Inhibition of temperature-dependent immobilization of fowl spermatozoa at body temperature by an increased intracellular pH

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Fowl sperm motility was measured by altering the extracellular pH (pH_e) at 30°C and 40°C. At 30°C, the motility of intact spermatozoa was vigorous in a medium in the wide pH_e range of 7.3–10.1. In contrast, intact spermatozoa were almost immotile at 40°C in medium below a pH_e of 8.1. However, the motility could be restored by increasing the pH_e ; maximum motility was obtained in medium at pH_e 9.4. Stimulation of the motility of demembranated spermatozoa at 40°C was also observed with an increased pH_e . However, demembranated spermatozoa at 40°C that had been stimulated by increasing the pH_e lost their motility when 1 mmol $CaCl_2$ l^{-1} was added. Motility was restored by the subsequent addition of 2 mmol EGTA l^{-1} . At a high pH_e at 40°C, the flagellar ATPase activity of crude dynein extract was not affected, regardless of the addition of $CaCl_2$ or EGTA. The intracellular pH (pH_i) of intact spermatozoa, estimated by measuring the accumulation of 9-aminoacridine fluorescence, increased with increasing pH_e at both 30°C and 40°C. These results demonstrate that the reversible temperature-dependent immobilization of fowl spermatozoa at 40°C is inhibited by an increased pH_i . Furthermore, it is possible that the effects of the increased pH_i may not act directly on dynein ATPase activity, but are mediated by a Ca^{2+} -related substance(s) on the axoneme.

Introduction

There is evidence that intracellular pH (pH_i) is a potential trigger for the activation of sperm motility of many species of invertebrates and vertebrates (for reviews, see Brokaw, 1987; Lindemann and Kanous, 1989; Majumder *et al.*, 1990; Morisawa and Morisawa, 1990). However, the detailed mechanisms of this phenomenon are unclear.

The activation of motility of sea urchin spermatozoa has been shown to depend on the presence of Na^+ in the medium, and is accompanied by a Na^+ -dependent release of H^+ from the cells (Nishioka and Cross, 1978) and by a rise of pH_i (Christen *et al.*, 1982; Lee *et al.*, 1983). It is therefore proposed that the requirement of Na^+ for the initiation of motility is attributed to an increase in the pH_i via the Na^+-H^+ -ATPase in rat and sea urchin spermatozoa (Wong *et al.*, 1981; Lee, 1984). Christen *et al.* (1983) showed that the intracellular alkalinization of sea urchin spermatozoa activates their respiration and induces a concomitant increase in the activity of dynein ATPase, suggesting that the dynein ATPase of the flagellar axoneme is the primary target for regulation by pH_i . Tombes and Shapiro (1985) postulated that energy for motility stimulated by an increased pH_i is provided by mitochondrial respiration; energy transport from the sperm mitochondria to the tail involves distinct isozymes of creatine kinase that effect a phosphorylcreatine shuttle.

However, Morisawa and Morisawa (1988) demonstrated that rainbow trout spermatozoa and chum salmon spermatozoa

acquire the potential for motility as a result of an increase in the seminal plasma pH due to the increasing bicarbonate concentration during passage through the sperm duct. Under these conditions, an increase in extracellular pH (pH_e) causes the rise in intracellular cAMP (Morisawa *et al.*, 1993). Similar results were obtained in bovine caput epididymal spermatozoa (Vijayaraghavan *et al.*, 1985). It has been suggested that the dependence of sperm motility on pH_i may play a significant role in maintaining the cAMP-dependent phosphorylated condition of the axoneme rather than directly activating dynein ATPase (Lindemann and Kanous, 1989), since the motility of demembranated bull spermatozoa without added cAMP is strongly dependent on alkaline pH ($pH > 7.5$), but with cAMP vigorous motility persists even at acidic pH (as low as pH 6.8) (Goltz *et al.*, 1988).

