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Average Membrane Penetration Depth of Tryptophan Residues of the Nicotinic Acetylcholine Receptor by the Parallax Method†

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ABSTRACT: The membrane penetration depths of tryptophan residues in the nicotinic acetylcholine receptor from *Torpedo californica* have been analyzed in reconstituted membranes containing purified receptor and defined lipids. Dioleoylphosphatidylcholine and three spin-labeled phosphatidylcholines with the nitroxide group at three different positions on the fatty acyl chain were used for reconstitution of the receptor. The spin-labeled phospholipids serve as quenchers of tryptophan fluorescence. Differential quenching of the intrinsic fluorescence of the acetylcholine receptor by the spin-labeled phospholipids has been utilized to analyze the average membrane penetration depth of tryptophans by the parallax method [Chattopadhyay, A., & London, E. (1987) *Biochemistry 26*, 39–45]. Analyses of the quenching data indicate that the tryptophan residues on the average are at a shallow location (10.1 Å from the center of the bilayer) in the membrane. In addition, the generally low levels of quenching imply that the majority of tryptophan residues are located in the putative extramembranous region of the receptor. These results are consistent with several proposed models for the tertiary structure of the acetylcholine receptor and are relevant to ongoing analyses of the overall conformation and orientation of the acetylcholine receptor in the membrane.

Due to the inherent difficulty of crystallizing membrane proteins, most analyses of membrane protein structure have utilized indirect biophysical techniques with an emphasis on spectroscopic methods. One such analysis involves determination of membrane penetration depth, the location of a molecule or a specific site within a molecule in relation to the membrane surface. Knowledge of the precise depth of a membrane-embedded group or molecule helps define the conformation and topology of membrane proteins and probes. Fluorescence has been one of the most widely used techniques to determine depth. Both long-range dipole–dipole (Forster)

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energy transfer (Shaklai et al., 1977; Koppel et al., 1979; Dewey & Hammes, 1980; Sklar et al., 1980; Johnson et al., 1984; Baird & Holowka, 1985; Kleinfeld, 1985) and shorter range fluorescence quenching caused by spin-labels [see reviews by London (1982) and Blatt and Sawyer (1985)] or brominated probes (Kao et al., 1978; Markello et al., 1985; Jain et al., 1985; Lala et al., 1988) have been used. Chattopadhyay and London developed a method for measurement of membrane penetration depth utilizing fluorescence quenching by spin-labeled phospholipids and applied it to determine penetration depths of the fluorescent groups in a series of 7-nitro-2,1,3-benzoxadiazol-4-yl (NBD)[1]-labeled lipids (Chattopadhyay & London, 1987; Chattopadhyay, 1990). The method involves determination of the parallax in the apparent location of fluorophores detected when quenching by phospholipids spin-labeled at two different depths is compared. The location of tryptophans in membrane-bound annexins, a class of calcium-dependent membrane binding proteins, has been determined by using this method (Meers, 1990). Advantages of this method are its wide applicability to reconstituted systems and the ability of spin-labels to quench virtually all types of fluorophores, including tryptophans (London, 1982).

The complexity of membrane protein structure is aptly exemplified by the *Torpedo californica* nicotinic acetylcholine receptor (AChR), a transmembrane glycoprotein with 5 subunits and a molecular weight of 290 000 [see Ochoa et al. (1989), Stroud et al. (1990), and Pradier and McNamee (1991)]. The AChR is a representative member of the superfamily of chemically gated ion channel receptors (Schofield et al., 1987). The receptor is an essential component in cholinergic synaptic transmission and serves a recognition, regulatory, and ion channel role at postsynaptic membranes. The relative ease with which AChR can be isolated and purified from *Torpedo* species electroplax in large quantities makes the *Torpedo* receptor an excellent model for detailed studies of both receptor function and membrane protein structure. Key biophysical studies on AChR have been performed by isolating and purifying the receptor from its native membrane and then reconstituting it into bilayers of defined lipid environments [see McNamee et al. (1986)].

