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# Inhibition of Ion Permeability Control Properties of Acetylcholine Receptor from *Torpedo californica* by Long-Chain Fatty Acids†

Terrence J. Andreasen‡ and Mark G. McNamee*

ABSTRACT: The characteristics of fatty acid inhibition of acetylcholine receptor function were examined in membrane vesicles prepared from *Torpedo californica* electroplax. Inhibition of the carbamylcholine-induced increase in sodium ion permeability was correlated with the bulk melting point of exogenously incorporated fatty acids. Above its melting temperature, a fatty acid could inhibit the large increase in cation permeability normally elicited by agonist binding to receptor. Below its melting temperature, a fatty acid was ineffective. None of the fatty acids altered any of the ligand binding properties of the receptor. Inhibitory fatty acids did not induce changes in membrane fluidity, as determined by electron paramagnetic resonance using spin-labeled fatty acids. The spin-labeled fatty acids also acted as inhibitors, and the extent of inhibition depended largely on the position of the nitroxide group along the fatty acid chain. Addition of noninhibitory fatty acid to the vesicle membranes did not protect the receptor from inhibition by spin-labeled fatty acids. The effects of free fatty acids on acetylcholine receptor function are attributed to the disruptions of protein-lipid interactions.

Membrane vesicles enriched in acetylcholine receptor (AcChR)[1] can be prepared from the electric tissue of *Torpedo californica*. The receptors in the vesicles retain important functional properties, such as agonist and antagonist binding and cation permeability control (Heidmann & Changeux, 1978). The AcChR normally exists in a resting but activatable state; pretreatment with agonist converts the AcChR into a desensitized state. This state is characterized by a 20–400-fold increase in affinity for agonist (Quast et al., 1978; Weiland et al., 1977), but agonist binding no longer results in the opening of a transmembrane cation channel. Both in vivo and in the in vitro vesicle system, the transition to the desensitized state is speeded by local anesthetics. [For reviews, see Heidmann & Changeux (1978) and Barrantes (1979).]

We previously reported that treatment of AcChR-enriched vesicles with phospholipase $A_2$ (EC 3.1.1.4) from *Naja naja siamensis* venom blocked AcChR activation (Andreasen & McNamee, 1977) and converted the AcChR into the desensitized state (Andreasen et al., 1979). Similar results with other phospholipases $A_2$ have also been reported (Hanley, 1978; Bon et al., 1979; Moody & Raftery, 1978). Further investigation revealed that certain fatty acids could cause an inactivation of AcChR which was apparently different from the desensitization phenomenon. These fatty acids did not affect any of the ligand binding properties of AcChR. However, they totally abolished the channel opening in response to bound agonist (Andreasen et al., 1979). The AcChR appeared to be in a nondesensitized but uncoupled state. The inhibition of AcChR function depended on fatty acid structure and concentration in the membrane. The rate of fatty acid uptake correlated with the rate of onset of inhibition and removal of fatty acids by bovine serum albumin restored AcChR function. Unsaturated fatty acids were much more effective inhibitors of AcChR function than saturated fatty acids.

Fatty acids have been found to modify membrane functions in a number of other systems. For example, they activate adenylate cyclase (Oryl & Schramm 1975; Hanski et al., 1979) and guanylate cyclase (Glass et al., 1977; Asakawa et al., 1976), promote fusion in vesicles (Kantor & Prestegard 1975) and cells (Ahkong et al., 1973), alter permeability control of zymogen granules (Schramm et al., 1967) and mitochondria (Wojtczak, 1976), and inhibit capping in lymphocytes (Klausner et al., 1980).

We report here results indicating that the inhibitory potency of fatty acids is directly correlated with the physical state of the fatty acids. AcChR inhibition is discussed in terms of specific disruption of protein–lipid interactions induced by the various fatty acids.

## Materials and Methods

*AcChR-Enriched Vesicles.* Vesicles were prepared from *T. californica* electroplax as described previously (Andreasen & McNamee, 1977). *T. californica* were obtained live (Pacific Biomarine, Venice, CA), and the electroplax was used immediately or stored in liquid nitrogen. Final pellets were suspended in vesicle diltuion buffer (VDB: 255 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, 1.5 mM sodium phosphate, and

† From the Department of Biochemistry and Biophysics, University of California, Davis, Davis, California 95616. *Received November 27, 1979.* Supported by Research Grant NS-13050 from the National Institute of Neurological and Communicative Diseases and Stroke.

‡ Present address: Department of Pharmacology, University of Washington, Seattle, WA 98195.

[1] Abbreviations used: AcChR, acetylcholine receptor; VDB, vesicle dilution buffer; carb, carbamylcholine chloride; Mops, 3-(*N*-morpholino)propanesulfonic acid; EPR, electron paramagnetic resonance.

