Translational Diffusion of Acetylcholine Receptor (Monomeric and Dimeric Forms) of *Torpedo marmorata* Reconstituted into Phospholipid Bilayers Studied by Fluorescence Recovery after Photobleaching†

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ABSTRACT: The translational diffusion of fluorescein isothiocyanate labeled acetylcholine receptor from *Torpedo marmorata* reconstituted, at lipid/protein molar ratios of ≥5000/1, into phospholipid bilayer membranes with and without cholesterol was examined by using fluorescence recovery after photobleaching. The receptor protein, free of nonreceptor peptides, was studied in the monomeric state (M_r ~250000) in multibilayers and large paucilamellar liposomes of (a) dimyristoylphosphatidylcholine (DMPC), (b) a 2/5 molar ratio mixture of cholesteryl hemisuccinate (a derivative of cholesterol) and DMPC, and (c) soya bean lipids. The dimeric protein, also free of nonreceptor peptides, was studied in paucilamellar liposomes and multibilayers of soya bean lipids. As a reference, the translational diffusion of the lipid probe *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)dimyristoylphosphatidylethanolamine (NBD-DMPE) was also studied in the same bilayer systems. In DMPC the translational diffusion coefficient (D_t) for the protein monomer was similar in multibilayers and liposomes at temperatures above 24 °C. In multibilayers the fluorescence recovery curves were apparently due to a single diffusing component with D_t of about 10^{-8} cm²/s above 24 °C, and a sharp transition occurred at 23 °C to multicomponent recoveries with D_t of the major component of less than 5×10^{-11} cm²/s. In large paucilamellar liposomes of DMPC, fluorescence recoveries were apparently due to a single diffusing component throughout, and a more gradual drop occurred below 22 °C from a fast diffusion (D_t of about 10^{-8} cm²/s) to a slow diffusion (D_t of about 10^{-10} cm²/s). D_t for the monomeric acetylcholine receptor showed a monotonic temperature dependence in the cholesteryl hemisuccinate containing DMPC membranes and soya bean lipid membranes over the temperature range examined (14–37 °C). Typical values of D_t for the receptor monomer in liquid-crystalline phase membranes were $(2.4 \pm 0.8) \times 10^{-8}$ cm²/s in DMPC, $(1.9 \pm 0.2) \times 10^{-8}$ cm²/s in the cholesteryl hemisuccinate–DMPC mixture, and $(3.3 \pm 0.7) \times 10^{-8}$ cm²/s in soya bean lipids, all at 36 °C. D_t for NBD-DMPE was $(8.8 \pm 1.3) \times 10^{-8}$ cm²/s in DMPC at 36 °C and $(10.8 \pm 0.9) \times 10^{-8}$ cm²/s in soya bean lipids at 37 °C. D_t for the dimeric protein in multibilayers of soya bean lipids was almost indistinguishable from that of the monomeric protein in the temperature range examined (14–37 °C).

The acetylcholine receptor (AchR)[1] is a transmembrane integral protein of the postsynaptic membrane which is believed to function as a cation-translocating complex in response to the binding of acetylcholine. The AchR purified from the electric organ of *Torpedo marmorata* has a molecular weight of about 250000 and is made up of four subunits termed α (M_r ~40000), β (M_r ~50000), γ (M_r ~60000), and δ (M_r ~66000). The stoichiometry of the subunits in the monomeric receptor protein is believed to be $\alpha_2\beta\gamma\delta$ [for reviews see Karlin (1980), Raftery et al. (1980), and Changeux (1981)]. Several oligomeric forms of the AchR occur, the dimer being the predominant species [for a comparative study, see Vandlen & Raftery (1979)]. In dimers two monomers are linked covalently to each other by a disulfide bond between their δ subunits (Chang & Bock, 1977; Hamilton et al., 1977). Techniques have been described for the functional reconstitution of the AchR into phospholipid bilayers [for a recent review, see Anholt (1981)]. The large size of this protein and the possibility of reconstituting it into phospholipid bilayers of defined chemical composition in the monomeric and dimeric states make the study of its diffusion attractive, particularly since the translational diffusion of this protein in natural membranes has been investigated (Axelrod et al., 1976b, 1978a,b; Poo, 1982; Tank et al., 1982).

