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Influence of Membrane Lipids on Acetylcholine Receptor and Lipid Probe Diffusion in Cultured Myotube Membrane†

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ABSTRACT: We measured how alterations of the fatty acyl chains in the plasma membranes of cultured chick embryo myotubes affect the lateral mobility of the fluorescent membrane lipid probe dioctadecylindocarbocyanine (diI) and of acetylcholine receptors (AChR) labeled with fluorescent tetramethylrhodamine-α-bungarotoxin (TMR-αBgt). Membrane phospholipid fatty acyl chains were altered by manipulating the fatty acid composition of the growth medium; lateral mobility was measured by the fluorescence photobleaching recovery (FPR) technique. In general, our results demonstrate that substantial membrane lipid acyl changes need not substantially affect the lateral mobility of cell-membrane components. We found that membrane fatty acyl changes affected lateral motion by less than a factor of 1.5 to 2 for diI and less than a factor of about 3 to 4 for AChR, at both low (12 °C) and high (31 °C) temperatures. For all the altered cell types tested, an increase of temperature from 12 to 31 °C resulted in (a) a threefold increase in the diI diffusion constant with almost all the diI mobile throughout the temperature range, and (b) an increase in the fraction of AChR which is mobile from about 20 to about 60%. At 31 °C, the diI diffusion constant is almost two orders of magnitude larger than the mobile AChR diffusion constant. The fatty acyl changes do not affect the optically observed distribution of either diI or AChR in the membrane.

Animal cell membranes possess a complex lipid composition consisting primarily of several different phospholipids and fatty acyl groups, cholesterol, and sphingolipids. The functional role of the diverse lipid composition remains, in large part, a mystery. One speculation is that it modulates the mobility and distribution of membrane components. The combination of two recent developments makes feasible the investigation of this possibility. First, techniques are now available for manipulating the lipid composition of cells grown in culture (Horwitz, 1977). Second, a method now exists for measuring the lateral mobility and distribution of fluorescence-labeled membrane components (Axelrod et al., 1976a; Koppel et al., 1976).

With these experiments, we observed that substantial membrane lipid fatty acyl changes in primary cultures of myotubes from chick did not substantially affect the lateral mobility of either a membrane lipid probe or the acetylcholine receptor (AChR), an integral membrane protein. We report the lateral mobilities of the lipid probe and AChR as a function of temperature.

We have altered the lipid composition of cultured cells by growing them in a medium depleted of lipid and biotin and supplemented with a selected fatty acid. The incorporation of those fatty acids that are not readily metabolized results in an appreciable alteration in the fatty acyl composition of the membrane phospholipids (Horwitz, 1977; Horwitz et al., 1978).

The exogenous phospholipid-like membrane fluorescent lipid probe employed was dioctadecylindocarbocyanine [diI-C_{18}-(3), or diI] (Sims et al., 1974; Schlessinger et al., 1977; Badley et al., 1973). AChR on the myotube surface was visualized by specific binding with fluorescent-tagged α-bungarotoxin (TMR-Bgt) (Ravdin and Axelrod, 1977; Axelrod et al., 1976b; Anderson and Cohen, 1974). We have measured lateral motion of fluorescent molecules on a cell surface by a technique called fluorescence photobleaching recovery (FPR) (Axelrod et al., 1976a, Koppel et al., 1976). In this technique, the fluorescence of a small region on the cell surface is bleached by a bright flash

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of laser light focused on the surface. The subsequent lateral motion of unbleached fluorophore from surrounding areas into the bleached area is determined by measuring the recovery of fluorescence excited by the same, although much attenuated, focused laser beam.

Materials and Methods

Culturing of Cells. Chick pectoral muscle was dissected from 11-day-old embryos, dissociated into single cells, and prepared for plating as described by Bischoff and Holtzer (1969), except that the trypsin treatment of the tissue was terminated with lipid-depleted growth medium (LDM). Other minor modifications are presented in Horwitz et al. (1978). Two milliliters of cells suspended in LDM (5×10^5 cells/mL) was added to 35-mm collagen-coated tissue culture dishes. Where appropriate, the addition of fatty acids (oleate $18{:}1_{cis}$ or elaidate $18{:}1_{trans}$) or biotin or replacement with media containing undelipidated serum was made after 3 h. Fatty acids were stored in ethanol at a concentration of 4.2 mg/mL; 10 μL was added to each dish. Biotin was stored in ethanol/water (1:1) at a concentration of 0.5 mg/mL; 5 μL was added to each plate. Five microliters of cytosine arabinoside was added on day 2 from a 0.4 mg/mL stock in phosphate-buffered saline (PBS). The cells were grown in an incubator containing a 10% CO_2 atmosphere.

