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Fluidity of the Lipids Next to the Acetylcholine Receptor Protein of Torpedo Membrane Fragments. Use of Amphiphilic Reversible Spin-Labels†

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ABSTRACT: Choline esters of spin-labeled fatty acids (long-chain acylcholines) were used to probe the hydrophobic environment of the acetylcholine receptor protein in membrane fragments from *Torpedo marmorata*. These spin-labels competitively inhibit the binding of [^{3}H]acetylcholine to the receptor site. Their inhibition constants (K_I) were close to 200 nM. At the high membrane concentration required for electron spin resonance (ESR) experiments, the apparent inhibition constants (K_I^{app}) differed from K_I determined by using dilute membrane concentration. This is due to the amphiphilic character of long-chain acylcholine. For most spin-labels used, only difference ESR spectroscopy provided reliable spectra corresponding to receptor-bound spin-labeled acylcholines. Acetylcholine receptor agonists and antagonists displaced the acylcholine from the receptor sites, whereas choline had only a weak effect. This produced a modification in the ESR spectra of the bound acylcholines and provided evidence that the acylcholines bound to the receptor sites in a specific manner. The interpretation of the spectra of receptor-bound spin-labels favored a strong barrier to the motion of the probe when attached to the middle of the acyl chain. However, when the probe was close to the methyl terminal of a stearoylcholine molecule a much greater fluidity was found. Short-range spin–spin interactions were created between spin-labels bound to the receptor site and spin-labels in a fluid phase. This indicates that lipids next to the receptor protein are not completely immobilized in spite of the semicrystalline organization of the proteins in the postsynaptic region.

The electric organ of Torpedo is an excellent source of membrane fragments rich in acetylcholine receptor protein (Cohen et al. 1972, Duguid and Raftery, 1973). X-ray diffraction and electron microscopy studies of these membrane fragments (Cartaud et al., 1973, Nickel and Potter, 1973, Dupont et al., 1974) have revealed a regular organization of the receptor protein in the plane of the membrane. If these proteins form crystalline arrays in the postsynaptic region, it is questionable whether the lipids present in that membrane can form fluid bilayers. Hence, it was important to determine whether such bilayers can exist in the vicinity of the acetylcholine receptor protein.

In order to study the lipid environment of the cholinergic receptor protein of Torpedo, we have used a spin-label method similar to that previously described to study mitochondrial and microsomal membrane-bound proteins (Devaux et al., 1975a,b). It was shown that if a spin-labeled fatty acid was linked to a polar group binding to specific membrane proteins, the ESR[1] spectra of the paramagnetic probe could provide valuable information on the state of the mobility of the hydrocarbon chains next to the proteins, as well as on the depth that the proteins penetrated into the hyprophobic part of the membrane.

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‡ Supported by the DGRST.

[1] Abbreviations used are: ESR, electron spin resonance; TETRAM, *O,O*-diethyl *S*-(*N,N*-diethylamino)ethyl phosphorothiolate.

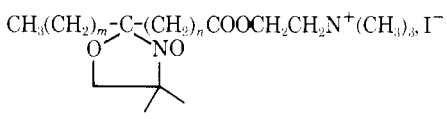
TABLE I: Characterization of the Membrane Fragments.

Fraction no. (1.5 mL)	Sucrose (%)	Toxin Binding Sites (μM)	mg/mL Protein	Organic Phosphate (mM)	Organic Phosphate Toxin Binding Sites (μM)	Sp Act. (nmol) of Toxin Binding Sites/g of Protein
1 and 2	37–39	0.66	0.65	0.9	1360	1015
3	36.6–37	2.65	0.75	0.7	264	3500
4, 5, and 6	35.4–36.6	3.05	1.75	1.0	327	1750
7 and 8	34.6–35.4	2.65	2.50	1.5	566	1050

In a previous report (Brisson et al., 1975a), we showed that a spin-labeled derivative of a long-chain acylcholine (8-doxylpalmitoylcholine) had a high affinity for the acetylcholine receptor protein in its membranous state. In the present work, we will show that, by using specific spin-labels, it is possible to obtain information on the hydrophobic phase next to the receptor protein.

Materials and Methods

Synthesis of Long-Chain Spin-Labeled Acylcholines. Spin-labeled acylcholines having the following general structure:



with $m + n + 3 = 16$ or 18 were synthesized from spin-labeled fatty acids and dimethylaminoethanol as described by Brisson et al. (1975a). The following improvements to the method were carried out. Dimethylaminoethanol, which had not reacted with fatty acid anhydride, was carefully washed out with water. Afterwards, the quaternization of the amino group by methyl iodide (stored with one drop of mercury) was performed in hexane. After 12 h, the precipitate was centrifuged. The unreacted fatty acid was almost completely recovered in the supernatant. The pellet was dissolved in minimum acetone before preparative thin-layer chromatography on silica gel G 60 (Merck) plates (silica gel washed with 0.1 M Na H_2PO_4) and eluted with methanol–acetone–1 N acetic acid (45:5:5, v/v). The band with R_F 0.3–0.4 (revealed by Draggendorf and iodine reagents) was scraped off and the spin-label acylcholine was extracted with 2,2,2-trifluoroethanol. The product was stored in acetone at −20 °C without any trace of hydrolysis after several months.

