Effect of temperature on the fluidity of boar sperm membranes*

A. T. Canvin and M. M. Buhr†

Department of Animal Science, University of Manitoba, Winnipeg, Manitoba, Canada R3T 2N2

Summary. Fluidity was used to assess changes in molecular organization of boar spermatozoa plasma membranes from (1) the head and (2) the rest of the sperm body and acrosome as a consequence of temperature. The initial fluidity of the head membranes at 25°C was less than that of the sperm body membranes ($P < 0.05$). When held at 25°C, the fluidity of the head membranes decreased for 105 ± 8 min and then stabilized for the remainder of the 160-min incubation. Calcium (10 mM) caused a significantly greater decrease in fluidity. The fluidity of the sperm body membranes increased slightly over time in the absence of Ca^{2+} , but decreased significantly with Ca^{2+} . Cooling from 25 to 5°C and subsequent heating to 40°C (0.4°C/min) caused marked alterations in the fluidity of each membrane. Cooling the head membranes prevented the fluidity increase seen at 25°C, while reheating caused a dramatic decrease in fluidity. Fluidity of the head membranes was now unaffected by Ca^{2+} . Lipid phase transitions, indicated by sharp break points in data curves, were detected at the onset of reheating ($7 \pm 3^\circ\text{C}$) and at $23 \pm 4^\circ\text{C}$ during reheating. Fluidity of the sperm body membranes decreased slightly and in a linear fashion with Ca^{2+} . Without Ca^{2+} , the sperm body membranes showed an additional lipid phase shift at $31 \pm 5^\circ\text{C}$, which led to a rapid fall in fluidity. These results suggest that the fluidity, and therefore the molecular structure, of sperm head and body membranes differ. The head plasma membranes, and to a lesser extent the sperm body membranes, undergo a fluidity change over time, which may reflect the structural reorganization of capacitation. This fluidity pattern is significantly disrupted by cooling and reheating.

Keywords: boar; spermatozoa; membranes; fluidity; temperature

Introduction

Communication between a cell and its environment is mediated by the plasma membrane. The fluid mosaic model predicts that such communication would be affected by the ability of the molecules within the membrane to interact with each other and with the cytoplasm and/or extracellular environment. Other mammalian tissues show a correlation of function with membrane structure. As luteal progesterone production drops, luteal membranes show an increase in gel phase lipid detected by X-ray diffraction (Buhr *et al.*, 1979) and a decrease in fluidity detected by fluorescence polarization (Carlson *et al.*, 1984).

Insemination with boar semen thawed after frozen storage reduces both the farrowing rate and the litter size (Johnson *et al.*, 1981). The fertility of such semen is not correlated with sperm motility, and is only poorly correlated with acrosomal morphology (Wilmot & Polge, 1977). Such loss of function may therefore be due to changes in the plasma membrane of the sperm head.

*Reprint requests to Dr M. M. Buhr.

†Present address: Department of Animal and Poultry Science, Ontario Agricultural College, University of Guelph, Guelph, Ontario, Canada N1G 2W1.

Ram spermatozoa have been shown to undergo temperature-dependent changes in both membrane structure and in the activity of membrane-bound enzymes (Holt & North, 1986). If temperature manipulations induce similar alterations in the plasma membrane surrounding the head of boar spermatozoa, some of the normal membrane-dependent events in capacitation and fertilization might be adversely affected. The head plasma membranes might undergo irreversible alterations in ultrastructure resulting from the cooling and reheating process. These alterations could be unique to the head plasma membrane, since this membrane is structurally different from those of the acrosome and the rest of the sperm body (Peterson & Russell, 1985; de Curtis *et al.*, 1986).

Materials and Methods

Sample preparation. Semen was collected by the gloved-hand technique from mature boars of known fertility. The whole ejaculate was strained through 2 layers of J-cloth into a vacuum flask maintained at 37°C. Semen was evaluated for motility and morphology and normal semen (>65% motility, <20% abnormal) was filtered twice at 25°C through Miracloth (Calbiochem, La Jolla, CA, USA) and diluted 1:1 (v/v) with Buffer I (5 mM Tris-HCl, 0.25 M-sucrose, pH 7.4) at 25°C. A 1:1 (v/v) mixture of 550 and 1107 silicon oils (Dow Corning, Mississauga, Ontario, Canada) was centrifuged (1500 g, 10 min), and the supernatant harvested. The buffered semen was layered on an equal volume of this supernatant and centrifuged (2500 g, 10 min) to remove seminal plasma and debris (P. F. Watson, personal communication).

