Journal of Supramolecular Structure 11:327–338 (1979)
Biological Recognition and Assembly 79–90

Pyrenesulfonyl Azide as a Fluorescent Label for the Study of Protein-Lipid Boundaries of Acetylcholine Receptors in Membranes

J. M. Gonzalez-Ros, P. Calvo-Fernandez, V. Šator, and M. Martinez-Carrion

Department of Biochemistry, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298

Acetylcholine receptor (AcChR) enriched membrane fragments from Torpedo californica electroplax were labeled by in situ photogenerated nitrenes from a hydrophobic fluorescent probe, pyrene-1-sulfonyl azide. Preferential photolabeling of membrane proteins, mainly AcChR, has been achieved and there is a pronounced exposure of the 48,000 and 55,000 molecular weight subunits of AcChR to the lipid environment of the membrane core.

Covalent attachment of the photogenerated fluorescence probe does not perturb the α-neurotoxins' binding properties of membrane-bound AcChR or the desensitization kinetics induced by prolonged exposures to cholinergic agonists. Non-covalent photoproducts can be conveniently removed from labeled membrane preparations by exchange into lipid vesicles prepared from electroplax membrane lipids. Fluorescence features of model pyrene sulfonyl amide derivatives, such as fine vibrational structure of emission spectra or fluorescence lifetimes, are highly sensitive to the solvent milieu. The covalently bound probe shows similar fluorescence properties in situ. PySA photoproducts have great potential to spectroscopically monitor neurotransmitter induced events on selected AcChR subunits exposed to the hydrophobic environment of membranes.

Key words: AcChR-enriched membranes, pyrenesulfonyl azide, fluorescent probes, photolabeling

Assignments of functional role(s) or topographical features of AcChR protein subunits have usually been limited to the 40,000 molecular weight subunit, which is implicated in processes involving ligand interactions with water exposed AcChR segments [1–5]. In addition, the very nature of the *affinity* labels employed in those experiments does not allow the possibility of their use in monitoring possible ligand-induced effects on the receptor protein.

Abbreviations used are: AcChR, acetylcholine receptor; PySA, pyrene-1-sulfonyl azide; α-Bgt, α-bungarotoxin; PySAH, N-(1-pyrenesulfonyl)hexadecylamine; BSA, bovine serum albumin; PMSF, phenylmethylsulfonyl fluoride; carb, carbamyl choline; SDS, sodium dodecyl sulfate.

Received April 3, 1979; accepted June 25, 1979.

0091-7419/79/1103-0327$02.30

On the other hand, an intact membrane environment plays an important role in maintaining the AcChR functional state. Minor perturbations of the membrane components produce AcChR which act as in a pharmacologically desensitized-like high affinity state for cholinergic agonists [6–9]. This lack of manipulative tools has hampered attempts to identify possible structural changes in the receptor assembly which may be implicated in the AcChR function.

We have previously reported [10] the advantages of pyrene as an adequate fluorescent probe that can be introduced, with minimum perturbation, into the lipid phase of AcChR-rich membranes. In this paper, we describe a fluorescent pyrene derivative, PySA, that shows great promise as a non-perturbing probe that can photogenerate covalent labels with the potential to monitor spectroscopically ligand-induced events at the strategic lipid-receptor boundary area.

METHODS

Torpedo californica electroplax was purchased at Pacific Biomarines Supply Co. and AcChR-enriched membrane fragments in Ca^{++} free-Ringer solution were obtained according to the procedures of Duguid and Raftery [11] and Lee et al [12].

Specific activities of the preparations (between 1 and 2 nmoles of α-Bgt binding sites/mg protein) were determined by a DEAE-cellulose filter disc assay procedure [13] using [^{125}I] α-Bgt prepared from α-Bgt purified from Bungarus multicinctus venom (Sigma Chemical Co.) [14] and iodinated by the solid phase Enzymobead (glucose-oxidase and lactoperoxidase) method (Bio Rad Laboratories). Protein concentrations were determined by the method of Lowry et al [15]. PySA and PySAH were purchased from Molecular Probes and [^{3}H] PySA was obtained by titration by the Whilzbach method (by ICN Corp.) and purified by TLC. The purified material shows no presence of impurities, as revealed by TLC using mixtures of chloroform/methanol (10:1, 5:1, and 1:1, by vol) or n-hexane/diethylether/acetic acid/methanol (60:40:1:1, by vol) as solvent systems. The specific activity of [^{3}H] PySA was 8 Ci/mole. All handling of PySA was performed in the dark and glassware covered with aluminum foil.

Cobratoxin was purified from Naja naja siamensis venom (Miami Serpentarium) [16] and used to prepare cobratoxin-sepharose affinity gel [17].

Absorption spectra were measured in a Cary model 15 spectrophotometer. Emission spectra and fluorescence lifetimes were measured at 20°C in an SLM model MC 320 subnanosecond fluorimeter after deaeration of the samples by bubbling through pure argon during 10 minutes.

