Pyrenesulfonyl Azide: A Marker of Acetylcholine Receptor Subunits in Contact with Membrane Hydrophobic Environment†

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ABSTRACT: A lipophilic photolabel, [^{3}H]pyrenesulfonyl azide, has been synthesized and employed to detect which portions of the acetylcholine receptor (AcChR) molecule may be in contact with the hydrophobic environment of *Torpedo californica* electroplax membranes. The probe preferentially partitions into the hydrophobic regions of membrane lipids or Triton X-100 micelles of detergent-solubilized membranes. When irradiated by UV light, the azide generates a nitrene compound which binds covalently, and preferentially, to membrane proteins. In Triton X-100 solubilized AcChR, the 40 000 and 48 000 molecular weight subunits are preferentially labeled, whereas in situ membrane labeling produces incorporation of the radioactive photoproduct in the 48 000 and 55 000 subunits isolated from the membrane. In both solubilized and membrane-bound receptor, the 68 000 molecular weight subunit is poorly labeled. The results suggest that in the membrane environment the 48 000 and 55 000 molecular weight subunits have a pronounced exposure to the membrane lipids, whereas the 68 000 subunit is protected from the label, possibly being partially enveloped by the other polypeptide chains from AcChR. Variations in labeling of receptor subunits in Triton X-100 and native membrane surroundings indicate that in these two environments there is an unequal accessibility of the photolabel probe to various regions of the receptor.

The nicotinic acetylcholine receptor (AcChR)[1] is a protein localized at some synaptic junctions and in the electric organ of certain fishes. AcChR isolated from *Torpedo californica* has a molecular weight of 270 000 (Martinez-Carrion et al., 1975a) and contains four different polypeptide chains with apparent molecular weights of 40 000, 50 000, 60 000, and 65 000 (Chang & Bock, 1977; Flanagan et al., 1976; Hucho et al., 1976; Karlin et al., 1975; Nickel & Potter, 1973; Raftery et al., 1976). There is increasing evidence that the 40 000 subunit is implicated in processes involving ligand interactions (Karlin et al., 1975; Hucho et al., 1976; Witzemann & Raftery, 1977, 1978; Hsu & Raftery, 1979), whereas there is very little knowledge about the functional role(s) or topography of the other subunits within the postsynaptic membrane.

Hydrophobic probes suitable for labeling membrane components, such as those containing photogenerated arylnitrenes or carbenes, have previously been designed, with mixed results. These probes appear to bind with varying efficiency to both protein and lipid components of membranes (Nieva-Gomez & Gennis, 1977; Bayley & Knowles, 1978a,b; Klip & Gitler, 1974; Bercovici & Gitler, 1978). Furthermore, very little is known about the selectivity of the photogenerated probes; arylnitrenes label both proteins and lipids (Klip & Gitler, 1974; Bercovici & Gitler, 1978), while carbenes show great promise as labels for both saturated and unsaturated fatty acids of lipid bilayers (Bayley & Knowles, 1978b). On the other hand, preparation of water-soluble photoaffinity labels for the binding sites of several receptors, including AcChR, has achieved greater success (Levy et al., 1977; Witzemann & Raftery, 1977, 1978; Bregman et al., 1978; Forbush et al., 1978).

We have previously reported (Šator et al., 1979) the advantages of pyrene as an adequate fluorescent probe that can be introduced into the lipid phase of AcChR-rich membranes. In this paper, we describe the synthesis and properties of a pyrene derivative as a photoprobe, suitable to enter the hydrophobic regions of the bilayer and, after photoactivation, be able to link to the polypeptide chains of AcChR presumably in contact with the lipid environment of the membrane fragments.

Materials and Methods

BSA, crystallized and lyophilized, was from Sigma Chemical Co., as was sodium azide; Carb and spectrograde acetone were from Aldrich Chemical Co. Protein standards for the determination of molecular weights by $NaDodSO_4$ gel electrophoresis were from Boehringer. *Torpedo californica* electroplax was purchased at Pacific Biomarines Supply Co. and pyrene-1-sulfonyl chloride was from Molecular Probes (Roseville, MN). α-Bgt was purified from *Bungarus multicinctus* venom (Sigma Chemical Co.) following published procedures (Clark et al., 1972) and the α-[^{125}I]Bgt was prepared by iodination by the solid-state lactoperoxidase method (David & Reisfield, 1974). Cobratoxin was purified from *Naja naja siamensis* venom (Miami Serpentarium) by the method of Ong & Brady (1974).

Synthesis and Purification of Radioactive PySA. To 0.4 g of pyrene-1-sulfonyl chloride dissolved in 10 mL of tetrahydrofuran, sodium azide (saturated solution in water) in 10 molar excess over pyrenesulfonyl chloride was added and the mixture stirred overnight. Subsequently, 15 mL of water was added and the precipitate was collected and treated again with sodium azide, under the same conditions, for another 6 h. The crude product was recrystallized from benzene, giving a total yield of about 80% in weight.

