Penetration by field vole spermatozoa of mouse and hamster zonae pellucidae without acrosome reaction

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Spermatozoa of the field vole (*Microtus montebelli*) that bound to the zona pellucida of field vole oocytes underwent the acrosome reaction before passing through it. In contrast, vole spermatozoa that bound to the zonae of mouse and hamster oocytes penetrated the zonae without any sign of the acrosome reaction. The presence or absence of proteinase/hyaluronidase inhibitors in the medium did not make any difference to zona penetration by acrosome-intact vole spermatozoa. These observations suggest that field vole spermatozoa can cross mouse and hamster zonae mechanically without assistance from zona-hydrolysing enzymes.

Introduction

Mammalian oocytes are each surrounded by a glycoprotein coat, the zona pellucida, which protects fragile oocytes and developing preimplantation embryos during their descent through the oviduct. Under natural conditions, the fertilizing spermatozoon must cross the zona before reaching and fusing with the plasma membrane of the oocyte. The mechanism by which spermatozoa penetrate through the zona is not fully understood. While it is a popular notion that acrosomal enzymes (for example, acrosin) tightly bound to the inner acrosomal membrane of acrosome-reacted spermatozoa depolymerize zona glycoproteins (for example, O'Rand *et al.*, 1986), the vigorous thrusting motion of the sperm head may play an equally important role in this process (Bedford, 1991; Yanagimachi, 1994).

We have reported that the spermatozoa of the field vole penetrate not only the zona of homologous species (Wakayama *et al.*, 1995a, b), but also zonae of heterologous species such as mice and hamsters (Wakayama *et al.*, 1993). Here, we report that while field vole spermatozoa cross the vole zonae after completing the acrosome reaction, they pass through mouse and hamster zonae without the acrosome reaction.

Materials and Methods

Animals

The field vole (*Microtus montebelli*) used in this study has been bred in the Japan's National Institute of Animal Health at Tsukuba for over 20 years. It is a herbivorous rodent that can

be used as an animal model for various physiological studies of herbivorous farm animals (Goto *et al.*, 1977; Goto and Hashizume, 1978; Kudo and Oki, 1984; Imai and Ogimoto, 1988; Obara and Goto, 1988). Voles were maintained in a temperature-controlled (25°C) room with 14 h light:10 h dark cycles. Females were used before reaching puberty (< 4 weeks after birth). Mice (ICR) and the golden hamsters maintained under the same conditions as described above were used after full maturity (> 2 and 3 months of age, respectively).

Reagents

All inorganic and organic compounds were purchased from Sigma Chemical Co. (St Louis, MO) unless otherwise stated. Myocrisin (gold sodium thiomaleate; hyaluronidase inhibitor) was the gift from Merck Sharp and Dohme Research Laboratory (Rahway, NJ). Soybean trypsin inhibitor (SBTI) and leupeptin (Wako Chemical, Tokyo) were used as proteinase inhibitors.

Medium

A modified Krebs'–Ringer's solution, mKRB (Toyoda and Chang, 1974), was used for handling vole oocytes and spermatozoa. In some experiments this medium was supplemented with 1 mmol hypotaurine l⁻¹. TYH medium (Toyoda *et al.*, 1971) was used for insemination of mouse oocytes with mouse spermatozoa.

Preparation of oocytes

Immature female voles and mature female mice and hamsters were induced to superovulate by consecutive

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injections of pregnant mares' serum gonadotrophin (PMSG) and hCG at an interval of 48 h. The doses of PMSG and hCG were 30 iu and 30 iu for the vole and hamster, and 5 iu and 5 iu for the mouse, respectively. Mature unfertilized oocytes of field voles and mice were collected from oviducts 13–16 h after hCG injection. Hamster oocytes were collected 15–17 h after hCG injection. Mouse zygotes (pronuclear eggs) and two-cell embryos were collected from oviducts of mated, superovulated mice 14 h and 38 h after hCG injection, respectively. Eggs (oocytes with cumulus cells, zygotes or two-cell embryos) were collected from oviductal ampullae and transferred into droplets (100 µl) of fresh mKRB which had been previously placed under mineral oil in a plastic Petri dish (35 mm diameter). The medium in the dish had been equilibrated with 5% CO₂ in air for at least 2 h at 37°C before use.

Preparation of spermatozoa

Vole and mouse spermatozoa were collected from caudae epididymides of mature males. The distal portion of each epididymis was cut using a blade and a dense sperm mass was squeezed out of the epididymis. Spermatozoa were allowed to disperse into 200 µl mKRB. The concentration of spermatozoa at this stage was approximately 1 × 10⁸ cells ml⁻¹. This sperm suspension was used for insemination immediately after preparation.

