Enhanced Molecular Diffusibility in Muscle Membrane Blebs: Release of Lateral Constraints

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ABSTRACT Measurements of lateral molecular diffusion on blebs formed on the surfaces of isolated muscle cells and myoblasts are reported. These blebbed membranes retain integral proteins but apparently separate from the detectable cytoskeleton. On blebs, acetylcholine receptors, concanavalin A receptors, and stearoyldextran extrinsic model receptor molecules are free to diffuse with a diffusion coefficient (D) $\sim 3 \times 10^{-9}$ cm^2/s, which is close to the value predicted for hydrodynamic drag in the lipid membrane. In contrast, diffusion of these typical receptors in intact cell membranes is constrained to $D \lesssim 10^{-10}$ cm^2/s with substantial fractions virtually nondiffusible ($D < 10^{-12}$ cm^2/s). Lipid analog diffusion is also slightly enhanced in blebs as expected of evanescent lipid protein interaction. This strong enhancement of membrane protein diffusion is attributed to release from unidentified natural constraints that is induced in some way by detachment of the bleb membrane.

The lateral diffusion of plasma membrane components has become routinely accessible to measurement by fluorescence photobleaching recovery (FPR) (1). Taken together, data accumulated for vertebrate cells in culture establish several distinctive common characteristics of lateral diffusibility of cell surface components (2–6): (*a*) A substantial fraction of the population of each cell surface protein is not detectably diffusible over distances of several micrometers during an experimental interval of hours. Thus the diffusible fraction (R) is said to be <100%, and it is often <75%. (*b*) Diffusion coefficients (D) of the diffusible fraction of cell membrane proteins do not generally exceed 1×10^{-10} cm^2/s and are frequently much smaller. (Implied diffusive transit time across a 10-μm cell: ½ h.) (*c*) Plasma membrane lipid diffusibility is virtually complete over distances of several micrometers (R = 100%, except possibly in a few compartmentalized cell types) with diffusion coefficients of the order of 1×10^{-8} cm^2/s, i.e., usually >100 times faster than membrane proteins. (Implied diffusive transit time across a 10-μm cell: ½ min.)

Certain proteins are virtually nondiffusible *in toto* (R = 0%) on the cell surface. For example, the acetylcholine receptor (AChR) localized at neuromuscular junctions and in patches on myotubes (7, 8), and fibronectin on the surface of cultured fibroblasts (9).

In contrast with the cell surface, protein molecules that are reconstituted into phospholipid model membranes diffuse nearly as fast as lipid molecules and with no immobile fraction. For example, in dimyristoyl phosphatidylcholine bilayers, we have glycophorin at 24°C with $D = 1.7 \times 10^{-8}$ cm^2/s, R = 100% (10), and gramicidin C at 30°C with $D = 4.0 \times 10^{-8}$ cm^2/s, R = 100% (11).

Saffman and Delbruck have developed an appropriate hydrodynamic model for a lipid bilayer membrane which yields a Stokes-Einstein relation that gives the diffusion coefficient of a molecule penetrating the membrane in terms of membrane viscosity and molecular size (12, 13). A distinctive result of the model calculation and of any two-dimensional hydrodynamic model is very weak (logarithmic) dependence of D on molecular size. Thus the diffusion coefficient of a membrane protein of 300,000 mol wt should be about half that of a lipid molecule, in clear disagreement with the measurements of protein diffusion on the cell surface.

These results indicate that cell surface protein diffusibility is not determined by lipid viscosity. Putative alternatives include binding interactions between membrane proteins, coupling to an immobile exoskeleton, coupling or anchorage to the cytoskeleton, and combinations thereof. Interestingly, the only case in which cell surface protein has been found to diffuse nearly as fast as membrane lipid is rhodopsin in visual disks of rod outer segments, where cytoskeletal components are absent from the planes of the disks and other membrane proteins are scarce (14, 15). The potential subtlety of the mechanisms responsible for modulation of protein diffusibility on the cell surface is suggested by the global decreases of the diffusion coefficients

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of receptors that can be induced by strictly localized binding of concanavalin A to its receptors on a small fraction of the cell surface (16, 17).

