Lateral Diffusion of the PH-20 Protein on Guinea Pig Sperm: Evidence That Barriers to Diffusion Maintain Plasma Membrane Domains in Mammalian Sperm

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Abstract. PH-20 protein on the plasma membrane ($PH\text{-}20_{PM}$) is restricted to the posterior head of acrosome-intact guinea pig sperm. During the exocytotic acrosome reaction the inner acrosomal membrane (IAM) becomes continuous with the posterior head plasma membrane, and $PH\text{-}20_{PM}$ migrates to the IAM. There it joins a second population of PH-20 protein localized to this region of the acrosomal membrane ($PH\text{-}20_{AM}$) (Cowan, A. E., P. Primakoff, and D. G. Myles, 1986, *J. Cell Biol.* 103:1289–1297). To investigate how the localized distributions of PH-20 protein are maintained, the lateral mobility of PH-20 protein on these different membrane domains was determined using fluorescence redistribution after photobleaching. $PH\text{-}20_{PM}$ on the posterior head of acrosome-intact sperm was found to be mobile, with a diffusion coefficient and percent recovery typical of integral membrane proteins ($D = 1.8 \times 10^{-10}$ cm²/s; %R = 73). This value of D was some 50-fold lower than that found for the lipid probe 1,1-ditetradecyl 3,3,3′,3′-tetramethylindocarbocyanine perchlorate (C_{14}diI) in the same region ($D = 8.9 \times 10^{-9}$ cm²/s). After migration to the IAM of acrosome-reacted sperm, this same population of molecules ($PH\text{-}20_{PM}$) exhibited a 30-fold increase in diffusion rate ($D = 4.9 \times 10^{-9}$ cm²/s; %R = 78). This rate was similar to diffusion of the lipid probe C_{14}diI in the IAM ($D = 5.4 \times 10^{-9}$ cm²/s). The finding of free diffusion of $PH\text{-}20_{PM}$ in the IAM of acrosome-reacted sperm supports the proposal that PH-20 is maintained within the IAM by a barrier to diffusion at the domain boundary. The slower diffusion of $PH\text{-}20_{PM}$ on the posterior head of acrosome-intact sperm is also consistent with localization by barriers to diffusion, but does not rule out alternative mechanisms.

THE ability of many cell types to regionalize components of the plasma membrane is now well established. However, the mechanisms by which the cell overcomes the randomizing effects of free diffusion in a fluid lipid bilayer are still largely unknown. Measurement of lateral diffusion rates has shown that the attainment of a nonrandom distribution of some membrane proteins is correlated with a restriction in diffusion or complete immobilization of the protein (Angelides, 1986; Stya and Axelrod, 1983). Immobilization has been suggested to occur by interaction with a plasma membrane-associated cytoskeleton (Golan and Veatch, 1980; Sheetz et al., 1980; Wu et al., 1982), although other means are possible (Cherry, 1979). In other cases, however, localized molecules can be freely diffusing (Myles et al., 1984), suggesting that other mechanisms exist to maintain the localized distribution.

The extreme structural polarity of the mammalian sperm cell provides a unique opportunity to study the mechanisms involved in initiating and maintaining an asymmetric membrane topography. At least five distinct surface regions can be identified morphologically on the mature sperm (Friend, 1977), and a substantial degree of differential partitioning of membrane components among the different regions has been demonstrated (Friend, 1982; Primakoff and Myles, 1983). Lateral diffusion rates of both lipid probes (Wolf and Voglmayr, 1984; Wolf et al., 1986*a*) and membrane proteins (Wolf et al., 1986*b*) also show marked regionalization over the sperm surface, indicating that the morphological regions exhibit distinct membrane structure. Furthermore, the mature sperm cell has essentially no synthetic capabilities, yet changes in its surface composition are temporally coordinated with developmentally regulated changes in surface functions (Nicolson and Yanagimachi, 1979; O'Rand, 1979; Bearer and Friend, 1982; Myles and Primakoff, 1984). At least some of these changes in surface composition occur by the topographical rearrangement of proteins already present within the membrane (Bearer and Friend, 1982; Myles and Primakoff, 1984).

We have examined the mechanism of localization of one membrane protein on the guinea pig sperm cell, the PH-20 protein. Previous evidence has indicated that this protein functions in sperm binding to the egg zona pellucida (Prima-