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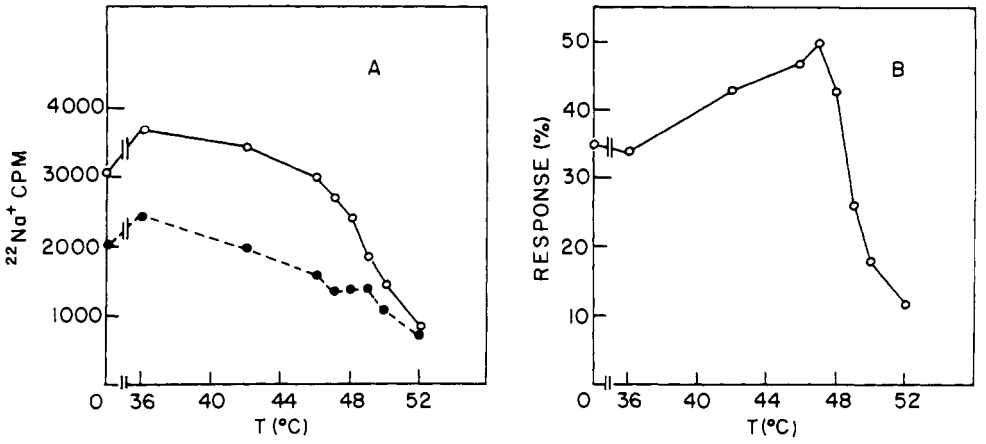



FIGURE 1: $^{22}Na^+$ efflux from *Torpedo* membranes incubated at various temperatures for 30 min prior to dilution into vesicle dilution buffer at the same temperature. (A) Vesicles were allowed to efflux for 0.25 min in the presence (●) or absence (○) of $2.5 \times 10^{-4}$ M carb and then filtered and counted (see Materials and Methods). cpm retained on the filter represent $^{22}Na^+$ still within the membrane vesicles. (B) Percent response to carb for data in (A): % response: [cpm (no carb) − cpm (+carb)]/cpm (no carb) × 100.

0.02% $NaN_3$, pH 7.0) at a protein concentration of ~10 mg/mL, determined according to the Lowry procedure (Lowry et al., 1951). Vesicles were used immediately or they were stored in liquid nitrogen. Some vesicles were analyzed by sucrose density gradient centrifugation. Vesicles (0.5 mL) were layered on top of 12 mL of a continuous 27–45% (w/w) sucrose gradient in VDB and centrifuged overnight at 4 °C at 35 000 rpm by using an SW40 rotor and a Beckman L5-65 centrifuge. Fractions of 0.4 mL were collected by using an ISCO fractionator. Typically, iodinated α-bungarotoxin was added before centrifugation (to saturate ~5% of the binding sites) in order to provide a convenient marker for AcChR.

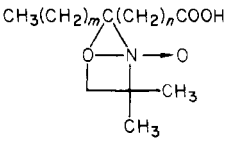
The toxin binding specific activity was determined by a DE-81 filter binding assay (Hamilton et al., 1979) using [$^{125}$I]-α-bungarotoxin instead of tritiated *N. naja siamensis* α-neurotoxin. The monoiodinated α-bungarotoxin was prepared and purified as referenced previously (Andreasen et al., 1979). The *rate* of iodinated bungarotoxin binding to AcChR was measured as described previously (Andreasen et al., 1979) except that 10 mM Mops buffer (pH 7.4) was used in place of 10 mM phosphate in the wash buffer. This change was made so that all the toxin binding assays used the same wash buffer. Filters were counted on a Packard γ counter optimized for $^{125}$I.

*$^{22}Na^+$ Efflux Assays.* AcChR-enriched vesicles (10 mg of protein/mL in VDB) were incubated overnight at 4 °C with $^{22}Na^+$ (New England Nuclear; carrier free) at a final $^{22}Na^+$ concentration of 20 μCi/mL. Assays were performed by diluting 10–20 μL of the vesicle solution into 1.5 mL of VDB. After 0.25 min of efflux, this solution was filtered through HAWP 2400 Millipore filters by using a Hoeffer filtration apparatus. The filters were washed 3 times with 3 mL of ice-cold VDB and counted in 10 mL of PCS (Amersham) by using a Beckman LS200 liquid scintillation counter. In the temperature studies reported here, the vesicles and the initial dilution buffer were maintained at the same temperature. The wash buffer was always kept on ice until used.

*Fatty Acid Treatments.* Aliquots of fatty acid stock solutions (50 mM in $CHCl_3$) sufficient to yield a final total concentration of 1–2 mM were added to glass test tubes and dried under nitrogen. Appropriate volumes of vesicles were added, and the mixture was incubated at 33 °C in a constant temperature bath. From this initial state, the vesicles were transferred to the temperature used in the particular assays. A 30-min incubation was performed at each subsequent temperature prior to the efflux assay.

Unlabeled fatty acids were obtained from Sigma Chemical Co. [$^3$H]Oleic acid [9,10-$^3$H(N); 5 Ci/mmol] was obtained from New England Nuclear, and [$^{14}$C]stearic acid (carboxyl-$^{14}$C; 55.5 mCi/mmol) was obtained from International Chem Nuclear Corp.