The recrystallized product was further purified by TLC on silica gel G, using chloroform as a solvent (R_f 0.74), and then eluted with chloroform in a sintered-glass funnel giving a final yield of 65%. Elemental analysis of the synthetic material was determined. Anal. Calcd for $C_{16}H_9N_3O_2S$: C, 63.41; H, 2.97; N, 13.87; S, 10.58. Found: C, 63.29; H, 2.95; N, 13.28; S, 10.42. IR spectra in KBr pellets show the presence of the band characteristic of sulfonyl azides (~2100 cm^{-1}). The purified material shows no presence of impurities, as revealed by TLC using chloroform and mixtures of chloroform/methanol (10:1, 5:1, and 1:1, by vol) or *n*-hexane/diethyl ether/acetic acid/

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[1] Abbreviations used: AcChR, acetylcholine receptor; BSA, bovine serum albumin; Carb, carbamoylcholine; α-Bgt, α-bungarotoxin; PySA, pyrenesulfonyl azide; TLC, thin-layer chromatography; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; $NaDodSO_4$, sodium dodecyl sulfate; DAPA, bis(3-aminopyridinium)-1,10-decane azide, ETA, ethidium azide.

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methanol (60:40:1:1, by vol) as solvent systems. In these solvents the synthetic compound comigrates with the same compound which is now commercially available (Molecular Probes).

A portion of the synthesized PySA was tritiated by the Whilzbach method (by ICN Corp.) to yield [^{3}H]PySA and purified by TLC as indicated above. After purification the radioactive material migrated as a single spot with the R_f equal to the unlabeled PySA and contained the total radioactivity applied to the TLC plate. The specific activity of the tritiated PySA was 8 Ci/mol.

All operations involving the synthesis and handling of PySA were performed in the dark with all glassware protected with heavy-duty aluminum foil. The azide was never exposed to room light.

Solubilized AcChR and AcChR-Enriched Membrane Fragment Preparations. Solubilized AcChR was isolated from *Torpedo californica* electroplax. The electric tissue was minced and homogenized at 4 °C in an equal volume of 10 mM sodium phosphate buffer (pH 7.4) containing 10^{-1} M NaCl, 10^{-3} M EDTA, 10^{-5} M $PhCH_2SO_2F$, and 0.02% NaN_3, using a Virtix homogenizer at 75% maximum speed for 5 min. The homogenate was centrifuged at 10000*g* for 20 min and the pellet was resuspended in 2 volumes of the above buffer without sodium chloride and recentrifuged. After the second centrifugation, the pellet was resuspended in the initial buffer containing 1% (v/v) Triton X-100 and stirred for 4 h, and the suspension was centrifuged at 100000*g* for 3 h. The supernatant was incubated overnight at 4 °C with cobratoxin–Sepharose 4B affinity gel. The affinity gel was prepared by adding the purified cobratoxin to the CNBr-activated Sepharose (Moore & Brady, 1977) and stirring for 20 h at 4 °C. The mixture was washed alternately with 0.1 M sodium acetate buffer, pH 4.0, and 0.1 M sodium borate buffer, pH 8.0 (both containing 1 M sodium chloride), until the coupling ligand was no longer eluted.

After incubation of the solubilized AcChR preparation with the affinity gel, the mixture was filtered through a sintered-glass funnel and washed successively with the initial sodium phosphate buffer containing 0.1% Triton X-100, the same buffer containing 1 M NaCl, and again with the buffer containing 0.1% Triton X-100. The AcChR was displaced from the cobratoxin–Sepharose 4B affinity gel by overnight incubation with 1 M Carb solution in the initial buffer, containing 0.1% Triton X-100. Recovery of the AcChR from the electric tissue was 30–40%. Preparations of AcChR used in this work had specific activities higher than 9 nmol of α-Bgt binding sites/mg of protein.

AcChR-enriched membrane fragments in Ca^{2+}-free Ringer solution were obtained by a small variation of the methods of Duguid & Raftery (1973) and Lee et al. (1977). The electric tissue was minced and homogenized (2:1, w/v) in Ca^{2+}-free Ringer solution (5 mM Tris buffer, pH 7.4, 262 mM NaCl, 5 mM KCl, 0.1 mM $PhCH_2SO_2F$, and 0.02% NaN_3). The homogenate was centrifuged at 3200*g* during 10 min and the supernatant was filtered through eight layers of cheesecloth. The filtered solution was centrifuged at 100000*g* for 1 h, the pellet resuspended in Ringer solution, and the solution recentrifuged twice. The final pellet was resuspended in Ringer solution containing 30% sucrose and layered on top of a discontinuous sucrose gradient (35, 39, and 50% in sucrose, w/v) and centrifuged in a swinging bucket Beckman rotor SW 27, at 24 000 rpm during 2.5 h. Fractions showing specific activities between 1 and 2 nmol of α-Bgt binding sites/mg of protein were used.

Protein concentrations were determined by the method of Lowry et al. (1951) and the α-[^{125}I]Bgt binding to the solubilized AcChR or AcChR-enriched membrane fragments was determined using a DEAE filter disk assay procedure (Schmidt & Raftery, 1973).

