Regulation of flagellar motility of fowl spermatozoa: evidence for the involvement of intracellular free Ca^{2+} and calmodulin

K. Ashizawa, H. Tomonaga and Y. Tsuzuki

Laboratory of Animal Reproduction, Faculty of Agriculture, Miyazaki University,
Miyazaki 889-21, Japan

The possible role of intracellular free Ca^{2+} and calmodulin in the regulation of fowl sperm motility was investigated using an intracellular Ca^{2+} chelator, 1,2-bis (2-aminophenoxy) ethane-*N,N,N',N'*-tetraacetic acid, tetraacetoxymethyl ester (BAPTA/AM) and calmodulin antagonists such as *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7), *N*-(6-aminohexyl)-1-naphthalenesulfonamide hydrochloride (W-5) and trifluoperazine. Intact fowl spermatozoa maintained vigorous movement in a Ca^{2+} -free medium at 30°C. In contrast, the motility of spermatozoa loaded with BAPTA/AM was negligible at 30°C, but could be instantly restored by the addition of 2 mmol $\text{CaCl}_2 \text{ l}^{-1}$. At this time, the intracellular free Ca^{2+} concentrations increased from 0 to about 100 nmol l^{-1} , as measured by the fluorescent Ca^{2+} indicator fura-2. At 40°C, neither control nor BAPTA/AM-treated spermatozoa were motile, but the motility of both kinds of spermatozoa was restored by the subsequent addition of 2 mmol $\text{CaCl}_2 \text{ l}^{-1}$. Even in the presence of 2 mmol $\text{CaCl}_2 \text{ l}^{-1}$, the addition of W-7 and trifluoperazine inhibited the motility of intact spermatozoa at 30°C and 40°C, and induced a concomitant decrease in the rate of oxygen consumption and ATP concentration, suggesting that energy depletion might be involved in the inhibition of motility. In contrast, the motility of demembranated spermatozoa was not inhibited by the addition of W-7 and trifluoperazine at 30°C. The addition of W-5, a weaker antagonist, did not appreciably affect the motility of either intact or demembranated spermatozoa. These results suggest that intracellular free Ca^{2+} is indispensable for the maintenance of fowl sperm motility. Calmodulin, which occurs in the cytoplasm or mitochondria but not in the axoneme, is a prominent candidate for the signal transducer in Ca^{2+} -stimulated motility.

Introduction

It is well known that Ca^{2+} is a signal transducer in the regulation of sperm motility (for review, see Tash and Means, 1983; Lindemann and Kanous, 1989; Majumder *et al.*, 1990). The effects of intracellular Ca^{2+} on sperm motility can be divided into two major classes: modification of wave form and oscillation frequency (Gibbons and Gibbons, 1973; Brokaw *et al.*, 1974; Brokaw, 1979; Lindemann *et al.*, 1987; Suarez *et al.*, 1993); and complete inhibition of motility at $> 1 \mu\text{mol Ca}^{2+} \text{ l}^{-1}$ (Gibbons and Gibbons, 1980; Mohri and Yanagimachi, 1980; Tash and Means, 1982).

Since the inhibition of mammalian sperm motility by high concentrations of intracellular Ca^{2+} is paralleled by a decrease in the overall phosphorylation of sperm proteins, some of which may be axonemal components (Tash and Means, 1982), it is logical to presume that the effects of Ca^{2+} on sperm motility may be partly regulated by protein phosphorylation. The inhibitory effect of Ca^{2+} on protein phosphorylation could be explained by a direct effect of Ca^{2+} on protein

kinase activity, Ca^{2+} -stimulated phosphoprotein phosphatase activity, or conformational changes within the axoneme that prevent enzyme–substrate interactions (Tash and Means, 1983).

However, fowl spermatozoa display a unique phenomenon: their motility is reversibly inhibited as the temperature is raised from 30°C to 40°C, the avian body temperature (Munro, 1938; Nevo and Schindler, 1968; Ashizawa and Nishiyama, 1978; Takeda, 1982; Ashizawa and Okauchi, 1984; Ashizawa and Wishart, 1987; Wishart and Ashizawa, 1987; Ashizawa *et al.*, 1989a). One of the possible mechanisms of this phenomenon involves a loss of intracellular Ca^{2+} to the suspending medium at 40°C, with a subsequent resequestration of Ca^{2+} at a lower temperature (Thomson and Wishart, 1989, 1991). Motility is restored by decreasing the temperature or by the addition of Ca^{2+} at 40°C (Wishart and Ashizawa, 1987; Ashizawa *et al.*, 1989a; Thomson and Wishart, 1989, 1991). In addition, the motility of demembranated fowl spermatozoa is also inhibited in Ca^{2+} -free medium (Ashizawa *et al.*, 1992a) and reactivation was observed with the addition of $10^{-3} \text{ mol Ca}^{2+} \text{ l}^{-1}$ (Ashizawa *et al.*, 1989b). In such a high concentration of Ca^{2+} , the motility of demembranated ram and hamster spermatozoa

was inhibited (White and Voglmayr, 1986; Feng *et al.*, 1988). It therefore seems likely that there is a species difference in sensitivity to Ca^{2+} , and Ca^{2+} is indispensable for the movement of fowl spermatozoa.

