REGULATORY SYSTEM OF TEMPERATURE-DEPENDENT FLAGELLAR MOTILITY IS RETAINED AFTER FREEZING AND THAWING OF DEMEMBRANATED FOWL SPERMATOZOA

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ABSTRACT

In the presence of adenosine 5'-triphosphate (ATP), the motility of demembranated fowl spermatozoa was vigorous at 30°C, both before and after rapid freezing at -70°C or -196°C without cryoprotectants. The rate of ATP consumption of the demembranated spermatozoa treated by freezing and thawing was, at approximately 200 nmol ATP hydrolysis/10^9 spermatozoa/min, also similar to that of the untreated spermatozoa. At 40°C, neither frozen-thawed spermatozoa nor control spermatozoa were motile, but motility could be restored by decreasing the temperature to 30°C or by the addition of inhibitors of protein phosphatases. These observations suggest that the substance(s) involved in the temperature-dependent immobilization of fowl spermatozoa are closely associated with the axoneme, and that physical damage to the demembranated spermatozoa, such as freezing and thawing, is not enough to remove the substance(s).

Key words: spermatozoa, motility, freezing, temperature, fowl

INTRODUCTION

While abundant evidence has shown that flagellar movement of spermatozoa is based on the active sliding of microtubules as a result of ATPase activity on the dynein arms of the outer doublet microtubules (13,16,18,21), much remains to be learned about how the sliding mechanism is controlled. For example, fowl spermatozoa display the unique phenomenon of reversible temperature-dependent immobilization: in simple salt solutions they become immotile at the avian body

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temperature of 40 to 41°C, but motility is restored by decreasing the temperature (1,2,17,19,20,23). 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 (3,4).

It has been proposed that the activation of protein phosphatase type 1 (PP1), one of the serine/threonine phosphatases, 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, specific inhibitors of PP1, inhibitors 1 and 2 (11), also stimulated the motility of demembranated spermatozoa at 40°C (7). Furthermore, the presence of not only 1-(5-chloronaphthalene-1-sulfonyl)-1H-hexahydro-1, 4-diazepine hydrochloride (ML-9), a specific inhibitor of myosin light chain kinase (MLCK) but also an MLCK substrate peptide, inhibited the motility of demembranated spermatozoa at 30°C (8). In addition, the presence of an MLCK substrate peptide decreased the phosphorylation levels of 30 kDa axonemal protein (9). In contrast, the motility of demembranated spermatozoa was not restored by the addition of cAMP at 40°C (4,5), and the presence of cAMP-dependent protein kinase substrate peptides did not appreciably affect motility at 30°C (8,9). From these results, it has been suggested that the phosphorylation-dephosphorylation of axonemal protein(s), at least by MLCK or an MLCK-like protein and PP1 rather than a cAMP-dependent protein kinase, may be involved in the regulation of fowl sperm motility (8). However, details of the mechanisms involved in the immobilization and restoration of motility remain unsolved.

If the regulatory system could be removed from the axoneme, then fowl spermatozoa might be expected to be motile even at 40°C. In other words, if the substance(s) involved in the temperature-dependent immobilization of fowl spermatozoa are closely associated with the axoneme, demembranated spermatozoa may be immotile at 40°C, even after the treatments which physically damage them such as rapid freezing and thawing without cryoprotectants. In this study, we attempted to ascertain whether or not the regulatory system of temperature-dependent immobilization survives rapid freezing and thawing and if this system is closely associated with the axoneme.

MATERIALS AND METHODS

Animals and Preparation of Spermatozoa

Commercial White Leghorn roosters (Babcock strain, Akagi Poultry Breeding Farm, Miyazaki, Japan) were used throughout the study. All birds were housed in individual cages and were fed ad libitum on a commercial breeder diet. They were exposed to a 14 h light : 10 h dark cycle.

Semen was collected by the method of Bogdonoff and Shaffner (10). Samples of semen pooled from 4 to 6 males were diluted approximately tenfold in 150 mM NaCl with 20 mM TES (*N*-Tris[hydroxymethyl]-methyl-2-aminoethanesulphonic acid) at pH 7.4 and centrifuged at 700 x g for 13 min at room temperature (20 to 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 to 4 ml were poured into 30-ml Erlenmeyer flasks with screw caps.

