Protein–Lipid Interactions and *Torpedo californica* Nicotinic Acetylcholine Receptor Function. 1. Spatial Disposition of Cysteine Residues in the γ Subunit Analyzed by Fluorescence-Quenching and Energy-Transfer Measurements†

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ABSTRACT: The nicotinic acetylcholine receptor from *Torpedo californica* was labeled with a fluorescent, lipophilic probe, *N*-(1-pyrenyl)maleimide, specific for sulfhydryls in a hydrophobic environment, and was found to alkylate Cys 416, Cys 420 and Cys 451 in the γ subunit [Li, L., Schuchard, M., Palma, A., Pradier, L., & McNamee, M. G. (1990) *Biochemistry 29,* 5428–5436]. The spatial disposition of the acetylcholine receptor-bound pyrene with respect to the membrane bilayer was assessed by a combination of fluorescence-quenching and resonance energy transfer measurements, under conditions of selective labeling of the γ subunit. Quenching of pyrene fluorescence by spin-labeled fatty acids with the doxyl group at positions C-5 and C-12 revealed that the former was more effective, with a Stern–Volmer quenching constant of 0.187 compared to 0.072 for the latter, suggesting that the fluorophore(s) are located closer to the membrane–water interface rather than the hydrophobic interior. Energy transfer was found to occur from tryptophan in the acetylcholine receptor to cysteine-bound pyrene with a distance of separation of ~ 18 Å. However, there was no energy transfer when pyrene-labeled AChR was reconstituted into membranes containing brominated phospholipids and cholesterol, suggesting that the fluorophore(s) responsible for energy transfer are located in the membrane domain. Thus, the *N*-(1-pyrenyl)maleimide can be used to monitor lipid–protein interactions of the AChR.

Studies on protein–lipid interactions have contributed significantly to our understanding of the structure and function of membrane proteins. A great deal of attention has been focused on the nicotinic acetylcholine receptor (AChR),[1] a prototype of several ligand-gated ion channels, which play a crucial role in synaptic transmission. The AChR is a heterologous, pentameric glycoprotein, made up of four different subunits in the stoichiometry $\alpha_2\beta\gamma\delta$ [for reviews, see Galzi et al. (1991), Stroud et al. (1990), and Pradier and McNamee (1991)].

Efforts to crystallize the acetylcholine receptor and obtain high-resolution data have not been successful, but it has been possible to view the receptor at low resolution by a combination of X-ray diffraction and electron microscopy using two-dimensional arrays (Mitra et al., 1989; Toyoshima & Unwin, 1988; Unwin, 1993). In the postsynaptic membrane, the large hydrophilic amino-terminal domain of the AChR, carrying the agonist binding site, is located in the synaptic side, while about 30% of the receptor traverses the bilayer, and the remaining portion is in the cytoplasmic domain.

cDNA cloning and complete sequencing of the AChR subunits from *Torpedo californica* have been carried out (Noda et al., 1983; Claudio et al., 1983). Hydropathy profiles suggest that the acetylcholine receptor subunits have a propensity to form multiple transmembrane crossings, most likely in the form of α helices. Of the models proposed for the transmembrane organization of the subunits (Noda et al., 1983; Devillers-Thiery et al., 1983; Guy, 1984; Finer-Moore & Stroud, 1984; Ratnam et al., 1986; Pedersen et al., 1990), the model with the transmembrane segments designated M1–M4 spanning the lipid bilayer four times and the amino and carboxyl terminals facing the synaptic cleft (Noda et al., 1983; Devillers-Thiery et al., 1983) has now gained more credence with converging results from different techniques. Investigators using photolabeling studies (Hucho et al., 1986; Revah et al., 1990) and site-directed mutagenesis coupled with electrophysiological data (Imoto et al., 1986, 1988) conclude independently that the M2 domains from each subunit line the wall of the ion channel along the central axis of the pseudosymmetric pentamer [see Galzi et al. (1991)]. Hydrophobic residues interspersed with polar noncharged amino acids appear to face the lumen, and three rings of negatively charged residues have been suggested to modulate the channel conductance and selectivity (Imoto et al., 1988). The M4 domain is the most hydrophobic and the least conserved and is believed to be in contact with the surrounding lipids on the basis of photolabeling studies of AChR using aryl azide derivatives of phosphatidylcholine (Giraudat et al., 1985) and phosphatidylserine (Blanton & Wang, 1990). Photolabeling of the receptor with the nonspecific membrane reagent 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine (TID) is also consistent with exposure of M4 domains to the lipid environment (Blanton & Cohen, 1992).

In continuation of our efforts to understand the significance of sulfhydryl and disulfide groups in the AChR, we have used chemical modifications to probe the functional role of free sulfhydryls in the γ subunit of the receptor. The cysteines

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[1] Abbreviations: AChR, nicotinic acetylcholine receptor; NPM, *N*-(1-pyrenyl)maleimide; BrPC, 1-palmitoyl-2-(6,7-dibromostearoyl)-*sn*-glycero-3-phosphocholine; Chol, cholesterol; CHS, cholesterol hemisuccinate; DiBrCHS, 5,6-dibromocholesterol hemisuccinate; DOPC, dioleoylphosphatidylcholine; DOPA, dioleoylphosphatidic acid; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; 5-FASL, 5-fatty acid spin label (5-doxylstearic acid); 12-FASL, 12-fatty acid spin label (12-doxylstearic acid); MOPS, 3-(*N*-morpholino)propanesulfonic acid; SDS, sodium dodecyl sulfate; TID, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine.

