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LATERAL DIFFUSION OF GRAMICIDIN *C* IN PHOSPHOLIPID MULTIBILAYERS

Effects of Cholesterol and High Gramicidin Concentration

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ABSTRACT   We have measured the lateral diffusion coefficient (D),[1] of active dansyl-labeled gramicidin *C* (DGC), using the technique of fluorescence photobleaching recovery, under conditions in which the cylindrical dimer channel of DGC predominates. In pure, hydrated, dimyristoylphosphatidylcholine (DMPC) multibilayers (MBL), D decreases from 6×10^{-8} cm^2/s at 40°C to 3×10^{-8} cm^2/s at 25°C, and drops 100-fold at 23°C, the phase transition temperature (Tm) of DMPC. Above Tm, addition of cholesterol decreases D; a threefold stepwise drop occurs between 10 and 20 mol %. Below Tm, increasing cholesterol increases D; a 10-fold increase occurs between 10 and 20 mol % at 21°C, between 20 and 25 mol % at 15°C, and between 25 and 30 mol % at 5°C. In egg phosphatidylcholine (EPC) MBL, D decreases linearly from 5×10^{-8} cm^2/s at 35°C to 2×10^{-8} cm^2/s at 5°C; addition of equimolar cholesterol reduces D by a factor of 2. Thus this transmembrane polypeptide at low membrane concentrations diffuses quite like a lipid molecule. Its diffusivity in lipid mixtures appears to reflect predicted changes of lateral composition. Increasing gramicidin *C* (GC) in DMPC/GC MBL broadened the phase transition, and the diffusion coefficient of the lipid probe *N*-4-nitrobenzo-2-diazole phosphatidylethanolamine (NBD-PE) at 30°C decreases from 8×10^{-8} cm^2/s below 5 mol % GC to 2×10^{-8} cm^2/s at 14 mol % GC; D for DGC similarly decreases from 4×10^{-8} cm^2/s at 2 mol % GC to 1.4×10^{-8} cm^2/s at 14 mol % GC. Hence, above Tm, high concentrations of this polypeptide restrict the lateral mobility of membrane components.

INTRODUCTION

Saffman and Delbruck have presented a model hydrodynamic calculation describing the diffusion coefficient D of a cylindrically shaped molecule in a biologically modeled membrane (Saffman and Delbruck, 1975). This hydrodynamic model supposes the bilayer to be an infinite sheet of isotropic viscous fluid (lipid) separating infinite regions of less viscous liquid (water). Each cylinder is assumed to move about in independent Brownian motion with its axis perpendicular to the membrane. The theory predicts that the diffusion coefficient of such a cylindrical molecule is only weakly dependent on its size because D depends upon molecular radius only through a logarithmic factor. Fur-

thermore, the diffusion coefficient of the membrane molecule is very insensitive to its protrusion into the surrounding aqueous phase of much lower expected viscosity. Measurements of protein and lipid diffusion in vertebrate tissue cell membranes contrast sharply with the above predictions. They have revealed that proteins diffuse at least 40 times slower than lipids (see Webb et al., 1981). This discrepancy between theory and experiment has prompted us to measure lateral diffusion of a protein of well-characterized physical properties (and of roughly cylindrical shape) that has been reconstituted into a well-defined model membrane system, where we can systematically study the effects of lipid composition (viscosity) and other physical parameters, such as temperature and concentration of the diffusant. For the experiments described in this paper, we have chosen the peptide channel dansyl gramicidin *C* as our model diffusant.

The antibiotic gramicidin (commerically available as gramicidin *D*, a mixture of the *A*, *B*, and *C* species) is known to form a dimer transmembrane channel in biological membranes, making the cell permeable to many cations.

As proposed by Urry (1971) and confirmed by x-ray diffraction studies (Koeppe et al., 1979), the channel

[1]Abbreviations used in this paper: DMPC, dimyristoylphosphatidylcholine; EPC, egg phosphatidylcholine; GC, gramicidin *C*; DGC, dansylgramicidin *C*; NBD-PE, *N*-4-nitrobenzo-2-diazole phosphatidylethanolamine; D, lateral diffusion coefficient; Tm, phospholipid phase transition temperature; MBL, multibilayers (multilammellar liquid crystals); FPR, fluorescence photobleaching recovery.

molecule is formed from the formyl-to-formyl hydrogen bonded dimerization of two helical monomers. The dimer has a molecular weight of ~4,000 and can be modeled, for our purposes, as a cylinder of 12 Å diameter and 26 Å length. The monomer is a pentadecapeptide; the species gramicidin C (GC) has an L-tyrosine at position 11, and fluorescence-labeled dansyl gramicidin C (DGC) can be synthesized by specific O-dansylation of this L-tyrosine (Veatch and Blout, 1976). Veatch et al. have shown this fluorescent analogue to be fully active, forming dimer channels in artificial lipid bilayers (Veatch et al., 1975).