Membrane Preparation. The membrane fragments enriched in acetylcholine receptor protein were prepared from fresh electric tissue of *Torpedo marmorata* using the method of Cohen et al. (1972), with the following modifications. After homogenizing the tissue in a Sorvall Omni-mixer at maximum speed (three times for 1 min), the crude extract was rehomogenized with a Potter–Elvejhem homogenizer at 1000 rpm (five runs). After low speed centrifugation, the supernatant was filtered on gauze and centrifuged for 90 min at 15 000 rpm in a Beckman R 10 rotor at 4 °C. The resulting pellet was resuspended in water (20 mL of distilled water for 250 g of fresh organ) and rehomogenized with the potter. Five milliliters of this preparation was layered on a 25-mL continuous sucrose gradient (30–46% sucrose) and centrifuged overnight at 25 000 rpm in a Beckman SW 27 rotor. Fractions (1.5 mL) were collected from the bottom of the gradient. Proteins were measured by the method of Lowry et al. (1951) using bovine serum albumin as standard. The concentration of α-toxin binding sites was estimated by titrating a known quantity of [^{3}H]α-toxin of *Naja nigricollis* (Weber and Changeux, 1974) with increasing amounts of membrane suspension. Phospholipids were measured by the method of Rouser et al. (1969) with the following modification: sucrose was removed and the centrifuged membrane fragments were resuspended in distilled water. Then, 10 or 20 μL of this suspension was added to 250 μL of 70% perchloric acid and allowed to stand for 1.5 h at 180 °C in an oil bath. Absorption was measured at 797 nm on a Beckmann Acta III spectrophotometer. Typical results of a membrane preparation are shown in Table I.

Biochemical Assays. Binding at equilibrium of [^{3}H]acetylcholine with or without inhibitor was determined by the centrifugation assay of Weber and Changeux (1974). Membrane fragments (3 μM of [^{3}H]α-toxin binding sites) were incubated for 1 h at 0 °C in the presence of 100 nM *O,O*-diethyl *S*-(*N,N*-diethylamino)ethyl phosphorothiolate (TETRAM), a potent acetylcholinesterase inhibitor (Eldefrawi et al., 1971), and then diluted to 100 nM of α-toxin binding sites before incubation for 20 min at 22 °C, with the desired concentration of acylcholine and [^{3}H]acetylcholine. Radioactivities of the media before and after centrifugation at 100 000 *g* for 90 min were counted on 200-μL samples in 10 mL of Unisolve 1 (Koch light) solution in an Intertechnique scintillation counter. The efficiency of counting was about 30%.

Electron Spin Resonance (ESR) Measurements. Standard ESR measurements were carried out in a 50-μL flat quartz cell, using a Varian E9 Century Line spectrometer with temperature-control accessory. Before the ESR experiments, the membranes, stored in sucrose, were resuspended in Torpedo physiological saline medium and preincubated with 10^{-4} M TETRAM (for more details see Brisson et al., 1975a). When very low concentrations of spin-labels were used (1 μM or below) and for difference ESR spectrometry, a multichannel Intertechnique (Didac 4000) was connected to the Varian spectrometer and used to store and accumulate the signal. Generally, 2-min scans were used and accumulation proceeded for 2 to 4 h. A spectrum corresponding to 100-G sweep was stored in 1000 channels. For difference ESR spectrometry where two consecutive spectra were subtracted, the lines were positioned on the sharp peaks arising from the spin-labels in water. That such lines could be cancelled after subtraction of two spectra (each accumulated for at least 2 h) was proof of the reproducibility of the consecutive tunings and of the stability of the field. No additional lock to the standard Varian spectrometer was necessary. Final spectra were displayed on the screen of the multichannel apparatus or plotted on a X–Y recorder. Samples exposed to microwave power (about 20 mW) for 2 to 4 h did not show any evidence of alteration, except occasional chemical reduction of the spin-label. For example, although the same sample was generally not used twice, difference spectra could be obtained after 3 h accumulation by mere addition of acetylcholine to the cell, in order to displace the bound acylcholine from the receptor site.

TABLE II: Association Constants of Various Acylcholines with Lipids and Acetylcholine Receptors.

Acylcholine	K_I (nM)[a]	K_p (molar ratio)	K_I^{app} (nM)[b]	α[c]
5-doxylpalmitoyl-choline	180 ± 50	9.4 ± 1.5 × 10^4	415	0.78
8-doxylpalmitoyl-choline	200 ± 50	1.6 ± 0.5 × 10^5	700	0.72
12-doxylstearoyl-choline	200 ± 50	6.7 ± 1 × 10^5	2650	0.51
16-doxylstearoyl-choline	260 ± 50	9.6 ± 0.8 × 10^5	3850	0.44

[a] K_I was determined from double-reciprocal plots, with experimental conditions described in Figure 1. [b] K_I^{app} was calculated by eq A1 from the experiment described in Figure 4. [c] α is the fraction of ligand bound to the acetylcholine receptor

Partition Coefficient Measurements. Quantitative ESR experiments designed to determine the partition coefficients of long-chain spin-labeled acylcholine between water and lipids were carried out in Teflon tubes (0.2-mm diameter), the samples being also previously mixed in Teflon tubes. We used Teflon because we found that low concentrations of acylcholine in water did not give stable signals in quartz cells or in plastic or glass tubes.

For such experiments, the amplitude of the high-field line was measured for a given concentration of spin-label in water in the presence or absence of membranes corresponding to a known phospholipid concentration (Butler et al., 1974). The difference in amplitude from the two consecutive experiments was used to determine the amount of acylcholine in the lipids (see Appendix). Torpedo membranes used for these experiments were preincubated with TETRAM (10^{-4} M) to prevent hydrolysis of the acylcholine and with toxin (10^{-5} M) to prevent binding to the acetylcholine receptor.

Results

Long-Chain Acylcholines Are Competitive Inhibitors of the Binding of [^{3}H]Acetylcholine to the Receptor. Figure 1 shows the effect of 16-doxylstearoylcholine on the binding of [^{3}H]acetylcholine to membrane fragments rich in acetylcholine receptors. Similar measurements were made with 5-doxylpalmitoylcholine, 8-doxylpalmitoylcholine, and 12-doxylstearoylcholine. Each compound was a competitive inhibitor of [^{3}H]acetylcholine binding as determined from double-reciprocal plots; the inhibition constants (K_I) for the various acylcholines were determined with membrane fragments containing 100 nM α-toxin binding sites. At these concentrations of receptor sites, Table II shows that the inhibition constants for the four acylcholines were almost identical (200 nM).