We report here some measurements of changes in diffusibility of membrane components in plasma membrane that has been physically detached from the cytoskeleton. The phenomenon of cell surface blebbing, the mechanical or chemically induced dissociation of plasma membrane from its subsurface attachments, provided a simple and ideal preparation for these experiments. Our blebbing treatments produced blister or bubblelike protuberances on the cell surface, similar to those observed by others (18), in which both cell surface protein and lipid compositions have been retained. Using FPR, we have measured the diffusibility of integral membrane proteins, lipids, and an extrinsic receptor analog in blebbed membrane for comparison with diffusion on the normal cell surface.

We find that for AChRs on flexor digitorum brevis (FDB) single muscle fibers and concanavalin A (Con A) receptors, and the receptor analog acetylrhodamine stearoyldextran (AcRSD) on L6 myoblasts, the molecules are released from whatever contraints limited their diffusion on normal cell surface so that they are completely diffusible on blebs, with diffusion coefficients approaching the viscous limit of the lipid.

A preliminary account of this work was presented at the 1981 Biophysical Society Meeting.

MATERIALS AND METHODS

Reagents

Rhodamine-labeled Succinyl-Con A (Rh-S-Con A) was purchased from E-Y Laboratories (Burmingham, Calif.). The fluorescence-labeled lipid N-4-nitrobenzo-2-oxa-1,3 diazole phosphatidylcholine (NBD-PC) was obtained from Avanti Biochemical, Inc. (Birmingham, Ala.). The fluorescence-labeled membrane receptor analog AcRSD was the gift of Pierre Henkart (National Institutes of Health). Bungarotoxin from Miami Serpentarium (Miami, Fla.) was labeled with tetramethylrhodamine by the technique of Rinderknecht (19). Monolabeled toxin (Rh-αBgt) was purified from the mixture of reacted species by the technique of Ravdin and Axelrod (20).

Cells and Cell Culture

Single muscle fibers from mouse FDB were prepared following the protocol of Bekoff and Betz (21), except that brief vortexing (~2-s duration) of the partially dissociated muscle in suspension followed trituration with a Pasteur pipette. This alteration produced more single fibers. L6 rat embryo myoblasts (22; obtained from T. Podlesky (Cornell University)) were grown on glass cover slips in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.).

Vesiculation

Membrane blebbing was induced on L6 cells following the techniques developed by Scott (23). Cells on glass cover slips were incubated for 25 min in a vesiculant (VES) which consisted of 25 mM formaldehyde and 2 mM dithiothreitol in Phosphate-buffered saline containing 0.9 mM Ca^{2+} and 0.5 mM Mg^{2+} (PBS+; Gibco Laboratories). Blebs on enzymatically dissociated FDB single muscle fibers could be induced using VES; however, incubation in PBS+ alone usually resulted in spontaneous bleb formation in a small percentage of the cells. Bleb formation was always induced before binding of fluorescence-labeled ligands to cell surface receptors or incorporation of fluorescence-labeled probes into the plasma membrane.

Probe Incorporation and Ligand Binding

AcRSD was incorporated into L6 plasma membranes by incubating cells for 15 min at 37°C with PBS+ containing 50 μg/ml AcRSD. The fluorescence-labeled lipid NBD-PC was incorporated into the plasma membrane of L6 and FDB cells by the ethanol injection technique; cells were incubated in Medium 199 (FDB) or PBS+ (L6) containing 1 μM NBD-PC and 1% ethanol. Con A receptors, on L6 cells, were fluorescently tagged by incubating cells with 20 μg/ml Rh-S-Con A in PBS+ for 15 min at 37°C. AChRs on FDB single muscle fibers were fluorescently tagged by incubating cells with 2.5×10^{-7} M Rh-αBgt in DMEM for 1 h at 37°C. All cells were washed several times with PBS+ before experiments.

Microscopy

A Nikon Optiphot epifluorescence microscope was used for visual examination of cells in phase contrast and fluorescence. Photomicrographs were recorded on Tri-X film, push-processed in Kodak D19 to ASA 3200. Excitation was at 535–550 nm for rhodamine, using a 580-nm barrier filter.

