Protein–Lipid Interactions and *Torpedo californica* Nicotinic Acetylcholine Receptor Function. 2. Membrane Fluidity and Ligand-Mediated Alteration in the Accessibility of γ Subunit Cysteine Residues to Cholesterol†

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Received April 26, 1993; Revised Manuscript Received August 25, 1993®

ABSTRACT: Fluorescence-quenching and energy-transfer measurements were carried out to further characterize lipid–protein interactions involving the nicotinic acetylcholine receptor (AChR) from *Torpedo californica* in reconstituted membranes. To assess the fluidity of the receptor microenvironment, *cis*- and *trans*-parinaric acids were used to take advantage of the preferential partitioning behavior of the *trans* isomer for the gel phase. A relatively higher extent of energy transfer from the intrinsic tryptophan fluorescence of AChR in dielaidoylphosphatidylcholine bilayers to *cis*-parinaric acid in both the gel and the fluid phase suggests that the AChR is surrounded by a relatively fluid annulus of lipids. The ability of AChR to accommodate and interact with specific lipids such as cholesterol and fatty acids in the vicinity of pyrene-labeled cysteine residues in the membranous domain and/or the membrane–water interface region of the γ subunit was assessed. Pyrene-labeled AChR prepared in (6,7-dibromostearoyl)phosphatidylcholine showed a 25% decrease in fluorescence as sites accessible to phospholipids were occupied; subsequent addition of dibromocholesterol hemisuccinate (DiBrCHS) caused further quenching by about 25%. This result is consistent with the presence of sites accessible to cholesterol, but not accessible to phospholipids, in the vicinity of the cysteine-bound pyrene in the membranous domain of the AChR. Quenching by DiBrCHS was sensitive to the presence of an AChR activator (carbamylcholine) but not a competitive antagonist (α-bungarotoxin). The Stern–Volmer quenching constant was 0.123 in the absence of added ligands and 0.167 and 0.134 in the presence of carbamylcholine and α-bungarotoxin, respectively, corresponding to accessibilities of 65%, 90%, and 70%.

A role for lipids in maintaining the structural and functional integrity of membrane proteins has emerged in recent years (Devaux & Seigneuret, 1985; Marsh, 1987). Effects mediated by lipids include long-range modulation of the motional dynamics of the bulk lipids and/or specific, lipid–protein interactions. Reconstitution of membrane proteins into defined lipid environments has provided a powerful means of studying specific interactions of an intrinsic protein with its surrounding media (Jones et al., 1987). Analysis of the nicotinic acetylcholine receptor (AChR)[1] in its native membrane form and in reconstituted systems (Fong & McNamee, 1986, 1987; Jones et al., 1988; Bhushan & McNamee, 1993) has suggested a requirement of specific lipids to support the function and structural integrity of integral membrane proteins.

The acetylcholine receptor from the electric tissue of the Pacific ray, *Torpedo californica*, is a chemically gated ion channel which becomes permeable to cations at the postsynaptic membrane upon binding of acetylcholine [for reviews, see Pradier and McNamee (1991), Galzi et al., (1990), and Stroud et al. (1990)]. It is a transmembrane glycoprotein whose primary sequence has been deduced from cDNA cloning and sequencing [see Noda et al. (1983) and Claudio et al. (1983)]. The *Torpedo* AChR subunits bear considerable sequence homology amongst themselves and with the receptor from the mammalian neuromuscular junction. Secondary structure predictions on the pentameric structure, $\alpha_2\beta\gamma\delta$, made from hydropathic analysis suggest four transmembrane crossings, M1–M4, of about 20 amino acid residues in each subunit, with a fifth amphipathic segment, MA, possibly in the cytoplasmic domain (Noda et al., 1983; Devillers-Thiery et al., 1983). Bundles of about 20 transmembrane segments, which are thought to be α helices, are presumed to be embedded in the hydrophobic milieu of lipids with the ligand binding site on the extracellular side. Point mutation (Imoto et al., 1986, 1988) and photoaffinity labeling studies (Hucho et al., 1986; Revah et al., 1990) indicate that the M2 segments from all subunits line the wall of the ion channel. There is mounting evidence that the M4 domain from each subunit faces the lipids (Giraudat et al., 1985; Blanton & Wang, 1990; Cohen & White, 1988; Blanton & Cohen, 1992). The M4 segment is the most hydrophobic and the least conserved of the four transmembrane domains. Radioactive arylazido derivatives of phosphatidylcholine (Giraudat et al., 1985) and phosphatidylserine (Blanton & Wang, 1990) preferentially label the M4 domain of all the subunits. Photoincorporation of 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine ([^{125}I]-TID), a hydrophobic, photoactivatable probe with a very high membrane partition coefficient, was studied in postsynaptic membranes from *Torpedo* (White & Cohen, 1988; McCarthy

† This research was supported by USPHS Grant NS 13050 to M.G.M.

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® Abstract published in *Advance ACS Abstracts*, October 15, 1993.

[1] Abbreviations: AChR, acetylcholine receptor; 6,7-BrPC, 1-palmitoyl-2-(6,7-dibromostearoyl)-*sn*-glycero-3-phosphocholine; α-BTx, α-bungarotoxin; Carb, carbamylcholine; Chol, cholesterol; CHS, cholesterol hemisuccinate; DiBrCHS, dibromocholesterol hemisuccinate; 6,7-DiBrStA, 6,7-dibromostearic acid; DEPC, dielaidoylphosphatidylcholine; DOPA, dioleoylphosphatidic acid; DOPC, dioleoylphosphatidylcholine; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; NPM, *N*-(1-pyrenyl)maleimide; OA, oleic acid; PnA, parinaric acid.

0006-2960/93/0432-12420$04.00/0

& Stroud, 1989). All subunits of AChR were labeled in the absence of the agonist, carbamylcholine, with strong preferential labeling of the γ subunit, while membranes labeled in the presence of carbamylcholine had dramatically reduced labeling. Analysis of the proteolytic digest of the α subunit suggested that the M4 segment was in maximal contact with the lipid (Blanton & Cohen, 1992).

