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FLUORESCENCE PROBES FOR THE STUDY OF ACETYLCHOLINE RECEPTOR FUNCTION

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INTRODUCTION

Our group initiated the use of pyrene derivatives for functional and structural studies of the membrane-bound Acetylcholine Receptor (AcChR) system. Our studies have taken two approaches. We have recently used a hydrophobic derivative (pyrene-1-sulfonyl azide) that is able to bind covalently to regions of the AcChR molecule accessible from the membrane lipid (1) to monitor ligand-induced effects at those hydrophobic regions of the AcChR protein exposed to the membrane matrix (2). The structural effects induced by ligand binding are interpreted in relation to the AcChR "desensitization" process. We have also used a hydrophilic pyrene derivative (1,3,6,8-pyrene tetra sulfonate [PTSA]) to develop a fast spectroscopic method for measuring AcChR functionality, i.e., the formation of a short-lived cation channel, within the physiological time scale. The procedure is based on the fluorescence quenching of intravesicularly entrapped PTSA by externally added Tl^+ which substitutes for the physiologically occurring Na^+. Addition of cholinergic agonist to preparations of AcChR membranes containing entrapped PTSA promotes the formation of the AcChR cation channel, which allows the added Tl^+ into the internal volume of the vesicles where collisional quenching between the cation and the entrapped fluorophore occurs. This Tl^+ influx is spectroscopically monitored as a time-dependent decrease of the fluorescence emitted by PTSA in the millisecond time scale.

In this communication, we use the latter, "stopped-flow/fluorescence quenching," procedure to examine several functional aspects of the membrane bound AcChR, including the effect of alkaline extraction of peripheral proteins from the AcChR membranes, and the effect of binding of specific anti-AcChR antibodies, which serves as a molecular model for the autoimmune disease *Myasthenia gravis*(4, 5).

Abbreviations used in this paper: AcChR, acetylcholine receptor; PTSA, 1,3,6,8-pyrene tetrasulfonate; EDTA, ethylenediaminetetracetic acid; PMSF, phenylmethanesulfonyl fluoride; α-Bgt, α-bungarotoxin; DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, 1-[4-(trimethylammonium)phenyl]-6-phenyl-1,3,5-hexatriene.

MATERIALS AND METHODS

AcChR-enriched membranes were purified for *Torpedo californica* electric tissue. The electroplax was minced and homogenized 1:2 (wt/vol) in ice-cold buffer (10 mM Tris, pH 7.4, 5 mM EDTA, 5 mM iodoacetamide, 0.5 mM PMSF and 0.02% $NaNO_3$). The homogenate was centrifuged at 3,500 rpm for 10 min in a GS-3 Sorvall rotor (DuPont Instruments-Sorvall, Newton, CT). The supernatant was filtered through cheesecloth and centrifuged at 30,000 rpm for 30 min in a Beckman 35 rotor (Beckman Instruments, Inc., Fullerton, CA). The resulting pellet, referred to as crude AcChR-membranes, was resuspended in HEPES buffer (10 mM HEPES, pH 7.4, 100 mM $NaNO_3$) at ~10 mg of protein/ml. This crude AcChR had specific activity values of 10 μg of α-Bungarotoxin (α-Bgt) bound/mg of protein. Routinely, three-fourths of that membrane preparation was submitted to alkaline extraction of the peripheral membrane proteins (5).

Protein concentration and α-Bgt binding of the membrane fractions were determined (1). The extraction, fractionation and characterization of lipids from the membranes were performed as previously described (6). Lipid vesicles from the different lipid extracts were formed through a detergent dialysis method (6). Steady-state fluorescence measurements were obtained as described (2, 6) using a SLM 4800-series subnanosecond spectrofluorometer.