Calmodulin, the Ca^{2+} -binding protein that modulates the activity of a number of key regulatory enzymes (Means *et al.*, 1982), has been identified in spermatozoa from a diverse range of species, from sea urchins to humans (Brooks and Siegel, 1973; Jones *et al.*, 1978, 1980; Garbers *et al.*, 1980; Burgess, 1983; Moore and Dedman, 1984; Yamamoto, 1985; Camatini *et al.*, 1986, 1991; Weinman *et al.*, 1986; Fouquet *et al.*, 1991; Kann *et al.*, 1991). It has been suggested that calmodulin regulates flagellar motility in dog and sea urchin spermatozoa by inducing wave-form changes (Tash and Means, 1982; Brokaw and Nagayama, 1985) or by inhibition in human spermatozoa (Hong *et al.*, 1985), as shown by anti-calmodulin drug treatment.

It may therefore be assumed that the Ca^{2+} -sensitive motility of fowl spermatozoa described above is mostly regulated by the Ca^{2+} -calmodulin complex rather than by Ca^{2+} directly. However, the presence of calmodulin or its role in the regulation of motility has not been demonstrated in fowl spermatozoa. In this study, attempts have been made to clarify the role of calmodulin in the regulation of flagellar motility and metabolic activity of fowl spermatozoa by using calmodulin antagonists. We have shown that fowl spermatozoa are rendered immotile at 30°C and 40°C by the addition of calmodulin antagonists, even in the presence of Ca^{2+} .

Materials and Methods

Animals and preparation of spermatozoa

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

Semen was collected by the method of Bogdonoff and Shaffner (1954). Samples of semen pooled from four to six males were diluted by a factor of approximately 10 in 150 mmol NaCl l^{-1} with 20 mmol Tes (*N*-Tris-[hydroxymethyl]-methyl-2-aminoethanesulphonic acid) l^{-1} at pH 7.4 and centrifuged at 700 *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^{-1} . Samples of 3–4 ml were poured into 30 ml Erlenmeyer flasks with screw caps.

Chemicals

1-(2-(5''-Carboxyoxazol-2''-yl)-6-aminobenzofuran-5-oxy)-2-(2'-amino-5'-methylphenoxy)-ethane-*N,N,N',N'*-tetraacetic acid pentaacetoxymethyl ester (fura-2/AM) and 1,2-bis (2-aminophenoxy) ethane-*N,N,N',N'*-tetraacetic acid, tetraacetoxymethyl ester (BAPTA/AM) were purchased from Dojindo Laboratories, Inc, Kumamoto. *N*-(6-Aminoethyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7) and *N*-(6-aminoethyl)-1-naphthalenesulfonamide hydrochloride

(W-5) were obtained from Seikagaku Kogyo Co, Ltd, Tokyo. Trifluoperazine, ATP, dithiothreitol, potassium glutamate, Tes, Triton X-100, Cremophor EL and desiccated firefly tails were purchased from Sigma Chemical Co, St Louis, MO. Other chemicals were of reagent grade from Nacalai Tesque, Inc, Kyoto.

Loading of spermatozoa with BAPTA/AM

Stock solutions of BAPTA/AM were made up to 50 mmol l^{-1} in dry dimethylsulfoxide (DMSO) and kept desiccated at 4°C in the dark until use. For the complete emulsification of BAPTA/AM, 1 μl 10% Cremophor EL was added to 2 μl BAPTA/AM, and 1 ml of Tes/NaCl buffer was then added. This solution was sonicated for 2 min on ice using an ultrasonic processor with a microtip (Sonics and Materials, Inc, Danbury, CT) at 50 W. This procedure subsequently gave optimal conditions for BAPTA/AM cellular loading and hydrolysis. Sperm suspension (0.1 ml) was added to 1 ml BAPTA/AM solution and shaken for 90 min at room temperature (20–25°C) in the dark. During this incubation, final concentrations of BAPTA/AM and spermatozoa were about 90 $\mu\text{mol l}^{-1}$ and $0.9 \times 10^8 \text{ ml}^{-1}$, respectively. Sperm preparations diluted with Tes/NaCl buffer to the same concentrations described above and shaken for 90 min at room temperature were used as controls.

Measurement of motility of intact and demembranated spermatozoa

In the first series of experiments, the sperm preparations with or without BAPTA/AM treatment were incubated aerobically in a shaking water bath at 30°C or 40°C. Five minutes after the start of incubation, 2 mmol $\text{CaCl}_2 \text{ l}^{-1}$ was added. During incubation of spermatozoa, the suspension of spermatozoa was placed in a microscope chamber (Sekisui Chemical Co, Ltd, Tokyo; UR-157 type; 0.07 mm in depth) and the motility of spermatozoa was recorded by videomicroscopy (magnification of the black and white monitor was approximately $\times 600$) at 30°C or 40°C on a thermostatically controlled warm plate (Katz and Overstreet, 1981). Measurements were made on 200–300 spermatozoa, distributed uniformly among the three or more fields, to determine percentage motility.

