Induction of physiological acrosome reactions in caput epididymal spermatozoa of mice

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Mouse caput spermatozoa are considered immature and thus unable to fertilize oocytes. In this study, we determined whether washing mouse caput spermatozoa increased their ability to acrosome react in response to a physiological stimulus. The results obtained showed that mouse caput spermatozoa incubated in Earles' modified medium containing calcium chloride and supplemented with BSA and pyruvate for 1 h at 37°C and then washed acrosome reacted in response to both solubilized zonae and immunoaggregation of a zona binding site. In addition, the material removed from caput spermatozoa by washing blocked induced acrosome reactions of cauda spermatozoa. The data indicate that mouse caput epididymal spermatozoa, if incubated and washed, can undergo physiological acrosome reactions.

Introduction

Spermatozoa leaving the testes are immature. During epididymal transit, they undergo a number of structural, biochemical and functional changes resulting in their ability to fertilize. Collectively, these changes constitute epididymal maturation (Orgebin-Crist, 1987; Yanagimachi, 1988). Maturation includes, among other events, the incorporation, release or modification of components of the plasma membrane. The plasma membrane is important in the study of maturation because it is directly involved in a number of significant prefertilization events: capacitation, zona binding, the acrosome reaction and sperm-egg fusion. The plasma membrane is the site of the most prominent changes occurring during epididymal maturation (Yanagimachi, 1981).

In mice, caput spermatozoa are immotile and when incubated under capacitating conditions cannot bind to zonae (Saling, 1982). Cauda spermatozoa, however, can bind to zonae even when their motility is compromised. It was considered that during epididymal maturation the ability to bind was due to an unmasking or transformation of zonae binding components already present (Saling, 1982) or the addition of zonae binding components from epididymal secretions (Peterson *et al.*, 1986). If caput spermatozoa are incubated under capacitating conditions and then washed, their ability to bind zonae is greatly enhanced (McLaughlin and Shur, 1987). During fertilization, spermatozoa bind to the zona and undergo a zona-induced acrosome reaction which facilitates zona penetration. Although caput spermatozoa can undergo ionophore-induced acrosome reactions, they are unresponsive to zona stimulation (Lakoski *et al.*, 1988). These spermatozoa were incubated under capacitating conditions, but not washed. Since washing increases binding ability, we

determined whether the washing would also increase the ability of spermatozoa to undergo zona-induced acrosome reactions.

Mouse caput epididymal spermatozoa, when incubated under capacitating conditions and washed, were shown to acrosome react to a zona stimulus as well as by immunoaggregation of a zona-binding site.

Materials and Methods

Animals

ICR mice, 6–12 weeks of age, were used throughout the study. These animals were subjected to controlled lighting (16 h light:8 h dark) and constant temperature ($22 \pm 2^\circ\text{C}$) and had free access to food and water.

Isolation, capacitation and washing of spermatozoa

The medium (M199-M) used for the isolation of spermatozoa and capacitations was Earles' modified medium 199 (Sigma, St Louis, MO) containing $1.8 \text{ mmol CaCl}_2 \text{ l}^{-1}$ and supplemented with 2 mg BSA ml^{-1} (Sigma) and $30 \mu\text{g pyruvate ml}^{-1}$ (Sigma). The epididymis was divided into segments as proposed by Pavlok (1974). The caput, as used here, consisted of the proximal caput and the proximal portion of the distal caput. The cauda segment consisted of the proximal, middle and distal caudal regions. Caput and cauda regions were removed and placed in separate dishes in M199-M. Spermatozoa were removed from the cauda region by exerting pressure on the tissue, forcing concentrated spermatozoa into the medium. Spermatozoa were removed from the caput region by the technique reported by McLaughlin and Shur (1987). Caput tissue was punctured with a 25-gauge needle and spermatozoa were allowed to

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disperse. Spermatozoa from both regions were filtered through Kimwipes and diluted to $2 \times 10^6 \text{ ml}^{-1}$ before use. Capacitation and incubations were carried out for 1 h at 37°C in a moist atmosphere of 5% CO_2 in air. In some cases, 1 ml samples of spermatozoa were centrifuged three times at 2000 g for 2 min each. The supernatants were removed and 1 ml of fresh M199-M was added to the pellet to wash the cells. Before the next washing, the cells were resuspended by gentle agitation. The unwashed samples were centrifuged and resuspended in their original supernatants. Washes from 2×10^6 caput spermatozoa were used to treat an equal number of cauda spermatozoa.