In contrast, Babcock *et al.* (1983) suggested that pH_i plays a role in regulating the motility and metabolic activity of bovine cauda epididymal spermatozoa that is not mediated by cAMP, since cAMP-dependent protein kinase activity ratios in cellular extracts are increased by the addition of caffeine but not by alkaline pH. Furthermore, Carr and Acott (1989) found at least four proteins from bovine epididymal spermatozoa that undergo changes in phosphorylation states in response to pH_i modulation, but these authors suggested that the changes in phosphorylation are probably not due to changes in the concentration of cAMP, since they have previously shown that increasing pH_i does not increase the amount of cAMP in bovine spermatozoa (Carr and Acott, 1984).

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Fowl spermatozoa provide an excellent system in which to examine the regulation of flagellar motility, as, unlike mammalian spermatozoa, fowl spermatozoa show reversible temperature-dependent changes in motility. In most synthetic diluents, they become immotile at the body temperature of 40–41°C, but motility is restored by decreasing the temperature (Munro, 1938; Ashizawa and Nishiyama, 1978; Ashizawa and Okauchi, 1984; Ashizawa and Wishart, 1987, 1992; Wishart and Ashizawa, 1987; Thomson and Wishart, 1989, 1991). The axoneme itself appears to be directly involved in this regulatory system, since the motility of demembranated fowl spermatozoa is, as with intact spermatozoa, negligible at 40°C and restored at 30°C (Ashizawa *et al.*, 1989a, b). However, the intracellular molecular cascade involved in immobilization and restoration of motility is unknown.

If changes in pH_i are involved in the above temperature-dependent regulatory system, fowl spermatozoa might be expected to become motile even at 40°C after an increase in pH_i . However, little information is available about the effects of pH on the motility of fowl spermatozoa (Terada *et al.*, 1981). In this study attempts were made to manipulate pH_i by changing pH_e , and the effects of increasing pH_i on the flagellar motility, metabolic activity and dynein ATPase activity of fowl spermatozoa were investigated at 30°C and 40°C.

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). Pooled semen was divided into seven aliquots and diluted by a factor of approximately ten in seven different buffers: 150 mmol NaCl l⁻¹ with 20 mmol N-tris-[hydroxymethyl]-methyl-2-aminoethanesulfonic acid (TES) l⁻¹ adjusted to pH 7.5, 8.0 and 8.5 at 20°C, or 150 mmol NaCl l⁻¹ with 20 mmol 3-[cyclohexylamino]-2-hydroxy-1-propanesulfonic acid (CAPSO) l⁻¹ adjusted to pH 9.0, 9.5, 10.0 and 10.5 at 20°C. The samples were centrifuged at 700 g for 13 min at room temperature (20–25°C), and the washed spermatozoa were reconstituted in the same buffer to give a final concentration of approximately 1×10^9 cells ml⁻¹.

Chemicals

ATP, cAMP, 9-aminoacridine, BSA, CAPSO, desiccated firefly tails, dibutyl- γ -cAMP, dithiothreitol, potassium glutamate, TES and Triton X-100 were purchased from Sigma Chemical Co., St Louis, MO. Other chemicals were of reagent grade from Nacalai Tesque, Inc., Kyoto.

Measurement of pH_i of intact spermatozoa at different pH_e values

The pH_i of spermatozoa was estimated by the method of Christen *et al.* (1982). Changes in fluorescence intensity induced

by the addition of 150 μ l of the sperm sample to 2.85 ml of the NaCl-TES or NaCl-CAPSO buffer containing 2.5 μ mol 9-aminoacridine l⁻¹ were monitored continuously in a fluorescence spectrophotometer (Shimadzu, Model RF-5000, Kyoto). The concentration of spermatozoa was approximately 0.5×10^8 cells ml⁻¹. An excitation wavelength of 398 nm was used and emitted fluorescence was measured at 456 nm. The temperature of the cuvette was maintained at 30°C or 40°C by a circulating water jacket, and the sperm suspension was mixed continuously with a magnetic stirring bar.