In this paper, we present the results of fluorescence photobleaching recovery (FPR) measurements that probe the effects of temperature and cholesterol concentration on the lateral diffusion of DGC in multibilayers (MBL) of egg phosphatidylcholine (EPC) and dimyristoylphosphatidylcholine (DMPC). Snyder and Freire (Snyder and Freire, 1980) reported that Monte Carlo calculations of bilayer configurations predict that cholesterol rich areas of the phospholipid-cholesterol bilayers suddenly become connected at 20 mol % cholesterol to form a network extending over the entire sheet. Experimentally, abrupt changes in lateral diffusion of phospholipid (Rubenstein et al., 1979) and reconstituted M13 coat protein (Smith et al., 1979) occur in this concentration region, however the exact cholesterol dependence is different in the two cases. We report here that diffusion of DGC in DMPC and EPC MBL exhibits a cholesterol dependence similar to that observed for phospholipids and different from that observed for M13 coat protein. We were also able to introduce large concentrations of GC into DMPC MBL in an effort to see if protein-protein and/or protein-lipid interactions will alter DGC lateral mobility or the mobility of lipid probes. At high GC concentrations, we find reduction of both DGC and lipid lateral mobility. This reduction commences at a GC concentration where scanning calorimetry studies reveal a change in membrane organization (Chapman et al., 1977), ^{2}H-NMR spectra reveal a higher proportion of disordered (boundary) lipid (Rice and Oldfield, 1979; Westermen et al., 1982) and where recent theoretical calculations (Freire and Snyder, 1982) predict the onset of a percolation process, the annular lipid domains surrounding a GC molecule becoming topographically connected, and the free lipid being disrupted into isolated compartments.

MATERIALS AND METHODS

Dansyl Gramicidin C

Gramicidin C (GC) was purified from the *Bacillus brevis* extract (Sigma Chemical Co., St. Louis, MO.) by the methods of Pepinsky and Feigenson (1978) using preparative thin-layer chromatography. The same support-solvent system was utilized for purification of O-dansyltyrosine Gramicidin C (DGC) from the reaction products obtained after dansylation of GC using the labeling protocol of Veatch and Blout (1976). Samples used in these experiments exhibited an absorbance ratio $A(350)/A(290)$ of 0.22, indicating monolabeling (Veatch and Blout, 1976).

Reagents

DMPC, EPC, and cholesterol were obtained from Applied Science (Division, Milton Roy Co., State College, PA). The fluorescence-labeled phospholipid NBD-PE, was from Avanti Biochemicals, Inc., Birmingham, AL. Lipids and cholesterol were dissolved in a 1:1 chloroform methanol mixture and used without further purification.

Preparation of Lipid Multibilayers

Details of the preparation of MBL have been described elsewhere (Wu et al., 1977). NBD-PE, DGC, and GC were added to the dissolved lipids before the evaporation step. The MBL films were hydrated in double-distilled water above the lipid-chain melting temperature (Tm). Oriented MBL were formed by pressing hydrated lipids between two glass plates. Before FPR measurements, samples were cycled through Tm several times to help eliminate defects.

Microscopy and Visual Observation

A Nikon Optiphot (Nikon, Inc., Instrument Div., Garden City, NY) epifluorescence microscope was used for visual observation of MBL samples in phase and fluorescence. Fluorescence excitation was at 330–380 nm for DGC and 410–485 nm for NBD-PE, using 420 and 515 nm barrier filters, respectively. Photomicrographs were recorded on Tri-X film push-processed in Kodak D19 to ASA 3,200 (Eastman Kodak Co., Rochester, NY).

