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EFFECT OF CHOLINERGIC LIGANDS ON THE LIPIDS OF ACETYLCHOLINE RECEPTOR-RICH MEMBRANE PREPARATIONS FROM TORPEDO CALIFORNICA

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Ion permeation, triggered by ligand-receptor interaction, is associated with the primary events of membrane depolarization at the neuromuscular junction and synaptic connections. To explore the possible sites of ion permeation, the long-lived fluorescent probe pyrene (fluorescence lifetime ~400 nsec) has been inserted into the lipid phase of acetylcholine receptor-rich membrane (AcChR-M) preparations from Torpedo californica. The pyrene probe is susceptible to both fluidity and permeability changes in the lipid bilayer. These changes are detected by variations in the rate of decay of the excited singlet state of pyrene after pulsation with a 10-nsec ruby laser flash. Variations of these lifetimes in the membrane preparations alone or in the presence of quenchers show that binding of cholinergic agonists and antagonists, neurotoxins, and local anesthetics to AcChR-M produces varying effects on the properties of the pyrene probe in the lipid phase.

It is concluded that binding of cholinergic ligands to the receptor does not significantly alter the fluidity or permeability of the lipids in the bilayer in contact with pyrene. On the other hand, local anesthetics do affect these properties.

INTRODUCTION

Our knowledge of primary molecular events leading to membrane depolarization at the synaptic or neuromuscular junction is progressing with the aid of in vitro and in situ approaches. The in vitro techniques have received a great impulse with the availability of large scale preparations of acetylcholine receptor (AcChR) from electric fish, particularly Torpedo californica (1, 2), and utilization of these AcChR preparations to study those factors determining recognition of neurotransmitters and their agonists and antagonists (1–3). In situ techniques are also progressing with the ability to prepare membrane vesicles rich in AcChR from the electric organ of T. californica (4) and Electrophorus electricus (5, 6). These membrane preparations are useful in the study of cation perme-

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Abbreviations: AcChR, acetylcholine receptor; AcChR-M, acetylcholine receptor rich membranes; α-Bgt, α-Bungarotoxin

ability (7) and specificity of ligand binding (8). Thus, considerable information on the properties of ligand affinity to isolated and membrane-bound AcChR is accumulating (1–8). On the other hand, little is known about the role of lipids and/or AcChR in cation translocation subsequent to cholinergic ligand binding to AcChR in the membrane.

One possible mechanism for ion translocation through excitable membranes is the change of the fluidity and/or ion permeability of the lipid bilayer upon binding of neurotransmitter to AcChR. If it is so, sensitive techniques which allow for the study of changes in fluidity and permeability of this lipid bilayer should be used to monitor these properties in AcChR-rich membrane vesicles in the absence and presence of cholinergic ligands. To this end there exists a potential array of elegant techniques, such as spin labels (9) and fluorescence polarization probes (10), developed for the study of membrane fluidity. On the other hand, we have chosen to monitor possible changes at the lipid bilayer of AcChR membranes by following the kinetics of fluorescence decay of the photoactive probe, pyrene, inserted in the hydrocarbon core of the bilayer. This method was selected because it has proven sensitive to the simultaneous changes in ambient fluidity, as well as permeability in model micelles, lipid bilayer, and membrane vesicles (11–14).

RESULTS

Pyrene Probe in AcChR-Rich Electroplax Membranes

Large-scale separation of membrane vesicles from T. californica electroplax can now be achieved (4). After the zonal centrifugation (Fig. 1), the large α-Bgt binding fraction contains membrane fragments in which 40%–50% of the total protein represents AcChR. The specific activity of the α-Bgt binding by this particle is about half the specific activity of AcChR isolated from the same tissue (2).

