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Changes in Membrane Lipid Order With Capacitation in Rhesus Macaque (*Macaca mulatta*) Spermatozoa

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Abstract

Lipophilic fluorescent dye merocyanine 540 is believed to stain cell membranes with increasing affinity as the lipid components become more disordered and has been associated with changes in membrane fluidity. The aim of this study was to determine whether membrane lipid disorder is associated with capacitation of macaque spermatozoa. To induce capacitation, spermatozoa from 5 rhesus macaques were incubated at 37°C (5% CO_2 in air) for 2 hours in a modified Biggers-Whitten-Whittingham medium containing 30 mg/mL bovine serum albumin and 36 mmol/L $NaHCO_3$. Caffeine (1 mmol/L) and dbcAMP (1.2 mmol/L) were added to the medium, and incubation was performed for an additional 30 minutes. Sperm motility was determined by computer-assisted sperm analysis, and membrane lipid order and sperm viability was determined by flow cytometry with merocyanine (2.7 µmol/L) and Yo-Pro-1 (25 nmol/L), respectively. Tyrosine phosphorylation of proteins in sperm tail was immunohistochemically examined by means of anti-phosphotyrosine (α-PY; clone 4G10). Capacitation resulted in a significant increase in the amplitude of lateral head displacement and beat cross frequency ($P < .005$) and a significant decrease in linearity and straightness in capacitated spermatozoa ($P < .005$), compared with control spermatozoa, which suggests the expression of hyperactivated motility. Animals in which capacitation was

induced had a significant increase in the number of spermatozoa showing tyrosine phosphorylation of tail proteins ($P < .0001$) and a significant increase in the intensity of merocyanine fluorescence ($P < .0001$), compared with control animals. The observed decrease in membrane lipid order after capacitation was induced was not associated with surface exposure of phosphatidylserine, as determined by flow cytometry with annexin V-Alexa Fluor 488. Merocyanine may be a useful tool for investigating the role of the plasma membrane on capacitation and other cytotoxic events in macaque spermatozoa.

Key words: Primate sperm, merocyanine 540, annexin-V, tyrosine phosphorylation

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Introduction

Freshly ejaculated mammalian spermatozoa are unable to fertilize an oocyte. To become competent for fertilization, spermatozoa must undergo a complex series of physiological changes, collectively termed "capacitation," that result in an array of cell changes, including development of a fusogenic membrane and hyperactivated motility, before they can fertilize an oocyte (Austin, 1951, 1952; Chang, 1951). Capacitation confers on spermatozoa the ability to undergo the acrosome reaction, penetrate the zona pellucida, and fuse with the oocyte (Yanagimachi, 1994). In vivo, capacitation occurs during transit through the female reproductive tract. However, capacitation can also be induced by incubation in an appropriate in vitro environment. Common components of in vitro capacitation media include bicarbonate and bovine serum albumin (BSA). Albumin facilitates cholesterol efflux from sperm membranes, which presumably increases membrane fluidity and fusogenic capacity (Cross, 1998; Langlais et al, 1998; Visconti et al, 1999). Bicarbonate activates a soluble adenylate cyclase in sperm (Okamura et al, 1985; Chen et al, 2000), resulting in an increase in cAMP levels and protein kinase A-dependent protein tyrosine phosphorylation, which is a key event in sperm capacitation (Aitken et al, 1995, 1998; Visconti et al, 1995a,b; Leclerc et al, 1996; Galantino-Homer et al, 1997). Harrison et al (1996) have also reported that bicarbonate induces a rapid change in the lipid architecture of boar sperm plasma membranes, as determined by an increase in merocyanine 540 fluorescence. Merocyanine, a lipophilic fluorescent dye, is believed to stain cell membranes with increasing affinity as their lipid components become more disordered and has been associated with changes in membrane fluidity (Williamson et al, 1983; Langner and Hui, 1993).

