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Annular and Nonannular Binding Sites for Cholesterol Associated with the Nicotinic Acetylcholine Receptor†

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ABSTRACT: Interactions between lipids and the nicotinic acetylcholine receptor from *Torpedo californica* have been measured in reconstituted membranes containing purified receptor and defined lipids. The ability of brominated lipids to partially quench the intrinsic fluorescence of the acetylcholine receptor has been exploited to monitor contacts between the protein and the surrounding lipid. Relative binding constants for lipid binding to the protein have been quantitatively determined by measuring quenching observed in mixtures of brominated and nonbrominated lipids by use of equilibrium exchange equations developed by London and Feigenson [London, E., & Feigenson, G. W. (1981) *Biochemistry 20*, 1939–1948] and by Simmonds et al. [Simmonds, A. C., Rooney, E. K., & Lee, A. G. (1984) *Biochemistry 23*, 1432–1441]. Dioleoylphosphatidylcholine and its dibromo derivative are the two principal lipids used in the reconstituted membranes to establish the quenching parameters. Competition studies between cholesterol and phosphatidylcholine indicate that cholesterol does not compete effectively for the phospholipid sites presumed to surround the membrane-embedded portions of the receptor (annular lipids). However, dibromocholesterol partially quenches the receptor and leads to additional quenching of receptor in pure dibromophosphatidylcholine membranes. The results are consistent with the presence of additional binding sites for cholesterol that are not accessible to phospholipids (nonannular sites). Similar results are obtained by using cholesterol hemisuccinate and its dibromo analogue, both of which can be introduced into membranes more easily than cholesterol because of their greater solubility in water. Fatty acids appear to compete for both annular and nonannular sites, and analysis of the quenching data suggests that there are 5–10 nonannular sites associated with the receptor. Cholesterol has been shown to play a critical role in both acetylcholine receptor structural stabilization and ion channel activity, and the results presented here provide additional information about cholesterol–receptor interactions.

Our understanding of biological membranes is largely based on the now classical "fluid-mosaic" model of Singer and Nicolson (1972) where membrane proteins are embedded either wholly or partially in a fluid lipid bilayer. In recent years it has become apparent that the role of the lipids is not merely passive, since the lipids may directly regulate a wide variety of cellular functions. While such regulation is not completely understood, it is likely to be of physiological significance since the lipid composition of cell membranes is known to be affected by changes in diet, disease, or other physiological stresses (Sarzala et al., 1975; Ansell et al., 1973; Lee, 1985). Since effects of lipids are exerted ultimately on the membrane proteins, much interest has been focused on the nature of lipid–protein interactions (Devaux & Seigneuret, 1985). One of the most successful approaches has been to use model membrane systems consisting of proteins that can be readily purified and reconstituted into defined lipid environments (Jones et al., 1987). Among the most extensively characterized systems are $(Na^+ + K^+)$-ATPase (Esmann & Marsh, 1985), $(Ca^{2+} + Mg^{2+})$-ATPase (Froud et al., 1986), rhodopsin (Baldwin & Hubbell, 1985), D-glucose transporter (Tefft et al., 1986), cytochrome *c* oxidase (Griffith et al., 1986), and the nicotinic acetylcholine receptor of *Torpedo californica*

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(AChR),[1] a ligand-gated ion channel (Fong & McNamee, 1986; McNamee et al., 1986a; Montal et al., 1986). The AChR is particularly suitable as a model membrane protein due to the ease with which it can be purified and reconstituted, and due to the full extent to which its biochemical and biophysical properties have been characterized (Popot & Changeux, 1984; McCarthy et al., 1986).

The receptor is a pentameric, transmembrane glycoprotein of molecular weight around 250 000 whose function is to facilitate cholinergic transmission by transducing the binding of acetylcholine into a large increase in the cation permeability of the postsynaptic membrane (Popot & Changeux, 1984; McNamee et al., 1986a; McCarthy et al., 1986). The AChR can be solubilized, purified by affinity chromatography, and subsequently reconstituted into lipid vesicles with full retention of both the ligand binding and ion permeability functions (Epstein & Racker, 1978; Anholt et al., 1981; Criado et al., 1984; McNamee et al., 1986a; Montal et al., 1986).

One feature emerging from studies of the reconstituted AChR is its sensitivity to the lipid environment (Ochoa et al., 1983; Criado et al., 1984; Fong & McNamee, 1986; McNamee et al., 1986b). It is known, for example, that the presence of a lipid environment is essential for preserving receptor function. Extensive delipidation of AChR in native membranes has been found to stabilize the receptor in a state of very low affinity for agonists (Chang & Bock, 1979). More recent evidence suggests that a minimum number of lipids per receptor (~45) are required to prevent irreversible loss of both the allosteric transitions and ion channel gating functions in native and reconstituted AChR (Jones et al., 1988). Additionally, the functional properties of AChR are also highly sensitive to the nature of its lipid environment.

In order to understand the role of lipids in determining AChR function, two approaches have been adopted. First, biochemical studies in which AChR has been reconstituted into a variety of lipid mixtures have been used in order to identify those components required for AChR function (Kilian et al., 1980; Criado et al., 1982, 1984; Fong & McNamee, 1986). Several lipid mixtures have been found that fully support both the ligand binding and ion channel functions of AChR, most notably those containing cholesterol and negatively charged phospholipids. Second, biophysical studies have attempted to quantify the interaction of AChR with particular lipids and correlate AChR function with the physical properties of the lipid environment (Ellena et al., 1983; Criado et al., 1982, 1984; Fong & McNamee, 1986, 1987; McNamee et al., 1986b). From EPR spectroscopy analyses, AChR appears to interact preferentially with those lipids having a marked effect on AChR function, notably phosphatidic acid, cholesterol, and fatty acids (Marsh & Barrantes, 1978; Marsh et al., 1981; Ellena et al., 1983). FTIR analyses indicate that the lipids that support ion channel activity increase the apparent α-helix and β-sheet content of the receptor (Fong & McNamee, 1987).

The cholesterol–AChR interaction is particularly interesting. Cholesterol is a common component of cell membranes, including those of neural origin where it comprises up to 50% of the total lipids on a molar basis (Deutsch & Kelly, 1981; Gonzalez-Ros et al., 1982; Yeagle, 1985). Cholesterol and other sterols have been reported to affect several aspects of receptor function including the allosteric binding transitions for activators and the ion channel events. Initially, cholesterol was thought to be an absolute requirement for the allosteric state transitions (Criado et al., 1982). However, several lipids and lipid mixtures have been shown to facilitate the transitions even in the absence of cholesterol (Fong & McNamee, 1986). By far the most prominent effect of cholesterol appears to be on the ion channel gating event. For example, cholesterol appears to enhance both the conductance and the cooperativity of the agonist-activated ion channel in reconstituted planar bilayers (Nelson et al., 1980; Schindler, 1982). An enhancement of agonist-induced cation flux by cholesterol has also been demonstrated in reconstituted vesicles depending on the type and composition of the other lipids present (Dalziel et al., 1980; Criado et al., 1982, 1984; Fong & McNamee, 1986).