In the second series of experiments, the effects of calmodulin antagonists on the motility of intact and demembranated spermatozoa were examined. The dose-response and time-course of motility were measured at 30°C after addition of various concentrations of W-5, W-7 and trifluoperazine. The effects of the addition of CaCl_2 before the addition of calmodulin antagonists were also examined at 30°C and 40°C to be compared with those of calmodulin antagonists alone. Sperm preparations were diluted with Tes/NaCl buffer as described above for the measurement of motility intact spermatozoa. Demembranation and reactivation of spermatozoa were performed at 30°C according to the method described by Ashizawa *et al.* (1989b). The extraction medium used consisted of 0.1% Triton X-100, 0.2 mol sucrose l^{-1} , 25 mmol potassium glutamate l^{-1} , 1 mmol $\text{MgSO}_4 \text{ l}^{-1}$, 1 mmol dithiothreitol l^{-1}

and 20 mmol Tris-HCl buffer l⁻¹ (pH 7.9). The reactivation medium consisted of 0.5 mmol ATP l⁻¹, 0.2 mol sucrose l⁻¹, 25 mmol potassium glutamate l⁻¹, 1.5 mmol MgSO₄ l⁻¹, 1 mmol dithiothreitol l⁻¹ and 20 mmol Tris-HCl buffer l⁻¹ (pH 7.9).

Measurement of intracellular free Ca²⁺ concentrations of intact spermatozoa

Determination of the intracellular free Ca²⁺ concentrations was carried out with the fluorescent indicator fura-2, essentially according to Grynkiewicz *et al.* (1985) but with some modifications (Ashizawa *et al.*, 1992a). Fluorescence intensity was measured with a dual-wavelength spectrofluorimeter (Shimadzu, Model RF-5000, Kyoto) with the sample chamber maintained at 30°C or 40°C by a circulating water jacket, and the sperm suspension was mixed continuously with a magnetic stirring bar. For the measurement of intracellular free Ca²⁺ concentrations, the excitation wavelength was set to 340 and 380 nm, respectively, and the emission wavelength was set to 500 nm. The ratio of fluorescence intensities at 340:380 nm was monitored continuously. Calculation of intracellular free Ca²⁺ was based upon the equation described by Grynkiewicz *et al.* (1985) where a dissociation constant (K_d) was 224 nmol l⁻¹.

Measurement of oxygen consumption and ATP concentrations of intact spermatozoa

Oxygen consumption of spermatozoa was determined polarographically with a Clark electrode by the method of Kielley (1963), using a YSI model 53 biological oxygen monitor (Yellow Springs Instrument Co, Inc, Yellow Springs, OH). The rate of oxygen consumption was expressed in terms of $\mu\text{l O}_2$ consumed 10^{-8} spermatozoa h⁻¹. Numbers of spermatozoa were estimated by the method of Wishart and Ross (1985), using a double-beam spectrophotometer (Shimadzu, Model UV-150-02).

ATP concentration in spermatozoa was assayed by firefly bioluminescence in a boiled extract (Wishart, 1982).

Statistical analysis

Statistical comparisons were performed using Student's *t* test.

Results

Effects of BAPTA/AM on the motility and intracellular free Ca²⁺ concentrations of intact fowl spermatozoa

Intact fowl spermatozoa that were not treated with BAPTA/AM (control) maintained vigorous movement at 30°C in the presence or absence of CaCl₂ in the suspending medium. By contrast, the motility of spermatozoa loaded with BAPTA/AM, an intracellular Ca²⁺ chelator, was negligible at 30°C but was instantly restored by the addition of 2 mmol CaCl₂ l⁻¹ (Fig. 1a). At 40°C, neither control nor BAPTA/AM-treated

Fig. 1. The time-course of motility of intact fowl spermatozoa (●) with or (■) without the intracellular Ca²⁺ chelator BAPTA/AM at (a) 30°C and (b) 40°C. (↓) 2 mmol CaCl₂ l⁻¹ was added (○, □). Each point represents the mean (± SEM) of five samples of spermatozoa. **P* < 0.01, compared with value when Ca²⁺ was not added at each incubation period.

spermatozoa were motile, but the motility of both preparations was restored by the subsequent addition of CaCl₂ (Fig. 1b).

The intracellular free Ca²⁺ concentration of intact spermatozoa before the addition of CaCl₂ was approximately 20 nmol l⁻¹ at 30°C and 40°C. After the addition of CaCl₂, the Ca²⁺ concentration increased rapidly and reached a plateau at approximately 200 nmol l⁻¹. In contrast, the intracellular free Ca²⁺ concentration of BAPTA/AM-treated spermatozoa before the addition of CaCl₂ was almost 0 nmol l⁻¹ at 30°C and 40°C. However, the addition of 2 mmol CaCl₂ l⁻¹ caused an influx of Ca²⁺ from the medium and the Ca²⁺ concentration increased gradually at 30°C. Similar results were obtained at 40°C (Fig. 2).

Effects of calmodulin antagonists on the motility of intact and demembrated fowl spermatozoa

The vigorous motility of intact spermatozoa at 30°C was inhibited in a dose-dependent manner by the addition of the calmodulin antagonists W-7 and trifluoperazine. In contrast, no inhibition of motility was observed following the addition of

Fig. 2. Changes in the intracellular free Ca^{2+} concentration in fowl spermatozoa with or without the intracellular Ca^{2+} chelator BAPTA/AM at (a) 30°C and (b) 40°C measured by fura-2 fluorescence. ($\downarrow$) 2 mmol $CaCl_2$ l^{-1} was added.