Spermatozoa, similar to all mammalian cell types, have a high degree of lipid asymmetry of the plasma membrane, with phosphatidylethanolamine and phosphatidylserine in the inner leaflet and phospholipids with choline as the head group (eg, phosphatidylcholine and sphinogomyelin) in the outer leaflet (Müller et al, 1994; Nolan et al, 1995; Gadella et al, 1999; de Vries et al, 2003). Although the precise molecular mechanism signaled by increased merocyanine fluorescence remains uncertain, similar changes in merocyanine binding of other cell types have been interpreted as indicating a collapse of phospholipid asymmetry concomitant with increased membrane destabilization (Verhoven et al, 1992; Aussel et al, 1993; Mower et al, 1994). Capacitation of boar spermatozoa has been associated with a collapse of plasma membrane phospholipid asymmetry (Gadella and Harrison, 2000, 2002), and this was related to a decrease in membrane lipid order, as determined by merocyanine 540 staining. Furthermore, bicarbonate-mediated phospholipid scrambling of boar spermatozoa was observed to have implications for the lateral membrane topology of cholesterol, which possibly enables the extraction of this lipid by albumin or cyclodextrin (Flesch et al, 2001). Merocyanine could be a potentially useful tool for investigating the role of the plasma membrane in

capacitation or cytotoxic events in spermatozoa.

Merocyanine 540 staining has revealed changes in membrane lipid order for macaque spermatozoa during epididymal transit (Sivashanmugan and Rajalakshmi, 1997), another important phase of sperm maturation and development. The aim of this study was to investigate phospholipid asymmetry and membrane lipid order of capacitated macaque spermatozoa.

Materials and Methods

Chemicals and Reagents

Merocyanine 540, Annexin V-Alexa Fluor 488, Propidium Iodide, and Yo-Pro 1 were obtained from Molecular Probes (Eugene, Oreg). H-89 (*N*-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide-dihydrochloride) was obtained from EMD Biosciences (La Jolla, Calif). Anti-phosphotyrosine antibody (α-PY clone 4G10) was obtained from Upstate Biotechnology (Lake Placid, NY). Hepes (21 mmol/L)–buffered Biggers-Whitten-Whittingham (Hepes-BWW) medium was obtained from Irvine Scientific (Irvine, Calif), and Dulbecco's phosphate-buffered saline (DPBS) was obtained from Gibco BRL (Grand Island, NY). All other reagents were obtained from Sigma Chemical Company (St Louis, Mo).

Semen Collection, Processing, and Capacitation

Semen samples were obtained by electroejaculation from 7 male rhesus macaques (*Macaca mulatta*) under chair restraint, as described previously (Sarason et al, 1991). Animals were housed at California National Primate Research Center and maintained according to institutional animal care and use committee protocols at the University of California. After collection, semen samples were diluted in 4 mL Hepes-BWW media containing 1 mg/mL BSA and were rocked for 5 minutes. The coagulum was removed, and the sample was maintained at room temperature for 10 minutes. The top 3.5 mL of semen was then transferred into a separate tube for determination of initial motility and sperm count and for subsequent processing. Two millimeters of semen were placed over 3 mL of 80% Percoll and centrifuged at 300 x *g* for 25 minutes. First, a 95% Percoll solution was prepared by adding 20 x Hepes-buffered saline to 100% Percoll (modified from the protocol of Tollner et al [2000]). This solution was diluted with Hepes-BWW containing 1 mg/mL BSA to produce the 80% Percoll. After centrifugation at 300 x *g* for 5 minutes, the supernatant was removed and the pellet washed twice in Hepes-BWW and 1 mg/mL BSA. Sperm were finally resuspended at a concentration of 20 x 10^6 sperm/mL in bicarbonate (36 mmol/L)–buffered BWW (Bicarb-BWW; Overstreet et al, 1980) containing 30 mg/mL BSA (for capacitation) or Hepes-BWW plus 1 mg/mL BSA (for the noncapacitated control [RT control]). The RT control was maintained in the dark at room temperature for 2.5 hours. Spermatozoa were capacitated according to the method reported by Yudin et al (2000) by incubation for 2 hours at 37 °C (5% CO_2 in air) followed by incubation for an additional 30 minutes with (for the capacitated spermatozoa) or without (for the incubated control spermatozoa) caffeine (1 mmol/L) and dbcAMP (1.2 mmol/L). Preliminary experiments determined no significant difference in merocyanine fluorescence or phosphatidylserine exposure between incubation (for 2 hours at 37 °C in 5% CO_2) in Hepes-BWW plus 1 mg/mL BSA, compared with incubation in Bicarb-BWW plus 30 mg/mL BSA. Consequently, the Bicarb-BWW sample without the addition of activators was selected as the incubated control.

