Transmembrane Topology of Acetylcholine Receptor Subunits Probed with Photoreactive Phospholipids†

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ABSTRACT: The domains of the acetylcholine receptor subunits that contact the lipid phase were investigated by hydrophobic photolabeling of receptor-rich membrane fragments prepared from *Torpedo marmorata* and *Torpedo californica* electric organs. The radioactive arylazido phospholipids used carry a photoreactive group, either at the level of the lipid polar head group (PCI) or at the tip of the aliphatic chain (PCII), and thus probe respectively the "superficial" and "deep" regions of the lipid bilayer. The four subunits of *T. marmorata* and *T. californica* acetylcholine receptor reacted with both the PCI and PCII probes and thus are all exposed to the lipid phase. Ligands known to stabilize different conformations of the acetylcholine receptor (nicotinic agonists, snake α-toxin, and noncompetitive blockers) did not cause any significant change in the labeling pattern. The acetylcholine receptor associated 43 000-dalton ν_1 protein did not react with any of the probes. A striking difference in labeling between *T. marmorata* and *T. californica* acetylcholine receptors occurred at the level of the α-subunit when the superficial PCI probe was used. An approximately 5-fold higher labeling of the α-subunit as compared to the β-, γ-, and δ-subunits was observed by using receptor-rich membranes from *T. marmorata* but not from *T. californica*. The same difference persisted after purification of the labeled receptors from the two species and was restricted to an 8000-dalton C-terminal tryptic peptide. The only mutation observed in this region of the complete α-subunit sequence of the two species is the substitution of cysteine-424 in *T. marmorata* by serine-424 in *T. californica*. The difference in labeling between the two species is tentatively assigned to this unique mutation and the transmembrane topology of the peptide segment which carries the mutation compared to current models of acetylcholine receptor tertiary structure.

The nicotinic acetylcholine receptor (AcChR)[1] from fish electric organ and vertebrate neuromuscular junction is one of the best known membrane-bound allosteric proteins. It carries the acetylcholine binding sites, contains the ion channel, and mediates the conformational transitions responsible for the regulation of ion translocation by acetylcholine [reviews in Changeux (1981), Conti-Tronconi & Raftery (1982), Karlin (1983), Stroud (1983), Lindstrom (1983), and Changeux et al. (1984)]. It is a heterologous pentamer made up of four different though homologous subunits with the stoichiometry $\alpha_2\beta\gamma\delta$ (Reynolds & Karlin, 1978). The complete primary structure of the subunits has been established by cDNA cloning and sequencing in *Torpedo californica* (α, β, γ, and δ) (Noda et al., 1982, 1983a,b; Claudio et al., 1983), *T. marmorata* (α) (Devillers-Thiéry et al., 1983), calf (α), and man (α) (Noda et al., 1983c), and models of the transmembrane organization of the mature subunits have been proposed on the basis of these sequences (Claudio et al., 1983; Noda et al., 1983b; Devillers-Thiéry et al., 1983; Finer-Moore & Stroud, 1984; Guy, 1984).

One approach to testing these models, which has already proved useful in other systems, involves identifying the domains of the molecule that interact with the lipid phase by photolabeling with hydrophobic probes. These probes contain a dark-stable photoreactive group, which can be converted by illumination to a highly reactive intermediate able to cross-link even to the poorly reactive aliphatic residues frequently found on protein surfaces exposed to lipids (Brunner, 1981; Bayley, 1983; Bisson & Montecucco, 1985a,b).

Previous experiments carried out by this method with the AcChR gave conflicting results as to the exposure of the various subunits to lipids (Sator et al., 1979; Tarrab-Hazdai et al., 1980; Middlemas & Raftery, 1983). In these studies the photoreactive group was linked to a lipophilic molecule that partitioned into the membrane phase. However, when such probes are used, absorption to hydrophobic pockets present in the protein hydrophilic domain must be taken into account, and a labeling not restricted to membrane might occur. Moreover, the localization of these small lipophilic molecules in the lipid bilayer remains uncertain since they can be excluded from areas of strong lipid interactions (Bisson & Montecucco, 1985a). For these reasons, in the present study we have used two highly radioactive phosphatidylcholine analogues that can be incorporated into the membrane like

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[1] Abbreviations: AcChR, acetylcholine receptor; PCI, 1-palmitoyl-2-(2-azido-4-nitrobenzoyl)-*sn*-glycero-3-[^{3}H]phosphocholine; PCII, 1-myristoyl-2-[12-[(2-nitro-4-azidophenyl)amino]dodecanoyl]-*sn*-glycero-3-[^{14}C]phosphocholine; MPTA, (4-maleimidophenyl)[^{3}H]trimethylammonium; $NaDodSO_4$, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; Temed, *N,N,N',N'*-tetramethylethylenediamine; ESR, electron spin resonance.