Diffusion Measurements

The FPR technique has been described in detail elsewhere (Webb, 1981). For these experiments, the laser beam (an Argon 350 nm line for DGC, and a Krypton 466 nm line for NBD-PE) was first shaped into a Gaussian intensity profile by a spatial filter and then split into bleach and monitor beams by transmission or multiple reflection within a fused quartz optical flat. The bleach beam, which can be pulsed by an electromechanical shutter, and the monitor beam were recombined by a second matched quartz optical flat. Partial reflectance coatings reduce the monitor intensity to 1/2,000 of the bleach beam. The combined beam was then focused through the vertical illuminator of a Zeiss Universal microscope (Carl Zeiss, Inc., NY) onto the MBL sample at the object plane. For the 40× objective used in these experiments, the illuminated spot was 1.1 μG Gaussian radius. The excited fluorescence produced, after passing a barrier filter, was monitored by a photomultiplier tube with photon-counting electronics. FPR curves—the fluorescence intensity vs. time before and after a brief bleaching pulse—were stored, averaged (if necessary), and analyzed by computer. Least-squares fitting of the average curve based on the analysis of Axelrod et al. (1976) gave both the percent fluorescence recovery (R), as a measure of the mobile fraction of the diffusing species, and the lateral diffusion coefficient (D). For all measurements reported in this paper, R - 100%, and flow of membrane, detectable by a characteristic alteration in the shape of the recovery curve, was never seen. Stated uncertainties in the presented values of D represent the full range of values obtained. A temperature-controlled microscope stage maintained sample temperatures to within 1°C.

RESULTS

DGC Incorporation in MBL as a Transmembrane Channel

It was easy to incorporate the channel-forming peptide DGC into our MBL samples used for diffusion measurements by simply including it at a nominal concentration in the organic solvent-lipid solution used in the initial step of MBL formation. Our samples were composed of flat regions, with surfaces resembling plateaus, each having a

nearly constant number of stacked bilayers. An MBL of pure DMPC containing DGC at 0.025 mol % is shown in the phase and fluorescence micrographs of Fig. 1. The dark lines visible in phase indicate large defects (oily streaks or disclinations) that separate the plateau regions of uniform bilayer thickness. This uniformity is seen in the fluorescence of DGC, Fig. 1 b, which is partitioned equally into each bilayer. Brighter regions correspond to a greater number of stacked bilayers. For FPR measurements, the focused laser beam was centered in one of these uniform regions.

In DMPC MBL samples containing cholesterol at >30 mol %, nonuniformity appeared in the DGC fluorescence, as shown in Fig. 2. We do not know what altered topological structures might be present; hence it is hard to determine if this represents an aggregation of DGC that is excluded from bilayer regions, or a more condensed structure that is thicker. FPR measurements in these ranges of cholesterol were limited to regions of uniform fluorescence intensity (plateaus) such as the one indicated by a star (⋆) in Fig. 2 a.
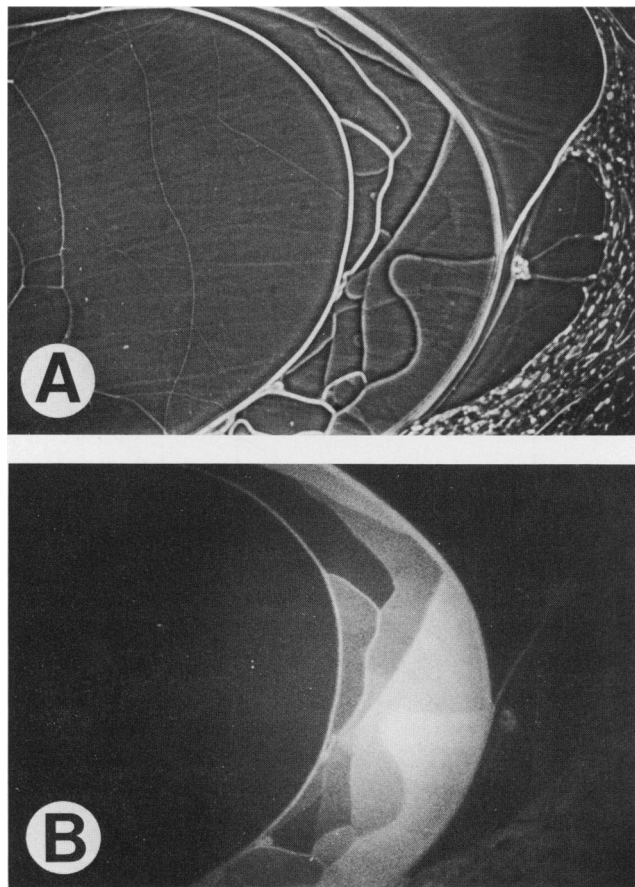

FIGURE 2 Phase (a) and fluorescence (b) micrographs of DMPC plus 40 mol % cholesterol MBL sample containing DGC. The bright fluorescent patches indicate that DGC is being partially excluded from the bilayer regions. Exclusion was only seen for MBL with >20 mol % cholesterol. All diffusion measurements on samples exhibiting exclusion represent the MBL regions of lower, uniform fluorescence intensity such as the region indicated by an (⋆) in the phase micrograph. Bar = 50 microns.