ESR conditions require the presence of membrane concentrations (1 μM of receptor sites) higher than those used in the binding experiments. We therefore investigated the effect of lipid concentration on the inhibition constants of the spin-labels.

The Lipid Concentration Influences the Binding of Long-Chain Acylcholines to the Acetylcholine Receptor. Since acylcholines are amphiphilic ligands, they partition between water and the hydrophobic phase in the membrane. It is possible to predict from general principles of three-state systems (such as the one experienced by acylcholines in the presence of the membrane-bound acetylcholine receptor protein) that

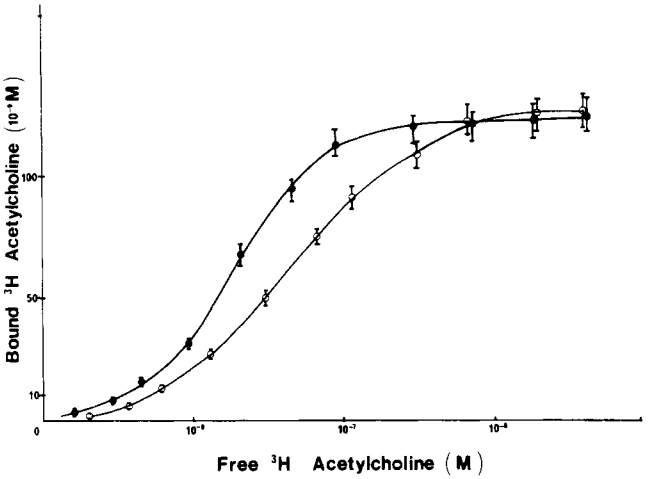


FIGURE 1: Competitive Inhibition of Acetylcholine Binding to the Acetylcholine Receptor of Torpedo by 16-Doxylstearoylcholine. Membrane fragments (3 μM α-toxin binding sites) were preincubated for 1 h in the presence of 100 μM TETRAM and then diluted to 100 nM α-toxin binding sites (28 mg/mL protein) in the physiological medium. (●) Control; (○) 16-doxylstearoylcholine 200 nM.

the apparent inhibition constant (K_I^{app}) of the acylcholines for the receptor sites is a function of lipid concentration. It can be shown that K_I^{app} has the following expression (see Appendix eq A1):

$$K_I^{app} = K_I(1 + K_p(n_l/n_w))$$

where K_I is the inhibition constant determined from very dilute membrane suspensions; n_l the number of moles of lipids, n_w the number of moles of water; and K_p the partition coefficient which is defined by the following expression:

$$K_p = \frac{n_{ach\text{-}l}/n_l}{n_{ach\text{-}w}/n_w}$$

where $n_{ach\text{-}l}$ and $n_{ach\text{-}w}$ are, respectively, the number of moles of acylcholine in the lipids and in the water.

From formulation (eq A1) one can predict that: (a) the proportion of bound spin-labeled acylcholines decreases as the lipid concentration increases. This becomes particularly evident at the high membrane concentration required for ESR experiments. To test this hypothesis, we measured the initial rates of binding of [^{3}H]α-toxin to the membrane fragments in the presence of 16-doxylstearoylcholine at various concentrations of lipid vesicles added to the incubation medium. Figure 2 shows that the addition of lipid vesicles increased the K_I^{app} of 16-doxylstearoylcholine for the receptor (reduction of the initial rate by half). It should be noted that the maximum concentration of lecithin used (0.54 mM) was in the range of the lipid concentration present during ESR experiments. (b) The proportion of bound probes decreases when K_p increases. Relative values of K_I^{app} for the different acylcholines must then depend on relative values of their partition coefficient.

For valid comparisons, K_p values for the different acylcholines must be measured under the same conditions and with good precision. The measurement is most accurate when the concentration of spin-label in the water approaches that in the membranes. The lipid concentration was generally chosen to fulfill this condition. In order to control the constancy of K_p, plots of $n_{ach\text{-}l}/n_{ach\text{-}w}$ vs. n_l/n_w were performed. They gave a linear dependence in the explored range. Figure 3 shows the results and Table II gives the values of the various K_p obtained in such conditions.

Theoretical Determination of the Proportion of Protein-

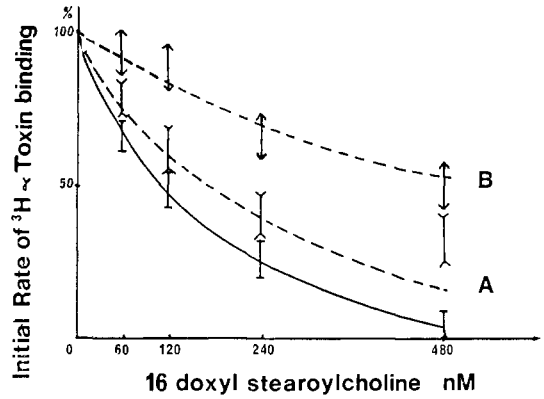


FIGURE 2: Effect of the Lipid Concentration on the Binding of 16-Doxylstearoylcholine to the Acetylcholine Receptor. 2.7 μM α-toxin binding site (0.75 mg/mL protein) membrane fragments in physiological salt solution were preincubated for 45 min with 10^{-4} M TETRAM and then diluted in the absence (control) or in the presence of phospholipid vesicles, to 2 nM α-toxin binding sites. Various concentrations of 16-doxylstearoylcholine were added 15 min prior to the addition of [^{3}H]α-toxin (1 nM). Quantities of [^{3}H]α-toxin bound to the membrane were determined by Millipore filtration. (|) control, 0.54 μM organic phosphate assimilated here to phospholipids; (↕) 33 μM phospholipids added as lecithin vesicles; (↑) 0.54 mM of phospholipids added as lecithin vesicles; (- - -) theoretical curves.