Fab fragments from polyclonal anti-AcChR IgG from immunized goats suffering from experimental autoimmune *Myasthenia gravis* were prepared following published procedures (3). For experiments involving the study of the anti-AcChR Fab on AcChR function, the AcChR membranes were incubated overnight at 4°C with a 30-fold molar excess of Fab over the α-Bgt binding sites. Unbound Fab fragments were removed by centrifugation of the membranes prior to resuspension in HEPES buffer for submission to the fluorophore-loading procedure described below.

Loading of the fluorophore PTSA (final concentration 4.3 mM) into AcChR membranes or lipid vesicles was accomplished through a rapid freezing/slow thawing technique (7). The external fluorophore was removed from the fluorophore vesicles by chromatography on a Sepharose 6 MB column.

Fast kinetic measurements of Tl^+ "influx" through the membranes were obtained using a Durrum D-110 stopped-flow spectrophotometer set up in the fluorescence mode. Excitation wavelength was 378 nm and emission of fluorescence was monitored using a Corning 3-75 filter (Corning Glass Works, Corning, NY). The flux process was initiated by rapid mixing of the fluorophore-loaded vesicles (dead time was ~3–4 ms) with an equal volume of a 10 mM HEPES, pH 7.4, 30 mM $TlNO_3$), 70 mM $NaNO_3$ buffer containing various concentrations of cholinergic agonists. The spectroscopic data was stored in a Physical Data model 514A transient recorder (Physical Data, Inc., Beaverton, OR) and visualized in a Tektronix oscilloscope (Tektronix, Inc., Beaverton, OR). Permanent storage and data analysis were performed by an interfaced Apple micro-computer. The spectroscopic traces were computer-fitted to the Stern-Volmer equation describing the collisional quenching process (8).

RESULTS AND DISCUSSION

Since the development of the alkaline extraction procedure (9) to remove periphal proteins associated with electroplax AcChR membranes, most researchers have utilized the alkali treatment to increase the AcChR-specific activities of their membrane preparations. As originally described, the alkaline treatment leads to AcChR-rich membranes having normal binding capabilities for cholinergic agonists and local anesthetics and having at least a qualitative retention of the cation permeability control by the AcChR molecule (9). However, further investigations revealed that the aggregation state and the distribution of the AcChR protein within the membrane were changed, probably as a consequence of the extraction of the 43,000 M_r polypeptide associated with the AcChR membranes. Also a greater rotational mobility of the AcChR protein following alkaline extraction has been reported.

Despite the data available on the effect of alkaline extraction on the AcChR protein, very little has been learned about the effect of this treatment on the properties of the membrane matrix. Thus we have studied the effect of the alkaline treatment on (*i*) the membrane lipid composition; (*ii*) the apparent "fluidity" of the native membranes and of lipid vesicles formed from plain lipid extracts; (*iii*) the permeability of those lipid vesicles and membranes for monovalent cations.

The lipid compositional features of AcChR-membranes before and after alkaline treatment (Table I) reveals that although an almost twofold increase in the lipid/protein ratio is produced by extracting peripheral proteins, the molar ratios of phospholipid/cholesterol remain unchanged. However, the proportions of the major phospholipid components are somewhat altered, revealing what could be a "selective loss" of phosphatidyl ethanolamine during alkaline extraction. In consequence, phosphatidylcholine becomes the major phospholipid component of the alkaline treated membranes. No significant changes could be detected in the degree of unsaturation of the acyl components of either individual phospholipids or the membranes as a whole.

The selective extraction of peripheral proteins as well as the slight loss of phosphatidyl ethanolamine subsequent to alkaline extraction does not promote significant changes in the apparent "fluidity" of the membranes. This is measured by the temperature-dependent fluorescence depolarization of two fluorophores serving as probes for different membrane domains, diphenylhexatriene (DPH) and its trimethyl ammonium derivative (TMA-DPH). Fluorescence polarization values appear to indicate only a slightly less fluid environment for the alkaline membranes at the DPH domain. Even those small differences are absent when plain lipid vesicles formed from lipid extracts of crude and alkaline-treated membranes are compared, suggesting total similarity in "fluidity" of both lipid media.