*Electron Paramagnetic Resonance (EPR).* Ethanol solutions of spin-labeled fatty acids of the structural formula




where $(m,n)$ = (12,3), (5,10), or (1,14) were added to glass tubes and dried under a stream of nitrogen. All of the spin-labels were purchased from Syva Co. (Palo Alto, CA). Appropriate amounts of vesicles were added to yield a final concentration of 0.2–1.0 mM spin-label. After 1-h incubation at room temperature, the suspensions were taken up into 50-μL glass capillaries, and the EPR spectra were recorded on a Varian E-4 EPR spectrometer equipped with a temperature-control device. When the spin-labels were used as a probe for fluidity, the native lipid to spin-label ratio was maintained above 30:1 to avoid spin–spin interactions (Eckstein et al., 1979). For these experiments a relatively high protein concentration was used (20–25 mg/mL).

## Results

*Temperature Dependence of $^{22}Na^+$ Efflux Response.* AcChR-rich vesicles, containing ~0.7–1 nmol of α-bungarotoxin binding sites/mg of protein, were preloaded with $^{22}Na^+$. The vesicles were then incubated for 15 min at various temperatures and assayed for $^{22}Na^+$ efflux at the incubation temperature in the presence or absence of $2.5 \times 10^{-4}$ M carb (Figure 1). The carb-induced increase in $^{22}Na^+$ release from the vesicles (the carb response) was measured after 0.25 min of efflux and was fairly constant up to 36 °C. The measured carb responses represent a time averaged release of $^{22}Na^+$ from a heterogenous population of vesicles undergoing both passive diffusion and the functional activation and densitization processes. Typically, 40–60% of the internal counts were specifically released by carb under the time and concentration conditions of the dilution–filtration assay described under Materials and Methods. Independent studies by rapid-flow filtration techniques have shown that most of the specific

Table I: Correlation of Fatty Acid Melting Points with Inhibition of Carb-Induced $^{22}Na^+$ Efflux[a]

| fatty acid | mp (°C) | assay temp (°C) | efflux inhibn |
|---|---|---|---|
| arachidonic (20:4) | −49.5 | 33 | + |
| | | 25 | + |
| | | 0 | + |
| vaccenic (18:1(11-trans)) | +13 | 33 | + |
| | | 25 | + |
| | | 0 | − |
| oleic (18:1(9-cis)) | +16 | 33 | + |
| | | 25 | + |
| | | 0 | − |
| petroselenic (18:1(6-cis)) | +30 | 33 | + |
| | | 25 | − |
| | | 0 | − |
| vaccenic (18:1(11-trans)) | +44 | 33 | + |
| | | 25 | − |
| | | 0 | − |
| stearic (18:0) | +69 | 33 | − |
| | | 25 | − |
| | | 0 | − |

[a] *Torpedo* vesicles preloaded with $^{22}Na^+$ were initially incubated with 2 mM (final concentration) of each fatty acid at 33 °C and subsequently incubated at 33, 25, or 0 °C. The carb efflux response was then measured at the incubation temperature. A "+" indicates that the fatty acid completely blocked any carb-induced increases in $^{22}Na^+$ flux. A "−" indicates that a full carb response was observed. Melting points of the fatty acids were obtained from technical information provided by Sigma.

carb-induced release of cations through AcChR ion channels occurs very rapidly, probably within 1 s (Hess et al., 1979). The 0.25-min efflux assay used in the experiments here represents a maximal response for a given carb concentration. Although our flux measurements cannot be directly related to the number of open channels or to the rates of channel opening and closing, they do give reproducible dose–response curves for agonist activation, and they are ideally suited for conveniently measuring channel blocking effects of antagonists or other agents (Andreasen et al., 1979; Heidmann & Changeux, 1978). Between 43 and 47 °C, the carb response actually increased, while above 47 °C the response declined. At 52 °C there was not only little response but also very few $^{22}Na^+$ counts were retained on the filters, indicating that many vesicles had been disrupted.

*AcChR Inhibition by Fatty Acids.* The ability of various long-chain fatty acids to inhibit carb response was measured as a function of temperature. Fatty acids were incorporated into $^{22}Na^+$-loaded AcChR vesicles at 33 °C, and the carb response was measured (Table I). These samples were then incubated at 25 °C for 30 min and then at 0 °C for 30 min. At each temperature, the carb response was again measured. These results indicated that at temperatures above their bulk melting points, fatty acids completely inhibited the carb response. At temperatures below their bulk melting points, the fatty acids did not inhibit the carb response. An exception was *trans*-vaccenic acid. While melting at 44 °C, this fatty acid was inhibitory at 33 °C. At 25 °C, however, it was not. A more detailed temperature analysis using petroselenic acid (mp 30 °C) emphasized the sharpness of the inhibition transition around the bulk melting point (Figure 2).

