory cytoskeletal components of fowl spermatozoa may be involved in the inhibition of motility.

Fowl spermatozoa provide an excellent model for investigating the regulatory mechanism of flagellar movement, since they uniquely display the phenomenon of reversible temperature-dependent motility inhibition: in simple salt solutions they become immotile at the avian body temperature of 40-41°C, but motility is restored by decreasing the temperature [1-6]. The axoneme and/or accessory cytoskeletal components appear 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 [7,8]. However, the intracellular molecular mechanisms of the immobilization and restoration of motility are not clearly understood at present.

Protein phosphorylation/dephosphorylation is a critical step in intracellular signal transduction for the regulation of a wide variety of cellular processes, including sperm flagellar motility (reviewed in [9-15]). While abundant information can be found concerning protein kinases and phosphorylation in relation to the regulation of sperm motility, relatively little attention has been paid to the potential role for protein phosphatases in this process [16]. However, the involvement of dephosphorylation by protein phosphatases in regulation of sperm motility has been reported in several animals. For example, the addition of a crude or partially purified protein phosphatase preparation from bovine cardiac muscle or rabbit skeletal muscle inhibited the motility of demembranated sea urchin spermatozoa, presumably due to its ability to cause dephosphorylation of axonemal proteins [17-19]. With regard to dog and human spermatozoa, it has been proposed that modulation of sperm motility by Ca^{2+} may be due to the activation of protein phosphatase type 2B (PP-2B), a Ca^{2+}/calmodulin-dependent protein phosphatase [20,21].

More recent work has demonstrated that the activation of PP-1, one of serine/threonine phosphatases, may be dominant in the involvement for the temperature-dependent inhibition of fowl sperm motility at 40°C, since, in addition to calyculin A and okadaic acid (specific inhibitors of PP-1 and PP-2A), the PP-1-specific inhibitors 1 and 2 [22] also stimulated the motility of demembranated spermatozoa at 40°C [23]. Furthermore, a functional PP-1 system may be present in bovine, human and rhesus monkey spermatozoa, and the immotility of caput epididymal spermatozoa may be due to higher PP-1 activity [24,25]. However, it should be noted that these assumptions were made from evidence obtained using phosphatase inhibitors, such as okadaic acid and calyculin A, and detailed mechanisms have still to be clarified. Moreover, to date, no identifi-

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cation of PP-1-substrate proteins involved in the regulation of motility has been reported. Therefore, the objectives of the present study were to investigate the axonemal substrates and mechanisms of action of PP-1 in fowl spermatozoa. We report here that PP-1 dephosphorylates axonemal and/or accessory cytoskeletal component proteins of Mr 116, 86, 79, 50 and 29-kDa, and propose that some of these proteins may be associated with the inhibition of flagellar movement of fowl spermatozoa.

MATERIALS AND METHODS

Chemicals. Recombinant protein phosphatase type 1 (PP-1) isolated from a strain of *E. coli* that carries a clone expressing the α-isoform of PP-1 from rabbit skeletal muscle was purchased from New England BioLabs Inc. (Beverly, MA). ATP, cAMP, dithiothreitol, potassium glutamate, *N*-Tris-[hydroxymethyl]-methyl-2-aminoethanesulphonic acid (TES) and Triton X-100 were obtained from Sigma Chemical Co. (St Louis, MO). SDS-PAGE molecular weight standards were purchased from Bio-Rad Laboratories (Hercules, CA). [γ-^{32}P]ATP was from Du Pont-New England Nuclear (Boston, MA). Other chemicals were of reagent 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 $\times$ *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 1×10^9 cells/ml. Samples of 3-4 ml were poured into 30 ml Erlenmeyer flasks with a screw cap.

Analysis of demembranated sperm motility. Demembranation and reactivation of spermatozoa were performed according to the method described previously [8]. 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). To examine the effects of PP-1, various concentrations of PP-1 were added to the reactivation medium. $MnCl_2$, cAMP and trypsin were also added to PP-1-treated spermatozoa. The reactivation medium, containing demembranated spermatozoa was incubated in a water bath at 30°C for 5 min. 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 12-inch black and white monitor was approximately $\times$ 600) at 30°C [26]. Measurements were made on a total of 200-300 spermatozoa, distributed uniformly among the three or more fields, to determine the percent of motile spermatozoa.