PySA Labeling Procedure. Solubilized AcChR at a concentration of 1.85 mg of protein/mL in 10 mM sodium phosphate buffer, pH 7.4, containing 0.1 mM EDTA, 0.01 mM $PhCH_2SO_2F$, 0.2 mM sodium azide, and 0.03% Triton X-100, was placed in a 5-mL quartz cuvette. PySA, dissolved in spectrograde acetone, was added to the receptor solution giving a final concentration of 2.3 mM PySA (less than 4% final acetone concentration). The suspension was flushed with nitrogen during 10 min, placed in a thermostated cell holder (20 °C) and exposed to UV light (>300 nm) from Mineral light UVS 58 (Ultraviolet Products Inc.), during 2.5 min under continuous gentle stirring. The distance between the sample cuvette and the light source was 3.5 cm.

When the suspension containing PySA and AcChR was irradiated in the presence of BSA, the concentration of BSA in the mixture was 6 mg of protein/mL; the remaining conditions were the same.

The AcChR-enriched membrane fragments to be labeled were suspended in Ca^{2+}-free Ringer solution (5 mM Tris buffer, pH 7.4, 262 mM NaCl, 5 mM KCl, 0.02% NaN_3, and 0.1 mM $PhCH_2SO_2F$) to give a final concentration of 8 mg of protein/mL and then placed into a tube containing PySA-coated glass beads. The mixture was stirred until the radioactivity per volume unit in the membrane suspension remained constant once separated from the glass beads (approximately 1 h). Nitrogen gas was bubbled through the samples during 10 min before irradiation. The final concentration of PySA in the mixture was in the range of 10^{-3} M and the exposure time to the UV light was 13 min.

The amounts of PySA incorporated into solubilized AcChR or AcChR-enriched membrane fragments, upon irradiation, were determined by measuring radioactivity (the presence of brownish yellow PySA photoproducts produce an apparent decrease of 12–20% in the radioactivity measurements by fluorescence quenching) using dioxane-based scintillation cocktail, in a Beckman SL 100 spectrometer, and by determining the absorbance at 347 nm. The latter method was used only as a rough estimate of the photoproduct concentration in membranes without loss of membrane material. Extinction coefficients of ~54 000 and ~20 000 M^{-1} cm^{-1} of the photoproducts in water solutions and membrane suspension were used. These values were calculated from radioactivity and absorbance measurements in water and membrane suspensions.

The absorption spectra of PySA and PySA photoproducts were measured in a Cary Model 15 spectrophotometer. Uncorrected emission spectra were measured in an Amin-co-Bowman spectrometer.

Removal of the Nonassociated Photolysis Products. After irradiation, the labeled, solubilized AcChR preparation was separated from unbound photolysis products by passage through a Sephacryl S-200 column (0.9 × 150 cm), equilibrated with 10 mM sodium phosphate buffer, pH 7.4, containing 0.1 mM EDTA, 0.01 mM $PhCH_2SO_2F$, 0.2 mM sodium azide, and 0.03% Triton X-100, at 4 °C (Figure 3). The specific activity of the labeled receptor was about 10^6 cpm/mg of protein.

The labeled AcChR membrane preparation was sedimented by centrifugation at 27000*g* during 30 min and the supernatant discarded. The pellet was resuspended in Ca^{2+}-free Ringer buffer containing 10 mg/mL of BSA and recentrifuged. The

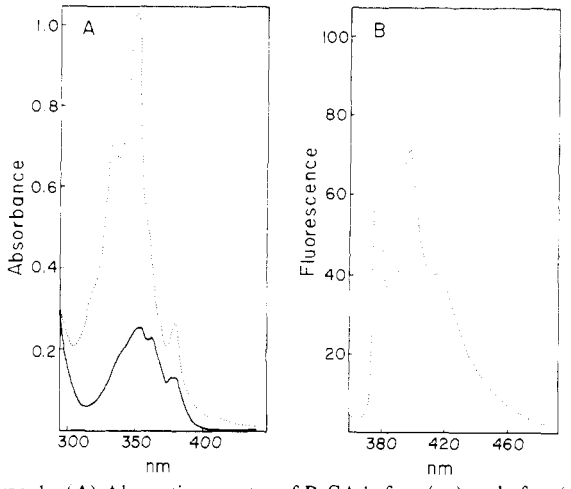

FIGURE 1: (A) Absorption spectra of PySA before (—) and after (···) irradiation. PySA (3×10^{-5} M) in 10 mM sodium phosphate buffer, pH 7.4, containing 0.03% Triton X-100, 0.02% NaN_3, and 3 mg/mL of BSA, was irradiated at 20 °C with long-wavelength ultraviolet light (see Materials and Methods). (B) Fluorescence spectrum of PySA in AcChR-enriched membrane fragments (1 mg of protein/mL) after irradiation with long-wavelength UV light for 15 min. Excitation was at 346 nm.

supernatant was discarded and the membranes were resuspended in BSA–Ringer solution. BSA remaining in the membrane preparations was removed by washing two times with Ca^{2+}-free Ringer buffer and, finally, resuspended to a protein concentration of approximately 1 mg/mL in 10 mM sodium phosphate buffer, pH 7.4, 3 mM EDTA, 0.1 mM $PhCH_2SO_2F$, 0.02% NaN_3, containing 1% Triton X-100. These disrupted membranes were used as a starting material to obtain labeled purified receptor by cobratoxin-affinity chromatography, as described above, for the isolation of solubilized AcChR. The specific activity of the labeled purified receptor proceeding from disrupted membranes was 1.5–2.0 $\times 10^5$ cmp/mg of protein.