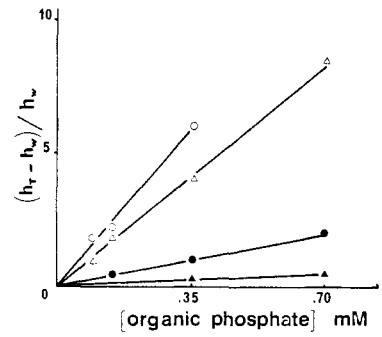


FIGURE 3: Measurement of Partition Coefficient between Water and Lipid of the Different Acylcholines by ESR Method. A membrane preparation (7 mM organic phosphate, 3 μM α-toxin binding sites) was preincubated with 2 μM toxin and 5 μM TETRAM. Acylcholines from a 3 mM stock solution in ethanol were added to make a final concentration of 10 to 30 μM. h_w and h_T are, respectively, the heights of the high-field peak in the presence or absence of membranes. (○) 16-Doxylstearoylcholine; (△) 12-doxylstearoylcholine; (●) 8-doxylpalmitoylcholine; (▲) 5-doxylpalmitoylcholine.

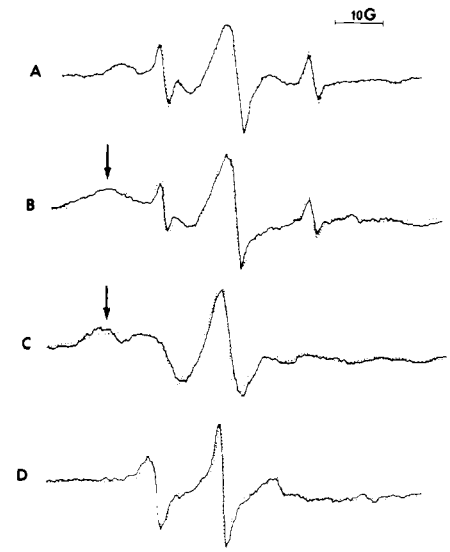


FIGURE 4: ESR spectra of labeled acylcholines in the presence of Torpedo membrane fragments. Receptor-site concentration: 3 μM; specific activity: 3500 nmol of toxin binding sites per gram of protein; spin-label concentration: 1.5 to 2 μM. Lines correspond to the directly recorded spectra. Dotted lines correspond to spectra obtained in the presence of 10^{-4} M acetylcholine. The amplitudes of two superposed spectra were normalized to the same midline amplitude. Each spectrum is obtained by one scan of 30 min. (A) 5-Doxylpalmitoylcholine; (B) 8-doxylpalmitoylcholine; (C) 12-doxylstearoylcholine; (D) 16-doxylstearoylcholine. Note that narrow lines, due to spin-labels in water, appear only with the first two compounds. This is consistent with the values obtained for the partition coefficient between lipids and water. Because of the existence of an equilibrium between lipids and water, the first two spectra cannot be due only to spin-labels bound to the receptor proteins.

Bound Spin-Labels in a ESR Experiment. The following conditions were used in a typical ESR experiment: specific activity of the receptor-rich membrane fragments, 3500 nmol of [^{3}H]α-toxin binding sites/g membrane protein; total concentration of [^{3}H]α-toxin binding sites, 5–10 μM; concentration of lipids, approximately 500 μM; concentration of spin-label, 2.5 μM. From the previous section, it is possible to deduce the proportion (α) of spin-labels that will be bound to the acetylcholine receptor site under these conditions (Table II, column 3). The values of α indicate that only a fraction of the spin-label will be bound to the receptor sites, even though the concentration of [^{3}H]α-toxin sites is two- to fourfold greater than that of spin-labels. Under these conditions, it is evident that the recorded spectrum will not represent that of a "pure" receptor-bound spin-label.

ESR Measurements of the Interaction of Long-Chain Spin-Labeled Acylcholine with Receptor-Rich Membrane Fragments of Torpedo. Figure 4 shows the ESR spectra of four different long-chain spin-labeled acylcholines in the presence of receptor-rich membrane fragments. The superposed dotted spectra represent spectra obtained with samples containing a high concentration (10^{-4} M) of acetylcholine added to the membrane fragments simultaneously with the spin-label. Similar spectra were recorded when tubocurarine-*d*, decamethonium, carbamylcholine (10^{-4} M) or toxin (6×10^{-6} M) was used instead of acetylcholine as the competitive agonist or antagonist. The latter spectra are identical to those obtained with spin-labeled fatty acids in such membrane preparations.

To determine the concentration of unlabeled ligand required to displace the spin-labeled acylcholines from their binding sites, the height of the low-field peak appearing with the 8-doxylpalmitoylcholine and the 12-doxylstearoylcholine was plotted vs. acetylcholine and choline concentrations. Figure 5 shows that the concentrations of acetylcholine and choline required to displace the bound acylcholine spin-label by 50% were, respectively, about 5×10^{-6} and 5×10^{-5} M.

