33. We wish to thank F. Kaye for helpful discussions throughout this study and for a critical reading of the manuscript. Thanks also to I. Magrath for the PA682 and CB cells, to J. Battey and K. Kelly for LY47 and BL37 DNA and for critical reading of the manuscript, to S. Abularach for technical assistance, C. Agnor for work in the initial identification of the PA682 clones, and to U. Siebenlist, F. Kern, S. Mackem, and L. Liotta for a critical reading of the manuscript. M.Z.-K. wishes to thank M. Lippman for continued encouragement and advice during the course of this work.

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Restricted Lateral Diffusion of PH-20, a PI-Anchored Sperm Membrane Protein

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The rate of lateral diffusion of integral membrane proteins is constrained in cells, but the constraining factors for most membrane proteins have not been defined. PH-20, a sperm surface protein involved in sperm-egg adhesion, was shown to be anchored in the plasma membrane by attachment to the lipid phosphatidylinositol and to have a diffusion rate that is highly restricted on testicular sperm, being more than a thousand times slower than lipid diffusion. These results support the hypothesis that lateral mobility of a membrane protein can be regulated exclusively by interactions of its ectodomain.

THE LATERAL DIFFUSION COEFFICIENTS (D) of most integral membrane proteins are much smaller than would be predicted on the basis of hydrodynamic size alone. Interactions beyond those mediated by lipid bilayer viscosity must be at work. The first indication of the possible nature of these interactions came out of studies on erythrocyte membranes; this work demonstrated the constraints on mobility imposed by interactions of cytoplasmic portions of the proteins with underlying cytoskeletal structures (*1*). Studies that made use of artificial lipopolysaccharides and genetically engineered plasma membrane proteins with shortened cytoplasmic domains indicated that noncytoplasmic interactions might be responsible for restricting the lateral diffusion of membrane molecules (*2–4*). The discovery that some proteins are anchored to the outer leaflet of the lipid bilayer by phosphatidylinositol (PI) rather than by a membrane-spanning stretch of hydrophobic amino acids suggested the possibility of measuring the mobility of proteins that are completely free from direct interaction with the cytoskeleton. Measurements on Thy-1, alkaline phosphatase, and decay accelerating factor, all PI-anchored, show that these proteins are freely diffusing at rates comparable to those of lipid probes (*5–7*). We show that the guinea pig sperm protein PH-20 is anchored in the plasma membrane by PI but that its rate of diffusion is highly restricted on testicular sperm. Because PH-20 is localized to surface domains at some stages of sperm differentiation, these findings also show that direct interaction with the cytoskeleton is not required for membrane protein localization.

The PH-20 membrane protein is involved in sperm adhesion to the glycoprotein coat of the egg, the zona pellucida, during the initial steps of fertilization (*8*). A potential connection has been pointed out between the function of certain proteins involved in cell adhesion and PI-anchoring in the membrane (*9, 10*). To determine whether PH-20 is anchored in the bilayer by PI, we treated cells treated with a PI-specific phospholipase C (PI-PLC), which has been shown to cleave PI-anchored plasma membrane proteins from other cells (*9–13*). Sperm were surface-iodinated and the release of PH-20 into the medium after treatment with PI-PLC was detected by immunoprecipitation (*14, 15*). Sperm at three progressive stages of differentiation were used: (i) testicular sperm, (ii) cauda epididymal sperm before sperm exocytosis (the acrosome reaction), and (iii) cauda epididymal sperm after the acrosome reaction. PH-20 was immunoprecipitated from the supernatants of sperm at all three stages after treatment of the cells with PI-PLC; little or no PH-20 protein was detected in the supernatants of untreated controls (Fig. 1).

