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Influence of alcohols, temperature, and region on the mobility of lipids in neuronal membrane

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Fluorescence recovery after photobleaching was used to examine lipid diffusibility in different regions of *Aplysia* neurons. Differences in diffusion of 1-acyl-2-(6-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)]aminohexanoyl)phosphatidylcholine (NBD-C_6-PC) in the cell body, axon hillock, and axon were not apparent. Lipid diffusibility during temperature variations and exposure to alcohols was also examined by photobleaching techniques. For these studies, all measurements were made on the cell body. Alcohols were found to be selective in their effects upon the diffusibility of lipid probes. Neither ethanol nor butanol affected the diffusibility of NBD-PC. However, at the same concentrations, both of these alcohols caused a significant increase in the diffusion coefficient (D) for rhodamine-phosphatidylethanolamine (Rho-PE). The diffusion coefficient for NBD-PC in the cell body plasma membrane did not increase with warming, between 4 °C and 25 °C. The fraction of lipid probe free to diffuse (per cent recovery; $\%R$) however, increased as temperature increased, within this range. The nonconventional relationship between temperature and D was even more pronounced for Rho-PE. As temperature increased, D became smaller for this probe, concurrent with an increase in $\%R$. These results suggest that immobile viscous lipid is recruited into a mobile fraction as temperature increases, resulting in the maintenance of constant diffusibility. The effects of temperature on D and $\%R$, and the selective effects of alcohols on lipid diffusibility suggest that the membrane is heterogeneously organized, on a submicroscopic scale, into domains. The implications of this organization for nerve function and responses of nervous systems to temperature and anesthetics are discussed.

Introduction

Neurons have a characteristic shape specialized for their integrative functions. Concurrent with morphological regionalization, the cells of the nervous system exhibit regionalization on the molecular level. Important examples of this regionalization are the localization of ionic currents to specific morphological regions of neurons [1,2], localization of acetylcholine receptors into hot spots in the endplates of the neuromuscular junction [3], and clustering of Na^+ channels on myelinated neurons [4]. Na^+ channels in muscle are localized and immobilized, and this morphology is dependent upon innervation of the muscle [5]. Cellular regionalization (morphologic, functional, and molecular), is characteristic of differentiated cells and, indeed, develops with cellular differentiation [6–13]. Thus, an understanding of the nature of membrane regionalizations and

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how they are produced is essential to an understanding of cellular differentiation. Membranes are largely composed of lipids and proteins. Thus, membrane organization results from the interaction of these molecules with each other and potentially with elements of the cytoskeleton and extracellular matrix [14,15].

The electrical activity characteristic of the nervous system results from the openings of voltage- and ligand-gated channels within the membranes of individual nerve cells. These gated channels are thought to be proteins which span the membrane [16], and whose conformational changes allow the selective passage of ions. The functioning of a number of membrane-bound proteins are affected by both pharmacological manipulation thought to affect their lipid environment, and by alteration of the lipid composition of the plasma membrane [17,18]. Variation of temperature [19,20] or exposure to anesthetics [21,22] dramatically affects channel function. Both of these manipulations have been suggested to act via the lipid matrix of the membrane. In *Aplysia* neurons, the electrophysiology of different voltage-dependent channels differ in their sensitivity to alcohols. For example, inward currents carried by calcium are far more sensitive to ethanol than are the delayed rectifier currents carried by potassium ions [23]. The effects of alcohols on membrane receptors and channels are dependent on a number of factors, such as chain length [18] or subtleties of structure, as demonstrated by the selective anesthetic effects of the isomers of tetradecanol [24]. Arrhennius plots of the effects of temperature on nerve cell channel function contain break points indicative of lipid involvement, and these occur at different temperatures for different channel populations [20]. This complexity suggests that if lipids are, in fact, the target for anesthetics and temperature, then the effects of these agents on protein function cannot be adequately explained in terms of a bulk membrane viscosity. Rather, they suggest that the membrane lipids are organized, on a submicroscopic scale, into domains of localized composition and viscosity.

In the present study, we examine the effects of alcohol and temperature upon lipid lateral diffusibility in the plasma membrane of *Aplysia* neurons. We use the technique of fluorescence photobleaching recovery (FPR) to measure the lateral diffusibility of fluorescent lipid analogues in neurons which have previously been examined by electrophysiological techniques. Specifically, our experiments address four questions about the relationship between membrane structure (on both the micro- and macroscopic level) and membrane function in differentiated nerve cells:

1. Is plasma membrane fluidity different in the morphologically distinct regions of the neuron?
2. Do different alcohols have different effects on the diffusibility of a given probe in the neuronal cell body?
3. Do alcohols selectively affect the diffusibility of different lipid probes in the neuronal cell body?
4. What effect does temperature have on membrane fluidity in the cell body?