Chemicals

Adenosine 5'-triphosphate (ATP), cAMP, desiccated firefly tails, dithiothreitol, *p*-nitrophenyl phosphate, potassium glutamate, TES and Triton X-100 were purchased from Sigma Chemical (St Louis, MO, USA). 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 also obtained from Sigma, while Lys-Lys-Arg-Pro-Gln-Arg-Ala-Thr-Ser-Asn-Val-Phe-Ser-NH_2 was purchased from Peninsula Laboratories (Belmont, CA, USA). Calyculin A and okadaic acid were obtained from Wako Pure Chemical Industries (Osaka, Japan). Other chemicals were of reagent grade and we purchased from Nacalai Tesque (Kyoto, Japan).

Measurement of Motility of Intact and Demembranated Spermatozoa

Demembranation and reactivation of spermatozoa were performed according to the method described by Ashizawa et al. (4). The extraction medium used consisted of 0.1% (v/v) 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).

A 100-µl portion of the reactivation medium containing demembranated spermatozoa in 1.5-ml microcentrifuge tubes was placed into a deep-freezer (-70°C) or plunged into liquid nitrogen (-196°C) directly, then stored for at least 1 h and thawed at room temperature (20 to 25°C). During freezing, the decreasing rate of temperature was monitored by a thermoelectric thermometer: freezing rates were approximately 20°C/min in the deep-freezer and 540°C/min in liquid nitrogen. Spermatozoa which were not exposed to freezing and thawing were used as the control. After thawing, demembranated spermatozoa were incubated in a water bath 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 (14). Measurements were made on a total of 200 to 300 spermatozoa, distributed uniformly among 3 or more fields, to determine the percentage of motility.

Extraction medium without Triton X-100 and reactivation medium without ATP were used for the measurement of motility for intact spermatozoa. The intact spermatozoa were treated in the same manner as demembranated samples described above.

Measurement of ATP Consumption of Demembranated Spermatozoa

The same reactivation medium was used without ATP. Freezing and thawing of demembranated spermatozoa were performed as described above. After thawing, reaction was initiated by the addition of 0.1 mM ATP into the reactivation medium. After incubation for 10 min at 30°C, the reaction was stopped by boiling the medium. The concentration of the remaining ATP was assayed by firefly bioluminescence (22). The rate of ATP consumption was expressed in terms of nmol ATP hydrolysis/10^9 spermatozoa/min.

Statistical Analysis

The percentage of motility was transformed using arc sine transformation. The results were analyzed by Duncan's multiple-range tests (12).

RESULTS

The percentage of motility of intact spermatozoa which were not exposed to freezing and thawing (control) was high. In contrast, rapid freezing to -196°C without cryoprotectants such as glycerol and dimethyl sulfoxide inhibited the motility of spermatozoa at 30°C completely, and the motility of spermatozoa frozen to -70°C was almost negligible (Fig. 1a). Unlike intact spermatozoa, the motility of demembranated spermatozoa was vigorous at 30°C, even after freezing to -70°C or -196°C and thawing, and there was no significant difference between control and frozen-thawed spermatozoa (Fig. 1b). Furthermore, the rate of ATP consumption of demembranated spermatozoa treated by freezing and thawing was approximately 200 nmol ATP hydrolysis/10^9 sperm/min, similar to that of the untreated spermatozoa (control; Fig. 1c).

Demembranated sperm motility was markedly affected by temperature (Fig. 2). During the first incubation at 30°C, spermatozoa showed approximately 75 to 80% motility before or after freezing and thawing, and even after a second incubation at 30°C, 50 to 60% motility was obtained. In contrast, motility of the same samples was almost negligible at 40°C, regardless of whether or not the samples were

treated by freezing at -70°C or -196°C.

Neither frozen-thawed spermatozoa nor control spermatozoa were motile following the addition of 1 mM EGTA to the reactivation medium at 30°C. However, spermatozoa regained their motility after the subsequent addition of 2 mM $CaCl_2$ (Fig. 3a). On the other hand, even in the presence of 1 mM EGTA and 2 mM $CaCl_2$, motility could not be restored at 40°C (Fig 3b).