Both the evidence and the explanations for the effects of cholesterol on membrane protein function are controversial (Simmonds et al., 1984; Yeagle, 1985). Cholesterol has a profound effect on the fluidity of phospholipid bilayers where it acts to increase the fluidity of lipids in the gel phase but decrease the fluidity of those lipids in the more physiologically relevant liquid-crystalline state (Yeagle, 1985). Consequently, many attempts have been made to ascribe the effect of cholesterol on protein activity to a decrease in fluidity of the surrounding bilayer. However, the effect of cholesterol on protein function might also be attributed to a more direct interaction of cholesterol with membrane proteins (Schubert & Boss, 1982). The association of steroid analogues with AChR and other proteins has been detected by EPR (Ellena et al., 1983; Devaux & Seigneuret, 1985). In early studies a preferential affinity of AChR for cholesterol compared to other lipids was detected by monolayer techniques (Popot et al., 1978). Very recently, Middlemas and Raftery (1987) have shown that all four receptor subunits can be labeled by a photoreactive cholesterol analogue. More selective labeling has been observed with a related cholesterol analogue (T. M. Fong and M. G. McNamee, unpublished observations). Studies on AChR-rich native membranes have suggested the existence of two pools of cholesterol: a fraction influencing the bulk lipid fluidity and a more tightly bound fraction thought to be associated with the receptor (Leibel et al., 1987). However, while some binding of steroids to proteins might be expected to occur at the lipid–protein interface, there is experimental evidence from another model membrane system, (Ca^{2+} + Mg^{2+})-ATPase, to suggest that cholesterol is excluded from binding at the lipid–protein interface (Warren et al., 1975; Simmonds et al., 1982, 1984; Silvius et al., 1984). Furthermore, theoretical arguments provide support for the exclusion of the rigid steroid molecule from the lipid–protein interface (Israelachvili et al., 1980).

In this paper we have attempted to define the cholesterol–AChR interaction using a fluorescence quenching method first described by Simmonds et al. (1982). Simmonds et al. (1984) and Froud et al. (1986) have shown that when (Ca^{2+} + Mg^{2+})-ATPase is reconstituted into bilayers containing brominated phospholipids, the intrinsic fluorescence of the protein is quenched as a result of the presence of brominated phospholipid at the lipid–protein interface. The fluorescence quenching properties of the brominated molecules can be used to detect displacement of phospholipids from the lipid–protein interface. For example, binding of nonbrominated molecules at the lipid–protein interface can be detected by an increase in protein fluorescence intensity due to displacement of the

[1] Abbreviations: AChR, nicotinic acetylcholine receptor; ATP, adenosine triphosphate; BRPC, 1,2-bis(9,10-dibromostearoyl)phosphatidylcholine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DOPC, dioleoylphosphatidylcholine; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EPR, electron paramagnetic resonance; FTIR, Fourier transform infrared; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; SDS, sodium dodecyl sulfate.

brominated lipid from the surface of the protein. Here we describe the applicability of the fluorescence quenching method in studying AChR–lipid interactions. We show that while the interaction of fatty acids and phospholipids with AChR is mutually exclusive at the lipid–protein interface, cholesterol does not displace phospholipids from AChR. However, cholesterol does appear to interact directly with AChR at other "nonannular" sites from which phospholipids may be excluded.

Materials and Methods

Materials. Dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidic acid (DOPA), and 1,2-bis(9,10-dibromostearoyl)phosphatidylcholine (BRPC) were purchased from Avanti Polar Lipids (Birmingham, AL). Cholesteryl hemisuccinate was from Sigma. Oleic acid was from Fluka. Nitroxide-labeled fatty acids were purchased from Molecular Probes. Cholesterol was obtained from Calbiochem.

Brominated Lipids. Bromination of oleic acid was achieved by addition of bromine (Mallinkrodt) to a solution of oleic acid in chloroform at −20 °C (Simmonds et al., 1982). The reaction mixture was then applied to a silicic acid column and free bromine eluted with chloroform. The brominated fatty acid was then eluted with chloroform–methanol (1:1), rotary evaporated, and stored at −20 °C. Cholesterol was brominated to give 5,6-dibromocholestan-3β-ol by adding bromine to a solution of cholesterol in diethyl ether–glacial acetic acid (2:1 v/v) in the presence of anhydrous sodium acetate (Simmonds et al., 1982). The crystalline product was filtered and free bromine removed by washing with cold glacial acetic acid; the product was then washed with cold methanol, dried under vacuum, and stored at −70 °C. The bromination of cholesterol hemisuccinate was achieved in essentially the same manner as that described for the preparation of cholesterol dibromide (Simmonds et al., 1984). The product ran as a single spot on thin-layer chromatography [petroleum ether (60–80 °C)–acetone, 9:2 v/v]. The bromine content of the steroid, phospholipid, and fatty acid analogues was determined by incubating the samples, or standards of 1,12-dibromododecane, in 5% (w/v) methanolic potassium *tert*-butoxide for 1 h at 125 °C (Markello et al., 1985). The solutions were acidified, SDS was added to a final concentration of 1% (w/v), and the free bromide was determined by the method of Tietz (1970) using gold chloride.

Purification of Acetylcholine Receptor. Acetylcholine receptor was purified from frozen electric tissue of *T. californica* by affinity chromatography exactly as described by Jones et al. (1987). Tissue was obtained from Dr. H. Wang, University of California at Santa Cruz. The lipid to protein ratio was adjusted to 100:1 by using DOPC or BRPC. Cholate and carbamylcholine were removed by dialysis, and lipid–AChR complexes were frozen in liquid nitrogen.

Reconstitution of Purified AChR in Membranes. Reconstitution of receptor into membranes consisting of mixtures of DOPC or BRPC with either cholesterol, cholesterol dibromide, or DOPA was achieved by adding DOPC–AChR or BRPC–AChR (0.5 mg) at a lipid to protein mole ratio of 100:1 (see above) to a solution containing the desired lipids dispersed in cholate at a final detergent concentration of 1% (w/v). The mixture was allowed to equilibrate on ice for 1 h and was then dialyzed at 4 °C against 6 L of buffer A (100 mM NaCl, 10 mM MOPS, 1 mM EDTA, 4 mM NaN_3, pH 7.4) for 48 h with two changes and then against 6 L of fluorescence buffer (100 mM NaCl, 40 mM MOPS, 1 mM EDTA, pH 7.4) for 24 h. As a precaution, samples with and without brominated lipids were dialyzed separately. Samples were not frozen and thawed but were used immediately. Samples containing cholesterol or cholesterol dibromide were prepared at a final lipid to protein mole ratio of 400:1. Samples containing mixtures of DOPA or DOPC with BRPC were prepared at a higher lipid to protein ratio of 600:1. The final concentration of AChR was 0.05–0.06 μM.

Fluorescence. Steady-state and fluorescence lifetime measurements were made on an SLM 4800 spectrophotofluorometer. Unless stated otherwise, an excitation wavelength of 280 or 295 nm was used. Emission was monitored through a 305-nm cutoff filter (Schott) at the wavelength of maximum fluorescence (336 nm). Excitation and emission slit widths were 4 nm. An internal correction was made for changes in lamp intensity by using a reference solution of rhodamine B (3 g/L) in ethylene glycol. All sample solutions had an optical density of less than 0.05. Fluorescence lifetime measurements were made by using the phase-modulation method at a modulation frequency of 18 or 30 MHz (Spencer & Weber, 1969). Phase and modulation data were measured relative to a reference fluorophore consisting of a solution of *p*-terphenyl in distilled ethanol (τ_0 = 1.05 ns) rather than the more usual glycogen scatterer, as recommended by Lakowicz et al. (1981).

The temperature of the sample solutions was maintained to within 0.5 °C by using a cuvette jacket fitted to a heating–cooling water bath (Haake Instruments). The sample temperature was maintained at 25 °C and checked before, and after, each measurement with a digital thermometer.