Pyrene, coated inside a test tube, can be introduced into the hydrophobic region of the membrane by gentle stirring at 25°C. The final concentration of the pyrene in the vesicles was measured spectrophotometrically at 337 nm using a molar extinction coefficient of 54,000 M^{-1} cm^{-1}. The absorption and emission spectra of the pyrene are shown in Fig. 2. The solid line corresponds to the singlet excited state of pyrene monomer while the dashed line represents the emission due to the excimer. This emission of the excimer increases with pyrene concentration indicating the values at which the excimer and, hence, aggregation becomes significant. Insertion of this probe does not affect the specific activity of the AcChR-rich membrane receptor for α-Bgt binding in any of the preparations.

Lifetime of the Pyrene Singlet Excited State in AcChR-Rich Membranes

The lifetime of the pyrene-excited singlet state was followed by fast kinetic spectroscopy (13) after a 10-nsec excitation pulse with a frequency doubled ruby laser (Korad) with an output of ~200 mJ. Excitation at 347.1 nm results in an emission due to the singlet excited state which decays after a few hundred nanoseconds into the ground state of pyrene. This first order process has a half-life of approximately 200 nsec in the AcChR-rich membrane preparations from T. californica at 25°C (Fig. 3). This technique is valuable to detect changes in membrane fluidity variations in the half-life of the excited state (12, 13).

The effect of temperature on the pyrene-singlet excited state lifetime is gradual and undramatic (Fig. 4) and within the temperature range of known stability for the AcChR does not show changes in slope which would indicate phase transitions. The half-life of

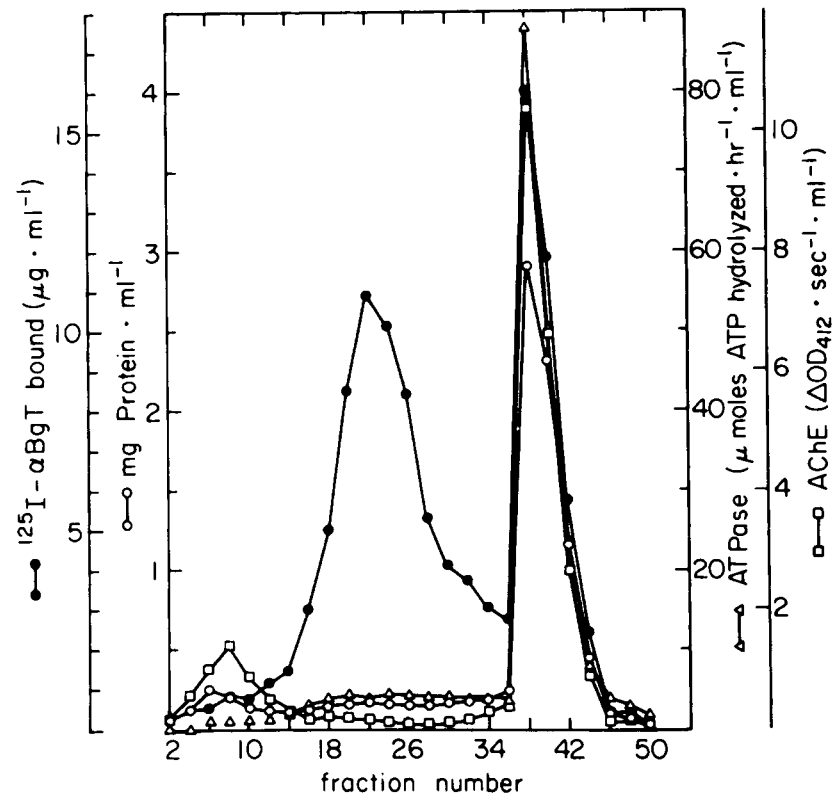

Fig. 1. Sucrose density gradient separation of AcCh esterase-rich and AcCh receptor-rich membrane fragments from T. californica electroplax tissue (for details see reference 4).

the excited state of pyrene in the AcChR-rich membranes is not altered by the presence of the cholinergic ligands at the receptor binding sites or by blocking the receptor with the α-Bgt.