*Uptake and Retention of Fatty Acids.* Loss of inhibitory activity could have been related to a temperature-sensitive removal of some free fatty acids from the membranes. Table II demonstrates that this was not the case. Uptake of [$^3$H]oleic acid or [$^{14}$C]stearic acid was measured at 25 °C, a temperature above the melting point of oleic but below that of stearic acid. Over a time course of 40 min, identical amounts of the two

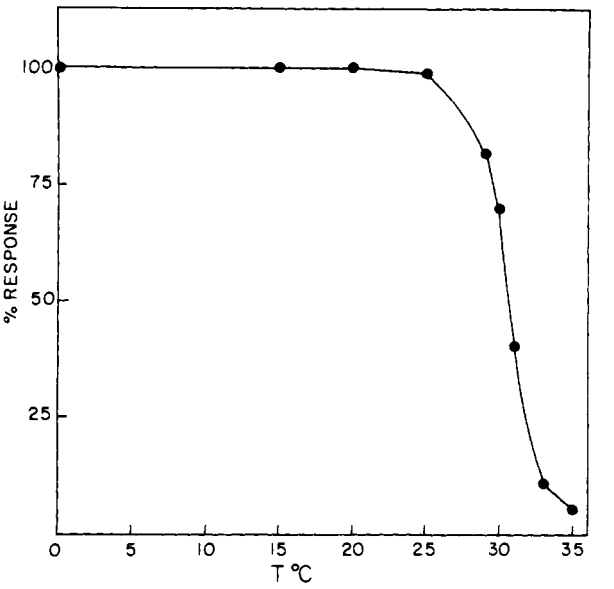



FIGURE 2: Relative carb response (percent) of *Torpedo* membranes containing 2 mM petroselenic acid as a function of temperature. The membranes were initially incubated at 35 °C, and the temperature was lowered to a new value for 30 min prior to the $^{22}Na^+$ efflux assay (Materials and Methods). Each point represents a separate incubation mixture. Controls with no petroselenic acid were run in parallel at each temperature. The percent response (●) is defined as carb response (+petroselenic acid)/carb response (−petroselenic acid) × 100. The carb response is defined in Figure 1 and for the controls was essentially constant over the 0–35 °C temperature range.

Table II: Uptake of Fatty Acids by *Torpedo* Membranes[a]

| fatty acid | incubn time (min) | pmol of fatty acid/pmol of α-toxin sites |
|---|---|---|
| stearic (18:0) | 10 | 191 |
| | 20 | 237 |
| | 40 | 375 |
| oleic (18:1) | 40 | 380 |

[a] The labeled fatty acid ([$^3$H] oleic acid or [$^{14}$C] stearic acid) was premixed with the corresponding unlabeled fatty acids prior to addition of membranes. Each time point represents a different incubation mixture. The uptake of fatty acid after 40 min corresponds to a final fatty acid concentration of ~1.5 mM. The protein concentration was 10 mg/mL, and the [$^{125}$I]-α-bungarotoxin binding activity was 700 pmol/mg.

acids were taken up by the vesicles to give a final fatty acid concentration of 1.5 mM. Moreover, lowering of the temperature below the melting point of oleic acid did not result in its removal. Thus, the failure of stearic acid to inhibit AcChR at 25 °C, or of oleic to inhibit at 0 °C, cannot be explained by their absence from the membranes.

*Effects of Fatty Acids on Membrane Fluidity.* The electron paramagnetic resonance (EPR) technique of spin-labeling was used to look for alterations in membrane fluidity induced by the fatty acids. Spin-labeled stearic acid derivatives, containing the oxazolidine nitroxide moiety at one of three different positions along the fatty acid chain, were used as probes. EPR spectra were obtained at several temperatures for native membranes and for membranes containing 2 mM petroselenic acid. Spectra for the (12,3) and (1,14) spin-labels are shown in Figures 3 and 4. For the (12,3) label the nitroxide is near the polar head-group region and for the (1,14) label the nitroxide is near the bilayer center.

For all three spin-labels, the order parameter, $S$, of the membrane was not altered by the presence of petroselenic acid, at temperatures both above and below the inhibition transition

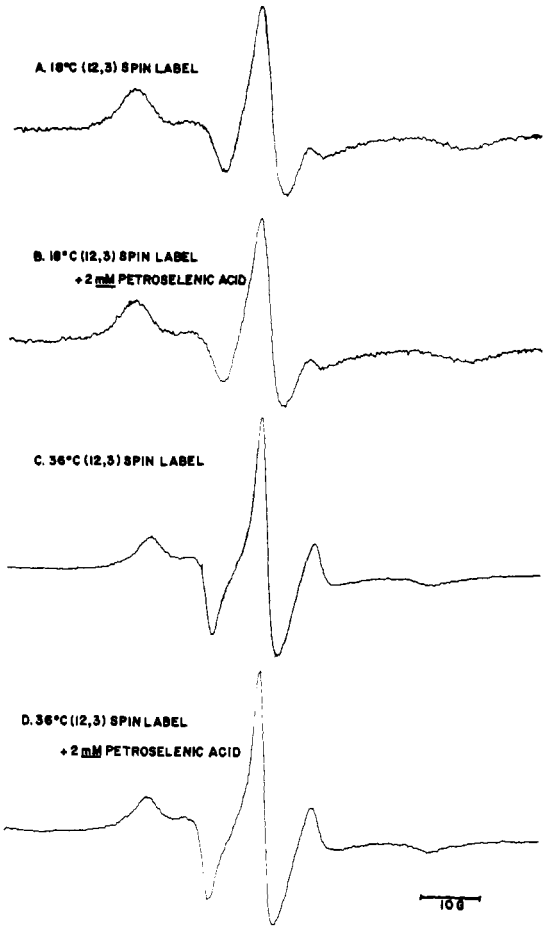



FIGURE 3: EPR spectra of *Torpedo* membranes containing the (12,3) fatty acid spin-label (0.5 mM) and 0 or 2 mM petroselenic acid at 18 and 36 °C. Further details on sample preparation are in the footnote of Table III.