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exist in locations of varying accessibilities and reactivities, which enables selective labeling of only those residues that are exposed. While functional significance was clearly attributed to several free sulfhydryl residues (Li et al., 1990; Yee et al., 1986; Clarke & Martinez-Carrion, 1986; Walker et al., 1984; Huganir & Racker, 1982), their exact location in the tertiary structure of the receptor is not well defined. Alkylation of the cysteine residues under nonreducing conditions resulted in inhibition of receptor ion flux activity without an effect on low- to high-affinity binding transitions induced by agonists (Yee et al., 1986). Peptide mapping of the cyanogen bromide and tryptic fragments of the labeled γ subunit indicated that the modified residues include Cys 416, Cys 420, and Cys 451. The Cys 416 and Cys 420 residues are located in an amphipathic helix (MA) that was once postulated to form the ion channel (Finer-Moore & Stroud, 1984). However, most models now place the MA domain in the cytoplasm. Cys 451 is located in the postulated M4 transmembrane domain.

A combination of site-specific mutagenesis and electrophysiological studies of the wild-type and mutant receptors expressed in *Xenopus laevis* oocytes has helped in further unraveling the functional significance of the sulfhydryls of the AChR (Pradier et al., 1989; Li et al., 1990). A double mutation of Cys 416 and Cys 420 in the γ subunit to Phe displayed a 30% decrease in the normalized activity, expressed as acetylcholine-induced channel conductance per femtomole of α-bungarotoxin binding sites, with little effect when both the residues were replaced by Ser (Pradier et al., 1989). However, mutation of Cys 451 to either Ser or Trp (Li et al., 1990) resulted in a decrease of normalized channel activity to about 50%.

The results of the mutation of Cys 451 (Li et al., 1990) suggest a functional role of this residue, probably in channel gating, indicating its presence in a location such as the protein–lipid and/or the membrane–water interface, whereby it could modulate the channel activity. Remarkably, a point mutation of a homologous cysteine on the α subunit (αCys 418) to Trp had a very large stimulatory effect on normalized channel activity (Li et al., 1992). Thus, residues at the protein–lipid interface seem to play a significant role in determining the functional properties of the receptor.

Here, we investigate the disposition of the cysteine residues in the γ subunit of acetylcholine receptor by using *N*-(1-pyrenyl)maleimide, a fluorescent label specific for sulfhydryls that can label cysteines in a hydrophobic environment. Spin-labeled fatty acids were used to cause differential quenching of the cysteine-bound pyrenes. Also, energy-transfer studies were carried out between tryptophan and cysteine-bound pyrenes in order to identify the most probable spatial relationship among membrane-associated side chains.

MATERIALS AND METHODS

Materials. *N*-(1-Pyrenyl)maleimide (NPM), cholesterol (Chol), cholesterol hemisuccinate(CHS), and fatty acid spin labels from Sigma (St. Louis, MO). Dioleoylphosphatidylcholine (DOPC), dioleolyphosphatidic acid (DOPA), and 1-palmitoyl-2-(6,7-dibromostearoyl)-*sn*-glycero-3-phosphocholine (6,7-BrPC) in chloroform were obtained from Avanti Polar Lipids (Birmingham, AL). Ultrol grade sodium cholate of very high purity was from Calbiochem (San Diego, CA). Affigel-10 was from Bio-Rad. Other chemicals used were of reagent grade.

Purification of Acetylcholine Receptor. The receptor was purified by affinity chromatography as described earlier (Ochoa et al., 1983; Bhushan & McNamee, 1990) from frozen *T. californica* electroplax tissue obtained from Winkler Enterprises (San Pedro, CA). Cholate was removed by dialysis for 48 h against three changes of buffer A (10 mM MOPS, 0.1 mM EDTA, 100 mM NaCl and 0.02% NaN_3, pH 7.4), and the receptor was either used immediately or frozen in liquid nitrogen until further use. AChR prepared in this manner had a lipid to protein mole ratio of about 400–500:1 (termed high L:P ratio). Preparation of AChR with a low lipid to protein ratio (low L:P ratio) was essentially the same as described by Ochoa et al. (1983), but included two more steps in washing the column with successively lower concentrations of DOPC, first with 0.62 mM DOPC and 0.5% cholate and subsequently with 0.12 mM DOPC and 0.5% cholate. Typically, lipid to protein mole ratios of about 50–100:1 were obtained by this procedure. The protein concentrations were determined by the Lowry method (Lowry et al., 1951), and the lipid concentration was determined by measuring the lipid phosphate content (McClare, 1971) using ammonium molybdate after complete digestion by perchloric acid. The samples were assayed for total ligand binding activity using equilibrium binding of α-[^{125}I]bungarotoxin (Jones et al., 1987).