In the most general sense, the purpose of these studies is to determine the extent to which the plasma membrane can be treated as having a bulk membrane fluidity. If the membrane can be treated as having such a property, then one would expect: (1) the same fluidity over all regions of the neuron; (2) all alcohols to have the same qualitative effect on lipid diffusibility; (3) all probes to show similar diffusibility characteristics, since they would probe the same fluid environment; and (4) both temperature and alcohols to affect all probes equally. As we shall see, such a simplification is not justified. In addition to probing the nature of the membrane lipid environment, we attempt to interpret some of the electrophysiological effects of alcohols and temperature on *Aplysia* cells in light of the FPR findings. These interpretations are directed toward an understanding of the nature of a putative lipid target for the alcohol molecule. We cannot ignore the possibility that the alcohols interact directly with channel proteins, since direct interaction between anesthetic molecules and membrane proteins have recently been documented [25,26].

Materials and Methods

Preparation of neurons

Aplysia californica (obtained from mariculture facilities at the Marine Biological Laboratory at Woods Hole, MA) were maintained in artificial sea water (ASW, Instant Ocean, Aquarium Sys-

tems, Inc., Eastlake, Ohio) at 15°C. The central nervous system was removed from the animal, and ganglia were incubated in 0.1% Protease (Type III, Sigma) for 45–60 min at 37°C. The softened connective tissue sheath was then removed by microdissection. Cells were dissociated from the ganglia by mild trituration in a constricted pipette. Electron microscopical examination of dissociated cells indicated that the glial coat of the neurons is removed by this treatment (unpublished data). We will refer to cells prepared in this fashion as 'dissociated' cells, to differentiate them from cells which have been maintained in culture after dissociation, becoming attached to culture dishes and exhibiting processes. This latter class of cells will be referred to as 'cultured' cells.

Preparation of Aplysia neuron cultures

Aplysia ganglia were removed from the animal and placed into Protease (Type IX, Sigma), 18 mg/2 ml), at 17°C for 18 h. After rinsing, the ganglion sheath was removed, and cells dissociated by mechanical disruption of the bath with pipette outflow. The culture medium contained filtered artificial sea water, glucose (400 mg/200 ml), L-glutamine (30 mg/200 ml), and pennicillin-streptomycin with Fungizone. The cells used were plated on Primaria culture dishes (Falcon).

Fluorescent probes

Two fluorescently-tagged lipids were used in these experiments. A phosphatidylcholine with a fluorescent nitrobenz-2-oxa-1,3-diazol-4-yl moiety conjugated via an amino linkage to a six-carbon acyl chain (NBD-C_6-PC) and phosphatidylethanolamine with a lissamine rhodamine B sulfonyl moiety conjugated to the amino terminus of the head (Rho-PE, Avanti Polar Lipids, Birmingham, AL). See Ref. 27 for the structure of these probes.

Labeling procedures

Controls (NBD-C_6)-PC. NBD-C_6-PC probe was added to the dissociated neurons in artificial sea water to give a final probe concentration of 6.67 mM, and neurons were maintained in this solution for 10 min at 22°C. The labeling solution contained 0.67% ethanol. The cells were washed three times with artificial sea water. Electrophysiological experiments show that the effects of ethanol are reversed by a similar washout protocol [23], strongly suggesting that ethanol is removed from the membrane by this procedure. Dissociated neurons were taken up by capillary action into 100 μm pathlength microslides (Vitro Dynamics) for FPR measurements. Cultured neurons were treated in the same manner, but instead of being placed into microslides, were viewed directly on the culture dishes.

Controls (Rho-PE). Neurons were incubated in 6.67 mM (dissociated) or 33.4 mM (cultured) Rho-PE in artificial sea water for 15 min at room temperature. The labeling solution contained 0.67% (dissociated) or 3.4% (cultured) ethanol. The cells were washed three times in artificial sea water and taken up into 100 μm pathlength microslides (dissociated) or were viewed directly on the dishes (cultured) for FPR measurements.

Ethanol-treated. After washing, labeled cells were rinsed with 5% ethanol in artificial sea water and incubated in 5% ethanol/artificial sea water for 15 min at room temperature. Cells (in 5% ethanol/artificial sea water) were then taken up into 100 μm pathlength microslides for FPR measurements.

Butanol-treated. Treatment was the same as described for ethanol, except that 0.5% butanol was used instead of 5% ethanol.

Temperature measurements

Capillary tubes containing labeled dissociated neurons were placed on a microscope slide and attached to a TS-2 Thermal Microscope Stage (Sensortech, Clifton, NJ) and subjected to a 4°C to 25°C temperature excursion in increments of 3°C. At each temperature the slide was equilibrated for at least five minutes before measurements were made. When using cultured cells, the entire culture dish was placed onto the stage and measurements were then made in the same way as described for the dissociated neurons.