A comparison of ^{125}I counts found in immunoprecipitable PH-20 released by PI-PLC into the medium with the total PH-20 extracted by NP-40 from untreated testicular sperm indicates that ~60% of the protein is released by 1 hour of exposure to PI-PLC. Incomplete release from intact cells might result from inaccessibility of some of the protein molecules to the enzyme, or it could indicate a population of PH-20 that is either not PI-anchored or has a PI anchor that is insensitive to this particular PI-PLC. Other PI-anchored proteins have also

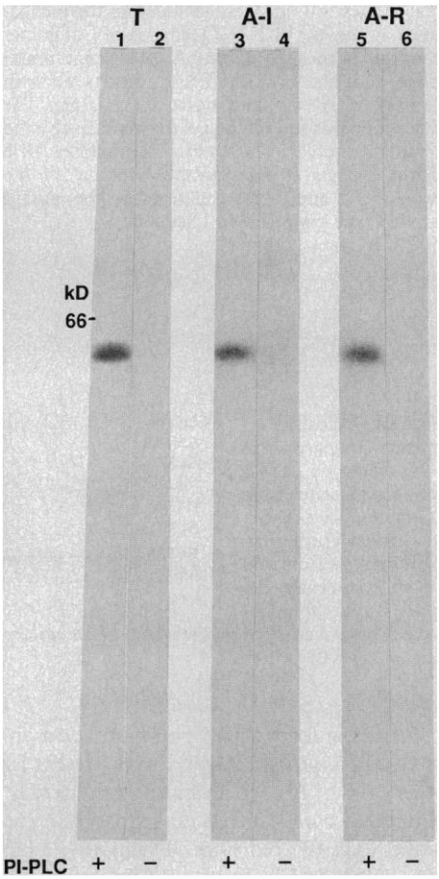

Fig. 1. Immunoprecipitation of PH-20 released from the surface of guinea pig sperm after exposure to PI-PLC. (Lanes 1 and 2) Immunoprecipitates of supernatants from 3×10^6 testicular (T) sperm per lane. (Lanes 3 and 4) Supernatants from 2×10^7 acrosome-intact (A-I) epididymal sperm per lane. (Lanes 5 and 6) Immunoprecipitates of supernatants from 2×10^7 acrosome-reacted (A-R) epididymal sperm per lane. Lanes 1, 3, and 5 are from sperm exposed to PI-PLC; lanes 2, 4, and 6 are from control sperm incubated without PI-PLC. Male Hartley guinea pigs (>700 g) were killed by CO_2 asphyxiation, and live sperm at 1×10^7 to 7×10^7 per milliliter were surface-iodinated with 1 to 4 mCi of $Na^{125}I$ with Iodogen (*15*). The labeled cells were washed with Mg^{2+}-Hepes buffer and evenly distributed to each of two flasks containing either buffer or buffer plus *Bacillus thuringiensis* PI-PLC (*13*) for 1 hour at 37°C. PH-20 was immunoprecipitated from the separated supernatants with either a rabbit polyclonal antiserum to PH-20 plus protein A–Sepharose beads or with a monoclonal antibody (PH-22) bound to Sepharose 4B beads. After boiling and removal of the beads, the samples were run on 10% SDS–polyacrylamide gel electrophoresis (*24, 25*).

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shown less than 100% release from intact cells by PI-PLC (*6, 9, 11, 16, 17*).

To verify that the release of PH-20 into the medium after exposure to PI-PLC was due to a specific enzyme-substrate reaction and not to a generalized disruption of the plasma membrane, we looked at the effect of PI-PLC on three other integral membrane proteins of guinea pig sperm. Little or no protein was detected in the supernatants of either PI-PLC–treated or untreated sperm (Fig. 2A), whereas all three proteins could be immunoprecipitated from the cell pellet after detergent extraction, regardless of exposure to PI-PLC (Fig. 2B).

The mobility of PH-20 was measured by fluorescence redistribution after photobleaching (FRAP) (*18*) on the anterior and posterior regions of the head of testicular sperm (*19*). No appreciable difference in the mobilities or the percent recovery was found between the two head regions. The measured diffusion coefficients of PH-20 ranged from 2.9×10^{-13} to 2.9×10^{-10} cm^2/s, with most of the measurements clustered between 9×10^{-12} and 4×10^{-11} cm^2/s (see box in Fig. 3A). The average D for the points within this grouping was $(1.9 \pm 0.31) \times 10^{-11}$ cm^2/s (Fig. 3B). The percent recovery of fluorescence for these cells was 72 ± 7.1; that is, 28% of the PH-20 molecules were immobile within the time scale of the experiment.