Labeling of Receptor with N-(1-Pyrenyl)maleimide. Purified AChR was labeled with 1 mM *N*-(1-pyrenyl)maleimide (NPM) in the presence of 1% cholate for 1 h at room temperature. NPM in DMSO was dried under vacuum, buffer A with 1% cholate was added, and the mixture was sonicated. Purified AChR (low L:P ratio) was added while the mixture was vortexed to a final protein concentration of about 1 mg/mL. The mixture was incubated at room temperature for 1 h in the dark with occasional vortexing and then centrifuged. The supernatant was either reconstituted in PC:PA:Chol (60:20:20, mole ratio) to a final lipid to protein ratio of 300–400:1 or dialyzed against buffer A for 48 h with three buffer changes to remove cholate.

Prelabeling of AChR with Iodoacetic Acid. Purified AChR (1 mg/mL) in buffer A was titrated to pH 8.0 with 5 N NaOH and treated immediately with the water-soluble iodoacetic acid (1 mM). The mixture was incubated under nitrogen for 15 min at room temperature, and the reaction was quenched by adding 5 mM dithiothreitol (DTT). The reaction mixture was dialyzed against buffer A for 2 days at 4 °C with three buffer changes.

Fluorescence-Intensity Measurements. Steady-state fluorescence measurements were performed on an SLM 8000C spectrofluorometer (SLM Instruments, Urbana, IL) operating in the ratio mode with a 1-cm-path-length quartz cuvette. For intensity measurements, 0.04–0.05 μM pyrene-labeled AChR in PC:PA:Chol was diluted in 2 mL of buffer A. Spin-labeled fatty acids were added directly to the above, with vigorous vortexing, from a stock ethanolic solution so that the final concentration of ethanol was always <0.5%. The spin-labeled fatty acids at the indicated concentrations, ranging from 0 to 10 μM, contributed up to 40 mol % of the total lipids. Fluorescence-intensity measurements were taken after the samples were incubated at room temperature for about 20 min. An excitation wavelength of 345 nm was used to excite the pyrene residues, and the fluorescence intensity was measured at an emission wavelength of 376 nm. Excitation and emission slit widths were 4 nm. The sample absorbance was always <0.1, thereby eliminating inner filter effects and light scattering. An internal correction was made automat-

ically for changes in lamp intensity by using a reference solution of rhodamine B (3 g/L) in ethylene glycol. All measurements were done at room temperature.

Energy-Transfer Measurements. For resonance energy transfer measurements, the pyrene-labeled AChR in cholate was loaded back onto the affinity column and washed extensively to ensure removal of non-covalently bound pyrene in the lipid bilayer. AChR was eluted as usual, and the recovery of protein was about 60%. The eluted fraction was reconstituted into PC:PA:Chol at a final lipid to protein ratio of about 250:1 and dialyzed extensively against buffer A for 48 h with three buffer changes. For fluorescence measurements about 0.06–0.08 μM pyrene-labeled AChR in 2 mL of buffer A was used. The same concentration of unlabeled receptor served as a control for measuring fluorescence intensity of donor alone (Trp in AChR) in the absence of acceptor. The fluorescence intensities were measured at an excitation wavelength of 290 nm for Trp, and the emission spectrum was scanned from 300 to 450 nm and monitored at 334 nm for Trp. The same samples were used for the absorption spectrum of pyrene bound to AChR, with the same amount of unlabeled AChR as reference to calculate the overlap integral (described under Theory). The actual concentration of bound pyrene was calculated using the molar extinction coefficient of 38 000 M^{-1} cm^{-1} and was found to be about 10 μM (stoichiometry of 3:1, pyrene:AChR). Quantum yield of AChR intrinsic fluorescence was calculated by comparing the areas of corrected fluorescence spectra of AChR in buffer A with that of a known concentration of tryptophan in butanol.

Reconstitution of NPM-Labeled or Unlabeled AChR with Brominated Lipids. Energy-transfer measurements were also carried out after the AChR was reconstituted using brominated lipids in order to quench the fluorescent groups in the membrane environment. Unlabeled or pyrene-labeled AChR was loaded back onto the affinity column after solubilization and washed with a high concentration of BrPC (2.5 mg/mL BrPC and 1% cholate in buffer A), and the column was allowed to stand for 3 h to ensure complete exchange of DOPC for BrPC. The column was then further washed with 1.5 mg/mL BrPC in 1% cholate, allowed to stand for 12 h, and finally washed with 0.1 mg/mL BrPC in 0.5% cholate to obtain a low lipid:protein ratio. The receptor was eluted with 10 mM carbamylcholine. The concentration of protein was about 0.5 mg/mL, and the lipid to protein mole ratio was 100:1. CHS or DiBrCHS was incorporated into BrPC–AChR membranes in the presence of 1% cholate by incubation on ice for 1 h followed by dialysis against buffer A for 2 days with three buffer changes.

Gel Electrophoresis. SDS–PAGE of the pyrene-labeled receptor was performed on 8% acrylamide gels (Laemmli, 1970). The subunits were visualized by examining the gels on a UV transilluminator and photographed.

Theory. (A) Quenching Parameters. Collisional quenching of fluorescence is described by the Stern–Volmer equation (Lakowicz, 1983):

$$F_0/F = 1 + k_q\tau_0[Q] = 1 + K_{SV}[Q]$$

where F_0 and F are the fluorescence intensities in the absence and presence of quencher, respectively, k_q is the bimolecular quenching constant, τ_0 is the lifetime of the fluorophore in the absence of quencher, [Q] is the concentration of quencher, and $K_{SV} = k_q\tau_0$ is the Stern–Volmer quenching constant. A plot of F_0/F vs [Q] yields a slope equal to K_{SV}.

