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Membrane Dynamics of Differentiating Cultured Embryonic Chick Skeletal Muscle Cells by Fluorescence Microscopy Techniques

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Changes in membrane fluidity during myogenesis have been studied by fluorescence microscopy of individual cells growing in monolayer cultures of embryonic chick skeletal muscle cells. Membrane fluidity was determined by the techniques of fluorescence photobleaching recovery (FPR), with the use of a lipid-soluble carbocyanine dye, and by fluorescence depolarization (FD), with perylene used as the lipid probe. The fluidity of myoblast plasma membranes, as determined from FPR measurements in membrane areas above nuclei, increased during the period of myoblast fusion and then returned to its initial level. The membrane fluidity of fibroblasts, also found in these primary cultures, remained constant. The fluidity in specific regions along the length of the myoblast membrane was studied by FD, and it was observed that the extended arms of the myoblast have the highest fluidity on the cell and that the tips at the ends of the arms had the lowest fluidity. However, since the perylene probe used in the FD experiments appeared to label cytoplasmic components, changes in fluidity measured with this probe reflect changes in membrane fluidity as well as in cytoplasmic fluidity. The relative change in each of these compartments cannot yet be ascertained. Tips have specialized surface structures, filopodia and lamellipodia, which may be accompanied by a more immobile membrane as well as a more rigid cytoplasm. Rounded cells, which may also have a more convoluted surface structure, show a lower apparent membrane fluidity than extended cells.

Key words: **plasma membrane, fluidity, skeletal muscle, myogenesis, laser, fluorescence photobleaching recovery, fluorescence depolarization, carbocyanine, perylene, fluorescence anisotropy, microviscosity**

The evolution of the concept of the cell membrane from a static interface to a dynamic fluid, lipid bilayer containing proteins with varying degrees of mobility [1–3] has been accompanied by the development of sensitive techniques for measuring membrane fluidity.

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Many membrane phenomena may involve fluidity changes, such as formation of specialized surface structures, hormonal modulation, endocytosis, and some aspects of cellular differentiation. These membrane functions may be clarified when the dynamic changes underlying them are illuminated.

Skeletal muscle cells provide a good system for study of the involvement of membrane recognition and fluidity in differentiation. During myogenesis, mononuclear myoblasts fuse to form myotubes, or multinucleate skeletal muscle fibers. Monolayer cultures of dissociated skeletal muscle cells also differentiate to produce striated, multinucleate muscle cells synthesizing the normal complement of muscle-specific proteins [4–6]. Fusion is accompanied by specific cell–cell recognition. When cells of different tissue type are cocultured, fusion of the plasma membranes occurs between skeletal muscle cells only, although skeletal muscle cells of different species will recognize each other and fuse [7]. The mechanisms for cell recognition, as well as for fusion, are still unclear. Transmission electron micrographs reveal a row of vesicles along the two apposed and fusing cell membranes [8–10]. One report, however, indicates that fusion is initiated at a single site, with a single, widening, cytoplasmic bridge forming between the two cells [11].

In molecular terms, cell recognition and fusion could be envisioned as requiring rigid complementary protein structures, much like the protein rosette targets to which secretory vesicles fuse [12]. Such assemblies could be sufficient for fusion. On the other hand, a transient increase in membrane fluidity could be required during fusion. Fusion in model systems is enhanced by agents that increase lipid fluidity. For example, fusion of bilayers will occur only when the temperature is above the liquid-gel transition temperature [13]. Agents that perturb the bilayer structure, such as lysolecithin [14], enhance fusion, whereas agents that decrease fluidity, such as cholesterol, inhibit fusion [13].

In the present study, one of these possibilities was explored by the measurement of lipid bilayer fluidity during myogenesis in monolayer cultures of embryonic chick skeletal muscle cells. In a previous study [15], it was reported that an increase in membrane fluidity preceded fusion. The method involved measuring rotational diffusion of a fluorescent probe in muscle cultures brought into suspension by trypsin digestion. There are, however, certain limitations in the use of cell suspensions, such as heterogeneity of the cell population and possible membrane damage caused by trypsin. In addition, the cellular location of the fluorescent probe must be defined to be certain that only the plasmalemma is being monitored. Finally, myoblasts are anatomically polarized cells, having a central ellipsoidal nuclear region, two long thin arms or extensions on opposite sides, and tips, with terminal foot pads. The fluidity of the membrane may not be uniform along the entire cell, and any changes during differentiation may be restricted to one of these regions.

To resolve these problems we undertook this investigation of muscle membrane fluidity by making measurements under the fluorescence microscope. The use of the microscope allows us to determine the location of the probe in the labeled cells and to choose specific regions of the cell for measurements of fluidity.

Fluidity of specific regions of myoblasts and fibroblasts was studied by two methods. Lateral mobility was measured by the technique of fluorescence photobleaching recovery (FPR), with a carbocyanine dye used as the fluorescent lipid probe. Rotational mobility was measured by a fluorescence depolarization (FD) technique, with perylene used as the probe [16].

MATERIALS AND METHODS

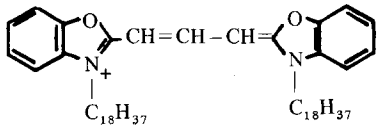
Cultures

Eleven-day chick embryo breast muscle tissue was dissociated by trypsin [17] and plated on 18 mm diameter collagenized coverslips (Calbiochem calf skin collagen or Sigma Type IV collagen). About $2-3 \times 10^6$ cells were plated on the coverslips in a 10 cm diameter Petri dish (Falcon) containing 5 ml medium 8:1:0.25 (Eagle's minimum essential medium with Earle's salts supplemented with 10% horse serum, 2.5% embryo extract, 0.2 mM glutamine, and 125 units/ml of penicillin and streptomycin). Medium components were obtained from Gibco. Material that can adsorb to collagen and produce a fluorescent background was removed from the medium by preincubation in a collagen-coated Petri dish at room temperature overnight. The cells were maintained in a humidified incubator with 5% CO_2: 95% air at 37°C, and the growth medium was changed daily.