The influence of the purity of the membrane preparation on the ESR results and, hence, the experimental proof of the dependence of the inhibition constants on lipid concentration are illustrated in Figure 6. Spectrum A in Figure 6 corresponds to 8-doxylpalmitoylcholine at low concentration (2 μM) in the presence of relatively pure membrane preparation (specific activity 1000 nM of α-toxin binding sites per gram of protein, concentration of sites 3.5 μM). Spectrum B in Figure 6 was obtained by adding to that preparation membranes of very low specific activity (100 nM α-toxin binding sites), in such a way that no modifications of the receptor concentration occured between A and B; only the lipid concentration was varied. The resulting spectrum B is identical to the "displaced" spectrum, shown in Figure 4, by adding 10^{-4} M acetylcholine to the sample. Therefore, K_I^{app} depends on the lipid concentration

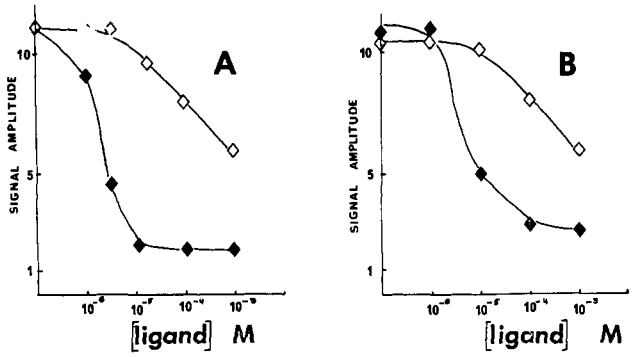


FIGURE 5: Comparison of the modifications induced by acetylcholine (♦) and by choline (◇) on the ESR spectra of the 8-doxylpalmitoylcholine (A) and 12-doxylstearoylcholine (B) in the presence of Torpedo membrane fragments. The amplitude of the signal at the position indicated by arrows on Figure 4 (curves B and C) is plotted vs. unlabeled ligand concentration.

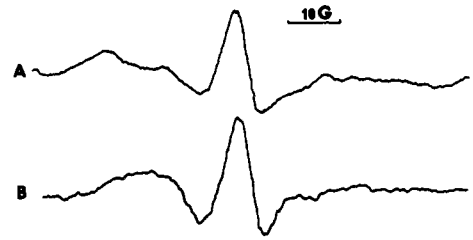


FIGURE 6: Modification of the spectra of 8-doxylpalmitoylcholine bound to the Torpedo membranes induced by the addition of lipids. Spectrum A: 8-doxylpalmitoylcholine, 2 μM, was added to a membrane preparation containing 3 μM α-toxin binding sites, 0.7 mM organic phosphate, and 0.75 mg/mL protein. Spectrum B: 2 μM 8-doxylpalmitoylcholine was added to a preparation containing 3 μM α-toxin binding sites, 12 mM organic phosphate, and 12 mg/mL protein.

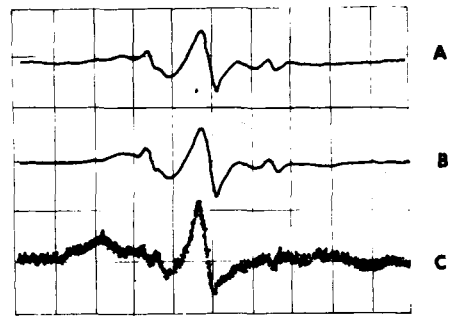


FIGURE 7: Difference ESR spectroscopy: 8-doxylpalmitoylcholine in the presence of the acetylcholine receptor of Torpedo membranes. Spectra of 8-doxylpalmitoylcholine in the presence of membrane fragments enriched in cholinergic receptor, obtained after 2.5-h accumulation at 24 °C (2-min scan time). Spectrum A: 10 μM acylcholine was added to a 3.5 μM α-toxin binding site membrane preparation. Spectrum B: same as A plus 10^{-4} M acetylcholine. Spectrum C: ten times magnification of the difference A − B. (Spectrum was redrawn from photograph of the screen.)

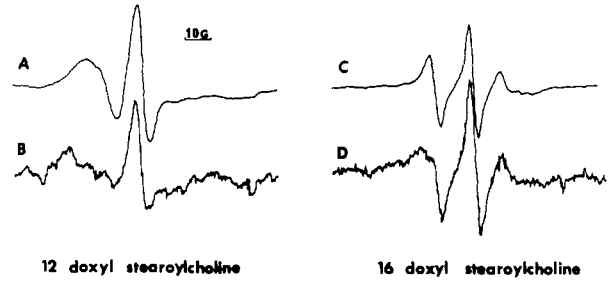


FIGURE 8: Difference ESR spectroscopy: 12-doxyl- and 16-doxylstearoylcholine. Spectra A and C were obtained by addition of 15 μM acylcholine to a 2 μM α-toxin binding site membrane preparation. Measurements were performed at 0 °C and correspond to 3-h accumulation (2-min scan time). Spectra B and D correspond to receptor-bound acylcholine molecules, and are obtained by the differential procedure described in the text.

for 8-doxylpalmitoylcholine.

The spectra in Figure 4 do not correspond to "pure" receptor-bound spin-labels. In order to obtain the "true" spectra of the spin-labeled acylcholines bound to the acetylcholine receptor of Torpedo, we used difference ESR spectroscopy. The principle of these experiments was described under Materials and Methods. A five- to tenfold excess in the concentration of acylcholine over receptor sites was used. A first spectrum of the spin-labeled acylcholine in the presence of the membranes was recorded and stored. Then, a second spectrum was obtained with spin-labeled acylcholine plus 10^{-4} M acetylcholine or decamethonium. The difference between the two spectra corresponds to the spectrum of the spin-label bound to the acetylcholine receptor. Figure 7 shows the three spectra obtained in this manner with 8-doxylpalmitoylcholine in Torpedo membrane fragments at 20 °C. The sharp lines appearing on spectra A and B in Figure 7 correspond to spin-labels in water. Since there is a constant ratio between acylcholines in water and in the lipids, the subtraction of spectrum B from spectrum A can be adjusted so that no "free signal" remains in the final spectrum (spectrum C, Figure 7). By this procedure, we are certain to exactly remove from the original spectrum A that portion which corresponds to the signal from acylcholine in lipids and water. In other words, this final correction takes into account any small error when preparing samples A and B. When using 8-doxylpalmitoylcholine (Figure 7), the final spectrum is similar to the spectrum (obtained directly) in Figure 4, which therefore corresponds mostly to the "bound" signal.