In contrast to PH-20's slow diffusion on testicular sperm, we had previously found much faster diffusion rates for PH-20 on sperm that have completed epididymal maturation. Prior to the acrosome reaction, PH-20 on epididymal sperm diffuses at a rate typical of integral membrane proteins [$D = (1.8 \pm 0.5) \times 10^{-10}$ cm^2/s; percent recovery, 73 ± 3], and after the acrosome reaction PH-20 is freely diffusing [$D = (4.9 \pm 2) \times 10^{-9}$ cm^2/s; percent recovery, 78 ± 9] (*20*). Thus, PH-20 diffusion on testicular sperm is, respectively, 10-fold and 250-fold slower than PH-20 diffusion at these two later stages of development, although it is PI-anchored at all three stages.

During the course of sperm maturation the surface distribution of PH-20 changes. PH-20 is found over the entire surface of testicular sperm, but is localized to the posterior head region of acrosome-intact epididymal sperm (*21*). After the acrosome reaction, PH-20 migrates to the newly exposed inner acrosomal membrane on the anterior head region (*22*). This demonstrates that a PI-anchored membrane protein, free from direct cytoskeletal constraints, can be localized. PH-20 diffusion is most restricted on testicular sperm where the protein is uniformly distributed and is less restricted or unrestricted when it is localized.

To test whether the restricted diffusion of PH-20 measured on testicular sperm could result from limited lateral diffusion of the membrane lipids compared to that of epididymal sperm, we measured the mobility of the lipid probe C_{14}diI on testicular sperm. The probe diffused at a rate of $D = (3.3 \pm 0.69) \times 10^{-8}$ cm^2/s, three orders of magnitude faster than the lipid-linked PH-20 protein (Fig. 3). This rate of diffusion is exceptionally fast for a lipid probe in a cell membrane. C_{14}diI diffusion on testicular sperm (3.3×10^{-8} cm^2/s) is almost four times as fast as that found with the same probe on the posterior head region of acrosome-intact sperm [$(8.9 \pm 2.1) \times 10^{-9}$ cm^2/s] where PH-20 is localized and approximately seven times the rate found over the anterior head region on acrosome-reacted sperm [$(5 \pm 1.7) \times 10^{-9}$ cm^2/s] (*20*). Thus, the rate of diffusion of the lipid probe is fastest on testicular sperm where PH-20 exhibits slow diffusion and slowest on acrosome-reacted sperm where PH-20 is freely diffusing. Figure 3B shows the difference in the rate of FRAP between the freely diffusing C_{14}diI lipid probe and the slowly diffusing lipid-anchored protein PH-20 on testicular sperm. These results indicate that changes in the phase state of the membrane lipids (gel to liquid crystalline) are not responsible for changes in the mobility of PH-