Lateral Mobility

Lateral mobility of membrane lipids was measured using the lipid probe N,N′-di(octadecyl)oxacarbocyanine (K-1) (Fig. 1a) generously supplied by A. S. Waggoner [18]. Fluorescence photobleaching recovery was performed in a manner similar to that previously described [19, 20]. A coverslip to which the cells were attached was washed twice with Dulbecco's phosphate-buffered saline (PBS), labeled in 0.3 ml PBS containing 13 μM K-1 and 1% ethanol at 22°C for 4 min, rinsed once in PBS plus 0.2% bovine serum albumin, and finally rinsed in PBS. The coverslip was secured face down to a glass slide by a silicone rubber gasket. One coverslip was used for measurements for no longer than one hour after removal from the incubator.

The apparatus for photobleaching was similar to that illustrated elsewhere [19], except that a helium/cadmium laser (Liconix model 4110) was employed, having an output of 10 mwatt and wavelength of 442 nm. The laser beam was focused onto a specific spot (2–3 μm diameter) on a cell through a 40X Pol objective on a Zeiss microscope under epi-illumination. Fluorescence was monitored by using the laser beam attenuated by three orders of magnitude by a neutral density filter. For bleaching, the attenuator was removed for 0.2–0.4 sec. The recovery of fluorescence into the bleached spot was detected and recorded with a photon counting system made from Ortec components consisting of a photomultiplier (model 9201), D-A convertor (9325), photon counter (9315), amplifier discriminator (9302), and strip-chart recorder.

a. 

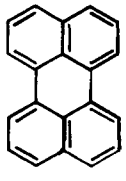
b. 

Fig. 1. Fluorescent probes: (a) N,N′-di(octadecyl)oxacarbocyanine (K-1); (b) Perylene.

Rotational Mobility

The laser beam is highly polarized and hence can be used to measure fluorescence depolarization, or anisotropy, at a single point on a cell through a microscope. The perylene probe used here is weakly oriented when embedded in a lipid bilayer and is thus suitable for measurement of microviscosity by fluorescence depolarization [21]. Cells were processed as above, except that they were labeled for 15 min at 37°C in PBS containing 10 μM perylene and 1% ethanol. A rotatable polarizer was placed in the path of the emitted beam, and the intensity was recorded with the polarizer parallel and perpendicular to the direction of polarization of the exciting light beam. Fluorescence anisotropies were corrected for light scattering, background fluorescence, and distortions introduced by 1) differences in transmission efficiencies through the microscope for light polarized parallel ($I_\parallel$) and perpendicular ($I_\perp$) to the direction of polarization of the incident beam and 2) depolarization introduced by the finite collection angle of the microscope objective. Light scattering and background fluorescence corrections were made by subtracting background fluorescence as determined from parallel and perpendicular intensities adjacent to cells under study. A correction factor α for transmission and collection angle artifacts was obtained by two different methods. In one, a thin film of perylene in a viscous oil was formed between a cover glass and microscope slide, and the directly measured, uncorrected ratio $(I_\perp/I_\parallel)_{OM}$ was determined under the microscope under conditions identical to those used in our cell experiments. The correct value of the polarization ratio, $(I_\perp/I_\parallel)_{OS}$, for perylene in the viscous oil had been previously determined in a steady-state spectrofluorimeter [22]. The corrrection factor is given by

$$\alpha = \left[\frac{I_\perp}{I_\parallel}\right]_{OS}\left[\frac{I_\parallel}{I_\perp}\right]_{OM} \tag{1}$$

The corrected ratio $(I_\perp/I_\parallel)^c$ for the cell experiments was obtained from experimental values of $I_\perp$ and $I_\parallel$ by the equation

$$R = \left[\frac{I_\perp}{I_\parallel}\right]^c = \alpha\left[\frac{I_\perp - I_\perp{}^b}{I_\parallel - I_\parallel{}^b}\right] \tag{2}$$

where $I_\perp{}^b$ and $I_\parallel{}^b$ are the perpendicular and parallel background intensities, respectively. Anistropy was calculated with the equation

$$A = \frac{1 - (I_\perp/I_\parallel)^c}{1 + 2(I_\perp/I_\parallel)^c} = \frac{1 - R}{1 + 2R} \tag{3}$$

In a second method for evaluating α, human red cell ghosts were labeled with perylene, and the value of $(I_\perp/I_\parallel)_{GS}$ of the ghost membranes was measured in a steady-state spectrofluorimeter using a membrane suspension. The $(I_\perp/I_\parallel)_{GM}$ was then measured under the microscope, corrected for background, and α was calculated with the expression

$$\alpha = \left[\frac{I_\perp}{I_\parallel}\right]_{GS}\left[\frac{I_\parallel}{I_\perp}\right]_{GM} \tag{4}$$

This procedure assumes that the weak orientation of perylene in the lipid bilayer does not significantly influence the values of the polarized intensities. Close agreement was obtained between the values of α determined by the two procedures described here. Furthermore, by calibrating in the steady-state spectrofluorimeter a series of solutions of varying microviscosities formed by mixing two solvents of different viscosities, we determined that α did not change in the range of anisotropies of interest here. The correction factor was found to be $\alpha = 1.50 \pm 0.04$. All measurements were made at 21–22°C.

RESULTS

Cell Cultures

Monolayer cultures of embryonic chick skeletal muscle cells grown under the conditions described here differentiate, as shown in Figure 2. Cells proliferate for the first 2 days. Myoblasts enlarge, elongate, and overlap in chains at about 30 h after plating. Fusion of many cells has occurred at about 36 h and is complete in the culture at about 60–70 h after plating. The myotubes enlarge, become striated, and develop spontaneous contractions after 5 days in culture.