Since a considerable portion of the exposed surface of the receptor in the transmembrane region is in contact with the surrounding lipids, it is not difficult to envisage that the composition and physical properties of the surrounding lipids should have an influence on their interaction with the peripheral regions of the receptor. Initially, electron paramagnetic resonance studies using spin-labeled sterol and fatty acids with AChR in native membranes from *Torpedo* revealed a layer of "immobilized" lipids, surrounding the receptor, in addition to the bulk lipids (Marsh & Barrantes, 1978). In reconstituted systems, sterol and phosphatidic acid spin-labeled analogs were found to have a higher affinity for the receptor (K of 4.3 and 2.7, respectively) when compared to phosphatidylcholine (K of 1.0) (Ellena et al., 1983). It was later shown that the structural and dynamic properties of the lipid environment are correlated with the functional activity of the AChR as assessed by the agonist-induced low- to high-affinity binding state transition and by an ion-flux response (Fong & McNamee, 1986; Sunshine & McNamee, 1992). Cholesterol and negatively charged phospholipids such as phosphatidic acid (Criado et al., 1982; Ochoa et al., 1983; Fong & McNamee, 1986) were found to be essential for the ion-gating activity, while they also supported the affinity-state transitions and retained an optimal fluidity. FTIR (Fourier transform infrared) spectroscopic studies lent further support to a hypothesis that cholesterol and phosphatidic acid have a direct effect on the secondary structure of AChR, the former by increasing the α-helical content and the latter by increasing the β sheet content (Fong & McNamee, 1987; Bhushan & McNamee, 1993; Butler & McNamee, 1993).

Two discrete classes of lipid binding sites on the AChR were identified on the basis of quenching of the intrinsic fluorescence of AChR using brominated phospholipids and brominated cholesterol (Jones & McNamee, 1988): "annular sites" at the protein–lipid interface having access to phospholipids and "nonannular sites" having unique access to cholesterol and/or fatty acids. Since the fluorophore (tryptophan) distribution in the AChR was not known, it was difficult to assign specific binding sites for cholesterol on the lipid-exposed surface of the receptor. In a later study the tryptophan residue(s) were estimated to be at an average distance of 10 Å from the center of the bilayer on the basis of differential quenching of the intrinsic fluorescence using spin-labeled phospholipids with the doxyl groups at three different positions on the fatty acid chain (Chattopadhyay & McNamee, 1991).

In this paper we have used the fluorescent reagent *N*-(1-pyrenyl)maleimide (NPM) to selectively label the γ subunit of AChR at specific cysteine residues. We examine the ability of brominated analogs of cholesterol and phosphatidylcholine to quench the fluorescence of cysteine-bound pyrene in the membranous portion. The specificity of labeling, the functional significance of the labeled site, and the approximate location of the probe relative to the membrane surface have been documented in previous work and in the accompanying manuscript (Li et al., 1990; Li et al., 1992; Narayanaswami et al., 1993). The results are consistent with a hypothesis that specific sites or pockets on the receptor may exist where cholesterol spends a considerably longer time during exchange with the bulk lipids.

MATERIALS AND METHODS

Materials. Cholesterol hemisuccinate was purchased from Sigma (St. Louis, MO). Parinaric acid (PnA, 9,11,13,15-octadecatetraenoic acid) isomers were obtained from Molecular Probes (Eugene, OR), and dielaidoylphosphatidylcholine (DEPC), 6,7-dibromostearoyl fatty acid (6,7-diBrStA), and 1-palmitoyl-2-(6,7-dibromostearoyl)-*sn*-glycero-3-phosphocholine (6,7-BrPC) were obtained from Avanti Polar Lipids (Birmingham, AL).

Purification and Labeling of Receptor. The acetylcholine receptor was purified on an affinity column from crude membrane preparations of the frozen electroplax tissue from *Torpedo californica*, using DOPC as the added lipid during purification (Ochoa et al., 1983; Bhushan & McNamee, 1990). The purified receptor was dialyzed 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 had a lipid to protein ratio of about 400–500:1. 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).

For studies with parinaric acid, AChR was purified as usual, but dielaidoylphosphatidylcholine (DEPC) was used at 1.25 mM concentration in the presence of 1% cholate instead of DOPC.

For quenching studies involving AChR in brominated phospholipids, purified AChR was prepared in DOPC at a lipid to protein ratio of 500:1 and labeled with 1 mM *N*-(1-pyrenyl)maleimide in the presence of 1% cholate [see Li et al. (1990)]. The samples were incubated for 1 h, centrifuged, and then loaded back onto the affinity column previously equilibrated with buffer A in 1% cholate. The receptor was washed extensively, first with 0.25 mM 6,7-BrPC containing 0.5% cholate and then with 0.06 mM 6,7-BrPC in 0.5% cholate, and eluted and dialyzed as described earlier (Ochoa et al., 1983; Bhushan & McNamee, 1990). A corresponding control was carried out where the labeled receptor was repurified in DOPC instead of 6,7-BrPC. The lipid to protein ratio of labeled receptor in 6,7-BrPC was about 100:1.

For experiments involving carbamylcholine-mediated functional changes, the purified receptor was labeled with NPM, loaded back onto the affinity column, washed with buffer A containing DOPC and cholate, and then reconstituted in DOPC:PA:Chol (60:20:20) to a final lipid to protein ratio of 200–300:1 as described earlier (Fong & McNamee, 1986).

Fluorescence Measurements. Buffer A was used for all fluorescence-intensity measurements. Parinaric acid, at the indicated concentrations, was added directly to membrane preparations containing AChR in DEPC at a lipid to protein mole ratio of about 500:1. For temperature-dependent studies, the concentration of parinaric acid was 2 μM. To 125 μL of AChR/DEPC (1.5 mg/mL) in 2.5 mL of buffer A was added 10 μL of 0.5 mM *cis*- or *trans*-PnA in ethanol ($\leq$0.5%, v/v) with vortexing to give a final concentration of about 2 μM. The lipid:PnA ratio was always at least 150:1. To ensure incorporation of the probes, the samples were incubated at 37 °C for 15 min and intensity measurements were made directly thereafter.

Fluorescence intensity of parinaric acid was measured at an excitation wavelength of 320 nm and an emission wavelength of 412 nm. Fluorescence energy transfer measurements were performed at an excitation wavelength of 290 nm for

tryptophan and an emission wavelength of 334 nm; excitation and emission slit widths were 4 nm. For temperature-dependent studies a circulating water bath was used to cool the cuvette holder in the spectrofluorometer from about 25 to about 2–3 °C at a rate of about 0.3 °C/min. A heating cycle was also done in some cases to check whether the process was reversible. The temperature was monitored directly by inserting a thermocouple into the cuvette, and the fluorescence intensity was measured simultaneously at a fixed wavelength of 334 nm in a slow time based aquisition mode. Fluorescence-intensity measurements were made using an SLM 8000C spectrofluorometer (Urbana, IL) and a 1-cm-path-length quartz cuvette. An internal correction was made for changes in lamp intensity by using a reference solution of rhodamine (3 g/L) in ethylene glycol. For spectral overlap studies, the absorption spectra of membrane-bound *cis*- and *trans*-parinaric acid in the presence of AChR were obtained using a Perkin-Elmer spectrophotometer with 125 μL of AChR in DEPC (1.5 mg/mL), 2.5 mL of buffer A and 2 μM parinaric acid, using the same amount of AChR in the reference cuvette at room temperature.