Diffusion Measurements

The FPR technique has been described in detail elsewhere (3). For these experiments, an Argon laser beam (488 nm for NBD-labeled probes, and 514 nm for rhodamine-labeled probes) was first shaped into Gaussian intensity profile by a spatial filter and then split into bleach and monitor beams by transmission or multiple reflection within a quartz optical flat. The bleach beam, which can be pulsed by an electromechanical shutter, and the monitor beam were recombined by a second matched quartz optical flat. Partial reflectance coatings reduce the monitor intensity to 1/2,000 that of the bleach beam. The combined beam was then focused through the vertical illuminator of a Zeiss Universal microscope and directed by a dichroic mirror onto the membrane sample at the object plane. With a × 100 (1.3 NA) objective, the illuminated spot was ~0.5 μm gaussian radius. The excited fluorescence, after passing a barrier filter, was monitored by a photomultiplier tube with photon counting electronics. FPR curves—the fluorescence intensity versus time before and after a brief bleaching pulse—were stored, averaged (if necessary), and analyzed by computer. Least squares fitting of the average curve based on the analysis of Axelrod et al. (1) gave both the percent fluorescence recovery (R), as a measure of the mobile fraction of the diffusing species, and the diffusion coefficient (D). Flow of membrane, detectable by a characteristic alteration in the shape of the recovery curve, was never seen in these experiments. Stated uncertainties in the presented values of D and R represent the full range of values obtained. All diffusion measurements were carried out at 22°C.

RESULTS

Diffusion on L6 Myoblasts and Blebs

When treated with VES for 25 min at 37°C, L6 cells growing on glass cover slips developed spherical protrusions, up to 20 μm in diameter, from the dorsal surface. The bleb surface area accounted for <30% of the total plasma membrane. By phase-contrast microscopy, bleb interiors appear devoid of cytoplasmic organelles and stress fibers. Rapid brownian motion of occasional particles suggests that the normal cytoplasmic architecture has been disrupted. Fig. 1 *a* and *c* are phase-contrast micrographs of a blebbing L6 cell: in *a* the plane of focus is at the substrate; in *c* it is above the cell at the level of the bleb's "equator."

We have incorporated the fluorescent lipid NBD-PC and the extrinsic model receptor molecule AcRSD into the plasma membranes of L6 blebs and cell surfaces; we have also bound Rh-S-Con A to Con A receptors on blebs and cell surface. In all cases, the fluorescence intensity on blebs was comparable to that of the normal cell surface from which they arose. Fig. 1 *b* and *d* show fluorescence micrographs of an L6 cell and bleb with incorporated AcRSD, the same cell and bleb as in Fig. 1 *a* and *c*. By focusing through a labeled cell-bleb and following the surface membrane in fluorescence, it was seen that the plasma membrane of an attached bleb was contiguous with the normal cell plasma membrane.

The diffusion coefficients and percent fluorescence recoveries of these probes on blebbed and normal L6 membranes were measured by FPR. Measurements were made on the top surface of blebs attached to VES-treated cells and also on normal (untreated) cell surface.

On normal L6 cell surfaces, Con A receptors with bound Rh-S-Con A have a diffusion coefficient of $D = 4.2 \times 10^{-11}$ cm^2/s with $R = 66\%$ (24). On blebs of L6, we see a dramatic

increase in both diffusion coefficient and diffusible fraction: $D = (4.0 \pm 1.0) \times 10^{-9}$ cm^2/s, $R = 102\% \pm 11\%$. An increase in diffusibility of AcRSD on blebs was also seen. On normal L6 membrane, we measured $D = (4.5 \pm 1.5) \times 10^{-10}$ cm^2/s, $R = 72\% \pm 10\%$; on blebs, $D = (3.0 \pm 1.1) \times 10^{-9}$ cm^2/s, $R = 101\% \pm 7\%$. An experiment on a VES-treated L6 cell showed that the mobility of AcRSD on nonblebbed regions of a blebbing cell was identical to that measured on the surface of a normal (untreated) cell. The diffusibility of the fluorescence-labeled lipid NBD-PC was also measured. In normal L6 membrane, $D = (4.4 \pm 1.5) \times 10^{-9}$ cm^2/s, $R = 101\% \pm 3\%$; on blebbed membrane $D = (1.2 \pm 0.4) \times 10^{-8}$ cm^2/s, $R = 99\% \pm 3\%$. These diffusion results are summarized in Table I.