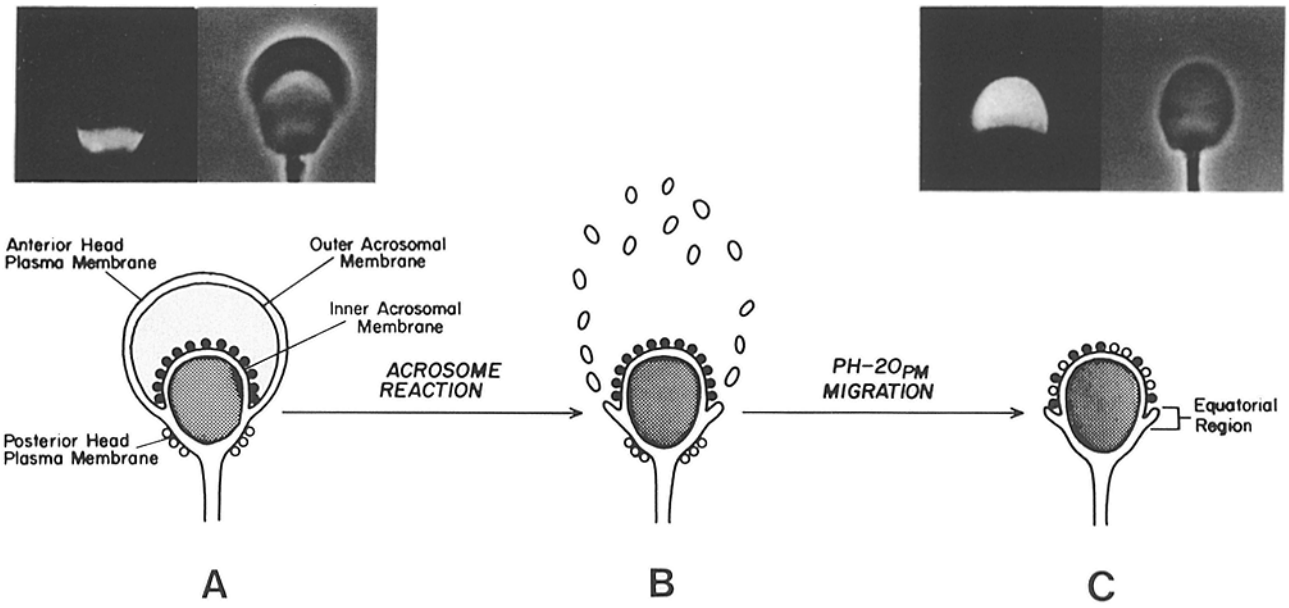

Figure 1. Distribution of PH-20 protein on acrosome-intact and acrosome-reacted sperm. The top panel shows the surface distribution of PH-20 protein revealed by indirect immunofluorescence of live acrosome-intact and acrosome-reacted sperm stained with intact PH-22 mAb and an RITC-labeled Fab second antibody. The bottom panel shows a schematic representation of the distribution of the two populations of PH-20 protein. On acrosome-intact sperm (*A*), PH-20_{PM} (*open circles*) is localized to the posterior head plasma membrane. PH-20_{AM} (*closed circles*) is initially intracellular and localized to a discrete region of the acrosomal membrane, the inner acrosomal membrane. During the acrosome reaction (*B*), the IAM is inserted into the plasma membrane and thus becomes continuous with the posterior head plasma membrane through the equatorial region. After the acrosome reaction (*C*), PH-20_{PM} migrates to the IAM, where both populations of PH-20 remain localized on the acrosome-reacted sperm. (Modified from Cowan et al., 1986.)

koff et al., 1985). Regulation of the surface distribution of PH-20 protein is complex, and involves a specific redistribution and an increase in surface expression of the protein in conjunction with the acrosome reaction (Myles and Primakoff, 1984; Cowan et al., 1986). The acrosome reaction is a calcium-regulated exocytosis that entails multiple fusions of the plasma membrane of the anterior head region with a region of the secretion vesicle membrane, the outer acrosomal membrane. This results in dispersion of the acrosomal contents and confluence of the inner acrosomal membrane (IAM)[1] with the plasma membrane. On acrosome-intact sperm, PH-20 protein on the plasma membrane (PH-20_{PM}) is localized to the posterior head region. Following the acrosome reaction, PH-20_{PM} migrates to the IAM, where it joins a second, initially intracellular, population of PH-20 protein that preexists on this region of the acrosomal membrane (PH-20_{AM}) (Fig. 1). All the PH-20 protein is thus confined to the IAM of the acrosome-reacted sperm.

To investigate the mechanism of localization of PH-20 protein, we have used fluorescence redistribution after photobleaching (FRAP) to measure the lateral diffusion of PH-20 on both the posterior head plasma membrane of acrosome-intact sperm and on the IAM of acrosome-reacted sperm. Our results show that PH-20_{PM} is mobile in both of these domains. On the IAM, diffusion of PH-20_{PM} is comparable with that of the lipid probe C_{14}diI, as would be predicted for free diffusion of the protein in a fluid bilayer. Thus, we suggest that localization of PH-20_{PM} to the IAM is accomplished by a barrier at the domain boundary that prevents diffusion of PH-20_{PM} onto the rest of the cell. Diffusion of PH-20_{PM} on the posterior head plasma membrane of acrosome-intact cells is some 30-fold slower. The slower rate of diffusion is still consistent with localization by barriers to diffusion, but also allows alternative mechanisms for localization of PH-20_{PM} to the posterior head domain.