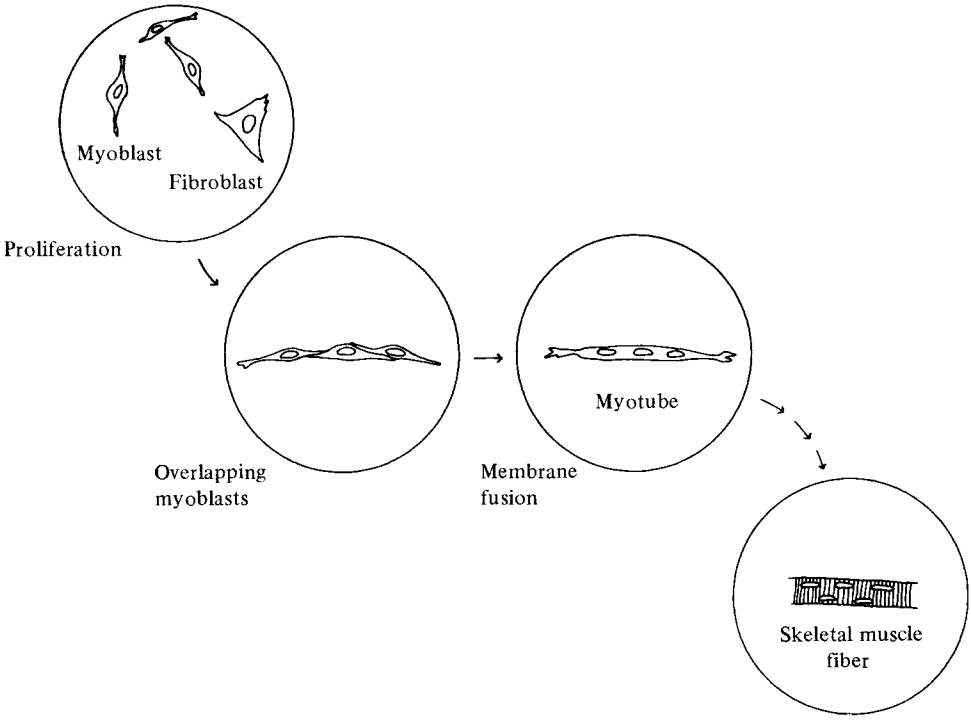


Fig. 2. Differentiation of embryonic skeletal muscle cells

Labeling of Cells With Fluorescent Probes

The fluorophore K-1 did not penetrate the plasma membrane of intact cells under our incubation conditions, as evidenced by distinct rim-labeling around the edges of the cells and a lack of labeling of internal membranous structures (Fig. 3). A striking observation was that the tips of the myoblast displayed much weaker fluorescence than the rest of the plasmalemma.

Two probes were tested for fluorescence depolarization (FD) measurements under the microscope. The probe 1,6-diphenyl-1,3,5-hexatriene (DPH) is widely used for studies of microviscosity in cell suspension and has been used in a previous study of suspended myoblasts [15]. Our observations under the microscope, however, indicate that DPH accumulates in very large spherical vacuoles in both myoblasts and fibroblasts. The fluorescence from these internal vacuoles represented a significant fraction of the total DPH fluorescence intensity. In addition, the cells deteriorated rapidly after labeling with DPH, probably due to the tetrahydrofuran solvent used to solubilize this fluorophore. We therefore examined another weakly oriented lipid probe, perylene, and found that this probe was also quickly internalized, but that the labeling procedure, which uses ethanol instead of tetrahydrofuran, did not damage the cells. With perylene, intense fluorescence was observed from small lipid granules around the nucleus, especially on the sides of the nucleus adjacent to the two cell extensions or arms (Fig. 3). Areas above the nuclei and in the arms and tips were more weakly fluorescent. In mature myotubes, fluorescence intensity displayed a striated pattern along the cell, probably due to internal labeling of the sarcoplasmic reticulum and transverse tubules. As with K-1, fluorescence intensity along the cell showed a marked decrease at the flattened, widening tips of the cells. In general, the lowest fluorescence intensity was seen at the tips of the cells and directly over the nuclei. The lower fluorescence intensity in the nuclear region seems to be due to the paucity of cytoplasm in this region, which decreases the fluorescence intensity from internalized probe. The intensity in this region is probably due mostly to fluorescence from perylene in the plasmalemma and nuclear membranes. The reason for the decreased fluorescence at the tips of the cells is not clear. By focusing the 2–3 μm illuminating light beam on specific areas above the nuclei or in the regions of the arms or tips, one can avoid exciting fluorescence from discernible lipid granules in adjoining areas.

Lateral Mobility

For measuring fluidity of membranes by photobleaching, the probe K-1 was incorporated into the plasma membranes of the cells. Figure 4 shows typical recordings from a single spot over the nucleus of a fibroblast successively bleached and allowed to recover fluorescence three times. The measured light intensity on the ordinate (counts per 0.1 sec) varied from cell to cell from 10^4 to 10^5 photons per 0.1 sec. The recovery of initial intensity was better than 85%. The 10% to 15% incomplete recovery of fluorescence intensity after a bleaching pulse could be due to internalization and labeling of slowly moving pinocytotic vesicles immediately below the membrane or to a small percentage of probe molecules immobilized by interaction with proteins. Our present experiments cannot distinguish between these two possibilities. Photo-cross-linking of probe molecules does not seem likely, since the percent fluorescence recovery was not dependent on the percent of bleach. For the recording in Figure 4, the bleached spot diameter was 2.5 μm. For most of the measurements, the spot diameter was 3.3 μm, to slow down the recovery times.