Subsequent membrane isolation was by a variation of the procedure of Gillis *et al.* (1978). Briefly, the sperm pellet was washed 3 times (2500 g, 10 min) and the final pellet brought up to 30 ml in Buffer I. The pellet was subjected to nitrogen cavitation in a Parr bomb at 116 kg/cm² N₂ for 10 min, extruded into 30 ml Buffer II (5 M Tris-HCl, 0.25 M-sucrose, pH 5.0) and shaken vigorously by hand for 1 min to separate membrane vesicles (Kaplan *et al.*, 1984). The cavitate was washed three times in Buffer II (1000 g, 10 min); the pellet contained intact spermatozoa minus much of the head membranes (Peterson *et al.*, 1980). This pellet was designated as sperm body membranes for fluorescence polarization. The combined supernatants were centrifuged (6000 g, 10 min) to remove remaining intact cells and the supernatant was centrifuged (365 000 g, 60 min). The resultant pellet was resuspended in Buffer III (10 mM Tris-HCl, 0.9% (w/v) NaCl, pH 7.4), centrifuged (365 000 g, 30 min) and the pellet of head plasma membranes was transferred in 2 ml Buffer II to a glass-Teflon homogenizer. After 10–15 strokes of the pestle, protein concentrations in the head and sperm body membranes were determined (Bradford, 1976) using bovine gamma globulin (Sigma, St Louis, MO, USA) as a standard. Membrane samples were diluted with Buffer III to a final concentration of 200 µg/ml.

Membrane analyses. To determine fluidity, fluorescence measurements were taken on a model LS5 Perkin Elmer spectrofluorometer fitted with a polarizing accessory, using excitation and emission slit widths of 10 nm and 5 nm, respectively. A stock solution (2 mM) of the probe molecule trans-parinaric acid (tPNA: Molecular Probes Inc., Junction City, OR, USA) was made fresh every 3 months and stored in the dark under N₂ at -20°C. At 1 h before use, it was diluted to 4 µM in Buffer III. Equal volumes (1.5 ml) of tPNA and sample were mixed and allowed to equilibrate for 10 min; final concentrations were 2 µM-tPNA and 100 µg protein/ml. Calcium was added as CaCl₂ to the appropriate samples with vigorous pipetting before the equilibration period; final concentration was 10 mM. Excitation and emission wavelength maxima were determined daily and were very consistent (324 ± 0.6 nm (mean ± s.e.) and 420 ± 0.5 nm, respectively). Sample temperature was controlled by a Lauda RC3 circulating water bath attached to the cuvette holder, and monitored directly with a thermocouple in a sample cuvette. Fluorescence was assessed continuously for 160 min at 25°C or while cooling from 25 to 5°C and heating to 40°C at an approximate rate of 0.4°C per min. Fluorescence intensities were transformed into polarization values using the Perrin equation (Shinitzky & Barenholz, 1978):

$$PV = \frac{I_{\parallel}/I_{\perp} - 1}{I_{\parallel}/I_{\perp} + 1}$$

with $I_{\parallel}$ and $I_{\perp}$ representing the emission intensities from photomultiplier tubes polarized parallel and perpendicular, respectively, to the plane of the incident light (excitation beam).

The activity of alkaline phosphatase, a marker enzyme for sperm head plasma membrane (Noland *et al.*, 1983), was assessed by the method of Linhardt & Walter (1963), using *p*-nitrophenyl-phosphate (Sigma) as a substrate.

Statistical analysis. Initial polarization values were analysed for differences using least square means and their predicted differences. Polarization values were adjusted within trials by subtracting the value at time zero from all values to reduce variation due to differences in initial values. Data were analysed by stepwise regression on a polynomial equation with time and powers of time as the dependent variables. If data were non-linear ($P < 0.05$) then a procedure to fit a series of straight-line segments was invoked. The NLIN procedure (SAS Inc., 1985) fitted the series of line segments simultaneously, using the criteria of minimum residual sums of squares to estimate the various slopes and intercepts. This procedure also estimated the points on the x-axis at which the lines changed direction (break points). If data were linear, slopes were compared using a *t* test for parallelism.

Results

Alkaline phosphatase activity of the head plasma membrane preparation was $421 \pm 55\%$ (mean $\pm$ s.e.) compared to 100% for intact spermatozoa and $93 \pm 10\%$ for sperm body membranes.