In general, receptor-containing samples were added to 2.5 mL of fluorescence buffer and incubated for 10 min. Aliquots of cholesterol hemisuccinate or fatty acids were added from concentrated stock solutions in methanol and allowed to equilibrate for at least 10 min. Fluorescence intensities were corrected for differences in protein concentration and dilution.

Table I: Intrinsic Fluorescence of Membrane-Associated AChR[a]

membranes	τ_0	λ_{em}(max)
DOPC–AChR (100:1)	3.41 ± 0.079 (6)	340
BRPC–AChR (100:1)	3.95 ± 0.019 (3)	340
native membranes	3.79 ± 0.070 (6)	338

[a] Fluorescence lifetimes and emission maxima for AChR in different lipid environments. Fluorescence lifetimes were determined by the phase shift method using *p*-terphenyl as a reference lifetime standard (see Materials and Methods). Values shown are the means [plus or minus (±) the standard deviation], and the number of determinations is shown in parentheses. The wavelength of fluorescence excitation was 280 nm.

Results

Interaction of AChR with Quenching Lipids. The acetylcholine receptor is a multisubunit protein containing 51 tryptophans and is thus intrinsically fluorescent. 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 (Table I). (A detailed dicussion of the fluorescence properties of AChR will be presented elsewhere.)

In previous studies it has been shown that the fluorescence of the AChR can be partially quenched by spin-labeled lipids incorporated into native membranes (Marsh & Barrantes, 1978). Replacement of AChR lipids by the quenching lipid (9,10-dibromostearoyl)phosphatidylcholine (BRPC) in reconstituted membranes also led to partial quenching. Following reconstitution into brominated lipid the receptor fluorescence was decreased to about 80% of that seen with AChR in DOPC (Figure 1). The quenching was not accompanied by a similar decrease in the fluorescence lifetime (Table I). As will be discussed later, such quenching is in-

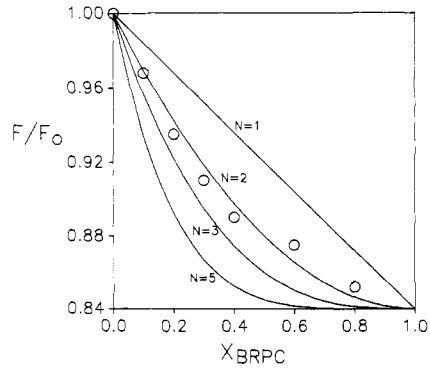

FIGURE 1: Effect of increasing the mole fraction of BRPC in BRPC–DOPC mixtures on the normalized fluorescence intensity (F/F_0) of AChR (O). The solid lines are the theoretical profiles for quenching assuming the model of London and Feigenson (1981) with different values of N (see eq 1). The lipid:protein molar ratio was 400:1, and the concentration of AChR was 0.05 μM.

dicative of quenching by a static mechanism (Lakowicz, 1983). When AChR was reconstituted into mixtures of brominated and nonbrominated lipids (for example, DOPC and BRPC), the fluorescence intensity of the receptor decreased as the mole fraction of the brominated lipid in the membrane increased (Figure 1). Since mixing of DOPC and BRPC is thought to be ideal (East & Lee, 1982), it is possible to relate the intrinsic fluorescence of the receptor to the mole fraction of quenching lipid in the mixture (X) by using the relationship given by London and Feigenson (1981):

$$F/F_0 = (F_0 - F_{min})(1 - X_{BRPC})^N + F_{min} \quad (1)$$

where F_0 is the fluorescence of the receptor seen in pure DOPC, while F_{min} is that found for the receptor in pure BRPC. The index N is the number of lipid sites around an average fluorescent residue. As shown in Figure 1, the experimental data are well described by eq 1 for a value of N of 2.0, with higher or lower values giving obviously worse fits to the data. The value of N is very similar to values found for quenching of $(Ca^{2+} + Mg^{2+})$-ATPase of 1.6 by BRPC (East & Lee, 1982) or 2.0 by nitroxide-labeled lipids (London & Feigenson, 1981).

As discussed by London and Feigenson (1981) and East and Lee (1982), it is possible to use the quenching profiles to determine the relative binding constants for lipids at the lipid–protein interface. The relative binding constant $K_{A/B}$ for lipid A relative to lipid B is given by the ratio of their intrinsic binding constants K_A and K_B such that:

$$K_{A/B} = K_A/K_B \quad (2)$$

The value of $K_{A/B}$ can be readily obtained from fluorescence quenching profiles similar to those in Figure 1. The fluorescence quenching profiles compare the ability of lipid A to displace BRPC or DOPC from the receptor. The binding constant for lipid A relative to DOPC ($K_{A\text{-}DOPC}$) is

$$K_{A\text{-}DOPC} = \frac{(X_A)(1 - X_{DOPC})}{(X_{DOPC})(1 - X_A)} \quad (3)$$

where X_A and X_{DOPC} are the mole fractions of lipids A and DOPC, respectively, which give the same levels of fluorescence quenching in mixtures with BRPC. Theoretical profiles for the quenching of AChR for lipids of different relative binding constants are shown in Figure 2. In order to experimentally validate the fluorescence quenching method, phosphatidic acid (PA) was employed as a test lipid since this lipid has been shown to interact preferentially with AChR with a binding

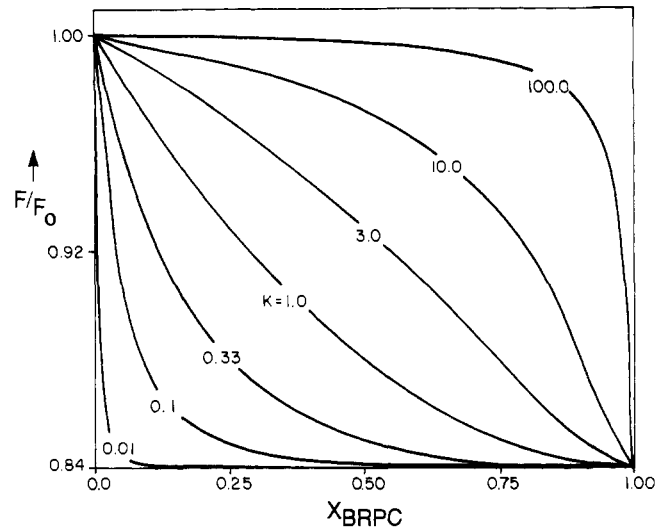

FIGURE 2: Theoretical fluorescence quenching profiles for AChR reconstituted into mixtures of BRPC and test lipids as a function of the binding constant of the test lipid relative to DOPC. The curves were calculated according to eq 3 as described in the text.

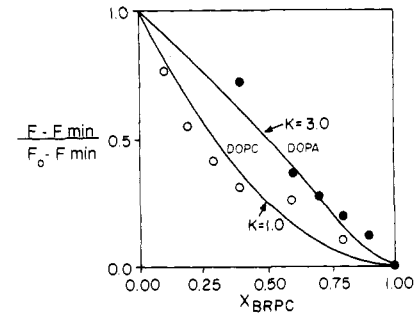

FIGURE 3: Fluorescence quenching of AChR by BRPC in the presence of the test lipid phosphatidic acid. The lipid to protein molar ratio was 400:1. The curves represent the theoretical profiles for quenching expected for the indicated relative binding constants, calculated according to eq 3. The AChR concentration was 0.05 μM.

constant of about 3.0 relative to DOPC (Ellena et al., 1983). The fluorescence quenching profile of AChR by BRPC when present in mixtures with phosphatidic acid is shown in Figure 3. The value for the relative binding constant was 2.5, in good agreement with that determined previously.