Insemination and examination of oocytes, zygotes or embryos in vitro

Insemination was performed by adding 1–10 µl of vole sperm suspension to 100 µl mKRB containing either vole or hamster oocytes or mouse oocytes, zygotes and two-cell embryos. Final sperm concentrations in the insemination medium were approximately either 1 × 10⁶ cells ml⁻¹ or 10 × 10⁶ cells ml⁻¹. In some experiments, hyaluronidase/

Fig. 1. Spermatozoa of the mouse (left) and the Japanese field vole (right). The field vole spermatozoon is slightly smaller than the mouse spermatozoon. Scale bar represents 5 µm.

proteinase inhibitors were incorporated into the insemination media. After incubation for 6 h at 37°C under 5% CO₂ in air, inseminated oocytes, zygotes or embryos were removed from the dishes and examined under a phase-contrast microscope for the presence or absence of spermatozoa within the perivitelline space. They were then fixed with 2.5% (v/v) glutaraldehyde in 0.1 mol phosphate buffer l⁻¹ for 2 h, stained with 0.25% (v/v) acetolacmoid (Toyoda *et al.*, 1971) and examined for evidence of sperm penetration into the ooplasm. Some mouse oocytes were fixed with 2.5% (v/v) glutaraldehyde for 2 h and processed for electron microscopy.

In a series of experiments, mouse oocytes that had been penetrated by vole spermatozoa were reinseminated with mouse spermatozoa. Mouse oocytes were first inseminated with field vole spermatozoa. Two hours later the oocytes with vole spermatozoa within their perivitelline space were selected under a dissecting microscope, washed with TYH medium and

Table 1. Penetration of field vole spermatozoa into field vole and mouse oocytes

Oocytes of	Concentration of vole spermatozoa in insemination medium × 10 ⁶ ml ⁻¹	Presence (+) or absence (-) of 1 mmol hypotaurine l ⁻¹ in medium	Total number of oocytes inseminated (Expt number)	Number (%) of oocytes with spermatozoa in:	
				Perivitelline space ^a	Vitellus ^b
Vole	1	—	69 (5)	33 (47.8)	33 (47.8)
		+	70 (5)	60 (85.7)	60 (85.7)
	10	—	68 (5)	15 (22.1)	1 (1.5)
		+	73 (5)	14 (19.2)	1 (1.4)
Mouse	1	—	93 (5)	15 (16.1)	0
		+	87 (5)	10 (11.5)	0
	10	—	90 (5)	87 (96.7)	0
		+	105 (5)	99 (94.3)	0

^a*P* < 0.05, low versus high concentrations of spermatozoa in the vole and mouse; presence versus absence of hypotaurine in the vole for low concentration of spermatozoa.
^b*P* < 0.05, low versus high concentrations of spermatozoa in the vole.

Fig. 2. (a) Field vole spermatozoon entering the zona pellucida of field vole oocyte 3.5 h after insemination. The sperm head is already within the zona. An arrow indicates the acrosomal ghost left on the zona. Scale bar represents 5 μm . (b) Semi-longitudinal section of field vole sperm head within the perivitelline space. An arrow indicates the inner acrosomal membrane of acrosome-reacted spermatozoon in the perivitelline space. V: oocyte vitellus; ZP: zona pellucida. Scale bar represents 1 μm .

Fig. 3. (a) Mouse oocytes inseminated with field vole spermatozoa 6 h after insemination. Scale bar represents 70 μm . (b) Mouse oocyte showing field vole spermatozoa within the perivitelline space. Scale bar represents 25 μm .

reinseminated with fresh mouse spermatozoa in 100 μl TYH medium. The concentration of mouse spermatozoa in reinsemination medium was 1×10^6 cells ml^{-1} . The oocytes were re-examined 6 h later.

Statistical analyses

Each experiment was repeated 2–7 times. The data were analysed with ANOVA (Table 1) or chi-squared tests (Tables 2,

3 and 4). In Table 1 and 2, data for mouse oocytes and vole oocytes were analysed separately.

Results

The outer diameter and thickness of the zona pellucida of a mature field vole oocyte are about 90 μm and 9–9.5 μm , respectively. Corresponding values for mouse and hamster

zonae are about 100 μm and 7.5 μm (mouse) and 115 μm and 13 μm (hamster), respectively. In other words, field vole zonae are smaller and thicker than mouse zonae and smaller and thinner than hamster zonae. The vole spermatozoon is smaller than the mouse spermatozoon (Fig. 1). The width (including hook) and length of sperm head and the length of tail are approximately: 3.0 μm , 7.5 μm and 110 μm for the vole; 3.5 μm , 8 μm and 120 μm for the mouse; and 7 μm , 9 μm and 180 μm for the hamster.