Experiment 1: Membrane Lipid Order and Capacitation in Macaque Spermatozoa

This experiment was replicated in 2 ejaculates from 5 monkeys each. Capacitation was confirmed by

evaluation of sperm motility for hyperactivation and tyrosine phosphorylation of sperm-tail proteins. Membrane lipid order and viability were determined by flow cytometry with merocyanine 540 and Yo-Pro 1, respectively.

Sperm Motility— Sperm motility was determined on at least 200 cells/sample in a minimum of 4 fields by means of computer-assisted sperm analysis (CASA) with HTM Ceros, version 12.2 g (Hamilton-Thorne Research, Beverly, Mass). Five microliters of sperm suspension was placed onto a prewarmed microscope slide that was overlaid with a 22-mm^2 prewarmed cover slip, and the slide was maintained at 37 °C during analysis by a heated slide holder (MiniTherm; Hamilton-Thorne Research). Sperm motility was videotaped using an AG-DV1000 digital video cassette recorder (Panasonic) and CCD video camera (Sony) mounted on a CH 30 microscope (Olympus) equipped with a 10 x negative phase-contrast objective and a 10 x projection ocular. The video tape was replayed for analysis at a subsequent time, and the playback feature was used to identify and delete aberrant tracks occurring when trajectories crossover each other or spermatozoa collide.

Percent total motility (TM), percent progressive motility (PM), straight line velocity (VSL), curvilinear velocity (VCL), average path velocity (VAP), linearity (LIN), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), and straightness (STR) were determined for sperm. Thresholds for defining hyperactivation were evaluated in a parallel study and are based on statistical comparisons between objectively selected tracks from incubated versus capacitated treatments and were as follows: VCL ≥ 130 µmol/L/sec, LIN ≤ 69%, and ALH ≥ 7.5 µmol/L (Baumber and Meyers, unpublished data). Spermatozoa exhibiting motility characteristics beyond all 3 thresholds and a trajectory with a minimum of 15 data points were subsequently defined as hyperactivated.

Instrument settings for the CASA analysis were as follows: frame rate, 60 Hz; frames acquired, 30; minimum contrast, 80; minimum cell size, 4 pixels; static VAP cutoff, 20 µ/s; static VSL cutoff, 10 µ/s; progressive VAP threshold, 25 µ/s; progressive STR threshold, 80%; static intensity limits, 0.6–1.4; static size limits, 0.6–2.31; and static elongation limits, 0–80.

Immunofluorescence Labeling for Tyrosine Phophorylation— After capacitation, sperm suspensions were centrifuged at 700 x *g* for 3 minutes and were washed in DPBS containing 1 mg/mL polyvinyl alcohol (DPBS-PVA), fixed for 10 minutes in 2% paraformaldehyde, washed twice in DPBS-PVA at 400 x *g* for 3 minutes, and then permeabilized with cold 95% ethanol for 10 minutes. Samples were washed twice again, incubated for 10 minutes in blocking solution (5% BSA in DPBS), washed, and resuspended in 4G10 monoclonal antibody (dilution, 1:500). Samples were then incubated at 4 °C overnight, washed, and then incubated with fluorescein-conjugated goat anti-mouse (Fab) IgG (dilution, 1:32) for 1 hour at room temperature in the dark. Cells were washed a final time in DPBS-PVA at 5000 x *g* for 3 minutes and resuspended in a drop of Vectashield fluorescence enhance (Vector; Burlingame, Calif). Immunofluorescence was visualized using oil immersion at 100 x magnification with an Olympus BX-60 fluorescence microscope using a fluorescein filter with an excitation wavelength of 480/30 nm and an emission wavelength of 535/40 nm. One observer determined the labeling patterns of at least 100 cells per treatment.