Measurement of motility of intact and demembranated spermatozoa at different pH_e values

Sperm samples were preincubated aerobically in a shaking water bath at 30°C or 40°C for 10 min. Intact spermatozoa were then diluted by a factor of approximately 250 in the same buffers, and the percentage of motile spermatozoa was measured at 30°C or 40°C. Dibutyl γ -cAMP, when incorporated, was present at a final concentration of 4 mmol l⁻¹.

Demembration and reactivation of spermatozoa were performed at 30°C and 40°C according to the method described by Ashizawa *et al.* (1989b). The extraction medium used consisted of 0.1% Triton X-100, 200 mmol sucrose l⁻¹, 25 mmol potassium glutamate l⁻¹, 1 mmol MgSO₄ l⁻¹, 1 mmol dithiothreitol l⁻¹ and 20 mmol Tris-HCl l⁻¹ (pH 7.5–8.5, adjusted at 20°C) or 20 mmol CAPSO l⁻¹ (pH 9.0–10.5, adjusted at 20°C). The reactivation medium consisted of 0.5 mmol ATP l⁻¹, 200 mmol 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 l⁻¹ (pH 7.5–8.5, adjusted at 20°C) or 20 mmol CAPSO l⁻¹ (pH 9.0–10.5, adjusted at 20°C). CaCl₂, EGTA and cAMP, when incorporated, were present at final concentrations of 1 mmol l⁻¹, 2 mmol l⁻¹ and 10 μ mol l⁻¹, respectively.

The suspension of intact or demembranated spermatozoa was placed on a microscope chamber (0.07 mm in depth, Sekisui Chemical Co., Ltd, Tokyo) and the motility of spermatozoa was recorded by videomicroscopy (magnification on the 12-inch black and white monitor was approximately $\times 600$) at 30°C or 40°C on a thermostatically controlled warm plate (Katz and Overstreet, 1981). The percentage of motile spermatozoa was determined by observing 200–300 spermatozoa, distributed uniformly among the three or more fields.

Measurement of oxygen consumption and ATP concentrations of intact spermatozoa at different pH_e values

Oxygen consumption of spermatozoa incubated in buffers of different pH was determined polarographically with a Clark electrode by the method of Kielley (1963), using a YSI biological oxygen monitor, model 53 (Yellow Springs Instrument Co., Inc., Yellow Springs, OH). The rate of oxygen consumption was expressed in terms of μ l O₂ consumed per 10⁸ 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).

The concentration of ATP in spermatozoa incubated in buffers of different pH was assayed by firefly bioluminescence in a boiled extract (Wishart, 1982).

Isolation of crude dynein extract and measurement of ATPase activity at different pH_e values

The method of McConnell *et al.* (1987) for the isolation of crude dynein from bovine spermatozoa was modified for use in fowl spermatozoa. Approximately 9×10^9 spermatozoa washed with NaCl-TES buffer (pH 7.4) were demembrated by resuspending the sperm pellet in 30 ml of cold extraction medium, as described above (pH 7.9). After centrifugation at 700 *g* for 10 min at 4°C, demembrated spermatozoa were washed once with cold extraction medium excluding Triton X-100 and recentrifuged. Axonemal ATPase was solubilized by resuspending the demembrated spermatozoa in 0.6 mol NaCl l^{-1} , 20 mmol Tris-HCl l^{-1} (pH 7.9), 1 mmol dithiothreitol l^{-1} and 4 mmol $MgCl_2$ l^{-1} for 10 min at 4°C. The supernatant obtained after centrifugation of the axonemal extract at 100 000 *g* for 30 min at 4°C was designated as crude dynein extract.