Alternately, the quenching profiles can be compared by the Lehrer, or modified Stern–Volmer, equation (Lehrer, 1971), which assumes the existence of two populations of fluorophore, one accessible to quenching, f_a, and the other inaccessible (or buried):

$$F_0/\Delta F = 1/(f_a K_{SV}[Q]) + 1/f_a$$

(B) Energy Transfer. The efficiency, E, of resonance energy transfer between the tryptophan donor in the AChR and cysteine-bound pyrene [see Stryer (1968)] is related to R, the distance separating the two species, by

$$R = R_0((1/E) - 1)^{1/6} \quad (1)$$

as described by Forster (1965) where R_0 is the distance at which the efficiency of transfer is 50%. R_0 (in units of Å) is calculated as

$$R_0 = 9.765 \times 10^3(\kappa^2 J Q_D \eta^{-4})^{1/6} \quad (2)$$

where Q_D represents the quantum yield of donor in the absence of acceptor, η denotes the refractive index of the medium between donor and acceptor, and κ^2 is the "orientation factor" which accounts for the relative spatial orientation of the donor emission and acceptor absorption transition dipoles. The overlap integral, J, represents the degree of resonance overlap between excited-state donor and acceptor dipoles and is evaluated as the integrated area of overlap between the donor emission spectrum, $I_D(\lambda)$, and the acceptor absorption spectrum, $\epsilon_A(\lambda)$ (as molar extinction coefficient) and is given by

$$J = \frac{I_D(\lambda)\epsilon_A(\lambda)\lambda^4\, d\lambda}{I_D(\lambda)\, d\lambda} \quad (3)$$

where λ is the wavelength in centimeters.

Typically, when the donor and acceptor species are at separate sites on the protein, the efficiency, E, of energy transfer is measured as the extent of reduction of donor quantum yield or fluorescence intensity, i.e.,

$$E = 1 - (I_{DA}/I_D) \quad (4)$$

where I_{DA} and I_D are the fluorescence intensities of the donor (D) in the presence and absence of acceptor (A), respectively.

RESULTS

Labeling AChR by N-(1-Pyrenyl)maleimide. Purified AChR was labeled with *N*-(1-pyrenyl)maleimide in the presence of cholate under conditions that label only the γ subunit as described previously (Li et al., 1990). SDS–PAGE of the pyrene-labeled receptor, followed by visualization under a UV illuminator, shows that the band corresponding to the γ subunit in the Coomassie-stained gel is labeled predominantly (Figure 1A); a densitometric scan of the gel (Figure 1B) revealed that about 99% of the label was on the γ subunit and about 1% was on the α subunit.

Quenching of Pyrene-Labeled AChR Fluorescence by Spin-Labeled Fatty Acids. The spatial relationship of AChR-bound pyrene with repsect to the membrane bilayer and to the rest of the protein was studied by fluorescence-quenching and resonance energy transfer measurements. Spin-labeled fatty acids (Mitra & Hammes, 1990; Blatt & Sawyer, 1985) and phospholipids (Chattopadhyay & London, 1987; Chattopadhyay & McNamee, 1991; Meers, 1990) with the doxyl group at different positions along the fatty acyl chain have been frequently used as molecular rulers to locate the depth of membrane-embedded fluorophores.

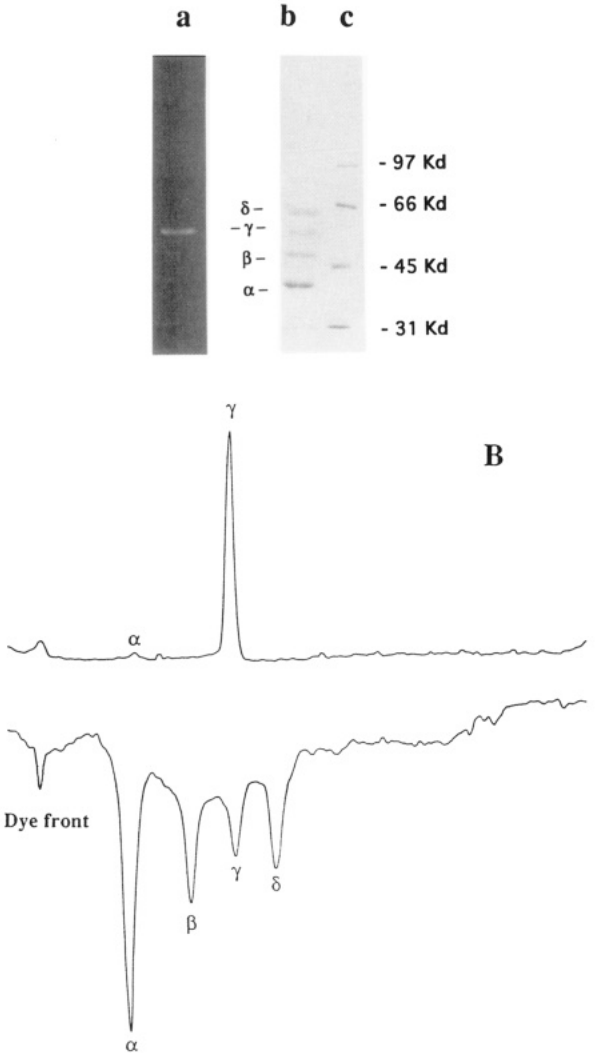

FIGURE 1: (**A**) SDS–PAGE of the pyrene-labeled AChR subunits on an 8% gel. Lane a: Fluorogram of the labeled gel as viewed on an UV illuminator. Lane b: Coomassie-stained gel showing the four subunits in the stoichiometry $\alpha_2\beta\gamma\delta$. Lane c: Molecular weight standards. Labeling conditions are described under Materials and Methods. (**B**) Densitometric scanning of the SDS–PAGE gel containing pyrene-labeled AChR. The upper trace is the fluorescence intensity, and the lower trace is the Coomassie Blue intensity. Less than 1% of the fluorescence was found to be incorporated into the α subunit.