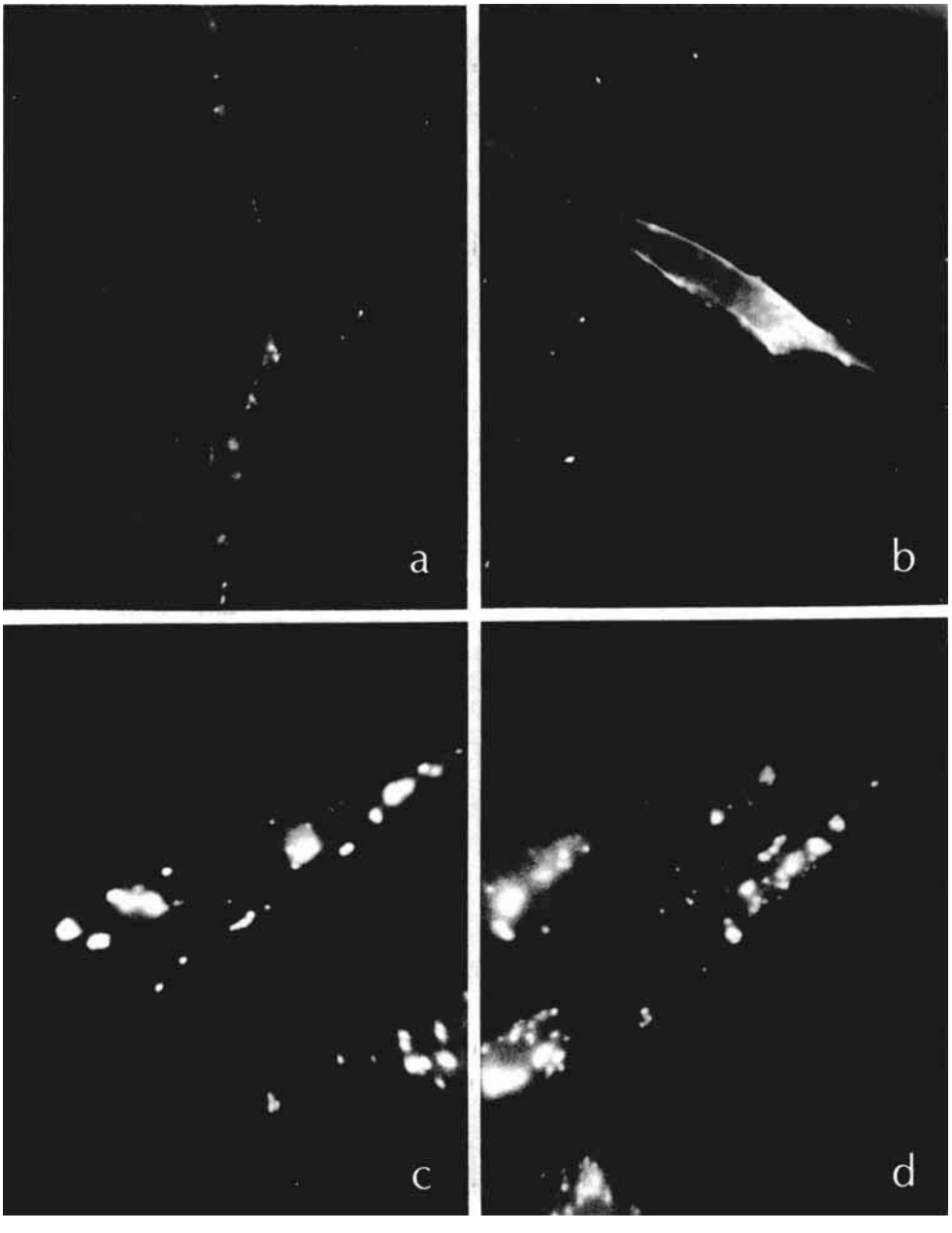


Fig. 3. Fluorescence micrographs of cells labeled with: (a,b) K-1; (c,d) Perylene.

The results of FPR measurements made at several intervals after plating the cells are shown in Figure 5. The $t_{1/2}$ ordinate represents the time after bleaching for half-maximal recovery of fluorescence and is plotted against age of the culture. These results indicate that the plasmalemma above the nuclear region of myoblasts underwent a 40% decline in $t_{1/2}$ over the first 3 days, or a 40% increase in the apparent rate of lateral diffusion of the lipid probe over this time. The $t_{1/2}$ value for a flat membrane is related to the diffusion coefficient of the fluorophore by the expression

$$t_{1/2} = \frac{\beta r^2}{4D} \tag{5}$$

where D is the diffusion coefficient and r is the effective $(1/e^2)$ radius of the bleaching beam [23]. For a laser bleaching beam with a Gaussian distribution of intensity along its diameter, β is a parameter that depends on the percent of bleached fluorophore. Its value ranges from 1.0 for a very low bleaching to about 1.7 for 90% bleaching. Values of $t_{1/2}$ are thus not directly comparable in general unless reduced to the same extent of bleaching. However, theoretical calculations indicate that for the range of bleaching intensities and precision in our experiments, a value of $\beta \sim 1$ is justified in all experiments. In this case, values of $t_{1/2}$ can be directly compared without corrections for percent bleach. Newly formed myotubes showed the same higher membrane fluidity as the unfused myoblasts. After a week in culture, the $t_{1/2}$ returned to its initial level for myoblasts that never fused, as well as for myotubes. It appears that fusion was not required for the changes that made the membrane more fluid, and that fusion was also not required to return the membrane to its initial state. Thus, the membrane changes are not coupled to fusion, although they may be required to make fusion possible.

The fibroblasts present in the culture maintained a constant plasma membrane fluidity in a region over their nuclei throughout the period examined. Their membranes appeared to be more fluid than those of myoblasts. A diffusion coefficient for the fibroblasts of 3×10^{-9} cm^2/sec was calculated using the relationship given above with $\beta = 1$.

