REGULATION OF FLAGELLAR MOTILITY BY TEMPERATURE-DEPENDENT PHOSPHORYLATION OF A 43 kDa AXONEMAL PROTEIN IN FOWL SPERMATOZOA

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Summary. Phosphorylation of fowl sperm proteins was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) after incubating the demembranated spermatozoa with [γ-^{32}P]ATP at 30°C or 40°C. A marked difference of phosphorylation between 30°C and 40°C was observed in a 43 kDa protein. This protein was slightly phosphorylated at 40°C, but strongly phosphorylated at 30°C in a cAMP-independent manner. The motility of demembranated spermatozoa was negligible at 40°C, but vigorous movement was observed at 30°C. These results showed that phosphorylation of a 43 kDa protein is likely to be a regulatory step in the maintenance of fowl sperm motility.

Unlike mammalian spermatozoa, fowl spermatozoa display a unique phenomenon: in most synthetic diluents, they become immotile at the avian body temperature of 40-41°C, but regain motility when the temperature is lowered below 35°C (1-9). It has been suggested that this phenomenon involves a loss of intracellular Ca^{2+} to the suspending medium at 40°C, with a subsequent resequestration of Ca^{2+} at lower temperatures (9,10). On the other hand, the axoneme itself appears to be directly involved in this immobilization, since the motility of demembranated fowl spermatozoa is, as with intact spermatozoa, negligible at 40°C and restored at 30°C (11,12). This phenomenon seems to be a superior model for the study of regulation of sperm motility, but detailed mechanisms on molecular levels have remained unsolved.

In othetr species, flagellar motility of spermatozoa may be controlled by a protein phosphorylation-dephosphorylation system (13-17). The phosphorylation of several integral axonemal proteins has been reported to be cAMP dependent. For example, phosphorylation of a 15 kDa protein is

indispensable for the initiation of trout sperm motility (18); the phosphorylation of a 55 kDa protein is apparently related to the motility state of bovine spermatozoa (19); and axokinin, a soluble 56 kDa phosphoprotein, seems to play a key role in mediating the cAMP response in dog spermatozoa (20,21). On the other hand, cAMP-independent phosphoproteins have also been identified in human (22), rat (23) and dog (24) spermatozoa.

It is possible that phosphorylation of axonemal proteins is also important for the regulation of motility in fowl spermatozoa, but little information is available. The present study was therefore undertaken to investigate the relationship between axonemal motility and protein phosphorylation of fowl spermatozoa at different temperatures.

Material and Methods

Chemicals. [γ-^{32}P]ATP was purchased from Du Pont-New England Nuclear. ATP, cAMP, dithiothreitol, potassium glutamate, TES and Triton X-100 were purchased from Sigma Chemical Co., St Louis, MO, USA. SDS-PAGE molecular weight standards were obtained from Bio-Rad Laboratories, Richmond, CA, USA. 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 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 cell/ml.

Phosphorylation of endogenous proteins. Demembranation of spermatozoa for phosphorylation was carried out essentially according to the same methods described previously (11), but with some modifications. Intact sperm suspensions were pre-incubated for 10 min at 30°C or 40°C. Then one volume of sperm suspension was mixed and stirred gently for 30 sec with 8.5 volumes of extraction-reactivation medium containing 0.1% Triton X-100, 200 mM sucrose, 25 mM potassium glutamate, 1 mM $MgSO_4$, 1 mM dithiothreitol, 20 mM Tris-HCl buffer (pH 7.9), 0.1 mM ATP and approximately 7000 cpm/pmol [γ-^{32}P]ATP. Cyclic AMP, when incorporated, was present at final concentration of 10 μM. Mixed solutions (demembranated spermatozoa and solubilized materials) were incubated for 2 min at 30°C or 40°C and the phosphorylation reaction was terminated by the addition of equal volumes of concentrated (2-fold) Laemmli (25) sample buffer and boiling for 5 min. To investigate the phosphorylation reaction of solubilized materials as a control, one volume of sperm suspension was mixed and stirred gently for 30 sec with 8.5 volumes of extraction-reactivation medium, excluding [γ-^{32}P]ATP, and then centrifuged at 15,000 x g for 1 min. [γ-^{32}P]ATP was added to the supernatant at the same concentration as that described above, and samples were subsequently treated in the same way as demembranated sperm preparations. All solutions, including extraction-reactivation medium and sample buffer, were pre-warmed at 30°C or 40°C.

Polyacrylamide gel electrophoresis and autoradiography. Before loading, all samples were centrifuged at 15,000 x g for 20 min to remove insoluble materials. The supernatant containing protein from approximately 1.3 x 10^6 spermatozoa was loaded on 8.5, 10 or 15% SDS-polyacrylamide slab gels and subjected to electrophoresis by the method of Laemmli (25). Gels were stained with Coomassie brilliant blue, and after destaining and drying, they were exposed to x-ray film (Kodak X-OMAT-AR, Rochester, NY, USA) with an intensifying screen (Lightning plus, Du Pont, Wilmington, DE, USA) at -80°C for 2-4 days.

Measurement of motility of demembranated spermatozoa. Intact spermatozoa were pre-incubated at 30°C or 40°C for 10 min. After the pre-incubation, demembranation and reactivation were performed under conditions identical with those for analysis of phosphorylation, but excluding [γ-^{32}P]ATP. The suspension of demembranated spermatozoa was viewed in a microscope slide chamber on a thermostatically-controlled warm plate, and the motility of spermatozoa was recorded by videomicroscopy at 30°C or 40°C (26). All data were subjected to statistical analysis using Student's *t*-test.