Spin-labeled stearic acids with the nitroxide group at the C-5 and C-12 positions (5-FASL and 12-FASL, respectively) were used to quench the extrinsic fluorescence of pyrene-labeled AChR, and the quenching profiles are shown in Figure 2. The decrease in fluorescence intensity at 376 nm (when excited at 345 nm) was monitored as a function of increasing concentration of spin-labeled stearic acids. The intensity of unlabeled receptor was used as a reference and subtracted suitably, and the intensity of pyrene-labeled receptor in the absence of added quencher served as the control, F_0. The 5-FASL quenches more effectively than the 12-FASL, indicating a more superficial location for the cysteine-bound fluorophores. The Stern–Volmer quenching constants and the accessibility data from Lehrer plots (described under Theory in Materials and Methods) are shown in Table I. It is clear that the fluorophores are more easily accessible to 5-FASL, which has a quenching constant of 0.187, than to 12-FASL with a quenching constant of 0.072.

Resonance Energy Transfer Measurements. Pyrene-labeled AChR reconstituted into PC:PA:Chol bilayers was used

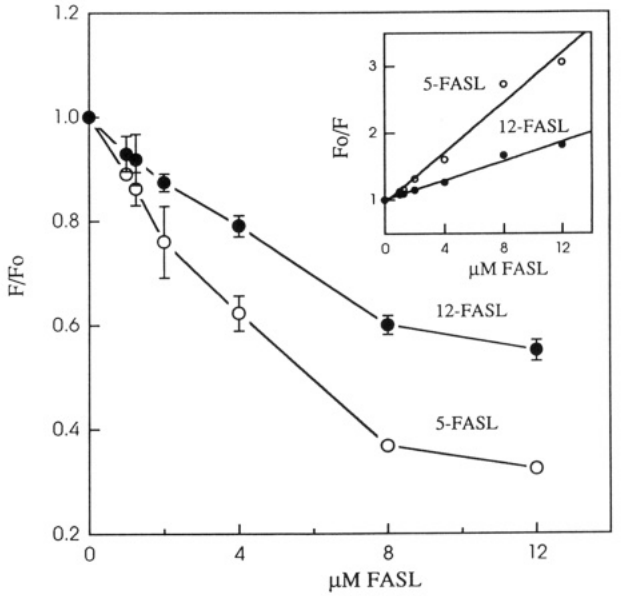

FIGURE 2: Estimation of transmembrane location of AChR-bound pyrene by quenching with 5- and 12-FASL. Shown is a plot of F/F_0 vs concentration of spin-labeled fatty acids, where F_0 and F are fluorescence intensities in the absence and in the presence of different concentrations of quenchers, respectively. The inset shows Stern–Volmer plots of F_0/F vs concentration. The fluorescence emission was measured at 376 nm at an excitation wavelength of 345 nm. Labeling conditions are described under Materials and Methods. Values are expressed as means ± SEM (n = 4).

Table I: Stern–Volmer Quenching Constants and Apparent Fraction of Accessible Fluorophores for Quenching of Pyrene-Labeled AChR Fluorescence by Spin-Labeled Fatty Acids[a]

FASL	K_{SV}[b]	$f_{a(app)}$[c]
5-FASL	0.187 ± 0.018	1.00 ± 0.09
12-FASL	0.072 ± 0.005	0.65 ± 0.04

[a] Values are averages ± SEM from three independent experiments with duplicates from each. [b] Stern–Volmer quenching constants, K_{SV}, from Stern–Volmer plots, Figure 2 (inset). [c] Apparent fraction of accessible fluorophore, $f_{a(app)}$, from modified Stern–Volmer plots of $F_0/\Delta F$ vs $1/[Q]$, as described under Theory in Materials and Methods.

for resonance energy transfer measurements to determine the distance between Trp residue(s) and cysteine-bound pyrene moieties. Tryptophan in AChR was the donor, with excitation at 290 nm and emission at 334 nm, and pyrene bound to cysteine was the energy acceptor, with excitation at 345 nm and emission at 376 nm. The donor–acceptor pair used in this system has the following advantages: pyrene has a fairly high extinction coefficient, labeling of the γ subunit in the receptor by *N*-(1-pyrenyl)maleimide is well defined, and there is an excellent degree of overlap between the emission spectrum of tryptophan and the absorption spectrum of pyrene. Noncovalently bound pyrene in the bilayer was removed by loading the labeled receptor back onto the affinity column followed by extensive washing with buffer containing DOPC in cholate. This ensures that the energy transfer observed is solely to the covalently bound pyrene on the AChR.