$NaDodSO_4$ Gel Electrophoresis. Distribution of the radioactive label on the AcChR subunits was measured after $NaDodSO_4$–polyacrylamide disk gel (7.5%) electrophoresis by the method of Osborn & Weber (1969). The polyacrylamide cross-linking agent was *N,N*-diallyltartardiamide instead of bisacrylamide (Anker, 1970). This modification allows for easy disolution of the gel in 2% periodic acid (0.15 mL per 1-mm gel slice) and efficient counting of radioactivity. Gels were cut into 1-mm slices beginning from the dye front, and the radioactivity was measured after dissolution of the slices with periodic acid.

The positions of the protein bands in the gels were determined in a Photovolt recording densitometer after staining with Coomassie Blue. The molecular weights were determined by comparison of electrophoretic mobility with protein standards.

Lipid Extraction and Fractionation. Total lipid extraction from PySA-labeled membranes was carried out by the procedure of Bligh & Dyer (1959). Different lipids were obtained from aliquots of the total lipid extracts after separation by TLC on 0.25-mm thick layers of silica gel G, using *n*-hexane/diethyl ether/acetic acid/methanol (60:40:1:1, by vol) as developing system. The lipid spots were visualized with iodine vapors and identified by means of known standards. After iodine elimination, the gel fractions were scraped off the plate and transferred to scintillation glass vials containing 10 mL of Triton X-100 based scintillation cocktail and counted.

Results

Properties of PySA. Absorption spectra of PySA are shown

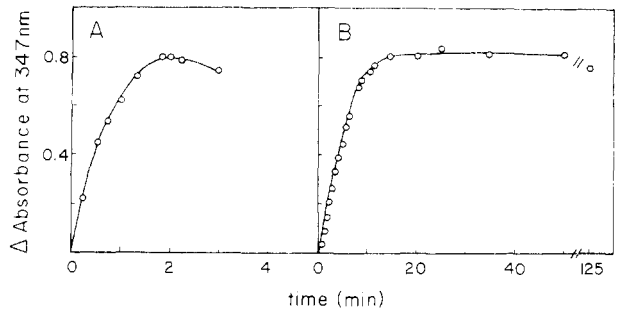

FIGURE 2: Time dependence of PySA photolysis. (A) Photolysis of PySA (3×10^{-5} M) in a solution containing 3 mg/mL of BSA and (B) the same solution irradiated through 1-cm path-length quartz cuvette containing a suspension of AcChR-enriched membrane fragments (1.6 mg of protein/mL). Experimental conditions as in Figure 1. The ordinate is expressed as a difference in the absorption of the photoproducts and the irradiated PySA at 347 nm.

in Figure 1A. Upon irradiation with long-wavelength UV light, there is a shift in absorption maxima and a fourfold increase in the absorbance of the resulting compounds. The molar extinction coefficient of PySA is 21 000 M^{-1} cm^{-1} at 355 nm (determined in benzene solution). Irradiation with UV light >300 nm allows the production of nitrene within short periods of time, thus avoiding possible damage to protein.

The fluorescence spectrum of the photoproducts of PySA in AcChR-enriched membrane fragments is given in Figure 1B. Upon excitation at 346 nm, the compound shows fluorescence maxima at 377, 396, and 416 nm. The fluorescence of the parent compound was not observed due to the rapid formation of photoproducts under the light source of the fluorimeter. A similar emission spectrum is obtained after irradiation of PySA in the presence of excess BSA.

Nonirradiated PySA is very soluble in organic solvents such as chloroform, ether, benzene, acetone, etc., showing very little solubility in water ($\sim 10^{-5}$ M). These solubility properties permit an almost complete incorporation (>96%) into the hydrophobic environment of AcChR membrane preparations.

Irradiation of PySA in Aqueous Solutions. The time necessary to obtain optimum conversion of PySA into a reactive nitrene is a function of the turbidity of the system under study. In clear solutions (containing only buffer and BSA), the conversion reaches a maximum within 2 min of exposure (Figure 2A), similar to the results obtained by Bercovici & Gitler (1978) with iodonaphthyl azide. Prolonged exposure to UV light causes a decrease of absorption at 347 nm, due to the fact that PySA photoproducts are themselves photolabile.

The optimum irradiation time, when PySA is incorporated into membrane fragments, was not possible to determine by absorption changes because of the high degree of light scattering. Changes in fluorescence were also difficult to follow because the light source of the fluorimeter caused further decomposition. For these reasons, we used an AcChR-enriched membrane fragment suspension in front of the sample containing the BSA solution to filter light in a manner similar to when PySA incorporated into membrane fragments is irradiated. The optimum irradiation time, under these conditions, was about 15 min and the resulting photoproducts seemed to be photostable over longer periods of time (Figure 2B) compared with the samples irradiated in the absence of membrane fragments as a filter.