Interaction of AChR with Cholesterol. Having shown that the fluorescence quenching method could be applied successfully to quantify the interaction of phospholipids with AChR, we attempted to measure the binding constants of cholesterol and its analogues to the phospholipid–protein interface. Surprisingly, even at high mole fractions, addition of cholesterol to both DOPC–AChR and BRPC–AChR showed little change in the fluorescence intensity (Figure 4). Consequently, the interaction of cholesterol at the lipid–protein interface appeared to be quite weak, with a relative binding constant less than 1.0. However, while these data are consistent with exclusion of cholesterol from the lipid–protein interface, a quite different result is obtained with the brominated analogue of cholesterol. As shown in Figure 4, reconstitution of AChR with a mixture of DOPC and dibromocholesterol resulted in fluorescence quenching which depended on the concentration of brominated steroid in the membrane. The simplest model to reconcile the apparent exclusion of cholesterol from the lipid–protein interface with the observed quenching by the brominated analogue is the existence of a second class of steroid binding sites other than those at the phospholipid–protein interface. An important

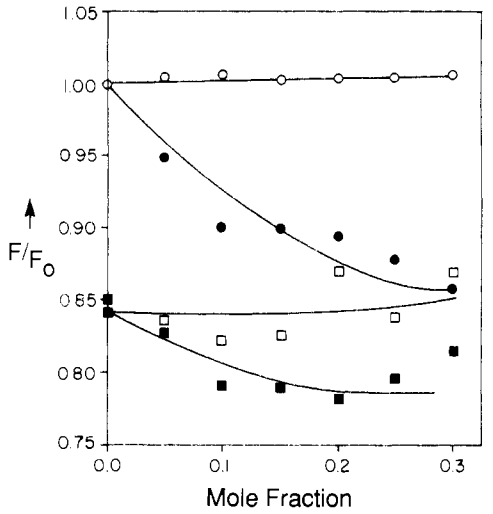

FIGURE 4: Effect of the mole fraction of cholesterol (○, □) or dibromocholesterol (●, ■) on the fluorescence intensity of AChR reconstituted into either DOPC (○, ●) or BRPC (□, ■). The total lipid to protein molar ratio was 600:1, and the concentration of AChR was 0.06 μM.

prediction of such a model would be the availability of sites for quenching other than those occupied by BRPC. Evidence for such sites is also provided in Figure 4 where reconstitution of dibromocholesterol with BRPC–AChR led to a further reduction in fluorescence intensity.

A major limitation for any detailed study of the receptor–steroid interaction using cholesterol was the need to use lengthy reconstitution procedures in manipulating the steroid content of the membranes. A convenient alternative was to use the more water soluble analogue cholesterol hemisuccinate (CHS). Addition of CHS directly to membranes from a concentrated stock solution in methanol results in almost total incorporation of steroid into membranes (Simmonds et al., 1984). Furthermore, although CHS lacks the 3βOH group, it retains the function and properties of cholesterol with respect to its interaction with both the membrane (Simmonds et al., 1984; Lai et al., 1985) and AChR (Criado et al., 1982, 1984). Fluorescence quenching profiles for CHS and its dibromo analogue are shown in panels A and B of Figure 5 for AChR reconstituted at lipid to protein molar ratios of either 100:1 or 45:1, respectively. Upon addition of cholesterol hemisuccinate to BRPC–AChR there was little increase in the fluorescence intensity, again consistent with the earlier observation that cholesterol binds only weakly at the lipid–protein interface. The fluorescence quenching caused by dibromocholesterol hemisuccinate to BRPC–AChR is also shown in Figure 5A. As previously observed for dibromocholesterol, dibromocholesterol hemisuccinate caused additional fluorescence quenching when added to BRPC–AChR.

Analysis of Cholesterol Interactions. To describe the steroid AChR interaction, it is necessary to analyze the binding events in terms of both the numbers and affinities of the various sites. Recently, Lee's group has shown that it is possible to obtain such binding constants from the fluorescence quenching data (Simmonds et al., 1984; Froud et al., 1986). In their analysis, binding of hydrophobic molecules is postulated to occur at three distinct classes of sites: the bulk lipids, the lipid–protein interface (annular sites), and sites on the protein (termed nonannular sites) from which phospholipids are excluded. Following their analysis, binding at the lipid–protein interface can be described by a series of displacement reactions:

$$PL + S \rightleftharpoons PS + L \qquad (4)$$

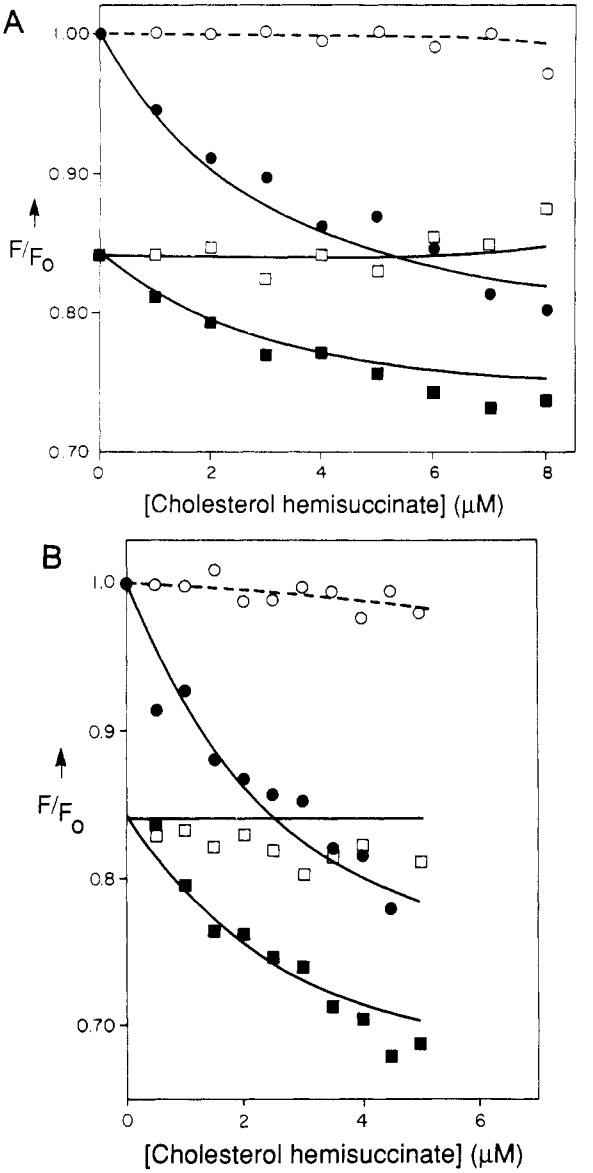

FIGURE 5: Effect of cholesterol hemisuccinate (○, □) or dibromocholesterol hemisuccinate (●, ■) on the fluorescence intensity of DOPC–AChR (○, ●) or BRPC–AChR (□, ■). The lipid to protein molar ratio (ϕ) was 100:1 (A) or 45:1 (B). The data for the cholesterol hemisuccinate–BRPC–AChR system were normalized with respect to the fluorescence intensity seen at an equal concentration of cholesterol hemisuccinate added to DOPC–AChR (Simmonds et al., 1984). The solid lines are theoretical calculations using the binding constants given in Table II. The concentration of AChR was 0.05 μM at a ϕ value of 45 and 0.08 μM at ϕ = 100.

where P, L, and S are protein, phospholipid (BRPC), and steroid, respectively. The displacement reaction can be described by an equilibrium dissociation constant K_D^{ANN} where

$$K_D^{ANN} = \frac{[PL][S]}{[PS][L]} \qquad (5)$$

The concentration of each species is also given by the corresponding conservation equation; for example, for BRPC:

$$[L] = [L_t] - [PL] \qquad (6)$$

where the subscript t refers to the total concentration of the brominated phospholipid.