W-5, a weaker antagonist used as a control drug for W-7, at concentrations of 0–500 μmol l^{-1} (Fig. 3).

The inhibition of motility 20 min after the addition of these calmodulin antagonists is shown (Fig. 3). The time course of inhibition of motility by calmodulin antagonists at 30°C is shown (Fig. 4). Inhibition of motility by trifluoperazine was irreversible. However, inhibition was reversible in spermatozoa treated with W-7: the motility could be restored by diluting the spermatozoa samples by a factor of 20 in W-7-free assay medium to decrease the concentrations of W-7 after exposure for 20 min. However, inhibition of motility by the addition of W-7 and trifluoperazine was not observed in demembrated and reactivated spermatozoa at 30°C (Fig. 5), and motility was maintained for at least 30 min in these spermatozoa as well as in control and W-5-treated spermatozoa (Fig. 6).

Even the presence of Ca^{2+} before the addition of W-7 and trifluoperazine could not prevent the inhibition of motility of intact spermatozoa at 30°C (Fig. 7a). At 40°C, the motility of

Fig. 3. Motility of intact fowl spermatozoa after addition of various concentrations of the calmodulin antagonists W-7 (●), W-5 (○) and trifluoperazine (■) at 30°C. The motility of spermatozoa was assessed 20 min after the addition of calmodulin antagonists at each concentration. Each point represents the mean ($\pm$ SEM) of five samples of spermatozoa. * $P < 0.01$ compared with value at 0 μmol l^{-1} .

Fig. 4. The time-course of motility of intact fowl spermatozoa at 30°C after addition of (●) 500 μmol W-7 l^{-1} , (○) 500 μmol W-5 l^{-1} and (■) 100 μmol trifluoperazine l^{-1} ; no addition of antagonist (control) (□). At arrow, sperm samples were diluted by a factor of 20 in antagonist-free assay medium to decrease extracellular antagonist concentrations. Each point represents the mean ($\pm$ SEM) of five samples of spermatozoa. * $P < 0.01$ compared with value at 0 min.

intact spermatozoa was negligible, but was restored immediately after the addition of 2 mmol $CaCl_2$ l^{-1} . However, the subsequent addition of W-7 and trifluoperazine inhibited the motility again (Fig. 7b). During this period, the intracellular free Ca^{2+} concentration maintained a constant value at approximately 200 nmol l^{-1} (data not shown). Furthermore, the motility of BAPTA/AM-treated spermatozoa could not be restored by the addition of both W-7 and $CaCl_2$ at 30°C and 40°C, but was instantly restored by the addition of $CaCl_2$ alone (Fig. 7c,d).

Effects of BAPTA/AM and calmodulin antagonists on the oxygen consumption and ATP concentrations of intact fowl spermatozoa

The oxygen consumption of spermatozoa that were treated with BAPTA/AM at 30°C and 40°C was 3.4 ± 0.1 and 4.2 ± 0.3 μl O_2 10^{-8} spermatozoa h^{-1} (mean $\pm$ SEM of five

Fig. 5. Motility of demembrated fowl spermatozoa after addition of various concentrations of the calmodulin antagonists (●) W-7, (○) W-5 and (■) trifluoperazine at 30°C. Spermatozoa were assayed for motility 20 min after the addition of calmodulin antagonists at each concentration. Each point represents the mean ($\pm$ SEM) of five samples of spermatozoa.

Fig. 6. The time-course of motility of demembrated fowl spermatozoa at 30°C after the addition of (●) 100 $\mu\text{mol W-7 l}^{-1}$, (○) 100 $\mu\text{mol W-5 l}^{-1}$ and (■) 50 $\mu\text{mol trifluoperazine l}^{-1}$; no addition of antagonist (□). Each point represents the mean ($\pm$ SEM) of five samples of spermatozoa.

samples), respectively. At both temperatures, there were significant differences ($P < 0.01$) between these spermatozoa and those not treated with BAPTA/AM (control) (5.4 ± 0.2 at 30°C and 6.2 ± 0.4 at 40°C).

Furthermore, the rate of oxygen consumption of intact spermatozoa increased slightly after the addition of 2 mmol $\text{CaCl}_2 \text{ l}^{-1}$ at 30°C and 40°C, but decreased significantly

Fig. 7. Motility of intact fowl spermatozoa after addition (↓) of (a) (○) 2 mmol $\text{CaCl}_2 \text{ l}^{-1}$, (●) 500 $\mu\text{mol W-7 l}^{-1}$, (■) 500 $\mu\text{mol W-5 l}^{-1}$ and (□) 100 $\mu\text{mol trifluoperazine l}^{-1}$ at 30°C, and (b) (○) 2 mmol $\text{CaCl}_2 \text{ l}^{-1}$, (●) 100 $\mu\text{mol W-7 l}^{-1}$, (■) 100 $\mu\text{mol W-5 l}^{-1}$ and (□) 50 $\mu\text{mol trifluoperazine l}^{-1}$ at 40°C. Motility of BAPTA/AM-treated intact fowl spermatozoa before (●) and after addition (↓) of (c) (□) 2 mmol $\text{CaCl}_2 \text{ l}^{-1}$ and (○) 2 mmol $\text{CaCl}_2 \text{ l}^{-1}$ + 500 $\mu\text{mol W-7 l}^{-1}$ at 30°C, and (d) (□) 2 mmol $\text{CaCl}_2 \text{ l}^{-1}$ and (○) 2 mmol $\text{CaCl}_2 \text{ l}^{-1}$ + 100 $\mu\text{mol W-7 l}^{-1}$ at 40°C. Each point represents the mean ($\pm$ SEM) of five samples of spermatozoa. * $P < 0.01$ compared with value when calmodulin antagonist was not added (control) at each period.