The mechanism for the apparent increase in myoblast membrane fluidity during the period of active fusion is not clear. One trivial possibility is a smoothing out of the myoblast membranes following trypsinization. A very convoluted membrane with many microvilli, blebs, and filopodia along it would have a larger surface area (and larger effective value of r in equation 5) within the bleached spot and hence a slower recovery time and lower apparent fluidity than a smooth surface. It is known that trypsin causes myoblast cells to round up, and scanning electron micrographs show that rounded cells produced by trypsinization or during mitosis have very irregular surfaces [24, 25]. Indeed, our direct comparisons of stretched out and rounded myoblasts or fibroblasts by FPR indicate an increase in $t_{1/2}$ as large as twofold for rounded cells. In the studies summarized in Figure 5, however, only extended cells were used. Scanning electron micrographs of cultured primary muscle cells have revealed relatively smooth surfaces on myoblasts and myotubes [26, 27]. Interestingly, primary chick fibroblasts have been shown to have very irregular surfaces [27], yet a higher rate of diffusion of K-1 was obtained on the fibroblast plasma membrane than on the smoother myoblast membrane. Furthermore, the fibroblasts in the culture were also trypsinized and yet did not exhibit the same large $t_{1/2}$ at early times after plating that the myoblasts did. Thus, changes in $t_{1/2}$ measured with K-1 in the region of a nucleus do not appear to be due to changes in surface topography but seem to reflect real changes in membrane fluidity.

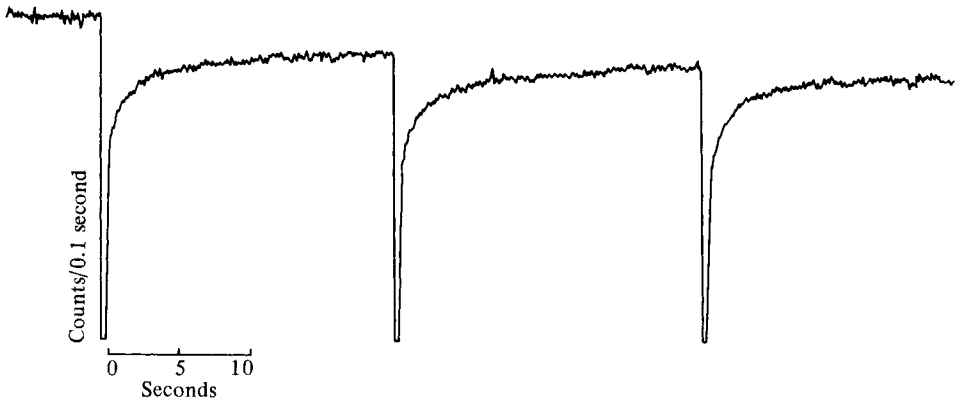


Fig. 4. Fluorescence photobleaching recovery record. A fibroblast labeled with K-1 was bleached on a 2.5 μm in diameter spot for 0.4 sec. Fluorescence recovery into the spot was monitored for three successive bleaches.

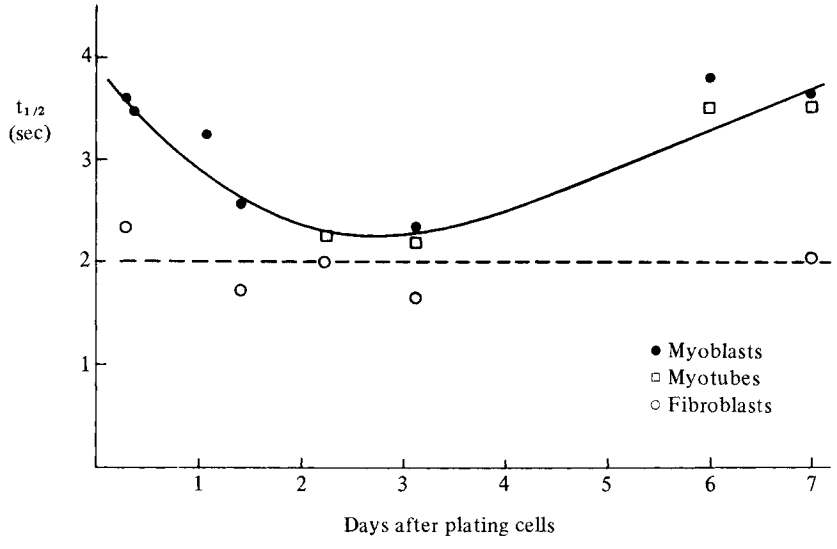


Fig. 5. Half-times for recovery of fluorescence as a function of time in culture. The $t_{1/2}$ were measured as described in Methods. All data from many experiments were averaged for these points. Between 9 and 38 data were averaged per point, with an average of 18 data per point. Standard deviations of the mean were less than ± 10%.

Rotational Mobility

As discussed above, the interpretation in terms of membrane fluidity of $t_{1/2}$ values obtained by the FPR technique requires some knowledge of the microtopography of the cell membrane. Moreover, FPR results obtained from geometrically narrow and irregular cell regions where the bleaching beam diameter is larger than the illuminated cell structure, such as tips of myoblasts, are difficult to interpret. These questions of surface and cell geometry led us to attempt to follow the time course of membrane fluidity by a technique of fluorescence depolarization (FD), which does not depend on such geometry. The tech-

nique measures the amount of rotational mobility displayed by a fluorescent probe during its lifetime, which is related to the membrane's fluidity, or microviscosity [21, 28]. Results obtained with weakly oriented probes such as perylene or DPH should be independent of the cell and surface geometry of the plasma membrane around or within the spot illuminated by the laser beam. Perylene was chosen rather than the commonly used DPH because it can be excited in the visible region, which minimizes autofluorescence, and because the labeling procedure does not damage cells, and it does not bleach as readily. It has the problem of not being as sensitive as DPH to changes in viscosity.

