Intracellular free Ca^{2+} concentration in fowl spermatozoa and its relationship to motility and respiration in spermatozoa

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Summary. The ability of fowl spermatozoa to accumulate and de-esterify the intracellular fluorescent Ca^{2+} indicator fura-2 was established. The cytosolic Ca^{2+} concentrations, measured by this technique, did not change after the addition of 1 mmol EGTA l^{-1} . Subsequently, addition of the calcium ionophore A23187 caused a reduction in cytosolic Ca^{2+} concentrations, presumably by efflux of Ca^{2+} from the spermatozoa. Intracellular free Ca^{2+} concentrations were then significantly increased by the addition of 1 mmol CaCl_2 l^{-1} . The motility of demembranated spermatozoa gradually decreased after the addition of EGTA alone or EGTA with A23187, but was instantly restored by the addition of CaCl_2 in the presence of both EGTA and A23187. Unlike demembranated spermatozoa, intact spermatozoa maintained their motility, even after the addition of EGTA, but their motility was reduced by the addition of A23187 in the presence of EGTA. The addition of A23187 also reduced the rate of oxygen consumption, but not the ATP concentrations in intact spermatozoa. These results demonstrate that the motility and respiration of fowl spermatozoa are strongly influenced by their intracellular Ca^{2+} concentrations.

Keywords: spermatozoa; calcium; fura-2; motility; oxygen uptake; fowl

Introduction

Flagellar movement of spermatozoa is based on the active sliding of microtubules as a result of ATP hydrolysis by dynein ATPase (for reviews, see Satir, 1979; Warner & Mitchell, 1980). Although this basic axonemal mechanism is fairly well accepted, the mechanism of control of this basic sliding is unknown. There are three major regulatory mechanisms that individually or synergistically appear to control sperm motility: calcium and calcium-associated compounds, cAMP and intracellular pH (Majumder *et al.*, 1990).

The role of exogenous Ca^{2+} in sperm motility is less straightforward; it has been observed that Ca^{2+} may have either an inhibitory or a stimulatory effect, depending on the species. External Ca^{2+} appears to have different effects from internal Ca^{2+} on sperm function, again depending on the species (Tash & Means, 1983). Intracellular Ca^{2+} or Ca^{2+} added to preparations of demembranated spermatozoa have shown a profound effect on flagellar motility in every species examined (Tash & Means, 1983). This effect can be divided into two major classes: (i) modification of wave form on reactivation in the presence and absence of Ca^{2+} (Gibbons & Gibbons, 1973; Brokaw *et al.*, 1974; Brokaw, 1979) and (ii) complete inhibition of motility at $> 1 \mu\text{mol Ca}^{2+} \text{l}^{-1}$ (Gibbons & Gibbons, 1980; Mohri & Yanagimachi, 1980; Tash & Means, 1982). The sperm plasma membrane has been shown to contain an active Ca^{2+} pump and a Na^+ – Ca^{2+} antiporter system that regulate the concentration of intracellular Ca^{2+} to maintain this profound influence on sperm

motility (Bradley & Forrester, 1980a, b). Calcium taken up by spermatozoa appears to be mainly accumulated in the mitochondria (Irvine & Aitken, 1986; Rigoni & Deana, 1986).

Unlike mammalian spermatozoa, fowl spermatozoa display a unique phenomenon: in most synthetic diluents, they become immotile at 40–41°C (Munro, 1938; Nevo & Schindler, 1968; Ashizawa & Nishiyama, 1978; Takeda, 1982; Wishart & Ashizawa, 1987). Motility is restored by reducing the temperature or by the addition of Ca^{2+} (Wishart & Ashizawa, 1987; Ashizawa *et al.*, 1989a), and Ca^{2+} is the major stimulatory factor in body fluids such as seminal plasma, or female peritoneal fluid taken at the time of ovulation, in which spermatozoa can maintain their motility even at 40°C (Ashizawa & Wishart, 1987, 1992). It is therefore possible that Ca^{2+} is a key regulator of sperm motility in fowl as well as in other species. However, in fowl spermatozoa, intracellular Ca^{2+} concentration has been estimated only spectrophotometrically with the indicator Arsenazo III, using disrupted spermatozoa (Thomson & Wishart, 1989). Techniques are therefore needed for the measurement of intracellular free Ca^{2+} concentration of the fowl spermatozoa *in situ*.