Despite these similarities, the extraction of peripheral proteins appears to perturb the passive cation permeability of the membranes. We measured rate-constant values corresponding to the pseudo-first–order fluorescence decay process due to slow Tl^+ leakage through the membranes upon rapid mixing in the stopped-flow spectrophotometer. A faster leakage of Tl^+ into alkaline-treated membranes was always observed even when measurements were done at different temperatures for both crude and alkaline-treated membranes. Nevertheless, when "leak" rates were

TABLE I
COMPOSITIONAL FEATURES OF THE LIPIDS FROM AcChR MEMBRANES BEFORE AND AFTER ALKALINE EXTRACTIONS*

	Crude AcChR-Membranes	Alkaline-treated AcChR-Membranes
Lipid/protein (wt/wt)	0.86	1.40
Phospholipids/cholesterol (molar ratio)	1.11	1.04
Fatty acid composition	identical (see ref. 6)	
Phospholipid composition‡ (molar percentage)	PC, 41%; PE, 44%	PC, 45%; PE, 39%

*Averages for two different preparations.
‡PC, phosphatidyl choline; PE, phosphatidyl ethanolamine.

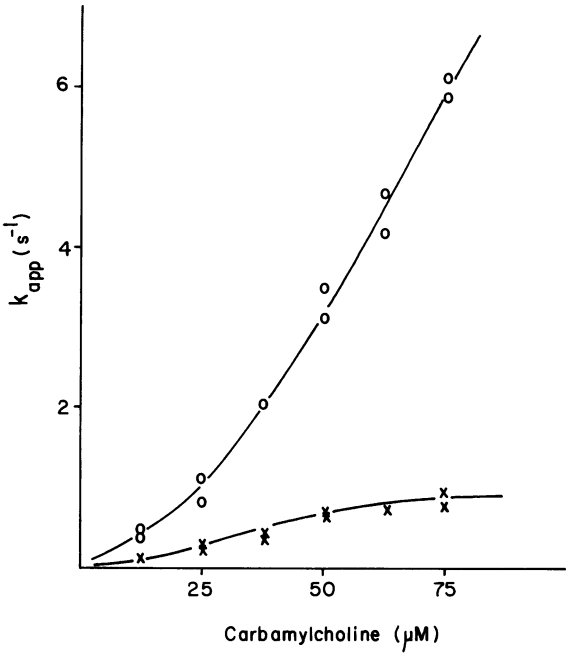

FIGURE 1 Dependence of the apparent Tl^+ flux rate on the concentration of carbamylcholine. Apparent rate constants were obtained from computer-fitting of the kinetic traces from the stopped-flow experiments to the Stern-Volmer equation. AcChR-enriched membranes with (x) or without (•, control) preincubation with anti-AcChR polyclonal Fab were used.

measured in plain lipid vesicles obtained from lipid extracts of both membrane types, there was a similarity of values for the rate constants, indicating that the observed increased permeability to cations of the alkaline treated membranes is due to a destabilizing effect of the absence of the peripheral proteins per se, and not to the perturbation of the lipid matrix.

In additional experiments we used the fast stopped-flow/fluorescence quenching technique to study the effect of binding of polyclonal anti-AcChR antibodies on the cation translocation properties of the AcChR (Fig. 1). Binding of anti-AcChR antibodies inhibits the AcChR-mediated cation flux. The specificity of the inhibition is undetermined because polyclonal Fab were used; the degree of saturation of possible selected sites in the AcChR molecule cannot be ascertained under these conditions where competition for antigenic sites on the AcChR has been shown to occur (4). Specific monoclonal antibodies will be used in the near future to achieve selectivity of binding. Meanwhile, the observed inhibition patterns apparently follow noncompetitive behavior between Fab and cholinergic ligands, in agreement with previous observations based on equilibrium binding studies (4).

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Note Added in Proof: Hill coefficients, maximum rate constants of the flux and apparent dissociation constants for cholinergic agonists were determined as described by Neubig et al. (10).

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