Table III: Effects of Petroselenic Acid on the Order Parameter ($S$) of *Torpedo* Membranes[a]

| spin-label | temp (°C) | [fatty acid] (mM) | $S$ |
|---|---|---|---|
| 12,3 | 36 | 1 | 0.54 |
| | | 0 | 0.54 |
| | 18 | 1 | 0.81 |
| | | 0 | 0.80 |
| 1,14 | 36 | 1 | 0.139 |
| | | 0 | 0.134 |
| | 17 | 1 | 0.23 |
| | | 0 | 0.23 |

[a] *Torpedo* membranes were preincubated at 33 °C with 0 or 1 mM (final concentration) petroselenic acid (18:1(6-cis)) for 30 min. Aliquots were then incubated with the (12,3) or (1,14) fatty acid spin-labels for 30 min at 33 °C (final spin-label concentration 0.2 mM). Spectra were then recorded at two different temperatures. Order parameters were measured from the observed splittings according to the procedure of Gaffney (1976).

temperature (Table III). The order parameter, which was readily calculated from the EPR spectrum, provided a measure of acyl chain motion in the region of the membrane near the probe (Gaffney, 1976).[2] Other fatty acids, including stearic

[2] For EPR spectra giving apparent order parameters as small as that calculated for the (1,14) label, the empirical method used here is not completely accurate (Gaffney, 1976). The essential point, however, is that the added fatty acid has no measurable effect on the line shape or spectral splittings.

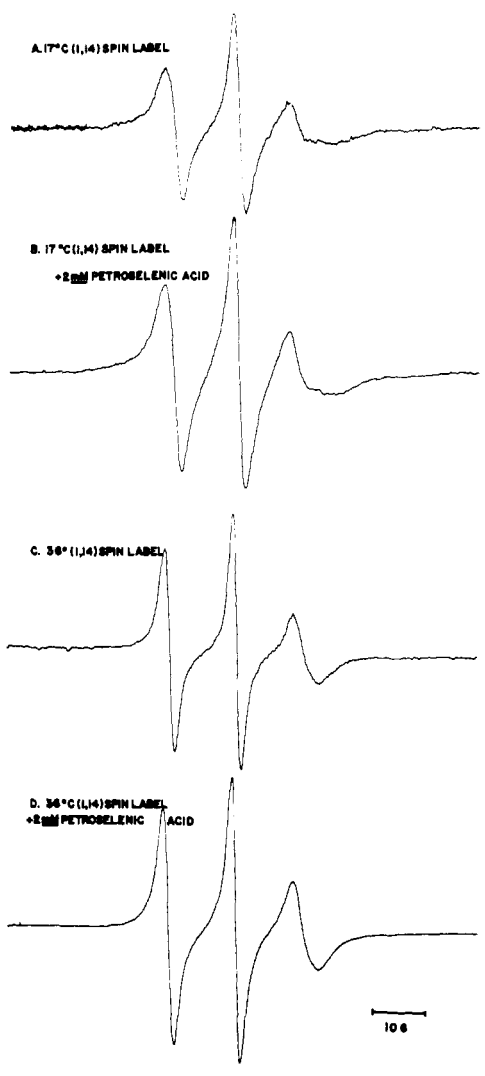



FIGURE 4: EPR spectra of *Torpedo* membranes containing the (1,14) fatty acid spin-label (0.5 mM) and 0 or 2 mM petroselenic acid at 17 or 36 °C.

Table IV: Effect of Spin-Labeled Fatty Acids on Carb Response[a]

| spin-label | temp (°C) | inhibn of carb response (%) |
|---|---|---|
| 12,3 | 33 | 100 |
| | 25 | 30 |
| | 0 | 0 |
| 5,10 | 35 | 49 |
| | 30 | 0 |
| | 25 | 0 |
| | 0 | 0 |
| 1,14 | 37 | 100 |
| | 33 | 67 |
| | 25 | 44 |
| | 0 | 50 |

[a] *Torpedo* membranes were preloaded with $^{22}Na^+$ and then incubated with 1 mM spin-label at 33 °C. $^{22}Na^+$ efflux was measured after a 30-min incubation at each temperature. The carb response is defined in Figure 1; and percent inhibition is 100% − percent carb response. Each point represents a separate incubation mixture.

acid, did not alter measured order parameters (data not shown).