Membrane Lipid Order— To avoid interference from BSA on the fluorescence of merocyanine (Harrison et al, 1996), samples were centrifuged at 700 x *g* for 5 minutes and resuspended in DPBS-PVA at 2 x 10^6 spermatozoa/mL for flow cytometry. Stock solutions of merocyanine (54 mmol/L) and Yo-Pro 1 (1 mmol/L) were made in DMSO, and working solutions (1.35 mmol/L merocyanine and 10 µmol/L Yo-Pro 1) were prepared by dilution in DPBS. Samples were incubated in the dark for 15 minutes at 37 °C with merocyanine (2.7 µmol/L) and Yo-Pro 1 (25 nmol/L) in a portable warmer (Minitube MT 35/42; Verona, Wis), during transit from the laboratory to the flow cytometry facility at the California

National Primate Research Center. Fluorescence of merocyanine and Yo-Pro 1 were detected by FL-2 (585/42 nm) and FL-1 (530/30 nm) respectively on a FACSCaliber flow cytometer (Becton-Dickinson; San Jose, Calif) equipped with an argonion laser for excitation (488 nm). A total of 10 000 gated events (based on the FS and SS of the sperm population) were collected per sample, with sample running rates of approximately 500 events/s.

Experiment 2: Aminophospholipid Asymmetry and Capacitation in Macaque Spermatozoa

Spermatozoa (1 ejaculate from 5 monkeys each) were processed and incubated as described above. Aminophospholipid asymmetry was evaluated by the exposure of phosphatidyl serine (PS) on the outer plasma membrane leaflet (detected by annexin V-alexa fluor 488) and sperm viability was determined by propidium iodide. After capacitation, spermatozoa were diluted to 2 x 10^6 cells/mL in prewarmed DPBS-PVA containing 2 mmol/L $CaCl_2$ for flow cytometry. Samples were incubated in the dark for 15 minutes at 37 °C with annexin-V (0.25 µg/mL) and propidium iodide (4.8 µg/mL) in a portable warmer (Minitube MT 35/42; Verona, Wis) during transit from the laboratory to the flow cytometry facility at the California National Primate Research Center. Fluorescence of propidium iodide and annexin V were detected as described above. As positive controls for annexin staining, spermatozoa were damaged by three cycles of flash freezing in liquid nitrogen followed by thawing at 37 °C or spermatozoa were fixed in 3% formalin for 30 minutes on ice.

Influence of Caffeine and dbcAMP Alone on Membrane Lipid Order and Hyperactivation

Spermatozoa (1 ejaculate from each of 4 monkeys) were processed as described above and incubated for 2 hours at 37 °C (5% CO_2 in air) at a concentration of 20 x 10^6 cells/mL in Bicarb-BWW plus 30 mg/mL BSA followed by an additional 30 minutes with caffeine alone (1 mmol/L), dbcAMP alone (1.2 mmol/L), or caffeine plus dbcAMP. Membrane lipid order was determined as described above.

Influence of the PKA Inhibitor H-89 on Membrane Lipid Order and Hyperactivation

Spermatozoa (1 ejaculate from 5 monkeys each) were processed as described above and incubated for 2 hours at 37 °C (5% CO_2 in air) at a concentration of 20 x 10^6 cells/mL in Bicarb-BWW plus 30 mg/mL BSA alone or with either H-89 (60 µmol/L) or DMSO control (1 µL; final concentration, 0.2%). This was followed by an incubation for additional 30 minutes with caffeine (1 mmol/L) and dbcAMP (1.2 mmol/L). Membrane lipid order was determined as described above.

Statistical Analysis

Data were analyzed by analysis of variance (ANOVA), and comparisons between individual means were performed with Fisher's protected least significant difference test by use of Statview statistical software, version 5.0 (SAS Institute Inc, Cary, NC). In experiment 1, correlations between tyrosine phosphorylation and membrane lipid order were determined by Fisher's r to z test. Data are presented as mean ± SEM. Differences with *P* values of <.05 were considered to be statistically significant.

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*Motility data after 2.5 hours in the respective buffers at room temperature or 37°C**

Results

Experiment 1: Membrane Lipid Order and Capacitation in Macaque Spermatozoa

Sperm Motility— Capacitation resulted in a significant increase in ALH ($P < .0001$) and BCF ($P < .005$) and a significant decrease in LIN ($P < .005$) and STR ($P < .005$), compared with incubated spermatozoa, which suggests the expression of hyperactivated motility following the addition of caffeine and dbcAMP (Table). Hyperactivated motility was expressed by 4% ± 0.7% of the incubated sperm population, compared with 60% ± 3.2% of the capacitated sperm population ($P < .0001$).