Measurements of the translational mobility of proteins in reconstituted model membranes are expected to provide a baseline for the evaluation of diffusion studies on more complex cellular membranes, as well as an experimental basis for comparison with theoretical models for diffusion in membranes (Vaz et al., 1982a). To date, most studies on the translational diffusion of proteins in membranes have used the FRAP technique, the most commonly used version of which is that described by Axelrod et al. (1976a). The method requires the preparation of large membrane surfaces such as large multibilayer domains, large liposomes, or planar bilayer membranes with cross-sectional dimensions of about 10 μm or greater. The preparation of artificial phospholipid membranes containing proteins and having these dimensions poses several technical difficulties. As a consequence, most studies on the diffusion of proteins in reconstituted model membranes have been restricted to small peptides which can be comixed with the lipid in organic solvents or added to the lipid in the aqueous phase during hydration of the lipid to give the desired membrane structures (Wu et al., 1978; Smith et al., 1979b; Vaz et al., 1979). Recently, we (Vaz et al., 1981) have described a technique for the preparation of large proteoliposomes and multibilayer domains which does not necessarily involve the premixing of protein and lipid in organic solvents. The method

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[1] Abbreviations: AchR, acetylcholine receptor; CHS, cholesteryl hemisuccinate; DMPC, dimyristoylphosphatidylcholine; D_t, translational diffusion coefficient; FRAP, fluorescence recovery after photobleaching; SBL, soya bean lipids (a commercial preparation of soya bean lipids containing about 20% phosphatidylcholine); EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; NBD-DMPE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)dimyristoylphosphatidylethanolamine.

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involves the reconstitution of proteins into small proteoliposomes by conventional techniques, including detergent dialysis methods, followed by fusion of the proteoliposomes to give multibilayers and large (30–100-μm diameter) proteoliposomes by using a controlled dehydration step followed by rehydration to conditions of a large water excess. Using this technique, we have been successfully able to reconstitute several large integral membrane proteins into phospholid bilayers suitable for FRAP experiments (Vaz et al., 1982b). In this paper we report our results on the translational diffusion of the AchR of *Torpedo marmorata* reconstituted into bilayers of DMPC, a 2/5 molar ratio mixture of CHS and DMPC, and SBL. The translational diffusion of the protein in the monomeric state was studied in bilayers of all of the above lipids and in the dimeric state in bilayers of SBL.

Materials and Methods

DMPC (99%) was from Fluka AG, Buchs, Switzerland, CHS (99+%) was a gift from Dr. M. Shinitzky, Weizmann Institute of Science, Rehovot, Israel, SBL (L-α-phosphatidylcholine from soybean, type II-S) and *N*-ethylmaleimide were from Sigma Chemie, Munich, F.R.G., sodium cholate was from Serva Feinbiochemica, Heidelberg, F.R.G., native α-bungarotoxin was from the Miami Serpentarium, Miami, FL, its tritiated derivative was from Amersham-Buchler, Braunschweig, F.R.G., dithiothreitol was from Calbiochem-Behring Corp., La Jolla, CA, and fluorescein 4-isothiocyanate (isomer I) was from Molecular Probes Inc., Plano, TX. All chemicals were used as received. The electric fish, *Torpedo marmorata*, was obtained from the Marine Biological Station, Arcachon, France.

Preparation of AchR in the Monomeric and Dimeric Forms. AchR-rich membrane fragments were prepared as described by Barrantes (1982). These membranes were suspended at 1 mg of protein/mL in 150 mM NaCl, 1 mM EDTA, 3 mM sodium azide, and 20 mM Tris-HCl, pH 8.3, and incubated for 30 min at room temperature with or without 10 mM dithiothreitol. The treatment with dithiothreitol was done to reduce the dimeric form of the protein to the monomeric form. After this treatment, the membrane fragments were centrifuged in a Beckman airfuge at 140000*g* for 5 min and washed twice. The reduced and unreduced membranes were then treated with 10 mM *N*-ethylmaleimide in 50 mM NaCl, 1 mM EDTA, 3 mM sodium azide, and 10 mM sodium phosphate, pH 7.4, and centrifuged once more.