For measurements with cholesterol hemisuccinate (CHS) and dibromocholesterol hemisuccinate (DiBrCHS), about 25–30 μg of labeled receptor (~0.5 mg/mL) in DOPC or 6,7-BrPC was taken up in 2 mL of buffer A, and DiBrCHS in slightly alkaline DMSO was added in 10-μL aliquots (<0.5%, v/v) with rapid vortexing to give a final concentration from 1 to 8 μM, contributing about 50 mol % of the total lipids at the maximal concentration used. After incubation at room temperature for 20 min, the fluorescence-intensity measurements were made directly in duplicate at an excitation wavelength of 345 nm and an emission wavelength of 376 nm for pyrene. In cases where agonists and antagonists were present, they were added before DiBrCHS. Carbamylcholine in buffer A was added in 10-μL aliquots to 2.5 mL of buffer A containing about 30 μg of labeled receptor in PC:PA:Chol, to give a final concentration of 1 mM, and the mixture was incubated for 20 min at room temperature; the indicated concentrations of DiBrCHS were then added as described above. Similarly, α-bungarotoxin in buffer A was added in 10-μL aliquots to give a final concentration of 0.4 μM.

Quenching and energy-transfer parameters are described under Materials and Methods in the accompanying paper (Narayanaswami et al., 1993).

RESULTS

Energy-Transfer Measurements with Parinaric Acid. The *cis* and *trans* isomers of parinaric acid are sensitive fluorescent probes that are useful in assessing membrane fluidity and monitoring protein–lipid interactions (Illsley et al., 1988; Sklar et al., 1979b; Kimelman et al., 1979). Unlike diphenylhexatriene, which gives a measure of the average fluidity of the bulk and boundary lipids, parinaric acid isomers have the advantage of selective partitioning behavior (Sklar et al., 1979a,b). *trans*-Parinaric acid has a partition coefficient, K_p[solid]/[fluid], of about 4.0, preferring the gel phase, while *cis*-parinaric acid has a partition coefficient of about 1.0, distributing fairly evenly between the gel and fluid phases. In addition, *trans*-parinaric acid has a quantum yield of 0.33 in the gel phase and 0.04 in the fluid phase, while *cis*-parinaric acid has a quantum yield of 0.22 and 0.06 in the gel and the fluid phase, respectively. Both the *cis* and *trans* isomers absorb at 320 nm and have a fluorescence emission maximum at 412 nm. Parinaric acid acts as a good energy-transfer acceptor from tryptophan residues in proteins which have a fluorescence

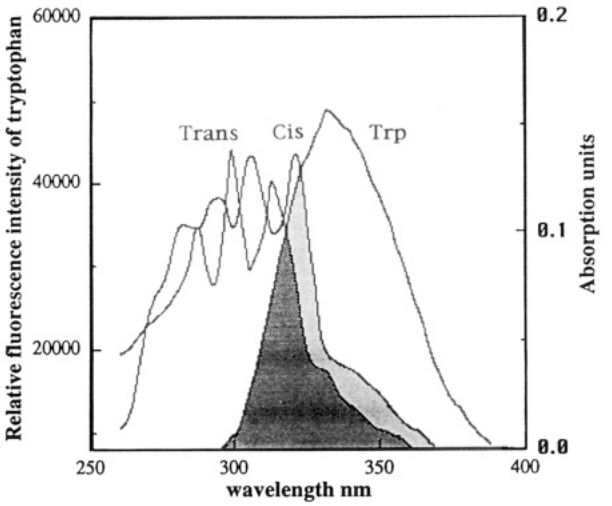

FIGURE 1: Spectral overlap between the emission spectrum of the intrinsic fluorescence of AChR and the absorption spectrum of parinaric acid in the presence of AChR. The emission spectrum is plotted as relative fluorescence intensity vs wavelength, at an excitation wavelength of 290 nm, and the absorption spectrum is plotted as absorbance units vs wavelength. The region of spectral overlap is shaded light gray for *cis*-PnA and dark gray for *trans*-PnA.

emission maximum at about 334 nm. Figure 1 shows the extent of overlap between the fluorescence emission spectrum of tryptophan in AChR and the absorption spectra of *cis*- and *trans*-parinaric acid in reconstituted membranes containing AChR in dielaidoylphosphatidylcholine (DEPC). AChR in DEPC without any added probe served as the blank for absorption spectrum measurements.

AChR was prepared in DEPC since the phase transition temperature of DEPC is at a convenient temperature of 7–8 °C; also, AChR reconstituted into DEPC has been shown to be functional in terms of affinity-state transitions (Fong & McNamee, 1986). The temperature dependence of energy transfer between tryptophan and *cis*- and *trans*-parinaric acid was studied in order to examine energy transfer as a function of average lipid fluidity. The fluorescence intensity of tryptophan is shown in Figure 2, after excitation at 290 nm, in the absence and in the presence of *cis*- and *trans*-parinaric acid. The temperature was decreased gradually from 25 to about 3 °C, using a circulating water bath to cool the cuvette holder. Maximum quenching by energy transfer was seen at temperatures above the phase transition temperature, and the degree of quenching diminishes with a decrease in temperature. Energy transfer is higher for *cis*-parinaric acid than for *trans*-parinaric acid at all temperatures.

Energy transfer was measured as a function of probe concentration at temperatures below (3 °C) and above (25 °C) the phase transition since preferential distribution of probes at different temperatures can affect energy-transfer efficiencies. Plots are presented as F/F_0 (a representative F/F_0 value is presented where F_0 and F are the intrinsic fluorescence intensities in the absence and the presence of the quencher parinaric acid) as a function of probe concentration in Figure 3. A decrease in the fluorescence intensity of tryptophan upon addition of both *cis*- and *trans*-PnA is observed, above and below the transition temperature. At 25 °C, at concentrations up to 0.5 μM, quenching by the *cis* and *trans* isomers is practically indistinguishable, and beyond this concentration *cis*-PnA quenches consistently, albeit only slightly, more than *trans*-PnA. At 3 °C the difference between the two isomers is observed at all concentrations, with greater quenching by *cis*-PnA than by *trans*-PnA. Energy-transfer