The use of fluorescent Ca^{2+} indicators quin-2 and fura-2 for the direct measurement of intracellular free Ca^{2+} has been described (Tsien, 1981; Tsien *et al.*, 1982; Grynkiewicz *et al.*, 1985) and applied to various mammalian spermatozoa (Aitken *et al.*, 1986, 1988; Irvine & Aitken, 1986; Mahanes *et al.*, 1986; Rigoni & Deana, 1986; Babcock & Pfeiffer, 1987; Simpson & White, 1988; Thomas & Meizel, 1988; Blackmore *et al.*, 1990; Vijayaraghavan & Hoskins, 1990), but no information is available for avian spermatozoa.

We therefore decided to determine the most suitable conditions for the measurement of intracellular free Ca^{2+} concentrations of fowl spermatozoa using fura-2. Attempts were also made to manipulate intracellular Ca^{2+} concentrations with EGTA and the calcium ionophore A23187; and the effects of these compounds on the motility, ATP concentrations and oxygen uptake of spermatozoa were investigated.

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 fed *ad libitum* on a commercial breeder diet. They were exposed to 14 h light per 24 h.

Semen was collected by the method of Bogdonoff & Shaffner (1954). Samples of semen pooled from four to six males were diluted approximately tenfold in 150 mmol NaCl l⁻¹ with 20 mmol TES (*N*-Tris-[hydroxymethyl]-methyl-2-aminoethanesulfonic 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 about 1×10^9 cells ml⁻¹. Samples of 3–4 ml were poured into 30 ml Erlenmeyer flasks with a screw cap.

Chemicals

Fura-2/AM was purchased from Dojindo Laboratories, Inc., Kumamoto, Japan. Adenosine 5'-triphosphate (ATP), adenosine 3':5'-cyclic monophosphate (cAMP), dibutylryl adenosine 3':5'-cyclic monophosphate (diB-cAMP), caffeine, dithiothreitol, firefly lanterns, potassium glutamate, TES, Triton X-100, Cremophor EL and ionophore A23187 were purchased from Sigma Chemical Co., St Louis, MO, USA. Other chemicals were of reagent grade from Nacalai Tesque, Inc., Kyoto, Japan.

Measurement of intracellular free Ca^{2+} concentrations of spermatozoa

Determination was carried out with the fluorescent indicator fura-2, according to Grynkiewicz *et al.* (1985), but with modifications. Stock solutions of fura-2 acetoxymethyl ester (fura-2/AM) were made up of 1 mmol l⁻¹ in dry dimethylsulfoxide (DMSO) and kept desiccated at –80°C until use. For the complete emulsification of fura-2/AM, 2 µl 10% Cremophor EL was added to 5 µl of fura-2/AM, and then 2 ml NaCl/TES buffer was added. This solution was sonicated for 30 s on ice with an ultrasonic processor with a microtip (Sonics & Materials, Inc., Danbury, CT, USA) at 50 W. This procedure gave optimal fura-2/AM cellular loading and hydrolysis; 1 ml of sperm suspension was added to fura-2/AM solution and shaken for 45 min at 30°C in the dark. During this incubation, final concentrations

of fura-2/AM and spermatozoa were $1.7 \mu\text{mol l}^{-1}$ and about $3.3 \times 10^8 \text{ ml}^{-1}$, respectively. After the incubation, the spermatozoa were pelleted by centrifugation at $700 g$ for 15 min and then washed twice to minimize the carry-over of extracellular fura-2/AM. The sperm pellet was finally resuspended in NaCl/TES buffer and sperm concentrations were adjusted to $1 \times 10^8 \text{ ml}^{-1}$, except for the investigations of the effects of sperm concentrations. These suspensions were incubated at 30°C in the dark before use.