The absorption spectrum of AChR-bound pyrene overlaps with the emission spectrum of the intrinsic fluorescence spectrum of AChR. The spectral relationship of the donor–acceptor system is shown in Figure 3. The fluorescence emission maximum of the unlabeled AChR at 334 nm indicates that, on the average, the tryptophan residue(s) are located in a hydrophobic environment (compared to 340 nm for tryptophan in butanol). The overlap integral J was calculated as described in the Theory section of Materials and Methods

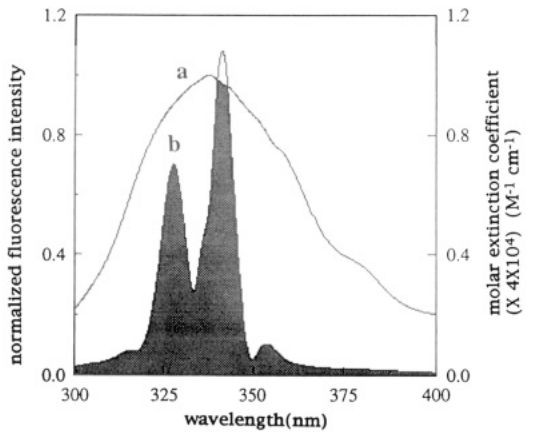

FIGURE 3: Overlap between the emission spectrum of tryptophan in AChR and the absorption spectrum of AChR-bound pyrene. (a) Normalized fluorescence emission spectrum of AChR tryptophan at an excitation wavelength of 290 nm. (b) Absorption spectrum of AChR-bound pyrene shown as the molar extinction coefficient in the units indicated. The concentration of AChR was 0.08 μM for both the measurements. The shaded area indicates the region of overlap. For calculation of the overlap integral, J, the area of overlap was integrated between 310 and 380 nm.

and was found to be 1.55×10^{-14} cm^6/mol. Figure 4A shows the emission spectrum of AChR in the absence of acceptor with a fluorescence intensity maximum at 334 nm (curve a), which decreases due to energy transfer to about 30% in the pyrene-labeled AChR (curve c). This is accompanied by the appearance of pyrene fluorescence emission at 376 and 396 nm. A transfer efficiency of 70% was calculated from eq 1 (see Theory in Materials and Methods). The quantum yield of tryptophan in AChR was found to be 0.033, using tryptophan in butanol as standard. Other spectroscopic parameters that were calculated are shown in Table II. The Forster distance, R_0, defined as the distance at which there is 50% transfer efficiency, was found to be 20 Å, using values of 1.4 for the refractive index in a fluid medium and 2/3 for the orientation factor. κ^2 has a maximum theoretical error of 20% (the theoretical range is 0–4) and is a potential source of uncertainty for calculating distances. Thus, the distance of closest approach between the *average* locations of cysteine-bound pyrene and tryptophan was calculated to be about 18 Å.

Energy-transfer measurements were also carried out using pyrene-labeled AChR reconstituted into BrPC and DiBrCHS (Figure 4A). In control experiments, the unlabeled AChR was reconstituted into DOPC/CHS (Figure 4A, curve a) or into BrPC/DiBrCHS (Figure 4A, curve b). A 30% decrease in the intrinsic fluorescence intensity was observed, which was in good agreement with previous results (Jones & McNamee, 1988). However, with pyrene-labeled AChR the brominated lipids eliminated fluorescence energy transfer to the pyrene (Figure 4A, curve d). These results suggest that extramembranous pyrene-labeled cysteines are probably not involved in the resonance energy transfer between tryptophan and pyrene. Figure 4A also demonstrates that excimer formation due to proximal pyrene rings does not occur since there is no peak at higher wavelengths in the 450–470-nm range (Vanderkooi & Callis, 1974).

The energy-transfer experiments outlined above using pyrene-labeled AChR reconstituted into brominated lipids strongly indicate that pyrene attached to Cys 451 is the primary species involved. All the current models of AChR organization place Cys 451 in the M4 helix in the membrane and Cys 416 and Cys 420 in the MA amphipathic helix in the cytoplasmic region. Preliminary efforts in our laboratory to discriminate