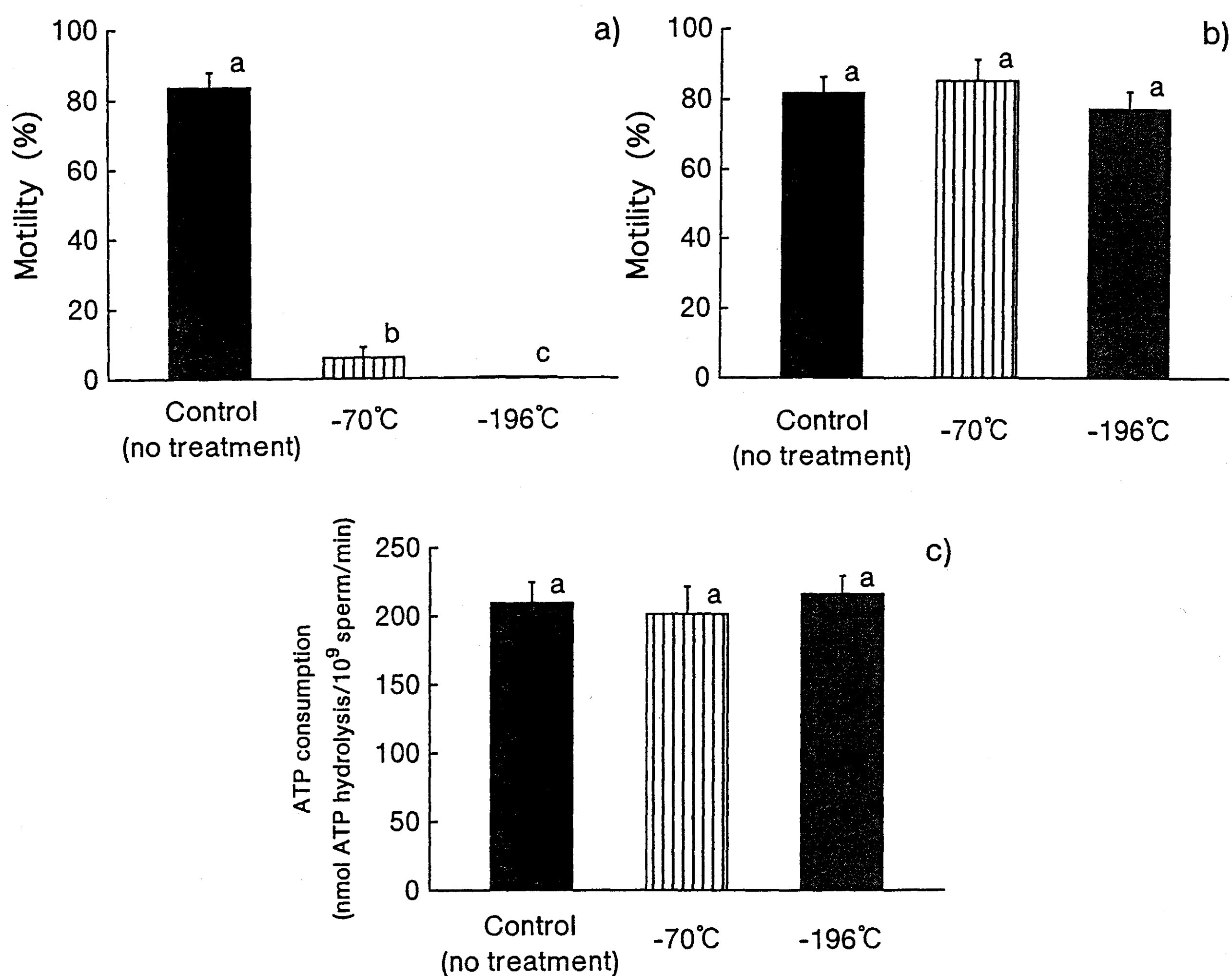

Figure 1. Motility of a) intact and b) demembranated fowl spermatozoa at 30°C and c) ATP consumption of demembranated fowl spermatozoa at 30°C after freezing and thawing. Each value represents the mean (±SEM) of 5 samples of spermatozoa. Values with different superscripts differ significantly ($P<0.01$).