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natural lipids. The two molecules employed carry a photoreactive group at two different positions, superficial (PCI) or deep (PCII) on one fatty acid chain (Bisson & Montecucco, 1981). Added in trace amounts, these molecules introduce a minimal perturbation to the biological membrane and may serve to probe different regions of the lipid bilayer. Evidence obtained with several other biological systems has indeed demonstrated that PCI and PCII label membrane proteins via the membrane phase; such studies provide information about the depth of their penetration into the lipid bilayer (Bisson et al., 1979a,b, 1982; Montecucco et al., 1980, 1983; Hoppe et al., 1983a).

In this paper, we report data obtained with these two phospholipid probes on the membrane-bound AcChR from *T. marmorata* and *T. californica*. The species specific difference observed in the labeling patterns is interpreted on the basis of a particular mutation known to exist in the C-terminal region of their α-subunits (Devillers-Thiéry et al., 1983). The data are compared to the current models of transmembrane organization of the AcChR subunits.

Materials and Methods

1-Palmitoyl-2-(2-azido-4-nitrobenzoyl)-*sn*-glycero-3-[^{3}H]-phosphocholine (PCI) (2.8 Ci/mmol) and 1-myristoyl-2-[12-[(2-nitro-4-azidophenyl)amino]dodecanoyl]-*sn*-glycero-3-[^{14}C]phosphocholine (PCII), (174 Ci/mol) were prepared as described in Bisson & Montecucco (1981). (4-maleimidophenyl)[^{3}H]trimethylammonium was synthesized by Dr. Pichat at the CEA (Saclay). Phencyclidine was a gift of Prof. M. Sokolowsky, erabutoxin *b* of Prof. Tamiya, and trimethisoquin of Dr. G. Waksman and B. Roques. Trypsin and protease inhibitors were from Sigma, $NaDodSO_4$ was from BDH, carbamoylcholine chloride was from K & K, tissue solubilizer was from Koch Light Laboratories, and acrylamide, bis(acrylamide), and Temed were from Kodak. Live *T. marmorata* electric organs were obtained from the Biological Station of Arcachon (France), and *T. californica* frozen electric organs were from Pacific Biomarine, Venice (CA).

Membrane Preparations. AcChR-rich membranes were routinely purified from freshly dissected *T. marmorata* electric organ as described in Saitoh et al. (1980). Alkaline treatment was done as in Neubig et al. (1979). For quantitative comparisons between species, membranes were prepared in parallel from frozen electric organs from each of *T. marmorata* and *T. californica*. Acetylcholine receptor sites were assayed with [^{125}I]-α-bungarotoxin as described in Weber & Changeux (1974).

Labeling with PCI and PCII. PCI and/or PCII probes were taken from stock ethanolic solutions, dried in a glass vial, and dispersed by sonication in either 50 mM Tris-HCl, 0.5 mM EDTA, pH 7.5, or *Torpedo* Ringer buffer (250 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 3 mM $MgCl_2$, and 5 mM sodium phosphate, pH 7.0); 25 μL of this suspension were added to an approximately 100-μg protein membrane sample (1–3 μmol of α-toxin binding sites/g of protein) in 75 μL of the same buffer and incubated in the dark at 4 °C overnight. All three operations were carried out under N_2. To measure the extent of incorporation of the probes into the membrane, an aliquot of the mixture was then diluted into 500 volumes of the same buffer and centrifuged 20 min at 10000*g*. The amount of probe associated with the membrane fragments or remaining in the aqueous phase was determined by counting the radioactivity present in the pellet and supernatant, respectively. When needed, cholinergic ligands were incubated with the membranes for at least 1 h at 4 °C. Samples were illuminated in a glass vial (internal diameter 12 mm) for 10 min at 0 °C with a long-wave UV lamp (UVL-56, Ultraviolet Products, San Gabriel, CA) equipped with a glass–water filter to cut down radiations below 300 nm. The distance between the lamp and the sample was 2 cm. Illumination did not change the number of [^{125}I]-α-bungarotoxin sites. Membranes were treated with 9 volumes of cold acetone and incubated for 30 min at −20 °C. After centrifugation, the protein pellets were resuspended in electrophoresis sample buffer containing 3% β-mercaptoethanol (Laemmli, 1970).