Effect of Quenchers on the Fluorescence Lifetime of the Pyrene Singlet Excited State in AcChR-Rich Membranes

The exploration of possible changes in permeability of the lipid bilayer of the membranes, whether due to opening in the ionic surface or by changes in the permeability of the membrane or in the freedom of access of the diffusible pyrene to the charged surface of the bilayer is reflected in a variation in the ease of quenching of the fluorescence lifetime of the singlet excited state of pryene. Figure 5 illustrates the effect of a quencher (CH_3NO_2) producing concentration-dependent variations in the lifetime of the pyrene fluorescence. Using the quencher (Q) at concentrations much higher than that of the excited pyrene molecules, the observed rate constant for fluorescence decay k is:

$$k = \frac{\ln 2}{\tau} + k_2 [Q]$$

where τ is the fluorescence lifetime of pyrene in the absence of quencher and k_2 the second order rate constant for the quenching reaction. Calculation of the second order

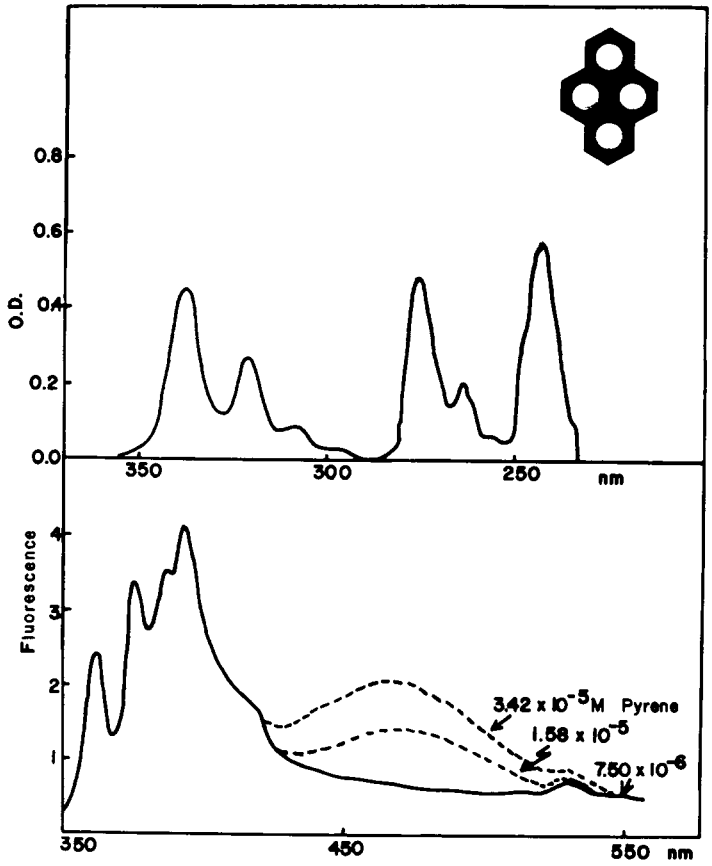

Fig. 2. Absorption and emission spectra of pyrene in cyclohexane.

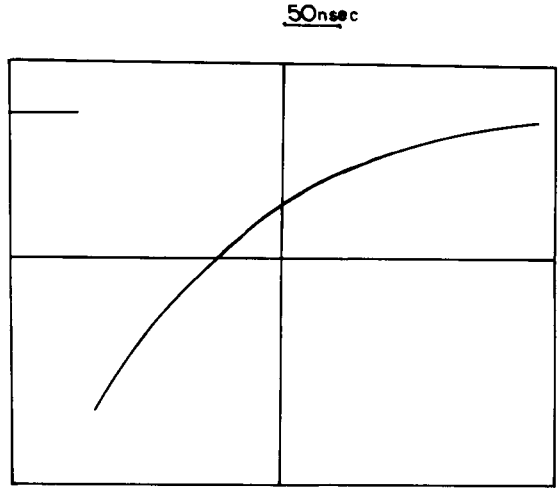

Fig. 3. The oscilloscope trace of pyrene fluorescence taken at 400 nm after excitation at 347.1 nm (for details see text).