Time course of microviscosity above nuclear region. Fluorescence anisotropy was measured by FD over the central nuclear region of myoblasts and fibroblasts. The anisotropy of the perylene emission was found to remain fairly constant on both cell types throughout the culture period examined. The ratio of anisotropies of myoblasts to fibroblasts ranged from 1.0 to 0.95 at 30 h after plating, with no clear trend of a change thereafter. The FPR results of increasing fluidity over the first 3 days and a subsequent decrease would have led to the prediction that the ratio of anisotropies of myoblasts to fibroblasts would have decreased and then increased, yet this was not clearly observed for the nuclear region. The results of the fluorescence anisotropy measurements, however, do not necessarily negate a similar change in fluidity, since calculations that we have made indicate that a 40% change in fluidity, as suggested by the FPR results, would produce only a 5% change in the ratio of anisotropies. Such a slight change would be difficult to detect in our experiments with the experimental precision which we have been able to achieve so far under the microscope with perylene. Moreover, the perylene probe, in contrast to K-1, is internalized, and some of the fluorescence registered in our experiments probably arises from perylene in the nuclear membrane as well as from the plasma membrane.

Fluorescence from the nuclear membrane would tend to obscure possible fluidity changes in the plasma membrane. To see if similar results are obtained at different locations along the length of the cells, fluorescence anisotropy at different regions of a myoblast were measured.

Microviscosity along length of myoblast. Figure 6 is a sketch of a myoblast and the letters a (tip), b (arm), and c (nuclear region) indicate the points on the cell at which fluorescence anisotropy measurements were made. The results for cells that had been cultured for about 50 h are given in Table I.

The arm of the myoblast appeared to have a lower fluorescence anisotropy, or higher rotational mobility (assuming a constant lifetime), than the nuclear region of the cell. The ratio of anisotropies was 0.41, suggesting that perylene in the arm region is in a relatively more fluid environment. However, since perylene penetrates into the cytoplasm, it is not possible at present to state to what extent changes in anisotropy in the arm region reflect changes in membrane versus cytoplasmic fluidity.

In all cases examined, the flattened, widened, or bulbous tips at the ends of the myoblasts had a higher fluorescence anisotropy, or lower rotational mobility, than the rest of the cell. The anisotropy of the tip is twice that of the nuclear region. It is possible that the specialized structures at the leading edge of migrating cells, the filopodia and lamellipodia, represent extensions of membrane that has somehow become more rigid. We have also done FPR measurements at the tips of myoblasts with K-1, which does not label the cytoplasm, and found a manyfold increase in $t_{1/2}$. The FPR results at the tips, however, are difficult to interpret because of the small size of these structures and the resulting uncertainty in the bleaching geometry.

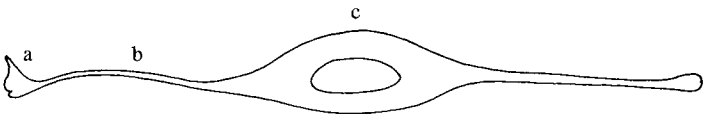


Fig. 6. An elongated myoblast. Letters indicate the positions assayed by the fluorescence depolarization technique: a. tip; b. arm; and c. nuclear region.

TABLE I. Fluorescence Anistropy Along the Length of a Myoblast

Location	$I_\perp/I_\parallel$[a]	Anisotropy	Ratio to nuclear region
a. Tip	0.78 ± 0.05 (9)	0.086	1.7
b. Arm	0.94 ± 0.02 (20)	0.021	0.41
c. Nuclear region	0.86 ± 0.02 (13)	0.051	

[a] $I_\perp/I_\parallel$ were corrected and anisotropy was calculated as described in the text. One standard deviation of the mean is given, as well as the number of measurements averaged (in parenthesis).

TABLE II. Fluorescence Anisotropy of Rounded vs Extended Myoblasts

	$I_\perp/I_\parallel$[a]	Anisotropy
Extended	0.92 ± 0.02	0.029
Rounded	0.78 ± 0.02	0.088
Ratio (extended/rounded)	1.2	0.32

[a] $I_\perp/I_\parallel$ as well as anisotropies for rounded and extended myoblasts were averaged from three experiments. One standard deviation of the mean is given.

Rounded vs extended cells. During mitosis the normally extended cells become rounded and develop many filopodia and microvilli [24]. Such cells are found at random in the tissue culture during the first 2 or 3 days. It was of general interest to study the microviscosity of these cells. Results with the FD technique are shown in Table II. Rounded cells had about a 20% lower ratio of $I_\perp/I_\parallel$, or a three times higher fluorescence anisotropy than extended cells. Hence, round cells seemed to show less rotational freedom for the lipid probe perylene. These findings may be related to the relatively lower rotational mobility found at the tips of myoblasts. Filopodia, and possibly other surface projections, may require relative immobilization of some structures in the region of the projections. In the case of membranes, one way this could be brought about is by increasing the protein concentration. This possibility could also account for our observation of lower fluorescence intensity at the tips of cells with the surface-specific lipid probe K-1, suggesting a lower percentage of lipids at these tips.

FPR also showed that rounded cells had a longer recovery time for fluorescence intensity after bleaching than extended cells, suggesting a lower fluidity. However, as mentioned above, this may be due in part to geometrical effects.