Fluorescence photobleaching recovery

The technique of fluorescence photobleaching recovery has been described in detail elsewhere [28,29]. Fluorescence photobleaching recovery provides us with two measures of diffusion: first, the fraction of the component that is free to

diffuse (per cent recovery, $\%R$) and second, the diffusion coefficient (D) of that fraction. Our instrumentation is similar to that of published designs. It consists of a Lexel 95-2 Argon Laser (Lexel Corp., Palo Alto, CA), a beam splitter attenuator similar to that described by Koppel [29], a Leitz Dialux fluorescence microscope with I2, D2, and N2.1 epillumination filter systems, and Leitz MPV photometry system (Kramer Scientific Corp., Yonkers, NY), modified to accept an EMI 9568 photomultiplier tube in a Products for Research, Inc. (Danvers, MA) solid CO_2 cooling housing with amplifier discriminator and electronic shutter from EMI. The image plane diaphragm of the Leitz MPV was always set to insure that light was collected only from the plasma membrane. This procedure is discussed in detail by Wolf and Edidin [30]. Photons are counted on a custom built scaler, which also interfaces the instrument to a Technico SS16 computer (Columbia, MD), which stores and analyzes the data on dual 8 inch floppy disks. Data are fitted by nonlinear least-squares programs after Bevington [31] according to algorithms described by Barisas and Leuther [32] and Wolf and Edidin [30]. Measurements were made using a Leitz 40×0.65 numerical aperature phase contrast objective (dissociated neurons) or a Zeiss Plan neofluor 25×0.8 numerical aperture water immersion objective (cultured neurons). The beam exp(−2) radius was $1.5 + 0.2$ μm for the $40\times$ objective and 2.4 ± 0.3 μm for the $25\times$ objective. Bleaching times were approx. 10 ms at approx. 2 mW at 514.5 nm for the rhodamine label and 488.0 nm for the NBD label. Monitoring intensities were approx. 0.2 μW. We used a counting interval of 30 ms.

Results

Labeling

The effectiveness of the enzyme treatment and subsequent 'rinsing' of the cell surface is illustrated in Fig. 1. For this illustrative example, a cluster of cells was removed from the buccal ganglion after enzyme treatment, but only one of the two prominent cells was rinsed with medium from a pipette tip. Thus, the washed cell (marked 'w') shows a clean cell surface, while the unwashed cell (marked 'u') shows the glial cells and debris still present. The fluorescent staining of both NBD-C_6-PC and Rho-PE produced a uniform ring stain in the neurons (as seen for NBD-C_6-PC in Fig. 2A) indicating that the probe had inserted into the membrane and was not internal. Cells were critically monitored throughout the experiment and discarded as soon as any internal fluorescence appeared.

Similar staining was observed in cultured neurons. The intensity of staining in the dissociated and cultured neurons was similar for NBD-C_6-PC, but was reduced considerably in the cultured cells for Rho-PE, even when 5-fold higher concentrations of probe and ethanol were used.

Is plasma membrane fluidity different over the morphologically distinct regions of the neurons?

In order to determine whether there were regional differences in lipid diffusion in neurons, we measured the diffusion of the fluorescent lipid NBD-C_6-PC in the plasma membrane of cell body, axon hillock, and axon of dissociated *Aplysia* neurons. Diffusion measurements were made at positions shown schematically in Fig. 2B. First, diffusion was measured at the center of the cell body. A second measurement was made on the cell body approximately midway to the axon hillock. Two measurements were made on the hillock and then a series of measurements were made along the axon. Diffusion coefficient and % recoveries normalized to the first cell body measurement are shown in Table I. We observed no significant differences between any of the three regions using Student's t-test. For these experiments mean diffusion coefficients for the cell body were $(3.7 \pm 0.5) \cdot 10^{-9} \mathrm{cm}^2/\mathrm{s}$ and mean $\%R$ values were 74.4 ± 4.2. These values are consistent with lipid diffusion values reported for other poikilothermic organisms [33–36]. The overall fluorescence measured in the membrane, indicated by counts per second (cps), did differ in different regions of the neuron, suggesting that all regions were not identical with respect to either incorporation of probe, and/or environment surrounding the probe.

Do different alcohols have different effects on the diffusibility of different lipid probes in the neuronal cell body?