Media Composition, The cells were grown in Dulbecco's modified Eagle's medium supplemented with either (a) 13% delipidized fetal calf serum containing avidin (2 μg/mL) (LDM) and oleate or elaidate or biotin or (b) 10% regular untreated fetal calf serum. All four media preparations (oleate, elaidate, biotin, regular) contained 1% penicillin (10 000 units/mL stock)-streptomycin (10 000 mg/mL stock) and 0.5% fungizone (250 mg/mL stock). Delipidated fetal calf serum was prepared with minor modifications as described previously for calf serum (Horwitz et al., 1974, 1976).

Test of Cell Membrane Lipid Composition. For lipid analyses, the growth media were aspirated, the cultures were washed twice with versene, and trypsin was added to remove the cells from the culture dish. The cells were centrifuged, and the pellet was washed with PBS and resuspended into versene. The lipids were extracted by the method of Bligh and Dyer (1959). The principal phospholipid classes, PE and PC, were isolated by thin-layer chromatography and transesterified for analysis by gas chromatography. Other details are presented elsewhere (Horwitz et al., 1978).

The plasma membrane fraction of our primary chick cultures was prepared by the method of Schimmel et al. (1973). Band I (in the nomenclature of Schimmel et al.) was used for these lipid analyses which were performed as described above, with the exception that the lipids were not fractionated.

Labeling of Cells. The cells were labeled with a phospholipid-like fluorophore, dioctadecylindocarbocyanine [diI-C_{18}-(3), or diI, a gift of Dr. Alan Waggoner] by incubating the tissue culture for 3 min at 37 °C in 1 mL of Hank's balanced salt solution (HBSS) to which 5 μL of 0.3 mg/mL diI in ethanol had been added. The cells were then rinsed several times in HBSS; the optical experiments were carried out with the cells in HBSS on 4-day-old cells.

The diI appeared to become incorporated into the plasma membrane (Schlessinger et al., 1977) in a spatially homogeneous distribution without significant internalization for about the first 20 min after labeling. At longer times, some labeling of internal hydrophobic regions became apparent if the cells were kept above room temperature. High-magnification fluorescence microscopy, with a depth of focus of ~1 μm, cannot resolve diI in the plasma membrane from diI in nearby cytoplasmic structures. However, the fluorescence of more deeply internalized diI can be distinguished from surface diI by its different position of focus in the microscope, its distinct exclusion from the nuclei, its delineation of internal organelles, and its lack of cell edge tangential brightening.

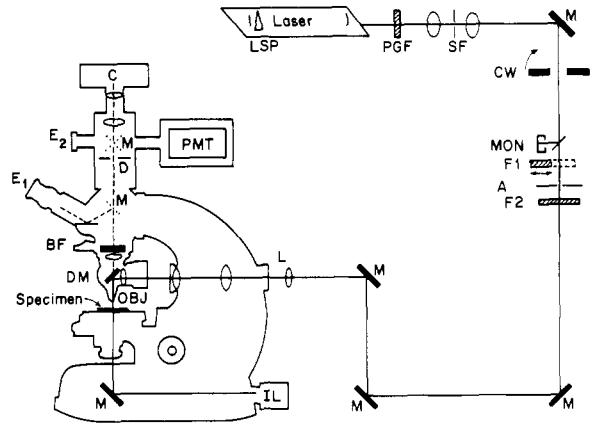

FIGURE 1: Optical apparatus. Abbreviations: LSP, laser prism; PGF, plasma glow filter; SF, spatial filter; M, mirror; CW, chopper wheel; MON, monitoring photodiode; F1, solenoid-mounted 3.9 OD filter; A, aperture; F2, neutral density filter; L, lens which ensures that laser beam is focused at the plane of the sample; DM, dichroic mirror; OBJ, microscope objective; BF, barrier filter; D, field diaphragm; E1 and E2, eyepieces; PMT, photomultiplier tube; C, camera; IL, tungsten illuminator.