FIGURE 1 Phase (a) and fluorescence (b) micrographs of a DMPC MBL sample containing 0.025 mol % DGC. The fluorescence intensity is proportional to the number of bilayers; hence, brighter areas are thicker. In phase, only the borders (large defects) between regions are visualized. Diffusion measurements were taken in the center of large areas of uniform fluorescence. Bar = 50 microns.
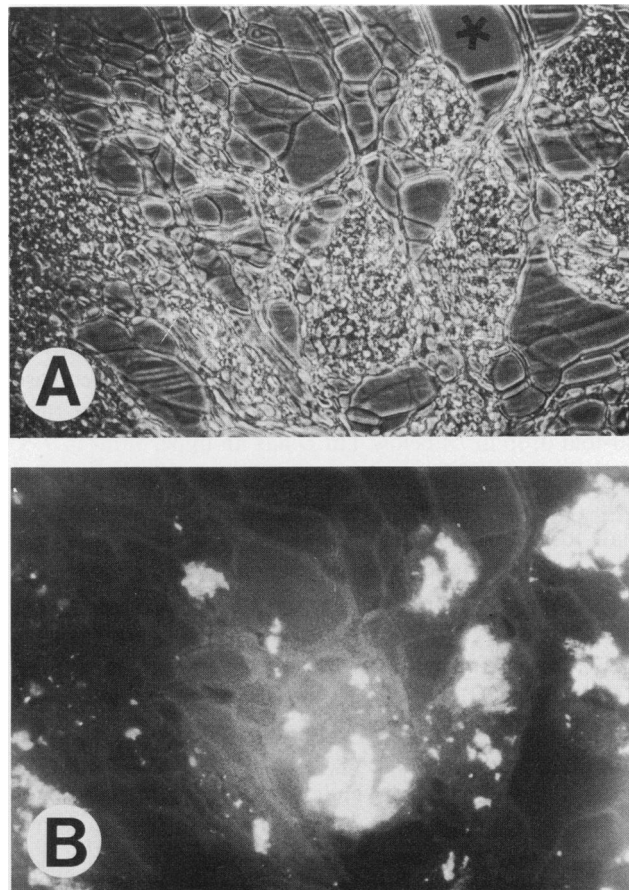
The gramicidin channel is a dimer that spans the bilayer. As calculated by Veatch et al. (1975) the gramicidin in the membrane is almost entirely in the dimeric form when K [gram] >> 1 where [gram] represents the membrane concentration of monomer in mol/cm^2 and K is the equilibrium constant for dimerization. In dioleylphosphatidylcholine black lipid membranes formed with decane, $K = 1 \times 10^{14} \, mol/cm^2$, for DGC. Also, K increases sharply as membrane thickness decreases: a 1,000-fold increase is observed in changing from a 47-Å thick glyceroester membrane to a 26-Å thick one (Veatch et al., 1975). Because a DMPC molecule is of shorter chain length than dioleylphosphatidylcholine and because our MBL samples contain no organic solvents that are known to swell the bilayer (see for example Waldbillig et al., 1979), the thickness of our bilayers is less than that of dioleylphosphatidylcholine/decane black lipid membranes, and therefore K for DGC in DMPC MBL is $>1 \times 10^{14} \, mol/cm^2$.

Because the minimum [DGC] used in our experiments

was 0.02%, which corresponds to [DGC] $> 2 \times 10^{-13}$ mol/cm^2, we have K [DGC] > 20 for all experiments and therefore satisfy the criteria that the channel is almost entirely in the dimer (transmembrane) state.

Temperature Dependence of D in DMPC and EPC MBL

In DMPC MBL, the diffusion of DGC shares many characteristics with lipid probe diffusion. As shown in Fig. 3, above 23°C, the Tm of DMPC, DGC diffuses rapidly in the range $2 - 8 \times 10^{-8}$ cm^2/s, showing a gradual decrease with decreasing temperature. At Tm there is a more than 100-fold drop in D; below Tm D has an upper limit of 7×10^{-11} cm^2/s. Our experimental FPR curves below Tm fitted accurately the theoretical curves representing a single diffusion coefficient; however, we did not have sufficient accuracy to rule out a second greater than 10-fold slower component of diffusion similar to that observed in a study of 3,3′-dioctadecylindolcarbocyanine (diI(3)C$_{18}$) diffusion below Tm in DMPC MBL samples (Chan et al., 1982).

