# Changes in Sperm Plasma Membrane Lipid Diffusibility after Hyperactivation during In Vitro Capacitation in the Mouse

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*Abstract.* We have used the technique of fluorescence recovery after photobleaching to measure the diffusibility of the fluorescent lipid analogue, 1,1′-dihexadecyl 3,3,3′,3′-tetramethylindocarbocyanine perchlorate on the morphologically distinct regions of the plasma membranes of mouse spermatozoa, and the changes in lipid diffusibility that result from in vitro hyperactivation and capacitation with bovine serum albumin.

We found that, as previously observed on ram spermatozoa, lipid analogue diffusibility is regionalized on mouse spermatozoa, being fastest on the flagellum. The bovine serum albumin induced changes in diffusibility that occur with hyperactivation are also regionalized. Specifically, if we compare serum incubated in control medium, which maintains normal motility, with those hyperactivated in capacitating medium, we observe with hyperactivation an increase in lipid analogue diffusion rate in the anterior region of the head, the midpiece, and tail, and a decrease in diffusing fraction in the anterior region of the head.

In general, ejaculated mammalian spermatozoa cannot fertilize ova. They gain this capacity from contact with the fluids of the female reproductive system, in particular with the ampullary fluid in the fallopian tube (1, 9; for more recent reviews see references 2, 6, and 40). Capacitation (9) results in two dramatic changes in sperm physiology: hyperactivation and acquisition of the ability to undergo the acrosome reaction and fertilize an oocyte. The midpieces of ejaculated spermatozoa are relatively inflexible. Their flagella exhibit low amplitude symmetric beating, which results in linear swimming patterns. In contrast, the midpieces of hyperactivated spermatozoa are highly flexible. Their flagella exhibit large amplitude, often asymmetric, beating, which results in circular swimming patterns. Often hyperactivation also results in altered beat frequency. The acrosome reaction refers to a fusion and vesiculation between the sperm plasma membrane in the anterior region of the head and the underlying acrosome. This results in a release of the acrosomal vesicle contents both lytic components and adhesion proteins (for review see reference 61) involved in fertilization, and exposes the inner acrosomal membrane. In most mammals, subsequent fusion of egg and sperm plasma membranes occurs at the equatorial region of the sperm surface (7). The precise molecular mechanism of this process of capacitation remains unknown. However, capacitation results in dramatic changes in plasma membrane lipid and protein composition as well as in the lateral distribution or regionalization of these components (5, 16, 21, 25, 29, 35, 39, 46, 51). Indeed, it has been suggested that lipid redistributions that result from capacitation provide the fusogenic domains required for vesiculation during the acrosome reaction (5).

In vitro capacitation can in some species be effected by incubation of spermatozoa in serum albumin (30). Once again, the precise molecular mechanism of this in vitro capacitation remains unknown. However, it has been demonstrated (30) that delipidation of albumin results in more effective capacitation and that the effect of relipidated albumin depends upon the particular fatty acid used.

These results are reminiscent of those from experiments in which albumin or vesicles are used to alter lipid composition of cells, particularly phospholipid-to-cholesterol ratios (34, 49, 52). Such alterations result in dramatic alterations in membrane fluidity (34, 46). Cholesterol-to-phospholipid ratios have been implicated as playing a controlling role in capacitation (11, 20, 27, 29, 43). Indeed, modification of cholesterol-to-phospholipid ratios with artificial membrane vesicles can reverse capacitation in vitro (12, 14, 20), and it has been suggested that seminal fluid vesicles play a similar role in the reversal of capacitation by seminal plasma (13).