The detailed molecular structure of the AChR is not known, and attempts to crystallize the protein in an active form are under way in various laboaratories [see Stroud et al. (1990)]. There have been several folding models proposed for the receptors, and these do not totally agree on the number of crossings of the transmembrane regions of the protein [see McCrea et al. (1987), DiPaola et al. (1989), and Pedersen et al. (1990)]. However, all these models propose at least four transmembrane α-helices per subunit, some of which are thought to form the ion channel. The five subunits are arranged in a rosette to form a central pore, which is presumed to serve as the ion channel.

In this study, the parallax method is used to analyze the penetration depth of the membrane-embedded tryptophan residues in AChR from *Torpedo californica* reconstituted into membranes containing varying amounts of dioleoylphosphatidylcholine (DOPC) and spin-labeled phospholipids. The results indicate that the tryptophans are at a shallow location in the membrane and that most of the tryptophans in AChR are located in the putative extramembranous region.

Materials and Methods

Materials. Spin-labeled phosphatidylcholines and DOPC were purchased from Avanti Polar Lipids (Birmingham, AL). Affi-Gel 10 was from Bio-Rad (Richmond, CA). Lipids were analyzed for purity by TLC on silica gel plates (Alltech Associates, Deerfield, IL) in chloroform/methanol/water (65:35:5 v/v). DOPC and spin-labeled PCs gave one spot with a phosphate-sensitive spray and subsequent charring (Dittmer & Lester, 1964). Very pure Ultrol-grade sodium cholate from Calbiochem (San Diego, CA) was used for reconstitution of AChR in lipid bilayers. All other chemicals used were reagent grade.

Purification of Acetylcholine Receptor. AChR was purified from frozen electric tissue of *Torpedo californica* by affinity chromatography as described by Jones et al. (1987) with some modifications. *Torpedo* electroplax tissue was obtained from Dr. Howard H. Wang, University of California, Santa Cruz. First, a crude membrane preparation partially enriched in AChR was prepared as described by Jones et al. (1987) with a slight modification of the homogenization buffer (20 mM MOPS, 5 mM EDTA, 5 mM EGTA, and 0.02% sodium azide, pH 8.0). AChR was purified from AChR-rich membranes by affinity chromatography using a modified Affi-Gel 10 resin that had been reacted with bromoacetylcholine (Bhushan & McNamee, 1990).

Crude membranes were diluted with buffer A (10 mM MOPS, 100 mM NaCl, 0.1 mM EDTA, and 0.02% sodium azide, pH 7.4) to a protein concentration of 2 mg/mL and solubilized by adding sodium cholate with gentle stirring, to a final concentration of 1.2% (w/v) cholate. The mixture was centrifuged in a Beckman type 35 rotor at 34 000 rpm for 45 min at 4 °C. The supernatant was filtered through four layers of cheesecloth, pooled, and applied to the affinity column. The column was preequilibrated with 3 column volumes of a solution containing 2 mg/mL DOPC and 1% cholate in buffer A and allowed to stand for 2 h to ensure complete exchange of native lipids with DOPC. The column was then washed with 50 mL of 1 mg/mL DOPC and 1% cholate in buffer A, followed by 50 mL of 0.1 mg/mL DOPC and 0.5% cholate in buffer A. AChR was then eluted by applying 50 mL of a final wash containing 10 mM carbamylcholine as eluent. Protein concentration was determined by monitoring the absorbance at 280 nm, and fractions were pooled to achieve a protein concentration of about 1 mg/mL. Cholate and carbamylcholine were removed by dialysis for 48 h at 4 °C against 4 L of buffer A with three changes of buffer. Lipid–AChR complexes were kept frozen in liquid nitrogen.