Inhibitor isolation and anti-trypsin activity

A proteinase inhibitor (SVI) was isolated from homogenized seminal vesicle tissues as described by Poirier and Jackson (1981). The procedure consisted of gel filtration (Sephadex G-75), affinity chromatography using bovine pancreatic trypsin inhibitor as the ligand and ion exchange chromatography (SP-Sephadex C-25). Once purified, the inhibitor solution was dialysed against phosphate-buffered saline and stored at -20°C until used. Chromatography materials were obtained from Pharmacia LKB Biotechnology, Inc. (Piscataway, NJ).

Measures of inhibitory activity were determined against porcine pancreatic trypsin with *N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA: Sigma) as the substrate in Ca^{2+} -free triethanolamine hydrochloride (TRA: Sigma) buffer, pH 7.8, 0.2 mol l^{-1} (Fritz *et al.*, 1974). Each 3.0 ml assay contained 6.0 μg trypsin (Sigma), 0.77 mmol BAPNA l^{-1} , and various amounts of inhibitor. An inhibitor unit (IU) is defined as the amount of inhibitor that reduces the hydrolysis of the substrate by $1 \mu\text{mol min}^{-1}$.

Zonae

Zonae were isolated from ovarian oocytes as described by Boettger *et al.* (1989). Zonae were solubilized just before use by incubation at 70°C for 30 min (Cholewa-Stewart and Massaro, 1972). The concentration of zonae used is expressed as zonae equivalents (ZE). One thousand zonae per 10^6 spermatozoa ml^{-1} represents 1 zona equivalent.

Antiserum

Antiserum to the seminal vesicle inhibitor used to induce acrosome reactions and verify inhibitor localization was raised in rabbits; its specificity has been established by Poirier and Jackson (1981), Irwin *et al.* (1983), Richardson *et al.* (1987) and Aarons *et al.* (1991).

Coomassie brilliant blue method for acrosome staining

Spermatozoa were scored for acrosome integrity by the Coomassie brilliant blue staining technique (Moller *et al.*, 1990) as modified by Aarons *et al.* (1991, 1992). Spermatozoa were fixed with equal amounts of 10% formaldehyde in phosphate-buffered saline (PBS) for 10 min, washed once with PBS at 6500 g for 1 min, and dried onto glass slides. The

cells were stained for 2 min in 0.05% Coomassie brilliant blue R-250 (BioRad Laboratories, Richmond, CA). The slides were rinsed with PBS and the spermatozoa covered with PBS: glycerol (1:1 by volume) and observed. Previous investigations (Aarons *et al.*, 1991) indicate that the Coomassie brilliant blue staining method, which stains the acrosomal cap, is as sensitive as the chlortetracycline assay (Leyton and Saling, 1989) in determining acrosome integrity after ionophore (A23187) or zonae treatment.

Induction of acrosome reaction

Spermatozoa were induced to acrosome react by immunoprecipitation of bound SVI as described by Aarons *et al.* (1991). Cells ($2 \times 10^6 \text{ ml}^{-1}$) were suspended in M199-M and incubated for 1 h to induce capacitation. Purified preparations of SVI in PBS were added to give a final inhibitor concentration of 30 mIU 10^{-6} sperm cells. After incubation for 30 min at room temperature, the sperm cells were then washed twice at 2000 g for 2 min in M199-M. Samples of the inhibitor-treated cells were incubated for an additional 30 min in a 1:40 dilution of anti-SVI serum. Controls consisted of substituting normal rabbit serum or PBS for the immune serum. These samples were then fixed and stained with Coomassie brilliant blue. Two zonae equivalent are sufficient to induce acrosome reactions in most incubated cauda spermatozoa (Boettger-Tong *et al.*, 1992). In the controls fucoidan or PBS were substituted for the zonae.

Indirect immunofluorescence

The details of the indirect immunofluorescent technique used to detect the presence of seminal inhibitor on spermatozoa were described by Aarons *et al.* (1991, 1992) and Boettger-Tong *et al.* (1992). Controls consisted of substituting PBS for SVI or substituting normal rabbit serum or PBS for the immune serum.