When the lipid to protein molar ratio is high (>500:1), the concentration of lipid associated with the protein (PL) is too

Table II: Binding Constants for Cholesterol Hemisuccinate Interactions with AChR[a]

	lipid binding		annular sites			nonannular sites			
membranes[b]	K_D^{LIP}	N_{LIP}	K_D^{ANN}	K_D^{EFF}	N	K_D^{NAN}	K_D^{EFF}	N	α[c]
AChR (100:1)	6.0	0.25	5.0	30	45	0.25	1.5	5–10	0.17
AChR (45:1)	6.0	0.25	>100	>600	45	0.25	1.5	5–10	0.30

[a] Binding constants (expressed in micromolar concentrations) and numbers of binding sites (N) calculated from fluorescence quenching curves by using cholesterol hemisuccinate (or its dibromo analogues) as shown in Figure 5 and described in the text. $K_D^{EFF} = K_D^{ANN}K_D^{LIP}$ (or $K_D^{NAN}K_D^{LIP}$) (Froud et al., 1986). [b] Membranes prepared at the phospholipid to protein mole ratios shown in parentheses at an AChR concentration of 0.05 μM. Both DOPC and BRPC were used as the phospholipids. [c] α represents the fractional amount of fluorescence remaining unquenched in BRPC–AChR that is available for quenching following binding to the nonannular sites.

small to significantly affect the free phospholipid concentration ($[L] \cong [L_t]$), and eq 5 can be simplified to

$$K_D^{ANN} = \frac{[PL][S]}{[PS][L_t]} = \frac{[PL]X_S}{[PS]} \quad (7)$$

where X_S is the molar ratio of added steroid to total phospholipid in the membrane ($[S]/[L_t]$). If it is assumed that the annular sites are identical and noncooperative, then the fractional occupancy of the annular sites by BRPC (f_{ANN}) is

$$f_{ANN} = \frac{[PL]}{[P_t^{ANN}]} = \frac{[PL]}{[PL] + [PS]} \quad (8)$$

where $[P_t^{ANN}]$ is the total concentration of annular sites ([PS] + [PL]). From eq 7 and 8, f_{ANN} can be expressed as

$$f_{ANN} = \frac{K_D^{ANN}}{X_S + K_D^{ANN}} \quad (9)$$

In the present study, however, the lipid to protein molar ratio is much lower and eq 7 no longer applies since it becomes necessary to account for the change in free phospholipid concentration caused by displacement of annular phospholipid by sterol. The expression for K_D^{ANN} is now

$$K_D^{ANN} = \frac{[PL][L_t]X_S}{[PS]([L_t] - [PL])} \quad (10)$$

Consequently, f_{ANN} is no longer a simple function of X_S and K_D^{ANN} but is found by substituting eq 8 into eq 10 and rearranging to give a quadratic in f_{ANN}:

$$f_{ANN} = -b - [(b^2 - 4ac)^{0.5}]/2a \quad (11)$$

where

$$a = K_D^{ANN}[P_t^{ANN}] \quad (11a)$$

$$b = -[X_S[L_t] + K_D^{ANN}([P_t^{ANN}] + [L_t])] \quad (11b)$$

$$c = K_D^{ANN}[L_t] \quad (11c)$$

The fractional occupancy of the nonannular sites (f_{NAN}) can also be calculated on the basis of the molar ratio of steroid in the lipid, X_S. By making the assumption that the quenching that arises from binding to the nonannular sites is directly proportional to their fractional occupancy, then f_{NAN} is given by an equation analogous to eq 9:

$$f_{NAN} = \frac{K_D^{NAN}}{X_S + K_D^{NAN}} \quad (12)$$

where K_D^{NAN} is the dissociation constant for binding to nonannular sites.

When considering binding of CHS to the lipid bilayer, complications arise due to partitioning of CHS between the aqueous phase and the membrane due to charge effects of the CHS (Simmonds et al., 1984). However, in a medium of high ionic strength such effects are minimized and binding of CHS can be described by an effective dissociation constant:

$$K_D^{LIP} = \frac{[L]_{free}[S]_{free}}{[S]_{bound}} \quad (13)$$

where $[S]_{free}$ and $[S]_{bound}$ are the free and lipid-bound concentrations of cholesterol hemisuccinate and $[L]_{free}$ is the concentration of unoccupied phospholipid "binding sites" in the phospholipid bilayer available for sterol incorporation. Simmonds et al. (1984) measured the equilibrium binding of CHS to DOPC membranes over a wide range of sterol and phospholipid concentrations and obtained effective K_D^{LIP} values that can be used in the analysis presented here.

By using equations analogous to eq 1, it is possible to relate fractional occupancy of the annular and nonannular sites to fluorescence quenching. Thus for annular binding we have

$$F/F_0 = 0.84 + 0.16(1 - f_{ANN})^2 \quad (14)$$

and for binding at nonannular sites:

$$F/F_0 = 0.84(1 - \alpha) + 0.84\alpha(1 - f_{NAN})^2 \quad (15)$$

where α is the fraction of the fluorescence remaining unquenched in BRPC–AChR that can be quenched by complete binding at the nonannular sites. As discussed by Froud et al. (1986), an identical value for N of 2 was chosen for the coefficient relating fluorescence quenching to occupancy for binding to both annular and nonannular sites.

Finally, the quenching of DOPC–AChR by brominated steroid the total fluorescence quenching is given by

$$F/F_0 = 0.84(1 - \alpha) + 0.84\alpha(1 - f_{NAN})^2 + 0.16(1 - f_{ANN})^2(1 - f_{NAN})^2 \quad (16)$$

In order to simulate the quenching data, the equations describing binding of steroid to lipid (eq 13) and to annular (eq 11) and nonannular (eq 12) sites were solved numerically by the bisection method of Bolzano (McCormick & Salvadori, 1964) for each concentration of added steroid. The extent of fluorescence quenching (α) was then calculated from the fractional occupancy of the annular and nonannular sites by using eq 14–16. In practice, the K_D^{ANN} for annular binding was determined by analyzing the quenching data obtained by the addition of various amounts of unbrominated steroid to BRPC–AChR. The K_D^{NAN} for nonannular binding was determined from the quenching data following addition of various amounts of brominated steroid to BRPC–AChR. The resulting binding constants were then used to fit the total quenching seen on addition of brominated steroid to DOPC–AChR. The total quenching curves generated in these latter experiments therefore served as a check on the internal consistency of the binding constants.