Solubilization of the membranes was carried out by suspending them in 2% sodium cholate, 3 mM EDTA, 1 mM EGTA, 0.1 mM phenylmethanesulfonyl fluoride, and 50 mM Tris-HCl, pH 7.5. The final mixture had 10 mg of sodium cholate/mg of protein. After 1 h at 4 °C the samples were centrifuged in the airfuge at 140000*g* for 5 min. The supernatant of this centrifugation was trace labeled with [^{3}H]-α-bungarotoxin, applied to a 5–20% continuous sucrose density gradient in the same buffer as used for solubilization, and centrifuged for 5 h at 50000 rpm in a Beckman SW50.1 rotor. Fractions of 0.15 mL were collected starting from the bottom of the gradients, and the distribution of radioactivity was determined. The peaks corresponding to the 9S "light" (monomer) and the 13S "heavy" (dimer) forms of the AchR were pooled and dialyzed overnight against 100 volumes of 1% sodium cholate and 10 mM sodium carbonate–bicarbonate buffer, pH 9.2.

The AchR was labeled by adding fluorescein isothiocyanate dissolved in 5% sodium cholate and 10 mM sodium carbonate–bicarbonate buffer, pH 9.2, to the dialysate described above. The final fluorescein isothiocyanate concentration was 1 mg/mL, and the mixture was incubated for 3 h at room temperature. Free dye was removed by dialysis against 500 volumes of 1% sodium cholate, 100 mM NaCl, 0.1 mM phenylmethanesulfonyl fluoride, and 10 mM sodium phosphate, pH 7.4, for 72 h at 4 °C with three buffer changes.

Reconstitution of AchR into Liposomes. Reconstitution was carried out by using procedures that maintain the agonist-induced affinity transitions of AchR (Criado et al., 1982). In addition, the ability of the fluorescein-labeled AchR in the reconstituted systems to bind [^{3}H]-α-bungarotoxin did not differ from that of unlabeled controls. Lipids were dissolved in chloroform, and the solvent was evaporated in vacuo for 2–3 h at 4 °C. The residue was dissolved in the cholate-containing solution of fluorescein-labeled AchR at a lipid concentration of 16 mg/mL, and the turbid solutions were submitted to one freeze–thaw cycle after which they became clear. The freeze–thaw cycle was not necessary in the case of DMPC. CHS was used as a substitute for cholesterol (Yuli et al., 1981), since the latter could not be dissolved in cholate micellar solutions. CHS, however, could be easily dissolved in 10% sodium cholate at pH 12 at a concentration of 20 mg/mL but not at neutral pH (Criado et al., 1982). Once CHS was dissolved in cholate micellar solutions, the pH was rapidly reduced to 7.6. The mixtures containing lipid, protein, and cholate were dialyzed over a period of 48 h against two changes of 500 volumes each of 100 mM NaCl, 0.1 mM phenylmethanesulfonyl fluoride, and 10 mM sodium phosphate, pH 7.4. The dialyzed material was diluted 5 times with the dialysis buffer and centrifuged in the airfuge at 140000*g* for 60 min. The pelleted proteoliposomes were resuspended in the same buffer and assayed for bungarotoxin binding ability according to the method of Schmidt & Raftery (1973) and for protein according to the method of Lowry et al. (1951).

Preparation of Slides for FRAP Experiments. Slides for FRAP experiments were prepared essentially by using the method of Vaz et al. (1981). A 50-μL aliquot of the soft proteoliposome pellet from the preceding section containing 2–4 mg of lipid was deposited on a cleaned microscope slide and allowed to dehydrate over a 12-h period at 35 °C in a water-saturated nitrogen atmosphere. The partially dehydrated film was brought back to conditions of excess water by dropping a cover slip over the residue with a hanging drop of 50 μL of distilled water. The excess water was drained with a filter paper wick, and the slide was sealed with wax and incubated for 12 h at 35 °C. Multilayer domains and large proteoliposomes formed after this incubation period. FRAP experiments were performed on the sealed slides a minimum of 12 h and a maximum of 4 days after sealing. Slides were stored at 35 °C for DMPC and 4 °C for all other lipids. One criterion used for determining whether a slide was "good" or not was the absence of patches of intense fluorescence due to labeled protein which did not recover their fluorescence intensity after photobleaching. Such patches were taken to be due to precipitation of the protein. When this criterion was used, it was not possible to prepare useable slides from proteoliposomes of DMPC containing the AchR dimer. The dimer, however, gave suitable slides when the host lipid was SBL.