At 40°C, the presence of calyculin A and okadaic acid, specific inhibitors of protein phosphatase type 1 (PP1) and type 2 (PP2A), or *p*-nitrophenyl phosphate, a nonspecific phosphatase substrate, permitted reactivation of control spermatozoa which had not been subjected to freezing and thawing. This stimulation was also observed in frozen-thawed spermatozoa. However, the addition of cAMP did not appreciably affect the motility of control and frozen-thawed spermatozoa. At 30°C, neither stimulation nor inhibition of motility of demembranated spermatozoa was observed following the addition of cAMP, phosphatase inhibitors or substrate (Table 1).

Demembranated spermatozoa, before or after freezing and thawing, maintained their motility at 30°C, even in the presence of Leu-Arg-Arg-Ala-Ser-Leu-Gly (Kemptide), a cAMP-dependent protein kinase substrate peptide; 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 pGlu-Lys-Arg-Pro-Ser-Gln-Arg-Ser-Lys-Tyr-Leu, a protein kinase C substrate peptide. In contrast, inhibition of motility was observed for control and frozen-thawed spermatozoa after the addition of Lys-Lys-Arg-Pro-Gln-Arg-Ala-Thr-Ser-Asn-Val-Phe-Ser-NH_2, an MLCK substrate peptide (Table 2).

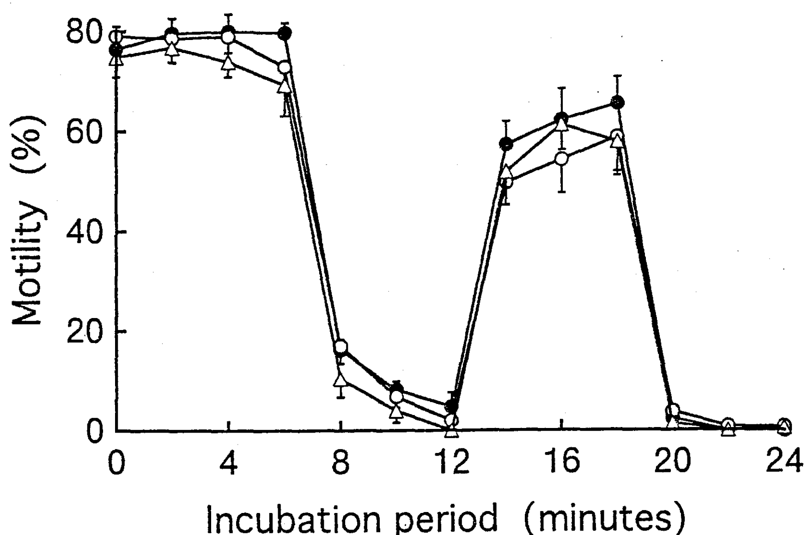

Figure 2. The time course of motility of demembranated fowl spermatozoa incubated at 30°C (0 to 6 and 14 to 18 minutes) and 40°C (8 to 12 and 20 to 24 minutes) after freezing and thawing (●, no treatment (control); ○, -70°C; △, -196°C). Each point represents the mean (±SEM) of 5 samples of spermatozoa. There was no significant difference among the 3 treatments at each period.