Statistical analysis

Contingency table analyses were used to test for significance of differences between proportions (Sokal and Rohlf, 1981). The level of significance was defined as *P* values < 0.05 . Differences were not significant if not indicated.

Results

Unwashed caput spermatozoa, incubated under capacitating conditions, did not acrosome react when presented with solubilized zonae (Fig. 1). However, if the capacitated samples were washed before the addition of the zonae, a significant decrease in the percentage of spermatozoa with intact acrosomes was seen. If fucoidan or PBS was substituted for zonae, no significant decrease was observed. Unwashed cauda spermatozoa responded to zonae, and washing also increased the percentage of these cells that reacted. No differences were observed between the washed and unwashed controls (PBS) for either caput or cauda spermatozoa, suggesting that the

Fig. 1. Caput and cauda spermatozoa were incubated under capacitating conditions then washed (W) or left unwashed (U), and induced to acrosome react with heat solubilized zonae (Z), fucoidan (F) or phosphate-buffered saline (PBS) (P). Fucoidan was used at a final concentration of 5 mg ml^{-1} and zonae at two zonae equivalents. The data represent the percentages of spermatozoa with intact acrosomes as determined by Coomassie blue staining and are the means of three separate trials counting 200 cells per treatment per trial. The horizontal lines represent the standard errors of the means. *Significantly different from the unwashed sample ($P < 0.05$).

Fig. 2. Caput and cauda spermatozoa were incubated under capacitating conditions then washed (W) or left unwashed (U) and induced to acrosome react with SVI anti-SVI treatment (S), SVI-normal rabbit serum (N) or phosphate-buffered saline (PBS)-PBS (P). The data represent the percentages of spermatozoa with intact acrosomes as determined by Coomassie blue staining and are the means of three separate trials counting 200 cells per treatment per trial. The horizontal lines represent the standard errors of the means. *Significantly different from the unwashed sample ($P < 0.05$).

procedures used were not detrimental to acrosome integrity. Similar results were obtained when the acrosome reactions were induced by immunoaggregation of a zona binding site (Fig. 2). Capacitated, unwashed caput cells did not acrosome react, while capacitated, unwashed cauda spermatozoa did react to SVI/anti-SVI treatment. Washing increased the ability of both caput and cauda spermatozoa to react to immunoaggregation. No motility was observed in the incubated caput spermatozoa after the washes.

The cells were treated with pertussis toxin and then induced to acrosome react to determine whether the acrosomes of caput and cauda spermatozoa react by similar mechanisms. Pertussis toxin blocked immunoaggregated acrosome reactions in both caput and cauda spermatozoa (Fig. 3). The effect was more dramatic with cauda spermatozoa.

As washing is necessary for incubated caput spermatozoa to acrosome react to zona or to an immunoaggregation stimulus, we determined whether washing alone, with no capacitation-incubation, could accomplish the same effect. Fresh caput and cauda spermatozoa were washed three times and tested for ability to acrosome react by immunoaggregation after each washing (Fig. 4). Washing alone did not prepare either caput or cauda spermatozoa for immunoaggregation-induced acrosome reactions.

Indirect immunofluorescence was used to determine when during the washings SVI could bind to the caput cells. SVI binds to the acrosomal cap region of both caput and cauda spermatozoa, albeit in much lower percentages for the caput cells (Irwin *et al.*, 1983). With successive washings the percentage of caput spermatozoa that bind SVI increased to equal that

Fig. 3. Capacitated caput and cauda spermatozoa were washed and induced to acrosome react by SVI anti-SVI in the presence (▨) or absence (□) of pertussis toxin ($10 \mu\text{g ml}^{-1}$). The data represent the percentages of spermatozoa with intact acrosomes as determined by Coomassie blue staining and are the means of three separate trials counting 200 cells per treatment per trial. The horizontal lines represent the standard errors of the means.

obtained for cauda cells (Fig. 5). No fluorescence was seen under any of the control situations. Spermatozoa were induced to acrosome react after each wash to determine when during

Fig. 4. Unincubated caput and cauda spermatozoa were induced to acrosome react by SVI anti-SVI treatment when unwashed (0) or after each of three (1, 2, 3) successive washes. The data represent the percentages of spermatozoa with intact acrosomes as determined by Coomassie blue staining and are the means of three separate trials counting 200 cells per wash per trial. The horizontal lines represent the standard errors of the means. The data points at the top of the bars represents control data with PBS substituted for SVI.