Distribution of PySA Photoproducts into Isolated AcChR and AcChR-Enriched Membrane-Fragment Preparations. Figure 3 shows the removal of non-protein-bound PySA photoproducts from solubilized AcChR preparations. About

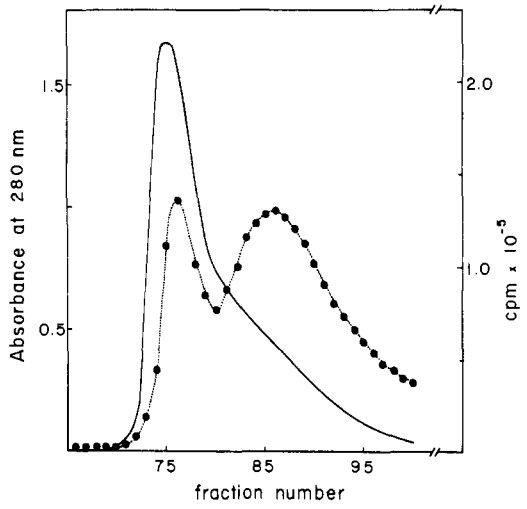

FIGURE 3: Removal of non-protein-bound PySA photolysis products from solubilized AcChR after irradiation. Gel-filtration chromatography in Sephacryl S-200 column (0.9 × 150 cm) (see Materials and Methods). Fractions of 1 mL were collected. The solid line represents the continuous monitoring of absorbance at 280 nm and the dotted line the elution pattern of radioactive photoproducts.

60–70% of the total radioactivity (PySA photoproducts) appeared to be associated with nonprotein components of the isolated AcChR solutions, presumably Triton X-100 micelles. Under the conditions, detailed in Materials and Methods, the specific activity of the PySA-labeled AcChR was about 0.12 μmol of PySA/mg of protein (10^6 cpm/mg).

When AcChR-enriched membrane fragments were irradiated in the presence of PySA, the removal of nonincorporated photoproducts was performed by several washes with Ca^{2+}-free Ringer buffer or Ca^{2+}-free Ringer buffer containing BSA (see Materials and Methods). BSA was used as a scavenger to eliminate free PySA photoproducts available in water solution. About 3–4% of the total radioactivity was present in the first Ringer buffer supernatant and a total of 10–12% was removed in the three BSA washes (depending on the concentration of PySA). The amount of radioactivity was not altered if BSA was present during the incorporation of PySA in the membrane-fragment suspension. Furthermore, the same amount of radioactivity was removed from membrane suspensions whether they were washed with Ca^{2+}-free Ringer, containing BSA, before or after the irradiation, which is an indication of the lack of partition of PySA or its photoproduct between membranes and buffer solvent after PySA is incorporated into the membrane.

The distribution of PySA photoproducts into components of AcChR-enriched membrane fragments is shown in Table I. About 92% of the incorporated PySA photoproducts are associated with the lipid components of the membrane fragments. This association is a noncovalent interaction of the PySA photoproducts with the lipid core (Table II) since about 95% of the lipid-associated photoproducts are not bound to any of the lipid classes present in the AcChR-enriched membrane fragments. These nonbound photoproducts produce several spots on the TLC plates. The small amount of lipid-bound PySA photoproducts (5% of the total amount in the lipid extracts) appears to be associated with the cholesterol ester fraction.

About 10% of the total amount of the photolyzed probe is present in the protein fraction that can be precipitated by treatment of the Triton X-100 solubilized membrane fragments with Cl_3CCOOH. Furthermore, most of the probe is associated with the AcChR protein (Table I).

Table I: Distribution of PySA Photoproducts into Components of AcChR-Enriched Membrane Fragments

	% radioactivity[a]
lipid extracts[b]	92.28
Cl_3CCOOH precipitate[c]	10.95
purified receptor from disrupted membranes[d]	9.72

[a] The radioactivity associated with the membrane components is expressed as percentages related to the specific activity of labeled membranes. [b] Lipids were extracted as described in Materials and Methods and radioactivity was determined in the organic extracts. [c] An aliquot of PySA-labeled membranes was disrupted with Triton X-100 (see Materials and Methods) and treated with 10% Cl_3CCOOH solution. The Cl_3CCOOH precipitate was Millipore-filtered and exhaustively washed and the radioactivity measured. [d] An aliquot of PySA-labeled membranes was disrupted with Triton X-100 and the AcChR was isolated by cobratoxin-affinity chromatography. Radioactivity and protein were determined as described in Materials and Methods.

Table II: Binding of PySA Photoproducts to Neutral and Polar Lipid Fractions from Labeled AcChR-Enriched Membrane Fragments

	% radioactivity[a]
neutral lipids	5.57 ± 1.00
polar lipids	0.0
nonbound photoproducts	94.43 ± 1.00

[a] Data are given in percentages of radioactivity of the total amount of PySA photoproducts present in the total lipid extracts. Total lipids were extracted and fractionated as detailed in Materials and Methods. PySA photolyzed in water was used as a standard to determine the radioactivity in each area of the plate.