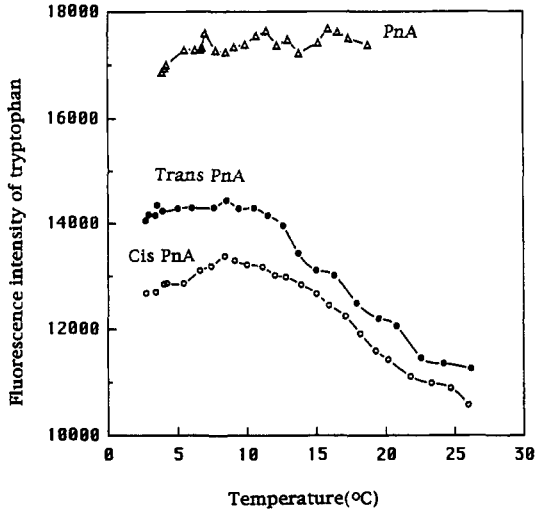

FIGURE 2: Temperature dependence of resonance energy transfer from Trp in AChR/DEPC to *cis*- and *trans*-parinaric acid. Fluorescence intensity was measured at 334 nm after excitation at 290 nm, in a slow time based acquisition mode. The cuvette holder was cooled from 25 to about 2–3 °C at a rate of about 0.3 °C/min. *cis*-PnA and *trans*-PnA were present at a concentration of 2 μM and were added to 2.5 mL of buffer A containing about 75 μg of AChR/DEPC. (△) Control without added PnA. (●) *trans*-PnA. (○) *cis*-PnA.

measurements restrict observations to the probes in the immediate vicinity of the receptor, due to the intrinsic distance dependence of the dipole–dipole energy-transfer process (Forster, 1965; Stryer & Haugland, 1967). On the basis of the above results, the receptor is thought to be preferentially surrounded by a fluid layer of lipids.

In Figure 4, the fluorescence intensities of *cis*- and *trans*-parinaric acid at 412 nm are measured directly at an excitation maximum of 320 nm and are plotted as a function of temperature and concentration of probes. The intensity, in this case, is the weighted average with contributions from the probes in both the annular and bulk domains. At 3 °C, when the bulk lipids are in a gel phase, *trans*-parinaric acid has a 2-fold higher fluorescence intensity compared to the *cis* isomer, with a maximum difference at a concentration of about 2 μM. At 25 °C, when the bulk lipids are in a more fluid phase, the fluorescence intensity of both isomers is roughly the same, with the *cis* isomer having a slightly higher fluorescence intensity at all concentrations.

Quenching of N-(1-Pyrenyl)maleimide-Labeled AChR. To further characterize the nature and specificity of the boundary lipids in a specified interfacial region of the receptor, quenching studies were carried out using an extrinsic fluorophore, *N*-(1-pyrenyl)maleimide (NPM), which labels cysteine residues in a hydrophobic environment. Earlier studies made use of the intrinsic fluorescence of membrane proteins, as in (Ca^{2+}–Mg^{2+})-ATPase (Simmonds et al., 1982, 1984) and AChR (Jones & McNamee, 1988), which is quenched when the protein is reconstituted into bilayers containing brominated phospholipids due to occupation of the phospholipid site. A similar strategy was used here with pyrene-labeled AChR, where the residues labeled have been earlier identified as γCys416 and γCys420 in the MA domain and γCys451 in the M4 domain (Li et al., 1990). Purified AChR in DOPC (high lipid to protein ratio) was labeled with *N*-(1-pyrenyl)-maleimide under conditions that label the γ subunit selectively. All the DOPC was exchanged for 6,7-BrPC, and pyrene-labeled AChR at a low lipid to protein mole ratio of about 100–150:1 was eluted and dialyzed. The fluorescence intensity of pyrene-labeled AChR was observed at 376 nm following

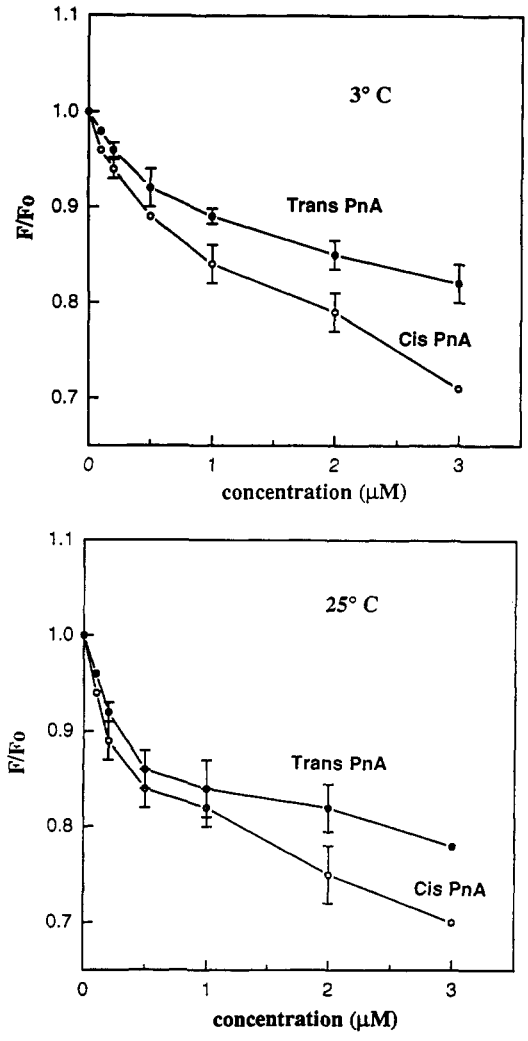

FIGURE 3: Resonance energy transfer as a function of parinaric acid concentration above and below the phase transition temperature of DEPC. Fluorescence intensity of Trp at 334 nm is plotted as F/F_0 vs parinaric acid concentration at 3 and 25 °C. F_0 and F are the fluorescence intensities in the absence and in the presence of parinaric acid, respectively. Excitation was at 290 nm. (●) *trans*-PnA. (○) *cis*-PnA. The concentration of AChR/DEPC was 75 μg/mL in buffer A. The samples were incubated at their respective temperatures (3 and 25 °C) before the fluorescence intensity measurements were taken, to ensure equilibration. Other conditions were as described in Materials and Methods. The lipid:PnA ratio was about 150:1 at the highest concentration of PnA.

excitation at 345 nm. Figure 5 shows the quenching profiles when cholesterol hemisuccinate and dibromocholesterol hemisuccinate were added at different concentrations to pyrene-labeled AChR in DOPC and in 6,7-BrPC.