Electrophoresis and Counting Conditions. Electrophoresis was performed as reported by Laemmli (1970) in either 10% acrylamide/0.13% bis(acrylamide) or 16% acrylamide/0.43% bis(acrylamide) gels. After Coomassie Blue staining, destaining and densitometric recording, the gels were cut into 1 mm thick slices. The slices were incubated with 200 μL of water–glycerol (2:1) for 2 h and then overnight with 400 μL of tissue solubilizer at room temperature; 4 mL of scintillation cocktail were added, and vials were counted after at least 24 h further incubation. Some gels were processed for fluorography as reported by Bonner & Laskey (1974).

Acetylcholine Receptor Purification. After labeling, the membrane sample (300 μg of protein) was centrifuged and then solubilized in 250 μL of 3% Triton X-100, 50 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA, and 3 mM NaCl, pH 7.0. The AcChR protein was purified by affinity chromatography on a bromoacetylcholine column following the microscale procedure described by Kaldany & Karlin (1983). The overall recovery of receptor as assayed by α-toxin binding was approximately 40%, a value similar to the one reported by these authors. After elution the AcChR protein was acetone precipitated and analyzed on $NaDodSO_4$ gels as described above.

Trypsin Digestion of AcChR-Rich Membranes. Labeled membranes were pelleted just after irradiation and resuspended in 0.1 M Tris-HCl, pH 7.5, and 1% Triton X-100 (2 mg/mL final protein concentration) and incubated at 25 °C with trypsin, as reported in the legend to Figure 4. Difluorophosphate was added (5 mM final concentration) to stop the reaction, and the membranes were acetone precipitated and electrophoresed as above. MPTA labeling was performed according to Sobel et al. (1977).

Results

Partition of PCI and PCII Probes into AcChR-Rich Membranes. The phospholipid probes in an ethanolic stock solution were dried, dispersed by sonication in aqueous buffer, and then added to AcChR-rich membrane fragments at final concentrations of 10–40 μM PC probe and 1 mg of protein/mL. After overnight incubation, the equilibrium distribution of the radioactive probes between the aqueous and membrane phases was measured by centrifugation. About 80% of PCI and 70% of PCII were incorporated into AcChR-rich membranes by simple incubation with the sonicated probes. Assuming a lipid/protein ratio of 0.5 (w/w) in the AcChR-rich membranes (Popot et al., 1978) and a 0.75 g/mL density for the hydrocarbon core, the concentrations of the two probes were estimated to be at least 3000 times higher in the membrane lipid bilayer than in the aqueous phase. Satisfactory partition of PCI and PCII probes into the membranes could thus be achieved without the complication of membrane reconstitution techniques (Bisson & Montecucco, 1981) or phospholipid exchange protein (Akeroyd & Wirtz, 1982).

Labeling Pattern of AcChR-Rich Membranes. Figure 1 shows the results of photolabeling experiments carried out after partition of PCI and PCII probes into *T. marmorata* AcChR-rich membrane fragments from which peripheral