*Inhibition by Spin-Labeled Fatty Acids.* The spin-labeled stearic acids could themselves act as inhibitors of carb response. The spin-labels were incorporated into the vesicle membranes at 33 °C, and carb responses were measured as the temper-

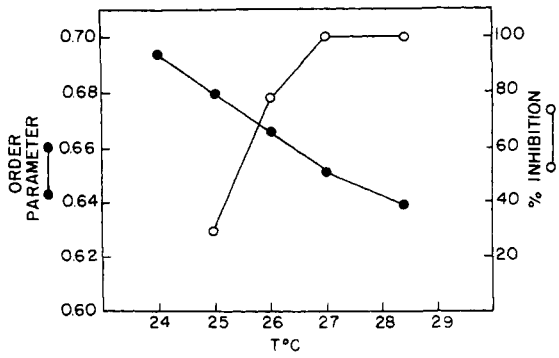



FIGURE 5: Variation in membrane order paramter ($S$) and percent inhibition of carb response with temperature. *Torpedo* membranes, preloaded with $^{22}Na^+$, were incubated with 1.0 mM (12,3) spin-label for 1 h at 33 °C. $^{22}Na^+$ efflux in the presence or absence of $2.5 \times 10^{-4}$ M carb was measured at the indicated temperatures. The EPR spectrum was recorded at each temperature and the order parameter was calculated.

ature was decreased (Table IV). For these experiments, the spin-labels were used at a concentration of 1 mM, corresponding to a 7:1 ratio of native lipids to spin-label at 10 mg of protein/mL. This concentration was considerably higher than that used in measuring the order parameters reported in Table III but was used to ensure that the maximum possible inhibition was obtained. Inhibition depended not only on temperature but also on the location of the nitroxide ring along the stearic acid backbone. When located on carbon 5 [(12,3) label], effective inhibition was seen at higher temperatures, but a full carb response could be elicited at 0 °C. With the label located on carbon 12 [(5,10) label], carb response was halved at 35 °C, but at lower temperatures full response was restored. Location of the label on carbon 16 [(1,14) label] resulted in full inhibition at 37 °C, with recovery to about half of the normal response at 0 °C.

A more detailed study of carb response in conjunction with membrane fluidity was undertaken using the (12,3) label as both the inhibitory agent and the probe. After incorporation of the label, vesicles were incubated at various temperatures. Aliquots were withdrawn for the $^{22}Na^+$ efflux assay and for obtaining the EPR spectrum at each temperature. The extent of inhibition by the spin-label and the corresponding order parameters as a function of temperature are shown in Figure 5. Under the conditions of this experiment, the spin-label concentration was higher (1 mM) than the optimal value for accurate measurement of order parameters. However, the measured values were within 2% of those measured at lower spin-label concentrations. No sharp break was observed in the order parameter which correlated with the onset of inhibition; rather, a smooth decline in the order parameter was found as inhibition fell from 100 to 30%. As stated earlier, EPR spectra revealed no change in the order parameter measured by using the (12,3) label in the presence of 1 mM stearic acid. In a separate experiment, the stearic acid, which was not inhibitory, was unable to prevent or modify the inhibition of carb response due to the (12,3) label.

*Location of Fatty Acid Spin-Label Probes.* Correlation of EPR spectral changes with functional properties of the AcChR requires localization of the probes in the functional membrane vesicles. The ability of the spin-labeled fatty acids to inhibit the carb response provides direct evidence that at least some of the spin-labels are in functionally important regions of the AcChR-rich membrane vesicles.

For determination of the distribution of label among various membrane subpopulations in the partially purified membranes used here, an aliquot of membranes was preincubated with

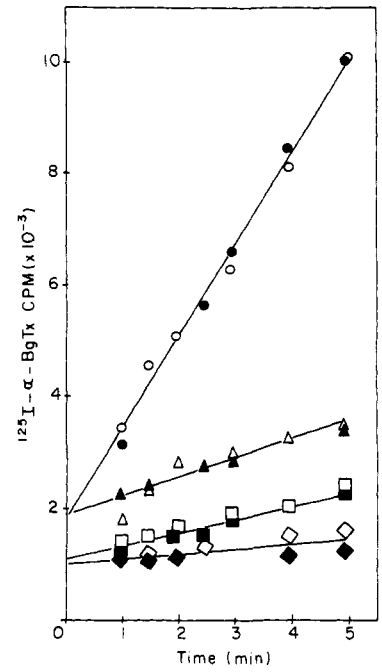



FIGURE 6: Effect of the (1,14) fatty acid spin-label on the initial rate of mono[$^{125}$I]-α-bungarotoxin binding to *Torpedo* membranes. Binding was measured to control (open symbols) membranes or to membranes preincubated for 60 min with 1 mM (1,14) spin-label (closed symbols). The assay was performed as previously described (Andreasen et al., 1979). (○ and ●) No carb present; (△ and ▲) 5 μM carb present in the assay mixture; (□ and ■) 2-min preincubation with 5 μM carb and 5 μM carb in the assay mixture; (◇ and ◆) 10-min preincubation with 5 μM carb and 5 μM carb in the assay mixture.

the (1,14) label and then analyzed by continuous sucrose density gradient centrifugation (27–45% w/w in VDB). Fractions were analyzed for total protein, bungarotoxin binding, and EPR spectral intensity. Two main peaks centered at 34.5 and 37% sucrose (w/w) were obtained for three different membranes preparations. For a membrane preparation with an initial bungarotoxin binding specific activity of 950 pmol/mg of protein, the 34.5 and 37% peaks, which contained approximately equal amounts of protein, had specific activities of 1250 and 1150 pmol of toxin sites/mg of protein. The spin-label concentration, as measured by the peak-to-peak amplitude of the central EPR line, was directly proportional to the protein concentration. In general, for membranes ranging in toxin binding specific activity from 300–2000 pmol/mg of protein, the spin-label intensity always was proportional to the protein concentration (and presumably to the total lipid concentration). Thus, there was no indication of selective incorporation or nonincorporation of spin-label into subpopulations of the AcChR-rich membranes.