Immunofluorescence Labeling for Tyrosine Phosphorylation— Capacitation of macaque spermatozoa resulted in a significant increase in tyrosine phosphorylated sperm tails (74% ± 3.3%), compared with incubated spermatozoa (19% ± 5.0%) and RT control spermatozoa (0.8% ± 0.33%; $P < .0001$). Incubated samples showed significantly greater tyrosine phosphorylation than the RT control samples ($P < .0005$). Fluorescence microscopy revealed that sperm tails were brightly fluorescent with minimal staining of the mitochondria and sperm head, and occasionally a bright region of fluorescence could be observed in the neck of the spermatozoa (Figure 1).

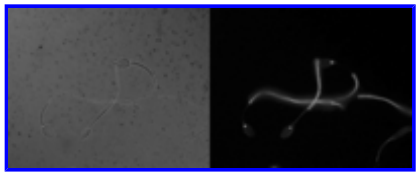

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Figure 1. Bright field **(left)** and epifluorescent **(right)** photomicrograhs of capacitated macaque spermatozoa showing tyrosine phosphorylation of sperm tail proteins.

Membrane Lipid Order— Capacitation of macaque spermatozoa resulted in a significant increase in the percentage of spermatozoa exhibiting a high intensity of merocyanine fluorescence (84% ± 3.6%), compared with incubated spermatozoa (11% ± 4.5%; $P < .0001$) and RT control spermatozoa (0.6% ± 0.1%; $P < .0001$) (Figure 2). Incubated samples showed a significantly greater number of spermatozoa exhibiting a higher intensity of merocyanine fluorescence than the RT control samples ($P < .05$). The percentage of spermatozoa exhibiting high-intensity merocyanine fluorescence was significantly correlated with the percentage of cells showing tyrosine phosphorylation of sperm tails ($P < .0001$; $r = 0.93$).

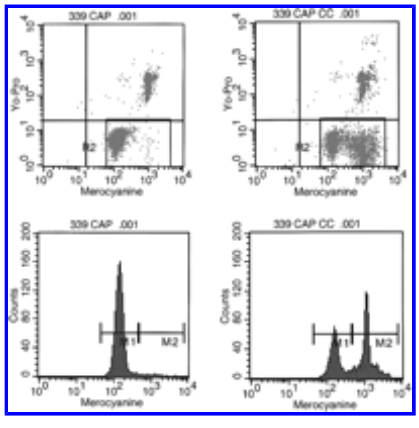

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Figure 2. Representative flow cytometry dot plots and histogram charts showing merocyanine and Yo-Pro 1 fluorescence for incubated **(left)** and capacitated **(right)** macaque spermatozoa. Live spermatozoa selected in region R2 of the dot plot are represented in the histogram chart below.

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Direct observation of merocyanine-stained live spermatozoa by fluorescence microscopy was difficult because of rapid fading of the probe. However, the entire spermatozoa appeared to be stained by merocyanine, with predominant labeling of the midpiece and acrosome (Figure 3A). The increase in merocyanine fluorescence was observed primarily in the midpiece and acrosome; the postequatorial region could be clearly delineated because of its reduced staining.

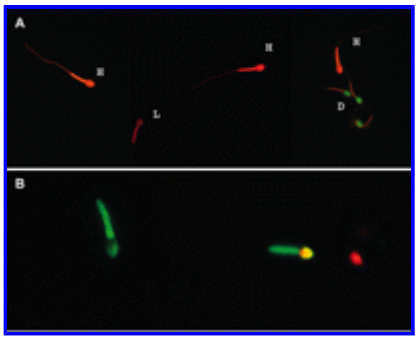

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Figure 3. **(A)** Epifluorescent photomicrograph of capacitated macaque spermatozoa; demonstrating live spermatozoa with both low (L) and high (H) merocyanine (red) fluorescence and dead (D; Yo-Pro 1 positive, green) spermatozoa. **(B)** Epifluorescent photomicrograh of capacitated macaque spermatozoa demonstrating live annexin-positive spermatozoan **(left)** and dead annexin-positive and annexin-negative spermatozoa **(right)**.

Preliminary experiments investigated merocyanine staining over time following the addition of caffeine and dbcAMP and concluded that a shift in merocyanine fluorescence can be observed within 5 minutes and increases to a maximum at 30 minutes. This is highly dependent on the individual monkey; certain monkeys immediately achieve a maximal response, whereas other monkeys show a gradual increase over time. The increase in merocyanine fluorescence in capacitated samples can be maintained for at least 2 hours.