Fluorescence intensity was measured with a dual-wavelength spectrofluorimeter (Shimadzu, Model RF-5000, Kyoto, Japan) with the sample chamber thermostatically controlled at 30°C by a circulating water jacket, and the sperm suspension was mixed continuously with a magnetic stirring bar. For the examination of fluorescence spectra, the emission wavelength was set at 500 nm, and the excitation wavelength was scanned over the range 250–450 nm, with an emission slit of 10 nm and an excitation slit of 1.5 nm. For the measurement of intracellular free Ca^{2+} concentrations, the excitation wavelength was set to 340 and 380 nm, respectively, and the emission wavelength was set to 500 nm. The ratios ($R_{340}:R_{380}$) of fluorescence intensities were monitored continuously, and Triton X-100 was then added to a concentration of 0.05% followed by EGTA to a concentration of 20 mmol l^{-1} , and the readings obtained were recorded as R_{max} and R_{min} , respectively. Calculation of intracellular free Ca^{2+} was based upon the equation described by Grynkiewicz *et al.* (1985) where the dissociation constant (K_d) was 224 nmol l^{-1} .

Measurement of motility, ATP concentrations and oxygen uptake in intact spermatozoa

The sperm preparations were incubated aerobically in a shaking water bath at 30°C . Three minutes after the start of incubation, $1 \text{ mmol EGTA l}^{-1}$ was added, and $3 \mu\text{mol}$ ionophore A23187 l^{-1} was added 6 min later. Fifteen minutes after the incubation, $1 \text{ mmol CaCl}_2 \text{ l}^{-1}$, $1 \text{ mmol NaHCO}_3 \text{ l}^{-1}$, $1 \text{ mmol caffeine l}^{-1}$ or $4 \text{ mmol dibutyl cyclic AMP (diB-cAMP) l}^{-1}$ was added. During incubation of spermatozoa, sperm motility, ATP concentrations and oxygen uptake were measured every 3 min.

The suspension of spermatozoa was placed on a microscope slide chamber and the motility of spermatozoa was recorded by videomicroscopy at 30°C on a thermostatically controlled warm plate as described previously (Ashizawa *et al.*, 1989c).

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

Oxygen uptake 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, Ohio, USA). The rate of oxygen uptake was expressed in terms of $\mu\text{l O}_2$ consumption per 10^8 spermatozoa h^{-1} . Numbers of spermatozoa were estimated by the method of Wishart & Ross (1985), using a double-beam spectrophotometer (Shimadzu, Model UV-150-02, Kyoto, Japan).

Measurement of motility of demembrated spermatozoa

Intact spermatozoa were demembrated at 30°C according to the method described previously (Ashizawa *et al.*, 1989c). The extraction medium used consisted of 0.1% Triton X-100, $0.2 \text{ mol sucrose l}^{-1}$, $25 \text{ mmol potassium glutamate l}^{-1}$, $1 \text{ mmol MgSO}_4 \text{ l}^{-1}$, $1 \text{ mmol dithiothreitol l}^{-1}$ and $20 \text{ mmol Tris-HCl buffer l}^{-1}$ (pH 7.9). The reactivation medium consisted of $0.5 \text{ mmol ATP l}^{-1}$, $0.2 \text{ mol sucrose l}^{-1}$, $25 \text{ mmol potassium glutamate l}^{-1}$, $1.5 \text{ mmol MgSO}_4 \text{ l}^{-1}$, $1 \text{ mmol dithiothreitol l}^{-1}$ and $20 \text{ mmol Tris-HCl buffer l}^{-1}$ (pH 7.9). EGTA, 1 mmol l^{-1} , $3 \mu\text{mol}$ ionophore A23187 l^{-1} , $1 \text{ mmol CaCl}_2 \text{ l}^{-1}$, $1 \text{ mmol NaHCO}_3 \text{ l}^{-1}$, $1 \text{ mmol caffeine l}^{-1}$ and $10 \mu\text{mol cAMP l}^{-1}$ were added at the same periods after the start of incubation as described for the intact spermatozoa. Sperm motility was assayed by the method described above.