Figure 8C,D shows the 0 °C spectra of 12-doxylstearoylcholine and 16-doxylstearoylcholine in Torpedo membranes obtained by difference ESR spectroscopy. Spectra A and B correspond to the original signals of the samples containing an excess of acylcholine. These two spectra are practically identical to the spectra one obtains with the corresponding fatty acids or the "displaced spectra" because of the large excess of acylcholine used.

The same procedure was applied to determine the shape of the spectrum corresponding to 5-doxylpalmitoylcholine bound to the receptor protein. The reason why the result does not appear on the figure is that, after subtraction, only the baseline could be seen. This is consistent with the fact that, when the first procedure was used to determine the shape of the "bound" spectrum of 5-doxylpalmitoylcholine (Figure 4), the observed spectrum was identical to the one obtained in the presence of an excess of unlabeled competitive ligand, such as acetylcholine.

Temperature Effect on ESR Spectra. 8-Doxylpalmitoylcholine gives rise to a "strongly immobilized" signal. Therefore, as one might expect, the shape of the spectrum depends very little on temperature. For example, between 0 and 30 °C the parameter $2T_{\parallel}'$ remains constant, close to 65 G. In contrast, the spectrum of the bound 16-doxylstearoylcholine recorded by difference ESR spectroscopy showed some sensitivity to temperature. For example, the splitting between the low- and high-field peaks increased by approximately 3 G between +25 and +2 °C. The ratio of the amplitude of the middle peak over that of the low-field peak changed from 2.00 to 1.92.

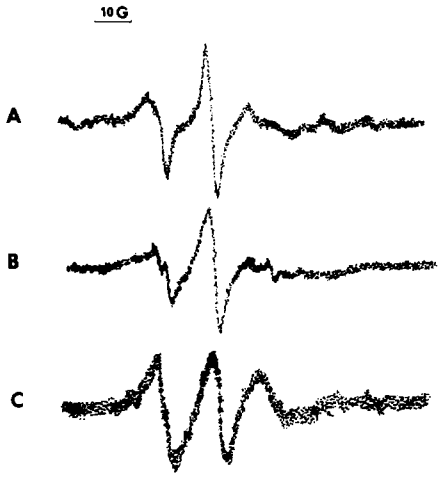


FIGURE 9: Effect of the increasing concentration of 16-doxylstearoylcholine on the difference spectra of that spin-label bound to the cholinergic receptor. Spectrum A: 15 μM acylcholine was added to a 2.5 μM α-toxin binding site membrane preparation. Spectrum B: 30 μM of acylcholine was added to a 2 μM α-toxin site membrane preparation. Spectrum C: 60 μM of acylcholine was added to a 1 μM α-toxin site membrane preparation. (Spectra were redrawn from photograph of the screen). Accumulation time: 3 h for each sample.

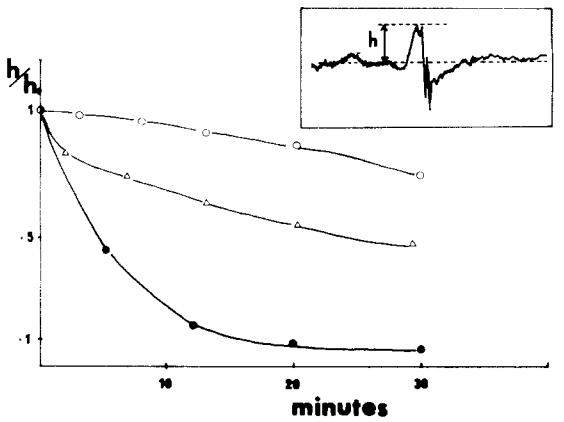


FIGURE 10: Rate of 8-doxylpalmitoylcholine nitroxide reduction by ascorbate at 0 °C. As shown in the inset, the spectrum of the 8-doxylpalmitoylcholine bound to the acetylcholine receptor is unaffected by the addition of 5 mM ascorbate (note the two sharp peaks created by ascorbate). h is proportional to the nitroxide concentration at time t, h_0 at time zero (before ascorbate addition); (●) reduction rate of 5 μM acylcholine in physiological medium by 5 mM ascorbate; (○) reduction rate of 5 μM acylcholine bound to the receptor (10 μM α-toxin binding sites) by 5 mM ascorbate; reduction rate of 5 μM acylcholine displaced from the receptor sites by acetylcholine (5 mM ascorbate).

Concentration Effects. In order to test the accessibility of the acetylcholine receptor protein from the bulk lipid phase of the membrane, spectra of protein-bound 16-doxylstearoylcholine were recorded in the presence of a concentration of spin-label in the membrane capable of inducing spin–spin interactions. The difference ESR technique was applied at 20 °C in the presence of increasing concentrations of the spin-labeled acylcholine. Figure 9 shows the spectra obtained with three different concentrations of 16-doxylstearoylcholine. Strong spin–spin broadening of the lines appear.

Kinetics of the Reduction by Ascorbate. The kinetics of reduction by sodium ascorbate were studied (at 0 °C) to determine the accessibility of protein-bound spin-labeled acylcholine from the aqueous phase. This study was undertaken only with the 8-doxylpalmitoylcholine because direct and rapid recording of the bound spectrum can be obtained only with that spin-label.

The addition of ascorbate (5 mM) does not introduce any modification in the *shape* of the spectrum. That is, there is no interaction of ascorbate with the binding properties of the acetylcholine receptor. The kinetics of reduction by ascorbate of a given concentration of 8-doxylpalmitoylcholine bound to the receptor with that displaced into the lipid phase are compared in Figure 10.