Fig. 8. Oxygen consumption ($\mu\text{l O}_2 \cdot 10^{-8}$ spermatozoa h^{-1}) of intact fowl spermatozoa after addition of (a) 2 mmol $\text{CaCl}_2 \text{ l}^{-1}$, 500 $\mu\text{mol W-7 l}^{-1}$, 500 $\mu\text{mol W-5 l}^{-1}$ and 100 $\mu\text{mol trifluoperazine l}^{-1}$ at 30°C, and (b) 2 mmol $\text{CaCl}_2 \text{ l}^{-1}$, 100 $\mu\text{mol W-7 l}^{-1}$, 100 $\mu\text{mol W-5 l}^{-1}$ and 50 $\mu\text{mol trifluoperazine l}^{-1}$ at 40°C. Each value represents the mean ($\pm$ SEM) of five samples of spermatozoa. * $P < 0.01$ compared with value when calmodulin antagonist was not added (control).

($P < 0.01$) following the addition of W-7 and trifluoperazine. In contrast, there was no significant decrease after the addition of W-5 or in the control experiment (Fig. 8).

The concentration of ATP in BAPTA/AM-treated spermatozoa decreased slightly at 30°C to $38.2 \pm 3.5 \text{ nmol } 10^{-9}$ spermatozoa (mean $\pm$ SEM of five samples) compared with those of untreated spermatozoa (control) (42.6 ± 4.7), but there was no significant difference between them. However, at 40°C, there was a significant difference ($P < 0.01$) between BAPTA/AM-treated spermatozoa and control (31.7 ± 2.9 in BAPTA/AM treatment versus 55.1 ± 6.0 in control).

In addition, the concentration of ATP in intact spermatozoa decreased slightly at 30°C and 40°C after the addition of CaCl_2 . The subsequent addition of W-7 and trifluoperazine in spite of the presence of Ca^{2+} caused a significant decrease ($P < 0.01$) in ATP concentration compared with that when no antagonist was added (control) or with W-5 treatment (Fig. 9).

Discussion

Unlike mammalian spermatozoa, fowl spermatozoa display a reversible temperature-dependent immobilization. This phenomenon might provide a unique model for basic studies on the cellular mechanisms involved in the regulation of flagellar movement. It is particularly useful for studying the stimulatory effect of Ca^{2+} , as at 40°C fowl sperm motility is negligible until the addition of millimolar concentrations of

Fig. 9. The concentration of ATP in intact fowl spermatozoa after addition (↓) of (a) (○) 2 mmol $\text{CaCl}_2 \text{ l}^{-1}$, (●) 500 $\mu\text{mol W-7 l}^{-1}$, (■) 500 $\mu\text{mol W-5 l}^{-1}$ and (□) 100 $\mu\text{mol trifluoperazine l}^{-1}$ at 30°C, and (b) (○) 2 mmol $\text{CaCl}_2 \text{ l}^{-1}$, (●) 100 $\mu\text{mol W-7 l}^{-1}$, (■) 100 $\mu\text{mol W-5 l}^{-1}$ and (□) 50 $\mu\text{mol trifluoperazine l}^{-1}$ at 40°C. Each point represents the mean ($\pm$ SEM) of five samples of spermatozoa. * $P < 0.01$ compared with value when calmodulin antagonist was not added (control) at each incubation period.

Ca^{2+} to the suspending medium after which 'maximal' motility is achieved (Wishart and Ashizawa, 1987; Ashizawa *et al.*, 1989a; Thomson and Wishart, 1989, 1991). However, detailed mechanisms of this process at the molecular level remain unresolved.

The present study shows that the motility of intact fowl spermatozoa loaded with an intracellular Ca^{2+} chelator, BAPTA/AM, is negligible at 30°C, even though spermatozoa are naturally motile at this temperature. However, addition of excess Ca^{2+} caused the intracellular free Ca^{2+} concentration to increase from 0 to about 100 nmol l^{-1} in BAPTA/AM-treated spermatozoa and motility was immediately restored, suggesting that intracellular free Ca^{2+} is indispensable for the maintenance of fowl sperm flagellar motility.

Calmodulin is a highly conserved, ubiquitous protein involved in mediating the influence of Ca^{2+} on such cellular processes as signal transduction, cell motility and membrane fusion through its ability to activate key intracellular enzymes (Means *et al.*, 1982). The results reported here suggest that calmodulin regulates fowl sperm motility at a point downstream from Ca^{2+} fluxes, as the addition of calmodulin

antagonists such as W-7 and trifluoperazine inhibited the motility of intact spermatozoa at 30°C and 40°C, even in the presence of Ca²⁺. Furthermore, although the motility of BAPTA/AM-treated spermatozoa could not be restored by the addition of both W-7 and Ca²⁺, it was instantly restored by the addition of Ca²⁺ alone. Our results also indicate that most of the calmodulin in fowl spermatozoa may be located in the cytoplasmic matrix, the mitochondria or the membrane, but not retained in the axoneme, since inhibition of motility of intact spermatozoa by the addition of W-7 and trifluoperazine was not observed in demembranated spermatozoa. These results were different from those reported by Brokaw and Nagayama (1985). These authors reported that calmodulin of sea urchin spermatozoa remains tightly bound to the axoneme after extraction with Triton X-100, and that millimolar concentrations of Ca²⁺ are required to produce 'potentially symmetric' flagella – although some calmodulin is readily solubilized during extraction, and may be either associated with the membrane or simply soluble in the cytoplasmic matrix.