Fluorescent labeling of cell-surface AChR by TMR-αBgt (Ravdin and Axelrod, 1977) was performed as previously published (Axelrod et al., 1976b). The binding of TMR-αBgt could be blocked completely by pretreatment of the cells with 5×10^{-8} M unlabeled αBgt for 1 h. Based on our visual observation, the surface lifetime of TMR-αBgt-AChR complexes exceeds several hours, and all AChR mobility experiments were completed well within this time limit. Optical measurements were performed on 4-day-old cells.

Data Analysis. Observation of the recovery curve shapes (Axelrod et al., 1976a) indicated that lateral molecular motion was predominantely diffusion-like for both diI and TMR-αBgt-AChR. The diffusion constants D were obtained by measuring the time required for the fluorescence to recover halfway from its immediate post-bleach value to its long-time asymptotic value (Axelrod et al., 1976a). These diffusion constants D characterize the motion of the mobile fluorescence molecules only and do not contain a contribution from an "immobile" fluorophore. If "immobile" fluorophore is present, the fluorescence at times long after bleaching will be less than the prebleach fluorescence; the mobile fraction f of the total fluorophore can be calculated according to the method of Axelrod et al. (1976a). The results for D and fractional mobility f of TMR-αBgt-AChR have a larger uncertainty than those for diI. One contribution to this uncertainty was occasional difficulty in determining the long-time asymptotic value of TMR-αBgt-AChR fluorescence due to slow cell motility.

Optics and Electronics. The optical apparatus for the FPR experiments shown in Figure 1 is a somewhat improved version of the FPR apparatus previously described (Koppel et al., 1976). A krypton ion laser beam (λ 520.8 nm for diI-lipid experiments; λ 568.2 nm for TMR-αBgt-AChR experiments) is spatially filtered and focused onto an adjustable-speed rotating wheel which chops the light into 10 or 15% duty cycle pulses. The duration of light pulses is 3 ms (15% duty cycle) and 400 ms (10% duty cycle) for the diI and TMR-αBgt experiments, respectively. A neutral density filter with an optical density of 3.9 mounted on a solenoid-operated lever attenuates the beam to a power of ~2 μW. The beam is focused through

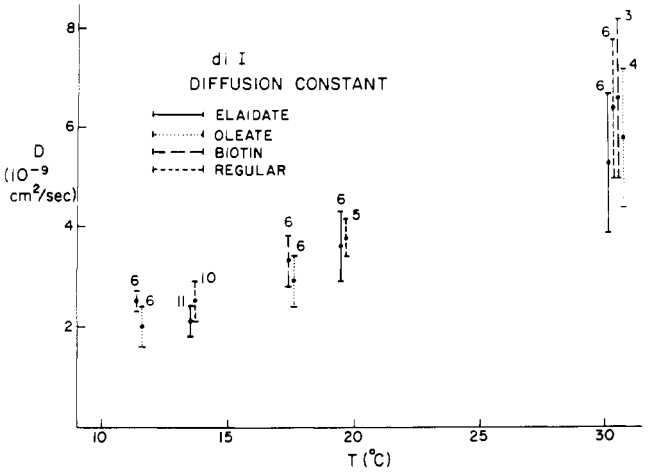

FIGURE 2: Diffusion constant D of dil vs. temperature on cells grown in the four media preparations indicated. The vertical bars represent uncertainty due to the standard deviation of an average of several measurements. The actual number of measurements being averaged is shown next to each bar. The diffusion constant D is for the mobile dil only, which constitute about 85–100% of the total dil.