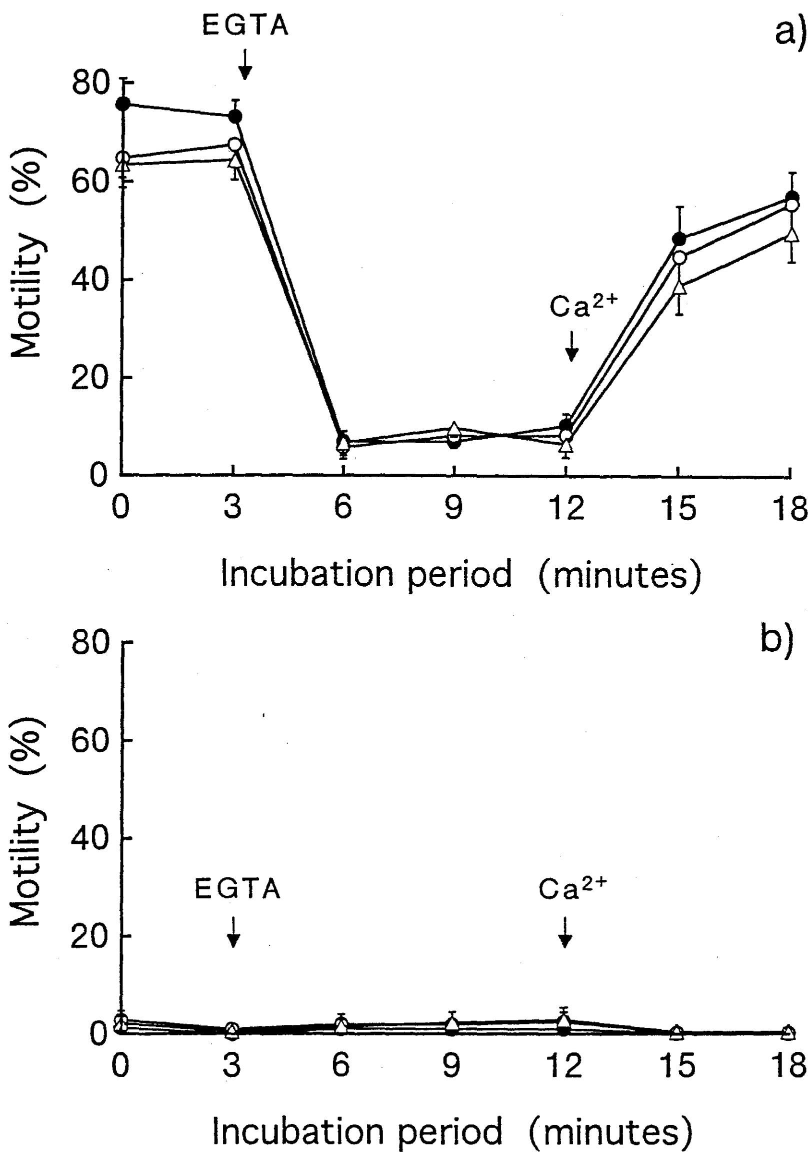

Figure 3. The time course of motility of demembranated fowl spermatozoa incubated a) at 30°C and b) 40°C after freezing and thawing (●, no treatment (control); ○, -70°C; △, -196°C). At arrows, 1 mM EGTA and 2 mM $CaCl_2$ were added. Each point represents the mean (±SEM) of 5 samples of spermatozoa. There was no significant difference among the 3 treatments at each period.

Table 1. Effects of cAMP, protein phosphatase inhibitors and substrate on the motility of frozen-thawed demembranated fowl spermatozoa at 30°C and 40°C.

Temperature	Substance	Motility (%)		
		Control (no treatment)	-70°C	-196°C
30°C	Control (no addition)	83.5 ± 3.0^{a}	81.5 ± 6.1^{a}	71.7 ± 6.4^{a}
	cAMP (10 μM)	82.0 ± 4.1^{a}	82.0 ± 4.3^{a}	72.8 ± 5.4^{a}
	Calyculin A (100 nM)	80.8 ± 2.6^{a}	83.2 ± 4.1^{a}	76.2 ± 2.9^{a}
	Okadaic acid (1000 nM)	82.5 ± 2.8^{a}	81.8 ± 3.5^{a}	78.8 ± 4.1^{a}
	p-Nitrophenyl phosphate (10 mM)	81.3 ± 4.0^{a}	78.0 ± 5.2^{a}	80.5 ± 4.0^{a}
40°C	Control (no addition)	2.3 ± 1.5^{a}	2.3 ± 2.3^{a}	1.7 ± 1.7^{a}
	cAMP (10 μM)	3.9 ± 1.8^{a}	2.8 ± 1.6^{a}	1.8 ± 1.8^{a}
	Calyculin A (100 nM)	59.7 ± 6.4^{b}	51.2 ± 5.8^{b}	50.2 ± 5.9^{b}
	Okadaic acid (1000 nM)	49.0 ± 6.2^{b}	55.4 ± 3.9^{b}	49.8 ± 7.4^{b}
	p-Nitrophenyl phosphate (10 mM)	49.3 ± 5.4^{b}	43.8 ± 5.0^{b}	44.0 ± 5.1^{b}

Each value represents the mean (±SEM) of 5 samples of spermatozoa. ^{a,b} Within columns and each temperature, values with different superscripts differ significantly ($P<0.01$). There was no significant difference among the 3 treatments at each row.

Table 2. Effects of protein kinase substrate peptides on the motility of frozen-thawed demembranated fowl spermatozoa at 30°C.