The fluorescence intensity of pyrene-labeled AChR in DOPC in the absence of the quenching moiety (Br) in cholesterol hemisuccinate (CHS) is used as the control (curve labeled as DOPC/CHS in Figure 5). Addition of dibromocholesterol hemisuccinate (DiBrCHS) to the labeled receptor in DOPC quenched the fluorescence (35%) in a concentration-dependent manner (curve labeled DOPC/DiBrCHS). A decrease in fluorescence intensity by 25% is seen in labeled receptors prepared in 6,7-BrPC, representing maximum quenching when the brominated phospholipids occupy all the sites accessible to phospholipids at the protein–lipid interface. When DiBrCHS was added to the labeled receptor in 6,7-BrPC, additional quenching (~25%) of the fluorescence occurred (curve labeled 6,7-BrPC/DiBrCHS). This result implies that some sites in the vicinity of cysteine-bound pyrene are occupied by cholesterol and that these sites are not

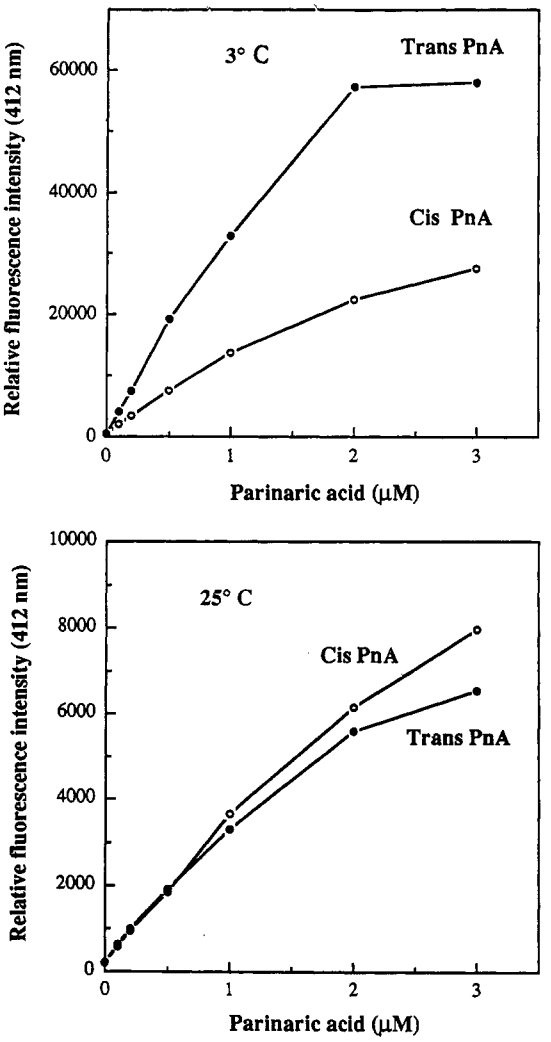

FIGURE 4: Fluorescence intensities of *cis*- and *trans*-parinaric acid in AChR/DEPC membranes as a function of their concentration above (25 °C) and below (3 °C) the phase transition temperature of DEPC. Fluorescence intensities of *trans*-PnA (●) and *cis*-PnA (○) were measured at 412 nm; the excitation wavelength was 320 nm. The concentration of AChR/DEPC was 75 μg/mL in buffer A. Parinaric acid was added at the different concentrations in 10 μL of ethanol as described in Materials and Methods. The lipid:PnA ratio was 150:1 at the highest concentration of PnA.

accessible to phospholipids. Addition of CHS to pyrene-labeled AChR in 6,7-BrPC (curve labeled 6,7-BrPC/CHS) led to a small increase in fluorescence, an observation also seen when oleic acid was added to the labeled receptor in 6,7-BrPC (Figure 6, curve labeled BrPC/OA), suggesting a nonspecific effect. Also, when brominated fatty acid, 6,7-dibromostearic acid (6,7-DiBrStA), was added to labeled AChR in 6,7-BrPC (curve labeled BrPC/6,7-DiBrStA), the level of quenching was very small (~5%).

Effects of Agonists and Antagonists on Quenching. The effects of AChR agonists and antagonists on quenching by DiBrCHS were studied to see if the accessibility to cholesterol is altered by ligand-induced conformational changes. Earlier work demonstrated that *N*-phenylmaleimide- (Yee et al., 1986) or *N*-(1-pyrenyl)maleimide-labeled receptors (Clarke & Martinez-Carrion, 1986) did undergo ligand-induced affinity-state transitions, although the ion flux activity was inhibited. Pyrene-labeled AChR was reconstituted in PC:PA:Chol, where cholesterol was added as cholesterol hemisuccinate at concentrations varying from 0 to 8–10 μM, amounting to about 50 mol % of the total lipids at the maximal concentration used; unlabeled receptor was used as the control. In control

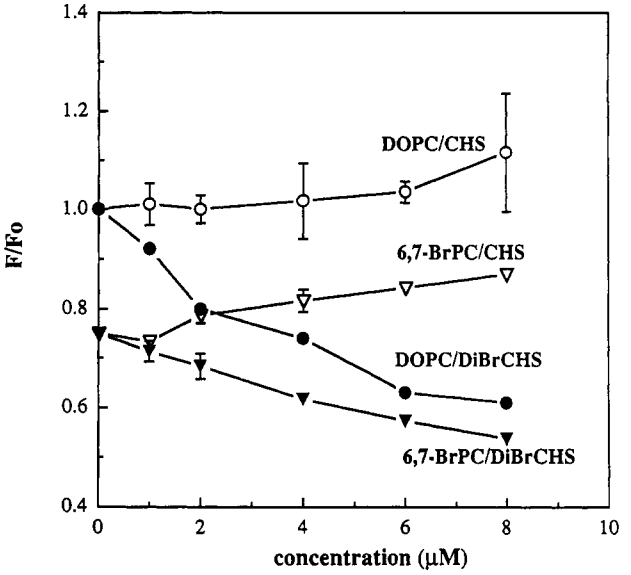

FIGURE 5: Fluorescence-quenching profiles of cholesterol hemisuccinate (CHS) (○, ▽) and dibromocholesterol hemisuccinate (DiBrCHS) (●, ▼) using pyrene-labeled AChR in DOPC (○, ●) and 6,7-BrPC (▽, ▼). Pyrene-labeled AChR, 25–30 μg (lipid/protein ratio about 100:1), was taken up in 2 mL of buffer A, and CHS and DiBrCHS were added directly at the indicated concentrations in 10 μL of DMSO. Fluorescence excitation was at 345 nm, and emission was measured at 376 nm. The data are plotted as F/F_0 vs concentration of CHS or DiBrCHS. Values are mean ± SEM (n = 3).