The effects of ethanol and butanol on the diffu-

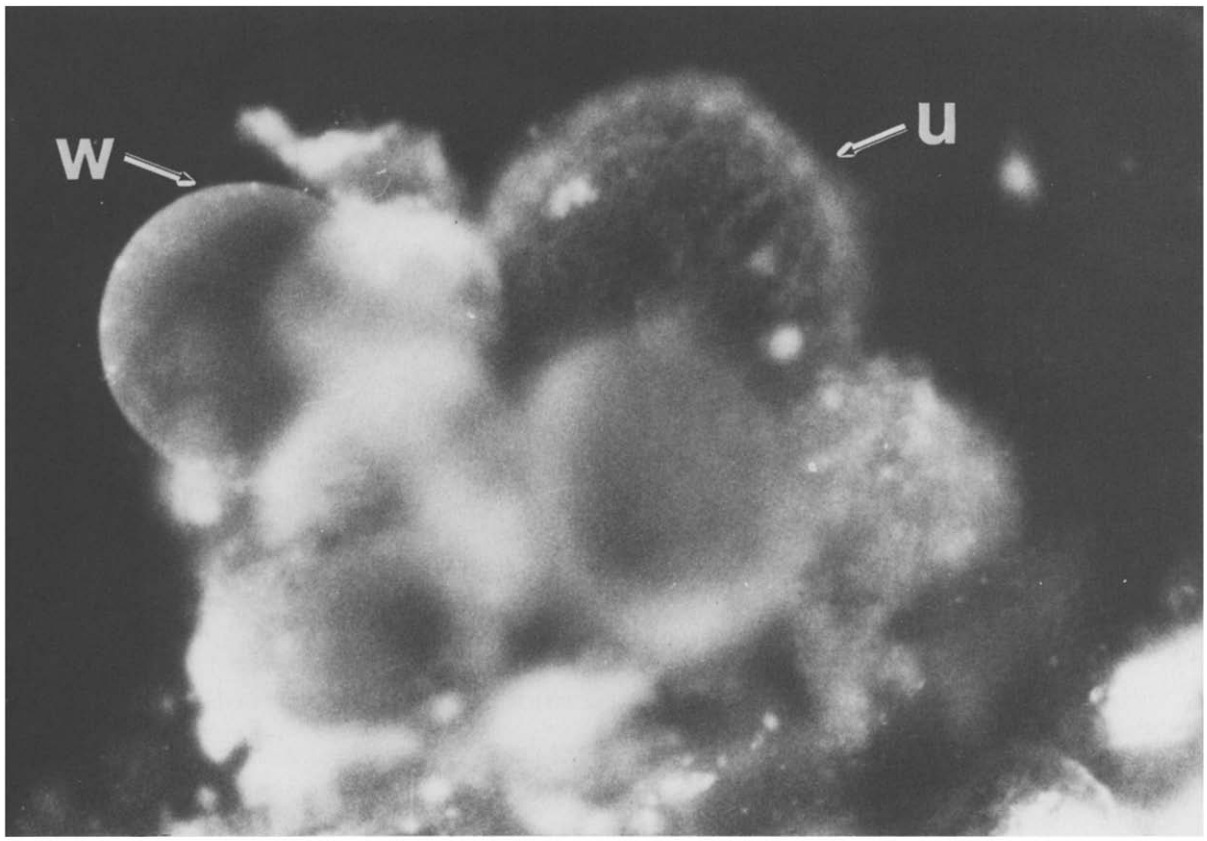

Fig. 1. Efficacy of treatment to obtain clean cell surfaces. A cluster of cells was removed from the buccal ganglion of *Aplysia* after enzyme treatment as described in Methods. Two prominent cells are shown. The cell marked 'w' was washed with a stream of sea water from a pipette tip, while the cell marked 'u' was left unwashed. The unwashed cell shows the glia and debris characteristic of unwashed cells. The cells have been exposed to NBD-C_6-PC probe.

sion of NBD-C_6-PC within the membrane of dissociated *Aplysia* neurons is shown in Table II. The concentrations chosen were used because ethanol and butanol produced roughly equivalent electrophysiological effects on bursting pacemaker activity in *Aplysia* neurons at these concentrations [37]. Analysis by Student's *t*-test between the controls and results obtained with each of the alcohols does not reveal significant differences between any of the groups. Thus, neither ethanol nor butanol affects the diffusibility of NBD-C_6-PC.

Do alcohols selectively affect the diffusibility of different lipid probes in the neuronal cell body?

It has previously been suggested [22,24] that alcohols may selectively partition into specific domains within the plasma membrane, and therefore affect not the bulk membrane fluidity, but fluidity of these local environments. Similarly, it has previously been suggested [33–35,38] that lipid analogues selectively partition into specific domains within the membrane and therefore report not on the bulk membrane fluidity but on the fluidity of local environments. To address this issue we considered the effects of alcohol on a diffierent lipid analogue, Rho-PE. In contrast to the results obtained with NBD-C_6-PC, we found the rate of diffusion of Rho-PE to increase significantly upon incubation with 5% ethanol ($P < 0.005$); the mobile fraction does not appear to be altered in the presence of ethanol. 0.5% butanol also affects the rate of diffusion of Rho-PE in a labeled neuron.