*α-Bungarotoxin Binding Studies.* As reported previously, the fatty acids had no effect on the number of α-bungarotoxin binding sites and no effect on the rate at which the toxin could bind to AcChR. Furthermore, the effects of carb on the rate of toxin binding were not affected by the fatty acids (Andreasen et al., 1979). The rate binding studies were repeated using *Torpedo* vesicles containing the spin-labeled fatty acids. As shown in Figure 6, coincubation of untreated AcChR membranes with 5 μM carb reduced the initial rate of monoiodinated α-bungarotoxin binding to AcChR. Preincubation of the AcChR with 5 μM carb for 10 min prior to toxin addition resulted in an even slower rate of toxin binding consistent with the shift of the AcChR into the high-affinity state for carb. Detailed analysis of toxin binding kinetics in this laboratory is consistent with a 400-fold increase in carb

affinity upon desensitization (J. W. Walker and M. G. McNamee, unpublished observations). If the rate of toxin binding was measured after 2 min of preincubation, an intermediate rate was observed, indicating that the shift to the high-affinity state was a moderately slow process, in accord with results of other groups (Weiland & Taylor, 1979; Quast et al., 1978; Heidmann & Changeux, 1978). In all cases, the (1,14) spin-labeled fatty acid had no effect on the observed rates (Figure 6), nor did the other two spin-labeled fatty acids (data not shown).

## Discussion

The results indicate that long-chain fatty acids specificially block the ion permeability control properties of the acetylcholine receptor in membrane vesicles isolated from the electric tissue of *T. californica*. The ability of a given fatty acid to block carbamylcholine-induced increases in $^{22}Na^+$ permeability is directly correlated with the physical properties of the fatty acids. Only above its bulk melting point is a fatty acid able to block ion permeability control.

Recent results from Hauser's group (Hauser & Guyer, 1979; Hauser et al., 1979) provide the basis for an explanation of the striking dependence of AcChR inactivitation upon *bulk* fatty acid properties. On the basis of electrophoretic analysis of fatty acid–phospholipid mixtures, Hauser et al. argue that fatty acids are unionized at neutral pH and form clusters within the membrane. It is thus reasonable to assume that some of the cooperative properties of the fatty acids (such as melting temperatures) may be retained in membranes. Since differential scanning calorimetry techniques did not detect the fatty acids as a separate phase, the fatty acid clusters are probably small, consisting of 10–20 molecules each (Hauser et al., 1979). Interestingly, spin-labeled fatty acids do not form clusters (Hauser et al., 1979), and the spectra of the spin-labeled fatty acids used here with *Torpedo* membranes are consistent with nonclustered distribution.

The effects of free fatty acids on the physical properties of phospholipids have been extensively studied. One consistent observation is that saturated fatty acids, such as myristic and palmitic acid, increase the phase transition temperature of the corresponding phosphatidylcholine bilayers (Jain & Wu, 1977; Mabrey & Sturtevant, 1977; Kantor & Prestegard, 1978; Usher et al., 1978). Usher et al. (1978) examined the effects of both saturated and unsaturated free fatty acids on dimyristoylphosphatidylcholine vesicles, using fluorescence and calorimetry. They found that saturated fatty acids stabilized the gel state but did not affect fluidity of the liquid-crystal lipids. Unsaturated fatty acids also did not affect the fluidity of the liquid-crystal state; however, they distorted the crystal lattice, increased the hydrophobic volume of gel-state lipid, and decreased the phase transition temperature. The range of the assay temperature was above the bulk gel → liquid-crystal transition temperature for the unsaturated fatty acids and below that for the saturated fatty acids. Seelig & Seelig (1977) found that palmitic acid increased the order parameter of fluid 1-palmitoyl-2-oleoylphosphatidylcholine, as measured by deuterium NMR. However, this order increase was not sensed by spin-labeled fatty acids.

The spin-labels used here did not detect any alteration in fluidity associated with fatty acid incorporation, even for fatty acids as different as stearic acid (18:0) and arachidonic acid (20:4). At 25 °C, the stearic acid was noninhibitory and the arachidonic acid completely blocked carb-induced $^{22}Na^+$ efflux, so there was no direct correlation between the bulk membrane fluidity measured by the spin-labels and the extent of AcChR inhibition. Since the spin-labeled fatty acids can also inhibit AcChR function, there is no doubt that the unlabeled and spin-labeled fatty acids are in the same types of functional membranes.