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 [27], 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 PP-1 on phosphorylation, PP-1 and other effectors for motility were added to the medium. At the end of the incubation, sodium pyrophosphate and unlabelled 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 the supernatant was discarded. Laemmli [28] sample buffer was added to the pellets and boiled for 5 min. Samples containing protein from approximately 2.2×10^6 spermatozoa were loaded on to 10% SDS-polyacrylamide slab gels, and subjected to electrophoresis. Autoradiography was performed at −70°C for 2-4 days exposure to X-ray film with an intensifying screen (Lightning plus, Du Pont, Wilmington, DE).

Statistical analysis. Percentage of motility was transformed using arc sine transformation. The results were analyzed by Duncan's multiple-range tests [29].

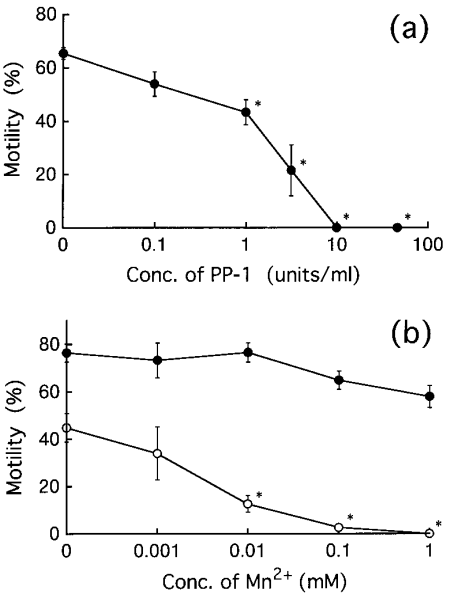

FIG. 1. The motility of demembranated fowl spermatozoa in the reactivation medium at 30°C after addition of various concentrations of (a) recombinant protein phosphatase type 1 (PP-1) supplemented with 0.2 mM $MnCl_2$ (●), and (b) $MnCl_2$ without PP-1 (●) or $MnCl_2$ with 10 units/ml PP-1 (○). Each point represents the mean (±S.E.M.) of five samples of spermatozoa. *$P<0.01$ compared with value of 0 unit/ml or 0 mM (control), respectively.

RESULTS

The percentage of motility of demembranated spermatozoa at 30°C was inhibited in a dose-dependent manner by the addition of PP-1 in the presence of 0.2 mM Mn^{2+} (Fig. 1a). No inhibition of motility was observed following the addition of Mn^{2+} alone, within the range 0-1 mM, but the motility of spermatozoa treated with 10 units/ml PP-1 decreased significantly ($P<0.01$) at higher concentration of Mn^{2+} (Fig. 1b). Fig. 1 shows the motility at 5 min after the addition of PP-1 and/or Mn^{2+}. The time course of motility in the presence of PP-1 and Mn^{2+} is shown in Fig. 2. Without the addition of PP-1 and Mn^{2+}, the motility was maintained for at least 15 min. In contrast, the motility was inhibited immediately after the addition of PP-1 and Mn^{2+}.

The addition of 10 μM cAMP to spermatozoa in the presence of PP-1 and Mn^{2+} did not restore motility (Fig. 3). This was also true for up to 1000 μM cAMP (data

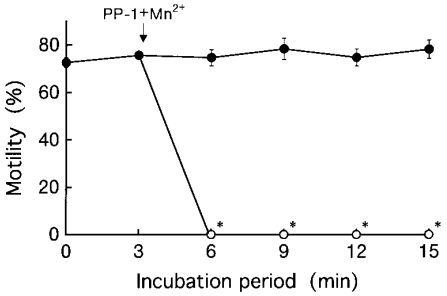

FIG. 2. The time course of motility of demembranated fowl spermatozoa in the reactivation medium at 30°C in the absence (●) or presence (○) of 10 units/ml PP-1 + 0.2 mM $MnCl_2$. Each point represents the mean (±S.E.M.) of five samples of spermatozoa. *$P<0.01$ compared with value of control (no addition) at each period.