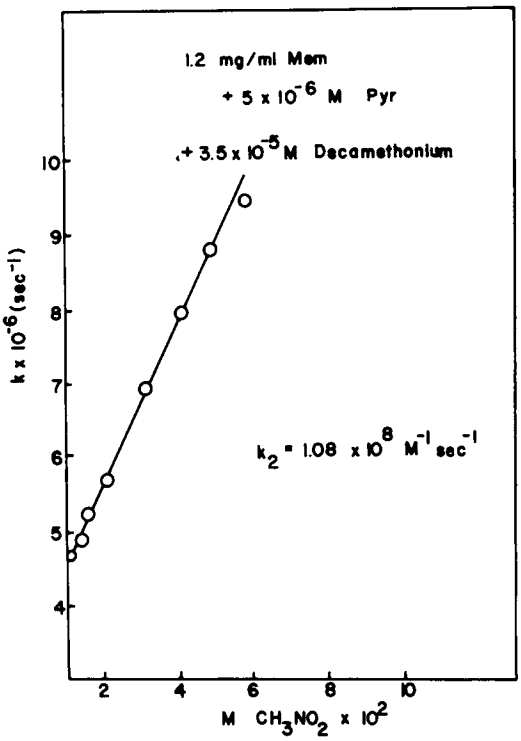

Fig. 4. Temperature dependence of lifetime of pyrene-excited singlet state in the AcChR-rich membrane vesicles.

rate constant from the rates of decay in the absence and presence of cholinergic ligands reflects any possible changes in quencher permeability due to ligand binding to the AcChR. Table I shows that none of the ligands tested nor an excess of α-Bgt produces a change in the quenching constant. Other quenchers such as I^- and O_2 produced effects similar to CH_3NO_2. A cationic quencher Tl^+ in the form of TlCl produces the same effect on the pyrene lifetime in the absence and presence of decamethonium with AcChR-rich membranes.

Effect of Anesthetics on the Fluorescence of the Singlet Excited State of Pyrene in AcChR-Rich Membranes

Local anesthetics change the order of the hydrocarbons in phospholipid bilayers (17) and decrease the Na^+ permeability in nerve cell membranes (18). When benzyl alcohol, procaine, and tetracaine were studied as possible modifiers of the fluorescence dynamics of pyrene in AcChR-rich membranes, it was observed (Table I) that a definite change in the half-life of the singlet excited state of the probe occurs. The order of effectiveness is benzyl alcohol $<$ procaine $\ll$ tetracaine. The permeability to quenchers, on the other hand, remained unaltered.

DISCUSSION

Pyrene is a probe which has been shown to enter the hydrophobic domain (13) of micelles (11), lipid bilayers (12, 14), and membranes (12, 15). It appears to diffuse

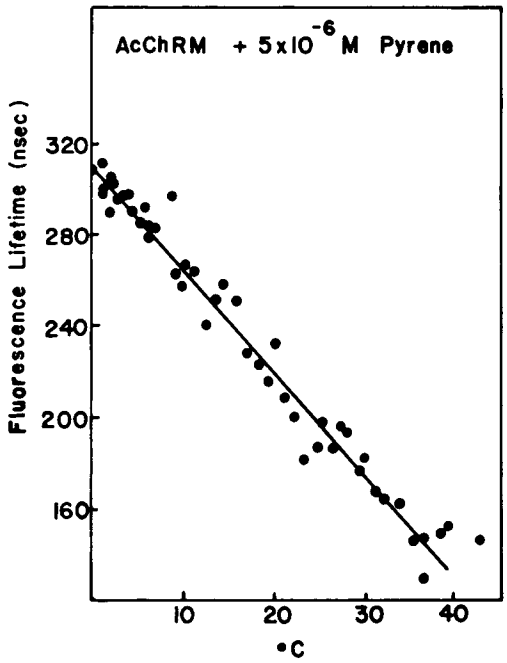

Fig. 5. Rate constant of pyrene fluorescence decay as a function of quencher concentrations.