The results of experiments in which vole and mouse oocytes were inseminated *in vitro* with vole spermatozoa are summarized (Table 1). Upon insemination, vole spermatozoa bound to the zona surfaces of both vole and mouse oocytes. Vigorous pipetting could not remove them.

The largest population of vole oocytes were fertilized when the insemination medium contained 1 mmol hypotaurine l^{-1} and sperm concentration was approximately 1×10^6 cells ml^{-1} . The presence or absence of hypotaurine and the concentration of the spermatozoa each significantly affected the fertilization rate in voles ($P < 0.05$). Zona penetration by spermatozoa began about 2 h after insemination. Acrosomal ghosts left by the penetrating spermatozoa were seen on zona surfaces (Fig. 2a). As far as we could observe ($n = 50$), the spermatozoa within the zona and in the perivitelline space were all acrosome-reacted (Fig. 2b). The relatively thin zona in the electron micrographs, as compared with the sperm head, appears to be due to zona shrinkage that occurs during dehydration and embedding of specimens.

Vole spermatozoa that bound to mouse zona pellucida (Fig. 3a) could pass through it (Fig. 3b), but they failed to penetrate into (fuse with) the vitellus of mouse oocytes (Table 1). The incidence of mouse zona penetration by vole spermatozoa was dependent on sperm concentration ($P < 0.05$), but not on the presence or absence of hypotaurine. The acrosomal status of several thousand spermatozoa could be observed ultrastructurally from about 300 mouse oocytes. Vole spermatozoa within the mouse zona (Fig. 4a) and in the perivitelline space (Fig. 4b) all had intact acrosomes, regardless of the concentration of spermatozoa. The presence or absence of hyaluronidase/proteinase inhibitors in the insemination medium had no significant effect on mouse zona penetration by vole spermatozoa (Table 2). In the homologous insemination these inhibitors apparently decreased the incidence of penetration of spermatozoa through the zona ($P < 0.005$) (Table 2). Vole spermatozoa also passed through the zonae of 10 (16%) of 61 hamster oocytes, without acrosome reaction, and never fused with the oocytes (data not shown). Vole spermatozoa could also penetrate the zonae of mouse zygotes and two-cell embryos (Fig. 5), although the zonae of two-cell embryos were less penetrable by spermatozoa than those of oocytes and zygotes ($P < 0.005$) (Table 3).

As mentioned, vole spermatozoa that had penetrated zonae could not penetrate into (fuse with) mouse vitellus. Mouse oocytes with vole spermatozoa within the perivitelline space were reinseminated with mouse spermatozoa. They penetrated both the zonae and vitelli of the majority of these oocytes (Table 4). The rates of fertilization (vitellus penetration) and polyspermy were not significantly different from those in oocytes without vole spermatozoa in the perivitelline space ($P > 0.05$).

Fig. 4. (a) Frontal section of an acrosome-intact field vole sperm head in the mouse zona pellucida. Scale bar represents 1 μm . (b) Transverse section of an acrosome-intact field vole spermatozoon in the perivitelline space of a mouse oocyte. An arrow indicates the intact acrosome. Scale bar represents 1 μm . The thinness of the zona pellucida is probably due to shrinkage during dehydration and embedding of specimens. V: oocyte vitellus; ZP: zona pellucida.

Discussion

The present study demonstrates that field vole spermatozoa, like the spermatozoa of other mammals, undergo the acrosome reaction before penetrating the zona pellucidae of homologous oocytes, whereas they cross the zonae of other species, the mouse and hamster, without undergoing the reaction. It was not surprising that such spermatozoa could not fuse with the mouse oocyte vitellus because acrosome-intact spermatozoa

Table 2. Penetration of vole spermatozoa through mouse and vole zona pellucida in the presence of proteinase/hyaluronidase inhibitors

Oocytes of	Inhibitor	Concentration	Total number of oocytes examined (Expt number)	Number (%) of eggs with spermatozoa in the perivitelline space
Vole	Leupeptin	0.1 mmol l ⁻¹	15 (2)	0 (0) ^a
	SBTI	0.1 mg ml ⁻¹	11 (2)	0 (0) ^a
	Myocristin	0.1 mg ml ⁻¹	20 (2)	3 (15.0) ^a
	None (Control)	—	15 (2)	11 (73.3) ^b
Mouse	Leupeptin	1 mmol l ⁻¹	72 (3)	63 (87.5)*
	SBTI	1 mg ml ⁻¹	68 (3)	61 (89.7)*
	Myocristin	1 mg ml ⁻¹	73 (3)	64 (87.7)*
	None (Control)	—	105 (3)	91 (86.7)*

^a versus ^b: $P < 0.005$.*No significant difference between values with asterisks ($P > 0.05$).