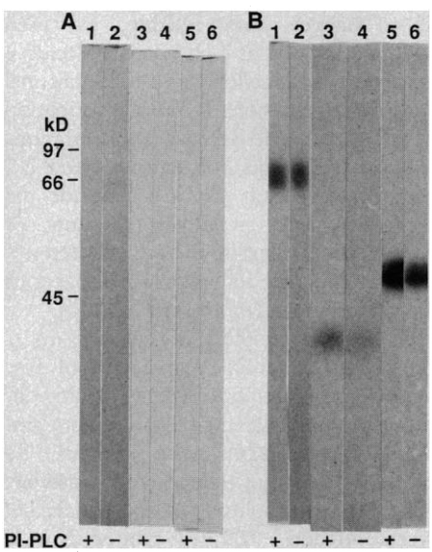

Fig. 2. Immunoprecipitation of three other membrane proteins from testicular sperm after exposure to PI-PLC. (**A**) Immunoprecipitates from supernatant. (**B**) Immunoprecipitates from detergent extract of cell pellet. (Lanes 1 and 2) PH-30 protein; (lanes 3 and 4) WH-1 protein; and (lanes 5 and 6) AH-20 protein. Testicular sperm cell pellets and supernatants were obtained as in Fig. 1. Cell pellets were extracted with 1% NP-40. The PH-30 protein was immunoprecipitated with a rabbit polyclonal antibody raised against affinity-purified PH-30 protein (*26*). The immunoprecipitation was done as described in Fig. 1; 3×10^5 input counts per minute were immunoprecipitated per lane. The AH-20 and WH-1 proteins were immunoprecipitated with their respective monoclonal antibodies (*14, 15*) as described (*8*); 1.75×10^5 input counts per minute were immunoprecipitated per lane.

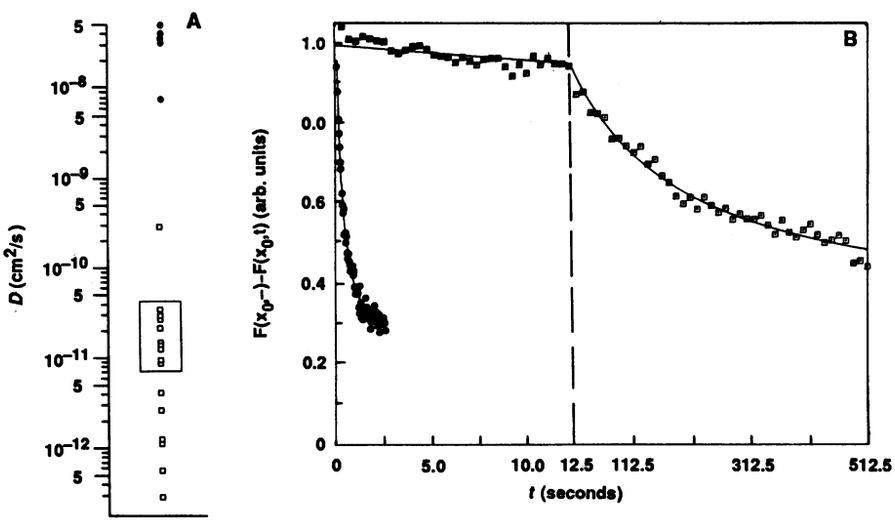

Fig. 3. Diffusion of PH-20 (squares) and C_{14}diI (circles) on testicular sperm. (**A**) Distribution of the measured rates of diffusion of C_{14}diI ($n = 5$) and PH-20 ($n = 16$) on testicular sperm. The FRAP apparatus and methods of data analysis have been described (*18, 20*). Immunofluorescent staining of PH-20 and labeling with the lipid probe C_{14}diI was as previously described (*20*). The C_{14}diI was used at 2.5 μg/ml. Only live cells, as evidenced by tail movement, were analyzed. (**B**) Decay of fluorescence depletion after photobleaching on testicular sperm averaged over all of the C_{14}diI data and all of the PH-20 data clustered in the box in (A). After the first 12.5 seconds the time scale for PH-20 was expanded 31-fold to cover the appropriate time range for the slow recovery. The solid lines are the computer-generated three-parameter (percent bleach, rate of recovery, and percent recovery) least-squares fitting of the data.

20 during sperm maturation. It is unlikely that changes in the density of PH-20 are responsible for the change in its mobility, since diffusion is fastest on acrosome-reacted sperm where PH-20 is the most dense (*23*). Rather, the present results indicate that restriction of lateral mobility of a membrane protein can be mediated exclusively by interactions of its ectodomain.