Materials and Methods

Antibodies

Preparation of the Fab′ fragment of the PH-22 mAb, one of three mAbs in our collection that recognize the PH-20 protein, has been described previously (Cowan et al., 1986). Fab′-PH-22 was free of $F(ab')_2$ fragments or intact IgG as determined from polyacrylamide gels stained with Coomassie Blue. To label cells with sufficient intensity for photobleaching, cells were fluorescently labeled indirectly using a univalent second antibody. A Fab′ fragment was prepared from a $F(ab')_2$ fragment of goat anti-mouse IgG [$F(ab')_2$-specific] (CooperBiomedical, Inc., Malvern, PA) by reduction with 20 mM cysteine followed by alkylation in 30 mM iodoacetamide. Fab′ was separated from $F(ab')_2$ on a Sephadex G-100 column. Purity of the Fab′ was assessed on polyacrylamide gels stained with Coomassie Blue. Rhodamine conjugation was carried out as described in Cowan et al. (1986), at a protein to dye ratio of 25 μg rhodamine isothiocyanate (RITC) per mg Fab′. The RITC-Fab′ second antibody was preabsorbed with sperm before use.

1. *Abbreviations used in this paper*: IAM, inner acrosomal membrane; FRAP, fluorescence redistribution after photobleaching; C_{14}diI, 1,1-ditetradecyl 3,3,3′,3′-tetramethylindocarbocyanine perchlorate; MgHepes, 0.14 M NaCl, 4 mM KCl, 4 mM Hepes pH 7.4, 10 mM glucose, 2 mM $MgCl_2$; mT, modified Tyrode's medium (109 mM NaCl, 2.8 mM KCl, 0.5 mM $MgCl_2$, 25 mM $NaHCO_3$, 5.6 mM glucose, 10 mM lactic acid, 1 mM sodium pyruvate, pH 7.6, containing 0.3% BSA); PH-20_{AM}, PH-20 protein localized to the inner acrosomal membrane; PH-20_{PM}, PH-20 protein on the plasma membrane; RITC, rhodamine isothiocyanate.

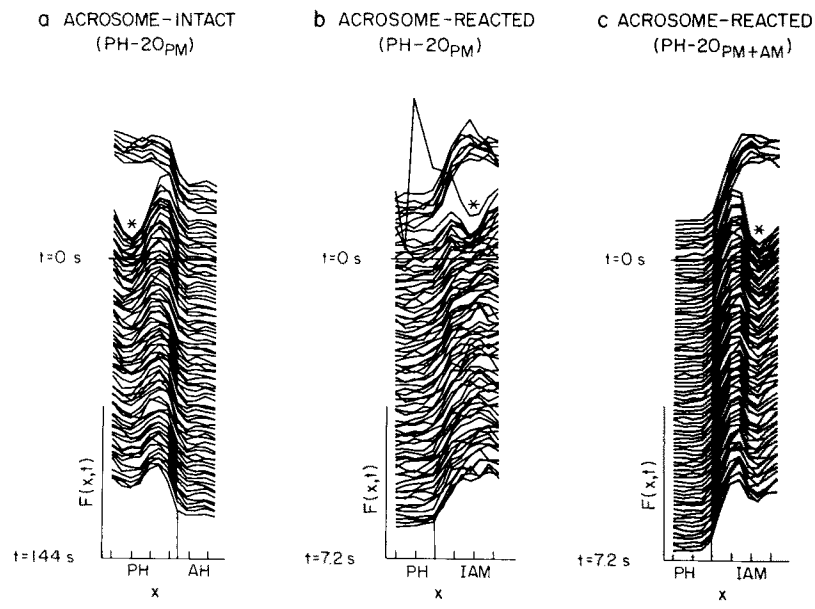

Figure 2. Fluorescence intensity measurements [$F(x,t)$] at twelve points aligned along the anterior-posterior axis (x) of the sperm head stacked as a function of time before and after photobleaching. The first eight scans show the initial distribution of labeled PH-20 on the sperm surface. The fluorescence at one point was then bleached (at $t = 0$) and subsequent scans show the recovery of fluorescence in the bleached regions. An asterisk indicates the bleached region in the first postbleach scan. During the bleach, the shutter to the photomultiplier is closed, thus the $t = 0$ scan line shows the closed-shutter 0 line for fluorescence intensity at $t = 0$. Note that the localized pattern of PH-20 fluorescence does not change during the time scale of the experiments. (*a*) $PH\text{-}20_{PM}$ on an acrosome-intact cell. (*b*) $PH\text{-}20_{PM}$ on an acrosome-reacted cell. (*c*) $PH\text{-}20_{PM+AM}$ on an acrosome-reacted cell. The time scale in *b* and *c* is reduced by a factor of 20 compared with *a*. The first postbleach scan in *b* is distorted by the tail of the decay of phosphorescence excited in the glass microscope slide by the photobleaching pulse. Such scans were not included in the data analysis.