In the present paper we investigate whether such fluidity changes occur during in vitro capacitation of mouse spermatozoa. Since sperm plasma membranes are known to be regionalized in both the distribution (15, 17, 25, 26, 33, 37, 38, 44, 45, 53, 60a) of their membrane components and in the fluidity of their plasma membranes (36, 58), we have used the technique of fluorescence recovery after photobleaching (FPR),[1] which enables us to measure the fluidity of the plasma

[1] *Abbreviations used in this paper:* $C_{16}$dil, 1,1′-dihexadecyl 3,3,3′,3′-tetramethylindocarbocyanine perchlorate; FPR, fluorescence recovery after photobleaching; %*R*, percent recovery.

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membrane on each of the major morphological regions of the spermatozoa (anterior and posterior regions of the head, midpiece, and tail). The fluorescent lipid analogue 1,1′-dihexadecyl 3,3,3′,3′-tetramethylindo-carbocyanine perchlorate ($C_{16}$diI) was used because it has been shown to behave as a typical lipid (for review see reference 41) and because its fluorescence properties make it particularly well suited for photobleaching studies (41, 57).

We have found that as previously observed on ram spermatozoa (58), lipid analogue diffusion is regionalized on mouse spermatozoa, being most rapid on the midpiece and tail; and that the BSA-induced changes in diffusibility that occur with hyperactivation are also regionalized. Specifically, if we compare sperm incubated in control medium that maintains normal motility to those hyperactivated in capacitating medium, we observe with hyperactivation an increase in the lipid analogue diffusion rate in the anterior region of the head, the midpiece, and tail, and a decrease in the diffusing fraction in the anterior region of the head.

Thus, in vitro capacitation conditions result in membrane changes in the anterior region of the head (perhaps required for induction of the acrosome reaction) and in the two regions of the flagellum (perhaps required for hyperactivated motility).

# *Materials and Methods*

## *Mice*

Mice used were retired male CD-1 breeders, 6–12 mo old, from Charles River Biological Laboratories, Inc., Wilmington, MA.

## *Spermatozoa*

Mice were killed by cervical dislocation, and immediately the ductus deferens and its proximal cauda epididymis were dissected out and placed into a petri dish containing Hanks' balanced salt solution. The spermatic artery and all adhering fat were removed from each duct and most of the cauda epididymis. The ducts were rinsed in clean Hanks' balanced salt solution with 0.4% wt/vol bovine serum albumin (BSA) and transferred to Whittingham's medium, pH 7.4, with 0.4% wt/vol BSA (54). By the use of two pairs of fine forceps, the sperm were squeezed from the ducts.

## *Hyperactivation/Capacitation*

Fresh cauda epididymal were placed in filtered Whittingham's medium with 0.4% wt/vol BSA and assayed immediately. Sperm were hyperactivated by incubation in Whittingham's medium with 3% wt/vol BSA for 3 h at 37°C. Control sperm were incubated for 3 h in Whittingham's medium with 0.4% wt/vol BSA.

## *Labeling with Lipid Probe*

Spermatozoa were labeled with the fluorescent lipid analogue $C_{16}$diI (Molecular Probes Inc., Junction City, OR). The carbocyanine dyes have been extensively studied and used as membrane probes, particularly for lateral diffusion measurements. Sperm were labeled with $C_{16}$diI as follows. Cells were washed three times in Hanks' balanced salt solution with 0.4% wt/vol BSA. They were then labeled by placing $10^6$ cells in 200 $\mu$l Hanks' balanced salt solution + 0.4% wt/vol BSA containing 5.5 $\mu$M $C_{16}$diI and 0.5% ethanol. Cells were incubated for 8 min at 22°C, then washed by overlaying onto 10% wt/vol BSA containing 0.2% wt/vol azide.

