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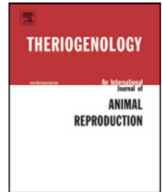

Regulation of fowl sperm motility: Evidence for the indirect, but not direct, involvement of dynein-ATPase activity on the reversible temperature-dependent immobilization

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Potential mechanisms of the reversible temperature-dependent immobilization of fowl sperm were investigated. At 30 °C, motility of demembranated fowl sperm was inhibited by adding 2 mM ethylene glycol-bis (2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), but restored immediately after the subsequent addition of 2 mM $CaCl_2$, whereas at 40 °C, such additions did not appreciably affect motility (which remained almost negligible). With intact sperm, 10^{-9} to 10^{-3} M Ca^{2+} had no effect on motility at 30 °C, which remained high. In contrast, intact sperm at 40 °C were almost immotile below 10^{-5} M Ca^{2+}, and then gradually recovered motility at higher Ca^{2+} concentrations. The negligible motility of demembranated sperm at 40 °C, and at 30 °C in the presence of EGTA, was stimulated by addition of 100 nM of the protein phosphatase inhibitor calyculin A. Dynein-ATPase activities of sperm at 40 °C in the presence of 2 mM EGTA, 2 μM $CaCl_2$, 2 mM $CaCl_2$, or 100 nM calyculin A were higher than those at 30 °C. Therefore, stimulation of fowl sperm motility by temperature, Ca^{2+}, and phosphatase inhibition was not simply associated with an increase of flagellar dynein-ATPase activity. Furthermore, Ca^{2+} was essential, at the axonemal level, for initiation of the 'intrinsic' motility of fowl sperm at 30 °C, but this Ca^{2+}-dependent mechanism might be different from that involved in restoration of motility of intact sperm at 40 °C. In addition, perhaps inhibition of protein phosphatase activity was involved in initiation of sperm motility, but acting at a location different from Ca^{2+} on the axoneme.
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1. Introduction

Although there is abundant evidence that flagellar movement of sperm is based on the active sliding of microtubules as a result of ATPase activity on the dynein arms of the outer doublet microtubules [1–4], much remains to be learned about how the sliding mechanism is controlled. Sperm are terminal products of one of the most spectacular differentiation processes, which are still able to undergo a series of dynamic changes before fertilization. It is thus plausible that protein phosphorylation, a major mechanism for regulation of many cellular processes in eukaryotic systems, has a pivotal role in the sequence of integrated events in which sperm are involved [5].

Intracellular cascade systems for regulation of sperm motility are triggered by several factors, mainly second messengers such as Ca^{2+} and cyclic nucleotides, which seem to converge on phosphorylation and dephosphorylation of proteins via several kinds of protein kinases and phosphatases which regulate axonemal movement [6,7]. Substantial evidence implicates a major role for protein phosphorylation and dephosphorylation systems in regulation of mammalian sperm movement; the predominant

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system involves protein kinase A (PKA), which is dependent on the second messenger, cyclic AMP (cAMP), and has been identified in several species [8–13].

Unlike that of mammalian sperm, fowl sperm motility is reversibly inhibited as the temperature is raised from 30 °C to 40 °C (avian body temperature). Motility was restored by decreasing temperature or by the addition of 2 to 6 mM Ca^{2+} at 40 °C [14–20]. Therefore, Ca^{2+} seems to have a key role in stimulation of motility of fowl sperm at avian body temperature. Indeed, Ca^{2+} was also shown to be the main stimulatory factor in seminal plasma [17] and in ovarian pocket fluid at ovulation [18]. In contrast, the motility of demembranated fowl sperm at 40 °C was not restored by the addition of Ca^{2+} or cAMP [21,22]. The PKA substrate peptide was also ineffective as an inhibitor of the motility of demembranated sperm at 30 °C [23,24]. Thus, it seems unlikely that phosphorylation of protein(s) by PKA is involved in maintenance of fowl sperm motility at avian body temperature.

However, it appears that dephosphorylation of regulatory axonemal proteins by protein phosphatase-type 1 (PP1) is involved in inhibition of fowl sperm motility at 40 °C, because addition of calyculin A or okadaic acid, specific inhibitors of PP1 and protein phosphatase-type 2A (PP2A), and small heat-stable proteins inhibitors 1 and 2, which inhibit PP1 activity only, stimulated the motility of demembranated sperm at 40 °C [25]. Furthermore, the motility of demembranated sperm at 30 °C decreased markedly after addition of recombinant PP1 supplemented with Mn^{2+} [26].