Cells

Sperm were isolated from the cauda epididymis of male Hartley guinea pigs (Buckberg Lab Animals, Inc., Tomkins Cove, NY). Acrosome-intact cells were isolated in MgHepes medium (0.14 M NaCl, 4 mM KCl, 4 mM Hepes pH 7.4, 10 mM glucose, 2 mM $MgCl_2$; modified from Green, 1978). The acrosome-intact cells were immobilized for FRAP experiments by embedding them in agar, as described by Myles et al. (1984). Using this procedure, some sperm are completely immobilized in the agar except for part of the posterior tail, which continues to beat in a pocket of liquid. Only sperm with actively beating tails were chosen for experiments.

Cells were acrosome-reacted by the addition of calcium to sperm previously capacitated in a modified Tyrode's medium (mT; 109 mM NaCl, 2.8 mM KCl, 0.5 mM $MgCl_2$, 25 mM $NaHCO_3$, 5.6 mM glucose, 10 mM lactic acid, 1 mM sodium pyruvate, pH 7.6 containing 0.3% BSA [BSA; Calbiochem-Behring Corp., La Jolla, CA; fraction V]) according to Fleming and Yanagimachi (1981). Briefly, cells were isolated in 0.9% NaCl at 10^8 cells/ml. 10^7 cells were diluted into 1.5 ml mT medium lacking Ca^{2+} in a 35 × 10 mm petri dish, overlain with mineral oil, and incubated overnight (16 h) at 37°C. The acrosome reaction was initiated by the addition of an equal volume of mT containing 4 mM Ca^{2+}. For FRAP experiments, a 5-μl aliquot of cells was applied to ethanol-washed slides and a coverslip whose edges were lined with vasoline applied. The head of acrosome-reacted sperm adhered tightly to the glass surface while the tail remained unattached and continued to beat vigorously with the characteristic motion of hyperactivated cells. Only actively motile sperm were used for FRAP experiments.

Labeling of Cells

To examine $PH\text{-}20_{PM}$ protein on the posterior head of acrosome-intact cells, 100 μl of sperm at 10^8 cells/ml were incubated with 100 μl of the Fab′ fragment of PH-22 mAb at 20 μg/ml in MgHepes medium for 20 min at room temperature. The cells were pelleted through MgHepes containing 3% BSA (Sigma Chemical Co., St. Louis, MO; fraction V), then incubated with 100 μl of RITC-Fab′ second antibody at 200 μg/ml for 20 min at room temperature. The cells were again pelleted through 3% BSA and resuspended in 100 μl MgHepes. To examine $PH\text{-}20_{PM}$ protein after its migration to the IAM, acrosome-intact sperm that had been capacitated overnight were pelleted, incubated for 20 min with Fab′–PH-22, washed through mT containing 3% BSA, then washed again in mT alone. The cells were then added to 3 ml mT medium containing 2 mM Ca^{2+} at 37°C and incubated for 15 min to allow the acrosome reaction to proceed. The cells were pelleted, stained for 20 min with RITC-Fab′ second antibody, washed through 3% BSA in mT containing 2 mM Ca^{2+}, and resuspended in mT containing 2 mM Ca^{2+}. For measurement of the combined population of $PH\text{-}20_{PM}$ and $PH\text{-}20_{AM}$, acrosome-reacted sperm were labeled for 20 min with Fab′–PH-22 in mT containing 2 mM Ca^{2+}, washed through 3% BSA in mT containing 2 mM Ca^{2+}, then stained for 20 min with RITC-Fab′ second antibody for 20 min at room temperature. The cells were again washed through 3% BSA and resuspended in mT containing 2 mM Ca^{2+}. To label only the $PH\text{-}20_{AM}$ population on the IAM of acrosome-reacted cells, migration of $PH\text{-}20_{PM}$ to the IAM was blocked by cross-linking $PH\text{-}20_{PM}$ protein on the posterior head with intact mAb and divalent second antibody before the acrosome-reaction (Cowan et al., 1986). The sperm were then acrosome reacted and labeled with PH-22 Fab′ and RITC-Fab′ second antibody.

For labeling with the lipid probe 1,1-tetradecyl 3,3,3′,3′-tetramethylindocarbocyanine perchlorate (C_{14}diI; Molecular Probes, Inc., Junction City, OR), 50 μl of sperm at 10^8/ml were incubated at room temperature for 15 min in 1 ml MgHepes (acrosome-intact sperm) or mT containing 2 mM Ca^{2+} (acrosome-reacted sperm) freshly mixed with 5 μl of a stock solution of C_{14}diI at 1 mg/ml in ethanol. The cells were washed by pelleting them through 3% BSA in MgHepes or mT containing 2 mM Ca^{2+}.