As discussed previously (Andreasen et al., 1979), the inhibition of AcChR by fluid fatty acids is similar in many respects to the inhibition brought about by both local and general anesthetics (Cohen et al., 1974; Weiland et al., 1977; Young et al., 1978). The apparent anesthetic effect of spin-labeled fatty acids was reported earlier by Brisson et al. (1975) in electrophysiological experiments. The inhibition by fatty acids appears to be different from anesthetic effects in one important respect, however. The fatty acids do not increase the rate at which AcChR agonists shift the receptor into a high-affinity desensitized state (Andreasen et al., 1979; see also Figure 6). By the binding criteria used here, the fatty acids have no effect on activator binding and thus completely uncouple ligand binding from ion permeability control.

There are numerous proposed unitary mechansims of anesthetic action, and many of them involve lipid alterations, such as increased fluidity (Lee, 1976), increased bilayer thickness (Haydon et al., 1977), decreased lipid cluster size (Tsong et al., 1977), or increased hydration (Finch & Kiesow, 1979). For acetylcholine receptors at postsynaptic membranes, analysis of ion conductance changes are consistent with a direct blockade of open ion channels by local anesthetic molecules (Lester et al., 1979; Adams, 1975). There is also evidence that some local anesthetics bind specifically to *Torpedo* AcChR (Krodel et al., 1979; Blanchard & Raftery, 1979). A recent report argues strongly for degenerate perturbations of protein function by the anesthetics and suggests that displacement or perturbation of annular lipids may be important (Richards et al., 1978).

Since the fatty acids are undoubtedly exerting their effects within the lipid phase and yet have no effect on bulk fluidity (as measured by spin–labeled fatty acids), we suggest that the site of action is the lipid-protein interface and that the mechanism may involve perturbation of receptor-associated lipids as suggested generally by Richards et al., (1978) and in more specific terms for axonal sodium channels by Lee (1976).

There is fatty acid spin-label evidence (Marsh & Barrantes, 1978) for immobilized (gel-like) and fluid lipid components in *Torpedo marmorata* membranes. Quantitatively, the immobilized component corresponds to a fraction of lipid larger than that required for a monomolecular lipid annulus about the AcChR and could represent lipids trapped within the interstices of the closely packed proteins (Marsh & Barrantes, 1978). The nature of annular and/or trapped lipids and the role of such lipid in membrane function are controversial issues now being intensively investigated by many techniques in several systems (Chapman et al., 1978; Kang et al., 1979; Favre et al., 1979; Lenaz, 1979; Sandermann, 1978). In earlier studies on AcChR using spin-labeled acylcholines and spin-labeled fatty acids (Bienvenuë et al., 1977), the spectra of acyl nitroxides reversibly linked to the AcChR through the choline residue could be detected. When the label was close to the terminal methyl of the acyl chain, the nitroxide gave a spectrum characteristic of a fluid environment (Bienvenuë et al., 1977). Motions of the bound nitroxides were more restricted further up the chain.

The spectra in Figures 3 and 4 do not show the immobilized components observed by Marsh & Barrantes (1978), but we have observed an immobilized component using the (1,14) label at lower temperatures (0–15 °C). In preliminary experiments, we have observed no effects of an inhibitory fatty acid (lino-

lenic acid, 18:3) or a noninhibitory fatty acid (stearic acid, 18:0) on either the fluid or immobilized spectral component of the (1,14) label at 4 °C (J. Ellena and M. McNamee, unpublished observations). We are planning to expand the sensitivity of such measurements in the near future by using saturation transfer techniques (Baroin et al., 1979) which provide more detailed information about the slow motions that are presumably characteristic of the immobilized lipids. The use of more highly purified, alkaline-extracted membranes (Neubig et al., 1979) and reconstituted membranes (Epstein & Racker, 1978) should also enhance the ability to identify specific AcChR–lipid interactions. However, in preliminary experiments petroselenic acid showed no effect on the spectrum of the (1,14) spin-label in alkaline-extracted membranes. Since completion of this work, Rousselet et al. (1979) have published EPR spectra of spin-labeled fatty acids in *T. marmorata* vesicles that clearly show the immobilized components at low temperatures, consistent with our observations and those of Marsh & Barrantes (1978). However, immobilized components were *not* observed for exogenously incorporated *phospholipids* containing a spin-labeled fatty acid chain. The authors argue that the free fatty acids are not detecting an immobilized lipid component about the AcChR but rather are interacting directly with the AcChR (Rousselet et al., 1979). Our results are consistent with such an interpretation.

The dramatic effects of fluid fatty acids on AcChR function suggest that the altered lipid–protein interactions are directly or indirectly affecting the ion channel of the AcChR. Unfortunately, there is virtually no detailed molecular information about the nature of the ion channel. Of the four subunits of *Torpedo* AcChR, only one (the 40 000 $M_r$ subunit) has a well-defined function (ligand binding). Further characterization of the ion channel, including its interactions with other subunits and with the lipids, is clearly required for a complete understanding of AcChR function.

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