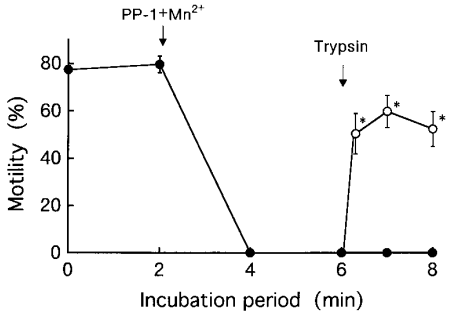

FIG. 4. The time course of motility of demembranated fowl spermatozoa in the reactivation medium at 30°C following addition of 10 units/ml PP-1 + 0.2 mM $MnCl_2$ and trypsin (●; no addition, ○; 50 ng/ml). Each point represents the mean (±S.E.M.) of five samples of spermatozoa. *$P<0.01$ compared with value of control (no addition) at each period.

not shown). However, the motility of spermatozoa inhibited by the addition of PP-1 and Mn^{2+} was instantly restored by the subsequent addition of 50 ng/ml trypsin at 30°C (Fig. 4). Similar results were obtained at 40°C. That is, the motility of demembranated spermatozoa was negligible before the addition of PP-1 and Mn^{2+}, because of the temperature-dependent immobilization. Even after the addition of PP-1 and Mn^{2+}, they still remained immotile. However, the subsequent addition of 50 ng/ml trypsin released the inhibitory effect within 30 sec.

No marked difference in the phosphorylation status of demembranated sperm proteins was observed between the control samples and samples treated with PP-1 alone: seven major phosphorylated protein bands of molecular weights approximately 116, 86, 79, 50, 43, 33 and 29-kDa were identified, together with several minor phosphorylated proteins. In contrast, dephosphorylation of 116, 86, 79, 50 and 29-kDa proteins was observed after the addition of PP-1 and Mn^{2+}. These results were the same as those obtained even in the presence of cAMP or trypsin (Fig. 5, denoted by arrows). In addition, when cAMP was incorporated, a 97-kDa protein was strongly phosphorylated.

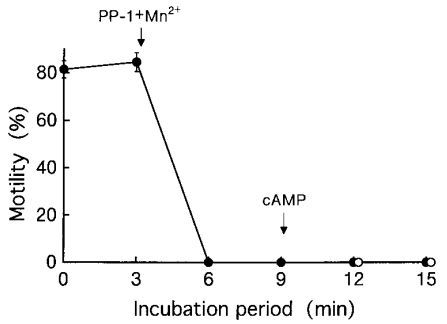

FIG. 3. The time course of motility of demembranated fowl spermatozoa in the reactivation medium at 30°C following addition of 10 units/ml PP-1 + 0.2 mM $MnCl_2$ and cAMP (●; no addition, ○; 10 μM). Each point represents the mean (±S.E.M.) of five samples of spermatozoa. There was no significant difference between 2 treatments at each period.

DISCUSSION

The activation of PP-1, present in the axoneme and/or accessory cytoskeletal components of fowl spermato-

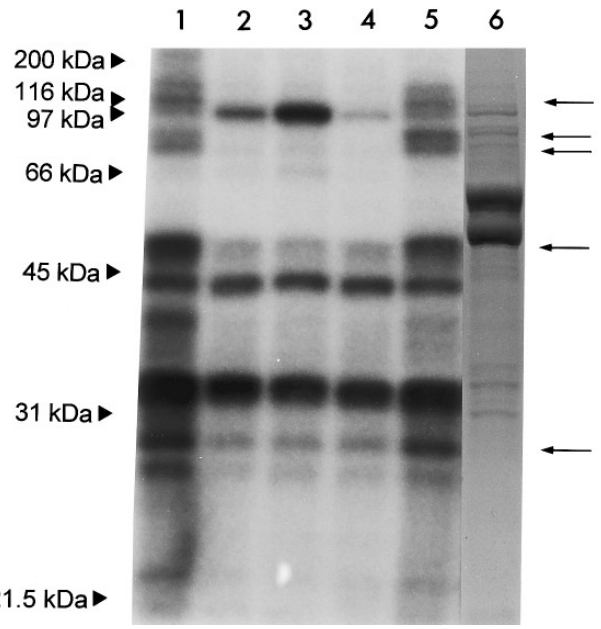

FIG. 5. 10% SDS-PAGE profile of demembranated fowl sperm proteins stained with the Coomassie blue (lane 6) and corresponding autoradiography of phosphorylated proteins (lanes 1-5). Triton X-100-extracted and reactivated samples were incubated for 2.5 min at 30°C. Approximately 7000 cpm/pmol [γ-^{32}P]ATP and 0.1 mM ATP were added in the medium. Samples containing protein from approximately 2.2×10^6 spermatozoa were loaded. Lane 1, presence of 10 units/ml PP-1 alone; lane 2, presence of 10 units/ml PP-1 + 0.2 mM $MnCl_2$; lane 3, presence of 10 units/ml PP-1 + 0.2 mM $MnCl_2$ + 10 μM cAMP; lane 4, presence of 10 units/ml PP-1 + 0.2 mM $MnCl_2$ + 50 ng/ml trypsin; lane 5, no treatment (control).