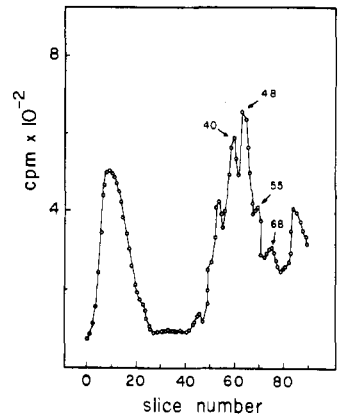

FIGURE 4: Radioactivity distribution after separation by $NaDodSO_4$ gel electrophoresis of isolated AcChR preparation labeled with photolyzed PySA (see Materials and Methods). The arrows represent the molecular weight values (in thousands) for the maximum absorbance detected for identical samples simultaneously run and scanned for protein band location after staining with Coomassie blue (see Materials and Methods). The protein distribution densitometer stains show the same resolution and distribution as in Figure 4a of Witzemann & Raftery (1978).

It is of interest that covalent labeling by the photogenerated nitrene with the receptor (both solubilized and membrane associated) does not interfere with the binding properties of the AcChR for the specific ligand α-[^{125}I]Bgt.

Photolabeling of AcChR Subunits. The distribution of the radioactive PySA photoproducts on the subunits of AcChR was determined after their separation by $NaDodSO_4$ gel electrophoresis (Figures 4 and 5). Qualitatively the distribution pattern of PySA photoproducts on the protein bands is independent of initial PySA concentration (experimental range of 10^{-5} to 5×10^{-3} M). However, both solubilized and

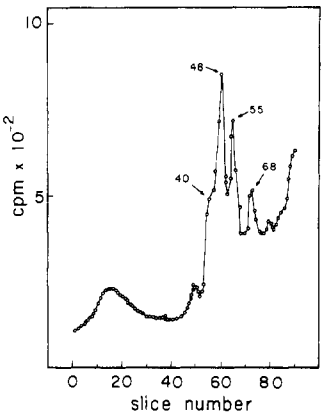

FIGURE 5: Distribution of radioactivity in PySA-labeled AcChR purified from membrane fragments enriched in AcChR. After labeling, receptor was solubilized from the membrane fragments and isolated as indicated in Materials and Methods. All the conditions are the same as in Figure 4.

membrane-bound sample solutions show increased covalent incorporation of radioactive PySA with increasing initial concentration of this compound within the concentration range tested. We have found that a faster and greater incorporation of PySA in detergent-solubilized receptor occurs when PySA is added in acetone solution instead of incorporation from PySA-coated glass beads; the latter method is preferred when handling membranes, as small concentrations of acetone perturb the properties of electroplax membranes by making them more permeable to sodium ions (data not shown). Nevertheless, the qualitative labeling patterns of solubilized receptors are identical with either of the two methods, although lower amounts of radioactivity are incorporated with the glass bead procedure. Association of the probe with the protein bands is observed after polyacrylamide electrophoresis only when PySA is irradiated in the presence of the protein samples. Control experiments, where nonirradiated or prephotolyzed (in buffer) PySA was used, showed no radioactivity associated with protein bands on the $NaDodSO_4$ gels; instead, the radioactivity traveled with the dye front.

Upon irradiation of solubilized AcChR and AcChR-enriched membrane fragments in the presence of PySA, several of the subunits of AcChR became labeled with this probe, although the patterns of the distribution of the label in both cases differ significantly. In the solubilized AcChR, most of the covalently bound PySA resides on the 40 000 and 48 000 molecular weight subunits, with very little label on the 55 000 and 68 000 (Figure 4).

In order to minimize the possibility of labeling of the receptor from the aqueous medium, we carried out the PySA labeling of the receptor in the presence of a threefold excess of BSA. Under these conditions, the extent and pattern of labeling of AcChR did not vary and less than 10% of the initial amount of the probe became associated with BSA.

When labeling was performed on AcChR-enriched membrane fragments, the distribution of the probe into the AcChR subunits (about 27 mol of PySA incorporated per mol of AcChR) was determined after solubilization of the membrane fragments with Triton X-100 and subsequent purification of the AcChR with cobratoxin-affinity column to eliminate other covalently or noncovalently associated PySA photoproducts to components of the membranes (see Materials and Methods). Figure 5 shows the pattern of radioactivity distribution under these labeling conditions. The majority of the label is associated with the 48 000 and 55 000 subunits, whereas the 40 000 subunit shows practically no incorporation.

In both, solubilized AcChR and AcChR-enriched membrane fragments, the smallest amount of radioactivity is always associated with the 68 000 subunit.