The relative binding constants giving the best fits to the quenching profiles in Figure 5A are shown in Table II. A fluorescence quenching study of the binding of steroid to AChR at a lower lipid to protein ratio of 45:1 was also made (Figure 5B) to enhance the quenching contribution of binding to nonannular sites. Using a centrifugation method, we have

Table III: Binding Constants for Fatty Acid Interactions with AChR[a]

membranes[b]	lipid partitioning K_D^{LIP}	annular sites K_D^{ANN}	annular sites K_D^{EFF}	annular sites N	nonannular sites K_D^{NAN}	nonannular sites K_D^{EFF}	nonannular sites N	α[c]
AChR (100:1)	17	2.0	68	45	0.4	14	5–10	0.17
AChR (45:1)	14	0.1	3	45	0.2	3	5–10	0.23

[a] Binding constants (expressed in micromolar concentrations) and numbers of binding sites (N) calculated from fluorescence quenching curves by using oleic acid or its dibromo analogues as shown in Figure 6 and described in the test. $K_D^{EFF} = K_D^{ANN}K_D^{LIP}$ (or $K_D^{NAN}K_D^{LIP}$) (Froud et al., 1986). [b] Membranes prepared at the phospholipid to protein mole ratios shown in parentheses at an AChR concentration of 0.05 μM. Both BRPC and DOPC were used as phospholipids. [c] α represents the fractional amount of fluorescence remaining unquenched in BRPC–AChR that is available for quenching following binding to the nonannular sites.

recently shown that it is possible to prepare membranes containing just enough phospholipid (45 per receptor) to maintain receptor function (Jones et al., 1988). In these membrane complexes the concentrations of annular and nonannular sites are comparable to the concentration of steroid required for maximum quenching since almost all the phospholipid is in contact with protein. Thus, it becomes possible to estimate the number of nonannular sites. As shown in Figure 5B, the addition of dibromocholesterol hemisuccinate to BRPC–AChR (45:1) and DOPC–AChR (45:1) results in fluorescence quenching. Addition of CHS to BRPC–AChR (45:1) results in a slight decrease in fluorescence. As before, the fluorescence quenching data can be fitted by using the above equations and give the binding constants, also shown in Table II. While it is only possible to obtain an approximate value for the number of nonannular sites (about 7), values much higher or lower gave significantly poorer fits to the data.

Interaction of Fatty Acids with AChR. Additional evidence for nonannular sites was provided by studying the interaction of fatty acids with AChR, again by using the quenching technique. As shown in Figure 6, addition of oleic acid to BRPC–AChR caused a small increase in fluorescence, a result consistent with some displacement of lipid from the protein. However, a further decrease in fluorescence was seen on adding dibromooleic acid to BRPC–AChR, again consistent with binding at sites other than at the lipid–protein interface. Following addition of brominated fatty acid to DOPC–AChR, quenching was also observed. Similar fluorescence quenching profiles were observed for membranes at the lower lipid to protein molar ratio of 45:1 (Figure 6B).

The fluorescence quenching curves were analyzed to obtain relative binding constants according to the same strategy described for CHS. Although equations describing the interaction of fatty acids with AChR are essentially identical with eq 1–16 above, there are two distinctions. First, in analyzing steroid binding to the AChR, it was assumed that each molecule of steroid binding at the lipid–protein interface caused a 1:1 displacement of phospholipid from the protein surface. For fatty acids, however, it seems more likely that two molecules of fatty acid are required to displace each molecule of phospholipid. For reasons discussed by Froud et al. (1986), it is probably more appropriate to express the molar ratio of fatty acid to phospholipid in the membrane, X_{FA}, on the basis of the number of acyl chains so that

$$X_{FA} = [FA]/2[L_t] \quad (17)$$

Second, there is strong evidence that fatty acids show unlimited binding to lipid bilayers (Froud et al., 1986) unlike the case with sterols where saturation of binding to the phospholipids occurs (Simmonds et al., 1984). Consequently, the concentration of fatty acids in the lipid bilayer $[FA]_{bound}$ was calculated for the case of simple partitioning by

$$[FA]_{bound} = \frac{[L_t][FA]_{TOT}}{[L_t] + K_D^{FA}} \quad (18)$$

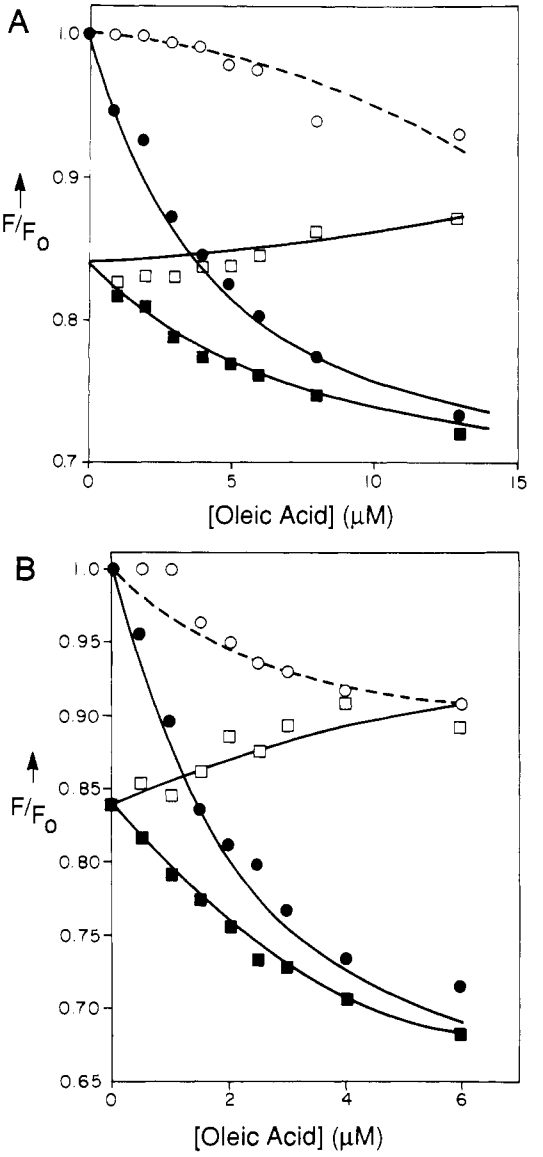

FIGURE 6: Effect of oleic acid (○, □) or dibromooleic acid (●, ■) on the fluorescence intensity of DOPC–AChR (○, ●) or BRPC–AChR (□, ■). The lipid to protein molar ratio (ϕ) was 100:1 (A) or 45:1 (B). The data for the oleic acid–BRPC–AChR system were normalized with respect to the fluorescence intensity seen at an equal concentration of oleic acid added to DOPC–AChR. The solid lines are theoretical calculations using the binding constants given in Table III. The concentration of AChR was 0.05 μM at ϕ = 45 and 0.08 μM at ϕ = 100.

where $[FA]_{TOT}$ is the total fatty acid concentration and $[L_t]$ is the total phospholipid concentration. The binding constants giving the best fits to the quenching profiles for the fatty acids are given in Table III.

Discussion

The fluorescence quenching data presented here strongly

suggest that two classes of lipid binding sites exist on AChR: *annular sites* at the lipid–protein interface and a set of *nonannular* sites, which occlude phospholipids. While cholesterol appears to bind strongly to the nonannular sites, it appears to bind much less well to the annular sites. In contrast, fatty acids appear to bind to both annular and nonannular sites.