FRAP

The optical and electronic systems used for photobleaching measurements have been described in detail elsewhere (Koppel, 1979). Briefly, a focused laser beam is sequentially centered at 12 discrete locations equally spaced 0.5 μm apart along the sperm head. Fluorescence intensity measurements are made at each of the 12 positions in a series of scans before and after bleaching by a short pulse (8 ms) with a high intensity beam. To accommodate different characteristic diffusion times, the time interval between scans could be varied, keeping the laser intensity and total sample exposure constant. Using this method, we obtained information regarding the fluorescence distribution over the entire head of the sperm and could thus document the localized distribution of PH-20 during the course of the experiment. The redistribution of fluorescence was analyzed as described by Koppel (1979), and the diffusion coefficient (D) calculated from $D = w_0^2/4\tau_D$, where w_0 is the 1/e radius of the initial post-bleach distribution of fluorescence depletion fitted to a Gaussian distribution, and τ_D is the characteristic half-time for fluorescence recovery. The initial distribution of fluorescence depletion was determined for each set of data by extrapolating the intensities measured at each position back to $t = 0$. τ_D and the percent recovery (%R) were determined by nonlinear least squares analysis of the traces of fluorescence depletion measured at the position of the bleaching pulse.

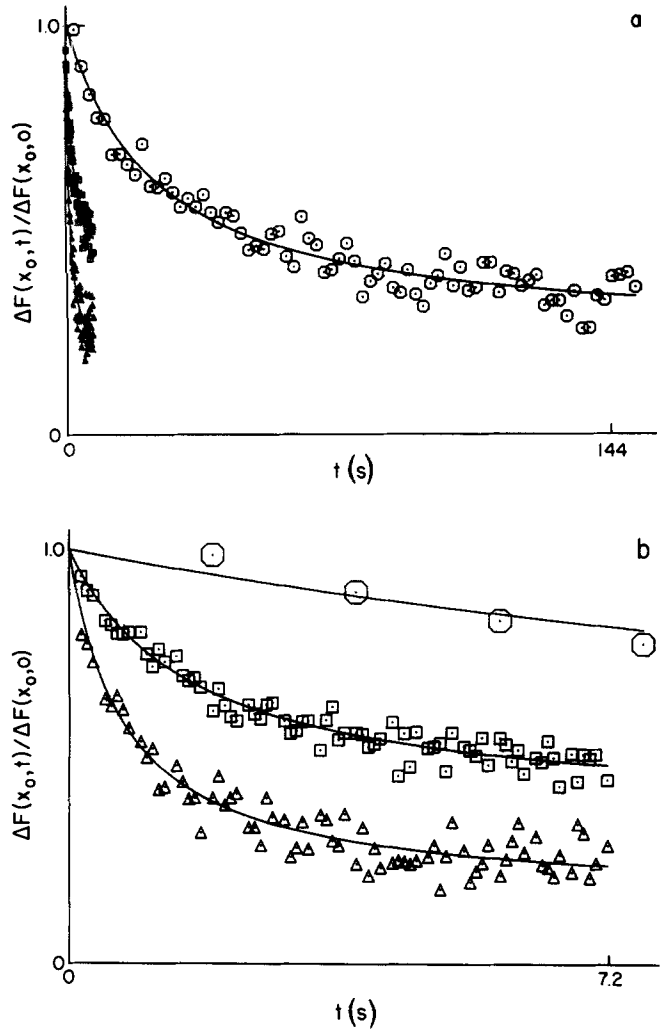

Figure 3. Decay of fluorescence depletion after photobleaching [$F(x_0,t)$], measured coincident with the position of the bleaching pulse (x_0), normalized by the computer-fitted initial postbleach depletion [$F(x_0,0)$]. (*a* and *b*) The same data for PH-20 plotted using different time scales. (*Open circles*) $PH\text{-}20_{PM}$ on the posterior head of acrosome-intact sperm; (*open triangles*) $PH\text{-}20_{PM}$ and (*open squares*) $PH\text{-}20_{PM+AM}$ on the IAM of acrosome-reacted sperm. The corresponding curves are computer-generated least squares fits to the data. The diffusion coefficient, D, is derived from both the decay of fluorescence depletion curves and the dimensions of the area bleached, determined from the scan data shown in Fig. 2.

Results

Diffusion characteristics of PH-20 protein in three different situations were investigated: (*a*) $PH\text{-}20_{PM}$ in the posterior head plasma membrane (Fig. 1 *A*); (*b*) the same population of $PH\text{-}20_{PM}$ after it has migrated to the IAM (Fig. 1 *C*, *open circles*); and (*c*) a mixed population of $PH\text{-}20_{PM}$ and $PH\text{-}20_{AM}$ in the IAM (Fig. 1 *C*, *open* and *closed circles*). For illustrative purposes, typical sets of FRAP data for each of these three cases are presented in Figs. 2 and 3. Fig. 2 shows each data set as a stack of individual fluorescence scans along the sperm head recorded before and after bleaching. In Fig. 3, fluorescence depletion measured at the location of the bleaching pulse is presented as a function of time after bleaching for each of the data sets in Fig. 2. Figs. 4 and 5 show similar sets of data for acrosome-intact and acrosome-reacted cells labeled with C_{14}diI. The results of computer analyses of many fluorescence depletion curves are summarized in Table I.