Discussion

The nicotinic AcChR is a macromolecular protein containing four different types of polypeptide chains. The polypeptides can be detected in solubilized and purified AcChR and in highly enriched membrane preparation from *Torpedo californica* electroplax (Karlin et al., 1975; Raftery et al., 1975, 1976; Hucho et al., 1976; Changeux et al., 1976). The functional roles and structural arrangement of these polypeptides within the quaternary assembly in the membrane core have not been clarified as yet. A partial assignment of the functions regarding binding of the effector ligands has been possible, and it is attributed to the polypeptide component of 40 000 molecular weight. This function has been clearly demonstrated using the cholinergic antagonist 4-(maleimido)-α-benzyltrimethylammonium bromide, a covalent label for reduced AcChR (Weil et al., 1974), bromoacetylcholine, a covalent analogue of the acetylcholine (Hsu & Raftery, 1979), and two types of photogenerated affinity labels, DAPA and ETA, azides of the parent cholinergic antagonists DAP and ethidium bromide (Witzemann & Raftery, 1977, 1978; Martinez-Carrion & Raftery, 1973; Schimerlik & Raftery, 1976). All these findings indicate that, in membranes, the 40 000 subunit has water-exposed portions bearing effector binding sites.

Hartig & Raftery (1977) have attempted to measure some topographical features of the AcChR protein regions in AcChR-enriched membrane fragments by performing selective iodination by the lactoperoxidase method. They have estimated that at least the 40 000, 50 000, and 60 000 subunits are easily accessible to iodination from the membrane surface. In our attempt to determine the portions of this AcChR macromolecule in contact with the lipid core of freshly prepared membrane fragments, we utilized PySA as a photogenerating hydrophobic covalent probe which does not show specificity for the AcChR binding sites (data not shown). This compound, like pyrene, apparently and almost quantitatively partitions into the hydrocarbon regions of membrane lipids. Once incorporated into the membrane fragments, extensive washing in the presence of excess BSA did not remove the incorporated probe, before or after conversion of the azide into its photoproducts. In addition, tetracaine, which is known to be a membrane perturbant with great accessibility to hydrocarbon regions of membranes (Koblin et al., 1975; Martinez-Carrion et al., 1975b) and which quenches significantly the fluorescence of pyrene incorporated into the AcChR-rich membrane fragments (Šator et al., 1979), appears to be an equally efficient quencher of fluorescence of membrane-incorporated PySA photoproducts (Gonzalez-Ros, in preparation). Judging from the chromatographic elution profile in Triton X-100 solutions, this probe seems to associate preferentially with micelles. Protection to PySA labeling of solubilized AcChR by the presence of an excess of BSA during the irradiation failed; indeed, the extent and the pattern of label distribution among the subunits of purified AcChR remains unchanged and less than 10% can be recovered as associated with BSA. From those results we conclude that the labeling is probably inflicted from an AcChR hydrophobic environment, not in exchange with the BSA in the medium. Since it is known that the AcChR binds a large amount of Triton X-100 (Šator et al., 1978) (45% w/w), which is an integral part of the large soluble AcChR structure (Šator et al., 1978; Hamilton et al., 1977), the receptor regions in contact with

"bound" Triton X-100 are the most likely candidates for providing an accessibility route to PySA.

It is of interest that, in the covalent binding to AcChR-enriched membrane fragments, the light-generated nitrene seems to have preference for proteins. Although only a relatively small fraction inserts into proteins, it is the predominant, if not the only, covalent interaction with membrane components (see Tables I and II). This may reflect the fact that, in these membrane fragments, protein is the main component (60%).

The most likely interaction of a water-soluble nitrene with membrane proteins is from the lipid core, where it is presumably photogenerated. Pyrene-1-nitrene in solution appears to be long-lived (Yamaoka et al., 1972). Sulfonylnitrene generated by photolysis of PySA should be even more stable than arylnitrenes and, hence, have a longer lifetime. These properties should allow pyrenesulfonylnitrene to diffuse to the surface of the membrane where the active nitrene group can become exposed to the aqueous environment and react with the solvent (water). 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. The electrophoretic pattern of AcChR subunits (protein stain), in both solubilized and membrane-bound states, is not changed upon photoaffinity labeling, which probably indicates that no apparent loss of polypeptide material occurred as a result of cross-linking or any other kind of polymerization or hydrolysis. On the other hand, there is marked contrast in the radioactivity patterns after $NaDodSO_4$–polyacrylamide electrophoresis of solubilized or in situ PySA labeled receptor. In the former, the labeled subunits are 40 000 and 48 000, whereas, in the membranes, the 48 000 and 55 000 molecular weight subunits are preferentially labeled. These differences suggest an unequal route of accessibility of the probe to various hydrophobic regions of the receptor in Triton X-100 aggregates compared with that in its native membrane environment. The extensive labeling of 48 000 and 55 000 subunits in the membranes should be expected if these two subunits come into contact with or span the lipid bilayer. The 40 000 subunit, the part of the receptor that binds specific ligands, appears to be predominantly exposed to aqueous surroundings. This interpretation is compatible with the variation in PySA labeling in solubilized preparation, compared with labeling in membrane preparations, suggesting that the 40 000 subunit is much less accessible to labeling when the attacking group arises from within the membrane bilayer. Changes in subunit labeling have also been detected by Witzemann & Raftery (1977, 1978) and Hartig & Raftery (1977) when using water-soluble (as distinct from hydrophobic) affinity labels. As a possible explanation, those authors suggested a change in the arrangement of the quaternary assembly upon solubilization of AcChR membranes. Therefore, the high degree of labeling of the 40 000 subunit in solubilized receptor preparations could be explained in terms of either of the two above-mentioned possibilities: a greater accessibility of the hydrophobic parts of the 40 000 polypeptide to the photogenerated nitrene mediated through these parts in contact with bound Triton X-100 or by the rearrangement of the subunit assembly during detergent solubilization of the membranes.