Experiment 2: Aminophospholipid Asymmetry and Capacitation in Macaque Spermatozoa

Capacitation of macaque spermatozoa did not result in a significant increase in the percentage of live spermatozoa exposing PS, compared with incubated and RT control spermatozoa (data not shown). All samples had less than 1% live annexin-positive spermatozoa. Fluorescence microscopy revealed live annexin-positive spermatozoa were nonmotile. Annexin predominantly labeled the midpiece and acrosome, with no staining of the postequatoral region (Figure 3B). A significant increase was not observed in the percentage of dead spermatozoa or dead annexin-positive spermatozoa in capacitated samples, compared with incubated or RT control spermatozoa (data not shown). Annexin-positive spermatozoa were readily observed in the positive controls (data not shown). Subsequent experiments involved observation of incubation for several hours and did not detect PS exposure in live cells (data not shown); the percentages of dead spermatozoa and dead annexin-positive spermatozoa do increase over time.

Influence of Caffeine and dbcAMP Alone on Membrane Lipid Order and Hyperactivation

The addition of caffeine (9.5% ± 6.3%) and dbcAMP (7.7% ± 3.8%) alone did not result in a significant increase in the percentage of spermatozoa exhibiting high merocyanine fluorescence, compared with the incubated sample (1.0% ± 0.5%). However, the combination of caffeine and dbcAMP

in capacitated samples resulted in a significant increase in the percentage of spermatozoa exhibiting high merocyanine fluorescence (68% ± 7.9%; $P < .0001$).

Influence of the PKA inhibitor H-89 on Membrane Lipid Order and Hyperactivation

The addition of H-89 resulted in a significant decrease in the percentage of spermatozoa exhibiting high merocyanine fluorescence, compared with the capacitated samples ($P < .01$) (Figure 4). However, H-89 (60% ± 6.2%) also resulted in a significant increase in the percentage of dead spermatozoa, compared with the incubated sample (25% ± 4.9%; $P < .05$) and the capacitated sample (37% ± 7.0%; $P < .05$).

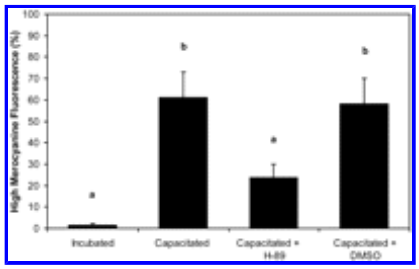

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Figure 4. Percentage of spermatozoa showing high merocyanine fluorescence after capacitation in the presence of H-89 (60 µmol/L) or DMSO control (1 µL). Spermatozoa were incubated for 2.5 hours at 37°C in Bicarb-BWW plus 30 mg/mL BSA with (capacitated) or without (incubated) the addition of 1.2 mmol/L dbcAMP and 1 mmol/L caffeine for the final 30 minutes of incubation. Mean values (± SEM) with different superscripts (^{a,b}) are statistically different ($P < .05$).

Discussion

Macaque spermatozoa do not capacitate spontaneously in vitro but require the addition of caffeine and dbcAMP to stimulate hyperactivated motility, binding to the zona pellucida, and fertilization (Boatman and Bavister, 1984; Vandevoort et al, 1992, 1994). Because macaque spermatozoa exhibit synchronous capacitation when subjected to a defined incubation protocol (Yudin et al, 2000), they may serve as a useful model to elucidate the physiological changes occurring during sperm capacitation. In this study, capacitation of macaque spermatozoa, which was confirmed by detection of hyperactivation and tyrosine phosphorylation, was associated with a significant decrease in membrane lipid order, as indicated by an increase in the fluorescence of merocyanine 540. The percentage of spermatozoa exhibiting a decrease in membrane lipid order was highly and significantly correlated with the percentage of cells showing tyrosine phosphorylation.