FRAP Experiments. These were performed on a microscope fluorometer essentially similar to that described by Axelrod et al. (1976a) and by Jacobson et al. (1976). Our instrument consists of a Zeiss Universal research fluorescence microscope fitted with an RCA C31034A photomultiplier operated at −30 °C. Data collection is done by photon counting. The light source is a Spectra Physics Model 165 argon ion laser (Spectra

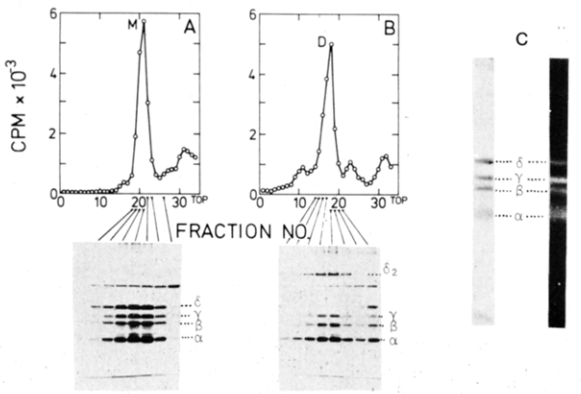

FIGURE 1: Isolation and purification of AchR monomer (panel A) and dimer (panel B) on a continuous 5–20% sucrose gradient in the presence of 1% sodium cholate. The sodium dodecyl sulfate–polyacrylamide gel electrophoretic patterns of relevant gradient fractions are shown below each panel. Panel C shows the electrophoretic pattern of fluorescein isothiocyanate labeled AchR monomer stained with Coomassie Blue and due to fluorescein fluorescence. It is seen that all four AchR subunits are labeled.

Physics, Darmstadt, F.R.G.) which was operated at 200 mW by using the 488-nm line. Beam attenuation is done by using a combination of two uncoated beam splitting plates as described by Koppel (1979). The bleaching beam is turned on and off by means of a shutter [Vincent Associates, Rochester, NY, Model 23X2A(Z)X5] which allows pulses of ≥1 ms to be used. Most photobleaching experiments used a Gaussian beam profile with a $1/e^2$ radius of 5.3 μm using a Zeiss Neofluar 16/0.4 objective. Evaluation of diffusion coefficients from the fluorescence recovery curves was done by using the three-point analysis method using half-times for complete fluorescence recovery as described by Axelrod et al. (1976a). Sample temperature was regulated by a thermoelectric microscope stage (Cambridge Thermionic Corp., Cambridge, MA) and was accurate to ±0.5 °C.

Results

Preparation of AchR-Containing Lipid Bilayers. The AchR protein used in the FRAP experiments was obtained in a three-step purification involving solubilization of the AchR-rich membranes with 2% sodium cholate, a short high-speed centrifugation, and a subsequent sedimentation of the extract in a linear 5–20% sucrose density gradient in the presence of 1% sodium cholate. Under such conditions, almost pure 9S monomer could be obtained from the dithiothreitol-reduced AchR membranes (Figure 1A), and predominantly 13S dimeric species was obtained from nonreduced, alkylated membrane extracts (Figure 1B). Both the monomer and dimer gradient peaks showed the four characteristic AchR subunits in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Figure 1). Only a small percentage of a contaminating high molecular weight polypeptide was present in both preparations. The main difference between the monomeric and dimeric preparations lay in the absence of the δ_2 band in the monomer and its presence in the dimer and, conversely, the absence of the δ subunit in the dimer. For labeling of the AchR with fluorescein isothiocyanate, only the three fractions of the gradient showing maximal radioactivity were used. In Figure 1C it is seen that labeling of the AchR in cholate micelles resulted in all four of its subunits being labeled. An average dye/protein molar ratio of about 19/1 was obtained on the basis of a molar extinction coefficient of 8.7×10^4 cm^{-1} for fluorescein. This high degree of labeling affected neither the ability of the AchR to bind [^{3}H]-α-bungarotoxin nor the oligomeric state (monomer–dimer) of the protein.