A successful interpretation of the fluorescence data in terms of lipid–protein interactions requires that quenching should only arise from those quencher lipids in contact with the protein at the moment of fluorophore excitation (London & Feigenson, 1981; East & Lee, 1982; Markello et al., 1985). From a consideration of the appropriate time scales it appears that this condition is satisfied. For example, the exchange of annular and bulk lipids appears to be slow on the EPR time scale ($V_{ex} < 10^7$ s^{-1}) although fast on the NMR time scale ($V_{ex} > 10^4$ s^{-1}) (Devaux & Seigneuret, 1985). In this study a fluorescence lifetime of around 4 ns was determined, in good agreement with that reported previously for AChR (Barrantes, 1978) and membrane proteins in general (Simmonds et al., 1982; Markello et al., 1985). Consequently, on the fluorescence time scale, exchange of molecules in and out of the annulus is slow. The observation of static quenching lends further support to such a conclusion. Static quenching is characterized by a reduction in fluorescence intensity without a change in the fluorescence lifetime, as a result of instantaneous deactivation of the fluorophore. Even when the fluorescence lifetime is affected, as in the case of dynamic quenching, the "caging" effect caused by the viscous lipid environment gives rise to expressions for quenching which predict that the analysis given by London and Feigenson (1981) is approximately correct (Keizer, 1981; Markello et al., 1985). In their study London and Feigenson (1981) employed nitroxide-labeled lipids as quenchers of tryptophan fluorescence to study lipid–protein interactions in (Ca^{2+} + Mg^{2+})-ATPase.

In our studies bromine-labeled lipids were chosen as quenchers of AChR fluorescence. There are several advantages to using bromine rather than nitroxide-labeled molecules as fluorescence quenchers. Simple bromination of the double bonds of DOPC or cholesterol gives compounds whose physical and chemical properties are similar to the parent molecules (East & Lee, 1982; Simmonds et al., 1982, 1984; Markello et al., 1985). In addition, the molecular volume of bromine is similar to that of a methyl group and causes little perturbation of the membrane (Markello et al., 1985). Finally, the high electron density of the bromine atom facilitates the determination of the location of the brominated molecule within the membrane by X-ray techniques (McIntosh & Holloway, 1987). In contrast, the presence of the nitroxide moiety may introduce significant polarity into the parent molecule, making it necessary to determine the precise location of the quenching group in the membrane (Chattopadhyay & London, 1987). In similar studies using either nitroxides or brominated lipids, the extent of fluorescence quenching of (Ca^{2+} + Mg^{2+})-ATPase by both nitroxide- and bromine-labeled lipids was similar (London & Feigenson, 1981; East & Lee, 1982).

A particularly important question is the location of the postulated nonannular sites. One possibility is that the nonannular sites lie on extramembranous portions of the receptor. An analogous set of binding domains for hydrophobic molecules has been well established for the water-soluble serum albumin family of proteins (Daniels et al., 1985). However, since the fluorescence quenching corresponding to binding at nonannular sites depends on the concentration of cholesterol or fatty acids in the lipid bilayer, this argues that the nonannular sites are accessed from the membrane. From studies on (Ca^{2+} + Mg^{2+})-ATPase, Simmonds et al. (1982) have suggested that there are two likely locations for the nonannular sites, at either inter- or intramolecular protein interfaces. As we prepare it, the AChR of *Torpedo* exists predominantly in a dimeric form of two AChR monomers (molecular mass ≅ 250 000 daltons) covalently linked by a disulfide bridge between the δ subunits (Hamilton et al., 1979). Consequently, the nonannular sites might reside at the monomer–monomer interface as suggested for (Ca^{2+} + Mg^{2+})-ATPase. This model is less attractive for AChR since the average intermonomer distance determined by electron microscopy appears to be too large to allow formation of a site capable of occluding phospholipids (J. P. Earnest, personal communication). An alternative possibility is that the nonannular sites may be formed at the interstices of the five receptor subunits. Although it is not possible to precisely define the number of binding sites, a range of 5–10 per 250 000-dalton monomer would be consistent with the data presented.

It is not possible to test the model without more detailed information on the fluorophore distribution within the AChR structure. Inspection of the primary amino acid sequence of AChR reveals a total of 51 tryptophan residues per 250 000-dalton unit (Noda et al., 1983). Since all five receptor subunits show considerable sequence homology, it follows that the distribution of tryptophans in the protein structure is likely to be similar among the subunits (Popot & Changeux, 1984; McCarthy et al., 1986). A common feature of all proposed models for AChR is the prediction that the majority of the tryptophan residues are located in putative extramembranous portions of the AChR. Relatively few tryptophan residues are predicted to occur within the membrane where they would be readily accessible to quenching by brominated lipids.

The above predictions are in qualitative agreement with the low levels of quenching (16–20%) seen with AChR reconstituted into BRPC and the inability of the brominated molecules to completely quench the residual fluorescence of BRPC–AChR. Any quantitative discrepancies between the extent of quenching and the predicted tryptophan distribution could be explained by the complexity of fluorescence processes in multifluorophore proteins (Lakowicz, 1983), and thus the quenching cannot be used directly to discriminate among different models.

Binding to the Lipid Bilayer. The interaction of cholesterol with phospholipids is presumably mediated by an interaction between the cholesterol 3βOH group and the phospholipid (Presti et al., 1982). Consequently, the presence of an esterified succinate group in cholesterol hemisuccinate might a priori be expected to significantly affect the interaction of the steroid with the phospholipid. However, the interactions of cholesterol hemisuccinate with the phospholipids appear to closely resemble those of cholesterol (Simmonds et al., 1984; Lai et al., 1985). The introduction of bromine groups into the steroid nucleus also appears to conserve the physical and chemical properties of the parent molecule (East & Lee, 1982; Simmonds et al., 1982, 1984; Markello et al., 1985). The steroid ring is likely to be located more or less perpendicular to the plane of the bilayer in such a way as to maximize van der Waals contacts with the phospholipid acyl chains (Cadenhead & Müller-Landau, 1979). The extent of binding of cholesterol and its succinate ester are somewhat different, however. Mixtures of cholesterol and phospholipids appear to be formed up to a 1:1 molar ratio, above which crystalline cholesterol is precipitated (Ladbrooke et al., 1968). In contrast, cholesterol hemisuccinate can be incorporated into

phospholipids far beyond the limit of the parent compound (Simmonds et al., 1984; Lai et al., 1985). In the present paper we have analyzed binding of cholesterol hemisuccinate to the lipid component in terms of a saturation model, outlined by Simmonds et al. (1984). In their analysis a steroid to lipid stoichiometry of 3:1 was used, which can be compared with the much lower values shown in Table II. There is some evidence for the formation of distinct clusters of lipid and sterol in membranes containing cholesterol (or cholesterol hemisuccinate) and excess phospholipids (Rogers et al., 1979; Presti et al., 1982). The most likely explanation for the differences in binding constants calculated here is the much lower lipid to protein ratio in the membranes. Since most lipid is in contact with the protein, there is little excess lipid available for cluster formation.

Binding at the Lipid–Protein Interface. Our results for phospholipids confirm the spin-label studies of Ellena et al. (1983), which showed that there is a preferential interaction of the negatively charged phosphatidic acid (PA) with AChR characterized by a threefold greater binding constant for PA than for DOPC. A relative binding constant of 2.5 was obtained by using the fluorescence quenching by BRPC in mixtures of BRPC and DOPA. Several factors may be involved in the preferential binding including electrostatic interaction between the lipid head groups and AChR (Ellena et al., 1983). An alternative explanation to account for the preferential interaction of PA with AChR is the possibility of lateral phase separation of lipids and/or protein within the plane of the membrane (Kouaouci et al., 1985). However, at the low lipid to protein mole ratios used here, the AChR forms membrane sheets that appear homogeneous when viewed under the electron microscope after negative staining.