Peptide	Motility (%)		
	Control (no treatment)	-70 °C	-196 °C
Control (no addition)	78.9±6.2[a]	73.5±6.4[a]	72.0±5.8[a]
Leu-Arg-Arg-Ala-Ser-Leu-Gly (Kemptide)	74.2±6.3[a]	75.3±4.2[a]	65.5±4.9[ab]
Pro-Leu-Ala-Arg-Thr-Leu-Ser-Val-Ala-Gly -Leu-Pro-Gly-Lys-Lys (Syntide 2)	56.4±4.3[ab]	60.3±3.4[ab]	47.1±4.2[b]
Lys-Lys-Arg-Pro-Gln-Arg-Ala-Thr-Ser-Asn -Val-Phe -Ser-NH_2 (MLCK substrate)	0.0±0.0[c]	0.0±0.0[c]	0.0±0.0[c]
pGlu-Lys-Arg-Pro-Ser-Gln-Arg-Ser-Lys-Tyr-Leu (PKC substrate)	46.4±4.5[b]	46.5±5.1[b]	45.2±6.3[b]

Each peptide was added in the amount of 0.5 mM to the reactivation medium. Each value represents the mean (±SEM) of 5 samples of spermatozoa. [a,b,c] Within columns, values with different superscripts differ significantly ($P<0.01$). There was no significant difference among the 3 treatments at each row.

DISCUSSION

Intact fowl spermatozoa lost their motility after freezing and thawing, presumably due to disintegration of the plasma membrane and loss of intracellular ATP, since no cryoprotectants except sucrose were added to the medium. In contrast, the motility of demembranated spermatozoa in the presence of ATP was vigorous at 30°C, even after rapid freezing to -70°C or -196°C and thawing. In these experiments, freezing of spermatozoa was carried out after their extraction and reactivation. However, similar results were obtained with demembranated spermatozoa in which the freezing was carried out before reactivation, i.e., after the extraction (data not shown). In these experiments, the rate of ATP consumption of demembranated spermatozoa treated by freezing and thawing was similar to that of controls. These results indicate that the basic sliding system of microtubules is preserved and that physical damage to the demembranated spermatozoa, such as freezing and thawing, is not enough to break this system. Similar observations were obtained in bull spermatozoa (15).

In demembranated fowl spermatozoa, several motility-regulating mechanisms have been identified involving Ca^{2+} (6); protein kinases (8,9) and protein phosphatases (7). Before we began our experiments, we suspected that effectors or inhibitors of each of these systems exhibited different activity in control and in frozen-thawed demembranated spermatozoa, since the regulatory systems could be removed from the axoneme due to freezing and thawing. Furthermore, it was assumed that demembranated spermatozoa could remain motile even at 40°C. In the present work, however, similar results were obtained in both control and frozen-thawed spermatozoa after the addition of various compounds involved in the regulation of flagellar movement. Moreover, the motility of demembranated spermatozoa, even after freezing and thawing, was markedly affected by temperature: spermatozoa which showed vigorous movement at 30°C became reversibly immotile at 40°C. It therefore seems that all the components which regulate flagellar movement of fowl spermatozoa are preserved even after freezing and thawing of demembranated spermatozoa. Regulatory components of flagellar movement appear to be closely bound to the axoneme.