DISCUSSION

The FPR and FD techniques are potentially powerful methods of unraveling the role of membrane fluidity and lateral mobility of membrane components in cell functions. The FPR technique can measure the lateral mobility of both fluorescent labeled lipids and proteins, while the FD technique can measure rotational mobility of lipids. Our extension of the FD technique to measurements through a microscope with a polarized laser beam makes it possible to use FD to measure fluidity in specific regions of anatomically polarized cells, and allows one to avoid exciting fluorescence from internal structures which can be discerned under the microscope. Insofar as mobility of lipids is concerned, the FPR and FD techniques complement or reinforce each other. With probes which are only weakly oriented in the membrane [29–31], the FD technique gives information on the average local microviscosity independent of the local cell geometry or topography of the cell membrane, including the presence of microvilli, filopodia, blebs, or lamellipodia, and aids in the interpretation of $t_{1/2}$ values of the FPR technique, which are sensitive to these parameters. In addition, the intense bleaching beam used in the FPR technique could alter membrane properties and yield artifactual results. The much lower intensities used in the FD measurements are not anticipated to alter membrane properties, and thus can be used to support or question results obtained by the FPR technique. The FD technique, on the other hand, is sensitive not only to microviscosity but also to the lifetime of the fluorophore. The lifetime of perylene is not highly sensitive to environmental changes but, in general, it must be ascertained that a change in polarized fluorescence is not due to changes in lifetime before it can be definitely interpreted in terms of fluidity changes.

The proper use of the FPR and FD techniques in the study of the lipid bilayer requires the availability of suitable fluorescent probes. K-1 labeled exclusively the plasmalemma of cultured muscle cells, unless they were damaged, in which case interior structures were labeled. It was thus an ideal lipid probe for FPR experiments with viable cells. However, we could not use it to study rotational mobility by polarized emission, because its short lifetime and relatively slow rotational motion in its surface location make its polarized emission insensitive to fluidity changes [32]. For FD experiments, DPH is a sensitive microviscosity probe, but its accumulation in large vacuoles produced in our cultures during the labeling steps precluded its use. Perylene is less sensitive to microviscosity, but labeling did not damage the cells. This fluorophore, however, is also internalized, which can complicate results of polarized fluorescence measurements. The source of the internal fluorescence can be the nuclear membrane, cytoplasmic membranes (mitochondria, sarcoplasmic reticulum, Golgi bodies, vesicles, etc), as well as components in the cytosol too small to be seen under the microscope (lipid droplets and lipophilic proteins, for example). Under the microscope, one can avoid areas where the major part of the fluorescence is clearly due to discernible internal organelles.

Although perylene proved to be the more useful, neither perylene nor DPH is an ideal probe for polarized fluorescence measurements under the microscope. An ideal probe should 1) not be internalized, 2) have a polarized emission that is highly sensitive to fluidity, 3) be only weakly oriented in a bilayer, 4) absorb in the visible region of the spectrum, 5) not be highly sensitive to bleaching, and 6) label without cell damage. To avoid internalization one must use an amphipathic probe [32]. This would seem to contradict the requirement of only weak orientation. However, covalent attachment of a fluorophore to the terminal region of a lipid hydrocarbon chain results in an amphipathic probe where the fluorescent moeity is weakly oriented [32]. Such probes may eventually allow much better use of the FD technique under the microscope than is possible with perylene and DPH.

With these caveats in mind, we have found that the fluidity of muscle plasma membranes appears to change during myogenesis, as revealed by FPR measurements in regions above nuclei. The fluidity in myoblasts apparently increases as cells become competent to fuse and returns to its initial level after all fusion is essentially complete.

In a previous study on membrane fluidity of myoblasts, Prives and Shinitzky [15] observed a 50% increase in membrane fluidity preceding fusion of muscle cells by following the rotational diffusion of the lipid probe DPH. However, their studies were done on cell suspensions obtained by trypsinization of cultured cells at various times after plating. They thus measured a population of cells that was heterogeneous and whose membranes may have been altered by trypsinization. In addition, our observations under the microscope indicate that the DPH probe used in their experiments is highly internalized and that the fluorescence intensity in a cell suspension may be dominated by structures other than the plasmalemma. However, as mentioned, our results with the surface-specific probe K-1, and using a microscope to assay the three cell types (myoblasts, myotubes, fibroblasts) individually while they were attached to their substratum, indicate that fluidity changes do indeed occur in the plasma membrane of the muscle cell population.

Edidin and Fambrough [33] used fluorescent antibody fragments of anti-muscle plasma membrane antibody to look at muscle membrane fluidity. Antibodies micropipetted onto cells formed a small spot whose rate of enlargement gave a rough diffusion coefficient on the order of $1-3 \times 10^{-9}$ cm^2/sec. Schlessinger et al [34] measured both lipid and protein fluidity by FPR on an established rat muscle cell line, L-6. Their lipid probe was another carbocyanine dye (diI) and gave a diffusion coefficient of $9 \pm 4 \times 10^{-9}$ cm^2/sec. This is a somewhat higher membrane fluidity than observed in our studies on primary chick cells. However, the small differences may be due to differences between 1) the 2 species, 2) established cell lines vs primary cell cultures, or 3) particular laboratory protocols.

Another study implicating an involvement of lipids in myoblast fusion was one in which treatment of cultures with phospholipase C was shown to inhibit fusion [35]. The lipid composition of plasma membranes can affect the rate of growth and fusion of myogenic cells [15, 36], and such changes may produce small changes in lipid mobility as measured by FPR [37]. In addition, tetrameric conconavalin A, which can patch certain glycoproteins and glycolipids, will inhibit fusion at high concentrations [38], and it has been suggested that mobility of some of these sugar-containing molecules is required. Sha'afi et al have measured the microviscosity of muscle sarcolemma preparations from 20-day-old chicks using FD with perylene. They obtained a membrane microviscosity of 61 ± 8 cP [39]. It is not clear, however, whether extraction can alter the membrane properties.