zoa, appears to be involved in the temperature-dependent inhibition of motility, since the immobilization of demembranated spermatozoa at 40°C can be reversed by the addition of inhibitors of PP-1, such as okadaic acid and calyculin A [23]. The present study supports this hypothesis, since the addition of recombinant PP-1 inhibited the motility of demembranated spermatozoa at 30°C. However, the presence of more than 0.1 mM Mn^{2+} was necessary for the complete inhibition. This result was consistent with the report that the recombinant PP-1 is identical to the native catalytic subunit of PP-1 from rabbit skeletal muscle, but requires the presence of Mn^{2+} for its full activity [30].

Most recently, the involvement of PP-1 in the regulation of sperm motility has also been proposed in mammals [24,25]. To date, however, no identification of the substrate protein(s) has been reported. In the study reported here, autoradiography of demembranated sperm proteins revealed that the inhibition of fowl sperm motility was associated with dephosphorylation of 5 proteins of Mr 116, 86, 79, 50 and 29-kDa proteins. The results suggest that dephosphorylation of some of these proteins may be involved in the inhibition of motility. Furthermore, it appears that these proteins are present in the axoneme and/or accessory cytoskeletal components, but not retained in the plasma membrane and/or cytoplasm.

There is substantial evidence that cAMP and cAMP-dependent protein kinase (PKA) is involved in the activation of sperm motility (reviewed in [9-15]). The inhibition of motility of demembranated sea urchin spermatozoa by the addition of protein phosphatase prepared from bovine cardiac muscle was reversed by the addition of cAMP or PKA [17,18]. In contrast, the present results showed that the motility of spermatozoa inhibited by the addition of PP-1 and Mn^{2+} could not be restored by the subsequent addition of various concentrations of cAMP, although the intense phosphorylation of a 97-kDa protein was observed. Therefore, it seems unlikely that cAMP and the phosphorylation of a 97-kDa protein is important for the maintenance of fowl sperm motility. This assumption can be supported by the previous results: the motility of demembranated fowl spermatozoa was not restored by the addition of cAMP at 40°C, and was maintained even in the presence of phosphodiesterase at 30°C [8]. Furthermore, PKA substrate peptide was also ineffective as a inhibitor of the motility of demembranated fowl spermatozoa at 30°C [31,32].

In contrast to cAMP, the addition of trypsin restored sperm motility which had been inhibited by the addition of PP-1 and Mn^{2+}. If the restoration of motility was due to the inactivation of exogenous PP-1 by trypsin, then spermatozoa would remain immotile at 40°C, because of the temperature-dependent immobilization. However, the motility of demembranated spermatozoa incubated with PP-1 and Mn^{2+} was instantly restored by the subsequent addition of trypsin even at 40°C. Thus, it is not possible that the restoration of motility at 30°C by trypsin results from inactivation of exogenous PP-1. Under trypsin-treated conditions, the phosphorylation state was similar to that of PP-1 and Mn^{2+} treated-sample. Therefore, there appear to be several regulatory systems that individually control the fowl sperm motility, and the motility may be modulated by a balance of these systems. In fact, protease activities were mainly thought to be involved in the catabolism of proteins, but recent evidence suggests that a protease activity with a Lys- and Arg-ester bound specificity is required for sperm motility [33]. Furthermore, Inaba and Morisawa [34] suggested that proteasomes (multicatalytic proteinase) associated with the axoneme may regulate sperm motility in chum salmon. We have also demonstrated that the motility of demembranated fowl spermatozoa was inhibited by the addition of protease inhibitors, such as aprotinin and PMSF, but the subsequent addition of trypsin released the inhibitory effect of inhibitors at 30°C [35]. These results suggest that at least, endogenous PP-1 and protease activities present in fowl sperm axoneme and/or accessory cytoskeletal components may be instrumental in the regulation of motility. Additional studies are therefore necessary to elucidate the relationship between them.

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