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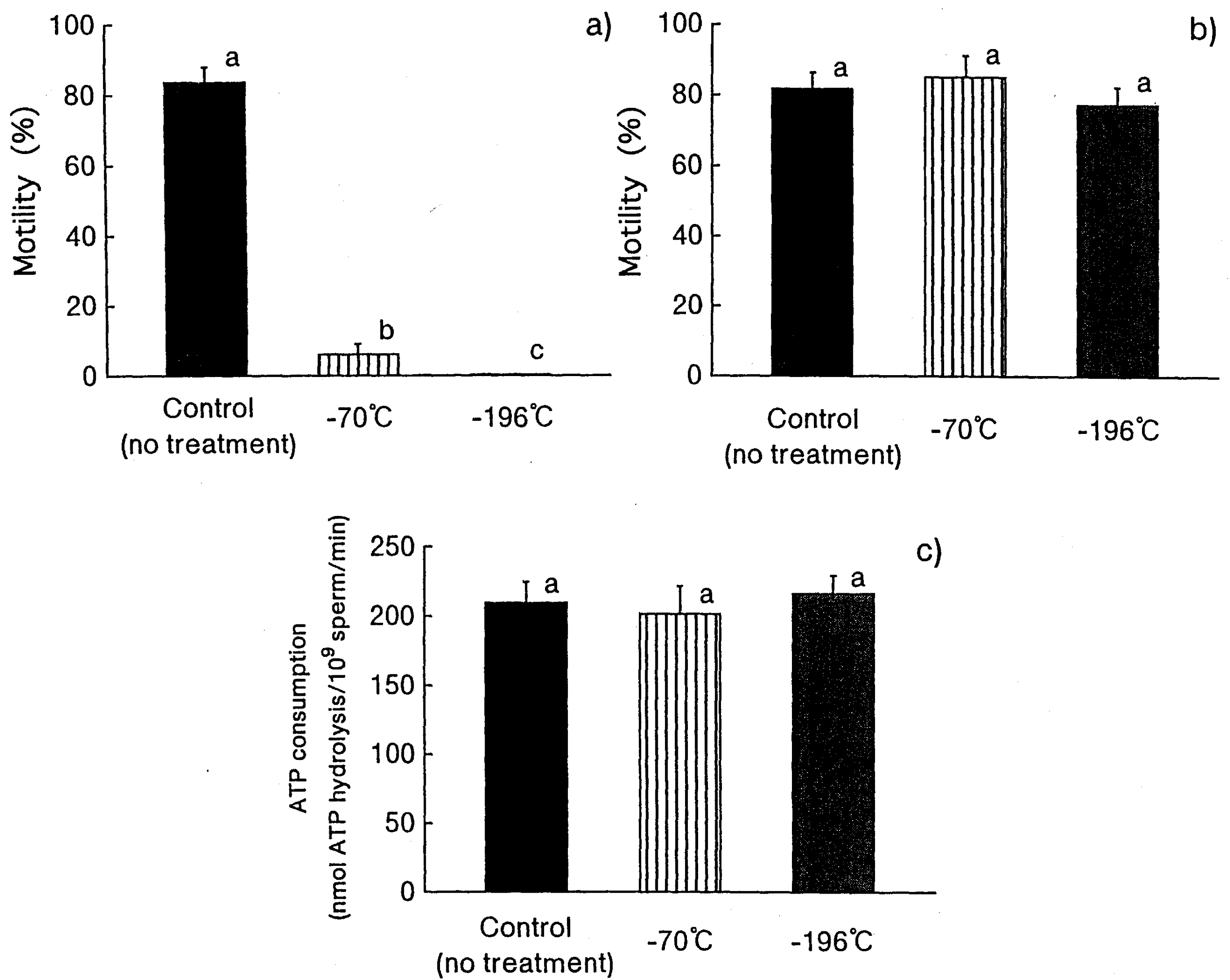

Figure 1. Motility of a) intact and b) demembranated fowl spermatozoa at 30°C and c) ATP consumption of demembranated fowl spermatozoa at 30°C after freezing and thawing. Each value represents the mean (±SEM) of 5 samples of spermatozoa. Values with different superscripts differ significantly ($P<0.01$).