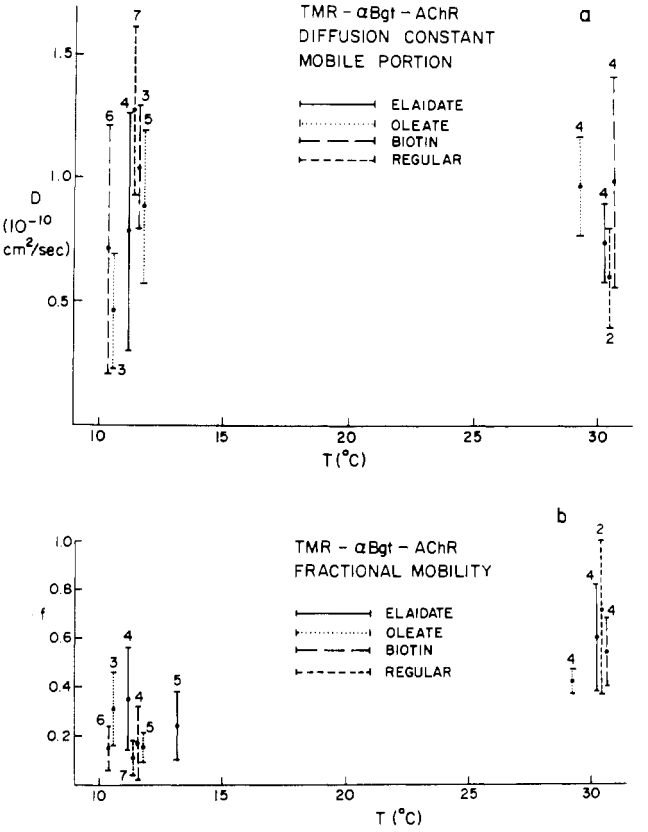

FIGURE 3: (a) Diffusion constant D of TMR-αBgt-AChR vs. temperature on cells grown in the four media preparations indicated. The vertical bars represent uncertainty due to the standard deviation of an average of several measurements. The actual number of measurements being averaged is shown next to each bar. (b) The fraction f of the total TMR-αBgt-AChR which is mobile vs. temperature.

the microscope objective to a small spot of Gaussian intensity profile with a 0.9 μm e^{-2} radius. Commercial colored glass barrier filters and custom-made dichroic mirrors (Valpey Co.) with appropriate spectral properties are employed to observe dil or TMR fluorescence while blocking scattered excitation light. Fluorescence is detected with an EMI 9658R photomultiplier tube thermoelectrically cooled to 0 °C.

In the FPR technique, the solenoid-mounted neutral density filter is briefly removed from the beam by activating the solenoid, thereby allowing exactly one bright (20 mW) unattenuated light pulse to photobleach the fluorescence in a small region on the sample. At the end of this bleaching pulse, solenoid activation is terminated, thereby returning the neutral density filter to the excitation beam path in order to permit observation of the subsequent fluorescence recovery with attenuated light pulses. Solenoid activation is controlled by a logic circuit triggered by the falling edges of the light pulses, as detected by a monitoring photodiode (shown in Figure 1) together with an enabling pulse from a manual "start" button. Simultaneous with the solenoid-activation pulse, a control pulse temporarily shuts off the operational high-voltage supply (Kepco Model OPS 2000) to the photomultiplier, thereby automatically protecting the photomultiplier from anode current overloading during the bright flash of fluorescence excited by the bleaching pulse.

Photon counting electronics (PAR) measures the number of fluorescence photons detected by the photomultiplier during each observation light pulse. These data are converted to an analog voltage and recorded either directly onto a strip chart recorder (TMR-αBgt experiments) or in a fast-scan recorder (PAR Model 4101, used in dil experiments) for subsequent slow output onto a strip chart recorder.

It is necessary to ascertain whether the experimental procedures for measuring diffusion could introduce excessive perturbations. According to rigorous calculations (Axelrod, 1977), it is unlikely that any local heating of the membrane during photobleaching is great enough to affect the results here. Furthermore, extensive control experiments on other labeled cells have established the invariance of the diffusion constants to repeated bleaching and the viability of cells exposed to a focused laser light of intensity ten times greater than that normally used in FPR experiments.

Results

The lateral diffusion constants D for dil in the lipid regions of the plasma membranes of various fatty acyl altered primary chick myotubes are shown as a function of temperature in Figure 2. We draw two main conclusions from these data: (1) the dil lateral-diffusion constant D increases by a factor of two to three as the temperature is raised from 12 to 31 °C; and (2) dil diffusion constants in normal myotubes and those enriched dramatically (see below) in either oleate or elaidate are indistinguishable, to within an overall uncertainty factor of 1.5 to 2. The mobile fraction f of dil is 85–100%, independent of temperature in the 12 to 31 °C range.