## *FPR Measurement*

The technique of FPR has been described in detail elsewhere (3, 28, 57). FPR provides two measures of lateral diffusion in the plane of the membrane: the fraction of the component that is free to diffuse (%$R$), and the diffusion coefficient ($D$) of that fraction. Typical lipid diffusion coefficients range from $10^{-9}$ to $10^{-8}$ cm$^2$/s, which means they diffuse 1 $\mu$m in ~1 s or less. The specifics for our instrument, which is similar to published instrument designs, have been described elsewhere (56). The beam, exp(-2) radius was determined (27) to be $0.9 \pm 0.1$ $\mu$m. Bleaching times were ~5 ms at ~10 mW at 514.5 nm. Monitoring intensities were ~1 $\mu$W. The counting interval was 30 ms. Data were fitted using nonlinear least squares programs after Bevington (8) to algorithms described by Barisas and Leuther (4) and Wolf and Edidin (57). Data were corrected for sperm geometry. The one- and two-dimensional solutions appropriate to the tail and head, respectively, can be found in the literature (3, 28). A solution appropriate for the midpiece was developed using standard procedures (3) and assuming a Gaussian beam and diffusion on a cylinder. Details will be published elsewhere (Wolf, D. E., manuscript in preparation). The validity of this solution has been checked experimentally using line bleaching where diffusion is one dimensional (27).

## *Hyperactivation Assay*

Sperm hyperactivation was assayed with a Zeiss standard microscope using dark field illumination with a 25×, 0.45 numerical aperture air objective. Hyperactivation was determined by observing frequency of beat, whether sperm were swimming straight or in "circles," whether the beat was symmetric, and the amplitude of the beat envelope. Data shown that document these patterns were taken on a Zeiss Universal microscope using a 40×, 0.75 numerical aperture air objective under phase contrast. A video recording of the sperm swimming pattern was made using a video camera (Dage-MTI, Wabash, MI) recorded on one-half inch video tape using a Panasonic reel-to-reel time lapse video recorder. A plastic sheet was then placed over the monitor screen, and the sperm were traced frame by frame onto the plastic using a marking pen.

## *Photomicroscopy*

Fluorescence photomicrographs were made using a Leitz Dialux microscope with a 100 W mercury lamp using standard N2.1 filters and a 63×, 1.4 numerical aperture plan apochromat. All photographs were taken on Ilford XP1-400 film developed to an effective ASA of 800.

# *Results*

## *$C_{16}$diI Labeling of Mouse Spermatozoa*

A caudal epididymal mouse spermatozoon stained with $C_{16}$diI is shown in Fig. 1. Staining within the major morphologically distinct regions is generally homogeneous (i.e., diffuse, not patchy); however, staining is regionalized in that some regions, particularly the anterior region of the head, stain more intensely. When labeling is done in the absence of $NaN_3$, sperm with this labeling pattern remain fully motile. Immotile sperm show blotchy staining patterns. Thus, when FPR is measured

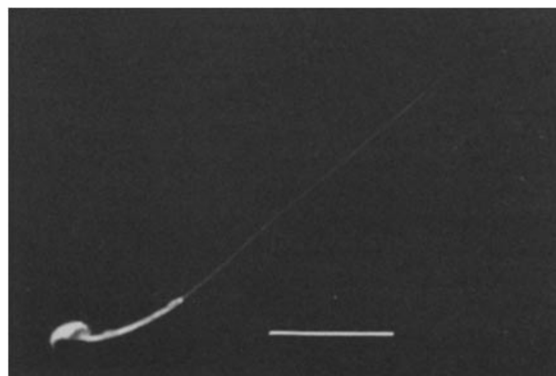

*Figure 1.* Fluorescence staining pattern of a mouse spermatozoon labeled with $C_{16}$diI as described in the text. Staining within the major morphologically distinct regions is generally homogeneous (i.e., diffuse, not patchy). However, staining is regionalized in that some regions, particularly the anterior region of the head, stain more intensely. Bar, 10 $\mu$m.

only nonpatchy stained sperm are chosen. Optical sectioning of sperm indicate that $C_{16}$diI labeling is ring stained and therefore largely confined to the plasma membrane. Furthermore, the $C_{16}$diI is completely accessible to the membrane impermeable quenching agent trinitrobenzene sulfonate, indicating that all of the $C_{16}$diI is present in the outer leaflet of the plasma membrane (55).