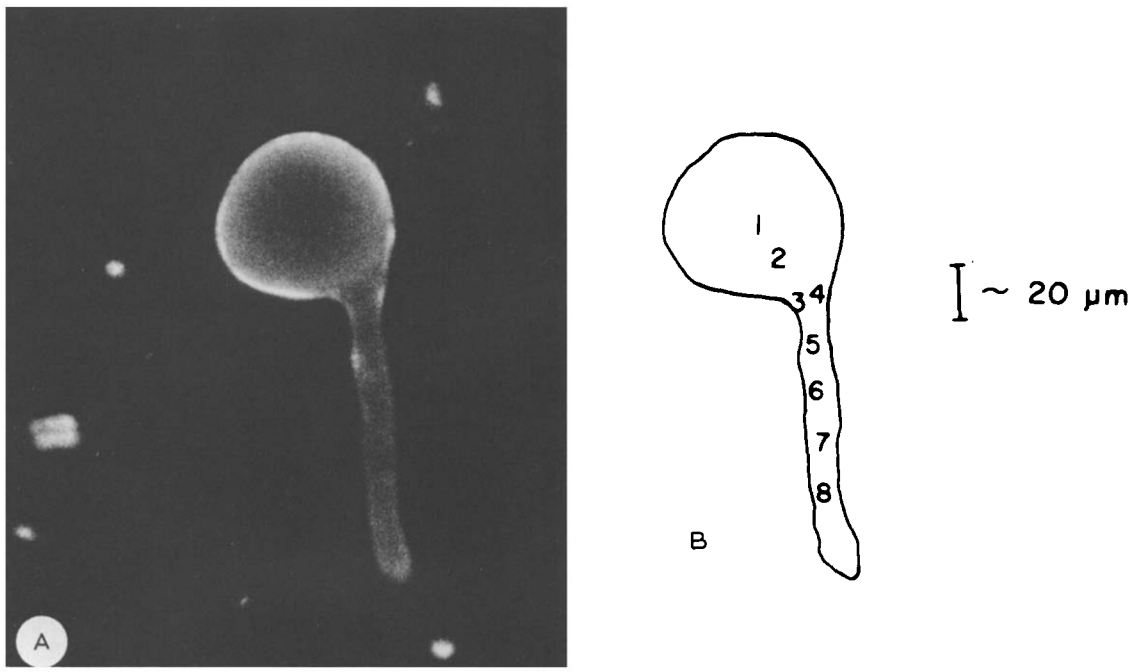

Fig. 2. (A) NBD-C_6-PC-labeled neuron. This neuron with attached axon was labeled in NBD-C_6-PC as described in Methods. A homogeneous ring stain is evident. (B) Diagram illustrating the sites on neuron shown in 2A where measurements were made in a typical experiment to determine regional diffusion. The numbers indicate the order of the measurements.

However, the variability of our results with butanol yielded data of much less reliability ($P < 0.025$) than those obtained with ethanol. Butanol also caused a decrease in $\%R$ which was statistically significant ($P < 0.005$). These results are also shown in Table II. Thus, in contrast to their lack of effect on NBD-C_6-PC diffusion, both ethanol and butanol increase the diffusion rate of Rho-PE.

TABLE I

DIFFUSION OF NBD-C_6-PC IN THE CELL BODY, HILLOCK, AND AXON REGIONS

This table represents data taken from the three regions of the cell, each measurement normalized to the first of two measurements taken on the cell body. The mean values obtained for these reference measurements in the cell body are $D = (3.7 \pm 0.5) \cdot 10^{-9} cm^2/s$ and $\%R = 74.4 \pm 4.2$. All data are shown with ± 1 S.E. The value in parentheses is the number of measurements taken in that data set. All measurements were made at room temperature (21–23° C).

	Cell body	Hillock	Axon
Diffusion	1.04 ± 0.08 (23)	1.20 ± 0.18 (20)	1.29 ± 0.11 (42)
% Recovery	1.03 ± 0.08 (23)	0.91 ± 0.06 (20)	1.08 ± 0.07 (42)
cps	1.03 ± 0.10 (23)	1.84 ± 0.93 (20)	1.57 ± 0.18 (42)

What effect does temperature have on membrane fluidity?

Neurons were labeled with NBD-C_6-PC and placed in capillary tubes as described above. They were then transferred to the microscope stage and equilibrated at least 5 min at 4° C. Several measurements were made near the center of the cell body at each temperature, as the cell was warmed and then cooled in 3Cdeg increments (the capillary equilibrated at least five minutes at each temperature before measurements were made). Fig. 3 shows D and $\%R$ in relation to temperature (average of 15 cells), as cells were warmed from 4° C to 25° C. The temperature range examined brackets that at which the animals normally live (14–18° C; unpublished results). D was not dependent on temperature. However, $\%R$ showed a statistically significant increase with elevated temperature. Fig. 4 shows the dependence of $\%R$ upon temperature

TABLE II

EFFECT OF ETHANOL AND BUTANOL ON DIFFUSION

All data are given with ±1S.E. values. The values in parentheses below each datum is the number of FPR measurements incorporated in data set. All measurements were made at room temperature (21–23°C).