The diffusion constants D and fractional mobility f of TMR-αBgt-AChR on these cells are shown in Figures 3a and 3b, respectively. Conclusions to be drawn from the data are: (1) the mobile fraction f increases by a factor of about two to four times from 12 to 31 °C; (2) the change with temperature of the lateral diffusion constant D is less than the ±50% uncertainty for each set of cell lipid types; and (3) the TMR-αBgt-AChR lateral mobilities of the different fatty acid treated cells are indistinguishable to within an overall uncertainty factor of about three to four. The uncertainties for each set of AChR results (ca. ±50%) are larger than those for dil (ca. ±25%) because of (a) the higher shot noise due to the lower density of TMR-αBgt-AChR labeling; (b) the real spatial variability of TMR-αBgt-AChR average mobility on the myotube surface; (c) occasional difficulty in determining the long-time TMR-αBgt-AChR fluorescence asymptote due to slow cell motility; and (d) the low fractional mobility of AChR, particularly at low temperatures.

In order to verify that our media nutrient enrichments do indeed greatly alter the fatty acyl composition of cell membranes in primary chick cultures, we have performed fatty acyl analyses on the phospholipids from both the plasma membrane fraction and from whole cells. In the plasma membrane fraction, 27 ± 3% of the fatty acyl chains in nonenriched "regular" cultures are oleate. However, in oleate-enriched cells the oleate fraction increases to 74 ± 3%, and in elaidate-enriched cells oleate decreases to 5.8 ± 2.8% with a concurrent appearance of elaidate chains from none to 84 ± 2%.[1] Total cell phospholipid alterations parallel those in the plasma-membrane fraction. For example, in unaltered cells, oleate occupies 31.1 ± 7.1% of the possible fatty acyl positions. In oleate-enriched cells this value increases to 67.2 ± 0.3%, whereas in elaidate-enriched cells oleate decreases to 13.5 ± 2.3% with a concurrent appearance of elaidate from none to 72.1 ± 9%. Analyses of the fatty acyl composition of cultures randomly selected from those used for the mobility experiments reported here are in accord with these values.

We did not observe "clusters" or patches (hot spots) (Sytkowski et al., 1973) of high AChR concentration on our 4-day-old cultures plated and grown according to the protocol described above.

Comparison of the diffusion constants D for the mobile portions only of dil and TMR-αBgt-AChR shows that AChR mobility is about two orders of magnitude lower than lipid probe mobility.

Discussion

We have shown that major nutrient-induced alterations of the myotube plasma-membrane phospholipid hydrocarbon chains do not affect the lateral motion of the lipid probe dil by more than a factor of 1.5 to 2 nor the AChR protein molecules by more than a factor of 3 to 4. This result contrasts with observations of other membrane properties on these and other cells in tissue culture upon lipid alteration (Horwitz, 1977). For example, in chick cultures prepared in these experiments, lipid alterations dramatically influence fusion of myoblasts into myotubes and the subsequent morphology of the myotubes (Horwitz et al., 1978). Fatty acyl alterations in mouse fibroblasts affect their lectin-mediated agglutinability (Horwitz et al., 1974). Substantial changes of the "critical" minimum temperature for rapid agglutinability accompany oleate or elaidate enrichment. These agglutination studies also reveal the complexity of membrane lipid–protein interactions, since fatty acyl induced changes in concanavalin A mediated agglutination differ from those for wheat germ agglutin-mediated agglutination. The temperature dependence of the partitioning of the spin-label Tempo exhibits several inflections at characteristic temperatures that vary with fatty acyl changes (Hatten et al., 1978). In addition, alterations of fatty acyl composition in model bilayers comprised of simple lipids can dramatically affect some aspects of the lipid fluidity, e.g., phase-transition temperature (Shimshick and McConnell, 1973).

Alteration of Plasma-Membrane Lipids. The indifference of our measured lateral-diffusion constants to lipid alteration would be fully explained if the composition of the plasma membrane was not altered by the enrichment procedures. However, despite the ambiguities inherent in preparing pure plasma-membrane fractions, our biochemical analyses of primary chick cells indicate that lipid enrichments do involve the composition of the plasma membrane. This conclusion is consistent with previous reports that large fatty acyl enrichments in the plasma membranes of several different tissue culture cell lines[2] are comparable in magnitude to those seen in analyses of whole cells (Hatten et al., 1978; Engelhard et al., 1976; Horwitz et al., 1978). In addition, the previously mentioned observations of alterations in plasma-membrane-associated activities (e.g., myoblast fusion in primary chick) induced by the fatty acyl replacements suggest compositional changes involving the plasma membrane on chick and other cell types.