With regard to the action of calmodulin antagonists such as W-7 and trifluoperazine, an alternative explanation should be noted: these antagonists could be interfering, not only with calmodulin, but also with membrane-bound, Ca²⁺-dependent protein kinase C. The activity of this kinase system is strictly independent of calmodulin and yet is susceptible to nonspecific suppression by calmodulin antagonists (Schatzman *et al.*, 1981, 1983). However, inhibition of both intact and demembranated fowl sperm motility at 30°C was not observed by the addition of H-7, the most potent and specific protein kinase C inhibitor (K. Ashizawa, S. Katayama, T. Kobayashi and Y. Tsuzuki, unpublished). The inhibition of fowl sperm motility by the addition of W-7 and trifluoperazine might therefore be due to the inhibition of Ca²⁺-calmodulin-regulated enzyme activities.

In these experiments, however, the target and precise mechanisms of the action of calmodulin in the regulation of sperm motility remain to be elucidated. One possibility is that the inhibition of motility is due to energy depletion, since our results have, for the first time, shown that the oxygen consumption and ATP concentrations of fowl spermatozoa decrease markedly after the addition of W-7 and trifluoperazine. It is still unclear whether energy depletion caused by adding W-7 and trifluoperazine is caused by either a calmodulin-regulated or some other, unidentified, process. It has been suggested that calmodulin may be involved in the control of axonemal function by regulating a number of key enzymes, including dynein ATPase, myosin light chain kinase, cyclic nucleotide phosphodiesterases and adenylate cyclase (Tash and Means, 1983). A possible explanation for the influence of calmodulin on sperm motility is that calmodulin in spermatozoa might stimulate the activity of adenylate cyclase responsible for generating cyclic AMP, which is known to be essential for the stimulation of mammalian sperm motility (Gross *et al.*, 1987). However, unlike mammalian spermatozoa, demembranated fowl sperm motility could not be restored by the addition of cyclic AMP at 40°C (Ashizawa *et al.*, 1989b, 1992b).

However, attempts to demonstrate a direct association of calmodulin with dynein have yielded conflicting results (Blum *et al.*, 1980; Gitelman and Witman, 1980). The high molar ratios of calmodulin to dynein required to alter dynein ATPase

activity (Hisanaga and Pratt, 1984) suggest either that conditions of axoneme or dynein preparation pivotal to preserving such an interaction have not been optimized or that calmodulin regulates motility through an indirect interaction with dynein via another component (Tash, 1989). Evidence for an intermediate calmodulin-regulated component has been obtained by the identification of calmodulin-binding proteins in mammalian spermatozoa (for example, Wasco *et al.*, 1989; Manjunath *et al.*, 1993).

Calmodulin appears to mediate many of the effects of Ca²⁺ on fowl sperm motility. However, it should be noted that not all effects of Ca²⁺ on fowl sperm flagellar movement might be mediated by calmodulin, since demembranated sperm motility was inhibited in Ca²⁺-free medium and was restored by the subsequent addition of Ca²⁺ alone at 30°C (Ashizawa *et al.*, 1992a), even though most of the calmodulin present was probably removed from the axoneme by demembranation.

The authors thank G. J. Wishart, Dundee Institute of Technology, Scotland, UK, for kindly reviewing the manuscript. This study was supported by a grant from the Ministry of Education, Science and Culture, Japan (No. 06660360).