	NBD-C_6-PC (all measurements on cell body)		
	Controls	5% ethanol	0.5% butanol
Diffusion ($\times 10^9$ s/cm^2)	1.80±0.17 (27)	1.73±0.16 (33)	1.69±0.19 (18)
Recovery	0.58±0.02 (27)	0.60±0.03 (33)	0.53±0.05 (18)
	Rho-PE (all measurements on cell body)		
	Controls	5% ethanol	5% butanol
Diffusion ($\times 10^9$ s/cm^2)	1.73±0.18 (33)	3.37±0.64 (22)	2.96±0.73 (21)
Recovery	0.75±0.02 (33)	0.71±0.04 (22)	0.62±0.04 (21)

for a single representative cell during a heating and cooling cycle, showing the reversibility of the temperature effect.

Toward the end of these experiments we were successful in routinely culturing *Aplysia* neurons, and we used these cultured cells to compare the effects of temperature on diffusibility of NBD-C_6-PC and Rho-PE in the plasma membrane. These data are summarized in Table III. The diffusion rates and per cent recoveries for NBD-C_6-PC in cultured cells are similar to those observed in dissociated neurons. As in the dissociated cells, temperature has no significant effect on D, but $\%R$ increases with temperature. In contrast, Rho-PE diffusion rate is faster in cultured neurons than in dissociated neurons. As mentioned above,

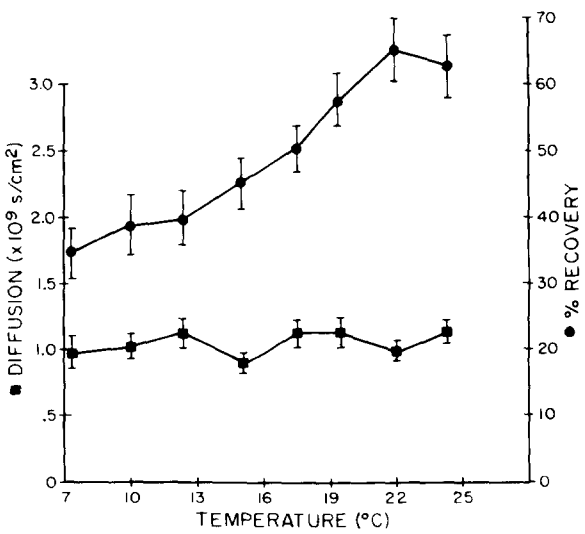

Fig. 3. Effects of temperature on diffusion of NBD-C_6-PC in *Aplysia* neurons. The graph indicates that diffusion is independent of temperature. In contrast, the mobile fraction shows a temperature-dependence.

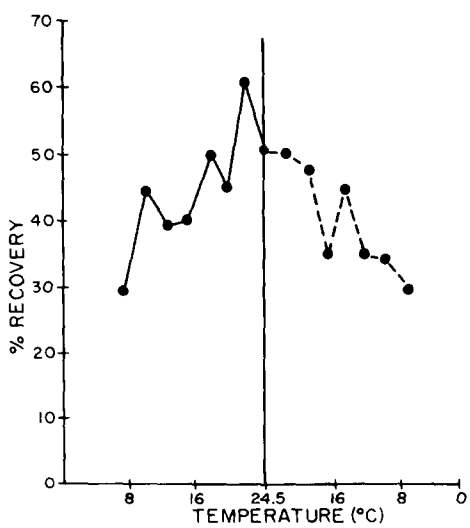

Fig. 4. Temperature-dependence of % recovery in an individual dissociated cell. The increase in mobile fraction produced by heating is reversed by a decrease in temperature.

TABLE III

EFFECT OF TEMPERATURE ON DIFFUSION IN CULTURED *Aplysia* NEURONS

All data are given with ±1 S.E. The values in parentheses below each datum is the number of FPR measurements incorporated in data set.

	NBD-C_6-PC (all measurements on cell body)			
	4°C	14°C	21°C	25°C
Diffusion ($\times 10^9$ s/cm^2)	3.06 ± 0.48 (21)	2.32 ± 0.20 (17)	2.49 ± 0.22 (23)	3.51 ± 0.44 (21)
Recovery	0.34 ± 0.03 (22)	0.42 ± 0.03 (17)	0.50 ± 0.02 (23)	0.56 ± 0.02 (21)
	Rho-PE (all measurements on cell body)			
	4°	14°C	21°C	25°C
Diffusion ($\times 10^9$ s/cm^2)	18.1 ± 2.6 (27)	–	–	9.4 ± 1.0 (41)
Recovery	0.31 ± 0.02 (30)	–	–	0.65 ± 0.02 (41)

the cultured cells were considerably more difficult to label with Rho-PE than were intact neurons. Increasing temperature did not cause an increase in D for Rho-PE. In fact, over the same temperature range as studied for NBD-C_6-PC diffusion, D decreased 2-fold for Rho-PE. The decrease in D was accompanied by an increase in $\%R$ for Rho-PE, analogous to the results seen with NBD-C_6-PC.