The lateral diffusion of DGC in EPC MBL is shown in Fig. 4. There is a weak logarithmic decrease in D with decreasing temperature, which is typical of the diffusion of lipid analogues in multicomponent lipid mixtures above Tm.
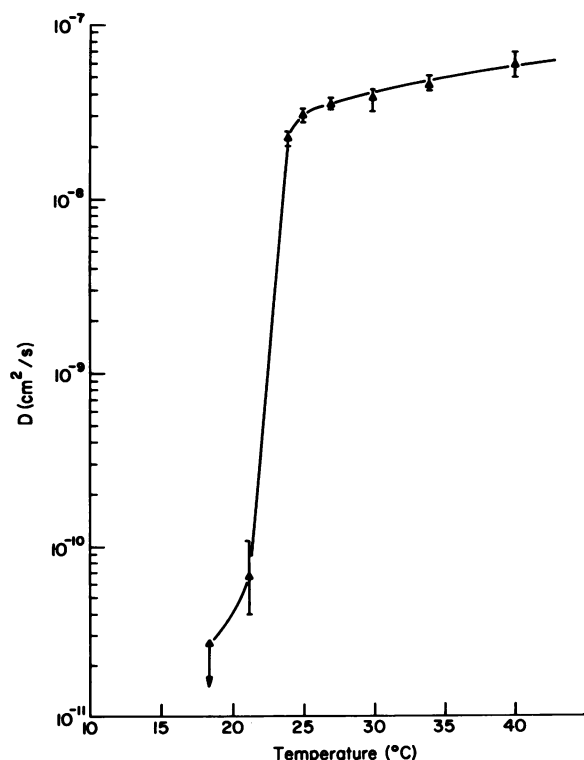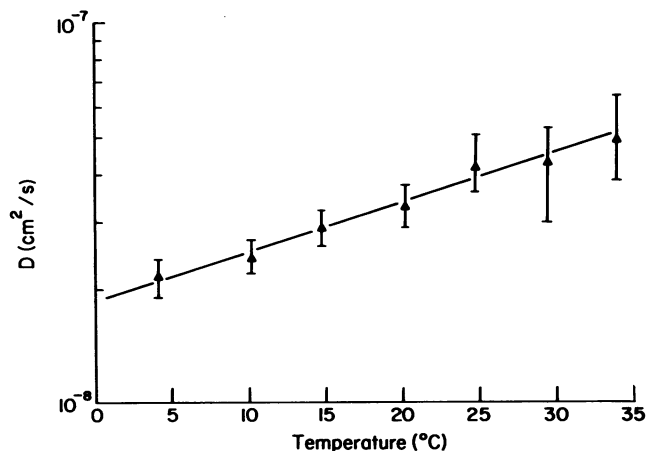
FIGURE 4   Lateral diffusion coefficient of DGC vs. T in MBL of EPC.

Cholesterol Dependence of D in DMPC and EPC MBL

The addition of cholesterol caused a striking change in D for DGC in DMPC MBL containing cholesterol. As shown in Fig. 5, we measured D in the range of 0–50 mol % cholesterol for several temperatures above and below Tm. Slightly above Tm, there was a sharp drop in D of about 3-fold between 10 and 20 mol % cholesterol. This was seen even at 35°C, 12°C above Tm.

FIGURE 3   Lateral diffusion coefficient of DGC vs. T in MBL of DMPC. The phase transition temperature Tm of DMPC is 23.8°C.
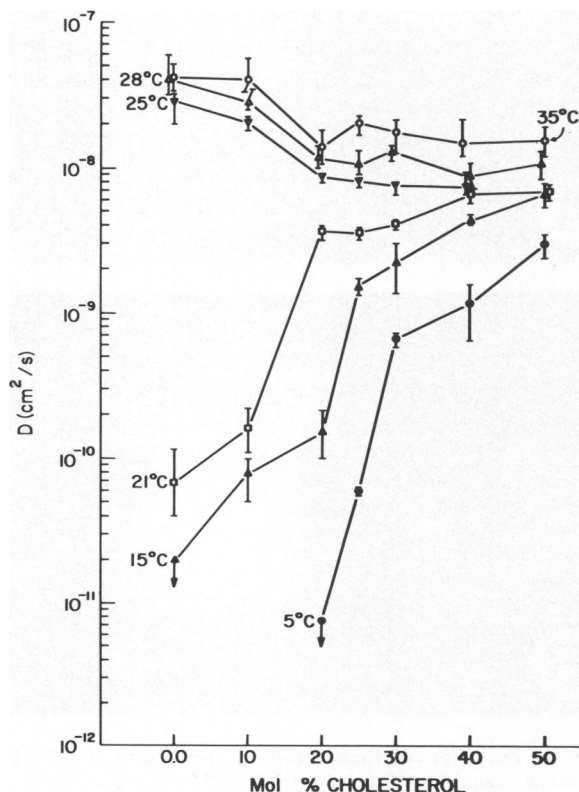
FIGURE 5   Lateral diffusion coefficient of DGC, at several temperatures, vs. mol % cholesterol in DMPC MBL.