References

- Aitken RJ, Clarkson JS, Hulme MJ and Henderson CJ (1988) Analysis of calmodulin acceptor proteins and the influence of calmodulin antagonists on human spermatozoa *Gamete Research* **21** 93–111
- Ashizawa K and Nishiyama H (1978) Effects of temperature on the vigour of motility, oxygen consumption and duration of motility of fowl spermatozoa under aerobic conditions *Japanese Poultry Science* **15** 264–266
- Ashizawa K and Okauchi K (1984) Stimulation of sperm motility and oxygen consumption of fowl spermatozoa by a low molecular weight fraction of seminal plasma *Journal of Reproduction and Fertility* **71** 593–598
- Ashizawa K and Wishart GJ (1987) Resolution of the sperm motility-stimulating principle of fowl seminal plasma into Ca²⁺ and an unidentified low molecular weight factor *Journal of Reproduction and Fertility* **81** 495–499
- Ashizawa K, Maeda S and Okauchi K (1989a) The mechanisms of reversible immobilization of fowl spermatozoa at body temperature *Journal of Reproduction and Fertility* **86** 271–276
- Ashizawa K, Suzuki Y and Okauchi K (1989b) Flagellar movement in demembranated preparations of ejaculated fowl spermatozoa *Journal of Reproduction and Fertility* **86** 263–270
- Ashizawa K, Hashiguchi A and Tsuzuki Y (1992a) Intracellular free Ca²⁺ concentration in fowl spermatozoa and its relationship with motility and respiration in spermatozoa *Journal of Reproduction and Fertility* **96** 395–405
- Ashizawa K, Katayama S and Tsuzuki Y (1992b) Regulation of flagellar motility by temperature-dependent phosphorylation of a 43 kDa axonemal protein in fowl spermatozoa *Biochemical and Biophysical Research Communications* **185** 740–745
- Blum JJ, Hayes A, Jamieson GA, Jr and Vanaman TC (1980) Calmodulin confers calcium sensitivity on ciliary dynein ATPase *Journal of Cell Biology* **87** 386–397
- Bogdonoff PD, Jr and Shaffner CS (1954) The effect of pH on *in vitro* survival, metabolic activity, and fertilizing capacity of chicken semen *Poultry Science* **33** 665–669
- Brokaw CJ (1979) Calcium-induced asymmetrical beating of Triton-demembranated sea urchin sperm flagella *Journal of Cell Biology* **82** 401–411
- Brokaw CJ and Nagayama SM (1985) Modulation of the asymmetry of sea urchin sperm flagellar bending by calmodulin *Journal of Cell Biology* **100** 1875–1883
- Brokaw CJ, Josslin R and Bobrow L (1974) Calcium ion regulation of flagellar beat symmetry in reactivated sea urchin spermatozoa *Biochemical and Biophysical Research Communications* **58** 795–800