The initial absolute, unadjusted polarization value indicated that the head plasma membrane was less fluid than the sperm body membrane. Calcium had no effect (Table 1). Fluidity changed over time at 25°C in a manner unique to each membrane type (Fig. 1). Fluidity of the head plasma membranes decreased over time, with Ca^{2+} significantly speeding the rate at which the overall fluidity declined (slope, Table 2). Break points were apparent in head plasma membrane slopes and were also affected by Ca^{2+} (Table 3). The polarization value of the sperm body membrane (Fig. 1) in the absence of Ca^{2+} described a bipartite line with an initially flat slope which subsequently decreased, indicative of increased fluidity. This differed significantly from the linear decrease in fluidity of the sperm body membrane in the presence of Ca^{2+} . Temperature significantly affected the fluidity characteristics of both the head and the sperm body membranes (Fig. 2). Cooling the head plasma membranes maintained the initial fluidity, while with onset of heating there was an immediate, rapid decrease in fluidity. The overall slopes and break points (Table 4) for the head plasma membranes were similar in the presence or absence of Ca^{2+} , although Ca^{2+} tended to increase fluidity. Fluidity of the sperm body membranes decreased gradually through cooling and reheating, with a rapid decrease in fluidity starting at $31 \pm 5^\circ\text{C}$ in the absence of Ca^{2+} (Table 3).

Table 1. Initial polarization values (least square means) of boar sperm membranes

Membrane	[Ca^{2+}]		s.e.m.	n
	0	10 mM		
Head	0.4259 ^a	0.4330 ^a	0.01065	18
Sperm body	0.3697 ^b	0.3792 ^b	0.01065	18

^{a,b}Means with different superscript letters differ ($P < 0.05$).

Table 2. Characteristics of fluidity curves of boar sperm membranes at 25°C

Membranes	Start (min)	Slope (polarization value /min $\times 10^{-4}$)	n
Sperm body			4
Slope 1	0	-0.5 ± 0.7	
Slope 2	88 ± 30	-2.6 ± 1.1	
Sperm body + Ca^{2+}			4
Total slope (linear)	0	5.6 ± 0.5	
Head			4
Slope 1	0	5.3 ± 0	
Slope 2	105 ± 8	0 ± 1.3	
Head + Ca^{2+}			4
Slope 1	0	19.2 ± 5.5	
Slope 2	44 ± 10	3.9 ± 1.0	

Values are mean $\pm$ s.e.

Table 3. Break points in fluidity curves of boar sperm membranes

Membranes	Break points		
	At variable temperature (<i>n</i> = 5)		At constant temperature (<i>n</i> = 4)
	Minutes	Equivalent °C	Minutes
Sperm body	25 ± 16 133 ± 5	16 (d) ± 5 31 (i) ± 5	88 ± 30
Sperm body + Ca ²⁺	ND	—	ND
Head plasma	64 ± 10 111 ± 11	7 (i) ± 3 23 (i) ± 4	105 ± 8
Head plasma + Ca ²⁺	62 ± 4 137 ± 6	6 (i) ± 1 32 (i) ± 2	44 ± 10

ND = non-detectable; (d) = decreasing temperature; (i) = increasing temperature.

Values are mean ± s.e.

Fig. 1. Changes in fluidity of boar sperm head plasma membrane (a) and the sperm body membrane (b) fraction at 25°C in the presence or absence of 10 mM-Ca²⁺. Each line was generated from data in 4 replicate trials. Increasing polarization value indicates decreasing fluidity.

Fig. 2. Changes in fluidity of boar sperm head plasma membrane (a) and sperm body membrane (b) during cooling (25–5°C) and subsequent reheating (5–40°C), in the presence or absence of 10 mM- Ca^{2+} . Increasing polarization value indicates decreasing fluidity. Each line was generated from data in 5 replicate trials.

Table 4. Characteristics of fluidity curves of boar sperm membranes at variable temperatures

Membranes	Start (min)	Equivalent °C	Slope (polarization value /min $\times 10^{-4}$)	n
Sperm body				5
Slope 1	0	25 (d) $\pm$ 0*	10.2 $\pm$ 6.6	
Slope 2	25 $\pm$ 16	16 (d) $\pm$ 5	1.6 $\pm$ 1.1	
Slope 3	133 $\pm$ 5	31 (i) $\pm$ 5	34.8 $\pm$ 8.6	
Sperm body + Ca^{2+}	0	25 (d) $\pm$ 0*	1.5 $\pm$ 0.3	5
Head plasma				5
Slope 1	0	25 (d)*	1.7 $\pm$ 1.7	
Slope 2	64 $\pm$ 10	7 (i) $\pm$ 3	13.2 $\pm$ 3.3	
Slope 3	111 $\pm$ 11	23 (i) $\pm$ 4	25.5 $\pm$ 3.3	
Head plasma + Ca^{2+}				5
Slope 1	0	25 (d) $\pm$ 0*	-0.7 $\pm$ 0.8	
Slope 2	62 $\pm$ 4	6 (i) $\pm$ 1	6.4 $\pm$ 0.6	
Slope 3	137 $\pm$ 6	32 (i) $\pm$ 2	15.7 $\pm$ 3.2	

(d) = decreasing temperature; (i) = increasing temperature.