Reconstitution of Purified AChR with Spin-Labeled Phospholipids. The above purification procedure typically yields membranes with a lipid to protein ratio of 200–300 (mol/mol). To reconstitute the purified AChR with varying amounts of spin-labeled phospholipids and DOPC, membranes were resolubilized and additional lipids were added to give membranes with higher lipid to protein ratios. By varying the relative amounts of additional lipids (DOPC and any one of the spin-labeled lipids), membranes with different amounts of spin-labeled lipids, but with the same overall lipid to protein ratio (400–600), were prepared. At this range of lipid to protein ratio, the AChR exists in bilayer sheets rather than in closed vesicles. For the preparation of such samples, calculated amounts of spin-labeled lipids and DOPC were dis-

[1] Abbreviations: AChR, nicotinic acetylcholine receptor; DOPC, dioleoyl-*sn*-glycero-3-phosphocholine; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EPR, electron paramagnetic resonance; ^{125}I-BgTx, ^{125}I-monoiodinated α-bungarotoxin; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NBD, 7-nitro-2,1,3-benzoxadiazol-4-yl; PC, phosphatidylcholine; 5-PC, 1-palmitoyl-2-(5-doxyl)stearoyl-*sn*-glycero-3-phosphocholine; 10-PC, 1-palmitoyl-2-(10-doxyl)stearoyl-*sn*-glycero-3-phosphocholine; 12-PC, 1-palmitoyl-2-(12-doxyl)stearoyl-*sn*-glycero-3-phosphocholine; TLC, thin-layer chromatography.

solved in buffer A containing 2% cholate and then mixed with the purified receptor in DOPC. The mixture was allowed to equilibrate on ice for 1 h and then dialyzed at 4 °C against 4 L of buffer A for 48 h with two changes and then against 4 L of fluorescence buffer (10 mM MOPS, 100 mM NaCl, and 1 mM EDTA, pH 7.4) for 24 h. As a precaution, samples with and without spin-labeled lipids were dialyzed separately. Samples were used immediately for fluorescence measurements instead of freezing and thawing.

Fluorescence. Steady-state fluorescence was measured with an SLM 4800 spectrofluorometer operating in the ratio mode and using 1-cm path-length quartz cuvettes. An internal correction was made for changes in lamp intensity by using a reference solution of rhodamine B (3 g/L) in ethylene glycol. The temperature of the samples was maintained at 25 ± 0.5 °C by a heating–cooling water bath (Forma Scientific, Marietta, OH). An excitation wavelength of 295 nm was used to selectively excite only the tryptophan residues (Teale, 1960; Longworth, 1971). Emission was monitored at 336 nm. Excitation and emission slits with a nominal band-pass of 4 nm were used. For fluorescence measurements, samples containing about 50 μg of AChR were diluted to 2.5 mL with fluorescence buffer and incubated for 10 min prior to spectral recording. Optical density and inner filter effects were negligible.

AChR Assays. The reconstituted samples were assayed for equilibrium binding of ^{125}I-monoiodinated α-bungarotoxin, as described previously (Jones et al., 1987). The function of the reconstituted AChR was assessed by monitoring the influx of $^{86}Rb^+$ into liposomes containing AChR in response to carbamylcholine, as described previously (McNamee et al., 1986). For these manual ion flux experiments, it was necessary to re-reconstitute the AChR with asolectin (Associated Concentrates, Woodside, NY) so as to generate morphologically closed vesicles with lipid to protein molar ratios of 6000–8000. The membranes containing additional asolectin were solubilized in 2% cholate, and then cholate was removed by dialysis, as described above.

Sucrose Density Gradient Centrifugation. After reconstitution, the incorporation of AChR into membranes was monitored by discontinuous flotation gradients. Sucrose gradients (20–55% w/w in buffer A) containing reconstituted membranes were prepared in Beckman SW-60 cellulose nitrate tubes. Less than 5% of the AChR α-bungarotoxin binding sites were labeled with ^{125}I-BgTx before the membranes were loaded at the bottom of the gradients. Samples were centrifuged for 20 h at 55 000 rpm in an SW 60 rotor, and 200-μL fractions were collected manually. Fraction densities were measured with a Bausch and Lomb refractometer. AChR was monitored by measuring the ^{125}I-BgTx cpm in a Packard γ scintillation counter. The gradient fractions were then diluted with water and dialyzed to remove sucrose (sucrose interferes with the lipid phosphate assay), and the lipid phosphate content was measured for each fraction (McClare, 1971).