At a fixed temperature below Tm, increasing the cholesterol concentration increased D in a stepwise fashion: at 21°C a greater than 10-fold increase occurred as the concentration was raised between 10 and 20 mol %; further increases, up to 50 mol %, only raised D by a factor of 2.

At lower temperatures, the position of the step increase was shifted to higher cholesterol concentrations: At 15°C the 10-fold increase occurred between 20 and 25 mol % and at 5°C this effect occurred between 25 and 30 mol %.

In MBL of EPC and cholesterol, we found that at a fixed temperature, increasing cholesterol concentration caused a gradual decrease in D for DGC. There was not an abrupt drop in D in the 10–20 mol % range as was seen in DMPC/cholesterol MBL above Tm. These data are presented in Fig. 6.

Effect of Increasing GC Concentration on D for DGC and NBD-PE

We were able to incorporate large quantities of GC into our MBL samples to see if increasing protein concentration in the bilayer affected the lateral transport of lipid and protein.

While keeping the DGC/lipid ratio at 1:5,000 we were able to form DMPC MBL samples containing up to 14 mol % GC in the nominal MBL forming solution. As presented in Fig. 7, D for DGC decreased from 4×10^{-8} cm^2/s at 2 mol % GC in DMPC MBL at 30°C to 1.4×10^8 cm^2/s at 14 mol % GC.

An even stronger effect was seen for the lipid probe NBD-PE, which was present at <1:1,000 probe:lipid in the DMPC/GC MBL. As shown in Fig. 7, D for this lipid decreased from 8×10^{-8} cm^2/s below 5 mol % GC to 2×10^{-8} cm^2/s at 14 mol % GC.

At GC concentrations >8 mol %, we began to see a nonuniformity of DGC fluorescence. As in the case of high cholesterol content in the membrane, we do not know whether this represents exclusion of DGC from bilayer regions; it might also represent a different topological structure (see for example, Van Echteld et al., 1981). However, x-ray diffraction experiments (Chapman et al., 1977) indicate that even at 33 mol % gramicidin A in
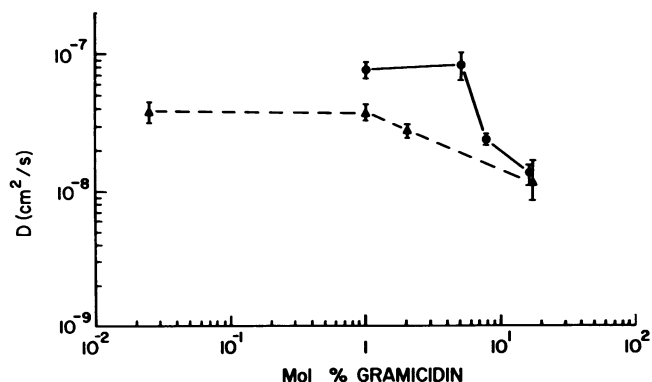

FIGURE 7 Lateral diffusion coefficient of NBD-PE (●) and DGC (▲) vs. concentration of gramicidin C in DMPC/MBL. High concentrations lower the mobility of both probes.

dipalmitoylphosphatidylcholine liposomes, a bilayer structure remains. Therefore the point at 14 mol % GC in Fig. 7 indicates only the nominal concentration used in MBL formation.

