Effects of taurine on the motility and intracellular free Ca^{2+} concentration of fowl spermatozoa *in vitro*

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The effects of taurine on the motility and intracellular free Ca^{2+} concentration of fowl spermatozoa were investigated *in vitro*. The addition of taurine, within the range of 0–5 mmol l^{-1} , did not appreciably affect the motility of intact fowl spermatozoa. Motility remained almost negligible at 40°C, while vigorous movement was observed at 25°C. Even with the addition of Ca^{2+} before the addition of taurine, neither stimulation nor inhibition of motility was observed compared with the control (no addition of taurine). Similar results were obtained by the addition of taurine and calyculin A, a specific inhibitor of protein phosphatases. There were no changes in intracellular free Ca^{2+} concentrations, measured by a fluorescent Ca^{2+} indicator, fura-2, in taurine-treated spermatozoa. These results suggest that taurine is not involved in the regulation of fowl sperm motility and metabolism by intracellular Ca^{2+} mobilization *in vitro*.

Introduction

The mechanism of storage within and release of spermatozoa from the sperm storage tubules (SSTs) is unknown. These phenomena are probably related to changes in sperm motility. Therefore *in vitro* studies have been performed to elucidate the physiological or biochemical processes that might influence the motility of fowl spermatozoa *in vivo* in the SSTs.

Spermatozoa survive without damage for days or weeks in the SSTs (van Drimmelen, 1946; Fujii and Tamura, 1963; Bobr *et al.*, 1964; Takeda, 1965). Lake (1975) suggested that substrates for maintenance, if provided, probably reach spermatozoa by diffusion from secretions of the cells of the SST, although there is no direct experimental evidence to support this assumption. Ashizawa *et al.* (1976) reported that fowl spermatozoa incubated at 41°C with cultured cells from the oviduct were almost immotile; however, at room temperature, their motility and fertilizing ability were maintained for several days *in vitro*. Furthermore, it is assumed that a low molecular weight factor(s) supplied by cultured cells effectively prolongs their motility (Ashizawa *et al.*, 1985; Ashizawa and Katayama, 1992).

Unlike mammalian spermatozoa, fowl spermatozoa show definitive temperature-dependent changes in their motility. In most synthetic diluents, spermatozoa become immotile at the avian body temperature of 40–42°C and it has been proposed that similar conditions may be responsible for their immobility and their prolonged survival in the SSTs (Munro, 1938; Ashizawa and Nishiyama, 1978; Wishart, 1989). Sperm motility at 40°C can be restored by decreasing the temperature (Munro, 1938; Ashizawa and Nishiyama, 1978;

Takeda, 1982; Ashizawa *et al.*, 1989; Thomson and Wishart, 1989) or by the addition of Ca^{2+} (Wishart and Ashizawa, 1987).

Taurine (2-aminoethanesulfonic-acid) is an acidic amino acid that is not involved in protein synthesis. Taurine and hypotaurine are the most concentrated of the free amino acids in the oviductal fluids of hens during the egg formation cycle. The maximum concentration of taurine in the oviduct was 3.26 mmol l^{-1} before oviposition and the minimum 0.36 mmol l^{-1} just after oviposition (Barna *et al.*, 1996). The question was raised of whether the concentration changes of taurine in the oviductal fluid are related to motility changes of fowl spermatozoa *in vivo* during their storage and release.

Taurine and hypotaurine are also present at high concentrations both in seminal plasma and in the oviductal secretions of other species (Gregoire *et al.*, 1961; Fahning *et al.*, 1967; Johnson *et al.*, 1972; Ibrahim and Boldizsár, 1981; Hernvann *et al.*, 1986). They are also known to be involved in capacitation, acrosome reaction and penetration (Van der Horst and Brand, 1971).

Results of several studies support the contention that taurine may act as a specific neurotransmitter in the mammalian central nervous system (CNS) (Shaw and Heine, 1965; Collins and Topiwals, 1974; Schmid *et al.*, 1975). Furthermore, evidence suggesting a modulator role of taurine for membrane excitability such as increased mitochondrial calcium uptake in the presence of taurine (Dolara *et al.* 1973b; Dolara *et al.*, 1973a) and enhancement of potassium transport by taurine (Dolara *et al.*, 1973a) have also been reported. According to Kuriyama (1980), taurine may not act as a neurotransmitter but as a modulator for membrane excitability by decreasing the concentration of intracellular free Ca^{2+} . If it acts in a similar way in fowl spermatozoa, it is possible that taurine would decrease sperm motility by decreasing the intracellular free calcium

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Revised manuscript received 27 May 1998.

content during egg formation, when its concentration is high and that as a result spermatozoa remain almost immotile in the SSTs. After oviposition, at a low taurine concentration, the intracellular calcium content of spermatozoa increases again, which activates sperm release from the SSTs. The presumed effect of taurine would also affect spermatozoa that are in the vicinity of the orifices of the SSTs.