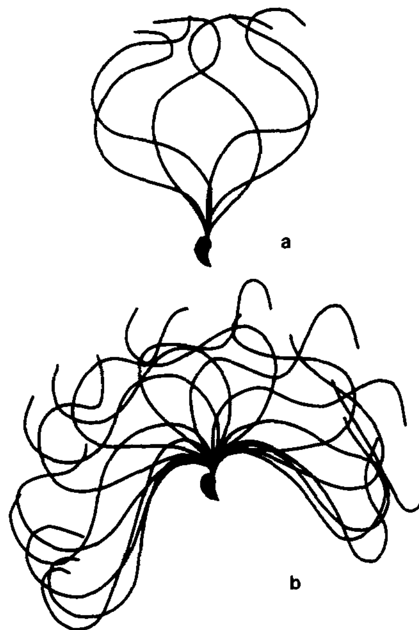


*Figure 2.* Beating patterns of mouse sperm collected from the cauda epididymis and incubated for 3 h in either control Whittingham's medium with 0.4% wt/vol BSA (*a*) or hyperactivating Whittingham's medium with 3% wt/vol BSA (*b*). As described in the text, these are tracings onto plastic from video recordings of motile sperm. Successive frames are separated by 1/60 s. Fresh spermatozoa can be seen to have low amplitude symmetric beats, whereas hyperactivated spermatozoa exhibit larger amplitude lower frequency beats.

*Table I. BSA Effects on Mouse Sperm Motility*

| Treatment | % Motile | % of motile hyperactivated | *n* |
|---|---|---|---|
| Cauda epididymal sperm | 48 ± 13 | 16 ± 18 | 4 |
| Control sperm 3 h in 0.4% wt/vol BSA | 30 ± 12 | 18 ± 19 | 4 |
| Sperm hyperactivated for 3 h in 3% wt/vol BSA | 35 ± 10 | 65 ± 14 | 17 |

In motility assays there is typically considerable animal-to-animal and day-to-day variation (31). The data reported here shows the results for *n* experiments. Values given are mean ± SEM, where the means were defined as the average of the values obtained for each experiment and the SEM is the SD of this average.

### *Hyperactivation Mouse Spermatozoa*

Fig. 2 shows the beating pattern of mouse spermatozoa freshly incubated for 3 h in control Whittingham's medium with 0.4% wt/vol BSA (Fig. 2*a*), or after a 3-h incubation in capacitating Whittingham's medium with 3% wt/vol BSA in Fig. 2*b*. Control sperm can be seen in Fig. 2*a* to have highly symmetric beat pattern with a narrow amplitude envelope. In contrast, spermatozoa incubated in capacitating medium with 3% wt/vol BSA for 3 h show the hyperactivated pattern shown in Fig. 2*b* typified by a wide amplitude envelope and reduced beating frequency. Often the wave patterns of hyperactivated sperm appear asymmetric. These beat patterns result in straight line swimming patterns for normal spermatozoa but "circular" swimming patterns for hyperactivated spermatozoa (11, 43). These beating and swimming patterns enable one to readily assay hyperactivation under dark field illumination. The sperm shown in Fig. 2, *a* and *b* have beat frequencies of 10 and 3.3 Hz, respectively. Using this video technique we determined the beat frequencies of a population of spermatozoa to be 9.7 ± 0.4 Hz for cauda epididymal sperm incubated in control medium and 6.2 ± 0.5 Hz for hyperactivated sperm. As shown in Table I we have found motile spermatozoa freshly collected from the vas deferens to exhibit essentially no hyperactivation, whereas sperm incubated for 3 h at 37°C in Whittingham's medium with 3% wt/vol BSA showed 65 ± 14% hyperactivation of motile spermatozoa. In contrast, maintenance of spermatozoa for 3 h at 37°C in control Whittingham's medium containing only 0.4% wt/vol BSA was not found to induce hyperactivated motility. Neither the 0.4% wt/vol nor 3% wt/vol BSA containing medium significantly altered the fraction of motile sperm. These results are similar to those previously published by others (30).