Because increasing GC concentration lowered D for both probes at $T = 30°C$ (above Tm) we measured D as a function of T for a DMPC MBL sample containing 8 mol % GC. As shown in Fig. 8 the presence of this amount of

FIGURE 6 Lateral diffusion coefficient of DGC, at 15°C (□) and 30°C (▲), vs. mol % cholesterol in EPC MBL.
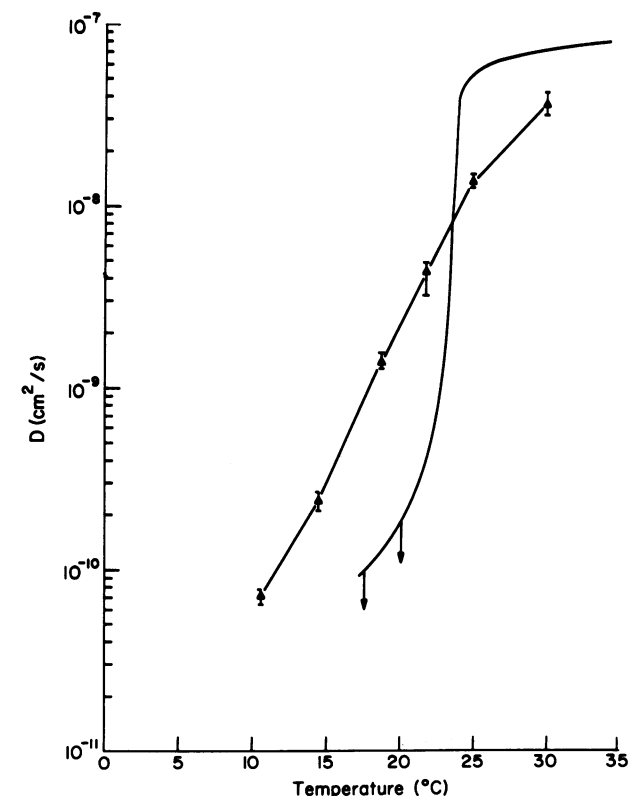

FIGURE 8 Lateral diffusion coefficient of NBD-PE (▲) vs. T in MBL of DMPC containing 7.7 mol % cholesterol (DGC/DMPC = 1/12). For comparison, D vs. T for NBD-PE in pure DMPC MBL is also shown (from Wu et al., 1977).
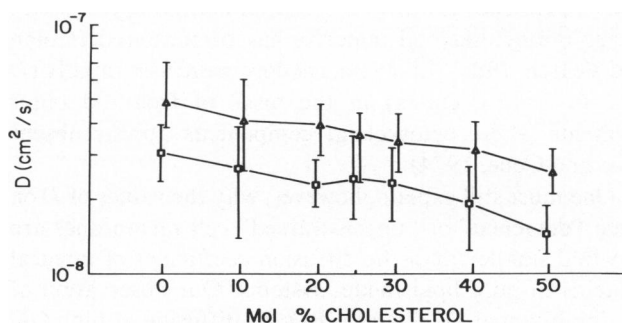
GC spread out the phase transition. For comparison the smooth curve of D for NBD-PE in pure DMPC MBL (from Wu et al., 1977) is shown.

DISCUSSION

Our results for the lateral diffusion of DGC in DMPC/cholesterol mixtures show that at low DGC membrane concentrations the lateral transport of this transmembrane channel closely resembles that of lipid probes. Above Tm we find about a threefold drop in D in the region 10–20 mol % cholesterol. This is similar to data for lateral diffusion of NBD-PE above Tm in the same system obtained by Rubenstein et al. (1979). Measurements of the diffusion of M13 coat protein (20,000 mol wt with 19 hydrophobic amino acid binding sequence; [Smith et al., 1980]) have yielded a different result: there is a pronounced dip in the region around 25 mol % cholesterol. In EPC/cholesterol MBL we see only a gradual decrease in D with increasing cholesterol, again similar to that observed for NBD-PE (Rubenstein et al., 1979), and different from the dip around 25 mol % observed for M13 coat protein in these MBL (Smith et al., 1980). From this we can conclude that the diffusion of this transmembrane protein closely resembles the diffusive behavior of a lipid molecule. Apparently the viscous drag on the transmembrane molecule DGC differs little from that on the lipid spanning one-half of the bilayer.