ATPase activities were assayed by the reduction of ATP concentrations determined by firefly bioluminescence. The assay solution consisted of 20 mmol Tris-HCl l^{-1} (pH 7.5–8.5, adjusted at 20°C) or 20 mmol CAPSO l^{-1} (pH 9.0–10.5, adjusted at 20°C), 4 mmol $MgSO_4$ l^{-1} and 0.1 mmol ATP l^{-1} . $CaCl_2$ and EGTA, when incorporated, were present at a final concentration of 1 mmol l^{-1} and 2 mmol l^{-1} , respectively. The reaction was initiated by mixing 150 μ l of the assay solution and 15 μ l of crude dynein extract. After incubation for 30 min at 30°C or 40°C, the reaction was stopped by boiling the mixture. The concentration of the remaining ATP was assayed by the method of Wishart (1982). Protein concentrations of crude dynein extract were determined using the method of Bradford (1976), with BSA as a standard. The rate of ATPase activity was expressed in terms of nmol ATP hydrolysis mg^{-1} protein min^{-1} .

Statistical analysis

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

Results

Effects of pH_e on the pH_i of intact fowl spermatozoa

The pH_i of intact spermatozoa, estimated by measuring the accumulation of 9-aminoacridine fluorescence, increased with increasing pH_e at both 30°C and 40°C. Regression equations were $y = 0.42x + 4.58$ ($r = 0.94$) at 30°C and $y = 0.46x + 4.00$ ($r = 0.93$) at 40°C. Both correlation coefficients were significant ($P < 0.01$); that is, at the same pH_e value (about 7.0–8.5), acidification of pH_i of spermatozoa by 0.25–0.30 pH units was observed at 40°C compared with the pH_i at 30°C (Fig. 1).

Effects of pH_e on the motility of intact and demembrated fowl spermatozoa

At 30°C, the motility of intact spermatozoa was high in medium with the wide pH_e range of 7.3–10.1. In contrast, intact spermatozoa were almost immotile at 40°C in a medium

Fig. 1. The correlation between pH_e and pH_i of intact fowl spermatozoa at (○) 30°C and (●) 40°C. Each point represents the mean ($\pm$ SEM) of five samples of spermatozoa.

below a pH_e of 8.1. However, the motility could be restored by increasing the pH_e . Maximum motility (67%) was obtained in a medium at a pH_e of 9.4 (Fig. 2a).

Stimulation of the motility of demembrated spermatozoa at 40°C was also observed if the pH_e was increased (Fig. 2b). However, demembrated spermatozoa at 40°C that had been stimulated by increasing the pH_e to 9.4 lost their motility when 1 mmol $CaCl_2$ l^{-1} was added. Motility was restored by the subsequent addition of 2 mmol EGTA l^{-1} (Fig. 3).

Furthermore, unlike mammalian spermatozoa (Goltz *et al.*, 1988), the addition of 4 mmol dibutyl cAMP l^{-1} to intact spermatozoa or 10 μ mol cAMP l^{-1} to demembrated spermatozoa did not appreciably affect their motility in all the ranges of pH_e examined, and similar values to those of control (no dibutyl cAMP or cAMP) were obtained at both 30°C and 40°C (Fig. 4a, b).

Effects of pH_e on the oxygen consumption, ATP concentrations and dynein ATPase activity of fowl spermatozoa

Below a pH_e of 9.4, the rate of oxygen consumption at 30°C and 40°C showed almost constant values (Fig. 5a).

The ATP concentrations at 30°C were fairly constant in the pH_e range of 7.3–10.1. In contrast, an increased pH_e at 40°C gradually decreased the ATP concentrations of spermatozoa (Fig. 5b).

The flagellar ATPase activity of crude dynein extract was stimulated by an increase in pH_e at both 30°C and 40°C, except at a pH_e of 9.8 at 40°C, although the activity at 40°C was higher than that at 30°C in all the ranges of pH_e examined (Fig. 5c). Furthermore, at a high pH_e of 9.4, this activity was not altered by the addition of 1 mmol $CaCl_2$ l^{-1} (59.8 ± 2.6 nmol ATP hydrolysis mg^{-1} protein min^{-1} ; mean $\pm$ SEM of five

Fig. 2. Motility of (a) intact and (b) demembrated fowl spermatozoa at different pH_e values at (○) 30°C and (●) 40°C. Each point represents the mean ($\pm$ SEM) of five samples of spermatozoa. * $P < 0.01$ compared with value at pH_e 7.3 at 30°C or pH_e 7.1 at 40°C.