Whereas displacement of annular lipids by phosphatidic acid can be detected by fluorescence quenching, a very different picture emerges for cholesterol and fatty acids. For both fatty acids and cholesterol the binding constants show that the interaction at the annular sites on the AChR is less strong than for phosphatidylcholine. For example, cholesterol is unable to increase the fluorescence of membranes prepared in BRPC, suggesting that it cannot displace BRPC from the protein surface. The poorer binding of cholesterol to the annular sites compared to DOPC most probably reflects differences in the flexibility of the two types of molecules. Cholesterol has a rigid planar structure (Yeagle, 1985) which probably does not interact with the rough surface of the protein as well as the more flexible phosphatidylcholine (Simmonds et al., 1984). The fluorescence results contrast sharply with the spin-label results obtained with a cholesterol analogue (Ellena et al., 1983). The spin-label studies were consistent with a relatively high binding constant for cholesterol, similar to the results obtained for phosphatidic acid. The discrepancy is probably explained by the existence of the nonannular sites. Since the EPR spectra of spin-labels bound to annular or nonannular sites would probably be similar, the presence of 5–10 nonannular sites could be interpreted as relatively high affinity binding to a larger number of annular sites.

Binding at the Nonannular Sites. An interesting feature of the postulated nonannular sites in both AChR and (Ca^{2+} + Mg^{2+})-ATPase is their ability to accommodate structurally dissimilar molecules. The observation of nonannular binding sites has been confirmed by using a variety of different spectroscopic techniques for (Ca^{2+} + Mg^{2+})-ATPase (Lee et al., 1982; Jones & Lee, 1985) which could, in principle, be applied to AChR.

Before discussing the possible functional significance of binding at either annular or nonannular sites, we wish to emphasize that the proposed models of lipid–AChR interactions are not intended to diminish the dynamic nature of the interactions. The rate of exchange of lipids into and out of the annulus is rather fast, consistent with a rather weak lipid–protein interaction and a homogeneous annulus (Ellena et al., 1983; East et al., 1985). This has two significant consequences: first, a consideration of the relevant time scales is important when considering any studies on the lipid–protein interaction. Second, it seems likely that the time-averaged composition of the annulus is the most important factor in determining the effect of the lipids on the activity of the membrane proteins. Since a complete environment of phospholipids is required for receptor function (Chang & Bock, 1977; Jones et al., 1988), one possible consequence of the extensive binding of molecules structurally dissimilar to phospholipids at the annular sites is inactivation of the protein, which at low concentrations might be reversible but at higher concentrations may lead to receptor denaturation. In support of this prediction, fatty acids have been shown to inactivate AChR-mediated ion flux (Andreasen & McNamee, 1980; Pjura et al., 1982).

Cholesterol-like molecules have been shown to be necessary for maintaining AChR ion channel function (Fong & McNamee, 1986). Since cholesterol shows extensive binding to the nonannular sites, we speculate that occupancy of nonannular sites may have an activating effect on receptor function. A dualistic effect on activity has been correlated with binding of a variety of hydrophobic molecules to (Ca^{2+} + Mg^{2+})-ATPase (Simmonds et al., 1984; Jones & Lee, 1985). Over the same concentration range where binding to the postulated nonannular sites occurred, ATPase activity was stimulated while inhibition of the ATPase activity was correlated to occupancy of the annular sites, depending on the lipid in the system. Another functional role of the nonannular sites may be to provide sites by which cholesterol might stabilize the receptor protein in the native membrane. Cholesterol has been shown to stabilize α-helices in membrane proteins (Rooney et al., 1984) including AChR (Fong & McNamee, 1987).

Binding of lipids at nonannular sites should also be considered in a wider context, for example, in facilitating insertion of membrane proteins into the lipid bilayer (Scotto & Zakim, 1986). Interestingly, the success of detergents containing a steroid nucleus, for example, CHAPS, digitonin, and cholate, in reconstituting multisubunit membrane proteins may be due, at least in part, to stabilization of the protein structure during solubilization. Evidence for competition between cholesterol and cholate for sites on AChR has been observed by using detergent-exchange experiments (J. P. Earnest, University of California, San Francisco, personal communication). An additional consideration is that the nonannular sites may be pharmacologically significant, particularly with respect to such membrane-active agents as insecticides and local and general anesthetics. A report has recently been presented that shows a competitive effect of cholesterol with the general anesthetic halothane on AChR channel kinetics (Lechleiter et al., 1986).

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Registry No. Cholesterol, 57-88-5; oleic acid, 112-80-1; cholesterol hemisuccinate, 1510-21-0; brominated oleic acid, 19117-94-3; 5,6-

dibromocholestan-3β-one, 25182-80-3; brominated cholesterol hemisuccinate, 88730-76-1.

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Mechanism of Agonist and Antagonist Binding to α_2 Adrenergic Receptors: Evidence for a Precoupled Receptor–Guanine Nucleotide Protein Complex†

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ABSTRACT: The α_2 adrenergic receptor (AR) inhibits adenylate cyclase via an interaction with N_i, a guanine nucleotide binding protein. The early steps involved in the activation of the α_2 AR by agonists and the subsequent interaction with N_i are poorly understood. In order to better characterize these processes, we have studied the kinetics of ligand binding to the α_2 AR in human platelet membranes on the second time scale. Binding of the α_2 antagonist [^{3}H]yohimbine was formally consistent with a simple bimolecular reaction mechanism with an association rate constant of 2.5×10^5 M^{-1} s^{-1} and a dissociation rate constant of 1.11×10^{-3} s^{-1}. The low association rate constant suggests that this is not a diffusion-limited reaction. Equilibrium binding of the α_2 adrenergic full agonist [^{3}H]UK 14 304 was characterized by two binding affinities: K_{d1} = 0.3–0.6 nM and K_{d2} = 10 nM. The high-affinity binding corresponds to approximately 65% and the low-affinity binding to 35% of the total binding. The kinetics of binding of [^{3}H]UK 14 304 were complex and not consistent with a mass action interaction at one or more independent binding sites. The dependence of the kinetics on [^{3}H]UK 14 304 concentration revealed a fast phase with an apparent bimolecular reaction constant k_+ of 5×10^6 M^{-1} s^{-1}. The rate constants and amplitudes of the slow phase of agonist binding were relatively independent of ligand concentration. These results were analyzed quantitatively according to several variants of the "ternary complex" binding mechanism. In the model which best accounted for the data, (1) approximately one-third of the α_2 adrenergic receptor binds agonist with low affinity and is unable to couple with a guanine nucleotide binding protein (N protein), (2) approximately one-third is coupled to the N protein prior to agonist binding, and (3) the remainder interacts by a diffusional coupling of the α_2 AR with the N protein or a slow, ligand-independent conformational change of the α_2 AR–N protein complex. The rates of interaction of liganded and unliganded receptor with N protein are estimated.

The study of the mechanism of activation and inhibition of adenylate cyclase by hormone receptors has shown remarkable progress recently (Gilman, 1984). Resolution, purification, and reconstitution of the individual proteins involved in these reactions has recently been achieved (Asano et al., 1984; May et al., 1985; Cerione et al., 1986). These advances allow detailed study of the interactions of the individual components in a well-defined system. To understand the implications of such interactions in reconstituted systems with respect to a less perturbed membrane environment, it would be useful to have probes that would be applicable in both systems. Extensive studies of adenylate cyclase activity in native membranes have resulted in models of the interaction of receptor (R)[1] and

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[1] Abbreviations: AR, adrenergic receptor; C, catalytic subunit of adenylate cyclase; GppNHp, guanosine 5′-(β,γ-imidotriphosphate); N_i, inhibitory guanine nucleotide binding protein; N protein, guanine nucleotide binding protein; UK 14 304, 5-bromo-*N*-(4,5-dihydroimidazol-2-yl)-6-quinoxalinamine; R, receptor; SS, sum of squared residuals; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

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