Statistical analysis

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

Results

Loading and de-esterification of fura-2/AM in fowl spermatozoa

Preliminary experiments were undertaken to check whether the fura-2 method of Grynkiewicz *et al.* (1985) was suitable for measuring intracellular free Ca^{2+} in fowl spermatozoa.

In this experimental procedure, for the complete emulsification of fura-2/AM, Cremophor EL was added and further sonication was performed. Even after these treatments, the wavelength of the excitation peak remained constant at 380 nm in the presence of various concentrations of Ca^{2+} (Fig. 1a). These results suggested that there was no disintegration from fura-2/AM to fura-2 during these treatments, since the fluorescence excitation of Ca^{2+} -bound fura-2 shifts to shorter wavelength (340 nm) (Grynkiewicz *et al.*, 1985).

Fig. 1. Excitation spectra of (a) 2.5 $\mu\text{mol fura-2/AM l}^{-1}$ and (b) fura-2 loaded fowl spermatozoa in the presence of various concentrations 0 (—), 1 ($\cdots$), 5 (— · —) and 10 (---) mmol CaCl_2 l^{-1} ; (c) fura-2 loaded fowl spermatozoa after consecutive additions of zero-control (—), 1 mmol CaCl_2 l^{-1} (— · —), 0.05% Triton X-100 (---) and 20 mmol EGTA l^{-1} ($\cdots$); (d) effects of sperm concentrations on the intracellular free Ca^{2+} concentrations in fowl spermatozoa. The excitation wavelength was set to 340 and 380 nm. The ratios (340:380 nm) of fluorescence intensities were monitored continuously. The emission wavelength for (a)–(d) was fixed at 500 nm.

In contrast, shifts of excitation spectra to 340 nm were observed by the addition of Ca^{2+} in the fura-2/AM loaded spermatozoa (Fig. 1b). It was thus apparent that fowl spermatozoa succeeded in accumulating fura-2/AM, de-esterified it to fura-2 free acid, and retained this latter molecule within the cell.

To calculate intracellular free Ca^{2+} concentrations, we determined the values of R_{max} and R_{min} . After the addition of Ca^{2+} , followed by Triton X-100 and EGTA, there was a shift in excitation spectra (Fig. 1c). The results conformed to the theoretical behaviour of fura-2 excitation spectra: after the disruption of spermatozoa by 0.05% Triton X-100, whole molecules of fura-2 were bound to intra- and extracellular Ca^{2+} and the fluorescence excitation spectrum shifted to shorter wavelength. Then, fluorescence intensity decreased by the addition of 20 mmol EGTA l^{-1} , and also the shift to longer wavelength was observed.

The ratios (i.e. intracellular free Ca^{2+} concentrations) were almost constant among the three different concentrations of spermatozoa ($0.5\text{--}2.0 \times 10^8$ cells ml^{-1}) (Fig. 1d). From these results, it was confirmed that intracellular free Ca^{2+} concentrations of fowl spermatozoa can be measured by this method.

Effects of EGTA and A23187 on the intracellular free Ca^{2+} concentration of fowl spermatozoa

Even after the addition of $1 \text{ mmol EGTA l}^{-1}$ to intact spermatozoa, the wavelength of the excitation peak did not change (Fig. 2). After the addition of $3 \mu\text{mol A23187 l}^{-1}$, the spectral maximum shifted to 360 nm , which is the absorption maximum of the free anion of fura-2 (Gryniewicz *et al.*, 1985). The subsequent addition of $1 \text{ mmol CaCl}_2 \text{ l}^{-1}$ shifted the spectral maximum of 340 nm , which is the absorption maximum of the Ca^{2+} complex of fura-2 (Gryniewicz *et al.*, 1985).