Results and Discussion

In the mixed fraction, in the absence of cAMP, five major phosphorylated protein bands of molecular weights of 62 kDa, 43 kDa, 35 kDa, 27 kDa and 18 kDa were identified together with several minor phosphorylated proteins (Figs. 1a,b). A marked difference of phosphorylation between 30°C and 40°C was obtained in a 43 kDa protein. This protein was slightly phosphorylated at 40°C, but strongly phosphorylated at 30°C (Fig. 1b). These results were the same as those obtained even in the presence of cAMP (Fig. 1c). In spite of the intense phosphorylation of the 43 kDa protein at 30°C, this protein was slightly stained with Coomassie brilliant blue (Fig. 1b), suggesting it to be a minor component. Since the motility of demembranated spermatozoa, analyzed under similar conditions to protein phosphorylation experiments, was negligible at 40°C with or without cAMP and vigorous at 30°C (Table 1), phosphorylation of a 43 kDa protein is likely to be a regulatory step in the maintenance of fowl sperm motility.

In the solubilized materials fraction, Pi was faintly incorporated into several proteins including 38 kDa and 29 kDa proteins in a temperature-independent manner. However, no phosphorylated 43 kDa proteins could be detected at either 30°C and 40°C.

Spermatozoa contain the major components known to be involved in the action of cAMP and Ca^{2+} in the control of both muscle and nonmuscle cell motility, namely cAMP-dependent protein kinase, calmodulin and myosin light chain kinase (13). Since these components regulate motility by producing changes in protein phosphorylation (27), it is logical to presume that the

effects of cAMP and Ca^{2+} on sperm motility may be in some part regulated by protein phosphorylation (13). In addition to cAMP-dependent phosphorylation, cAMP-independent phosphorylation of spermatozoa has been reviewed (13,16). The 42.7 kDa phosphoprotein of rat spermatozoa undergoes phosphorylation in the absence of cAMP (23). Three major cAMP-independent phosphoproteins of 98 kDa, 43 kDa and 26 kDa have been identified in dog spermatozoa (24). Thus, it has been assumed that the 42.7 kDa phosphoprotein in rat spermatozoa may be the same or similar to the 43 kDa protein obtained in dog spermatozoa (13). In the present study, phosphorylation of the 43 kDa protein in fowl spermatozoa was not also effected by the addition of cAMP. Based on molecular weights and the nature, the 43 kDa protein identified in this study is likely to be the same or similar to those obtained in rat (23) and dog (24) spermatozoa. However, in these species the relationship between the phosphorylation of these proteins and the motility of spermatozoa was not investigated. From the present study, phosphorylation of the 43 kDa protein appears to be associated with the maintenance of motility. The finding that phosphorylation of this protein is cAMP-independent is consistent with the lack of effect of phosphodiesterase (11) or cAMP (Table 1) on demembranated fowl sperm motility.

To clarify the localization of this 43 kDa protein in spermatozoa is important for the involvement of sperm motility. Since the demembranated spermatozoa washed free from the solubilized supernatant materials broke up on centrifugation and could not restore the motility, these could not be used to determine the source of the 43 kDa protein as axonemal. However, the 43 kDa protein was only found in detergent extracts of whole spermatozoa and not in the 15,000 x g supernatants, suggesting that the 43 kDa protein is in fact associated with the axoneme. Furthermore, it has been shown that the temperature-dependent reversible inactivation of motility is an axonemal phenomenon (11). It is thus interesting that the 42.7 kDa phosphoprotein of rat spermatozoa also appears to be axonemal in origin (23) and may well fulfill the same function.

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Footnot:

Abbreviations: ATP, adenosine 5'-triphosphate; cAMP, adenosine 3':5'-cyclic monophosphate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TES, *N*-Tris-[hydroxymethyl]-methyl-2-aminoethanesulphonic acid; Tris, Tris-[hydroxymethyl]-aminomethane

Legends for Figure:

Fig. 1. 15% (a) and 8.5% (b) SDS-PAGE of demembranated fowl sperm proteins phosphorylated at 30°C or 40°C. Triton X-100 extracted and reactivated samples (mixed fraction: demembranated spermatozoa and solubilized materials) were incubated at 30°C (lane 1) or 40°C (lane 2) for 2 min. Solubilized materials fraction was also incubated at 30°C (lane 3) or 40°C (lane 4). Lanes 5 and 6 depict the Coomassie blue-stained sperm protein pattern for mixed fraction and solubilized materials fraction, respectively. (c) 10% SDS-PAGE of mixed fraction (demembranated spermatozoa and solubilized materials) of fowl sperm proteins phosphorylated at 30°C or 40°C in the presence or absence of 10 μM cAMP. Lane 1, 30°C in the absence of cAMP; lane 2, 30°C in the presence of cAMP; lane 3, 40°C in the absence of cAMP; lane 4, 40°C in the presence of cAMP; lane 5, the Coomassie blue-stained sperm protein pattern.

Table 1. Motility of demembranated fowl spermatozoa in the presence or absence of 10μM cAMP at 40°C and 30°C

Treatment of spermatozoa	Motility (%)	
	40°C	30°C
−cAMP	0	63.2 ± 0.9[a]
+cAMP	0	64.5 ± 1.7[a]

Each value represents the mean (±s.e.m.) motility of 5 samples of spermatozoa.
Within columns, values with different superscripts differ significantly ($P<0.01$) from each other.