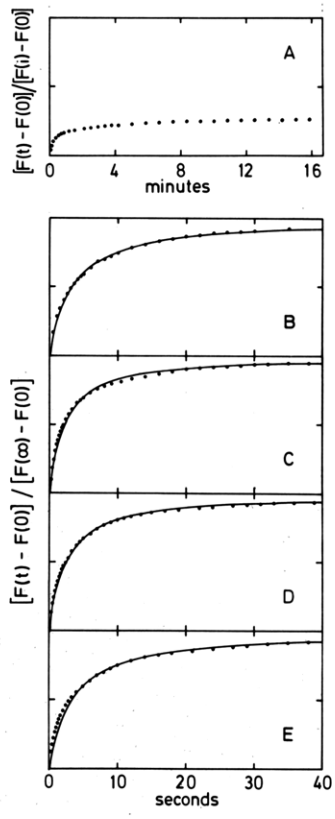

FIGURE 2: Typical fluorescence recovery curves after photobleaching. (A) AchR monomer in DMPC multibilayers at 22.8 °C; (B) AchR monomer in DMPC multibilayers at 30 °C; (C) AchR monomer in DMPC multibilayers which contain 45 mol % CHS at 30 °C; (D) AchR monomer in SBL multibilayers at 30 °C; (E) AchR dimer in SBL multibilayers at 30 °C. The points are experimental and the lines are theoretical recovery curves for one diffusing component. $F(i)$, $F(0)$, $F(t)$, and $F(\infty)$ are fluorescence intensities before photobleaching, immediately after photobleaching, at time t after photobleaching, and at "infinite" time after photobleaching, respectively. $F(0)/F(i)$ values were between 0.36 and 0.54. $F(\infty)$ varied between 0.95 and 1.0. The scale on the y-axis is from 0.0 to 1.0 in all cases.

At the high lipid/protein ratios used in the reconstitution by cholate dialysis, all of the added protein sedimented with the phospholipid. The final molar ratio of lipid/protein in the resultant proteoliposomes was between 5000/1 and 10000/1. No differences were observed up to this stage in the reconstitution of the dimeric and monomeric AchR. In attempts to prepare slides for FRAP experiments, however, the proteoliposomes of dimer in DMPC showed a marked tendency of the protein to aggregate. This occurred even when *N*-ethylmaleimide was present throughout the experimental procedure. The aggregation behavior of the dimeric protein in DMPC is, therefore, probably not due to cross-linking of dimers through free sulfhydryl groups. A similar observation has been made by us in the case of the sarcoplasmic reticulum Ca^{2+}-activated adenosinetriphosphatase in phosphatidylcholine bilayers (W. L. C. Vaz and V. M. C. Madeira, unpublished results). The reason for this behavior, seen with some proteins in some lipid systems and not in others, is not yet clear. This difficulty was not encountered in the case of the AchR monomer in all the lipids reported on here or with the dimer in SBL.

FRAP Measurements. FRAP measurements were mostly done on large (>100 μm across) multilayer domains but were also sometimes done on large liposomes which appeared to be paucilamellar according to criteria described earlier (Vaz et al., 1981). Typical curves for fluorescence recovery after photobleaching are shown in Figure 2. In all the bilayers,

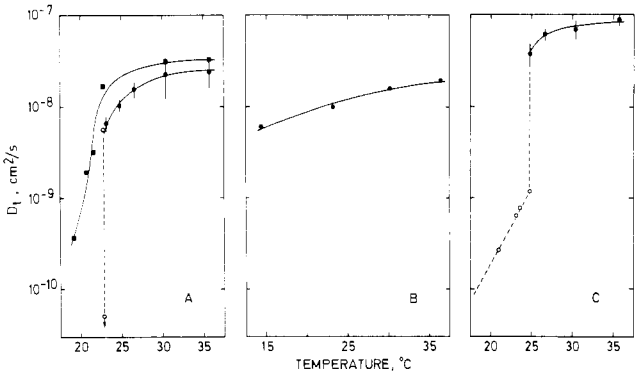

FIGURE 3: Temperature dependence of D_t for (A) the AchR monomer in DMPC multibilayers (●) and paucilamellar liposomes (■), (B) the AchR monomers in multibilayers of DMPC containing 45 mol % CHS, and (C) the lipid probe, NBD-DMPE, in DMPC multibilayers. In (A) and (C), the open symbols are to indicate that the fluorescence recovery curves in these cases were multicomponent and the half-times for fluorescence recovery were used to calculate D_t. These values do not, therefore, represent the true values of D_t. The broken lines are used to indicate this fact.