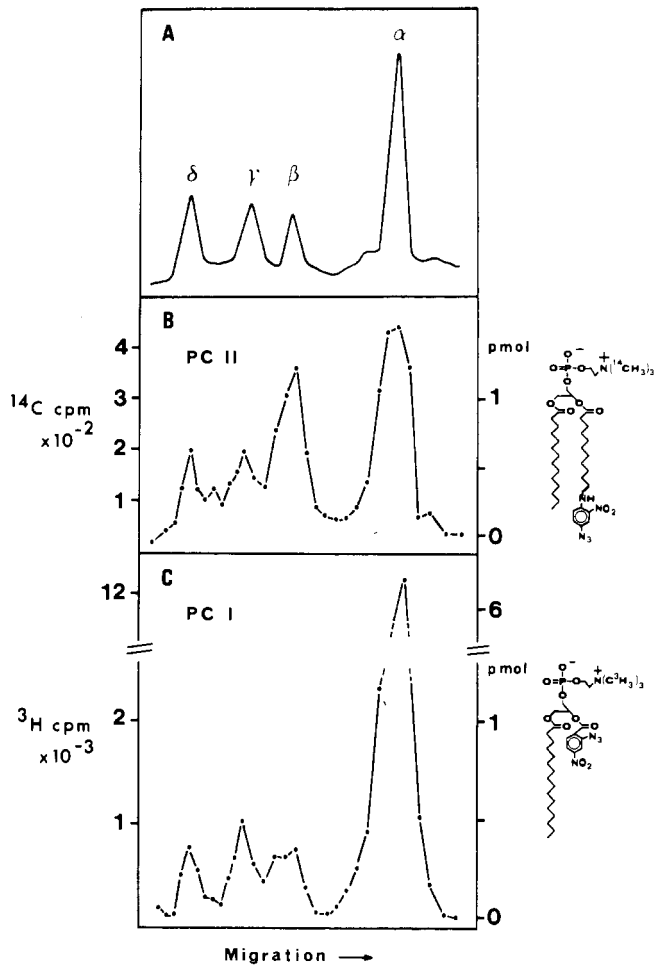

FIGURE 1: Labeling with PCI and PCII of alkaline-treated AcChR-rich membranes from *T. marmorata*. (A) Coomassie Blue staining pattern of the membrane fragments after analysis on a denaturing 10% polyacrylamide gel. Only the region of the gel containing the AcChR subunits is shown here. Corresponding patterns of labeling of *T. marmorata* membranes with 40 μM PCII (B) or 35 μM PCI (C), as determined by gel slicing and counting, are presented. Results are expressed in experimentally counted cpm (left-hand scale) and in picomoles of probe incorporated above the background level (right-hand scale). The structural formulas of the probes are shown.

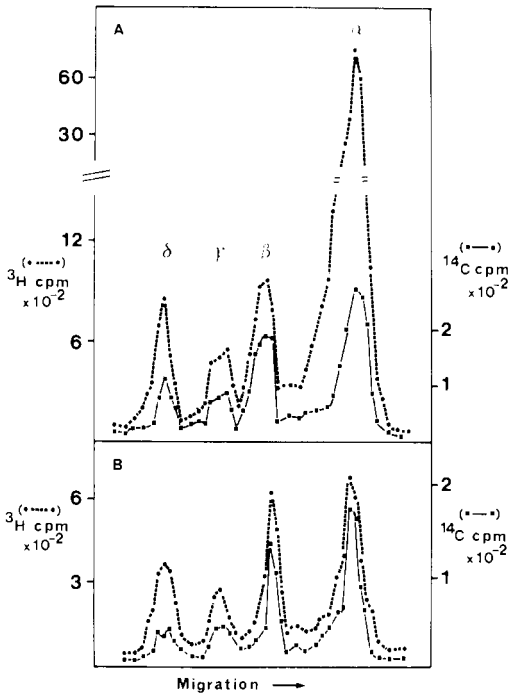

FIGURE 2: Comparison of PCI and PCII incorporation into the subunits of *T. marmorata* and *T. californica* AcChR proteins after purification by affinity chromatography on a bromoacetylcholine column. AcChR-rich membrane fragments were prepared in parallel from the two species. Samples of membranes, each containing approximately 300 μg of protein (~1.1 μmol of α-toxin binding sites/g of protein), were labeled in parallel with a mixture of 31 μM PCI and 74 μM PCII. After irradiation, the AcChR proteins from both species were purified by affinity chromatography on a bromoacetylcholine column. The purified AcChR proteins (~7 μmol of α-toxin binding sites/g of protein) were then analyzed on a 10% polyacrylamide gel. Radioactivity associated with the different subunits was measured by gel slicing and counting. The results presented here concern PCI and PCII labeling of *T. marmorata* (A) and *T. californica* (B) receptor-rich membranes. With both probes, the yield of protein labeling was slightly higher with all *T. marmorata* subunits; this difference can be attributed to the observed higher lipid/protein ratio of this *T. californica* membrane preparation.

proteins had been removed by alkaline treatment (Neubig et al., 1979). After UV irradiation, the labeled polypeptides were analyzed by NaDodSO$_4$–polyacrylamide gel electrophoresis (see Materials and Methods). All the subunits of *T. marmorata* AcChR reacted with both the "superficial" PCI (Figure 1C) and "deep" PCII (Figure 1B) probes. However, whereas the β-, γ-, and δ-subunits incorporated comparable amounts of PCI and PCII, there was about 5 times more PCI than PCII probe (in moles per gram of protein) cross-linked to *T. marmorata* α-subunit.

Interestingly, this preferential labeling of *T. marmorata* α-subunit by PCI (Figure 1C) was not observed with *T. californica* AcChR-rich membranes (data not shown). The labeling pattern of *T. californica* membranes was investigated and compared to that of *T. marmorata* by using four different membrane preparations prepared in parallel from frozen electric organs from each species. Whereas the labeling of β-, γ-, and δ-subunits was essentially the same as in *T. marmorata*, the *T. californica* α-subunit reproducibly incorporated 4–5 times less PCI probe than the *T. marmorata* one (data not shown).