- Brooks JC and Siegel FC (1973) Calcium-binding phosphoprotein: the principal acidic protein of mammalian sperm *Biochemical and Biophysical Research Communications* **55** 710–716
- Burgess WH (1983) Characterization of calmodulin and calmodulin isotypes from sea urchin gametes *Journal of Biological Chemistry* **257** 1800–1804
- Camatini M, Anelli G and Casale A (1986) Immunocytochemical localization of calmodulin in intact and acrosome-reacted boar sperm *European Journal of Cell Biology* **41** 89–96
- Camatini M, Colombo A and Bonfanti P (1991) Identification of spectrin and calmodulin in rabbit spermatogenesis and spermatozoa *Molecular Reproduction and Development* **28** 62–69
- Feng B, Bhattacharyya A and Yanagimachi R (1988) Ca^{2+} is essential for the motility of plasma membrane-intact, but not of demembrated, hamster spermatozoa *Andrologia* **20** 155–162
- Fouquet JP, Fraile B and Kann ML (1991) Sperm actin and calmodulin during fertilization in the hamster: an immune electron microscopic study *Anatomical Record* **231** 316–323
- Garbers DL, Hansbrough JR, Radany EW, Hyne RV and Kopf GS (1980) Purification and characterization of calmodulin from sea urchin spermatozoa *Journal of Reproduction and Fertility* **59** 377–381
- Gibbons BH and Gibbons IR (1973) The effect of partial extraction of dynein arms on the movement of reactivated sea urchin sperm *Journal of Cell Science* **13** 337–357
- Gibbons BH and Gibbons IR (1980) Calcium-induced quiescence in reactivated sea urchin sperm *Journal of Cell Biology* **84** 13–27
- Gitelman SE and Witman GB (1980) Purification of calmodulin from *Chlamydomonas*: calmodulin occurs in cell bodies and flagella *Journal of Cell Biology* **87** 764–770
- Gross MK, Toscano DG and Toscano WA, Jr (1987) Calmodulin-mediated adenylate cyclase from mammalian sperm *Journal of Biological Chemistry* **262** 8672–8676
- Gryniewicz G, Poenie M and Tsien RY (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties *Journal of Biological Chemistry* **260** 3440–3450
- Hisanaga S and Pratt MM (1984) Calmodulin interaction with cytoplasmic and flagellar dynein: calcium-dependent binding and stimulation of adenosine triphosphatase activity *Biochemistry* **23** 3032–3037
- Hong CY, Chiang BN, We P, Wei YH and Fong JC (1985) Involvement of calcium in the caffeine stimulation of human sperm motility *British Journal of Clinical Pharmacology* **19** 739–743
- Jones HP, Bradford MM, McRorie RA and Cormier MJ (1978) High levels of a calcium-dependent modulator protein in spermatozoa and its similarity to brain modulator protein *Biochemical and Biophysical Research Communications* **82** 1264–1272
- Jones HP, Lenz RW, Palevitz BA and Cormier MJ (1980) Calmodulin localization in mammalian spermatozoa *Proceedings of the National Academy of Sciences USA* **77** 2772–2776
- Kann ML, Feinberg J, Rainteau D, Dadoune JP, Weinman S and Fouquet JP (1991) Localization of calmodulin in perinuclear structures of spermatids and spermatozoa: a comparison of six mammalian species *Anatomical Record* **230** 481–488
- Katz DF and Overstreet JW (1981) Sperm motility assessment by videomicrography *Fertility and Sterility* **35** 188–193
- Kielley WW (1963) Preparation and assay of phosphorylating submitochondrial particles: sonicated mitochondria *Methods in Enzymology* **6** 272–277
- Leclerc P, Langlais J, Lambert RD, Sirard MA and Chafouleas JG (1989) Effect of heparin on the expression of calmodulin-binding proteins in bull spermatozoa *Journal of Reproduction and Fertility* **85** 615–622
- Lindemann CB and Kanous KS (1989) Regulation of mammalian sperm motility *Archives of Andrology* **23** 1–22
- Lindemann CB, Goltz JS and Kanous KS (1987) Regulation of activation state and flagellar wave form in epididymal rat sperm: evidence for the involvement of both Ca^{2+} and cAMP *Cell Motility and the Cytoskeleton* **8** 324–332
- Majumder GC, Dey CS, Halder S and Barua M (1990) Biochemical parameters of initiation and regulation of sperm motility *Archives of Andrology* **24** 287–303
- Manjunath P, Chandonnet L, Baillargeon L and Roberts KD (1993) Calmodulin-binding proteins in bovine semen *Journal of Reproduction and Fertility* **97** 75–81
- Means AR, Tash JS and Chafouleas JG (1982) Physiological implications of the presence, distribution and regulation of calmodulin in eukaryotic cells *Physiological Reviews* **62** 1–39
- Mohri H and Yanagimachi R (1980) Characteristics of motor apparatus in testicular, epididymal and ejaculated spermatozoa. A study using demembrated sperm models *Experimental Cell Research* **127** 191–196
- Moore PB and Dedman JR (1984) Calmodulin, a calmodulin acceptor protein, and calmedins: unique antibody localizations in hamster sperm *Journal of Cellular Biochemistry* **25** 99–107
- Munro SS (1938) Fowl sperm immobilization by a temperature-media interaction and its biological significance *Quarterly Journal of Experimental Physiology* **27** 281–287
- Nevo AC and Schindler H (1968) Reversible inactivation of cock spermatozoa by temperature *Proceedings of the 6th International Congress of Animal Reproduction and Artificial Insemination, Paris Vol II* pp. 1637–1640
- Schatzman RC, Wise BC and Kuo JF (1981) Phospholipid-sensitive calcium-dependent protein kinase: inhibition by anti-psychotic drugs *Biochemical and Biophysical Research Communications* **98** 669–676
- Schatzman RC, Raynor RL and Kuo JF (1983) *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), a calmodulin antagonist, also inhibits phospholipid-sensitive calcium-dependent protein kinase *Biochimica et Biophysica Acta* **755** 144–147
- Suarez SS, Varosi SM and Dai X (1993) Intracellular calcium increases with hyperactivation in intact, moving hamster sperm and oscillates with the flagellar beat cycle *Proceedings of the National Academy of Sciences USA* **90** 4660–4664
- Takeda A (1982) Studies on reversible inactivation of cock spermatozoa by temperature. I. Effects of several factors on reversible inactivation *Japanese Poultry Science* **19** 26–36
- Tash JS (1989) Protein phosphorylation: the second messenger signal transducer of flagellar motility *Cell Motility and the Cytoskeleton* **14** 332–339
- Tash JS and Means AR (1982) Regulation of protein phosphorylation and motility of sperm by cyclic adenosine monophosphate and calcium *Biology of Reproduction* **26** 745–763
- Tash JS and Means AR (1983) Cyclic adenosine 3', 5' monophosphate, calcium and protein phosphorylation in flagellar motility *Biology of Reproduction* **28** 75–104
- Tash JS, Krinks M, Patel J, Means RL, Klee CB and Means AR (1988) Identification, characterization, and functional correlation of calmodulin-dependent protein phosphatase in sperm *Journal of Cell Biology* **106** 1625–1633
- Thomson MF and Wishart GJ (1989) Elucidation of the mechanism responsible for the temperature-dependent reversible inactivation of the motility of fowl spermatozoa *British Poultry Science* **30** 687–692
- Thomson MF and Wishart GJ (1991) Temperature-mediated regulation of calcium flux and motility in fowl spermatozoa *Journal of Reproduction and Fertility* **93** 385–391
- Wasco WM, Kincaid RL and Orr GA (1989) Identification and characterization of calmodulin-binding proteins in mammalian sperm flagella *Journal of Biological Chemistry* **264** 5104–5111
- Weinman S, Ores-Carton C, Rainteau D and Puszkun S (1986) Immunoelectron microscopic localization of calmodulin and phospholipase A_2 in spermatozoa *Journal of Histochemistry and Cytochemistry* **34** 1171–1179
- White IG and Voglmayr JK (1986) ATP-induced reactivation of ram testicular, cauda epididymal, and ejaculated spermatozoa extracted with Triton X-100 *Biology of Reproduction* **34** 183–193
- Wishart GJ (1982) Maintenance of ATP concentrations in and of fertilizing ability of fowl and turkey spermatozoa *in vitro* *Journal of Reproduction and Fertility* **66** 457–462
- Wishart GJ and Ashizawa K (1987) Regulation of the motility of fowl spermatozoa by calcium and cAMP *Journal of Reproduction and Fertility* **80** 607–611
- Wishart GJ and Ross FH (1985) Characterization of a spectrophotometric technique for the estimation of fowl and turkey sperm motility *Gamete Research* **11** 169–178
- Yamamoto N (1985) Immunoelectron microscopic localization of calmodulin in guinea pig testis and spermatozoa *Acta Histochemica et Cytochemica* **18** 199–211