Table I. Diffusion Coefficients and Percent Recovery for PH-20 and C_{14}diI

	Region	D × 10^9 (cm^2/s)	Percent recovery	*n*
Acrosome-intact				
$PH\text{-}20_{PM}$	Posterior head	0.18 ± 0.05	73 ± 3	4
C_{14}diI	Posterior head	8.9 ± 2.1	95.2 ± 0.3	5
	Anterior head	10.5 ± 3.8	89.9 ± 6.6	5
Acrosome-reacted				
$PH\text{-}20_{PM}$	IAM	4.9 ± 2.0	78 ± 9	8
$PH\text{-}20_{PM+AM}$	IAM	1.7 ± 0.5	64 ± 17	5
C_{14}diI	IAM	5.0 ± 1.7	85.0 ± 3.6	5
C_{14}diI	Posterior head	5.4 ± 1.4	88.6 ± 3.0	5

FRAP of acrosome-intact sperm revealed that $PH\text{-}20_{PM}$ in the posterior head domain (Fig. 1 *A*) is mobile, as evidenced by the recovery of fluorescence in the bleached region shown in Fig. 2 *a*, and 3, *a* and *b*. From the decay of depletion curves such as those shown in Fig. 3, *a* and *b*, a diffusion coefficient $D = (1.8 \pm 0.5) \times 10^{-10}$ cm^2/s was obtained (Table I). A high percentage of the protein is mobile, as shown by the near complete recovery of fluorescence (%R = 73 ± 3; Table I). From the scan data shown in Fig. 2 *a*, it is clear that the localized distribution of $PH\text{-}20_{PM}$ is maintained while the protein is diffusing.

After migration of $PH\text{-}20_{PM}$ to the IAM (Fig. 1 *C*), the diffusion coefficient for $PH\text{-}20_{PM}$ increased by nearly 30-fold to $D = (4.9 \pm 2.0) \times 10^{-9}$ cm^2/s (Table I; Figs. 2 *b* and 3, *a* and *b*). Most of the $PH\text{-}20_{PM}$ in the IAM was mobile, as evidenced by the high percent recovery (%R = 78 ± 9; Table I), even though it maintains a localized distribution (Fig. 2 *b*).

FRAP measurements of the combined populations of $PH\text{-}20_{PM}$ and $PH\text{-}20_{AM}$ in the IAM were made on sperm that were labeled with antibody after the acrosome reaction (Fig. 1 *c*). Diffusion of the combined populations was similar to that of $PH\text{-}20_{PM}$ alone, with $D = (1.7 \pm 0.5) \times 10^{-9}$ cm^2/s (Table I; Figs. 2 *c* and 3, *a* and *b*). The percent recovery observed for the combined population of PH-20 on the IAM was more variable than observed for $PH\text{-}20_{PM}$ alone, ranging from 45 to 86%, with an average of 64% (Table I). Previous experiments have shown that $PH\text{-}20_{AM}$ accounts for approximately two-thirds of the total number of PH-20 molecules in the IAM of acrosome-reacted sperm (Cowan et al., 1986). From the result that 80% of the $PH\text{-}20_{PM}$ molecules are mobile, it can be deduced that approximately one-half of the $PH\text{-}20_{AM}$ in the IAM is mobile. Attempts to measure the mobility of the $PH\text{-}20_{AM}$ alone by preventing migration of $PH\text{-}20_{PM}$ to the IAM after the acrosome reaction (see Materials and Methods) yielded highly variable percent recoveries, and were not pursued further.

To investigate whether the observed difference in mobility of PH-20 protein in the different domains of the sperm might be due to differences in lipid viscosity (Saffman and Delbruck, 1975), diffusion coefficients were measured for the