Discussion

Interpretation of the ESR Spectra of Spin-Labeled Long-Chain Acylcholines Bound to Membrane-Bound Acetylcholine Receptor Protein. Of the four long-chain spin-labeled acylcholines used, only three generated ESR spectra different from that of the corresponding fatty acids. The reason why 5-doxylpalmitoylcholine, in spite of its high affinity for the acetylcholine receptor, does not give rise to a spectrum indicating some feature of receptor binding is not clear. The nitroxide may be selectively reduced when it is positioned at a certain distance from the receptor site (Brisson, 1975), possibly by a chemical reaction with SH groups (for example, obtained by a reduction of the disulfide bridge known to exist next to the acetylcholine binding site (Karlin, 1969)) or by quenching by a paramagnetic ion. There is no evidence, however, for the existence of such an ion.

Both 8-doxylpalmitoylcholine and 12-doxylstearoylcholine give rise to "strongly immobilized signals". Such high immobilization or ordering of the probes would be inconsistent with a direct contact with the aqueous phase. The kinetics of reduction by ascorbate of the bound 8-doxylpalmitoylcholine demonstrate that, indeed, the nitroxide is not immobilized at the hydrophilic surface of the protein. Rather, it is embedded in some hydrophobic region around or in the protein. At this point, we cannot discriminate between these two hypotheses.

The receptor-bound 16-doxylstearoylcholine gives rise to a very different and interesting spectrum (Figure 8D and Figure 9A). It is difficult to measure an accurate order parameter on such spectra, but it appears very clear that the order parameter of 16-doxylstearoylcholine, even at 0 °C, is much smaller than for the two spin-labels described above. Furthermore, the lines appear relatively narrow, particularly at room temperature. In other words, there appears to be more fluidity in the vicinity of the latter probe.[2] This evidence strongly suggests the presence of fluid lipids close to the receptor protein. However, the present results do not demonstrate the continuity of the lipids close to the protein with a fluid bilayer. The estimated order parameter of 16-doxylstearoylcholine bound to the receptor is slightly greater than that of the same spin-label in the lipid bilayer. This may be due to some hindrance in the motion of the probe caused by the protein itself, or to a small difference in the fluidity of the lipids surrounding the protein (boundary lipids) compared to the bulk lipids.

We shall now examine the results obtained with increasing concentrations of 16-doxylstearolycholine (Figure 9). Spec-

[2] Our real purpose is to compare the signals of spin probes in the direct lipid environment of a protein with their signals in the bulk lipid phase, as explored by ordinary spin-labeled fatty acids. Whether the nitroxide creates some perturbation or not is irrelevant to our point. As indicated by Seelig (1976), "the spin probes indicate the ease of hydrocarbon chain distortions". That obviously is related to the fluidity of the unperturbed system.

trum A in Figure 9 was obtained by using a concentration of 2 μM α-toxin binding sites in a highly purified membrane preparation and 15 μM of the spin-labeled acylcholine. Under these experimental conditions, we find that the percentage of occupied sites should be 90% or higher (by using K_I^{app} estimated from Figure 2). As a consequence, any increase in the concentration of the spin-labeled acylcholine should not substantially increase the number of receptor-bound spin-labels. This means that the observed spin-spin interactions appearing in spectrum B and C (Figure 9) do not come from the spin-spin interactions between spin-labels bound to the acetylcholine receptor sites. One is forced to interpret such interactions as being induced by the interaction of spin-labels bound to the receptor site with excess spin-labels present either in the bulk lipid phase or in a boundary layer.[3]

Since line broadening increases with increasing acylcholine concentration, the number of molecules interacting with a receptor-bound molecules also increases. Therefore, it seems unlikely that the long chain of the acylcholine bound to the receptor site is embedded in a small hydrophobic pocket of the receptor protein. The most likely interpretation is that spin-labels from the bulk lipid phase can interact directly with spin-labels bound to the receptor sites.

The Structure and Dynamics of the Lipid Environment of the Membrane-Bound Acetylcholine Receptor of Torpedo marmorata. Our spin-label study shows that a nitroxide probe can detect an environment corresponding to low order and, very likely, to high fluidity close to the receptor protein. This result would not be expected with membranes formed by a tight array of crystallized proteins. For example, artificial membranes can be formed with cytochrome oxidase and small amount of lipids. In such systems, 16-doxylstearic acid, in close contact with the protein, gives rise to a strongly immobilized signal (Griffith and Jost, 1976). Similarly, if inner mitochondrial membranes are progressively delipidated, the membrane proteins are artificially clustered and 16-doxylstearic acid incorporated in such membranes (as well as other spin-labeled fatty acids) gives rise to spectrum indicating a strong immobilization (Vignais et al., 1975). In Torpedo membrane vesicles, the lipids experience a certain degree of fluidity even close to the receptor protein. Although the existence of a lipid bilayer close to the receptor protein is not demonstrated by our results, the degree of motion observed strongly suggests such structure. If such bilayers exist, we cannot measure their surface area, nor do we know if the proteins are completely surrounded by such fluid lipids.[4]

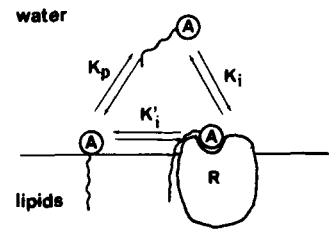


FIGURE 11: Schematic representation of the three-state equilibrium experienced by amphipatic ligands in the presence of membranes.

Acknowledgments

We thank Drs. Menez, Morgat, Fromageot, and Boquet for the gift of *N. nigricollis* [^{3}H]toxin. Drs. A. Sobel and M. Weber are acknowledged for their advice concerning the membrane preparation. We thank B. Bouabdallah for help during membrane preparations and binding experiments, and Dr. J. Cartaud for doing the electron microscopy of membrane preparation. Dr. J. P. Changeux is acknowledged for helpful discussions.