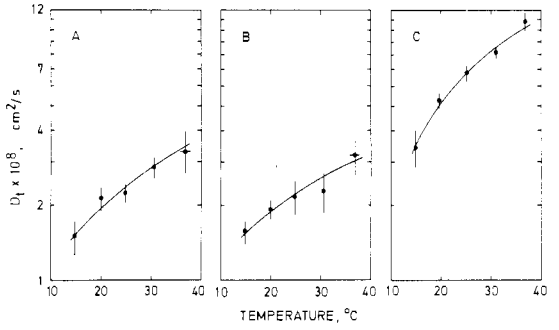

FIGURE 4: Temperature dependence of D_t for (A) the AchR monomer in SBL multibilayers, (B) the AchR dimer in SBL multibilayers, and (C) the lipid probe, NBD-DMPE, in SBL multibilayers.

examined in the liquid-crystalline phase, the experimental recovery curves were reasonably well fit by theoretical curves for fluorescence recovery due to redistribution of a single diffusing species. In multibilayers of DMPC at 22.8 °C, i.e., below the gel–liquid-crystalline phase transition temperature, however, this was not the case (see Figure 2A). This behavior has been reported for lipid probe diffusion (Derzko & Jacobson, 1980) and for the diffusion of other integral membrane proteins (Vaz et al., 1981).

Diffusion in DMPC Bilayers. Figure 3A shows the temperature dependence of D_t for the AchR monomer in pure DMPC multibilayers and large (>30 μm diameter) paucilamellar proteoliposomes. In the liquid-crystalline phase ($T >$ 24 °C), there was no significant difference between protein diffusion rates in the liposomes and in multibilayers. D_t changed continuously from $(2.3 \pm 0.8) \times 10^{-8}$ cm²/s at 36 °C to $(1.0 \pm 0.1) \times 10^{-8}$ cm²/s at 25 °C. In multibilayers, a change from fast diffusion to slow diffusion occurred abruptly between 23 and 24 °C. At 22.8 °C in the multibilayers, the multicomponent fluorescence recovery curve showed D_t of less than 5×10^{-11} cm²/s for about 75% of the diffusant and D_t of about 7×10^{-9} cm²/s for about 25%. In the paucilamellar proteoliposomes, the transition from the fast diffusing state to the slow diffusing state occurred over a broader range of temperatures and was not yet complete at 19 °C. The reason for this difference is not clear. In previous work we have argued in favor of an "ice-breaker" effect to explain rapid protein diffusion in the lipid gel phase (Vaz et al., 1981, 1982a). We speculate that the reason why the ice-breaker effect is not evident in multilayer systems is that the reduction of the interlamellar space and increase in rigidity of the phospholipid bilayers in the gel phase may squeeze the rather large hydrophilic portions of membrane-bound proteins between two nonplastic gel phase bilayers and consequently result in an abrupt change in its diffusion behavior. Figure 3B shows D_t for the AchR monomer as a function of temperature in multilayers of DMPC containing 45 mol % CHS. D_t changes monotonically in this case from 6×10^{-9} cm²/s at 14 °C to 2×10^{-8} cm²/s at 36 °C. As expected from the known effects of cholesterol upon phospholipid bilayers, and as specifically reported for CHS (Criado et al., 1982), no phase transition behavior is seen in this case. The values of D_t in liquid-crystalline multibilayers of DMPC are similar to the values of D_t in CHS-containing DMPC multilayers at comparable temperatures. A similar effect was observed for the diffusion of the M-13 viral coat peptide with 45 mol % cholesterol in DMPC by Smith et al. (1980). For comparison we have shown the temperature dependence of D_t for the lipid probe NBD-DMPE in pure DMPC multibilayers in Figure 3C. Our value for lipid probe D_t at 30 °C in pure DMPC bilayers is about 7×10^{-8} cm²/s. This agrees reasonably well with values reported by other authors for other lipid probes (Wu et al., 1977; Rubenstein et al., 1980; Derzko & Jacobson, 1980; Chang et al., 1981). For lipid diffusion we observe, in agreement with the observations of previous workers, that the change in D_t for lipid from values which are characteristic for the liquid-crystalline phase to those that are characteristic for the gel phase occurs sharply at about 25 °C. Below 25 °C (data not shown) the lipid fluorescence recovery curves showed multicomponent behavior. This was reported earlier by Derkzo & Jacobson (1980).