Membrane Fragments Labeling Procedure

AcChR-enriched membrane fragments were suspended in Ca^{++} free-Torpedo Ringer solution (5 mM Tris buffer, pH 7.4, NaCl 262 mM, KCl 5 mM, NaN_3 0.02% and PMSF 0.1 mM) to give a final concentration of 8 mg protein/ml and transferred into a tube containing PySA-coated glass beads. The mixture was stirred until the radioactivity per ml of suspension remained constant (∼ 1 h). Final concentration of PySA in the range of 10^{-3} M. The suspension was flushed with nitrogen during 10 minutes, placed in a thermostated cell holder (20°C) and irradiated with UV light (Mineral light UVS 58, Ultraviolet Products Inc.) during 13 minutes under continuous stirring. After irradiation, the labeled AcChR membrane preparation was sedimented by centrifugation at 27,000 × g during 10 minutes and the supernatant was discarded. The pellet was resuspended twice in Ca^{++} free-Ringer buffer containing 10 mg/ml BSA and recentrifuged. BSA remaining in

the suspension was removed by washing twice with Ca^{++} free-Ringer buffer and, finally, resuspended to give a protein concentration of about 1 mg/ml. A portion of this membrane preparation was disrupted in 10 mM sodium phosphate buffer, pH 7.4, 3 mM EDTA, 0.1 mM PMSF, 0.02% NaN_3, containing 1% Triton X-100, to obtain labeled purified receptor by cobratoxin-affinity chromatography. The AcChR was displaced from the cobratoxin-sepharose 4B affinity gel by overnight incubation with 1 M carb solution in 10 mM sodium phosphate buffer, pH 7.4, containing 0.1 M NaCl, 1 mM EDTA, 0.01 mM PMSF, 0.02% NaN_3 and 0.1% Triton X-100. The specific activity of the labeled purified receptor proceeding from disrupted membranes was $1.5–2.0 \times 10^5$ cpm/mg protein. Distribution of the radioactive label on the AcChR subunits was measured by scintillation counting after SDS polyacrylamide disc gel (7.5%) electrophoresis [18] using N,N-diallyltartardiamide as polyacrylamide cross-linking agent [19] to allow for easy dissolution of gel slices (1 mm thick) in 2% periodic acid, and efficient counting. The position of protein bands in the gels was elucidated after staining with Coomassie blue. The molecular weights were determined by comparison of electrophoretic mobility with protein standards.

Time Courses of [^{125}I] α-Bgt Binding to AcChR-Enriched Membrane Fragments

Time dependence of [^{125}I] α-Bgt binding to AcChR-rich membranes was followed basically according to Quast et al [20]. In all assays the concentration of AcChR was 10^{-7} M and [^{125}I] α-Bgt concentration was 2.5×10^{-7} M. Exposures of 30 minutes to carb (10^{-6} M) prior to the addition of [^{125}I] α-Bgt were performed. Upon 40-fold dilution of carb containing sample, the rate of α-Bgt binding reverts, within a period of 15 minutes, to that observed in the absence of carb pretreatment.

Lipid Extraction and Fractionation

Total lipids from PySA-labeled or native AcChR-rich membranes were extracted by the Bligh and Dyer procedure [21]. Lipid classes were fractionated by TLC on 0.25 mm thick layers of silica gel G using n-hexane/diethylether/acetic acid/methanol (60:40:1:1, by vol) as solvent systems. Lipid spots were visualized with iodine vapors and identified by comparison with standards. Relevant gel zones were scraped off the plate and radioactivity was determined.

Removal of Non-Covalently Bound PySA Photoproducts From Labeled Membrane Preparations

Non-covalently bound photoproducts contained in labeled AcChR-rich membrane fragments were partially removed by exchange with lipid vesicles prepared from AcChR-rich membrane fragments total lipid extracts. Total lipids were previously dissolved in benzene and evaporated to dryness. Deaerated Ca^{++} free-Ringer buffer, containing 2 mM MgCl, was added to give a final proportion of 8 mg total lipids/ml Ringer and the mixture was sonicated at 20°C, under nitrogen, in a Sonifier Cell Disruptor model W 140 (Heat Systems-Ultrasonics Inc.) for 6 periods of 3 minutes. The lipid suspension was then centrifuged at 800 × g for 5 minutes to eliminate possible titanium particles from the sonicator probe. A certain volume of lipid vesicles was added to the labeled AcChR-rich membranes preparation in such a way that the ratio between lipids in the vesicles/lipids in the membranes preparation was approximately 2:1. The mixture was incubated at room temperature for 2 h, with gentle stirring, layered on top of 10–20 ml 5 mM Tris buffer, pH 7.4, containing 0.5 M sucrose and centrifuged 1 h at 35,000 × g. The pellet was resuspended in Ca^{++} free-Ringer buffer and radioactivity contents, protein concentration, and α-Bgt binding activity were assayed in this fraction and in the supernatant.