*Start temperature.

Values are mean $\pm$ s.e.

Discussion

The two membrane fractions described here as the head plasma membrane and sperm body membranes differ dramatically. The activity of the head plasma membrane marker enzyme alkaline phosphatase (Noland *et al.*, 1983) was considerably enriched in the head membrane fraction. The known structural differences between the head and sperm body membranes (Vijayasaraty & Balaram, 1982) also were evident in fluidity measurements. Fluorescence polarization is a sensitive test of the fluidity of the membrane lipid bilayer, which reflects the molecular organization of the membrane in the vicinity of the probe molecule (Shinitzky & Barenholz, 1978). These pig sperm head membranes initially were less fluid than the body membranes, in contrast to bull sperm plasma membranes, which are more fluid than acrosomal membranes (Vijayasaraty *et al.*, 1982). In both species, however, the fluidity and therefore molecular structure of the head membranes differ from those of the membranes of the rest of the spermatozoon. The differences in absolute values could result from the varied components in the sperm body membranes, procedural differences in membrane preparation or species differences.

The sperm body membranes also differed from head membranes in fluidity patterns at 25°C. Motility of fowl spermatozoa (Ashizawa & Wishart, 1987) and activity of ATPase in flagellar membranes of bovine spermatozoa (Vijayasaraty & Balaram, 1982) require Ca^{2+} and Ca^{2+} also is bound specifically to acrosomal membranes of ejaculated boar spermatozoa (Berruti & Franchi, 1986). Calcium in the current study caused a steady decrease in fluidity, perhaps due to these functions of the varied components of this fraction. Temperature-dependent Ca^{2+} sensitivity also differs between the sperm head and body membranes, both in the nature of the temperature-induced fluidity change and in the retention of Ca^{2+} sensitivity by the body membranes in all circumstances. The sperm head and body membranes therefore differ in enzyme activity and fluidity characteristics in the presence or absence of Ca^{2+} .

Capacitation and the acrosome reaction involve extensive changes in the organization of head plasma membrane domains and vesiculation of the membranes. Plasma membrane proteins are re-organized during sperm maturation and ejaculation (Saxena *et al.*, 1986) and during ejaculation (Ahuja, 1984) and lipid diffusibility changes during hyperactivation (Wolf *et al.*, 1986). The fluidity of the head membranes decreased with time at 25°C, either reflecting or actually monitoring the membrane dynamics of the final achievement of fertilizing ability. Calcium caused a further, bipartite decrease in fluidity, perhaps indicative of a biphasic effect of a relatively high Ca^{2+} concentration on sperm fertilizing ability. Fraser (1987) noted that exposure of mouse spermatozoa to 3.6 mM- Ca^{2+} accelerated capacitation; increased exposure decreased fertilizing ability with no further effect on acrosomes or motility.

Cooling and reheating the head plasma membrane of boar spermatozoa significantly shifted these fluidity patterns. Logically, cooling should decrease lipid fluidity and heating increase it. Holt & North (1986), using the probe diphenylhexatriene (DPH), found that higher temperatures did increase the fluidity of mixed head and tail membranes from ram spermatozoa. In the current study, cooling head membranes from boar spermatozoa prevented the normal decrease in fluidity and heating decreased fluidity at a higher rate and in a different pattern. The tPNA probe molecule preferentially inserts into gel phase lipids (Sklar *et al.*, 1979a). It monitors the most ordered areas of the membrane and can detect phase variations undetectable by DPH (Carlson *et al.*, 1984). Lipids shifting to the gel phase would be preferentially monitored by tPNA, which suggests that these temperature-induced fluidity changes could either be occurring in specific domains within the membrane or throughout the membrane.

Cooling and reheating also affected the onset of lipid phase shifts in the boar sperm head membrane, indicated by the different break points in the constant and variable temperature regimens (Sklar *et al.*, 1979b). Holt & North (1986) detected thermotropic phase shifts in mixed membranes from ram spermatozoa. Their phase shifts appeared to be of considerably lower magnitude than those described here. This may be due to the different membrane fraction, different

species, different probe, or temperature variations which occurred during the storage and analysis of the ram membranes. The current study isolated all boar membranes at 25°C, analysed them on the day of isolation and controlled the rate of temperature change (0.4°C/min) during the fluorescence analysis. The resultant data clearly demonstrate a differential temperature sensitivity between head and body membranes from boar spermatozoa.

This study has demonstrated that the fluidity, and therefore the molecular organization, of plasma membranes from the head and body of boar spermatozoa differ. The head plasma membrane is particularly dynamic, and its structure naturally changes over time at a constant temperature. Cooling and reheating significantly alter membrane molecular organization and may thereby alter membrane and sperm function.

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