Other Assays. The concentrations of the spin-labeled PCs and DOPC were checked by phosphate assay subsequent to total digestion with perchloric acid (McClare, 1971). The actual number of spins per spin-labeled lipid was calculated by doubly integrating the EPR signal of these lipids in an organic solvent and by comparison to spin-label standards, as described previously (Chattopadhyay & London, 1987). Protein concentration was determined by the Lowry method (Lowry et al., 1951).

Electron Microscopy. Samples were examined by negative-stain electron microscopy to determine the morphology of reconstituted membranes. Ten-microliter samples were applied to 400-mesh copper grids which had been coated with parlodian, shadowed with carbon, and glow-discharged to render them hydrophilic. The samples were blotted, washed, stained with 0.5% uranyl acetate, and examined in a Philips TEM-400 electron microscope at 100 kV.

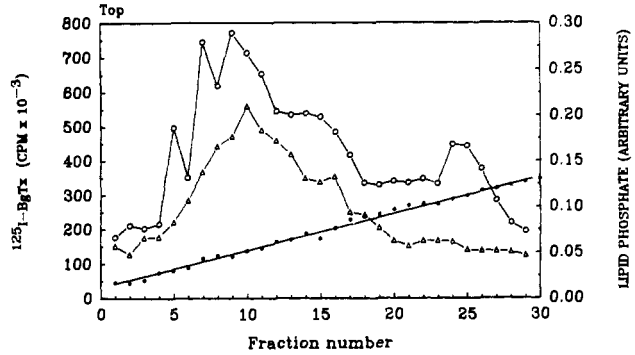

FIGURE 1: Sucrose density gradient analysis of reconstituted membranes. (●) Percent sucrose (w/w) was determined by the refractive index and ranged from 0.7% to 42.2% at the end points; (○) AChR concentration determined by the ^{125}I-BgTx equilibrium binding assay. (△) Lipid concentration determined by phosphate assay. The lipid to protein molar ratio was 450:1. See Materials and Methods for other details.

Results

Characterization of Reconstituted Membranes. Incorporation of AChR into membranes was determined by subjecting the reconstituted samples to discontinuous sucrose density gradients. It has been shown that in reconstituted membranes of variable lipid composition at a low lipid to protein ratio AChR is indeed associated with lipids (Ellena et al., 1983; Fong & McNamee, 1986). To verify that the same is true in reconstituted membranes supplemented with spin-labeled phosphatidylcholines, reconstituted membranes containing 50 mol % spin-labeled lipid were analyzed by 20–55% sucrose density gradient centrifugation. The sucrose density gradient pattern of the reconstituted sample is shown in Figure 1. The gradient exhibits coincident peaks of both α-bungarotoxin binding activity and total phosphate, indicating that AChR is firmly associated with the added lipids.

The morphology of the reconstituted membranes was studied by electron microscopy of negatively stained samples. At low lipid to protein ratio, the receptor appears in membrane patches, termed "bilayer sheets", with no evidence for a population of membrane vesicles (Earnest et al., 1987; Jones et al., 1988). The reconstituted membranes, when reconstituted with DOPC as the additional lipid, showed typical bilayer sheet structure. On reconstitution with any of the spin-labeled lipids (e.g., 5-PC or 10-PC) instead of DOPC, almost identical electron micrographs were obtained without any change in the basic morphological features (data not shown). There was very little contamination by either free receptors or lipid vesicles devoid of receptors, which are often observed in samples made at higher lipid to protein ratios (Earnest et al., 1987; Jones et al., 1988).

The functional integrity of AChR reconstituted with spin-labeled lipids and DOPC was assessed by the manual ion flux assay using $^{86}Rb^+$ as the tracer cation (McNamee et al., 1986). Sealed vesicles are required to detect the ion-gating activity of AChR-containing membranes in such an assay. However, samples used in our fluorescence quenching experiments were in the range of lipid to protein ratios in which the reconstituted membranes were predominantly bilayer sheets with no measurable trapped volume (Earnest et al., 1987) and thus