RESULTS

Properties of PySA and Derivatives

PySA is a hydrophobic fluorescence compound, soluble in organic solvents and with poor water solubility (10^{-5} M).

Irradiation of PySA with long wavelength (>300 nm) UV light in solutions containing BSA (10 mM sodium phosphate buffer, pH 7.4, 0.03% Triton X-100, 0.02% NaN_3 and BSA 3 mg/ml) produces reactive nitrenes. Photoproducts' appearance is easily followed by measuring absorbance at 347 nm, which is 4-fold increased during the process (Fig. 1). On the other hand, when PySA is incorporated into membrane fragments, absorbance changes associated to photolysis are hard to follow because of the high degree of light scattering. For this reason, the optimum irradiation time is monitored using an AcChR rich membrane fragments suspension in front of the sample containing the BSA solution to filter light in a manner similar to that when PySA incorporated into membrane fragments is directly irradiated (Fig. 1B). Under these conditions, the optimum irradiation time is about 15

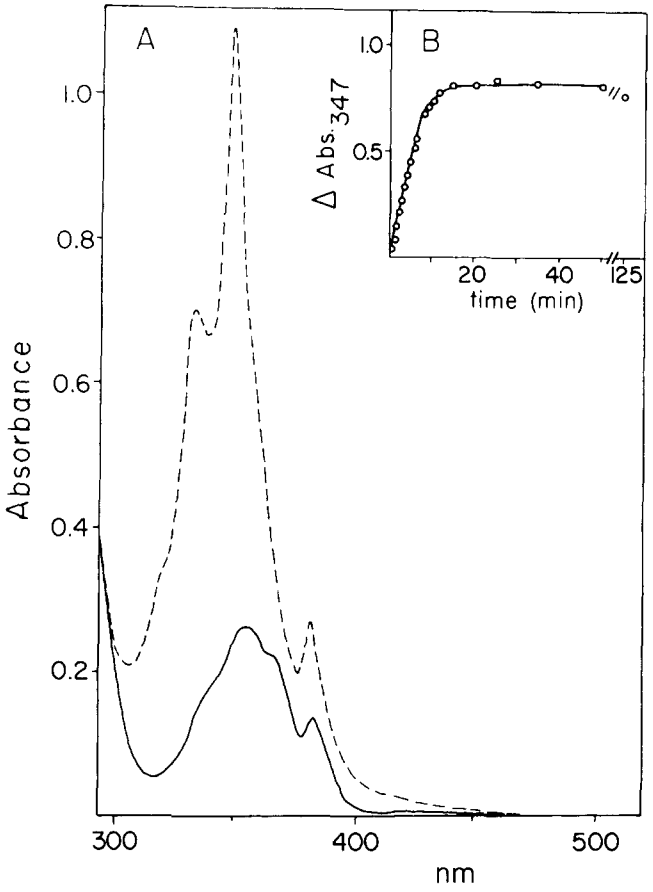

Fig. 1A) Absorption spectra of PySA before (——) and after (· · ·) irradiation. PySA (3×10^{-5} M) in 10 mM sodium phosphate buffer, pH 7.4, containing 0.03% Triton X-100, 0.02% NaN_3 and 3 mg/ml BSA, was irradiated at 20°C with long wavelength ultraviolet light. B) Time dependence of PySA photolysis when it was irradiated through a 1 pathlength quartz cuvette containing a suspension of AcChR-enriched membrane fragments (1.6 mg protein/ml). Experimental conditions as in Figure 1A.

minutes and the resulting photoproducts appear to be photostable up to 2 h of additional irradiation.

Figure 2A shows the emission fluorescence spectrum of PySA photoproducts resulting by photolysis when incorporated into AcChR-enriched membrane fragments. Upon excitation at 346 nm, the compound, probably a blend of pyrenesulfonyl amide derivatives, shows fluorescence maxima at 379, 398, and 416 nm. Fluorescence of the parent compound cannot be observed because of the rapid formation of photoproducts under the light source of the fluorimeter.

Figure 2B shows the solvent dependence of the emission fluorescence spectra of a pyrenesulfonylamide derivative, PySAH. Upon excitation at 346 (the absorption maximum of PySAH in the solvents assayed), the fluorescence maxima are at similar wavelengths to those observed for emission maxima of PySA photoproducts in the membrane preparations. On the other hand, the fluorescence emission intensities depend on the nature of the solvent environment (Figs. 2 and 3). In addition, other fluorescence parameters, such as the lifetime of the excited state, undergo dramatic changes dependent on the solvent (Table I).