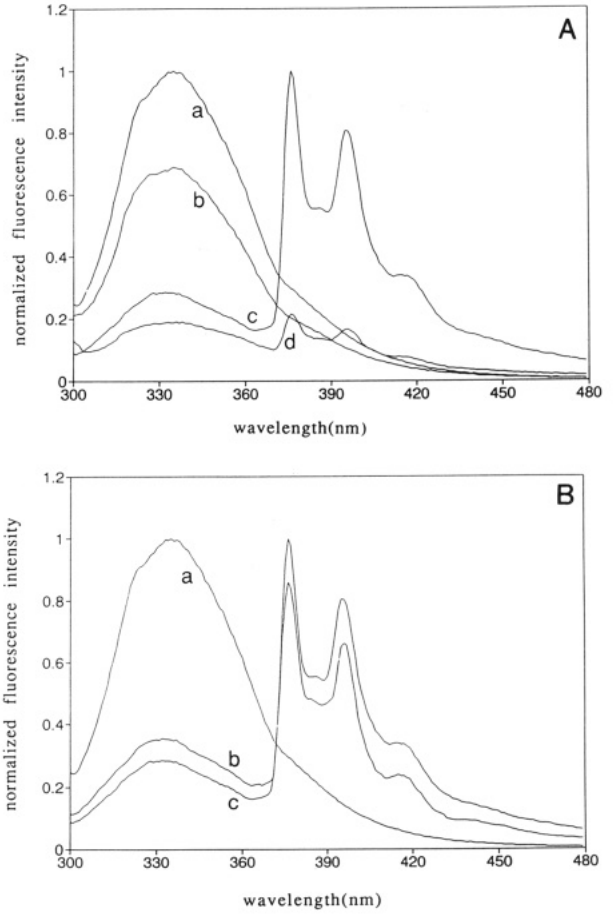

FIGURE 4: (**A**) Resonance energy transfer from tryptophan to pyrene in AChR reconstituted in DOPC/CHS or BrPC/DiBrCHS. (a) Control fluorescence emission spectrum of unlabeled AChR in DOPC/CHS in the absence of an acceptor, 0.08 μM in buffer A. (b) Fluorescence emission spectum of unlabeled AChR in BrPC/DiBrCHS. (c) Fluorescence emission spectrum of pyrene-labeled AChR in DOPC/CHS. (d) Fluorescence emission spectrum in pyrene-labeled AChR in BrPC/DiBrCHS. Excitation wavelength was 290 nm. (**B**) Resonance energy transfer from tryptophan to pyrene in pyrene-labeled and iodoacetate-prelabeled, pyrene-labeled AChR. (a) Fluorescence emission spectrum of AChR alone, 0.08 μM in buffer A, in the absence of acceptor. (b) Fluorescence emission spectrum of 0.08 μM pyrene-labeled AChR. (c) Fluorescence emission spectrum of iodoacetate-prelabeled, pyrene-labeled AChR. The excitation wavelength was 290 nm. The conditions for reconstitution, prelabeling, and labeling are described under Materials and Methods.

Table II: Parameters for Resonance Energy Transfer from Trp to AChR-Bound Pyrene[a]

quantum yield, Q_D[b]	0.033
orientation factor, κ^2[c]	0.67
overlap integral, J[d]	1.55×10^{-14} cm^6/mol
distance at 50% efficiency, R_o[e]	20.0 Å
energy-transfer efficiency, E[f]	0.7

[a] Values are averages of two independent experiments. Labeling of AChR was performed as described under Theory in Materials and Methods. [b] Quantum yield based on a value of 0.2 for 30 mM tryptophan in butanol. [c] Orientation factor taken as 2/3, the dynamic average. [d] Calculated as described under Theory in Materials and Methods using eq 3. [e] Calculated from eq 2 as described under Theory in Materials and Methods, using a refractive index of 1.4. [f] Calculated from eq 4 both from quantum yield (Integrated areas under curve) and from fluorescence-intensity measurements in the presence and the absence of acceptor.

among the pyrene-labeled cysteine sites were not completely successful. However, when fluorescence measurements were made using iodoacetate-prelabeled, pyrene-labeled AChR, in order to preferentially alkylate cysteines in hydrophilic regions

with a nonfluorescent group (Figure 4B, curve c), the effect on energy transfer was insignificant. The energy-transfer results are consistent with the expectation that Cys 451 in the membrane-embedded portion of the receptor is primarily responsible for the observed transfer.

DISCUSSION

The results from the present investigation offer a refined view of the location of cysteine-bound pyrene residues in the γ subunit of the acetylcholine receptor. We made use of spin-labeled fatty acids to quench the extrinsic fluorescence of the pyrene moiety attached to specific cysteine residues and energy-transfer measurements to evaluate the distance between tryptophan and cysteine residues in the AChR.

Under the labeling conditions, cysteines in the γ subunit were labeled predominantly, with negligible labeling of the α subunit. The sulfhydryls on the γ subunit are relatively more accessible and prone to labeling by sulfhydryl reagents compared to those on the α subunit. We have taken advantage of this differential reactivity of the SH groups to manipulate the labeling so as to obtain enhanced labeling of either the γ or the α subunit. Marquez et al. (1989) have used the same probe to label the AChR in its native membrane environment in the absence of cholate and have concluded that only one of seven Cys residues in the α subunit exists as a free SH, most likely αCys 222 on the M1 segment. While two pairs, αCys 128–αCys 142 and αCys 192–αCys 193, are located in the synaptic region as disulfide bridges, it is thought that the third pair, namely, αCys 412–αCys 418, in the M4 segment either may be involved in a thioester linkage or are acylated, and do not exist as free sulfhydryls or as disulfides (Mosckovitz & Gershoni, 1988). The importance of αCys 418 in the M4 segment has been demonstrated by Li et al. (1992) by site-directed mutagenesis. Conversion of this single residue to a Trp results in increased open channel time.

The distance between the cysteine-bound pyrene and tryptophan residues estimated by energy-transfer measurement is consistent with that calculated between γCys 451 and γTrp 453 in an α-helical conformation (Figure 5). Molecular modeling was done on the M4 segment of the γ subunit, assuming an α-helical secondary structure for a section of the sequence from γM4 Ala 450 to Leu 468. Trp 453 and Cys 451 are located diametrically opposite each other, and the distance between the two residues was calculated to be about 13 Å. If the bulky nature of the pyrene ring system contributes about 3–5 Å, the resultant distance between the residues would be in better agreement with the calculated distance. The distances between these two residues were found to be 13–14 Å in an α helix, 5–6 Å in a β strand, and 9–10 Å in an extended structure based on standard dihedral angles by considering three possible conformations of the M4 transmembrane region.