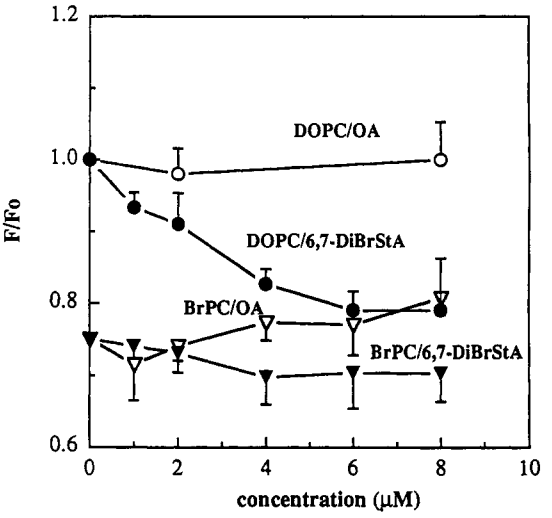

FIGURE 6: Fluorescence-quenching profiles of oleic acid (OA) (○, ▽) and 6,7-dibromostearic acid (6,7-DiBrStA) (●, ▼) using pyrene-labeled AChR in DOPC (○, ●) and 6,7-BrPC (▽, ▼). Pyrene-labeled AChR, 25–30 μg, (lipid/protein ratio about 100:1), was taken up in 2 mL of buffer A, and OA and 6,7-DiBrStA were added directly at the indicated concentrations in 10 μL of DMSO. Fluorescence excitation was at 345 nm, and emission was measured at 376 nm. The data are plotted as F/F_0 vs concentration of OA or 6,7-DiBrStA. Values are mean ± SEM (n = 3).

experiments in the absence of any added ligand, buffer A was added so that the final volume remained the same, and there were no dilution effects. Table I shows the quenching constants and the fraction of accessible fluorophores, obtained from Stern–Volmer [see Lakowicz (1983)] and Lehrer plots (Lehrer, 1971), respectively, when DiBrCHS was added to both pyrene-labeled and unlabeled receptor. In pyrene-labeled AChR there was about 65% accessibility to cholesterol with a quenching constant, K_{SV}, of 0.123. In the desensitized state, in the presence of 1 mM carbamylcholine, the quenching constant increased to 0.167 and the accessibility to cholesterol

Table I: Stern–Volmer Quenching Constants and Apparent Fraction of Accessible Fluorophores for Quenching of Pyrene-Labeled AChR and Intrinsic AChR Fluorescence by Dibromocholesterol Hemisuccinate in the Presence of Agonists and Antagonists

ligand	K_{SV}[a]	$f_{a(app)}$[b]
AChR-NPM		
control (–carb)[c]	0.123 ± 0.010	0.65 ± 0.05
+carb (1 mM)	0.167 ± 0.015	0.90 ± 0.08
+α-Btx (0.4 μM)	0.134 ± 0.012	0.70 ± 0.07
AChR-Trp		
control (–carb)	0.043 ± 0.003	0.27 ± 0.01
+carb (1 mM)	0.032 ± 0.003	0.23 ± 0.01
+α-Btx (0.4 μM)	0.037 ± 0.003	0.31 ± 0.05

[a] Stern–Volmer quenching constants calculated from plots of F_0/F vs quencher concentration. [b] Accessibility calculated from plots of $F_0/\Delta F$ vs $1/[Q]$. Values are averages from three independent experiments with duplicates from each. [c] Average from about 10 different experiments.

increased by about 20–25%. However, 0.4 μM α-bungarotoxin did not have any significant effect in terms of changing the accessibility. The change triggered by carbamylcholine, as sensed at the protein–lipid interface in the bilayer environment, is statistically significant.

In the case of the unlabeled AChR, it is seen that quenching by cholesterol is much less, with quenching constants about one-fourth that seen with pyrene-labeled receptor. The presence of carbamylcholine appears to have an opposite effect when compared to the data obtained for the labeled receptor. K_{SV} decreased by about 25%, and the percent of accessible fraction decreased by about 15%. α-Bungarotoxin had no significant effect.

DISCUSSION

We have used two classes of fluorophores to examine membrane dynamics and specific lipid–protein interactions: (1) parinaric acid, which partitions into the lipids and can be used to probe the immediate environment surrounding the AChR by means of energy transfer, and (2) *N*-(1-pyrenyl)-maleimide, which labels specified cysteine residues on the receptor. Direct receptor contact with the lipids can be monitored by suitable use of quenchers such as cholesterol and fatty acid derivatives which would partition preferentially into lipid bilayers.

Parinaric acid is useful as a probe for studying the lipid domains around the receptor since the *cis* and *trans* isomers are differentially sensitive to the fluidity of the lipids. *trans*-Parinaric acid is sensitive even to a few percent gel phase of lipids, due to its enhanced quantum yield and preferential partitioning in the solid phase (Sklar et al., 1979a,b). As an acceptor of energy transfer from the emission of tryptophan in the receptor, parinaric acid is used to sense contacts with the membrane protein at very low concentrations, although it is difficult to estimate how far into the boundary layer of lipids energy transfer can take place. All studies with parinaric acid were done with AChR preparations in DEPC (the 9-*trans* isomer of DOPC), which has a phase transition temperature at about 7–8 °C. The receptor was found to be functional in this lipid environment in terms of agonist-induced affinity-state transitions (Fong & McNamee, 1986). The higher degree of energy transfer from tryptophan to *cis*-parinaric acid, especially at temperatures below the phase transition temperature, reflects a relatively fluid phase surrounding the receptor, at least in the vicinity of the tryptophan. This is supported by lower levels of quenching of tryptophan fluorescence by *trans*-parinaric acid. If there exists a relatively fluid layer of lipids around the receptor, the *trans* isomer, which has a preference for the gel phase, would be excluded from the fluid phase, thereby resulting in decreased energy transfer, as observed. Interestingly, of the 51 Trp residues in the AChR (Noda et al., 1983), only one, namely, Trp 453 in the putative M4 helix of the γ subunit, is located in the bilayer region, possibly at the protein–lipid interface, about 10 Å from the center of the bilayer (Chattopadhyay & McNamee, 1991).

That the probe is sensing the immediate environment of the receptor in energy-transfer experiments and not the bulk lipid is seen in the complementary set of experiments where the fluorescence intensity of the probes at 412 nm was measured at different temperatures (Figure 4). The fluorescence intensity would have contributions from the probes in both the bulk and the immobilized phase. A few points are noteworthy: (1) at 3 °C, when the bulk lipids are in the gel phase, the fluorescence intensity of *trans*-PnA is at least 8-fold higher compared to its intensity at 25 °C, while for *cis*-PnA it is about 3-fold higher; (2) at 3 °C, the fluorescence intensity of the *trans* isomer is about 3-fold higher than that of the *cis* isomer at the maximum concentration studied; and (3) at 25 °C, when the bulk lipids are in a fluid phase, the fluorescence intensity of *cis*-PnA is slightly higher than that of *trans*-PnA. Similar results indicating that a relatively fluid layer of lipids surrounds membrane proteins have been reached using Ca^{2+}-ATPase in sarcoplasmic reticulum (Blazyk et al., 1985) and the M13 virus coat protein (Kimelman et al., 1979). The presence of a fluid microenvironment around the receptor probably explains the functional response in DEPC (Fong & McNamee, 1986) in terms of agonist-mediated conformational changes from a low- to a high-affinity state, at temperatures above and below the phase transition temperature, and supports the optimal fluidity hypothesis on a more spatially localized level.