Discussion

Membrane lipids and proteins appear to be organized on a submicroscopic scale into domains of nonaverage composition and viscosity [7,22,24, 33–35,38]. In this paper we have posed four questions to explore plasma membrane complexity in *Aplysia* neurons, and to determine the extent to which this membrane can be treated as having a bulk membrane fluidity: (1) Is plasma membrane fluidity different in the morphologically distinct regions of the neuron? (2) Do different alcohols have different effects on the diffusibility of a given probe in the cell body? (3) Do alcohols selectively affect the diffusibility of different lipid probes in the neuronal cell body? (4) What effect does temperature have on membrane fluidity in the cell body?

Before considering our diffusion results, we must consider the issue of probe intercalation into the bilayer. NBD-C_6-PC and Rho-PE exist in several forms [39]: (1) intercalated into the bilayer; (2) as free monomer in solution; (3) as micelles or bilayers either in solution or possibly bound to the surface. Because of the low critical micelle concentrations, extremely little probe is present as free monomer. In any event, both monomers and micelles are essentially non-fluorescent forms [39,40]. For solution monomers this is because of the low quantum efficiency in the aqueous phase, while for the micelles, it is because of concentration quenching. Thus, fluorescence photobleaching recovery is measuring only the fluorescent forms within the bilayer.

The diffusion coefficients observed in *Aplysia* neurons for both NBD-C_6-PC and Rho-PE of approx. $2 \cdot 10^{-9}$ cm^2/s are similar to those reported in other cell systems [33–35,44]. It is worth noting that these values indicate that it would take 40 min for a lipid molecule to diffuse from cell body to axon tip for a 0.1 mm long neuron and 7 h for a 1 mm long neuron. Large immobile lipid fractions are not generally observed on homeothermic cells, the notable exception being spermatozoa [41,42]. They do however appear to be a trademark of poikilothermic cells [33–36]. The cause of immobile lipid remains uncertain. It could either reflect gel phase domains in the membrane, or possibly, lipid adhering to immobile protein in the membrane. The presence of a non-diffusing fraction of lipid, whatever its causes, means that

the membrane cannot be treated as a bulk fluid.

Studies of lipid lateral diffusibility on spermatozoa plasma membranes [41,42] have shown that the fluidity of the membrane is different over the morphologically distinct regions of this highly differentiated cell (i.e., anterior and posterior regions of the head, midpiece, and tail). Since neurons are also highly differentiated cells with regions of dramatically distinct morphology, we have addressed this same question in the *Aplysia* nervous system, measuring lateral diffusion of NBD-C_6-PC in the cell body, the axon hillock, and the axon. Since the cell diameter was much greater than the laser beam in all regions of the neuron, geometric corrections of diffusion coefficients, such as those made on spermatozoa [41,41] were unnecessary. In contrast to regional differences in diffusibility observed on spermatozoa, no significant differences were observed on neurons. This does not necessarily imply that these regions are compositionally identical, as is indicated by differences in the intensity of staining (reflected in cps values) of the different regions with NBD-C_6-PC. Statistically significant differences in intensity were observed when the cell body was compared with the axon. Although this could represent differences in surface folding (surface amplification), it most probably reflects true compositional differences. Either the compositional differences result in more NBD-C_6-PC binding to the axon compared to the cell body, or compositional differences result in increased fluorescence quantum efficiency for the NBD-C_6-PC in the axon. Data obtained for the hillock is obscured by the considerable variability of cps in this region. For the hillock such variability could indeed reflect membrane infolding. This region is known as the trophospongium, and the cell is actually fragmented in this region, before reuniting to form the axon [43].

The *Aplysia* neuron plasma membrane shows large-scale homogeneity, at least as measured by NBD-C_6-PC lateral diffusibility. However, our alcohol and temperature data suggest that the membrane is submicroscopically heterogeneous and cannot be treated as having a bulk membrane fluidity. It is therefore possible that other lipid analogues would show differences in lateral diffusibility between different regions of the neuron.

The finding that neither alcohol affected NBD-C_6-PC diffusion, while producing increases in D of Rho-PE suggests that Rho-PE and NBD-C_6-PC probe different ensembles of the lipid domains within the membrane. Such an interpretation has been suggested to explain the selective anesthetic effects of the isomers of *n*-tetradecenol [24] and of the physiological effects of free fatty acids [38]. This interpretation may be relevant to observations on the effects of alcohols on the electrophysiology of *Aplysia* neurons, such as the greater sensitivity to ethanol of calcium channels when compared to other channels within the same cell membrane [23]. If we focus on the lipids as the anesthetic target, then this result might be expected if specific channels inhabit different domains which are selectively perturbed by alcohols and other anesthetic molecules. It is essential in interpreting these experiments not to assume that NBD-C_6-PC probes native PC while Rho-PE probes native PE. The fluorescent group in both cases alters the molecules in ways that potentially alter their partitioning into lipid enivronments. The critical point is that within the context of a homogeneous fluid membrne, the qualitative behavior of all probes and the qualitative effects of all alcohols should be the same.