PySA Labeling of the Components of AcChR-Enriched Membrane Fragments

The removal of possible non-membrane incorporated PySA photoproducts was performed by washing with Ca^{++} free-Ringer buffer or Ca^{++} free-Ringer buffer containing BSA as a scavenger. About 3–4% of the total radioactivity was present in the first Ringer buffer

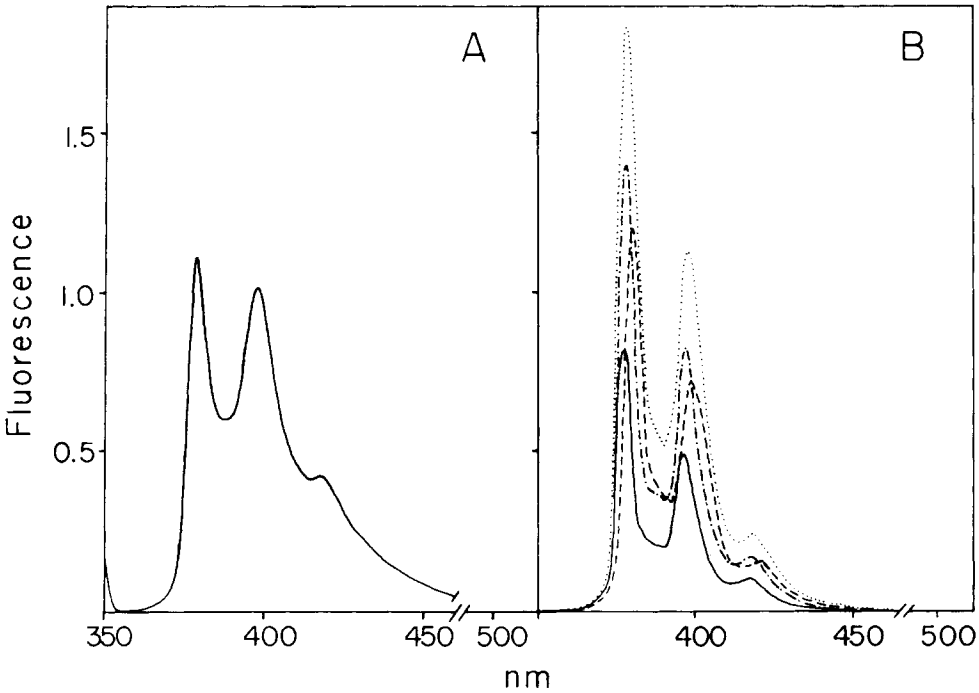

Fig. 2A) Fluorescence emission spectrum of PySA (10^6 cpm/mg protein) in AcChR-enriched membrane fragments (1.94 mg protein/ml) after irradiation with long wavelength ultraviolet light for 13 minutes. 100 μl of the membrane suspension was diluted to 1.5 ml in 5 mM Tris buffer, pH 7.4, containing 0.5 M sucrose and 0.02% NaN_3. Samples were purged with argon and fluorescence measured. Excitation at 346 nm. B) Fluorescence emission spectra of PySAH (2×10^{-6} M) in spectra grade hexane (——), chloroform (- - -), cyclohexane (----), and methanol (· · ·). Excitation at 346 nm.

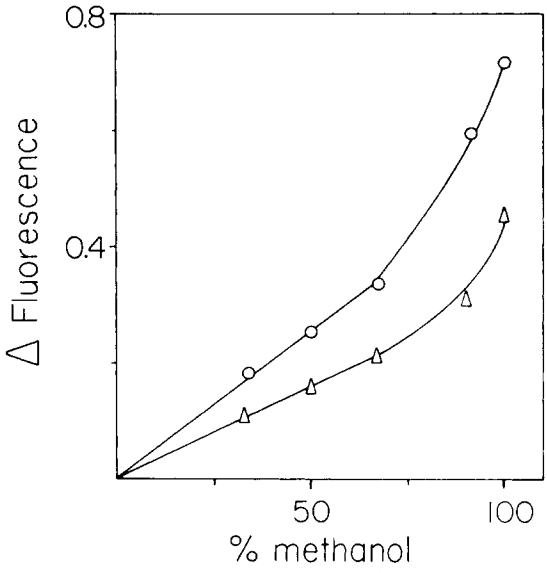

Fig. 3. Variation of fluorescence intensity of PySAH (2×10^{-6} M) at 337 (○) and 396 (△) nm in chloroform/methanol mixtures at different proportions.

TABLE I. Fluorescence Lifetimes Values of PySAH (2×10^{-6} M) in Different Solvents

	Florescence lifetime (nsec)[a]
Methanol	14.54 ± 0.03[b]
Chloroform/methanol (1:9, v/v)	14.15 ± 0.05
Chloroform/methanol (1:2, v/v)	13.21 ± 0.09
Chloroform/methanol (1:1, v/v)	12.15 ± 0.04
Chloroform/methanol (2:1, v/v)	10.91 ± 0.07
Chloroform	9.28 ± 0.06
Hexane	12.36 ± 0.09
Cyclohexane	18.84 ± 0.15

[a]Emission filters: Corning 4-96 and 3-144.
[b]Standard deviations from 4 different determinations.

supernatant and amounts no greater than 10–12% were removed in the 3 BSA washes. The extent of the removal was not altered if BSA was present during the incorporation of PySA into membranes or if the membranes were washed with Ca^{++} free-Ringer buffer containing BSA before or after the irradiation, which indicates the lack of partition of PySA or its photoproducts into water solvent and membranes.