Although fowl sperm provided an excellent model for investigating the regulatory mechanisms of sperm motility, no information is available concerning the involvement of dynein-ATPase activity directly on the reversible temperature-dependent immobilization. Additionally, details regarding the potential involvement of Ca^{2+} and PP1 on the regulation of ATPase activity remain to be elucidated. In the following experiment, therefore, attempts were made to investigate the effects of Ca^{2+} and calyculin A on motility, membrane fluidity and dynein ATPase activity of fowl sperm.

2. Materials and methods

2.1. Animals and preparation of sperm

Commercial White Leghorn roosters (Dekalb strain, Amuse Poultry Breeding Farm, Miyazaki, Japan) were used throughout the study. All birds were housed in individual cages and fed a commercial breeder diet *ad libitum*. They were exposed to a photoperiod of 14 hours light:10 hours darkness. The experimental protocols were approved by the Animal Experiment Committee of University of Miyazaki, and this study followed the animal experimentation guidelines of the university (Permission No. 2010-002-2).

Semen was collected as described [27]. Samples of semen pooled from four to six males were diluted approximately 10-fold in 150 mM NaCl with 20 mM *N*-Tris-[hydroxymethyl]-methyl-2-aminoethanesulfonic acid (TES) at pH 7.4 and centrifuged at 700 × *g* for 13 minutes at room temperature (20 °C to 25 °C). Washed sperm were reconstituted in the same buffer to give a final concentration of approximately 3×10^9 sperm per mL.

2.2. Chemicals

Adenosine 5′-triphosphate (ATP), aprotinin, bovine serum albumin, 1,6-diphenyl-1,3,5-hexatriene (DPH), dibucaine hydrochloride (cell-permeable activator of calcium-dependent calpain), dithiothreitol (DTT), ethylene glycol-bis (2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), firefly tails, phenylmethylsulfonyl fluoride (PMSF), potassium glutamate, sodium orthovanadate, and TES were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA), and Val-Ala-Ile-Thr-Val-Leu-Val-Lys (CALP 1) and Val-Lys-Phe-Gly-Val-Gly-Phe-Lys (CALP 3), cell-permeable calmodulin agonists, were purchased from Tocris Bioscience (Bristol, UK). Calyculin A, an inhibitor of PP1 and PP2A, was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and was dissolved in DMSO as a stock solution and stored at −30 °C until used. Bicinchoninic acid protein assay regent was from Pierce Chemical Co. (Rockford, IL, USA). Other chemicals were of reagent grade from Nacalai Tesque, Inc. (Kyoto, Japan).

2.3. Analysis of motility of sperm

Approximately 5×10^6 per mL sperm samples in TES/NaCl buffer (pH 7.4) at various concentrations of free Ca^{2+} were preincubated aerobically in a water bath at 30 °C or 40 °C for 10 minutes. Numbers of sperm were estimated as described [28], using a double-beam spectrophotometer (Shimadzu, Model UV-150-02, Kyoto, Japan). Free Ca^{2+} concentrations in diluents were adjusted using EGTA and $CaCl_2$ [29], and after preincubation, numbers of motile sperm were measured at 30 °C or 40 °C, and also after the addition of CALP 1, CALP 3, dibucaine, and calyculin A.

Demembranation and reactivation of sperm were performed as described [21]. The extraction medium consisted of 0.1% (vol/vol) Triton X-100, 200 mM sucrose, 25 mM potassium glutamate, 1 mM $MgSO_4$, 1 mM DTT, 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 DTT, and 20 mM TRIS-HCl buffer (pH 7.9). Even though double-distilled water was used to make extraction or reactivation medium, a ‘trace’ of calcium ion was present in these media (perhaps from glassware) before the addition of EGTA. Free Ca^{2+} concentrations in the reactivation medium were adjusted using EGTA and $CaCl_2$ as well as those mentioned above. The effects of the presence of calyculin A were also examined.

The sperm suspension was placed into a microscope slide chamber (Sekisui Chemical Co., Ltd., UR-157 type, Tokyo, Japan) on a thermostatically-controlled warm plate, and sperm motility was recorded by videomicroscopy (magnification on the 12-inch black and white monitor was approximately × 600) at 30 °C or 40 °C [30]. Motility measurements were done on a total of 200 to 300 sperm, distributed uniformly among three or more fields.

2.4. Isolation of crude dynein extract and measurement of ATPase activity of sperm

The method of McConnell et al. [31] for isolation of crude dynein from bovine sperm was modified for use in

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