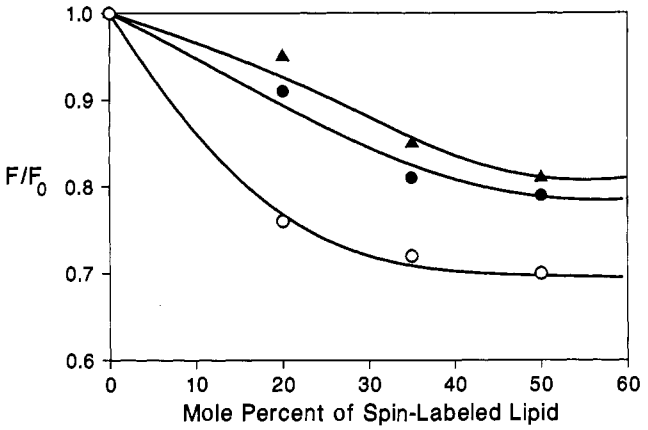

FIGURE 2: Fluorescence quenching of AChR reconstituted into bilayers containing one of the three spin-labeled phospholipids and DOPC. The spin-labels used were 5-PC (○), 10-PC (●), and 12-PC (▲), respectively. The abscissa is the mole fraction of spin-labeled lipid in the reconstituted sample. The ordinate is the ratio of fluorescence in the presence (F) and absence (F_0) of the spin-labeled lipid. See Materials and Methods for details of sample preparation. The total lipid to protein molar ratio was 470:1, and the concentration of AChR was 0.07 μM.

could not be used directly. Samples were re-reconstituted in asolectin at a higher lipid to protein ratio by the cholate dialysis procedure. These samples showed flux responses typical of fully functional receptor, indicating that the spin-label treatments did not lead to any irreversible changes in receptor structure.

Interaction of AChR with Spin-Labeled Lipids. The acetylcholine receptor is a multisubunit protein containing 51 tryptophans and 92 tyrosines (Noda et al., 1982, 1983a,b) and is thus intrinsically fluorescent. The fluorescence of the AChR is predominantly due to its tryptophan residues (Barrantes, 1978). Nevertheless, we used an excitation wavelength of 295 nm to selectively excite only the tryptophan residues (Teale, 1960; Longworth, 1971). The fluorescence properties of the receptor in reconstituted membranes are similar to those in the native lipid environment and are typical of those found for integral membrane proteins (Jones & McNamee, 1988).

In previous studies, it has been shown that the fluorescence of AChR can be partially quenched by spin-labeled fatty acids incorporated into native membranes (Marsh & Barrantes, 1978; Barrantes, 1978) and by brominated lipids in reconstituted membranes (Jones & McNamee, 1988). Such quenching in the membrane is predominantly static in nature (London, 1982). Figure 2 shows the fluorescence quenching curves obtained with AChR reconstituted in membranes containing varying mole percents of DOPC and one of the three spin-labeled lipids. Points above 50 mol % of spin-labeled lipids are not shown since very little additional quenching was obtained. Maximum quenching of tryptophan fluorescence of the AChR occurs with 5-PC as the quenching lipid. The least amount of quenching is obtained with 12-PC, whereas the quenching behavior of 10-PC is intermediate. This differential quenching behavior implies that the tryptophan residues, on the average, are at a shallow location in the membrane. This conclusion is reinforced by depth calculations.

Depth of Membrane-Embedded Tryptophan Residues. Depth of fluorophore was calculated by use of the equation developed by Chattopadhyay and London (1987):

$$z_{cF} = L_{c1} + \{[(-1/\pi C) \ln (F_1/F_2) - L_{21}^2]/2L_{21}\}$$

where z_{cF} = the depth of the fluorophore from the center of the bilayer, L_{c1} = the distance of the center of the bilayer from the shallow quencher 1, L_{21} = the difference in depth between