Diffusion in SBL Bilayers. Figure 4A shows the temperature dependence of D_t for the AchR monomer in bilayers of SBL. These lipids show no phase transition in the temperature range examined in this work. The values of D_t for the protein were quite similar to those seen in liquid-crystalline phase DMPC at the same temperatures. A monotonic change in D_t from $(1.5 \pm 0.2) \times 10^{-8}$ cm²/s at 15 °C to $(3.3 \pm 0.7) \times 10^{-8}$ cm²/s at 37 °C was seen. Figure 4B shows the diffusion behavior of the AchR dimer in the same lipid. It is noteworthy that the values of D_t for the monomeric and dimeric proteins were very similar in the entire temperature range studied. It cannot be ruled out that the monomeric protein associates in a noncovalent manner so as to form oligomeric species in the lipid bilayers. However, as seen in Figure 1, no tendency for aggregation of the monomer was evident in cholate micelles. Also, electron microscopic studies on AchR reconstituted at high lipid/protein ratios indicate that aggregation of monomers to oligomers is not likely (Cartaud et al., 1980). We therefore consider the dimerization of the reduced and alkylated monomeric AchR in SBL bilayer to be unlikely. In Figure 4C the temperature dependence of D_t for NBD-DMPE in SBL bilayers is shown for comparison. The value of D_t for this lipid probe changed monotonically from $(3.4 \pm 0.6) \times 10^{-8}$ cm²/s at 15 °C to $(1.1 \pm 0.1) \times 10^{-7}$ cm²/s at 37 °C. These values of lipid D_t are similar to those seen in liquid-crystalline phase DMPC bilayers at the same temperatures (see Figure 3C).

Discussion

At high dilution the rate of diffusion of the AchR protein, in its monomeric and dimeric states, in liquid-crystalline phase lipid bilayer membranes is quite high. Typical values for the protein D_t lie between 1×10^{-8} and 3×10^{-8} cm²/s in the temperature range examined in this work. These values are in agreement with the diffusion rates that have been reported

for other membrane-bound proteins in model systems [for a review, see Vaz et al., (1982a); see also Chang et al. (1981); Peters & Cherry, 1982; Vaz et al., 1982b]. In bilayers of SBL, the AchR monomer and dimer have similar values of D_t over the entire temperature range examined (14–37 °C). An attempt to understand this result must take into account what is known about the structure of this protein. On the basis of low-angle neutron scattering results on the AchR monomer in micelles of Triton X-100, Wise et al. (1979) have suggested that the most likely structure of this protein may be modeled by three stacked concentric cylinders with diameters of 90, 30, and 60 Å and heights of 40, 55, and 20 Å, respectively. The portion of the protein that lies embedded in the lipid bilayer, according to this model, is a cylinder with diameter and height of 30 and 55 Å, respectively. These dimensions are compatible with studies on the rotational diffusion of this protein in native membranes (Bartholdi et al., 1981). Neutron-scattering experiments on AchR dimers in detergent solutions also suggest that the center to center spacing between the monomers is about 80 Å (Wise et al., 1979). Therefore, the protein dimer could be visualized as a structure in which there could be sufficient space between the membrane-bound portions of the constituent monomers to give a "porous" structure through which the molecules of the lipid "solvent" could drain more or less freely [for a treatment of the diffusion of porous particles, see Wiegel (1980)]. Alternatively, if it is assumed that the draining of the lipids is not possible in the case of the dimer, the fact that the monomeric and dimeric protein molecules have similar values of D_t, notwithstanding their large difference in size, may be taken as evidence in support of the applicability to membranes of the diffusion model of Saffman and Delbrück (Saffman & Delbrück, 1975; Saffman, 1976). This model proposes that the translational diffusion of cylindrical particles in fluid viscous sheets, where the particle is oriented with its long axis parallel to the normal of the sheet and where the height of the particle is equal to the thickness of the sheet, is a relatively insensitive function of particle radius. The assumption has to be made in this case that the frictional drag experienced by the hydrophilic portions of the protein is negligible in comparison with the frictional drag experienced by the membrane-bound portion. Some evidence exists to indicate that this may be true for diffusion in membranes (Smith et al., 1979a).