Fig. 2. Excitation spectra of fura-2 loaded fowl spermatozoa after the consecutive addition of none (—), $1 \text{ mmol EGTA l}^{-1}$ (···), $3 \mu\text{mol A23187 l}^{-1}$ (---) and $1 \text{ mmol CaCl}_2 \text{ l}^{-1}$ (— · —). Emission wavelength was fixed at 500 nm .

Changes in intracellular free Ca^{2+} concentrations were negligible after the addition of $1 \text{ mmol EGTA l}^{-1}$ (Fig. 3a, b). Subsequently, the addition of $3 \mu\text{mol A23187 l}^{-1}$ caused a decrease in the intracellular free Ca^{2+} concentrations. Intracellular free Ca^{2+} concentrations were then rapidly increased by the addition of $1 \text{ mmol CaCl}_2 \text{ l}^{-1}$ (Fig. 3a). In contrast, the addition of $1 \text{ mmol CaCl}_2 \text{ l}^{-1}$ in the absence of A23187 only increased the intracellular free Ca^{2+} concentration slightly (Fig. 3b). In both cases, actual extracellular free Ca^{2+} concentrations were approximately $10\text{--}20 \mu\text{mol l}^{-1}$, from the calculation of Bartfai (1979).

Effects of EGTA and A23187 on the motility, ATP concentrations and oxygen uptake of intact fowl spermatozoa

During the incubation, spermatozoa maintained their motility at about 60% even after the addition of EGTA alone, but the motility decreased gradually with the addition of A23187 in the presence of EGTA, and after 15 min of incubation the motility was about 20%. The subsequent addition of Ca^{2+} restored the motility to 50–60%. In contrast, the motility was not restored by the addition of HCO_3^- , caffeine and diB-cAMP (Fig. 4a).

Fig. 3. Changes of intracellular free Ca^{2+} concentrations in fowl spermatozoa measured by fura-2 fluorescence: (a) 1 mmol EGTA l^{-1} , 3 $\mu\text{mol A23187 l}^{-1}$ and 1 mmol $\text{CaCl}_2 \text{l}^{-1}$ were added at arrows; (b) 1 mmol EGTA l^{-1} and 1 mmol $\text{CaCl}_2 \text{l}^{-1}$ were added at arrows.

The ATP concentrations (nmol 10^{-9} spermatozoa) were fairly constant (41.3–43.2) during incubation in the presence of EGTA alone. The addition of A23187 slightly decreased the ATP concentrations, but there was no significant difference between the two treatments. After the addition of Ca^{2+} , the ATP concentrations decreased rapidly, but were not significantly changed by the addition of HCO_3^- , caffeine and dibutyl-cAMP (Fig. 4b).

The rate of oxygen uptake was not changed by the addition of EGTA, but decreased significantly ($P < 0.01$) following the addition of A23187 in the presence of EGTA. It was almost restored after the addition of Ca^{2+} , but not by addition of HCO_3^- , caffeine and dibutyl-cAMP (Fig. 5).

Effects of EGTA and A23187 on the motility of demembrated fowl spermatozoa

In the absence of EGTA and A23187, the motility of reactivated spermatozoa was maintained 65–75% during incubation for 15 min. In contrast, the motility of demembrated spermatozoa decreased gradually by the addition of either EGTA alone or EGTA + A23187. Subsequently, the motility was instantly restored by the addition of Ca^{2+} in the presence of both EGTA and A23187, but the addition of HCO_3^- , caffeine and cAMP were not effective for the restoration of motility (Fig. 6).