Previous work from our laboratory focused attention on the average locations of tryptophan residues in the membrane-embedded portion of the AChR by the parallax method (Chattopadhyay & McNamee, 1991). By making use of the differential quenching of tryptophan fluorescence by spin-labeled phospholipids, with the nitroxide groups attached at different positions along the acyl chain, we concluded that the Trp residue(s) were located about 10 Å from the center of the bilayer, which places them close to the headgroup interface. It was postulated that Trp 453 in the M4 segment of the γ subunit of AChR (which is the only Trp present in the transmembrane segment, of the total of 51 tryptophan residues in the receptor) is the most likely candidate for such a location. This was in agreement with earlier studies (Marsh & Barrantes, 1978; Barrantes, 1978) using spin-labeled fatty acids and AChR-rich membranes from *Torpedo marmorata*, where a superficial location of Trp residues at the polar–apolar interface was implied. In the present study, the quenching data suggest that the average vertical depth of the pyrene labeled site(s) on cysteine(s), using spin-labeled fatty acids as molecular rulers, is closer to 6 than to 15 Å from the head-group interface in accordance with the higher level of quenching by 5-FASL. The penetration depth for the doxyl group is assumed to be about 6 and 15 Å for 5- and 12-FASL, respectively, from the head-group interface (Voges et al., 1987).

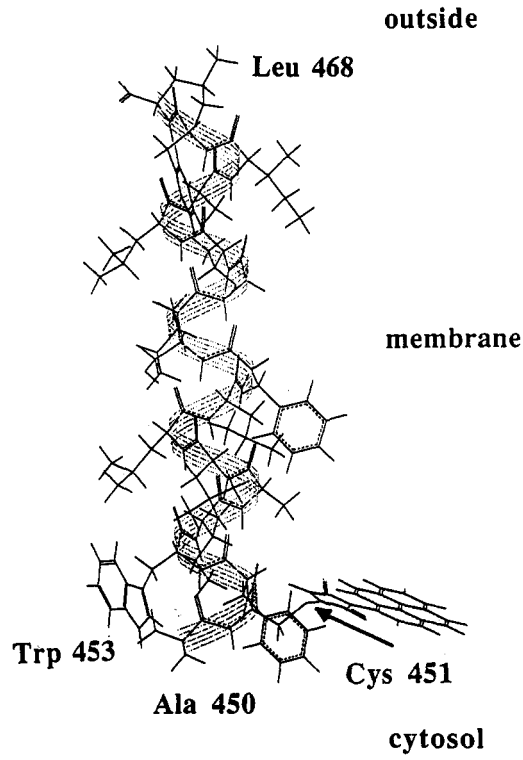

FIGURE 5: Schematic representation of the M4 helical segment of the γ subunit of the AChR. The residues from Ala 450 to Leu 468 in the M4 segment of the γ subunit are shown assuming an α-helical secondary structure for the segment and with a pyrene moiety attached to Cys 451. The distances were calculated by using the program MIDAS (University of California, San Francisco) on an IRIS workstation (Silicon Graphics). The sulfhydryl of Cys 451 and three sites on Trp 453 were found to be separated by 12.4, 12.5, and 9.0 Å.

The quenching data do not rule out a contribution of Cys 416 and Cys 420 and thus cannot be used to precisely assign the depth of a specific residue within the membrane. However, the energy-transfer results provide a more precise indication that Cys 451 is the primary group involved in the fluorescence measurements. Tryptophan 453 in the γ subunit appears to be closest to Cys 451, and pyrene bound to this cysteine residue is the most likely candidate to account for the observed results. If the four-crossing model is considered, then Cys 451 in the γ subunit will be toward the inner, cytoplasmic side of the membrane on the M4 helix and Cys 416 and Cys 420 in the MA helix will be located in the extramembranous domain. The results from the energy-transfer experiments with brominated lipids and iodoacetate-prelabeled receptor minimize the possibility that the latter two residues participate in the energy-transfer process.

There is a body of evidence accumulating in favor of the proposal that the M4 segment of the subunits of AChR faces the lipid milieu (Giraudat et al., 1985; Blanton & Wang,

1990; White & Cohen, 1988). The M4 segment is the most hydrophobic and the least conserved of the four transmembrane crossings and appears to be important in expression of the receptor (Tobimatsu et al., 1987). Hydrophobicity profiles of M4 show that one-half of the segment is relatively more hydrophobic, while the other half has more polar, uncharged residues such as Thr, Ser, and Cys, somewhat surprisingly. The latter side has been proposed to face the lipid environment on the basis of photolabeling studies with TID (Blanton & Cohen, 1992). Energy-transfer data (Table II) and modeling studies (Figure 5) suggest a helical structure for this segment. Also, the accessibility to quenching by lipid derivatives argues against it being buried in the interior of the protein. The accompanying paper (Narayanaswami & McNamee, 1993) addresses the availability of sites for binding cholesterol and fatty acids in the vicinity of the pyrene-labeled cysteine residues on the γ subunit of AChR and the effects of agonists and antagonists on their accessibility.

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