In these experiments our assay was hyperactivation. The conditions we have used are standard and have been shown by others to result in capacitation assayed by in vitro fertilization. To verify this further, Dr. Carol Ziomek has run parallel experiments with us and achieved 70 to 80% in vitro fertilization of CD-1 mouse ova with our hyperactivated spermatozoa.

*Table II. Hyperactivation of Mouse Sperm with BSA: Diffusion of $C_{16}$DiI*

| | Head | | | |
|---|---|---|---|---|
| | Anterior | Posterior | Midpiece | Tail |
| | | *Diffusion coefficient × $10^9$ s/cm²* | | |
| A. Fresh cauda epidermal | 2.11 ± 0.21 (25) | 1.91 ± 0.17 (26) | 5.65 ± 0.58 (32) | 5.04 ± 0.66 (29) |
| Control | 1.24 ± 0.21 (17) | 1.31 ± 0.26 (13) | 2.85 ± 0.58 (11) | 1.65 ± 0.34 (9) |
| Hyperactivated | 2.65 ± 0.29 (23) | 2.08 ± 0.34 (19) | 6.06 ± 1.1 (18) | 7.77 ± 1.5 (13) |
| | | *% Recovery* | | |
| B. Fresh cauda epididymal | 61 ± 2 (31) | 57 ± 3 (31) | 58 ± 4 (34) | 77 ± 4 (30) |
| Control | 60 ± 4 (17) | 53 ± 4 (16) | 49 ± 5 (14) | 69 ± 9 (14) |
| Hyperactivated | 45 ± 3 (29) | 46 ± 4 (26) | 45 ± 5 (23) | 53 ± 6 (21) |

Values given are mean ± SEM. Number of measurements is given in parentheses.

### *Lipid Analogue Diffusibility Changes with Capacitation on Mouse Spermatozoa*

The results of FPR measurements of the diffusibility of the fluorescent lipid analogue $C_{16}$dil on anterior and posterior regions of the head, the midpiece, and tail of mouse spermatozoa freshly collected from the vas deferens, hyperactivated in vitro in Whittingham's medium with 3% wt/vol BSA, or incubated in control Whittingham's medium with 0.4% wt/vol BSA are given in Table II. The results of a $t$ test comparison of hyperactivated with fresh or control incubated spermatozoa are given in Table III. $C_{16}$dil diffusion rate is the same on the two regions of the head of fresh cauda epididymal sperm ($\sim 2 \times 10^{-9}$ cm$^2$/s) and faster on the midpiece and tail ($\sim 5 \times 10^{-9}$ cm$^2$/s). About 60% of $C_{16}$dil is free to diffuse on the two regions of the head and on the midpiece with nearly 80% free to diffuse on the tail. Lipid analogue diffusion is thus seen to be regionalized on mouse spermatozoa, as was previously reported on ram spermatozoa. Such regionalization is also observed on hyperactivated and control incubated spermatozoa. Hyperactivation results in no significant differences in diffusion rate on any region of the sperm surface when compared with fresh cauda epididymal sperm. Significant decreases in the fraction diffusing, however, occur on both the anterior region of the head and the tail. Since hyperactivation requires a 3-h incubation in medium with 3% wt/vol BSA, a more relevant comparison is with control incubated sperm maintained for 3 h in 0.4% wt/vol BSA. As noted above, maintenance of sperm in 0.4% wt/vol BSA does not affect motility. As shown in Table II, however, these sperm show reduced diffusion rate in all regions except the posterior region of the head and no change in $\%R$ on any region, when compared with fresh cauda epididymal sperm. When these control sperm are compared with hyperactivated sperm, we observe that hyperactivation results in significant increases in diffusion rate on all regions except the posterior region of the head and a decrease in $\%R$ on the anterior region of the head. Thus, as one might expect, in vitro capacitation results in membrane changes in the anterior region of the head (perhaps related to induction of the acrosome reaction) and in the flagellum (perhaps related to onset of hyperactivation).