About 90% of the membrane incorporated PySA photoproducts are extracted with the lipid components of the membrane fragments. This association, however, is by non-covalent interaction of the PySA photoproducts with the lipid core, since about 95% of these lipid-associated photoproducts are not bound to any of the lipid classes extracted. Comparison of the radioactivity distribution in TLC plates of lipid extracts from labeled AcChR-enriched membranes and a standard of PySA photolyzed in water shows that non-bound PySA photoproducts produce several spots on the TLC plates. Only a small amount

(~5%) of the total PySA photoproducts present in the lipid extracts shows any appearance of being lipid-bound PySA photoproducts as they comigrate with cholesterol esters.

About 10% of the membrane-contained PySA photoproducts are detected in the protein fraction that can be precipitated by treatment of the Triton X-100 solubilized membrane fragments with trichloroacetic acid. AcChR protein was isolated by cobratoxin-affinity chromatography from Triton X-100 solubilized labeled membrane fragments and it contains (in terms of specific activity per milligram protein) most of the protein-bound PySA photoproducts. The molar ratio of bound PySA photoproducts to AcChR under the conditions described in the Methods section is about 27 ± 7.

Figure 4 shows the distribution of PySA photolabeling on the AcChR protein subunits determined after their separation by SDS-gel electrophoresis. The majority of the label is associated with the 48,000 and 55,000 molecular weight subunits, whereas the 40,000 and 68,000 subunits show practically no PySA photoproducts attachment. Different initial concentrations of PySA were assayed (10^{-5} to 5×10^{-3} M) without any substantial modification of the qualitative labeling pattern. In addition, control experiments in which non-irradiated or previously photolyzed (in buffer) PySA was used showed no radioactivity associated with protein bands on the SDS gels; instead, the radioactivity traveled with the dye front. The electrophoretic pattern of AcChR subunits (protein stain) is not modified upon photolabeling, which indicates that no apparent loss of polypeptide material occurred as a result of cross-linking or any other kind of polymerization or hydrolysis.

Removal of Non-Covalently Bound PySA Photoproducts

Removal of non-covalently bound PySA photoproducts contained in labeled AcChR-enriched membrane preparations was attempted in order to increase the relative proportion of AcChR covalently bound label to free PySA photoproduct in the membrane. Figure 5 shows that up to 70% of the radioactivity due to the presence of non-protein-bound tritiated PySA photoproducts can be removed from the electroplax PySA-labeled membranes. Total lipid extracts from AcChR-enriched membrane fragments, instead of commercially available lipid mixtures, as well as short periods of incubation time, were used in order to avoid the possibility of lipid composition modification by exchange between the labeled membrane fragments and the "acceptor" lipid suspensions. Under these conditions (Fig. 5) the resulting "clean" membranes retain most of the initially present total amount of proteins as well as both the specific activity (in molar terms of α-Bgt binding sites) and susceptibility to desensitization by a cholinergic agonist.

Binding Properties of PySA-Labeled AcChR-Enriched Membrane Fragments

The covalent attachment of PySA photoproducts to AcChR by photolysis within AcChR-enriched membrane fragments does not affect the extent of [^{125}I] α-Bgt binding; therefore, the specific activity of the membrane preparations in molar terms of α-Bgt binding sites remains substantially unchanged.

In addition, the kinetics of transitions from low to high carb affinity states of AcChR-enriched membrane fragments [20, 22, 23] are not modified by covalent labeling with PySA nitrene. In this regard the modified membranes display a kinetic behavior similar to the well-known "desensitization" kinetics of freshly prepared (control) membrane preparations.