Fig. 3. Motility of demembrated fowl spermatozoa at 40°C at (○) pH_e 7.6 and (●) pH_e 9.4. At arrows, 1 mmol $CaCl_2$ l^{-1} and 2 mmol EGTA l^{-1} were added. Each point represents the mean ($\pm$ SEM) of five samples of spermatozoa. * $P < 0.01$ compared with value at 0 min.

samples) or by the addition of 1 mmol $CaCl_2$ l^{-1} followed by 2 mmol EGTA l^{-1} (58.5 ± 3.0) at 40°C compared with the activity when neither $CaCl_2$ nor EGTA was added (59.7 ± 3.2), even though the motility of demembrated spermatozoa was inhibited in the presence of 1 mmol $CaCl_2$ l^{-1} alone and was stimulated in the presence of 1 mmol $CaCl_2$ l^{-1} plus 2 mmol EGTA l^{-1} at 40°C (Fig. 3).

Discussion

Gatti *et al.* (1991, 1993) demonstrated a linear relationship between pH_i and pH_e for ejaculated boar and ram spermatozoa.

Fig. 4. Motility of (a) intact fowl spermatozoa at different pH_e values at (□, ○) 30°C and (■, ●) 40°C (■, □) with or (○, ●) without 4 mmol dibutyryl-cAMP l^{-1} , and (b) demembrated fowl spermatozoa at different pH_e values at (□, ○) 30°C and (■, ●) 40°C (□, ■) with or (○, ●) without 10 μ mol cAMP l^{-1} . Each point represents the mean ($\pm$ SEM) of five samples of spermatozoa. At both 30°C and 40°C, no significant difference was observed between motility in the presence and absence of dibutyryl cAMP or cAMP at each pH_e .

In the present study, we also showed that the pH_i of ejaculated fowl spermatozoa increases with increasing pH_e at both 30°C and 40°C. However, while the pH_i changed by only approximately 1.3 pH units, the pH_e was changed by 3.0 units. Under these conditions, at the same pH_e value, the pH_i at 40°C was 0.25–0.30 units lower than that at 30°C. This suggests that such an acidification might play some role in the temperature-dependent immobilization of fowl spermatozoa, since an increase in the pH_i by 0.36 units upon dilution of sea urchin semen into seawater is sufficient to trigger the initiation of sperm motility (Johnson *et al.*, 1983).

Our results also indicate that the temperature-dependent immobilization of intact fowl spermatozoa at 40°C was inhibited by a high pH_e , although the motility could be restored in a medium above pH_e 8.5. However, the mechanism by which changes in pH stimulate sperm motility is not known. As the pH_e is raised, a much larger stimulation of respiration of sea urchin spermatozoa occurs with a given amount of H^+ efflux (Repaske and Garbers, 1983). Both respiration and motility of sea urchin and bovine cauda epididymal spermatozoa increase, when the pH_i is high (Christen *et al.*, 1982; Babcock *et al.*, 1983). However, in the present study, the rate of oxygen consumption at 40°C showed almost constant values below pH_e 9.4, in spite of motility being stimulated at this pH_e value. Under these conditions, the concentration of ATP in spermatozoa gradually decreased with an increased pH_e , suggesting

Fig. 5. (a) Oxygen consumption, (b) ATP concentration and (c) ATPase activity of crude dynein extract of fowl spermatozoa at different pH_e values at (○) 30°C and (●) 40°C. Each point represents the mean (± SEM) of five samples of spermatozoa. **P* < 0.01 compared with value at pH_e 7.3 at 30°C or pH_e 7.1 at 40°C.

that the concentration of ATP probably reflects coupling of ATP hydrolysis by the axoneme to the rate of respiration and oxidative phosphorylation.