In the above discussion the term "boundary layer" is used to indicate the layer of lipids perturbed by the receptor and would encompass both the annular and the nonannular sites, as described by Simmonds et al. (1982). A minimum of 45 mol of lipid per 1 mol of receptor was suggested to be essential to maintain the functional integrity of AChR (Jones et al., 1988). Further, two classes of mutually exclusive lipid binding sites were believed to exist (Jones & McNamee, 1988) on the basis of the quenching of intrinsic fluorescence by brominated phospholipids and cholesterol: annular sites at the protein–lipid interface that accomodate phospholipids and nonannular sites which bind cholesterol and exclude phospholipids. Fatty acids were found to compete for both annular and nonannular sites.

Using the same strategy, but with extrinsic fluorophores attached to specific cysteine residues on the γ subunit, similar sites that are unique to cholesterol and not accessible to phospholipids are implicated in the present study. In addition, the fluorophore distribution is better understood since the NPM labeling is restricted to a few cysteine residues in the γ subunit, only one of which is believed to be in a transmembrane domain. In the accompanying paper (Narayanaswami et al., 1993), we have indicated that the cysteine-bound pyrene is at a superficial location, close to the membrane/water interface; an average distance of about 18 Å was measured by energy-transfer studies between tryptophan residue(s) and cysteine-bound pyrene residues. As cholesterol hemisuccinate has been shown (Simmonds et al., 1984) to be readily incorporated into membranes up to a molar ratio of 3:1 (cholesterol:phospholipids), it is probably sensing the cysteine-bound pyrene in the transmembrane region. Inter-

stitial spaces at the receptor's surface such as regions of subunit contact or sites wedged between tilted transmembrane α helices appear to be likely locations for binding sterol moities (Jones & McNamee, 1988).

An increase in accessibility and quenching constant for the quenching of pyrene-labeled AChR by dibromocholesterol hemisuccinate in the presence of carbamylcholine (Table I) was observed. The agonist-mediated alterations from the resting to the desensitized state probably involve a conformational change wherein buried and/or partially exposed residues are more exposed, resulting in increased access to quenching. Interestingly, this is accompanied by a decrease in quenching constant and accessibility to quenching of the *intrinsic* fluorescence by dibromocholesterol hemisuccinate. In the membranous portion, if the M4 segment is considered to be an α helix, then γCys 451 and γTrp 453 would be on opposite sides of the helix, and such complementary changes may be expected to occur. Gonzales-Ros et al. (1983) showed that carbamylcholine altered the accessibility of a soluble quencher to receptor that had been labeled under similar conditions in native *Torpedo california* membranes.

Cryoelectron microscopy images (Unwin et al., 1988) of flattened vesicular crystals grown from postsynaptic membranes of *Torpedo marmorata*, obtained at about 18-Å resolution, show that carbamylcholine induces a quarternary rearrangement of the AChR, involving a tangential displacement of the δ subunit by about 10° with respect to the receptor axis and a radial displacement of the γ subunit away from the axis toward the periphery of the receptor. Similar localized conformational changes during receptor desensitization involving the γ subunit have been implicated by photolabeling studies, using triphenylmethylphosphonium (Hucho et al., 1986) and chlorpromazaine [see Galzi et al. (1991)], while the δ subunit has been suggested to play a role in channel gating (Sakmann et al., 1985) from electrophysiological studies using chimeric receptors in *Xenopus* oocytes.

From the data presented here, it is difficult to assign the side of the M4 domain that faces the lipid and the side that faces the rest of the helical bundle. An axial projection of the M4 segment of the γ subunit is shown as a helical wheel plot in Figure 7A. The M4 domain of all the subunits of AChR has been recognized as the most hydrophobic of the four transmembrane segments. Point mutation studies (Li et al., 1990) where the γCys 451 was mutated to either Ser or Trp indicated a decrease in normalized channel activity by about 50%. If the hydrophobicities of the M4 domain residues are considered, it is possible that the side of the helix containing amino acids with aliphatic side chains faces the lipid environment (Eisenberg et al., 1982). On the other hand, it has been shown (Blanton & Cohen, 1992) that the polar uncharged side of the M4 domain faces the lipid environment, while it has been observed (Gray & Mathews, 1984) that about 75% of Ser, Thr, and Cys residues occurring in the transmembrane region in an α helix have a tendency to form intrahelical hydrogen bonding with the carbonyl group $n - 3$ or $n - 4$ residues away, toward the N-terminus, enabling that side to face the hydrophobic milieu. This would mean that the side of the M4 domain containing the residues Ser, Thr, and Cys is buried in the lipid domain of the membrane. From our observations it appears possible that Trp and Cys, being located on diametrically opposite sides of the helix and having similar nonannular sites, are at or near regions of interhelical contact. The cholesterol binding domains are probably located at intersubunit sites and/or interhelical sites (Figure 7B), which

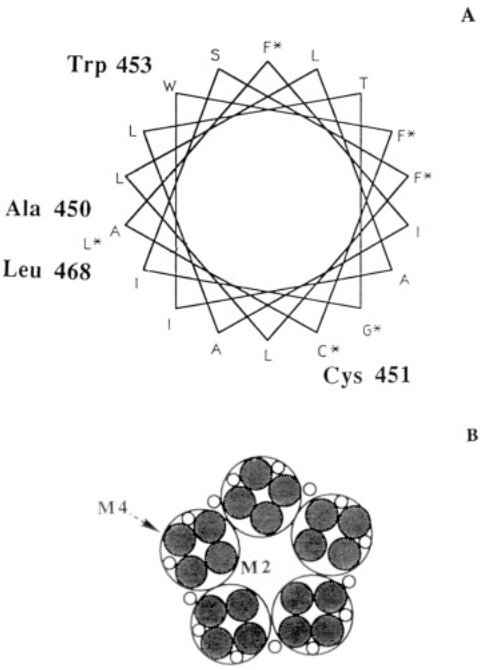

FIGURE 7: (A) Helical wheel plot of the putative M4 helix of the γ subunit of *Torpedo* AChR, from Ala 450 to Leu 468, represented as single-letter amino acid codes. Conserved residues are shown with an asterisk (*). (B) Model of the AChR subunits (big circles) showing the four transmembrane domains (shaded medium circles) and the possible cholesterol binding sites (small open circles). The domains believed to face the ion channel and the lipid milieu, M2 and M4, respectively, are indicated.

confer a critical role for the sterol moities in modulating the receptor function.