Diffusion on FDB Muscle Cells and Blebs

When freshly isolated FDB single muscle fibers were incubated in PBS+, a small fraction of the cells exhibited spontaneous blebbing. Blebbing of fibers mounted on a glass slide was enhanced following compression of the cells by an unsupported cover slip; prolonged vortexing during preparation also enhanced blebbing. This leads us to believe that bleb formation is induced by the mechanical damage. Shear stress and sonication are known to induce surface vesiculation in macrophages (25).

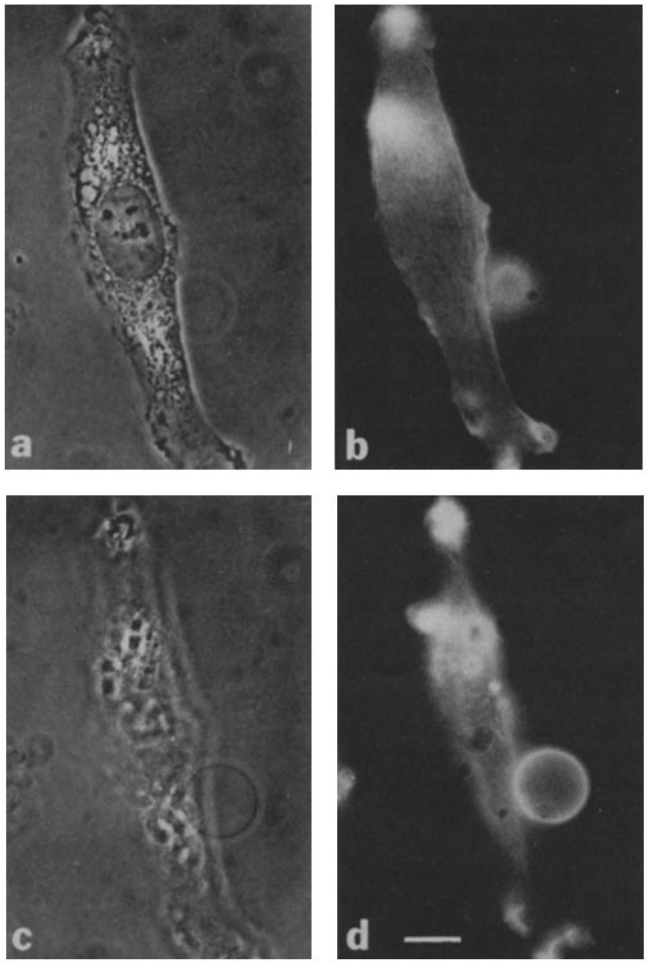

FIGURE 1 Photomicrographs of a blebbling L6 cell with AcRSD incorporated into its plasma membrane. (*a*) Phase contrast, focused at the level of the glass cover slip. (*b*) Fluorescence of AcRSD at the same level, showing labeling of normal cell surface. (*c*) Phase contrast, at the midsection of a bleb forming on the dorsal surface. (*d*) Fluorescence of AcRSD at the same level—the bleb exhibits a ring of stain. Bar, 12 μm. × 750.

Incorporation of NBD-PC into the plasma membrane of blebbing FDB cells introduced uniform surface fluorescence, the fluorescence intensity of all of the blebs was comparable to that of the normal cell surface. There was, however, some labeling out of the plane of the cell surface in what we inferred to be the elaborate invaginations of the T-tubule membrane system expected in adult muscle.

Each muscle cell contains a single motor endplate which is easily visualized in fluorescence after binding Rh-αBgt to AChRs. In contrast to the uniform NBD-PC fluorescence of all blebs, Rh-αBgt bound to AChRs showed fluorescence only in the blebs forming at the endplate region. Fig. 2 *a* and *b* show this effect; they are phase-contrast and fluorescence micrographs of an FDB cell with blebs forming above the motor endplate, which is near the center of the field of view.