We also observed distinct changes when different locations on a myoblast were assayed by FD using perylene. The tip of the cell was found to be the least fluorescent and least fluid. Fibroblasts in these cultures, when examined by FD using perylene, were also found to have anisotropies at their tips and ruffling membranes that were twice the value measured over their nuclear regions. Although these results indicate interesting changes in fluidity, we cannot at present, using perylene, separate changes in plasma membrane fluidity from changes in cytoplasmic fluidity. Since the same lower intensity was found with the surface-specific probe K-1, the results seem to reflect, at least in part, membrane properties. On the other hand a variety of observations from other laboratories indicate that the cytosol in the tips may be in a state of low fluidity. Thus it has been reported that actin filaments in motile cells form a network at the cell edges and within lamellipodia [40–42]. It has been proposed that the network is in the form of a gel containing actin as well as gelation proteins [43]. If such a gel network should indeed exist in the myoblast tips, it is quite

possible that it could be sensed by internalized probe. It should finally be noted that changes in plasma membrane and cytoplasm microviscosities are not mutually exclusive but may, in certain instances, go hand in hand; that is, changes in microviscosity in the cytoplasm might be reflected in similar changes in the adjacent plasma membrane. To determine whether indeed such a correlation exists in the myoblast tips, we are examining various fluorophores as probes of cytosol microviscosity [32].

The arms of the myoblasts showed the highest levels of fluidity as determined from FD measurements. The interpretation here is again limited by the unknown contribution of fluorescence from internal membranes and the cytoplasm. If these contributions are not significant, then it is interesting that the region of higher fluidity coincides with the region where fusion first occurs [11, Fig. 9]. The intermediate level of fluidity over the nuclear region measured with perylene is probably an average between that of the nuclear membrane and the sarcolemma, although their relative contributions are not known.

The observations presented here are that myoblast fusion is accompanied by a higher membrane fluidity, as revealed by FPR measurements with the membrane-specific probe K-1. The membrane changes, however, do not seem to require fusion since they occur in the entire population of muscle cells, even those that do not fuse. In addition, the tips of the cells appear to be more rigid than the other regions of the cell as revealed by FD measurements with the penetrating probe perylene, but it is not clear whether these reflect changes in the plasma membrane, the cytoplasm, or both. The advantages of fluorescence microscopy methods are that single cells can be assayed and that specific locations on the membrane can be selected for assay with a finely focused laser beam.

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REFERENCES

1. Frye LD, Edidin M: J Cell Sci 7:319, 1970.
2. Hubbell WL, McConnell HM: J Am Chem Soc 93:314, 1971.
3. Singer SJ, Nicolson GL: Science 175:720, 1972.
4. Holtzer H, Abbott J, Lash J: Anat Rec 131:567, 1958.
5. Cooper WG, Konigsberg IR: Anat Rec 133:368, 1959.
6. Shainberg A, Yagil G, Yaffe D: Dev Biol 25:1, 1971.
7. Yaffe D, Feldman M: Dev Biol 11:300, 1965.
8. Hay ED: Z Zellforsch 59:6, 1963.
9. Shimada Y: J Cell Biol 48:128, 1971.
10. Rash JE, Fambrough D: Dev Biol 30:166, 1973.
11. Lipton BH, Konigsberg IR: J Cell Biol 53:348, 1972.
12. Beisson J, Lefort-Tran M, Pouphile M, Rossignol M, Satir B: J Cell Biol 69:126, 1976.
13. Papahadjopoulos D, Poste G, Schaeffer BE, Vail WJ: Biochim Biophys Acta 352:10, 1974.
14. Poole AR, Howell JI, Lucy JA: Nature 227:810, 1970.
15. Prives J, Shinitzky M: Nature 268:761, 1977.
16. Elson HF, Yguerabide J: J Supramol Struct 9:116, 1979.
17. Bischoff R, Holtzer H: J Cell Biol 44:134, 1970.
18. Yguerabide J, Stryer L: Proc Natl Acad Sci USA 68:1217, 1971.
19. Yguerabide J: Presented at Biophysical Society Meeting, 1971.

20. Koppel DE, Axelrod D, Schlessinger J, Elson EL, Webb WW: Biophys J 16:1315, 1976.
21. Shinitzky M, Dianoux A-C, Gitler C, Weber G: Biochemistry 10:2106, 1971.
22. Kehry M, Yguerabide J, Singer SJ: Science 195:486, 1977.
23. Wode B, Yguerabide J, Feldman J (in preparation).
24. Porter K, Prescott D, Frye J: J Cell Biol 57:815, 1973.
25. Follett EAC, Goldman RD: Exp Cell Res 59:124, 1970.
26. Shimada Y: Dev Biol 29:227, 1972.
27. Papadimitriou JM, Dawkins RL: Cytobios 8:227, 1973.
28. Perrin F: J Phys Radium 7:390, 1926.
29. Kawato S, Kinosita K Jr, Ikegami A: Biochemistry 16:2319, 1977.
30. Chen LA, Dale RE, Roth S, Brand L: J Biol Chem 252:2163, 1977.
31. Yguerabide J, Foster MC: In Grell E (ed): "Membrane Spectroscopy." New York: Springer-Verlag (in press).
32. Yguerabide E, Yguerabide J: unpublished results.
33. Edidin M, Fambrough D: J Cell Biol 57:27, 1973.
34. Schlessinger J, Axelrod D, Koppel DE, Webb WW, Elson EL: Science 195:307, 1977.
35. Nameroff M, Trotter JA, Keller JM, Munar E: J Cell Biol 58:107, 1973.
36. Horwitz AF, Wight A, Ludwig P, Cornell R: J Cell Biol 77:334, 1978.
37. Axelrod D, Wight A, Webb W, Horwitz A: Biochemistry 17:3604, 1978.
38. Sandra A, Leon MA, Przybylski RJ: J Cell Sci 28:251, 1977.
39. Sha'afi RI, Rodan SB, Hintz RL, Fernandez SM, Rodan GA: Nature 254:525, 1975.
40. Abercrombie M, Heaysman JEM, Pegrum SM: Exp Cell Res 67:359, 1971.
41. Lazarides E: J Cell Biol 68:202, 1976.
42. Lazarides E: J Supramol Struct 5:531, 1976.
43. Brotschi EA, Hartwig JH, Stossel TP: J Biol Chem 253:8988, 1978.