In conclusion it can be said that the AChR is surrounded by a relatively fluid layer of lipids which probably maintains an optimal fluidity especially at localized regions, facilitating conformational changes. There appear to be sites for cholesterol binding in the vicinity of cysteine-bound pyrene on the receptor in the membranous portion, the access to which increases in the presence of carbamylcholine but not α-bungarotoxin. The ligand-induced conformational changes are sensed at the protein–lipid interface, supporting a possible functional linkage between the membrane environment and ligand-mediated ion conductance changes.

ACKNOWLEDGMENT

We thank Jordan Scalo for purifying the acetylcholine receptor. We thank Dr. David Deamer for use of the SLM 8000C spectrofluorometer and Dr. Owen Jones for the brominated cholesterol hemisuccinate.

REFERENCES

Bhushan, A., & McNamee, M. G. (1990) *Biochim. Biophys. Acta 1027*, 93–101.

Bhushan, A., & McNamee, M. G. (1993) *Biophys. J. 64*, 716–723.

Blanton, M. P., & Wang, H. H. (1990) *Biochemistry 29*, 1186–1194.

Blanton, M. P., & Cohen, J. B. (1992) *Biochemistry 31*, 3738–3750.

Blazyk, J., Wu, C.-J., & Wu, S.-C. (1985) *J. Biol. Chem. 260*, 4845–4849.

Butler, D., & McNamee, M. G. (1993) *Biochim. Biophys. Acta 1150*, 17–24.

Chattopadhyay, A., & McNamee, M. G. (1991) *Biochemistry 30*, 7159–7164.

Clarke, J. H., & Martinez-Carrion, M. (1986) *J. Biol. Chem. 261*, 10063–10072.
Criado, M., Eibl, E., & Barrantes, F. J. (1982) *Biochemistry 21*, 3622–3629.
Eisenberg, D., Weiss, R. M., & Terwilliger, T. C. (1982) *Nature (London) 299*, 371–374.
Ellena, J. F., Blazing, M. A., & McNamee, M. G. (1983) *Biochemistry 22*, 5523–5535.
Fong, T. M., & McNamee, M. G. (1986) *Biochemistry 25*, 830–840.
Fong, T. M., & McNamee, M. G. (1987) *Biochemistry 26*, 3871–3880.
Forster, T. (1965) in *Modern Quantum Chemistry* (Sinanoglu, O., Ed.) Part 3, pp 93–137, Academic Press, New York.
Galzi, J.-L., Revah, F., Bessis, A., & Changeaux, J.-P. (1991) *Annu. Rev. Pharmacol. 31*, 37–72.
Giraudat, J., Montecucco, C., Bisson, R., & Changeaux, J.-P. (1985) *Biochemistry 24*, 3121–3127.
Gonzales-Ros, J. M., Farach, M. C., & Martinez-Carrion, M. (1983) *Biochemistry 22*, 3807–3811.
Gray, T. M., & Mathews, B. W. (1984) *J. Mol. Biol. 175*, 75–81.
Hucho, F., Oberthur, W., & Lottspeich, F. (1986) *FEBS Lett. 205*, 137–142.
Illsley, N. P., Lin, H. Y., & Verkman, A. S. (1988) *Biochemistry 27*, 2077–2083.
Imoto, K., Methfessel, C., Sakmann, B., Mishina, M., Mori, Y., Konno, T., Fukuda, K., Kurasaki, M., Bujo, H., Fujita, Y., & Numa, S. (1986) *Nature (London) 324*, 670–674.
Imoto, K., Busch, C., Sakmann, B., Mishina, M., Konno, T., Nakai, J., Bujo, H., Mori, Y., Fukuda, K., & Numa, S. (1988) *Nature (London) 335*, 645–648.
Jones, O. T., & McNamee, M. G. (1988) *Biochemistry 27*, 2364–2374.
Jones, O. T., Eubanks, J. H., Earnest, J. P., & McNamee, M. G. (1988) *Biochemistry 27*, 3733–3742.
Jost, P. C., Griffith, O. H., Capaldi, R. A., & Vanderkooi, G. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 480–484.
Kimelman, D., Tecoma, E. S., Wolber, P. K., Hudson, B. S., Wickner, W. T., & Simoni, R. D. (1979) *Biochemistry 18*, 5874–5880.
Lakowicz, J. R. (1983) in *Principles of Fluorescence Spectroscopy*, Plenum Press, New York.
Lehrer, S. S. (1971) *Biochemistry 10*, 3254–3263.
Li, L., Schuchard, M., Palma, A., Pradier, L., & McNamee, M. G. (1990) *Biochemistry 29*, 5428–5436.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. (1951) *J. Biol. Chem. 193*, 265–275.
Marquez, J., Iriarte, A., & Martinez-Carrion, M. (1989) *Biochemistry 28*, 7433–7439.
McClare, C. W. F. (1971) *Anal. Biochem. 39*, 527–530.
Narayanaswami, V., Kim, J., & McNamee, M. G. (1993) *Biochemistry* (preceding paper in this issue).
Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Kikyotani, S., Furutani, Y., Hirose, T., Takashima, H., Inayama, S., Miyata, T., & Numa, S. (1983) *Nature 302*, 528–532.
Ochoa, E. L. M., Dalziel, A. W., & McNamee, M. G. (1983) *Biochim. Biophys. Acta 727*, 151–162.
Pradier, L., Yee, A. S., & McNamee, M. G. (1989) *Biochemistry 28*, 6562–6571.
Sakmann, B., Methfessel, C., Mishina, M., Takahashi, T., Takai, T., Kurasaki, M., Fukuda, K., & Numa, S. (1985) *Nature (London) 318*, 538–543.
Simmonds, A. C., East, J. M., Jones, O. T., Rooney, E. K., McWhirter, J., & Lee, A. G. (1982) *Biochim. Biophys. Acta 693*, 398–406.
Simmonds, A. C., Rooney, E. K., & Lee, A. G. (1984) *Biochemistry 23*, 1432–1441.
Sklar, L. A., Miljanich, G. P., & Dratz, E. A. (1979a) *Biochemistry 18*, 1707–1716.
Sklar, L. A., Miljanich, G. P., Bursten, S. L., & Dratz, E. A. (1979b) *J. Biol. Chem. 254*, 9583–9591.
Stroud, R. M., McCarthy, M. P., & Shuster, M. (1990) *Biochemistry 29*, 11009–11023.
Stryer, L., & Haugland, R. P. (1967) *Proc. Natl. Acad. Sci. U.S.A. 58*, 719–726.
Sunshine, C., & McNamee, M. G. (1992) *Biochim. Biophys. Acta 1108*, 240–246.
Unwin, N., Toyoshima, C., & Kubalek, E. (1988) *J. Cell Biol. 107*, 1123–1138.
White, B. H., & Cohen, J. B. (1988) *Biochemistry 27*, 8741–8751.
Yee, A. S., Corey, D. E., & McNamee, M. G. (1986) *Biochemistry 25*, 2110–2119.