Fig. 5. Caput (□) or cauda (▨) spermatozoa incubated under capacitating conditions were tested for their ability to bind SVI as determined by indirect immunofluorescence when left unwashed (0) or after each of three (1, 2, 3) successive washes. The data represent the percentages of spermatozoa that bind SVI at the acrosomal cap region and are the means of three separate trials counting 200 cells per treatment per trial. The horizontal lines represent the standard errors of the means.

the washing procedure capacitated caput spermatozoa could react to immunoaggregation (Fig. 6). A progressive decrease in the percentage of intact acrosomes was observed for both the

Fig. 6. Caput or cauda spermatozoa incubated under capacitating conditions were induced to acrosome react by SVI anti-SVI treatment when unwashed (0) or after each of three (1, 2, 3) successive washes. The data represent the percentages of spermatozoa with intact acrosomes as determined by Coomassie blue staining and are the means of three separate trials counting 200 cells per treatment per trial. The horizontal lines represent the standard errors of the means. The data points at the top of each clear bar represent control data with PBS substituted for SVI.

caput and cauda cells. No significant decrease in control (PBS or normal rabbit serum treated) samples was observed.

As washing effectively increased the percentage of caput spermatozoa that could bind SVI and react to immunoaggregation, the ability of the caput washes to block acrosome reaction induction in cauda spermatozoa was determined. Incubated, washed cauda spermatozoa were mixed with supernatants removed from incubated caput spermatozoa samples and induced to acrosome react by immunoaggregation. These supernatants effectively reduced the number of incubated, washed cauda cells that acrosome reacted (Fig. 7). The supernatants, however, did not completely block SVI/anti-SVI induced acrosome reactions.

The washes contained trypsin inhibitory activity (Table 1) as determined by the BAPNA assay. The amount of inhibitory activity in the supernatants was reduced with each wash for both the caput and cauda cells. No differences were seen in the amount of trypsin activity when caput and cauda supernatants were compared.

Discussion

Capacitation has been defined as the biochemical modifications that spermatozoa undergo before they can be induced to acrosome react (Zaneveld *et al.*, 1991) in response to a zona agonist (Florman and Babcock, 1991). The ability to acrosome react in response to zonae, the physiological acrosome reaction, is considered a distinguishing characteristic of the fertile state

Fig. 7. Incubated washed cauda spermatozoa were treated with supernatants obtained from washing incubated caput spermatozoa. Supernatants (1 ml) obtained from the first wash of 2×10^6 caput spermatozoa were used to treat 2×10^6 cauda spermatozoa. The cauda cells were treated with either SVI, supernatant (SUP) plus SVI or PBS and then induced to acrosome react with anti-SVI. The data represent the percentages of spermatozoa with intact acrosomes, as determined by Coomassie blue staining and are the means of six separate trials counting 200 cells per treatment per trial. The horizontal lines represent the standard errors of the means.

Table 1. Trypsin-inhibitor activity of supernatants removed during three successive washes of mouse epididymal spermatozoa

Epididymal region	Wash	Inhibitor activity (mIU ml ⁻¹) ^{ab}
Caput	1	15.2 ± 8.4
	2	9.0 ± 4.5
	3	6.8 ± 1.5
Cauda	1	14.2 ± 10.9
	2	7.4 ± 3.0
	3	6.7 ± 1.2

^aThe *N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) assay was used to determine the trypsin-inhibitor activity of the supernatant from each washing, based on removal of 1 ml washes from 2×10^6 cells; ^bthe data represent the means and SEM from three separate investigations.