REFERENCES AND NOTES

1. D. E. Golan and W. Veatch, *Proc. Natl. Acad. Sci. U.S.A.* *77*, 2537 (1980); D. E. Koppel, M. P. Sheetz, M. Schindler, *ibid.* **78**, 3576 (1981).
2. D. E. Wolf, P. Henkart, W. W. Webb, *Biochemistry* **19**, 3893 (1980).
3. M. Ediden and M. Zuniga, *J. Cell Biol.* **99**, 2333 (1984).
4. E. Livnen *et al.*, *ibid.* **103**, 327 (1986).
5. A. Ishihara, Y. Hou, K. Jacobson, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 1290 (1987).
6. M. Noda, K. Yoon, G. A. Rodan, D. E. Koppel, *J. Cell Biol.* **105**, 1671 (1987).
7. J. Thomas, W. Webb, M. A. Davitz, V. Nussenzweig, *Biophys. J.* **51**, 522a (1987).
8. P. Primakoff, H. Hyatt, D. G. Myles, *J. Cell Biol.* **101**, 2239 (1985).
9. H.-T. He, J. Barbet, J.-C. Chaix, C. Goridis, *EMBO J.* **5**, 2489 (1986).
10. M. L. Dustin, P. Selvaraj, R. J. Mattaliano, T. A. Springer, *Nature* **239**, 846 (1987).
11. M. G. Low and P. W. Kincade, *ibid.* **318**, 62 (1985).
12. M. A. Davitz, M. G. Low, V. Nussenzweig, *J. Exp. Med.* **163**, 1150 (1986).
13. R. Taguchi, Y. Asahi, H. Ikezawa, *J. Biochem.* **97**, 911 (1981).
14. D. G. Myles, P. Primakoff, A. R. Bellvé, *Cell* **23**, 433 (1981).
15. P. Primakoff and D. G. Myles, *Dev. Biol.* **98**, 417 (1983).
16. M. G. Low and J. B. Finean, *FEBS Lett.* **82**, 143 (1977).
17. M. E. Medoff, E. I. Walter, W. L. Roberts, R. Haas, T. L. Rosenberry, *Biochemistry* **25**, 6740 (1986).
18. D. E. Koppel, *Biophys. J.* **28**, 281 (1979).
19. Because of the nature of the FRAP technique, we are measuring the diffusion of labeled membrane proteins. We do not know if the presence of the Fab' label alters the intrinsic diffusion coefficient of the protein. Nevertheless, our conclusion that the diffusion of PI-anchored proteins can be limited by interactions with the ectodomain remains valid.
20. A. E. Cowan, D. G. Myles, D. E. Koppel, *J. Cell Biol.* **104**, 917 (1987).
21. B. M. Phelps and D. G. Myles, *Dev. Biol.* **123**, 63 (1987).
22. D. G. Myles and P. Primakoff, *J. Cell Biol.* **99**, 1634 (1984).
23. A. E. Cowan, P. Primakoff, D.G. Myles, *ibid.* **103**, 1289 (1986).
24. Testicular sperm were enriched to between 80% and 90% by density separation through an isotonic 52% Percoll gradient before ^{125}I labeling. Epididymal sperm were isolated from the distal tubules of the cauda epididymis (*25*). After surface iodination, >90% of the epididymal sperm were induced to undergo the acrosome reaction by the addition of the calcium ionophore A23187 (2 μg/ml) (*23*). Because the sperm were in each case iodinated while they were acrosome-intact, we observed only the population of PH-20 on the plasma membrane and not the additional internal pool that becomes exposed on the surface after the exocytotic acrosome reaction (*23*).
25. D. G. Myles, H. Hyatt, P. Primakoff, *Dev. Biol.* **121**, 559 (1987).
26. P. Primakoff, H. Hyatt, J. Tredick-Kline, *J. Cell Biol.* **104**, 141 (1987).

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The α Subunit of the GTP Binding Protein Activates Muscarinic Potassium Channels of the Atrium

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It has been debated whether the potassium channel of the atrium is activated by the α subunit or by the βγ subunits of guanine nucleotide binding (G) proteins, which dissociate on activation with guanosine triphosphate (GTP). Therefore, the channel-activating effectiveness of these subunits on isolated guinea pig atrial cells was tested. The activated α_K subunit from human erythrocytes activated the channel in subpicomolar concentrations. The βγ dimer from bovine brain activated the channel in nanomolar concentrations. These results support the view that, physiologically, the α subunit activates the channel.