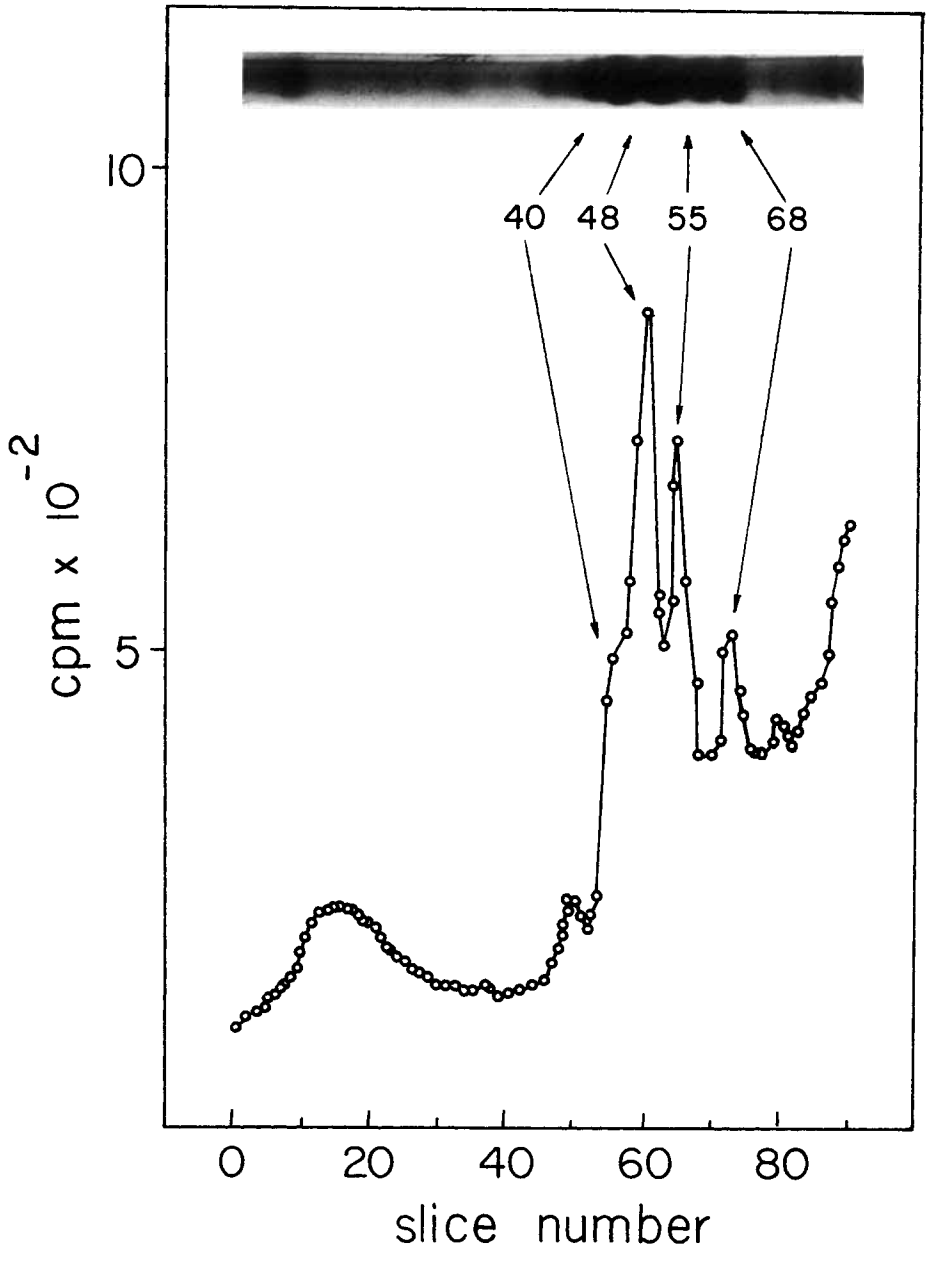

Fig. 4. Distribution of radioactivity in AcChR purified from PySA labeled AcChR-enriched membrane fragments (see Methods). The protein electrophoretic pattern is shown in the upper part of the figure. Numbers within the figure indicate molecular weight values in thousands.

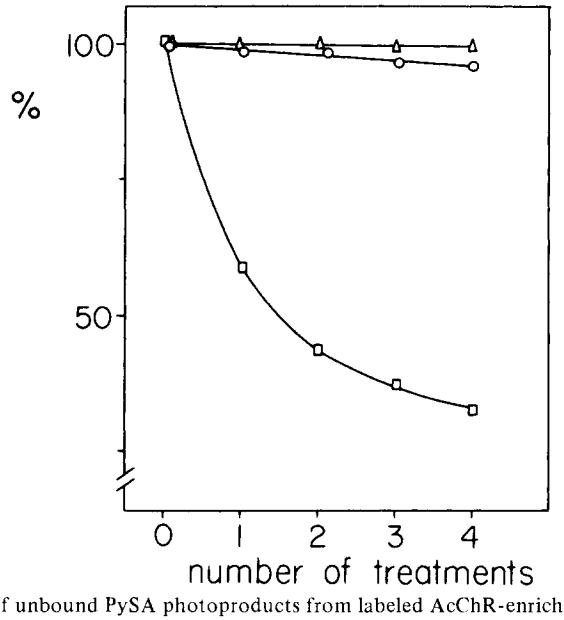

Fig. 5. Removal of unbound PySA photoproducts from labeled AcChR-enriched membrane fragments. Membranes were treated with lipid vesicles as many times as indicated in the figure (see Methods) and protein concentration (○), specific activity of the preparation in molar terms of α-Bgt binding sites per milligram of protein (△), and radioactivity contents (cpm/mg of protein) due to the presence of radioactive PySA within the membranes (□), were determined after every treatment. Data expressed as percentages of initial values.