SBTI: Soybean trypsin inhibitor.

Fig. 5. Mouse two-cell embryo with field vole spermatozoa within the perivitelline space. Scale bar represents 25 μ m.

cannot fuse even with homologous oocyte plasma membranes (Yanagimachi, 1988).

Zona penetration by spermatozoa is not strictly species-specific as evidenced by the occurrence of hybrids between closely related species (Gray, 1954), but in general spermatozoa of remote species cannot bind to and penetrate through zonae of other species. Evolutionary distance, however, is not necessarily related to the success or failure of sperm–zona binding or zona penetration by spermatozoa (Yanagimachi, 1994). Ram spermatozoa, for example, can penetrate through bovine zonae (Slavik *et al.*, 1990); goat spermatozoa can cross bovine zonae (Slavik and Fulka, 1992) and human spermatozoa can penetrate through hamster zonae (Yoshimatsu *et al.*, 1988). In none of above cases, however, was the acrosomal status of penetrating spermatozoa investigated, perhaps due to the

Table 3. Penetration of field vole spermatozoa through zonae pellucidae of mouse oocytes, zygotes and two-cell embryos

	Total number of eggs inseminated (Expt number)	Number (%) of eggs with spermatozoa in the perivitelline space
Mouse eggs		
Unfertilized	216 (7)	199 (92.1) ^a
Zygote	197 (7)	185 (93.9) ^a
Two-cell	173 (7)	90 (52.0) ^b

^a versus ^b: $P < 0.005$.**Table 4.** Penetration of mouse spermatozoa into the vitelli of mouse oocytes previously inseminated with field vole spermatozoa

Mouse oocytes with (+) or without (–) vole spermatozoa within perivitelline space	Total number oocytes inseminated (Expt number)	Number (%) of vitelli penetrated	Number (%) of polyspermic eggs
+	103 (5)	85 (82.5) ^a	5 (4.9) ^a
–	74 (5)	58 (78.4) ^b	3 (4.1) ^b

^a versus ^b: $P < 0.05$.

presumption by previous investigators that only acrosome-reacted spermatozoa are able to pass through the zona. The study reported here shows that field vole spermatozoa can cross the zona of mouse and hamster oocytes without first undergoing an acrosome reaction. It is generally assumed that zona penetration by mammalian spermatozoa is achieved by zona-hydrolysing acrosomal enzymes, zona lysins (for example, acrosin), which are presumably bound tightly on the inner acrosomal membrane of acrosome-reacted spermatozoa

(O'Rand *et al.*, 1986). Since the frontal edge of acrosome-reacted sperm head is sharply pointed, a thrusting power (shearing force) generated by the vigorously motile sperm flagellum, which is transmitted to the head, may be sufficient to push through the meshwork of the zona matrix without enzyme activity (Baltz *et al.*, 1988; Bedford, 1991; Yanagimachi 1994). The fact that acrosome-intact vole spermatozoa can penetrate mouse zonae even in the presence of proteinase/hyaluronidase inhibitors seems to suggest that mechanical force is enough for vole spermatozoa to cross the zonae without using hydrolysing enzymes (for example, acrosin and hyaluronidase) that are present on the sperm plasma membrane over the acrosome (Talbot, 1985; Overstreet *et al.*, 1995). We cannot explain why acrosome-intact vole spermatozoa penetrate mouse zonae very efficiently, whereas the same spermatozoa penetrate vole zonae only after undergoing the acrosome reaction. Perhaps mouse zonae are 'softer' than vole zonae and the high concentration of the vole spermatozoa (1×10^7 cells ml^{-1}) may increase the chance of their making slits through the mouse zona. Hamster zonae, which are penetrable by vole spermatozoa but to a lesser degree than mouse zonae, may have intermediate stiffness. This point must be investigated further. What we want to stress in this paper is that under special circumstances spermatozoa can penetrate zonae without the assistance of the acrosome reaction or the presence of hydrolysing enzymes on sperm surfaces. Under ordinary circumstances mechanical force alone is not sufficient for successful zona penetration by spermatozoa, but it must be an essential component of this fertilization process (Yanagimachi, 1994).

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