AChRs are completely immobile at a normal adult endplate: $D \gtrsim 10^{-12}$ cm^2/s, $R = 0\%$ (7). However, we measured the D of AChR in blebs of FDB cells to be at least 1,000-fold larger, with complete diffusibility: $D = (2.0 \times 0.8) \times 10^{-9}$ cm^2/s, $R = 104\% \pm 9\%$.

On FDB cell blebs containing AChR, during visual observation of the Rh-αBgt fluorescence by the focused laser beam many small highly fluorescent "speckles" diffused through the illuminated spot, suggesting that the AChRs are present as a distribution of receptor clusters. Thus the measured values of D represent an average over the distribution. The FPR recovery curves could be fitted accurately by theoretical curves representing a single diffusion coefficient suggesting that D is insensitive to the cluster size.

We have also measured the mobility of NBD-PC on FDB cell blebs and normal plasma membranes. On blebs, we find $D = (1.5 \pm 6) \times 10^{-8}$ cm^2/s, $R = 100\% \pm 6\%$. On the normal cell surface, we measure $D = (1.0 \pm 0.5) \times 10^{-9}$ cm^2/s, $R = 92\% \pm 5\%$, although this value should be considered a lower limit since the known irregularity of the muscle cell surface with its T-tubule system could reduce the apparent value of D. These diffusion results on FDB single muscle fibers are summarized in Table II.

DISCUSSION

We have utilized the phenomenon of membrane blebbing to provide plasma membrane preparations that retain membrane macromolecules but are physically detached from the cytoplasmic cytoskeleton. The essential feature of our results is the discovery that slowly diffusing, fractionally immobile receptors on the cell surface are released, in blebs, from lateral constraints and diffuse rapidly at the hydrodynamic limit. We have measured this effect for three membrane macromolecules: the extrinsic model receptor AcRSD and the Con A receptor on cultured L6 myoblasts, and AChR on FDB single muscle cells. Consideration of the structure and cell surface behavior of these three receptors provides some insight into the possible mechanisms of modulation of cell surface diffusibility.

AcRSD is an extrinsic model receptor molecule consisting of an 82,000-dalton dextran molecule to which, on average, 1.3 stearoyl hydrocarbon tails and 1.0 rhodamine molecules have been covalently linked (26). The acetyl groups can be replaced by a hapten, trinitrophenol, for antibody crosslinking experiments. The diffusion of these model receptors has previously been studied on model membranes and cell surfaces. The molecule readily attaches to pure lipid bilayers or to cell

surfaces by insertion of its hydrocarbon tails into the bilayer. Wolf et al. (26) have found that stearoyl dextrans diffuse at $D = (3.0 \pm 1.8) \times 10^{-10}$ cm^2/s, $R = 65\% \pm 17\%$, on 3T3 cell plasma membranes, and that hapten-labeled derivatives could be patched or capped by antibody-induced cross-linking. On solvent-free, egg phosphatidylcholine Large Unilammelar Vesicles (LUVs), AcRSD, at similar surface concentrations, diffused with complete recovery ($R = 100\%$) at 2×10^{-9} cm^2/s (27). These authors conclude that the immobile fraction seen on 3T3 cells and the slower diffusion (on 3T3 cells compared to LUVs) is due to the coupling of stearoyldextran to some immobilizing structure on the cell surface, possibly through saccharide interactions (steric and/or specific) between stearoyldextran and other surface components. No direct interaction with cellular (subplasmalemmal) cytoskeleton was evident. We find that AcRSD diffuses on normal L6 membranes with diffusion coefficient and immobile fraction close to that found on 3T3 cells, but on blebs its diffusion coefficient is enhanced by a factor of 9, and the immobile fraction is eliminated; thus, its diffusibility on blebs becomes identical to that on LUVs. This indicates that the immobilizing structures that inhibit AcRSD diffusion on cell surfaces either are excluded from blebs or are disabled, perhaps by loss of their anchorage, so that they are also freely diffusing.