However, taurine and hypotaurine support the motility of hamster spermatozoa *in vitro* (Mrsny *et al.*, 1979; Boatman *et al.*, 1990). No details of the precise molecular mechanism by which they exert their effects on sperm motility and viability were given, although taurine and hypotaurine have been shown to protect against loss of motility by inhibiting lipid peroxidation in rabbit spermatozoa (Alvarez and Storey, 1983). Taurine has also been shown to protect cells, including mammalian spermatozoa, from osmotic stress by stabilizing cell membranes (Osaza and Gould, 1982). These conflicting reports related to taurine indicate that it has several physiological functions in different animal species and justify the objective of this study: to determine the role played by taurine in the intracellular Ca^{2+} mobilization of fowl spermatozoa and resultant changes in sperm metabolism and motility. Elucidation of this question may provide new information about the mechanism of sperm storage and release in the hen oviduct, since these processes are likely to involve changes in sperm motility.

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 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 15 min at room temperature (20–25°C). The washed spermatozoa were reconstituted in the same buffers 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 a screw cap.

Chemicals

TES and Triton X-100 were purchased from Sigma Chemical Co., St Louis, MO. Calyculin A was obtained from Wako Pure Chemical Industries, Ltd., Osaka. 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) was purchased from Dojindo Laboratories Inc., Kumamoto. Taurine and other chemicals were of reagent grade from Nacalai Tesque Inc., Kyoto.

Analysis of sperm motility

Sperm samples were preincubated aerobically in a shaking water bath at 25°C or 40°C for 10 min. After the preincubation, the dose-response of motility of intact spermatozoa was measured at 25°C or 40°C after addition of various concentrations of taurine. The effects of the addition of CaCl_2 or calyculin A, an inhibitor of protein phosphatase-type 1 and -type 2, before or after the addition of taurine, were also examined at 25°C and 40°C. Diluent for the measurement of sperm motility was TES–NaCl buffer (pH 7.4) described above.

The suspension of spermatozoa was placed into a microscope slide chamber (Sekisui Chemical Co., Ltd, UR-157 type, Tokyo) on a thermostatically controlled warm plate, and the motility of spermatozoa was recorded by video microscopy (magnification on the 12 inch black and white monitor was approximately $\times 600$) at 25°C or 40°C (Katz and Overstreet, 1981). Measurements were made on a total of 200–300 spermatozoa, distributed uniformly among three or more fields, to determine the percentage of motility.

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

Determination of the intracellular free Ca^{2+} concentrations was carried out with the fluorescent indicator fura-2, essentially according to Grynkiewicz *et al.* (1985), but with some modifications (Ashizawa *et al.*, 1992). Fluorescence intensity was measured with a dual-wavelength spectrofluorometer (Shimadzu, Model RF-5000, Kyoto) with the sample chamber regulated at 25°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 Ca^{2+} concentrations, the excitation wavelength was set to 340 and 380 nm, and the emission wavelength was set to 500 nm. The ratio (340:380 nm) of fluorescence intensity was monitored continuously. The calculation of intracellular free Ca^{2+} was based upon the equation described by Grynkiewicz *et al.* (1985), in which the dissociation constant (K_d) was 224 nmol l^{-1} .

Statistical analysis

Percentage motility was transformed using arc sine transformation. The results were analysed by Duncan's multiple-range tests (Duncan, 1955).

Results

Effects of taurine on the motility of fowl spermatozoa

The vigorous motility of intact fowl spermatozoa at pH 7.4 was not inhibited or stimulated after the addition of taurine at 25°C, within the range 0–5 mmol l^{-1} . At 40°C, the addition of taurine did not appreciably affect motility of spermatozoa, and sperm motility remained almost negligible (Fig. 1).

Fig. 1. Motility of fowl spermatozoa at pH 7.4 after addition of various concentrations of taurine at (●) 25°C and (○) 40°C. Each point represents the mean ($\pm$ SEM) of five samples of spermatozoa. There was no significant difference in sperm motility in the presence or absence of taurine at either 25°C or 40°C.