DiI as a Membrane Probe. The lateral diffusion of dil in lipid membranes is known to be sensitive to the phase state of the lipids in its immediate vicinity. In hydrated lipid multilayers and in bilayers forming the walls of large bilayer vesicles of pure phosphatidylcholine, a sharp change in lateral-diffusion constant occurs at the phase-transition temperature (Wu et al., 1977; Fahey and Webb, 1977; Fahey and Webb, unpublished). Since the phase-transition temperature varies with acyl-chain composition in the multilayer system, the diffusion constant D shows a strong fatty acyl chain dependence at certain temperatures. Therefore, the lack of effect of fatty acyl changes on dil diffusion in chick myotubes must reflect both a lack of change in the phase state of the membrane lipid regions in which dil is dissolved and a weak dependence of D on acyl-chain composition.

For the FPR experiments to assay lateral mobility in the plasma membrane, the fluorescence-labeled molecules must be confined there. One reason we chose dil as a lipid probe instead of phospholipids labeled with fluorescent rhodamine or NBD groups was an observation (Axelrod, unpublished) that various cultured cell types internalize dil less rapidly than they internalize labeled phospholipids. Internalized fluorescent lipid probes become bound to internal organelles where they decorate these structures in an easily recognized pattern, as discussed under Materials and Methods. Although noticeable internalization of dil does occur in times as short as 0.5 h at high (37 °C) temperature, the FPR experiments reported here were performed while dil still appeared smoothly distributed in or near the cell surface. The depth of focus of the microscope is much larger than the thickness of plasma membrane, so optical observation cannot unambiguously place the dil exclusively in the plasma lipid bilayer. However, it seems likely that any dil that is pinocytosed or associated with subsurface cytoskeletal structures would move laterally at rates far slower than those reported here. Indeed, the small fractions of immobile dil (up to 15%) that we detected may simply indicate the onset of some internalization. The intimate association of substantial amounts of dil with membrane proteins also seems unlikely, since this too would greatly slow dil diffusion. Our conclusion that the dil diffusion rate is mainly determined by its interaction with lipids is consistent with the observed approximate similarity between the dil diffusion coefficient in cells and in lipid model membranes.

[1] Plasma-membrane fraction phospholipid analyses results are as follows, with fatty acyl chain type followed by its molar concentration percent of all fatty acyl chains. (a) Normal cells, average of three trials: 14:0, 2.3 ± 0.8; 16:0, 34 ± 0.5; 16:1, 3.7 ± 1.6; 18:0, 30.8 ± 0.3; 18:1$_c$, 26.6 ± 2.9; 18.1$_t$, negligible; 18:2, 2.6 ± 0.8; 18:3, trace. (b) Oleate enriched at 10.4 μg/mL, average of three trials: 14:0, 1.2 ± 0.7; 16:0, 13.7 ± 2.3; 16:1, 1.4 ± 0.3; 18:0, 9.4 ± 0.4; 18:1$_c$, 74.4 ± 2.8; 18:1$_t$, negligible; 18:2 and 18:3, negligible. (c) Elaidate enriched at 10.4 μg/mL, average of two trials: 14:0, 0.3 ± 0.1; 16:0, 4.2 ± 0.1; 16:1, 1.7 ± 0.7; 18:0, 2.9 ± 1.4; 18:1$_c$, 5.8 ± 2.8; 18:1$_t$, 84.4 ± 2.4; 18:2, 0.6 ± 0.1.

[2] On one of these cell lines in which a plasma membrane analysis has been made, rat L6, we have performed dil lateral-mobility experiments similar to those for primary chick cells reported in detail here. For these rat L6 cells we again found no significant change of dil lateral motion upon various fatty acyl enrichments (Axelrod and Horwitz, unpublished results).

DiI and a related carbocyanine dye, diO-C_{18}-(3), have been used to label phospholipid bilayers and multilayers (Fahey and Webb, 1977; Wu et al., 1977). Both of these long-chain, water-insoluble carbocyanines uniformly label planar membranes without segregating both above and below phase transitions, unlike some other membrane probes. Their molecular structure, consisting of a polar head group coupled to two long hydrocarbon tails, is similar to phospholipid structure. Therefore, the favored molecular orientation of diI in lipid bilayers is parallel to that of phospholipids. Fluorescence polarization studies of carbocyanine dyes in bilayers and multilayers show this preferred orientation (Badley et al., 1973; P. Dragsten, private communication).