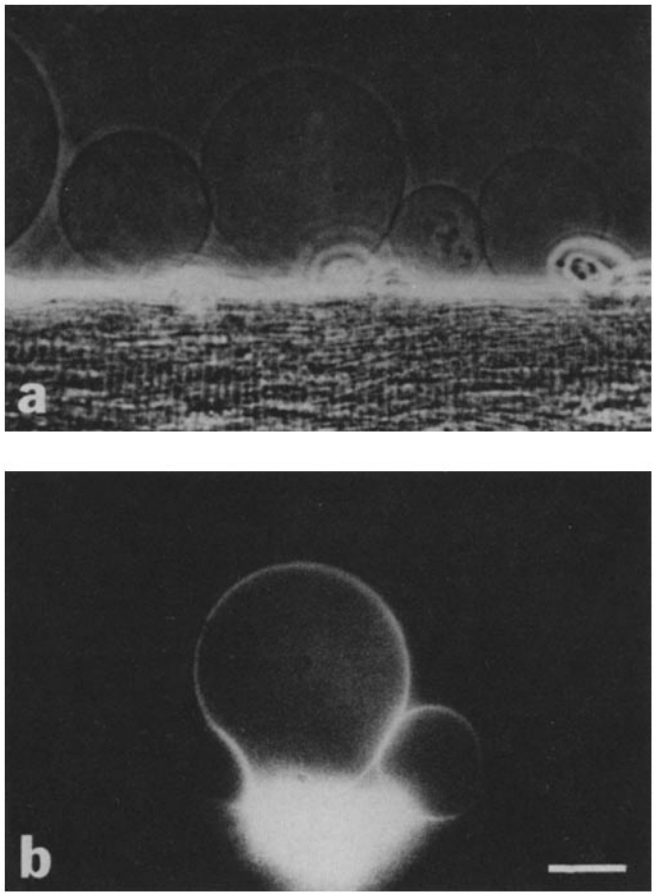

FIGURE 2 (*a*) Phase-contrast micrograph of the edge of a blebbing FDB cell. (*b*) Rh-αBgt fluorescence of same field as in *a*. The endplate is edge-on and hence appears as a blurred triangle. Only blebs forming at the endplate region fluoresce. Bar, 12 μm. × 750.

Con A receptor mobility on L6 plasma membranes has been measured by Schlessinger et al. (24). Immediately after labeling with the tetravalent ligand R-Con A, these receptors diffused with $D = 2.9 \times 10^{-11}$ cm^2/s, $R = 66\%$; D decreased with increasing incubation time. Eventually, complete immobilization and internalization occurred. Receptors labeled with the bivalent ligand S-Con A diffused initially at $D = 4.2 \times 10^{-11}$ cm^2/s, $R = 66\%$, with R independent of the dose and incubation; high S-Con A concentrations slowed D somewhat. As with AcRSD, the receptor exhibited an appreciable immobile fraction and slow diffusion, and cross-linking, in this case by the multivalent lectins, further decreased diffusibility. Evidence that Con A receptor mobility can be modulated by cellular components (possibly cytoskeletal) is provided by experiments done in our laboratory by Schlessinger et al. (16) who found that binding of Con A-covered platelets to 3T3 cells decreases Con A receptor mobility at cell surface points distant from the platelet. This type of global surface modulation has recently been found on lymphocytes also (17). In contrast to the slow diffusion found by Schlessinger et al. (16) on normal cell surface, we find that on L6 blebs, S-Con A-labeled receptors diffuse at $D = (4.0 \pm 1.0) \times 10^{-9}$ cm^2/s, $R = 102\% \pm 11\%$.

TABLE I

Diffusion on L6 Cells and Blebs

	Cell surface		Bleb	
Probe	D	R	D	R
	cm^2/s	%	cm^2/s	%
Con A receptors*	$(4.2 \pm 0.8) \cdot 10^{-11}$‡	66‡	$(4.0 \pm 1.0) \cdot 10^{-9}$	102 ± 11
AcRSD	$(4.5 \pm 1.5) \cdot 10^{-10}$	72 ± 10	$(3.0 \pm 1.1) \cdot 10^{-9}$	101 ± 7
NBD-PC	$(4.4 \pm 1.5) \cdot 10^{-9}$	101 ± 3	$(1.2 \pm 0.4) \cdot 10^{-8}$	99 ± 3

* Labeled with Rh-S-Con A.
‡ From Schlessinger et al. (24).