Our results demonstrate a decrease in membrane lipid order with capacitation in macaque spermatozoa and are similar to those described in equine (Rathi et al, 2001) and boar (Harrison et al, 1996) spermatozoa incubated with bicarbonate. Bicarbonate is a common constituent of capacitation media for mammalian sperm and plays a major role in the induction of sperm capacitation in vitro (Boatman and Robbins, 1991; Suzuki et al, 1994; Shi and Roldan, 1995). Bicarbonate has been shown to activate a soluble sperm adenylate cyclase (Okamura et al, 1985; Garty and Salomon, 1987; Chen et al, 2000), resulting in an increase in intracellular cAMP levels (Harrison and Miller, 2000). In turn, cAMP acts as an important second messenger by stimulating cAMP-dependent protein kinase A (PKA), which, in turn, phosphorylates specific target proteins (Walsh and Van Patten, 1994; Urner and Sakkas, 2003). Boar spermatozoa appear to be especially sensitive to the effects of bicarbonate on membrane

lipid order; a concentration of 15 mmol/L was sufficient to promote an increase in merocyanine fluorescence. The capacitation media used in this study contained 36 mmol/L bicarbonate; however, a shift in merocyanine fluorescence was only observed after the addition of caffeine and dbcAMP. Because the addition of these activators essentially synchronizes capacitation in macaque spermatozoa, a longer period of incubation may be required to affect a shift in merocyanine fluorescence with bicarbonate alone. Preliminary experiments determined that bicarbonate and BSA were not required in the media to effect a shift in merocyanine fluorescence with the addition of dbcAMP and caffeine. Although the addition of caffeine and dbcAMP would negate the inclusion of bicarbonate anyway, because they presumably act to increase cAMP downstream of the action of bicarbonate. Our results support those of Harrison et al (1996) and Gadella and Harrison (2000), because BSA was not required in the media to observe a shift in merocyanine fluorescence. Thus, in macaque spermatozoa, the shift in merocyanine fluorescence was dependent on the addition of activators (caffeine and dbcAMP) and could be elicited in basic media, which suggests that the change in merocyanine fluorescence may not be related to capacitation per se but simply to activation of a cAMP pathway. Preliminary experiments also determined that caffeine and dbcAMP did not cause a shift in merocyanine fluorescence when spermatozoa were incubated at room temperature, suggesting that the change in lipid architecture detected by merocyanine is temperature dependent.

Both caffeine and dbcAMP are presumed to elicit the same effect—an increase in intracellular levels of cAMP—when used at concentrations suitable for in vitro fertilization. If caffeine and dbcAMP were acting through similar mechanisms in macaque sperm, both compounds should not be necessary to promote a change in membrane lipid order. In this study, when caffeine and dbcAMP were added separately, there was no associated increase in merocyanine fluorescence. It is possible that caffeine and dbcAMP together are more efficient at increasing cAMP levels than they are individually. Harrison et al (1996) reported that, in boar spermatozoa, 1 mmol/L caffeine could cause a small but significant increase in merocyanine fluorescence similar to that elicited by bicarbonate, whereas dbcAMP did not. However, in a subsequent study, cBIMPS (a highly specific and phosphodiesterase-resistant cAMP analog) caused dose-dependent increases in merocyanine fluorescence (Harrison and Miller, 2000). Perhaps a longer incubation time or a higher concentration may be required with macaque spermatozoa to promote membrane lipid disorder when the activators are used separately, or it could be dependent upon the cAMP analog used.

H89 is a specific protein kinase A (PKA) inhibitor that operates by competing with ATP at the catalytic subunit of the kinase (Engh et al, 1996). Evidence of its PKA-inhibiting properties have been reported in sperm cells (Leclerc et al, 1996; Galantino-Homer et al, 1997; Harrison and Miller, 2000). Our results support those of Harrison and Miller (2000) and Wang et al (2004), in that addition of H-89 causes a significant reduction in merocyanine fluorescence. Results of these studies support a role for cAMP in the control of membrane changes indicated by shifting merocyanine fluorescence.