Stimulation of the motility of demembrated fowl spermatozoa was also observed with an increased pH_e, that is, pH_i, suggesting that the effects of the increased pH_i may act directly on the axoneme. If the pH_i affects the axoneme directly, then the question of its locus of action is raised. The coupled activation of respiration and motility through an increased pH_i seems to be due to the activation of dynein ATPase (Christen *et al.*, 1983). We also showed that the flagellar ATPase activity of crude dynein extract was stimulated by an increased pH_e at both 30°C and 40°C. Nevertheless, this rise in ATPase activity may not be a direct result of the inhibition of temperature-dependent immobilization of fowl spermatozoa at 40°C, since the motility of both intact and demembrated fowl spermatozoa was almost negligible at 40°C between pH_e 7.1 and 8.1, even though the ATPase activity at 40°C was more than two times higher than that at 30°C. Furthermore, the magnitude of dynein ATPase activity at pH_e 9.4 did not decrease with the addition of Ca²⁺ at 40°C, although the motility of demembrated spermatozoa was inhibited. These data suggest that the action of the increased pH_i may not be directly on dynein ATPase activity, but may rather be mediated by other substance(s) on the axoneme.

Vijayaraghavan *et al.* (1985) provided evidence that a developmental increase in pH_i, in addition to a rise in the intracellular concentration of cAMP, is required for the expression of motility in bovine caput epididymal spermatozoa. Morisawa *et al.* (1993) proposed that the increases in seminal

pH affect membranous adenylate cyclase, which synthesizes cAMP, and this may be a factor inducing the acquisition of sperm motility in salmonid fishes. As similar results have been obtained in porcine spermatozoa (Tajima *et al.*, 1987), it seems likely that intracellular cAMP is the primary target for regulation of sperm motility by pH_i in these species. In fact, Goltz *et al.* (1988) demonstrated that the motility of demembrated bull spermatozoa without added cAMP is strongly dependent on alkaline pH (pH > 7.5), whereas in the presence of cAMP vigorous motility persists even at acidic pH (as low as pH 6.8); this suggests that cAMP-dependent activation is crucial for motility at low pH (pH 6.4–7.1), which is the actual pH_i of bull spermatozoa. In contrast, Babcock *et al.* (1983) suggested that cAMP may not be involved in initiating the motility of bovine cauda epididymal spermatozoa through an increase in pH_i, as no significant difference in cAMP-dependent protein kinase activity was observed between pH 6.8 and 7.8. The results presented here support Babcock's hypothesis, since the motility of both intact and demembrated fowl spermatozoa was not appreciably affected by dibutyl cAMP or cAMP, and sperm motility was inhibited at low pH_e but activated at higher pH_e at 40°C.

It should be noted that Ca²⁺ is indispensable for the maintenance of motility of demembrated fowl spermatozoa at 30°C (Ashizawa *et al.*, 1992). In contrast, when the pH of reactivation medium is increased, the following observations were made: spermatozoa that had been stimulated by increasing the pH at 40°C lost their motility when 1 mmol CaCl₂ l⁻¹ was added. Motility was restored by the subsequent addition of 2 mmol EGTA l⁻¹. The reason for this is not well

understood, but similar results were obtained by adding calyculin A or okadaic acid, specific inhibitors of protein serine–threonine phosphatases (K. Ashizawa, G. J. Wishart, H. Tomonaga, K. Nishinakama and Y. Tsuzuki, unpublished). It is therefore possible that the increased pH_i may act directly on axonemal phosphoprotein(s), mediated by a Ca^{2+} -related substance(s). Carr and Acott (1989) found at least four proteins from bovine spermatozoa that have altered phosphorylation states in response to pH_i modulations in a cAMP-independent manner. Additional studies are therefore necessary to examine whether such a phosphoprotein(s) is involved in the stimulation of fowl sperm motility by an increasing pH_i .

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