TABLE II

Diffusion on FDB Cells and Blebs

	Cell surface		Bleb	
Probe	D	R	D	R
	cm^2/s	%	cm^2/s	%
AChR*	$\gtrsim 10^{-12}$‡	0‡	$(2.0 \pm 0.8) \cdot 10^{-9}$	104 ± 9
NBD-PC	$(1.0 \pm 0.5) \cdot 10^{-9}$	92 ± 5	$(1.5 \pm 0.6) \cdot 10^{-8}$	100 ± 6

* Labeled with Rh-αBgt.
‡ From Axelrod et al. (7).

Again, receptor diffusion on blebs appears to be free of constraints and limited only by viscous membrane hydrodynamics.

AChRs, on adult mouse muscle fibers, are known to be localized at the neuromuscular junction at the tops of the junctional folds (28). We find that only those blebs forming at the neuromuscular junction region of FDB single muscle fibers contain AChR. This result suggests that the junctional folds are released from their mechanical restraint and that their membranes incorporated into the forming bleb. The diffusion coefficient of AchRs at the normal mouse neuromuscular junction is $D \gtrsim 10^{-12}$ cm^2/s with $R = 0\%$ (7). On blebs, we find $D = 2.5 \times 10^{-9}$ cm^2/s, $R = 100\%$, again fast diffusion in accord with the limitation of lipid membrane viscosity. The fluorescence "speckle" observed during laser spot illumination of these blebs indicates that a distribution of receptor aggregates is present; this suggests that some of the original molecular interactions between receptor molecules at the endplate may be retained. However, the residual interaction is evidently not sufficient to prevent lateral diffusion of the receptors on the blebs, where aggregates are observed to diffuse rapidly. Diffusion of such "islands" of receptor at rates comparable to single receptors in a lipid bilayer has been predicted by an elaboration of the Saffman-Delbruck theory by F. Wiegel (29). This suggests that AChR immobilization in motor endplates cannot be attributed to aggregation of AChR alone but requires additional anchorage mechanisms.

The fact that membrane receptors and macromolecules diffuse unrestrained on blebs but not on normal cell surface indicates that molecular interactions which impede diffusion and cause immobilization are absent on blebs. The process of bleb induction by VES is not well understood, but it is known from the work of Scott (23) to be an energy-dependent process requiring monovalent and divalent cations at near physiological temperature and pH. Scott et al. (18) have also biochemically characterized blebs detached from 3T3 cells by treatment with VES. The normal subplasmalemmal architecture (including microtubules and microfilaments) is not present. Freeze-fracture analysis shows an intramembrane particle density in blebs similar to that of the intact cell surface. SDS PAGE showed ~20 major polypeptides with molecular weights from 10,000 to 200,000. Lipid analysis by Perkins and Scott (30) shows that VES-induced blebbed membrane has cholesterol, sphingomyelin, phospholipid, and fatty acyl chain distribution similar to that of other plasma membrane preparations.

Ben-Ze'ev et al. (31) have reported that following extraction of cells with Triton X-100 a cytoskeletal framework remains which is bordered by a continuous sheet or lamina containing specific protein components of the plasma membrane. Similarly, Mescher et al. (32) have presented evidence that a nonionic detergent-insoluble protein matrix is present on the inner surface of murine tumor and lymphoid cells, consisting primarily of actin and four other unidentified proteins. With the help of the F-actin-specific stain NBD-phallacidin (2), we have observed, on several cell types, that the bulk of the F-actin submembrane cytoskeleton and the stress fibers are eliminated from blebs, but we have not determined how much of the membrane-associated matrix or lamina, or of the exoskeleton remains.

Like the erythrocyte, where the spectrin-containing cytoskeleton has been implicated in modulation of diffusibility of cell surface receptors (33, 34), it is possible that an analogous structure exists in all eucaryotic cells that can restrict the lateral mobility of membrane components. If a membrane-associated matrix is responsible for modulation of surface topography and component diffusibility, then our results indicate that its structural characteristics may have been altered in bleb formation. This conjecture suggests an approach for future investigations.