DiI Lateral Motion. In view of the confirmed alterations of plasma-membrane lipid composition, the properties of diI as a membrane lipid probe discussed above, and the essential constancy of the lateral diffusion rate of diI upon fatty acyl alteration reported here, we believe that the phase states of regions visited by diI are almost independent of lipid acyl chain composition over the temperature and composition region explored. The presence of cholesterol, which comprises about 35–40% of the plasma-membrane lipid, provides one explanation for this conclusion. Cholesterol in the membrane should suppress sharp acyl-chain transition in the temperature region investigated, leaving only a continuous structural change spread over a wide temperature range and insensitive to changes in fatty acyl chain composition. This behavior has been observed in sonicated vesicles and in certain phospholipid multilayers, where the rapid change of lateral diffusion coefficient with temperature in the vicinity of the phase transition is reduced dramatically upon addition of 50% cholesterol (Wu et al., 1977). In biological membranes, solute transport (Hale et al., 1977) and two physical properties closely related to lipid structure, microviscosity (Esko et al., 1977) and fatty acid chain ordering (Hatten et al., 1978), also show only small, although experimentally significant, effects upon certain lipid compositional changes. We note, however, that the presence of cholesterol does not block acyl chain alterations from affecting certain other membrane physical properties, as discussed above.

Other possible models also could account for our results. (a) The membrane might contain "solid" lipid domains from which diI is excluded. Thus, if lipid alterations induce changes in the amount of lipid contained in these "solid" domains, diI and the mobile AChR fraction might still maintain a relatively constant lateral mobility in the remaining "fluid" lipid regions. The "solid" domains, if present in our experiments, must be smaller than the resolution limit of light microscopy ($\sim$0.5 μm) in order to escape detection and spaced apart sufficiently so as not to impede the diffusive motion of diI or AChR molecules around them. However, such domains have not been found in lipid bilayer model membranes composed of simple lecithin–cholesterol mixtures (Fahey et al., 1977; Fahey and Webb, 1977). Furthermore, as mentioned above, diI does not segregate upon "freezing" phospholipid multilayers. In addition, with the extent of lipid enrichments seen here, it is highly likely that virtually every phospholipid carries at least one altered fatty acyl chain, so that no domains could remain completely unaltered. (b) Lateral diffusion in cell membranes might be regulated by changes in concentrations of lipid molecules other than altered phospholipids. However, direct analyses of cellular lipid on other cell lines indicate that major secondary alterations of other lipid classes in response to the deliberate fatty acyl change do not occur (Horwitz, 1977; Horwitz et al., 1978).

We observed a significant temperature dependence in the diffusion rate of diI despite the insensitivity of this quantity to lipid modification. The significant slowing of diI with a decrease in temperature from 31 to 12 °C certainly reflects a change in the membrane's physical properties or in the distribution of diI in the membrane. For example, the slowing may be due to a change in either the membrane lipid viscosity or relative changes in the quantities of diI associated with membrane components having a range of different mobilities.

AChR Lateral Motion. There appear to be two general classes of AChR whose relative population depends on temperature: mobile and immobile. The molecular relationship between these two classes is not clear. One possibility is that the "immobile" class is a submicroscopic self-aggregated state of AChR in temperature-dependent equilibrium with the mobile state. Another possibility is that there exist three distinct classes of AChR which are inserted into the membrane or attached to cytoplasmic or surface components differently. One class may be mobile at both high and low temperatures; a second class may be mobile only at high temperatures; and a distinct third class of AChR may be immobile at both high *and* low temperatures. It is even possible that the molecular structures of these putative distinct classes of AChR are not the same.

The constancy of the diffusion rate of mobile AChR upon lipid acyl changes may, of course, be explained by the constancy of lipid mobility as measured by diI in at least substantial regions of the membrane. If the remainder of the AChR is immobilized by "solid" lipid domains (which, if they exist, presumably change their size and composition with fatty acyl changes as discussed above) then it is curious that the immobile fraction f of AChR is also insensitive to fatty acyl changes. It seems more likely that mobility of at least some of the AChR is controlled by mechanisms other than the "fluidity" of the surrounding lipids (Axelrod et al., 1978).