*Table III. t Test Comparisons of Hyperactive, Control, and Fresh Sperm*

| A. $t$ Tests for diffusion coefficient | Fresh cauda epididymal | | | |
|---|---|---|---|---|
| | ah | ph | m | t |
| Hyperactivated | | | | |
| ah | NS | NS | <0.0005 | <0.0005 |
| ph | NS | NS | <0.0005 | <0.0005 |
| m | <0.0005 | <0.005 | NS | NS |
| t | <0.005 | <0.0005 | NS | NS |
| | Fresh cauda epididymal | | | |
| Control | | | | |
| ah | <0.005 | NS | <0.0005 | <0.0005 |
| ph | NS | NS | <0.0005 | <0.0005 |
| m | <0.01 | <0.01 | <0.005 | NS |
| t | NS | NS | NS | <0.0005 |
| | Hyperactivated | | | |
| Control | | | | |
| ah | <0.0005 | NS | <0.0005 | <0.005 |
| ph | NS | NS | <0.005 | <0.0005 |
| m | <0.01 | <0.01 | <0.01 | NS |
| t | NS | NS | NS | <0.0005 |
| | Fresh cauda epididymal | | | |
| B. $t$ Tests for $\%R$ | ah | ph | m | t |
| Hyperactivated | | | | |
| ah | <0.0005 | NS | NS | <0.0005 |
| ph | NS | NS | NS | <0.0005 |
| m | NS | NS | NS | <0.005 |
| t | NS | NS | NS | <0.005 |
| | Fresh cauda epididymal | | | |
| Control | | | | |
| ah | NS | NS | NS | <0.0005 |
| ph | NS | NS | NS | <0.0005 |
| m | NS | NS | NS | <0.005 |
| t | NS | NS | NS | NS |
| | Hyperactivated | | | |
| Control | | | | |
| ah | <0.005 | NS | NS | NS |
| ph | NS | NS | NS | NS |
| m | NS | NS | NS | NS |
| t | NS | NS | NS | NS |

$t$ test comparisons are here set up as a matrix. Consider for instance the first matrix which compares diffusion coefficient on cauda epididymal to that on hyperactivated sperm. Diagonal elements give the $p$ value comparing a given region on the two types of sperm. Elements above the diagonal give interregional comparisons on cauda epididymal sperm, and those below the diagonal give interregional comparisons on hyperactivated sperm. NS, not significant. ah, anterior head. ph, posterior head. m, midpiece. t, tail.

## Discussion

Our data extend to the mouse the previous observation on ram spermatozoa (57) that lipid "fluidity," as measured by lipid analogue lateral diffusibility, is regionalized over the morphologically distinct regions of the sperm plasma membrane. Ultrastructural studies suggest the possibility of barriers to diffusion in mouse (50) and other mammalian sperm (19). Also extended to the mouse is the observation that carbocyanine dyes show significant immobile fractions on ram (58) and guinea pig sperm (Koppel, D. E., personal communication). That changes in lipid diffusibility result from incubation in BSA are not surprising, especially if BSA is modifying membrane composition. Since membrane composition is regionalized, it is not surprising that fluidity changes are also regionalized. Thompson and Axelrod have shown that delipidation of erythrocytes with BSA alters membrane "fluidity" (52) and we have found (unpublished results) that a 1-h incubation of Y1 cells in tissue culture in serum free medium results in a doubling of $C_{16}$dil diffusion rate.