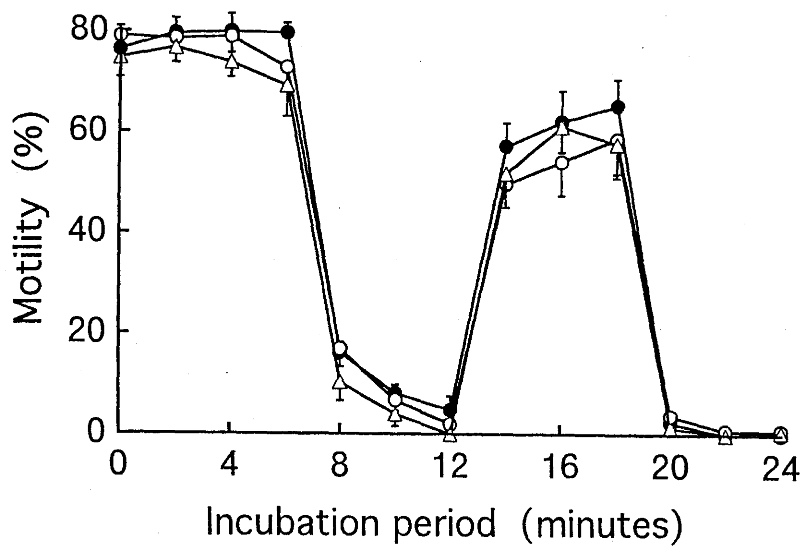

Figure 2. The time course of motility of demembranated fowl spermatozoa incubated at 30°C (0 to 6 and 14 to 18 minutes) and 40°C (8 to 12 and 20 to 24 minutes) after freezing and thawing (●, no treatment (control); ○, -70°C; △, -196°C). Each point represents the mean (±SEM) of 5 samples of spermatozoa. There was no significant difference among the 3 treatments at each period.

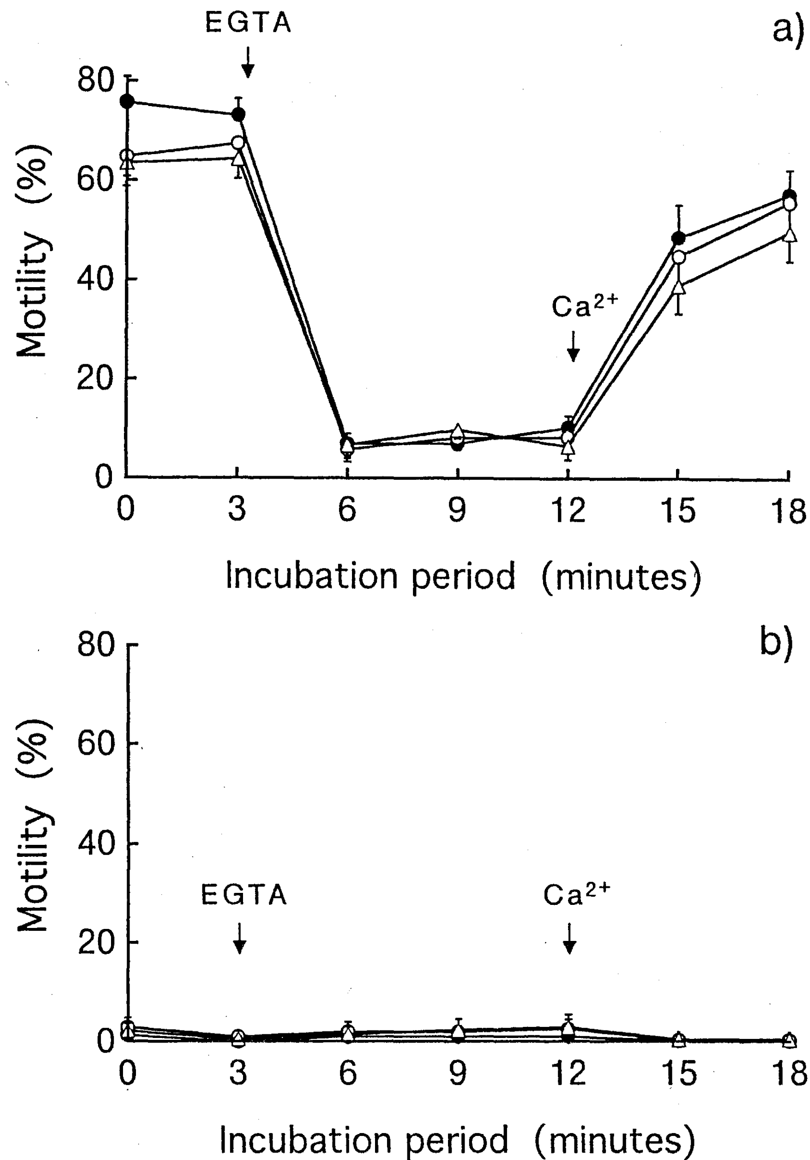

Figure 3. The time course of motility of demembranated fowl spermatozoa incubated a) at 30°C and b) 40°C after freezing and thawing (●, no treatment (control); ○, -70°C; △, -196°C). At arrows, 1 mM EGTA and 2 mM $CaCl_2$ were added. Each point represents the mean (±SEM) of 5 samples of spermatozoa. There was no significant difference among the 3 treatments at each period.