TABLE I. Lifetimes and Rate Constant of Quenching of Pyrene (1.5×10^{-3} M) Excited State in Torpedo Californica Membrane Preparations

Ligand	CH_3NO_2 initial lifetime	$k \times 10^{-8}$
	ns	$M^{-1}\ sec^{-1}$
None	212 ± 6	1.29 ± 0.11
Carbamylcholine (10.3×10^{-4} M)	214 ± 5	1.32 ± 0.13
Decamethonium (3.5×10^{-5} M)	217 ± 6	0.97 ± 0.11
Hexamethonium (7.5×10^{-4} M)	214 ± 5	1.08 ± 0.11
α-Tubocurarine (1.04×10^{-5} M)	210 ± 6	1.18 ± 0.12
α-Bungarotoxin (4.4×10^{-8} M)	213 ± 6	0.92 ± 0.12
Benzylalcohol (100 mM)	167 ± 5	1.3 ± 0.18
Procaine (100 mM)	145 ± 4	1.16 ± 0.12
Tetracaine (0.5 mM)	124 ± 5	0.8 ± 0.15

freely in the models as well as in biological membranes. When more than one molecule of pyrene is incorporated per micelle or membrane, it tends to aggregate, forming excimers whose ease of detection by their distinctive fluorescent emission has been exploited to study the fluidity of the membrane and lipid bilayers (12, 13, 14).

In addition to lateral diffusion through the hydrophobic milieu an excited pyrene molecule can approach the hydrophilic surface of the bilayer where its interaction with the charged surface leads to fluorescence quenching, which manifests itself in a higher rate constant of fluorescence decay (16). Since the lifetime of the excited singlet state

of pyrene in AcChR-rich membranes does not change in the presence of a variety of cholinergic ligands, as well as α-Bgt, it can be inferred that lateral and surface mobility of the pyrene through the fluid lipid domain remains unaffected by occupation of the "active sites" of the AcChR in the T. californica membranes. The hydrophobic medium in itself appears to be a quite fluid continuum that is consistent with X-ray diffraction patterns of these membrane fragments where the lipids at temperatures above 2°C are above phase transition (8).

Movement of molecules across a water membrane interface can be detected by the use of quenchers. The rate at which the quencher enters the membrane and/or affects the fluorescent probe diffusing in the hydrophobic core determines the kinetics of the quenching process. Because we cannot detect changes in the kinetic behavior of pyrene or the quenching constant in the presence of ligands bound to the AcChR, we must conclude that no significant changes in permeability or fluidity of the lipids in the membrane bilayer take place upon binding of the ligands to the AcChR. That this is observed with a neutral quencher (CH_3NO_2) as well as with anionic (I^-) and cationic (Tl^+) quenchers indicates that neither the overall permeability nor permeability localized into positive or negatively charged patches of the outside surface is changing, assuming that the pyrene may have access to these regions by free diffusion through a fluid lipid domain.

The order of effectiveness of the local anesthetics as affectors of the dynamic processes of pyrene fluorescence is significant. These compounds bind to the outside of the lipid bilayer and vary in their degree of perturbation of the hydrophobic core, tetracaine being the most effective on both counts (19). Tetracaine is also the best perturber of pyrene fluorescence and it is considerably more efficient than procaine in shortening the lifetime of the singlet excited state of pyrene. Since procaine interacts primarily with the hydrophobic region of the lipid bilayer, it appears that the decreased lifetime in the presence of tetracaine is the result of an increased fluidity of the hydrophobic core resulting from the penetration of this anesthetic into the hydrophobic domain of the AcChR-rich membrane vesicles. Because the local anesthetics do not effect the quenching constant, it must also be inferred that penetration of anions, cations, or neutral quenchers through the lipid domain does not increase by binding of local anesthetics to AcChR-rich membranes of T. californica.

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