DISCUSSION

Four different types of polypeptide chains have been previously detected in solubilized and purified AcChR and in highly enriched membrane preparations from Torpedo californica electroplax [1, 2, 24–27]. Nevertheless, the functional roles and structural arrangement of these protein subunits in the quaternary assembly of the AcChR within the membrane core is poorly understood. Only a partial assignment of the functions regarding the binding of some ligands to the 40,000 molecular weight subunit located in the external side of the membrane has been possible [3–5, 28]. Furthermore, selective iodination by the lactoperoxidase method [29] shows that the 40,000, 50,000, and 60,000 molecular weight subunits are easily iodinated from the outside membrane surface.

In our attempt to determine the portions of this AcChR macromolecule exposed to the lipid core of AcChR-enriched membrane fragments, we utilize PySA, a photogenerating hydrophobic covalent probe without specific AcChR binding sites. This compound, like pyrene [10], apparently, and almost quantitatively, partitions into the hydrocarbon regions of membrane lipids. Once incorporated into the membrane, extensive washing with excess of BSA does not remove the incorporated probe, before or after conversion of PySA into its photoproducts. In addition, tetracaine, which is known to be a membrane perturbant with great accessibility to hydrocarbon regions of membranes [30, 31], and which quenches significantly the fluorescence of pyrene incorporated into the AcChR-rich membrane fragments [10], appears to be an equally efficient quencher of fluorescence of membrane incorporated PySA photoproducts [Gonzalez-Ros, in preparation].

In the covalent binding to AcChR-enriched membrane fragments, the light-generated nitrene seems to have a preference for proteins. In general, different hydrophobic photolabels such as those generating arylnitrenes or carbenes have shown variable efficiency by labeling both protein and lipid components of different membrane components [32–36]. In our case, although only a relatively small fraction (∼10%) inserts into proteins, it is the predominant, if not the only, covalent interaction with the membrane components, a fact that can be partially explained by the peculiar composition of these membrane fragments in which protein is the main component (60%). Furthermore, it is now apparent [37] that AcChR is the major, integral protein present in AcChR membrane fragments isolated from Torpedo electroplax. The most likely interaction of water-insoluble nitrene with membrane proteins is from the lipid core, where it is presumably photogenerated. Nitrenes, produced in the lipid core in the vicinity of (or on) the protein hydrophobic segments in contact with lipids, rapidly react with protein components. Therefore, the extensive labeling of 48,000 and 55,000 molecular weight subunits in the membrane fragments should be expected if these two subunits come into contact, or expand, the lipid bilayer. These two polypeptide chains are known to be the only ones devoid of carbohydrates within the AcChR molecule [38], which probably indicates their "buried" character within the lipid bilayer. On the other hand, the absence of PySA labeling on the 40,000 subunits agrees with the results obtained by others [3–5, 28] using water-soluble (as distinct from hydrophobic) affinity labels.

A troublesome consequence of experimental tampering with electroplax membrane is the frequent final isolation of membranes in a "desensitized" state as an unfortunate consequence of handling these AcChR-rich membranes in the presence of membrane perturbants [6–9, 23]. From this work, it is apparent that not all perturbations of membrane architecture necessarily lead to loss of cholinergic ligand-induced, low to high, affinity state transitions of the receptor. As in the case of introduction of pyrene into the membrane lipid core [10], in situ, covalent labeling, with PySA, of the AcChR subunits in contact with the lipid phase does not affect the affinity state transitions either. The question of why pyrene compounds do not affect the sensitization-desensitization affinity states while other perturbations of the membrane do remains unclear.

These non-perturbing effects of PySA labeling on the activity of AcChR-bound membranes in conjunction with the ability to remove unbound photoproducts from the membrane preparations by exchange with lipid suspensions, allows us to obtain a functionally "unperturbed" receptor membrane preparation enriched in AcChR with a covalently bound probe at the protein-lipid boundary area.

Ideally, spectroscopic probes of molecular events in a protein must a) be identified with at least general regions of the protein; b) be non-perturbing as to the biological functions of the protein; and c) possess highly sensitive spectral properties which will reflect on the nature of its microenvironment. From the above discussion, it is apparent that certain hydrophobic domains of AcChR can be covalently labeled by photoproducts of PySA meeting criteria a) and b). Our studies with the model compound PySAH are a good indication that similar compounds, to be created as covalent photoproducts, should show equivalent fluorescent behaviors. The sensitivity of these compounds' fluorescence emission and lifetime of the excited singlet state to solvent polarity are very promising as to their potential for use in the monitoring of the probes' environment. Of particular interest is their potential for the detection (through physical means) of the transformation of protein-lipid boundary area in the AcChR from the sensitized to desensitized states.

Ligand binding induced conformational transition(s) of AcChR has been proposed [39]. Bonner et al [40] and Barrantes [41] monitored slight changes in intrinsic fluorescence of the receptor during its association with various agonists, and Witzemann and Raftery [4] have observed an increase of the AcChR labeling with a water-soluble probe, ethidium azide, in the presende of cholinergic ligands. Postulated conformational transitions may not only involve water-exposed regions of the receptor molecule but may also encompass the subunits in contact with the hydrocarbon core of the membrane. PySA appears advantageous as a generator of a non-perturbing covalent fluorescence tool to monitor events at this most interesting boundary.