No stimulation or inhibition of motility of intact spermatozoa was observed even in the presence of CaCl_2 before the addition of taurine at 25°C (Fig. 2a). At 40°C, the motility of intact spermatozoa was negligible, but the motility was restored immediately after the addition of

2 mmol CaCl_2 1^{-1} . However, subsequent addition of taurine did not affect the motility (Fig. 2b). The presence of calyculin A did not appreciably affect the motility at 25°C (Fig. 3a), but permitted restoration of motility of intact spermatozoa at 40°C, and this effect lasted for at least 30 min. However, stimulation of motility by calyculin A was not inhibited by the subsequent addition of taurine (Fig. 3b).

Effects of taurine on the intracellular free Ca^{2+} concentrations of fowl spermatozoa

Intracellular free Ca^{2+} concentrations at pH 7.4, measured by the fluorescent Ca^{2+} indicator fura-2, increased rapidly after the addition of 2 mmol CaCl_2 1^{-1} at 25°C and 40°C. Subsequently, the addition of taurine maintained almost the same Ca^{2+} concentration in the spermatozoa as that of the control (no addition of taurine) (Fig. 4).

Discussion

The effects of the addition of CaCl_2 or calyculin A before and after the addition of taurine on sperm function were

Fig. 2. Motility of fowl spermatozoa at pH 7.4 after addition (at arrows) of (●) 2 mmol CaCl_2 1^{-1} and (○) 5 mmol taurine 1^{-1} at (a) 25°C and (b) 40°C. Each point represents the mean ($\pm$ SEM) of five samples of spermatozoa. At both 25°C and 40°C, there was no significant difference in the presence or absence of taurine at each incubation period.

Fig. 3. Motility of fowl spermatozoa at pH 7.4 after addition (at arrows) of (●) 100 nmol calyculin A 1^{-1} and (○) 5 mmol taurine 1^{-1} at (a) 25°C and (b) 40°C. Each point represents the mean ($\pm$ SEM) of five samples of spermatozoa. At both 25°C and 40°C, there was no significant difference between the presence or absence of taurine at each incubation period.

Fig. 4. Intracellular free Ca^{2+} concentrations of fowl spermatozoa at pH 7.4 after addition (at arrows) of 2 mmol $\text{CaCl}_2 \text{ l}^{-1}$ and taurine (0 and 5 mmol l^{-1}) at (a) 25°C and (b) 40°C, measured by means of a fluorescence Ca^{2+} indicator fura-2.

investigated. Similar results were obtained: the addition of taurine did not appreciably affect the function of spermatozoa.

The neurotransmitter amino acids (aspartic acid, glutamic acid, glycine and gamma amino butyric acid (GABA)) are known to affect the transport of Na^+ , K^+ and Cl^- in the CNS and in other organs (Erdő, 1991; Boldizsár *et al.*, 1991) and thus affect the membrane potential of the cells and by this presumably – in the case of spermatozoa – their motility. We had previously proposed that neurotransmitter free amino acids found in the hen oviduct, which altered motility of fowl spermatozoa *in vitro* (Barna and Boldizsár, 1996), might also have a role in the sperm inactivation–reactivation processes *in vivo* during their storage and release in the oviduct. This action may occur via transmitter functions, by change of the pH of the environment, or by decreasing the concentration of intracellular free Ca^{2+} (Kuriyama, 1980).

Calcium is acknowledged to be a major regulator of sperm physiology, including motility (Morton *et al.*, 1974), membrane function (Bradley and Forrester, 1980) and metabolism (Peterson and Freund, 1976). It is involved in capacitation, acrosome reaction and fertilization in different species (Van der Horst and Brand, 1971; Johnson *et al.*, 1972; Yanagimachi and Usui, 1974). The role of the intra- and extracellular Ca^{2+} in the motility and respiration of fowl spermatozoa was also reported in several studies (Ashizawa

and Wishart, 1987; Wishart and Ashizawa, 1987; Thomson and Wishart, 1989; Ashizawa *et al.*, 1992, 1994a,b; 1995). On the basis of studies on the action of Ca^{2+} in the CNS, we presumed that taurine may decrease the intracellular free Ca^{2+} content of fowl spermatozoa and thus reduce their motility, although taurine was also known to stimulate mammalian sperm motility (Mrsny *et al.*, 1979; Boatman *et al.*, 1990). In the present investigations neither of the roles of taurine could be verified since under the described *in vitro* conditions even 5 mmol taurine l^{-1} did not affect intracellular free calcium or motility of spermatozoa.

Although we could not demonstrate a direct effect of taurine on these parameters, its high concentrations in the oviduct during egg shell formation and the decreased concentration at the time of oviposition and ovulation (Barna *et al.* 1996) indicate that taurine does have a function.

This study was supported by a Grant-in-Aid for Scientific Research from the Ito Memorial Foundation and by a grant from the Ministry of Education, Science and Culture, Japan (No. 08660348).

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