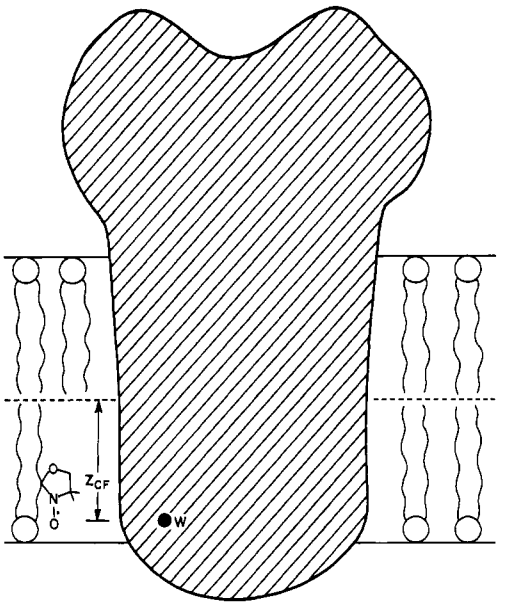

FIGURE 3: Schematic diagram illustrating the parallax method as applied to the AChR. The dashed line in the middle indicates the center of the bilayer. W corresponds to the average location of membrane-embedded tryptophans in AChR, and z_{cF} is the average depth of tryptophans from the center of the bilayer.

Table I: Average Depth of Accessible Tryptophan Residues in AChR[a]

spin-labeled PC pair used for quenching analysis	calcd distances from bilayer center, z_{cF} (Å)	av z_{cF} (Å)
5-PC/12-PC	10.6	
5-PC/10-PC	11.5	10.1
10-PC/12-PC	8.2	

[a] The spin-labeled probes are 1-palmitoyl-2-(*n*-doxyl)stearoyl-*sn*-glycero-3-phosphocholines, which are phosphatidylcholines with the doxyl nitroxide group at the 5th, 10th, or 12th position along the fatty acid chain. The distances were calculated from the quenching curves of Figure 2 as described under Results and illustrated in Figure 3.

the two quenchers, and C = the two-dimensional quencher concentration in the plane of the membrane. Here, F_1/F_2 is the ratio of F_1/F_0 and F_2/F_0 in which F_1 and F_2 are fluorescence intensities of two sets of samples (one set containing the shallow quencher 1 and the other containing the deep quencher 2), both at the same quencher concentration C; F_0 is the fluorescence intensity in the absence of any quencher. All the bilayer parameters used were the same as described earlier (Chattopadhyay & London, 1987). A schematic diagram illustrating the parallax method as applied to AChR is shown in Figure 3.

Table I shows the average depth of penetration of the tryptophan residues in the reconstituted AChR calculated from these quenching curves. For each quenching pair, depths were calculated corresponding to points on the quenching curve, and the means were calculated as shown in Table I. The average membrane penetration depth is 10.1 Å from the center of the bilayer. Since for the lipids used in these experiments the thickness of the hydrocarbon region is 30 Å (Lewis & Engelman, 1983; Chattopadhyay & London, 1987), a depth of 10.1 Å from the center of the bilayer is indeed shallow, and actually corresponds to the level of the seventh carbon atom of the fatty acyl chain. These depths thus confirm the quenching pattern.

Of the three quenching pairs used for depth analysis, the depth calculated from the 10-PC/12-PC pair is the one that

is most sensitive to errors because small errors in data could cause rather large errors in calculation of depth. The depths calculated from this quenching pair could thus be an overestimation. Nevertheless, the calculated depths are largely independent (within 20%) of the choice of the pair of spin-labels for the quenching analysis.

Another striking feature of the quenching curves is that in no case was more than 30% of the total fluorescence quenched. This is in sharp contrast to fluorophores that lie entirely in the membrane where it is usual to have more than 80% of the fluorescence quenched at high quencher concentrations (London & Feigenson, 1981; Chattopadhyay & London, 1987). These low levels of quenching imply that the majority of tryptophan residues are located in the extramembranous region of the AChR.