Although merocyanine indicates a dynamic change in the membrane lipid architecture of spermatozoa, the specific molecular mechanism signaled by increased merocyanine fluorescence and the physiological significance remains uncertain. Flesch et al (2001) sorted boar spermatozoa, which were subjected to capacitating conditions, by flow cytometry according to low and high merocyanine fluorescence; the spermatozoa exhibiting high merocyanine fluorescence demonstrated a 30% reduction in cholesterol levels. Loss of cholesterol from the plasma membrane would result in acyl chain disorder and increased affinity for merocyanine within the bilayer, represented by increased merocyanine fluorescence. However, BSA is not required in the media to affect a shift in merocyanine fluorescence (Harrison et al, 1996; Gadella and Harrison, 2000; Wang et al, 2004), so the loss of membrane lipid order may not be related to a loss of membrane cholesterol. Similar changes in

merocyanine binding in other cell types have been interpreted as indicating a collapse of the phospholipid asymmetry in the plasma membrane (Verhoven et al, 1992; Aussel et al, 1993; Mower et al, 1994). In boar (Gadella and Harrison, 2002) and human (de Vries et al, 2003) spermatozoa, the decrease in membrane lipid order after incubation with bicarbonate was correlated with PS exposure measured by annexin V. Therefore, Flesch et al (2001) proposed a model in which bicarbonate induces phospholipid scrambling in the sperm head that enables albumin mediated efflux of cholesterol. It would be informative if the decrease in membrane lipid order of spermatozoa sorted for high merocyanine fluorescence could be directly related to a loss of phospholipid asymmetry. However, potential changes occurring in the sperm plasma membrane because of flow sorting of spermatozoa limit the subsequent analysis of exposed PS.

In the present study, capacitation of macaque spermatozoa and the associated increase in merocyanine fluorescence was not accompanied by a loss of phospholipid asymmetry, as determined by labeling of exposed PS with annexin V. The shift in merocyanine fluorescence described in boar spermatozoa (which is essentially complete approximately 5–15 minutes after bicarbonate exposure) occurred much faster than the exposure of PS, the latter being maximal at approximately 60–90 minutes (Harrison et al, 1996; Gadella and Harrison, 2000). Our experiments determined that, in macaque spermatozoa, the fluorescence of merocyanine also increased rapidly after addition of caffeine and dbcAMP. However, no change in PS exposure was observed during longer incubation periods of 3–4 hours (data not show). The lack of an association between high merocyanine fluorescence and PS exposure in macaque spermatozoa, compared with that for boar (Gadella and Harrison, 2002), human (de Vries et al, 2003), and mouse (Wang et al, 2004) spermatozoa, may relate to species specific differences or to capacitation protocols and methodology. Although live annexin-positive cells were not observed in capacitated cells, annexin-positive cells were readily observed in dead spermatozoa and in the positive controls.

Early phases of disturbed membrane functions are associated with a loss of phospholipid asymmetry. Translocation of PS from the inner to the outer leaflet of the plasma membrane is one of the earliest events for cells undergoing apoptosis. Several groups have reported evidence that PS exposure in live sperm cells represents an early sign of cellular damage (Glander and Schaller, 1999; Ramos and Wetzels, 2001; Muratori et al, 2003, 2004). An increase in merocyanine fluorescence has also been attributed to an increase in membrane permeability, possibly related to penetration of merocyanine to the more fluid inner membrane structures (Falke and Lazarides, 1980; Szabo et al, 1989; Berthiaume and Frangos, 1994) In this study, dead spermatozoa exhibited high merocyanine fluorescence, which was no doubt related to the loss of membrane integrity. Merocyanine has also been used as an marker of apoptosis (Reid et al, 1996; Laakko et al, 2002) and as an indicator of membrane potential (Waggoner, 1979). Changes in merocyanine fluorescence and phospholipid asymmetry should therefore be interpreted with caution.

Muratori et al (2004) reported that neither merocyanine 540 or annexin V were able to detect capacitatation-related membrane modifications in human spermatozoa. They found that merocyanine stained "M540 bodies," round elements in seminal plasma devoid of nuclei. These elements resembled apoptotic bodies observed by electron microscopy in human semen (Bacetti et al, 1996; Gandini et al, 2000). In this study, spermatozoa were separated from seminal plasma by percoll gradient, and we confirmed staining of spermatozoa with merocyanine by microscopy; no "M540 bodies" or apoptotic bodies were observed.

In conclusion, we report a significant decrease in membrane lipid order, measured by increased merocyanine 540 fluorescence, with capacitation in rhesus macaque spermatozoa. This shift in membrane lipid order was not associated with a loss of phopholipid asymmetry, as determined by

labeling of exposed PS with annexin V, although it was dependent on a cAMP signaling pathway. Further experiments are required to determine the molecular mechanism responsible for increased merocyanine fluorescence and the physiological significance of changing membrane lipid order during sperm capacitation.

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