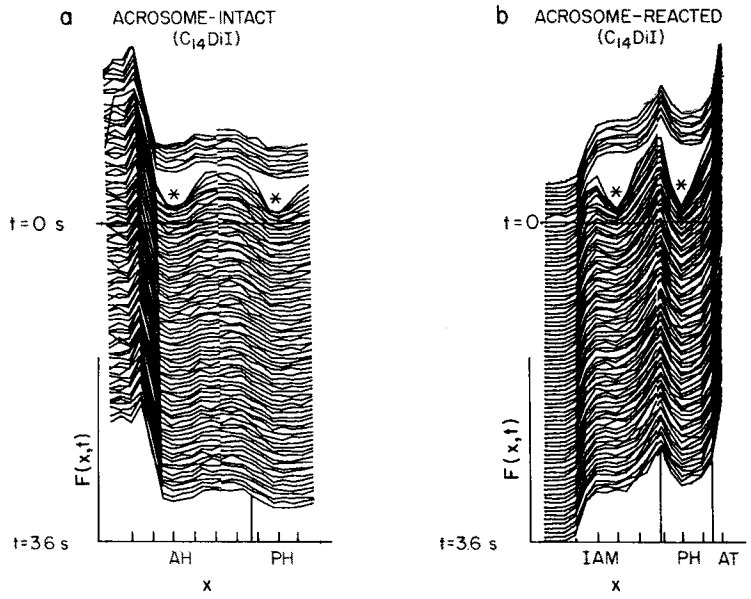

Figure 4. Stack diagrams of fluorescence intensity measurements of sperm stained with C_{14}diI before and after photobleaching. Each cell was aligned in the microscope so that the posterior head region could be analyzed, then the cell was realigned, and the anterior head (acrosome-intact cell) or IAM (acrosome-reacted cell) of the same cell was analyzed. The scans from the two measurements of each cell are shown on one graph by aligning the scans near the equatorial region of the sperm cell. (*a*) Acrosome-intact cell; (*b*) acrosome-reacted cell. The peak of fluorescence in *a* is due to an edge-effect at the anterior border of the cell. The peak in fluorescence in *b* marks the folded membrane of the equatorial region. Also visible in *b* are intensity measurements made on the anterior tail, which exhibits brighter fluorescence than the head of the sperm cell. The bleached region in the first postbleach scan is marked by an asterisk.

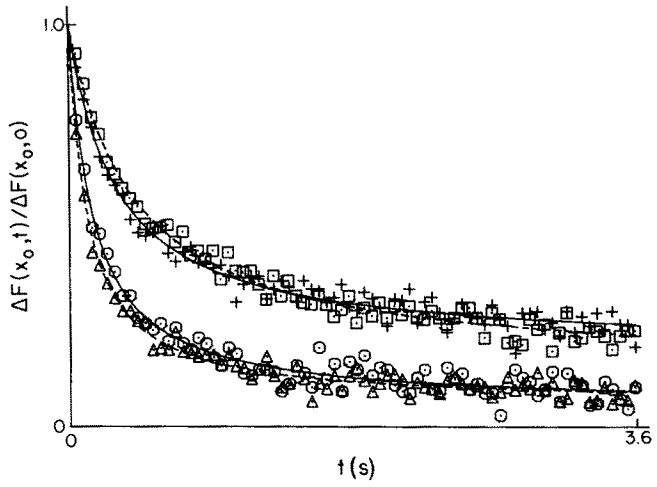

Figure 5. Decay of fluorescence depletion after photobleaching sperm labeled with C_{14}diI. (*Open circles*) Posterior head and (*open triangles*) anterior head of an acrosome-intact cell; (*open squares*) posterior head and (+ —— +) IAM of an acrosome-reacted cell. Although the decay of depletion curves for C_{14}diI and for PH-20_{PM} on the IAM are different, the calculated D is similar because of compensating differences in the dimensions of the bleached areas.

lipid probe C_{14}diI in different regions of acrosome-intact and acrosome-reacted sperm. FRAP measurements on both the posterior and anterior head of acrosome-intact sperm revealed no significant difference in lipid mobility between these two regions, with $D = (8.9 \pm 2.1) \times 10^{-9}$ cm²/s for the posterior head region and $(10.5 \pm 3.8) \times 10^{-9}$ cm²/s for the anterior head (Figs. 4 *a* and 5; Table I). Similarly, no difference in lipid mobility between the posterior head plasma membrane and the IAM of acrosome-reacted sperm was observed, with $D = (5.4 \pm 1.4) \times 10^{-9}$ cm²/s for the posterior head and $(5.0 \pm 1.7) \times 10^{-9}$ cm²/s for the IAM (Figs. 4 *b* and 5; Table I). In all cases near complete recovery was observed, indicating that there is no substantial immobile lipid fraction. Comparing the posterior head plasma membrane of acrosome-intact sperm with the IAM of acrosome-reacted sperm, there is only an ~two-fold reduction in lipid mobility, in contrast to the increase in diffusion of the PH-20_{PM} protein between these two regions.

Discussion

We have shown that PH-20 protein is mobile on two different localized domains of the guinea pig sperm cell: the posterior head plasma membrane of acrosome-intact sperm and the IAM of acrosome-reacted sperm. The diffusion of PH-20_{PM} in the IAM of acrosome-reacted cells ($D = 4.9 \pm 2.0 \times 10^{-9}$ cm²/s) is comparable with the rate of diffusion observed for the lipid probe C_{14}diI in this region ($D = 5.0 \pm 1.7 \times 10^{-9}$ cm²/s), as would be predicted for unrestricted diffusion of protein in a fluid lipid bilayer (Saffman and Delbruck, 1975). The finding that PH-20 protein is freely diffusing within its domain on the IAM rules out mechanisms of localization that require immobilization or reductions of protein mobility. Rather, our findings support the hypothesis that maintenance of PH-20_{PM} protein within the IAM domain occurs by barriers at the domain boundary that prevent diffusion of PH-20 onto the rest of the cell membrane.

A similar diffusion rate ($D = 1.7 \pm 0.5 \times 10^{-9}$ cm²/s) was observed for the combined population of PH-20_{AM} and PH-20_{PM} on the IAM of acrosome-reacted sperm. Although an immobile fraction of PH-20_{AM} was detected, the data indicate that about half of the PH-20_{AM} diffuses at a rate only slightly slower than PH-20_{PM}. A barrier to diffusion is the most straightforward hypothesis to account for such localization of a freely diffusing membrane protein, but other mechanisms are possible. For example, localization might occur by thermodynamic partitioning of the protein into a physicochemically distinct fluid lipid phase.