Our results enable us to consider lipid diffusibility in three physiological states: ($a$) cauda epididymal sperm with normal flagellar activity, ($b$) control incubated sperm also with normal flagellar activity, and ($c$) BSA-treated sperm with hyperactivated flagellar activity. In all cases diffusion is fastest on the two regions of the flagellum. Taken alone this observation suggests a need for greater "fluidity" in the motile regions of the sperm surface. However this hypothesis is not supported by the comparison of lipid diffusibility on cauda epididymal and control incubated spermatozoa. Although these two "states" of the sperm have identical motilities, their lipid

diffusibilities are quite different. Thus, whereas fluidity may be a requirement for motility, motility is supported over a wide range of fluidities. Similarly, a comparison of lipid analogue diffusion rates on control with those on hyperactivated sperm suggests a requirement of increased fluidity on the midpiece and tail to support hyperactivated motility. The observation that cauda epididymal sperm, with motility identical to that of control incubated sperm, however, show no significant differences in lipid analogue diffusion rate as compared with hyperactivated sperm argues against a simple relationship between hyperactivated motility and fluidity. Indeed, to see any statistically significant differences in lipid analogue diffusibility between cauda epididymal sperm and hyperactivated sperm, one must consider the diffusing fractions as well as the diffusion coefficients.

The failure of simple arguments about fluidity to relate physiological membrane transformations to changes in the molecular motions of membrane components is widespread (22, 24, 59, 60). This suggests subtle complexities in membrane organization. For instance, just as the major morphological regions of the sperm plasma membrane represent domains of different membrane composition and fluidity, each region may be further divided on a submicroscopic scale into domains (22, 23, 24, 48, 59, 60). Such domains are a common feature of synthetic membranes composed of multiple lipids (22, 32, 48). Significant amounts of gel-phase phospholipid domains could account for observed nondiffusing fractions (22). It has been suggested that physiological transformations do not represent a change in bulk membrane fluidity but rather in the ensemble of domains present within the membrane (59, 60). As discussed above, it has been suggested that the role of BSA in in vitro capacitation is to modulate membrane cholesterol to phospholipid ratios and that a similar event occurs in vivo (12–14, 16, 20). Cholesterol is known to alter dramatically the lateral domain organization of synthetic bilayers (32). Ultrastructural evidence suggests that similar reorganizations may be involved in capacitation (5, 17). Such reorganizations can be expected to affect the functioning of membrane proteins. It is possible for instance that hyperactivation results from lipid reorganizations that alter the conductivity of ionic channels within the membrane. A likely candidate is a membrane $Ca^{++}$ channel, since cytoplasmic $Ca^{++}$ has been implicated as regulating hyperactivated motility (18, 47, 62). Upon capacitation the sperm plasma membrane acquires the ability to transport $Ca^{++}$ (42). This transport may not be via an active mechanism (10).

While the precise cause and effect relationship between BSA-induced changes in sperm motility and sperm membrane lipid diffusibility remains to be determined, several conclusions can be drawn from these FPR measurements: First, as previously observed on ram spermatozoa (56, 60a) lipid diffusibility is regionalized on mouse spermatozoa; second, BSA, which induces hyperactivation of these sperm, and which in this and other systems (20, 34, 49, 52) is known to alter lipid composition, particularly cholesterol-to-phospholipid ratios, also induces changes in the physical state of the membrane as measured by lipid analogue diffusibility; and finally, these physical changes in the membrane are also localized to specific morphological regions of the sperm surface. These physical changes occur on the anterior region of the head and the two regions of the flagellum, the midpiece and the tail; the regions one would expect to be involved in the physiological transformations of the acrosome reaction and hyperactivated motility.

These results suggest several directions for future investigation that we are pursuing actively: Do similar changes in membrane lipid diffusibility occur in vivo? What lipid diffusibility changes occur when delipidated or selectively relipidated BSA is used? Can some or all aspects of capacitation be induced by nonprotein lipid transfer systems?

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