Fig. 4. (a) Motility and (b) ATP concentration in intact fowl spermatozoa after addition ($\downarrow$) of 1 mmol EGTA l^{-1} ($\circ$), 3 μmol A23187 l^{-1} ($\bullet$), 1 mmol CaCl_2 l^{-1} ($\blacksquare$), 1 mmol NaHCO_3 l^{-1} ($\blacktriangle$), 1 mmol caffeine l^{-1} ($\triangle$) and 4 mmol diB-cAMP l^{-1} ($\square$). Each point represents the mean ($\pm$ SEM) of six samples of spermatozoa. * $P < 0.01$ compared with value at 0 min.

Discussion

These experiments demonstrate that fura-2 can be easily loaded into fowl spermatozoa as the acetoxymethyl ester and that loadings of up to several $\mu\text{mol l}^{-1}$ of fura-2/AM are not toxic to fowl spermatozoa, as judged by motility (data not shown). Fura-2 has therefore proved to be a suitable indicator of intracellular free Ca^{2+} concentration of fowl spermatozoa.

Control of intracellular Ca^{2+} concentration appears to be important in mammalian spermatozoa for modulating the function of the flagellar axoneme (Lindemann & Kanous, 1989). Although the mechanism of regulation has not been completely elucidated, it is likely that optimal Ca^{2+} concentration depends on the balance between Ca^{2+} uptake by mitochondrial pump and Ca^{2+} extrusion by plasma membrane pump or Na^+ - Ca^{2+} antiporter, or both (Simpson *et al.*, 1987). However, only limited information about the relationship between Ca^{2+} fluxes and motility is available for avian spermatozoa (Thomson & Wishart, 1989, 1991). In this study, intracellular Ca^{2+} concentrations of fowl spermatozoa were manipulated with EGTA and ionophore A23187. A23187, an ionophorous antibiotic, increases permeability to Ca^{2+} , not only for plasma membrane, but also for mitochondrial and endoplasmic reticulum membrane (Reed & Lardy, 1972) and

Fig. 5. Oxygen uptake ($\mu\text{l O}_2 10^{-8}$ spermatozoa h^{-1}) of intact fowl spermatozoa after addition of 1 mmol EGTA l^{-1} , 3 μmol A23187 l^{-1} , 1 mmol CaCl_2 l^{-1} , 1 mmol NaHCO_3 l^{-1} , 1 mmol caffeine l^{-1} and 4 mmol diB-cAMP l^{-1} . Each value represents the mean ($\pm$ SEM) of six samples of spermatozoa. * $P < 0.01$ compared with control (no addition).

Fig. 6. Motility of demembrated fowl spermatozoa after addition of 1 mmol EGTA l^{-1} (Δ), 3 μmol A23187 l^{-1} ($\bullet$), 1 mmol CaCl_2 l^{-1} ($\blacksquare$), 1 mmol NaHCO_3 l^{-1} ($\blacktriangle$), 1 mmol caffeine l^{-1} (∇) and 10 μmol l^{-1} cAMP ($\square$); ($\circ$) control (no addition). Each point represents the mean ($\pm$ SEM) of six samples of spermatozoa. * $P < 0.01$ compared with value at 0 min.

provides a useful probe for the study of Ca^{2+} fluxes in spermatozoa (Simpson *et al.*, 1987). The influx of Ca^{2+} into cells following treatment with A23187 occurs when the extracellular Ca^{2+} concentrations are higher than that of intracellular Ca^{2+} . In contrast, when the concentrations of Ca^{2+} in the medium are lower than that of intracellular levels, an efflux of accumulated Ca^{2+} from the cells occurs after the addition of A23187. We have thus produced Ca^{2+} -depleted spermatozoa by treatment with A23187 in the presence of EGTA. Changes of intracellular free Ca^{2+} concentrations were almost negligible after the addition of EGTA alone, but the concentrations became nearly zero by the subsequent addition of A23187 (Fig. 3a). During this latter experiment, sperm motility decreased gradually, but was restored by the addition of exogenous Ca^{2+} which increased

intracellular free Ca^{2+} concentrations (Fig. 4a). Since the motility of demembranated spermatozoa could be restored by Ca^{2+} in the presence of A23187, it appeared that axonemal activity was not directly inhibited by the ionophore. Thus, the inhibitory effect of A23187 on intact sperm motility must result from changes in non-axonemal, presumably Ca^{2+} transport, mechanisms.