In conclusion, the nitrene, photogenerated from the lipophilic PySA, appears to be a useful probe to determine the exposure of receptor subunits to the hydrophobic core of AcChR-enriched membrane fragments, contributing to a better understanding of the arrangement of the receptor within the membrane environment. Furthermore, because of its non-perturbing effects and fluorescence properties, PySA shows promise as a spectroscopic tool to detect events at the strategic area of receptor-lipid interfaces on AcChR-enriched membrane fragments.

REFERENCES

1. Karlin A, Weill CL, McNamee MG, Valderrama R: Cold Spring Harbor Symp Quant Biol 40:193, 1975.
2. Hucho F, Layer P, Kiefer HR, Bandini G: Proc Natl Acad Sci USA 73:2624, 1976.
3. Witzemann V, Raftery MA: Biochemistry 16:5862, 1977.
4. Witzemann V, Raftery MA: Biochemistry 17:3599, 1978.
5. Hsu HPM, Raftery MA: Biochemistry 18:1862, 1979.
6. Brisson AD, Scandella CJ, Bienvenue A, Devaux PF, Cohen JB, Changeux JP: Proc Natl Acad Sci USA 72:1087, 1975.
7. Weiland G, Georgia B, Lappi S, Chignell CF, Taylor P: J Biol Chem 252:7648, 1977.
8. Andreasen TJ, McNamee MG: Biochem Biophys Res Commun 76:958, 1977.
9. Young AP, Brown FF, Halsey MJ, Sigman DS: Proc Natl Acad Sci USA 75:4563, 1978.
10. Sator V, Thomas JK, Raftery MA, Martinez-Carrion M: Arch Biochem Biophys 192:250, 1979.
11. Duguid JR, Raftery MA: Biochemistry 12:3693, 1973.
12. Lee T, Witzemann V, Schimerlik M, Raftery MA: Arch Biochem Biophys 183:57, 1977.
13. Schmidt J, Raftery MA: Anal Biochem 52:349, 1973.
14. Clark DG, Macmurchie DD, Elliot E, Wolcott RJ, Laudel AM, Raftery MA: Biochemistry 11:1963, 1972.
15. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: J Biol Chem 193:265, 1951.
16. Ong DE, Brady RN: Biochemistry 13:2822, 1974.
17. Moore WM, Brady RN: Biochim Biophys Acta 498:331, 1977.
18. Osborn M, Weber K: J Biol Chem 244:4406, 1969.
19. Anker HS: FEBS Lett 7:293, 1970.
20. Quast V, Schimerlik M, Lee T, Witzemann V, Blanchard S, Raftery MA: Biochemistry 17:2405, 1978.
21. Bligh EG, Dyer WJ: Can J Biochem Physiol 39:911, 1959.
22. Weber M, David-Pfeuty T, Changeux JP: Proc Natl Acad Sci USA 72:3443, 1975.
23. Weiland G, Georgia B, Wee VT, Chignell CF, Taylor P: Mol Pharmacol 12:1091, 1976.
24. Chang HW, Bock E: Biochemistry 16:4513, 1977.
25. Flanagan SD, Barondes SH, Taylor P: J Biol Chem 251:858, 1976.
26. Nickel E, Potter LT: Brain Res 57:508, 1973.
27. Raftery MA, Vandlen RL, Michaelson D, Bode J, Moody T, Chao Y, Reed K, Deutsch J, Duguid J: J Supramol Struct 2:582, 1974.
28. Weill CL, MacNamee MG, Karlin A: Biochem Biophys Res Commun 61:997, 1974.

29. Hartig PR, Raftery MA: Biochem Biophys Res Commun 78:16, 1977.
30. Koblin DD, Pace WD, Wang HH: Arch Biochem Biophys 171:176, 1975.
31. Martinez-Carrion M, Thomas JK, Raftery MA, Sator V: J Supramol Struct 4:373, 1975.
32. Nieva-Gomez D, Gennis RN: Proc Natl Acad Sci USA 74:1811, 1977.
33. Bayley H, Knowles JR: Biochemistry 17:2414, 1978.
34. Bayley H, Knowles JR: Biochemistry 17:2420, 1978.
35. Klip A, Gitler C: Biochem Biophys Res Commun 60:1155, 1974.
36. Bercovici T, Gitler C: Biochemistry 17:1484, 1978.
37. Neubig RR, Krodel EK, Boyd ND, Cohen JB, Proc Natl Acad Sci USA 76:690, 1979.
38. Vandlen RL, Wu WCS, Eisenach JC, Raftery MA: Biochemistry 18:1845, 1979.
39. Nachmansohn D: "Harvey Lectures, 1953/1954." New York: Academic, 1955, vol 49, p 57.
40. Bonner R, Barrantes FJ, Lovin TM: Nature 263:429, 1976.
41. Barrantes FJ: Biochem Biophys Res Commun 72:479, 1976.