It is of interest to compare our results on the translational diffusion of the AchR protein in model membrane systems with reports on its translational diffusion behavior in natural membranes (Axelrod et al., 1976b, 1978a,b; Poo, 1982; Tank et al., 1982). In the plasma membranes of developing rat and chick muscle fibers, two AchR populations are observed under the light microscope (Axelrod et al., 1976b): a diffusely distributed population which shows a D_t of about 5×10^{-11} cm^2/s and a "patched" population which apparently does not diffuse. These values were obtained by using the FRAP technique at 22 °C. Axelrod and co-workers have also shown that treatment of the myotubes with agents that disrupt the cytoskeleton does not have any effect upon diffusion of the AchR in diffuse zones (Axelrod et al., 1978a). Also, alteration of the membrane lipid composition was shown to be without effect (Axelrod et al., 1978b). In all this work, diffuse zones were defined as those that showed a uniform fluorescence distribution upon labeling with fluorescently labeled α-bungarotoxin. Since the criterion for diffuseness was an optical one, the occurrence of considerably large AchR aggregates in these diffuse zones, not visible as such under the light microscope, cannot be ruled out. Two recent studies on the diffusion of the AchR in natural membranes (Tank et al., 1982; Poo, 1982) have appeared. In "blebs" of myoblast plasma membranes, Tank et al. (1982) report D_t for this protein to be 3×10^{-9} cm^2/s. Using an electrophysiological approach to detect diffusion, Poo (1982) has reported D_t for the AchR in the plasma membrane of *Xenopus* embryonic muscle fibers to be about 2.6×10^{-9} cm^2/s. Both these reports differ from the D_t reported by Axelrod et al. (1976b, 1978a,b) by a factor of about 50×. The possible reasons for this difference have been discussed elsewhere (Tank et al., 1982; Poo, 1982). In any case, D_t for the AchR at high dilution in model membranes is at least a factor of 10 × higher than the highest value of D_t reported for this protein in natural membranes. Two possibilities for this difference are suggested: First, the presence of cholesterol in the plasma membranes could alter the fluidity characteristics of these membranes so that a pure phosphatidylcholine membrane may not be an adequate model. However, our results show that D_t for the protein in DMPC bilayers which contain 45 mol % of the cholesterol derivative, CHS, is not significantly different from D_t for the protein in pure DMPC bilayers. This is possibly the case for all integral membrane proteins [see, for example, Smith et al. (1980)]. We, therefore, do not believe that cholesterol alone can account for the difference in D_t for the protein in the model systems and natural membranes. Second, the model system work reported on here was done at high dilutions of the protein in the lipid bilayers (molar ratios of lipid/protein between 1000/1 and 10000/1). In contrast, plasma membranes of cells generally have considerably high protein contents (about 50 wt %). The differences between our results on the one hand and the results of Poo (1982) and Tank et al. (1982) on the other may be predominantly due to differences in membrane protein concentrations in the two cases. A rigorous test of this hypothesis was not possible in the reconstituted systems since protein precipitation in the bilayers began to become evident at lipid/protein molar ratios below 1000/1. In this respect, we also find that there is about a 6-fold difference between D_t for bovine rhodopsin at high dilution in liquid-crystalline phase phosphatidylcholilne bilayer membranes (Vaz et al., 1982b) and the value of D_t for this protein in visual disk membranes (Poo & Cone, 1974; Wey et al., 1981). Similarly, Chang et al. (1981) report a value of D_t of about 2×10^{-8} cm^2/s for erythrocyte band 3 protein in liquid-crystalline phase DMPC bilayers, a value which is about 10-fold higher than D_t for this protein in erythrocyte ghost membranes in the absence of the spectrin network (Sheetz et al., 1980; Golan & Veatch, 1980). From these results, we suggest that diffusion of membrane proteins with molecular radii of about 15–25 Å will, in the absence of other interactions, show a value of D_t of about $(1–3) \times 10^{-8}$ cm^2/s at high dilution in phospholipid membranes and values about 10-fold lower at protein concentrations in the membrane similar to those found in natural membranes.

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