WHEN ACETYLCHOLINE (ACh) OR adenosine activates the sarcolemmal potassium channel (K^+_{ACh} channel) of mammalian or chicken atria, receptor occupation is coupled to channel opening via guanine nucleotide binding (G) proteins (*1–4*). These G proteins are composed of three subunits, α, β, and γ, and it is thought that binding of the agonist to the receptor leads to binding of guanosine triphosphate (GTP) to the α subunit and that activated α (α*) in turn dissociates from the βγ dimer (*5, 6*). After dissociation of the αβγ trimer, the channel is opened (*1*), but whether opening results from interaction with the α* subunit or with the βγ dimer is a matter of controversy. Logothetis *et al.* unexpectedly failed to demonstrate activation of the K^+_{ACh} channel (from chicken) with the α subunit, but they were able to show K^+ channel opening with nanomolar concentrations of the βγ dimer (*7*). Brown and co-workers reported activation of mammalian K^+_{ACh} channels by picomolar concentrations of the α subunit (α^*_K) from human erythrocytes (*6, 8, 9*). Whether K^+_{ACh} channels are activated by α or βγ, or both, is important for understanding receptor-effector coupling.

We therefore studied activation of K^+_{ACh} channels in atrial myocytes from adult guinea pigs (*10*). The isolated cells were bathed in a medium containing 140 m*M* KCl, 1 m*M* K_2EGTA, and 3 m*M* magnesium adenosine triphosphate (ATP) that was adjusted to *p*H 7.4 by 5 m*M* Hepes-tris buffer. Single channel currents were recorded at room temperature (22° to 23°C) by gigaohm-seal patch clamping (*11*). The patch pipettes were filled with the bath medium but without MgATP.

In a first series of control experiments, we activated the K^+_{ACh} channels in cell-attached patches by adding 0.1 μ*M* carbachol, the muscarinic agonist, to the pipette. From 12 experiments, we evaluated an open channel conductance of 41 ± 5 pS (slope conductance between −40 and −100 mV) and a mean lifetime of the open state of 2.9 ± 0.4 ms (mean ± SEM). For the second series of control experiments, we excised the patch from the cell. In the resulting inside-out configuration, channel activity gradually disappeared within 1 to 5 min, but it reappeared when we added 100 μ*M* GTPγS [guanosine-5'-O-(1-thiotriphosphate)] to the bath (*1, 7, 8*). The GTPγS-activated channel (n = 10) had a conductance of 37 ± 3 pS and a mean channel open time of 2.1 ± 0.2 ms. Thus, the K^+_{ACh} currents activated by GTPγS are indistinguishable from those resulting from receptor occupation by carbachol (*1*). Because GTPγS activated the K^+_{ACh} channels very reliably, we used GTPγS activation as a criterion to decide if the protein was ineffective or if the channel was lost, for example, by vesicle formation in the patch. The GTPγS concentrations below 0.1 μ*M* did not activate the K^+_{ACh} channel; this result seems to exclude the possibility that picomolar amounts of GTPγS, contaminating the preactivated α^*_K, are responsible for activation of endogenous G proteins and K^+_{ACh} channels.

The activating effect of 0.4 p*M* α^*_K is shown by the on-line registration of Fig. 1A. The computer trace (Fig. 1C) demonstrates that the currents have a unitary amplitude of about 4 pA (patch potential −80 mV). In six patches treated with 4 p*M* α^*_K, we evaluated amplitude at potentials between −40 and −100 mV, plotted them in a current-voltage (I-V) curve and obtained from the slope a conductance of 41 ± 4 pS. The mean channel open time evaluated from histograms (Fig. 1E) was 2.2 ± 0.2 ms. Conductance and open time are indistinguishable from their counterparts measured under activation by carbachol or by GTPγS or from the values reported in the literature (*1, 4, 7, 9*). Thus the α^*_K subunit of the G protein effectively opens the K^+_{ACh} channel at picomolar concentrations.

This conclusion confirms the results of Brown and colleagues (*9*). We have seen this

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