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Steps Involved in the Processing of Common Precursor Forms of Adrenocorticotropin and Endorphin in Cultures of Mouse Pituitary Cells†

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ABSTRACT: The initial steps in the processing of the common precursor to adrenocorticotropin (ACTH) and endorphin in mouse pituitary tumor cells (AtT-20) have been investigated. Three forms of the precursor have been resolved by sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoresis with apparent molecular weights of 29 000 (29K ACTH-endorphin), 32 000 (32K ACTH-endorphin) and 34 000 (34K ACTH-endorphin). These forms have a similar peptide backbone, but their carbohydrate content differs. In particular, a tryptic glycopeptide has been observed in 32K ACTH-endorphin which is not present in 29K ACTH-endorphin and has been identified as the tryptic peptide containing the α(22–39) sequence of ACTH. Similar heterogeneity in carbohydrate has been observed in some of the smaller molecular weight forms of ACTH which are resolved by $NaDodSO_4$ gel electrophoresis. Pulse chase and continuous labeling studies using radioactive amino acids and sugars suggest that the 29K ACTH-endorphin is converted to 32K and 34K ACTH-endorphin by the addition of carbohydrate. The glycopeptide and pulse chase studies suggest that 29K ACTH-endorphin is at a branch point in the processing pathways. It can either be converted to 4.5K ACTH by proteolytic processing or to 32K ACTH-endorphin by the further addition of carbohydrate. The 32K ACTH-endorphin can then be converted to 13K ACTH, the glycosylated form of 4.5K ACTH (Eipper, B. A., & Mains, R. E. (1977) *J. Biol. Chem. 252,* 882), by proteolytic processing. A comparison of the distribution of the different molecular weight forms of ACTH and endorphin in mouse pituitary extracts and in the mouse pituitary tumor cells reveals that the pituitary contains all of the forms of ACTH and endorphin seen in the tumor cells, including the three forms of the ACTH-endorphin precursor. However, the molecular weight distribution of the forms in the anterior lobe is very different from that in the intermediate lobe of mouse pituitary.

The pituitary hormone, adrenocorticotropin (ACTH),[1] and the opiate peptide, β-endorphin [β-(61-91)LPH], have recently been shown to be synthesized as part of a much larger precursor molecule in a mouse pituitary tumor cell line (AtT-$20D_{16v}$ line) (Mains et al., 1977; Roberts & Herbert, 1977a,b) and in beef pituitary (Nakanishi, et al., 1977). When mRNA

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[1] Abbreviations used: ACTH, adrenocorticotropin; β-LPH, β-lipotropin; $NaDodSO_4$, sodium dodecyl sulfate; Dulbecco-Vogt MEM, Dulbecco-Vogt minimal essential medium; SAC, *Staphylococcus aureus* Cowan I; YADH, yeast alcohol dehydrogenase; BSA, bovine serum albumin; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; IAA, iodoacetamide; Tos-$PheCH_2Cl$, tosylphenylalanyl chloromethyl ketone; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; DNP, 2,4-dinitrophenyl; MSH, melanocyte stimulating hormone; Bis, *N,N'*-methylenebis(acrylamide); RIA-ACTH, ACTH activity by radioimmunoassay expressed as ng of purified porcine ACTH with equivalent activity. The forms of ACTH and endorphin are designated by their apparent molecular weights as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The precursor forms that contain both ACTH and endorphin are: 28.5 ACTH-endorphin, the product synthesized in the cell-free system and 29K, 32K, and 34K ACTH-endorphin, the glycosylated forms of the precursor synthesized in the tumor cells. Other forms of ACTH or endorphin are designated: 21K, 23K, and 26K ACTH for the intermediate forms of ACTH; 13K and 4.5K ACTH for the end products of processing of ACTH as seen in tissue culture medium; 12–15K ACTH for the pool of glycosylated ACTH end products seen in intact tumor cells; 11.5K endorphin for the β-LPH-like molecule and 3.5K endorphin for the β-endorphin-like molecule. These molecular weight designations are for identification purposes only, since it is well known that accurate molecular weights of glycoproteins cannot be otained by this method (Segrest & Jackson, 1972).

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