PySA labeling of the 68 000 subunit in both solubilized AcChR and AcChR-enriched membrane fragments is relatively inefficient. This subunit was not iodinated by the lactoperoxidase method (Hartig & Raftery, 1977) where the labeling of water accessible protein fragments may be favored. On the other hand, since the 68 000 subunit has been proposed as the site of phosphorylation by protein kinase (Gordon et al., 1977), it must exhibit some contact with other membrane proteins. All these observations are consistent with a model in which the 68 000 subunit is in some manner, possibly by being partially enveloped by polypeptide chains, protected from both the outside surface hydrophobic medium and the intrinsic membrane lipid components.

In conclusion, the nitrene, photogenerated from the lipophilic PySA, appears to be a useful probe to covalently label the protein subunits in proximity to the hydrophobic core of AcChR-enriched membrane fragments. This information contributes to a better understanding of the arrangement of the receptor subunits assembly in its membrane environment and opens the way for the selective introduction of spectroscopic probes at the strategic area of protein–lipid interfaces.

Acknowledgments

We wish to thank Dr. Michael A. Raftery for many helpful suggestions in the methodology of membrane preparations and Dr. Richard P. Haugland from Molecular Probes for valuable suggestions regarding the purification of pyrenesulfonyl azide.

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Purification and Characterization of a Thyrotropin-Releasing Hormone Deamidase from Rat Brain†

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ABSTRACT: This report describes the purification of a rat brain thyrotropin-releasing hormone (TRH) deamidating enzyme to apparent homogeneity. Criteria for purity include sodium dodecyl sulfate and disc gel electrophoresis, as well as isoelectric focusing (p*I* = 4.5). Enzyme purification was facilitated by development of a rapid and sensitive continuous assay using the substrate L-pyroglutamyl-N^{im}-benzylhistidyl-L-prolyl-β-naphthylamide, which, upon hydrolysis of the naphthylamide, results in the appearance of the fluorescent product, β-naphthylamine (βNA). With this substrate the homogeneous enzyme had a specific activity of 14.5 μmol of βNA min^{-1} mg^{-1}. The only peptide product formed was shown to be L-pyroglutamyl-N^{im}-benzylhistidyl-L-proline. Hydrolysis of [L-*prolyl*-2,3-^{3}H]TRH was shown to yield L-pyroglutamyl-L-histidyl-L-proline as the only radiolabeled product. Characterization of the brain deamidase by gel filtration chromatography and sodium dodecyl sulfate gel electrophoresis indicated that the enzyme consists of a single polypeptide chain having molecular weights of 70 000 and 73 500, respectively. Rat brain TRH deamidase has an apparent K_m of 34 μM, and a pH optimum between 7 and 8 using L-pyroglutamyl-N^{im}-benzylhistidyl-L-prolyl-β-naphthylamide as a substrate. With this substrate, TRH was shown to be a competitive inhibitor with an apparent K_i of 120 ± 20 μM.

Thyrotropin-releasing hormone (TRH)[1] is secreted from neurons located in the hypothalamus and travels via the portal vein to the anterior pituitary. There it interacts with receptors on the anterior pituitary to ultimately release thyrotropin, prolactin, and, under certain conditions, growth hormone (Reichlin et al., 1976). In addition to its localization in the hypothalamus, TRH has been demonstrated by bioassay and radioimmunoassay to be present in the extrahypothalamic areas of the brain (Jackson, 1978). The function of the extrahypothalamic TRH activity is unclear, although it has been suggested that it may function as a neurotransmitter (Schaeffer et al., 1977).

Several investigators have reported the presence of TRH inactivating enzymes in the hypothalamus, as well as other areas of the brain (Bauer & Kleinkauf, 1974; Taylor & Dixon, 1976; Prasad & Peterkofsky, 1976, and references therein). The role these TRH peptidases play in regulating the intra- and extracellular concentrations of the hormone is not yet understood. In an effort to begin to understand the role TRH-peptidases play in hormone regulation, this laboratory has purified to apparent homogeneity a TRH-deamidating

† From the Department of Biochemistry, Purdue University, West Lafayette, Indiana 47906. *Received September 28, 1978.* This work was supported in part by a Research Grant, AM 18849, from the National Institutes of Arthritis, Metabolism, and Digestive Diseases. This is Journal Paper No. 7351 from the Purdue University Agricultural Experiment Station, West Lafayette, IN 47906.

‡ John H. Rupnow is a postdoctoral National Institutes of Health trainee (AM 0594601).
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[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; pGlu, pyroglutamic acid; TRH, thyrotropin-releasing hormone (pGlu-His-Pro-NH_2); TRH(Bz-His)-βNA, pGlu-(N^{im}-benzyl-L-His)Pro-β-naphthylamide; EDTA, (ethylenedinitrilo)tetraacetic acid disodium salt; βNA, β-naphthylamine.

0006-2960/79/0418-1206$01.00/0