(Florman and Babcock, 1991). Thus the incubated, washed caput spermatozoa, as used in experiments presented here, are capacitated. As the incubations and washing did not increase the motility of the caput cells, it is difficult to consider them fertile. Fucoidan, a sulfated L-fucose polymer with antitrypsin activity was used as a negative control for the zona based experiments. Although fucoidan can block sperm–zona binding in a number of species (Töpfer-Peterson and Henschen, 1988), it cannot induce acrosome reactions in either caput or cauda

cells. Capacitated unwashed caput spermatozoa will acrosome react when treated with an ionophore, a non-physiological stimulus (Lakoski *et al.*, 1988). This finding suggests that the inability of these cells to react to zona is due to a block somewhere proximal to the Ca^{2+} influx step. Washing appears to remove the block but only from cells incubated under capacitating conditions. The fact that pertussis toxin effectively blocks SVI/anti-SVI induced reaction in both caput and cauda cells suggests that a G protein is involved in the exocytosis in both groups of cells. Pertussis toxin also blocks zona induced acrosome reactions of cauda spermatozoa (Endo *et al.*, 1987). The fact that caput and cauda spermatozoa react to the same stimuli by similar mechanisms suggests that caput acrosome reactions are true reactions. Pertussis toxin has no effect on the ability of mouse spermatozoa to bind SVI (Boettger-Tong *et al.*, 1992).

Unwashed capacitated cauda spermatozoa can acrosome react, but washing is necessary for induction of the acrosome reaction in capacitated caput spermatozoa. The ability to induce acrosome reactions in caput cells may be a function of dilution rather than washing. The caput region of the epididymis has only 2–5% of the spermatozoa found in the cauda region (McLaughlin and Shur, 1987). To achieve equal concentrations, as used in the assays presented here, the cauda cells were diluted much more than were the caput cells. Thus components removed from caput spermatozoa by washing may be removed from cauda spermatozoa by dilution.

It has been proposed that physiological acrosome reactions could be induced without zona by immunoaggregation of zona binding sites (Saling, 1989). Mouse cauda spermatozoa, incubated under capacitating conditions, will acrosome react when stimulated by zona (Bleil and Wassarman, 1983) or by immunoaggregation of zona binding sites (Aarons *et al.*, 1991, 1992; Leyton and Saling, 1991; Macek *et al.*, 1991). Acrosome reactions induced in cauda cells by solubilized zona and SVI/anti-SVI are similar in a number of respects; both are sensitive to pertussis toxin and proteinase inhibitors and are inducible by nanomolar quantities (Boettger-Tong *et al.*, 1992). In addition, incubated washed caput spermatozoa will effectively react to both stimuli.

Washing removes a component(s) that allows caput spermatozoa to bind to whole zona (McLaughlin and Shur, 1987), as well as allowing them to bind SVI and undergo physiologically induced acrosome reactions. Components in the washings removed from caput cells will block zona binding (McLaughlin and Shur, 1989) as well as SVI/anti-SVI induced acrosome reactions. Thus, inherent proteinase inhibitors may be at least partially responsible for the inability of the caput cells to react. Mouse epididymides contain at least two proteinase inhibitors that are distinct from that of the seminal vesicle (Poirier and Jackson, 1981). Thus, it is not surprising that the washes should contain anti-trypsin activity. However, since caput and cauda washes contain approximately the same amount of trypsin inhibitory activity, the blocking ability of the cauda supernatant is not due to total inhibitory activity; but may be due to the type of inhibitor or component present or to components without anti-trypsin activity that only secondarily block SVI binding sites.

It has been suggested that epididymal spermatozoa are covered with decapacitation factors that protect zona binding

sites and inhibit sperm motility (Bleil, 1991). Some of these components may limit membrane fluidity, thus preventing spermatozoa from undergoing premature acrosome reactions. Epididymal components that block zonae binding (McLaughlin and Shur, 1987) or stabilize the acrosome (Thomas *et al.*, 1984) are known. These and similar observations along with the data presented here support the suggestion of negative regulation: caput or even testicular spermatozoa have some features necessary for successful fertilization, for example functional zona binding sites and mature second message systems that are masked by negative regulators (Florman and Babcock, 1991). Epididymal maturation may then be thought of, at least in part, as an increase in the efficiency of regulator removal. The data suggest that incubation under capacitating conditions can induce changes in the plasma membrane of caput cells that are similar to those induced in cauda spermatozoa. However, physiological acrosome reactions cannot be induced in these cells because the zona binding sites are blocked by negative regulators. The regulators normally removed during epididymal transit can be removed from capacitated caput spermatozoa by washing. Washing also has a small effect on the ability of the cauda cells to react, suggesting that not all the regulator is removed during the epididymal transit.

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