The effects of 5% ethanol and 0.5% butanol on NBD-C_6-PC and Rho-PE lateral diffusion are small. The largest effect is that of ethanol on Rho-PE diffusion coefficient (a 2-fold increase). However, similar concentrations of ethanol and butanol produce profound effects on the electrophysiological characteristics of some channels [23]. This suggests that channel function may be sensitive to short-range order in the membrane, such as the ability of the membrane to accommodate channel rotation or conformational changes, rather than long-range order as measured in a fluorescence photobleaching recovery experiment. It is possible that other probes might show greater effects. While alcohols are known to interact with lipid bilayers, the relatively small changes which we report may alternatively suggest that alcohols also act directly on protein gate-channel complexes. If that is the case then conformational changes in channels, which result from this direct action on proteins could produce a second order effect on the lipid environment. The experiments reported here cannot distinguish between these

possibilities. A more complete understanding awaits measurements of short-range order, such as the extent and rates of rotational freedom for lipids and channels for these membranes (see, for instance, Ref. 54). The relative sensitivities of lateral and rotational lipid diffusion measurements is discussed in some detail in Ref. 55. This study of fatty acid effects on diffusion reported an influence on local rotational diffusion, but not on long-range lateral diffusion as measured by fluorescence photobleaching recovery and pyrene excimer formation. Again, these results indicate that the lipid matrix cannot be viewed as having a bulk viscosity in which rotational and lateral diffusion are derived by simple fluid dynamics. Rather, one must consider the specifics of the particular lipids and proteins within the bilayer.

Studies of changes in lipid lateral diffusion with temperature on intact, ghosted, or lipid extracted homeothermic membranes [45–48] as well as fluid phase model membranes [48–51] have consistently shown increasing D with increasing temperature. In the case of the poikilotherm *Aplysia's* neurons (both dissociated and cultured), we have found D for NBD-C_6-PC to be independent of temperature from 4°C to 25°C. Over this temperature range, however, $\%R$ steadily increases. This deviation from expected results is even more pronounced for Rho-PE, which also shows increasing $\%R$ with temperature, but in which D actually decreases with increasing temperature. As discussed above, immobile lipid could reflect gel phase domains. This raises the appealing hypothesis that immobile viscous lipid is recruited as temperature increases, so as to maintain homeoviscosity in lipid regions probed by NBD-C_6-PC and increased viscosity in regions probed by Rho-PE. Thus, these results complement our alcohol data, indicating that perturbations observed in one lipid pool need not be universal for all lipid pools, as would be expected if the membrane were describable as a bulk fluid. Differences in the temperature dependence of lipid probe diffusion, as a function of structure, has been reported in sea urchin eggs by Weaver [34]. Physiologically, our results suggest that a channel or other membrane protein's function will be sensitive to local lipid fluidity rather than an average membrane fluidity, provided that these proteins are also confined to specific lipid pools. Thus, different channels within the same cell potentially have different temperature sensitivities, reflecting the variability of the temperature sensitivity of different local lipid environments. Certain *Aplysia* neurons exhibit a characteristic electrical activity pattern known as bursting pacemaker activity, in which bursts of action potentials are separated by periods of interburst hyperpolarizations. The bursting pattern is highly temperature-sensitive [56], showing a sharp threshold for the appearance and disappearance of burst activity, and this may reflect the temperature sensitivity of voltage-dependent channels underlying the activity, although it may also reflect the contribution of metabolic processes. In contrast, the channels underlying the action potential itself do not show radical changes in the same temperature range [56].

Our data demonstrate that culturing neurons can alter membranes so as to drastically affect their ability to incorporate specific lipid analogues, and also affects the diffusibility of these analogues within the membrane. The dependence of lipid diffusibility upon culture conditions has previously been reported for mammalian cells [42,52,53].

A persistent controversy in anesthesia research has focused on whether the target for anesthetic molecules is the relevant membrane protein, or whether these proteins are secondarily affected as a result of perturbation of membrane lipid. An argument against the idea that anesthesia is the result of a disruption of lipid fluidity is that the small changes in fluidity that anesthetics reportedly cause would be mimicked by a small temperature change, which should then have profound anesthetic effects [57]. This argument assumes a bulk membrane fluidity, and our data indicate that this may not be accurate. The pool of lipids probed by Rho-PE are disparately affected by temperature and alcohols. Increasing temperature decreases diffusibility, but increases the diffusible fraction, while ethanol and butanol increase the diffusion rate but do not change the diffusible fraction of this pool.

In conclusion, our results indicate a complexity of organization within the *Aplysia* neuronal plasma membrane inconsistent with a homogeneous membrane describable by a bulk membrane fluidity. While plasma membrane lipid analogue diffusibil-

ity appears to be the same over the morphologically distinct regions of the neuron, there do appear to be compositional differences between the membrane on the cell body and the axon. Immobile lipid fractions are observed which for one pool of lipid, appear to melt out with increasing temperature, so as to maintain a state of constant diffusibility, and for another, to decrease diffusibility. Alcohol effects on lipid analogue diffusibility are selective, in that a given alcohol has different effects on the diffusibility of different probes.

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