On acrosome-reacted sperm, the boundary of the IAM lies within the equatorial region. This region consists of three

overlapping folds of membrane, the outermost originally derived from the anterior head plasma membrane, next a small stretch of the outer acrosomal membrane, and innermost a short proximal region of the IAM (see Fig. 1 *C*). Stable structures bridge the extracellular gap between the IAM and the outer acrosomal membrane, and appear to be embedded in the membrane based on a crystalline pattern of intramembraneous particles seen in freeze fracture and surface replicas (Phillips, 1977; Bedford et al., 1979; Russell et al., 1980). These specializations may be a morphological representation of a barrier in the membrane that prevents the diffusion of PH-20 from the IAM to the rest of the cell.

We have no idea what type of structure might constitute a diffusion barrier in the equatorial region of acrosome-reacted sperm. Our evidence indicates only a barrier to diffusion of the PH-20 protein; we do not know whether the diffusion barrier applies to lipids and/or other membrane proteins. In another region of the sperm cell, the junction between the anterior and posterior tail region has been proposed to contain a barrier to prevent movement of the freely diffusing PT-1 protein from the posterior tail region into the anterior tail region (Myles et al., 1984). Freeze-fracture electron microscopy shows that this boundary is delineated by a ring of tightly packed integral membrane proteins (Friend and Fawcett, 1974), which have been proposed to represent a structural barrier within the plane of the membrane. A similar model in epithelial cells suggests that tightly packed integral membrane proteins are involved in forming a barrier between the apical and basolateral domains (van Meer and Simons, 1986; van Meer et al., 1986).

Diffusion of PH-20_{PM} in the posterior head plasma membrane is significantly slower than its diffusion in the IAM. The different rates of diffusion exhibited by PH-20_{PM} could result from significant differences in the environment of PH-20 protein between the posterior head plasma membrane of acrosome-intact cells compared with the IAM of acrosome-reacted cells. Our results with the lipid probe C_{14}diI suggest that the 30-fold difference in PH-20_{PM} mobility in these two regions is not related to changes in lipid viscosity. However, differences in the extracellular and intracellular matrices of these regions have been documented (Fawcett, 1975) and could account for the difference in diffusion rates. Alternatively, the difference in diffusion rates could result from a specific, developmentally regulated modification of the PH-20_{PM} protein itself that may alter potential associations with other membrane components or with itself.

Because PH-20_{PM} on the posterior head of acrosome-intact sperm is mobile, maintenance of the localized distribution on the posterior head by barriers to diffusion would be entirely consistent with our results. However, due to the slower diffusion of PH-20_{PM} in this region, other mechanisms of localization that would result in a decrease in the long-range diffusion coefficient as measured by FRAP can not be ruled out. For example, transient interactions with an immobile species could account for both the slower diffusion and localization of PH-20_{PM} on the posterior head. According to this scenario, a major fraction of the PH-20_{PM} would be localized by binding to an immobile element in the posterior head region, while the remaining fraction of PH-20_{PM} would be free to diffuse throughout the cell membrane at a fast rate (e.g., $D = 10^{-9}$ cm^2/s). If the exchange rate of the bound PH-20_{PM} is fast compared with the time scale of the FRAP measurements (the so-called "diffusion limit"), the diffusion measurements in the region of transient immobilization would show a restricted time-averaged diffusion rate (Koppel, 1981), consistent with our data. The slower mobility of PH-20_{PM} on the posterior head plasma membrane cannot, in itself, account for the maintenance of a localized distribution. Models that posit slower diffusion due to steric hindrance by obstacles within the membrane (Schindler et al., 1980; Saxton, 1982; Eisinger et al., 1986) or by extramembraneous elements (Koppel et al., 1981) predict uniform distributions at equilibrium. Given the diffusion coefficient of $\sim 2 \times 10^{-10}$ cm^2/s, it can be estimated that PH-20 would redistribute to the anterior head plasma membrane with a characteristic time of ~10 min.

The observation that PH-20_{PM} is freely diffusing on the IAM of acrosome-reacted cells has specific implications regarding the mechanism of its migration from the posterior head plasma membrane after the acrosome reaction. Migration by diffusion from the posterior head region with subsequent trapping through immobilization in the IAM cannot be occurring unless the protein is released from immobilization after a diffusion barrier is set up at the equatorial region. Trapping without immobilization could occur, however, if the PH-20 protein is modified on the IAM and only the modified form of PH-20 respects the diffusion barrier. This form of a trap would allow free diffusion of the PH-20 protein in the IAM even during the process of migration. Alternatively, an active mechanism to transport PH-20 protein through a diffusion barrier to the IAM is possible. These models predict different diffusion characteristics for PH-20_{PM} during migration, thus making it possible to investigate these hypotheses using FRAP to characterize the diffusion of PH-20_{PM} during its migration.

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