Appendix

Influence of the Partition of Amphiphilic Ligands between Lipids and Water on their Affinity Constant to Membrane-Bound Receptors. Let us consider the equilibrium represented in Figure 11. We can define an equilibrium constant for each type of exchange:

$$A_{water} \overset{K_I}{\rightleftharpoons} A_{receptor\ bound}$$

$$A_{lipids} \overset{K_I'}{\rightleftharpoons} A_{receptor\ bound}$$

$$A_{water} \overset{K_p}{\rightleftharpoons} A_{lipids}$$

To express the equilibrium constants in such a heterogeneous system, we will use the number of moles, rather than the concentrations. Let n_{Aw}, n_{Al}, and n_{Ar} represent, respectively, the number of moles of ligands in water in the lipids or bound to the receptor sites. n_R is the number of receptor sites unoccupied, n_w and n_l are the number of moles of water and lipids, respectively. Then:

$$K_I = \frac{n_{Aw} n_r}{n_w n_{Ar}}$$

$$K_I' = \frac{n_{Al} n_R}{n_l n_{Ar}}$$

while:

$$K_p = \frac{n_{Al} n_w}{n_{Aw} n_l}$$

From simple thermodynamic considerations, it is clear that at equilibrium the three constants cannot be independent.

If we only differentiate, as in most biochemical assays, two types of ligands, those bound to the receptors and the others, the apparent equilibrium is the following:

$$A_{bound} \overset{K_I^{app}}{\rightleftharpoons} A_{not\ bound}$$

K_I^{app} is the effective constant for the apparent equilibrium. It is easy to show that:

$$K_I^{app} = K_I(1 + K_p n_l / n_w) \qquad (A1)$$

K_I appears as the limit of K_I^{app} for very dilute membranes.

[3] In order to see the spin-spin interactions, the ratio of spin-labeled acylcholines to phospholipids has to be at least a few percent. Since long-chain acylcholines, like most amphiphilic molecules, are likely to have detergent properties, one may argue that the receptor protein is not in its normal hydrophobic environment. However, it has been shown that the pharmacological response of membrane-bound receptor to a classical detergent, namely, Triton X 100, shows evidence of solubilization only above 50 μM, while working at low membrane concentration (Brisson et al., 1975b). Therefore, in the conditions described for ESR experiments, it is believed that no solubilization of the protein occurs.

[4] There is no evidence from our results that unlabeled lipids in close contact with the membrane proteins are much more immobilized or ordered than the bulk lipids. The strong immobilization seen with the 8-doxylpalmitoylcholine and the 12-doxylstearoylcholine may be attributed to steric hindrance between the oxyoxazolidine ring and the rigid protein. This feature is very convenient for detecting the protein but may very well be due only to the probe. Furthermore, the probe can be close to the protein (16-doxylstearoylcholine) and yet detect a high fluidity. Hence, we cannot say that lipids surrounding the protein form a continuous boundary layer with immobilized chains.

It is important to remark that at equilibrium the population of any state of A is independent of the path molecules A take. For example, if the in plane reaction is forbidden, it does not change any of our results. Only the kinetics would be modified.

Added in Proof

An equation similar to eq A1 has been proposed recently by Parry et al. (1976).

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The Electrochemical Proton Gradient in *Escherichia coli* Membrane Vesicles†

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ABSTRACT: Membrane vesicles isolated from *Escherichia coli* grown under various conditions generate a transmembrane pH gradient (ΔpH) of about 2 pH units (interior alkaline) under appropriate conditions when assayed by flow dialysis. Using the distribution of weak acids to measure ΔpH and the distribution of the lipophilic cation triphenylmethylphosphonium to measure the electrical potential ($\Delta\Psi$) across the membrane, the vesicles are demonstrated to develop an electrochemical proton gradient ($\Delta\bar{\mu}_{H^+}$) of almost -200 mV (interior negative and alkaline) at pH 5.5 in the presence of reduced phenazine methosulfate or D-lactate, the major component of which is a ΔpH of about -120 mV. As external pH is increased, ΔpH decreases, reaching 0 at about pH 7.5 and above, while $\Delta\Psi$ remains at about -75 mV and internal pH remains at pH 7.5-7.8. The variations in ΔpH correlate with changes in the oxidation of reduced phenazine methosulfate or D-lactate, both of which vary with external pH in a manner similar to that described for ΔpH. Finally, ΔpH and $\Delta\Psi$ can be varied reciprocally in the presence of valinomycin and nigericin with little change in $\Delta\bar{\mu}_{H^+}$ and no change in respiratory activity. These data and those presented in the following paper (Ramos and Kaback, 1976) provide strong support for the role of chemiosmotic phenomena in active transport and extend certain aspects of the chemiosmotic hypothesis.

Membrane vesicles isolated from *Escherichia coli* retain the same polarity as the membrane in the intact cell and catalyze active transport of various solutes by a respiration-dependent mechanism that does not involve the generation or utilization of ATP or other high-energy phosphate intermediates (Kaback, 1972, 1973, 1974b; Kaback and Hong, 1973; Stroobant and Kaback, 1975). Although the precise means by which energy released from the oxidation of D-lactate or reduced phenazine methosulfate (PMS)[1] is coupled to transport in this system are unknown, an increasing accumulation of evidence indicates that chemiosmotic phenomena play a central role in the process (Hirata et al., 1973; Altendorf et al., 1975; Kaback, 1974b; Schuldiner and Kaback, 1975; Patel et al., 1975). As visualized by the Mitchell hypothesis (Mitchell,

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[1] Abbreviations used: $\Delta\bar{\mu}_{H^+}$, the electrochemical gradient of protons; ΔpH, the proton gradient across the membrane; $\Delta\Psi$, the electrical potential across the membrane; PMS, phenazine methosulfate; DMO, 5,5-dimethyloxazolidine-2,4-dione; $TPMP^+$, triphenylmethylphosphonium (bromide salt); CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; glucose-6-P, glucose 6-phosphate; Tris, tris(hydroxymethyl)aminomethane.