Discussion

The location of tryptophans in AChR-rich membranes from *Torpedo marmorata* was previously investigated by differential quenching of tryptophan fluorescence by nitroxide-labeled fatty acids (Barrantes, 1978; Marsh & Barrantes, 1978). In our study, we have used spin-labeled phospholipids (rather than fatty acids) with the nitroxide group attached to different positions of the acyl chain. There are several limitations in using fatty acid probes as lipid analogues (Chattopadhyay, 1987). First, free fatty acids are not normal membrane components, and if used in high enough concentrations (as is often required in quenching studies), they may exert the lytic effects of detergents. Second, the possibility of varying degrees of ionization of fatty acids probes must always be considered; the degree of ionization varies with pH, and consequently the location of these probes in the bilayer may be pH-dependent. Third, fatty acids are much more water-soluble than phospholipids, and some of the labeled fatty acid will partition into the aqueous phase. This partitioning depends on the attachment site of the spin-label group on the acyl chain and anomalous quenching of anthroyloxy probes has been attributed to this type of complication (Thulborn & Sawyer, 1978). Fourth, fatty acids have been shown to perturb the structure and function of membrane proteins (Klausner et al., 1980; Pjura et al., 1982); in fact, fatty acids have been shown to inhibit the AChR-mediated ion flux (Andreasen & McNamee, 1980). In these studies, we have also used purified receptor reconstituted into membranes of defined lipid composition (as opposed to AChR-rich membrane fragments). To ensure that the exchange of native lipids with DOPC was complete and the reconstituted receptor was in a defined lipid environment, we have included several steps of equilibration with buffers containing varying amounts of DOPC in our AChR purification procedure (see Materials and Methods).

One problem with the parallax method, especially for proteins having multiple tryptophan residues in the membrane-embedded portion, is that only an average depth is obtained. Nevertheless, it is still useful since it represents the minimum depth of penetration. Another consideration is the orientation of the reconstituted AChR. Fortunately for AChR, this is not a major problem since previous studies using toxin binding to the receptor have shown that the reconstituted receptor is predominantly in the right-side-out orientation in the membrane (Huganir & Racker, 1982; Lindstrom et al., 1980; Ochoa et al., 1983). This natural ability of AChR to correctly assemble in reconstituted membranes is probably due to the inherent asymmetry of the AChR molecule and does not depend on the nature of phospholipids used for reconstitution (Criado et al., 1982). Even if there is a randomization of orientations, it will not have any significant effect on the depth of penetration, since the spin-labeled lipids are distributed in both leaflets of the bilayer.

The results of our experiments imply that the tryptophan residues in AChR on the average are at a shallow location in the membrane. This is in agreement with previous studies (Barrantes, 1978; Marsh & Barrantes, 1978) in which it was concluded, on the basis of the accessibility of quenchers to fluorophores, that the tryptophans in AChR are located close to the apolar–polar interface of the membrane. Such an interfacial location for tryptophan residues in membrane proteins in general has been recently suggested (Jacobs & White, 1989; Meers, 1990). In addition, low levels of quenching obtained from our experiments suggest that the majority of tryptophan residues are located in the putative extramembranous region of the AChR. Reconstitution of AChR with brominated phospholipids results in a 20% decrease in fluorescence (Jones & McNamee, 1988). The inability of quenchers to quench the residual fluorescence is a source of error in calculations of depth since the depth analysis is strictly valid for a homogeneous, membrane-embedded fluorophore.

Inspection of the primary amino acid sequence of AChR shows that there are 51 tryptophan residues in 1 molecule of AChR (Noda et al., 1982, 1983a,b). Since all receptor subunits show considerable sequence homology, it follows that the distribution of tryptophans is likely to be similar among the subunits (Stroud et al., 1990). A common feature of all proposed models for AChR is the prediction that the majority of tryptophan residues are located in the putative extramembranous regions of the AChR. Relatively few tryptophan residues are predicted to occur within the membrane where they would be accessible to quenching by membrane-embedded quencher molecules. In fact, Trp-453 on the γ subunit is the only Trp located in one of the postulated membrane-spanning helices. The location of Trp-453 is consistent with the results here since this Trp is in the M4 helix which may be at the lipid–protein interface. It is also located approximately 10 Å from the center of the bilayer. This is supportive of the low levels of quenching obtained when AChR is reconstituted with brominated lipids or spin-labeled lipids and the inability of the quenchers to quench the residual fluorescence. Further quantitative interpretation of the low levels of quenching is difficult due to the complexity of fluorescence processes in multi-tryptophan proteins and the differences in quantum yields among various tryptophans due to environmental sensitivity of fluorescence. However, if γ Trp-453 is the only Trp that is accessible to quenching in the membrane, it may be possible to use the quenching as a selective indicator of protein conformational change.

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