The effect of membrane protein at high concentration on the lateral transport of membrane components has been anticipated, but there is a paucity of experimental data. The GC/DMPC MBL system offered an ideal opportunity to test the effect of high protein concentration on the lateral diffusion of both the protein itself and the lipid. Our data show that a decrease in lateral diffusibility occurs for both probes when the GC concentration rises above ~1–5 mol % in DMPC at temperatures above Tm; at 15 mol % GC (~30% by weight) the reduction in D for lipids can reach fivefold. Previous experiments anticipate the onset of distinctive interactions in this system at ~5 mol % gramicidin. Chapman et al. (1977) attributed a change in the concentration dependence of the entropy of the gel-to-liquid crystal transition at 5 mol % gramicidin to an aggregation process. If aggregation of the gramicidin species (trace DGC with high GC) is responsible for the 2–4-fold reduction in DGC diffusion we observe at high GC concentrations and if we assume a logarithmic dependence of D on the molecular size of the diffusant (Saffman and Delbruck, 1975), then the diffusing aggregate islands must contain ~100–5,000 gramicidin dimers. Although these numbers are not physically improbable, the parallel reduction in lipid diffusion (NBD-PE) that we observe suggests that aggregation of gramicidin may not be responsible. Rice and Oldfield (1981) interpret deuterium resonance data on lipid probes in terms of an increase in the proportion of probe occupying a disordered (boundary)

environment at gramicidin concentrations above ~6 mol %. Consistent with these observed changes in lipid environment, theoretical studies using Monte Carlo modeling of proteins in lipid bilayers (Freire and Snyder, 1982) find that the point-to-point lateral connectivity representing composition variations in the membrane decreases enormously at 5–7 mol % for a ~4,000 mol wt transmembrane protein (the size of the dimer channel). If the dynamical property of the lateral diffusivity reflects the instantaneous connectivity predicted in these studies (see Freire and Snyder, 1982), then one would expect a break in the diffusion coefficient at these concentrations as we have observed.

Rapid diffusion ($D \sim 10^{-8}$ cm^2/s of reconstituted proteins in pure lipid model membranes above the phase transition temperatures has now been observed for several systems, including Apolipoprotein CIII (Vaz et al., 1979), M13 coat protein (Smith et al., 1979), Cytochrome P-450 (Wu et al., 1980) and glycophorin (Wu et al., 1981 a). This fast diffusion, usually less than a factor of 3 slower than lipid probes in the same system is consistent with the hydrodynamic model of Saffman-Delbruck (1975): a 100,000 dalton integral protein should diffuse laterally only ~2 times slower than the lipid.

It is well known that the diffusion of many proteins and/or receptors on the intact cell surface is orders of magnitude slower than in model systems (see, for example, Webb et al., 1981) and this discrepancy has led to the idea that cell surface lateral constraints may involve cyto- or exoskeletal-membrane interactions. Our recent measurements of enhanced lateral diffusibility of identified receptors on cell surface membrane that has been physically decoupled from the cytoplasm by the formation of blebs (Tank et al., 1981; Tank et al., 1982; Wu et al., 1981 b; Webb et al., 1981; Barak and Webb, 1981) demonstrates that large integral membrane proteins can diffuse, provided that they are unrestrained, at rates ($D \sim$ 2–5 $\times$ 10^{-9} cm^2/s) only slightly slower than reconstituted proteins in model systems. Hence, on blebs, coefficients approach their viscous drag limit. It has also recently been shown that enhanced diffusibility of band III protein ($D \sim 0.5$–2 $\times$ 10^{-9} cm^2/s occurs on spherocytic erythrocytes lacking spectrin (Sheetz et al., 1980) and on normal erythrocytes where the cytoskeletal integrity has been altered (Golan and Veatch, 1980). Likewise, rhodopsin diffuses rapidly ($D \sim$ 2–6 $\times$ 10^{-9} cm^2/s) in the disks of frog rod outer segments where cytoskeletal components appear absent (Poo and Cone, 1974).

One must still explain, however, why the values of D on these "detached" or "unconstrained" cell membranes are 2–5-fold smaller than the diffusion coefficient of integral proteins in pure lipid model systems. Our observation of the inhibition of DGC and NBD-PE diffusion at high GC concentrations is perhaps relevant. For a cell membrane with a multicomponent lipid system well above the phase transition temperature, we would anticipate that lateral

diffusion would be slowed below that observed in dilute model systems by the high membrane protein concentrations. For example, the residual constraint on the lateral diffusion of rhodopsin in frog visual disks may be caused by the presence of 60% protein (by weight) in the frog rod outer segment (Eichberg and Hess, 1967). We suggest that the residual inhibition of lateral transport observed in cell belbs is also attributable to the effects of membrane protein concentrations. In any case, it seems likely that diffusibility on the cell surface is not determined by pure lipid viscosity although the detailed molecular picture of the mechanism responsible for the lateral constraints on eukaryotic cell surfaces is unknown at this time.

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