ATP concentrations were fairly constant even after treatment with A23187 in the presence of EGTA, which reduced sperm motility considerably (Fig. 4b). Since the rate of oxygen consumption was also reduced by this treatment, the constancy of the ATP concentration probably reflects coupling of ATP hydrolysis by the axoneme to the rate of respiration and oxidative phosphorylation. This assumption was supported by the finding that ATP concentrations decreased soon after the addition of Ca^{2+} during which time motility increased rapidly in the absence of a significant increase in the rate of oxygen consumption (Figs 4 and 5).

When intact hamster spermatozoa were suspended in a medium completely free of Ca^{2+} , most spermatozoa quickly lost their motility and remained motionless until they were transferred back to Ca^{2+} -containing medium. However, demembranated hamster spermatozoa exhibited active movement in Ca^{2+} -free medium, and their motility was inhibited by Ca^{2+} (Feng *et al.*, 1988). In view of these results, it was suggested that the increase in flagellar beating observed during hyperactivation was probably due to the activation of Ca^{2+} -dependent adenyl cyclase with a resultant increase in cAMP concentrations, rather than an increase in intracellular Ca^{2+} concentrations (Feng *et al.*, 1988). In contrast, demembranated fowl sperm motility was inhibited in Ca^{2+} -free medium (in the presence of EGTA) and restoration was observed with the addition of Ca^{2+} (Fig. 6). These observations are different from those reported by Feng *et al.* (1988). Further study is needed to determine whether this could be due to a species difference in sensitivity to Ca^{2+} .

Fowl spermatozoa show a reversible inhibition of their motility as the temperature is raised from 30 to 40°C (Munro, 1938; Nevo & Schindler, 1968; Ashizawa & Nishiyama, 1978; Takeda, 1982; Wishart & Ashizawa, 1987). Motility is restored by decreasing the temperature or by the addition of Ca^{2+} (Wishart & Ashizawa, 1987; Ashizawa *et al.*, 1989a). Thomson & Wishart (1989) suggest that this phenomenon involves a loss of sperm Ca^{2+} to the suspending medium at 40°C, with a subsequent resequestration of Ca^{2+} at 30°C. On the other hand, we assumed that the mechanisms involved in the reversible temperature-dependent immobilization involve the axonemes themselves, since the motility of demembranated fowl spermatozoa was negligible at 40°C in the presence of $10^{-7} \text{ mol l}^{-1} \text{ Ca}^{2+}$ and the same samples restored their motility at 30°C (Ashizawa *et al.*, 1989c). In this work, however, the motility of both intact and demembranated spermatozoa was inhibited at 30°C in the absence of Ca^{2+} . As mentioned above, it is therefore apparent that Ca^{2+} is indispensable for the regulation of flagellar motility of fowl spermatozoa. Perhaps, in addition to such a basic regulatory system by Ca^{2+} , the mechanism responsible for the reversible inactivation predominates at 40°C and overrides the stimulatory effect of Ca^{2+} on fowl flagellar movement.

Fowl sperm motility is also stimulated by the addition of bicarbonate and caffeine at 40°C (Nevo & Schindler, 1968; Wishart & Ashizawa, 1987; Ashizawa *et al.*, 1989b). In this study, however, such stimulators were not effective for the restoration of motility, after the removal of Ca^{2+} (Figs 4a and 6). From these results, it may be suggested that the presence of Ca^{2+} is essential for the motility of fowl spermatozoa, and that stimulation of motility by bicarbonate and caffeine requires Ca^{2+} . This is in agreement with the proposal that caffeine stimulates fowl sperm motility by altering sperm intracellular Ca^{2+} flux (Thomson & Wishart, 1991).

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