Although the lipid composition of blebs appears similar to that of normal cell surface, differences in organization, such as changes in the asymmetry of distribution between inner and outer leaflets, are possible; such rearrangement might alter effective bilayer viscosity. To determine whether decreasing membrane lipid viscosity was alone responsible for the changes that we observed in receptor and macromolecule diffusion, we have measured the diffusion of a lipid probe in cell and bleb membranes. On the cell surface, our diffusion results for NBD-PC are similar to those reported for this probe on cultured Chinese hamster fibroblasts (35). On blebs of L6 and FDB, we find that D has increased 2.7-fold (L6) and <15-fold (FDB) over the mobility on the normal cell surface, to yield similar values: $D = (1.2 \pm 0.4) \times 10^{-8}$ cm^2/s on L6, and $D = (1.5 \pm 0.6) \times 10^{-8}$ cm^2/s on FDB, both with complete recovery. These diffusion coefficients for NBD-PC on blebs are encompassed by the range of our measurements of a variety of lipid analogs in lipid bilayer model membranes at physiological temperatures in multicomponent lipid mixtures (36). From these measurements we can conclude that membrane viscosity changes alone are not responsible for the changes of many orders of magnitude that we have found for the diffusibility of AChRs on FDB cells and Con A receptors on L6 cells, although the relatively small ninefold increase in the diffusion coefficient of AcRSD on L6 blebs may be partially attributable to the 2.7-fold decrease in viscosity as witnessed by the lipid probe diffusion.

Several factors may contribute to the observed increases of lipid probe diffusion coefficients on blebs. Results from our laboratory on lipid diffusion in model membranes in the presence of high concentrations of the peptide gramicidin C show that lipid diffusion coefficients decrease at membrane protein concentrations approaching 20–30% by weight (11). If protein concentrations are less in blebbed membrane than in normal cell surface, protein-lipid interactions would be reduced by protein dilution so that we might expect an increase in NBD-PC mobility. Consistent with this, Jacobson (37) has shown that lipid diffusion on normal cell surface is often slower than in model membrane systems constructed from lipids extracted from the same cell type. In general, the diffusion of lipid molecules interacting with membrane proteins will be slowed more on normal cell surfaces where the interaction is with stationary proteins than on blebs where the proteins are unrestrained. The effects of coupling between fast and slowly diffusing species on their diffusion in solutions has been demonstrated by Magde et al. (38, 39, 40). Smoothing out of the plasma membrane during bleb formation is probably not responsible for the increase in lipid diffusion on L6 cells since the normal cell surface is smooth. Because the FDB muscle cell surface is elaborated by the T-tubule system, the results in that case should be taken as a lower limit. Theoretical calculations by Aizenbud and Gershon indicate that the D does not change by more than a factor of two in nonplanar (sinusoidally varying) topography; this has been observed for lipid diffusion on lymphocytes (41) and on mouse egg cells with or devoid of microvilli (B. M. Aizenbud, N. D. Gershon, D. E. Wolf, A. H. Handyside, and M. E. Edidin, private communication).

It is not yet possible to attribute the increase in diffusibility that we see on blebbed membrane to the release of any

particular molecular interaction. We think that this phenomenon is quite general since other work in our laboratory, to be reported elsewhere, has shown this effect for low density lipoprotein receptor on human fibroblasts, for IgE (Fc) receptor on rat basophilic leukemia cells and for Con A receptor on lymphocytes. The simplest hypothesis is that bleb formation has mechanically broken the connections anchoring (directly or indirectly) membrane receptors to the cytoskeleton. Although we may be reasonably confident that the normal subplasmalemmal cytoskeleton has been removed by bleb formation, we do not yet know whether exoskeletal or a membrane-associated protein matrix, similar to that seen by Mescher et al. (32), have been retained. In any case, the complex interactions between membrane components, matrix, and cytoskeleton, suggested by observations of global modulation of receptor diffusion, preclude simplistic conjectures at this time. However, the mechanisms responsible for modulation of membrane component diffusibility may eventually be revealed by systematic biochemical analysis of bleb preparations.

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