The possibility was then considered that the different labeling patterns observed between the two species were due (1) to nonreceptor polypeptides comigrating with the AcChR subunits or (2) to denaturation of a fraction of *T. marmorata* AcChR which, as a consequence, would be more heavily labeled. Membrane fragments from both species were thus incubated with PCI and PCII probes and irradiated, and the AcChR protein was purified from these membrane preparations by affinity chromatography. As shown in Figure 2, whereas the PCII labeling profiles were very similar, the large difference in PCI radioactivity associated with the α-subunit as compared to β, γ, and δ became even more evident. Such species specific differences in labeling might thus result from amino acid permutations which are known to exist at least between the available sequences of the α-subunits from the two species of *Torpedo* (Devillers-Thiéry et al., 1983) (see following sections).

When native AcChR-rich membranes were used instead of alkaline-treated ones, the incorporation of PCI (Figure 3B) and PCII (data not shown) into *T. marmorata* AcChR subunits resembled that found with the alkali-stripped ones. The resolution of the radioactive peaks corresponding to the β-, γ-, and δ-subunits slightly decreased because of the presence of labeled nonreceptor polypeptides in that region of the gel. In these native membranes, the 43 000-dalton ν_1 protein was found strongly associated with the AcChR in almost equimolar amounts (Sobel et al., 1978). However, neither PCI (Figure 3B) nor PCII (data not shown) labeled this polypeptide in spite

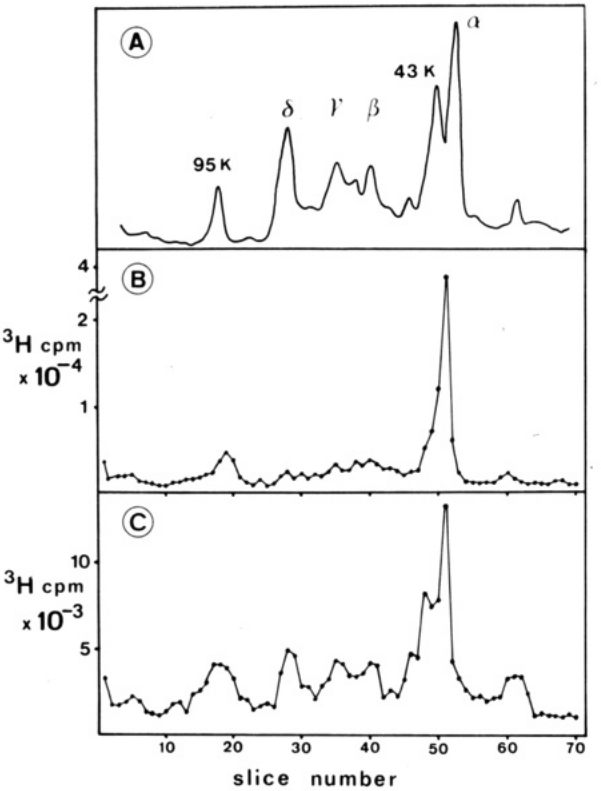

FIGURE 3: Labeling using PCI of native AcChR-rich membranes from *T. marmorata*. (A) Coomassie Blue staining pattern after electrophoresis on a denaturing 10% polyacrylamide gel. (B and C) Corresponding patterns of PCI incorporation. Note the scale difference with Figure 1C. The labeling was performed with 35 μM PCI in standard conditions (B) or 7 μM PCI in the presence of 0.2% NaDodSO$_4$ (C).

of the fact that it contains amino acid residues such as cysteines (Sobel et al., 1978) which are highly reactive with nitrenes. In the presence of NaDodSO$_4$, however, a clear incorporation of PCI (Figure 3C) and PCII (data not shown) into the ν_1 protein was observed. The absence of significant labeling of ν_1 under nondenaturing conditions in which all receptor subunits react with the lipid probes is consistent with the view that ν_1 is a peripheral protein (Neubig et al., 1979) that may not have a direct access to the lipid bilayer [see, however, Porter & Froehner (1985)].

Finally, it was observed that both probes were incorporated into the 95K polypeptide chain which is usually interpreted as representing the membrane spanning α-subunit of Na^+,K^+-ATPase.

Labeling in the Presence of Pharmacological Effectors of the AcChR. Membrane fragments were also labeled in the presence of various pharmacological ligands known to affect AcChR conformational and functional states. The agonist carbamoylcholine (1 mM), the competitive blocker erabutoxin *b* (50 μM), and the noncompetitive blocker phencyclidine (0.1 mM) did not modify the labeling either by PCI or by PCII. Increasing concentrations of the noncompetitive blocker trimethisoquin (from 25 μM to 2.5 mM in the presence of 1 mM carbamoylcholine) decreased the amount of PCI probe covalently bound to the receptor subunits. However, in the range of concentrations explored, the amplitude of the observed effect was proportional to the amount of trimethisoquin added and most likely resulted from quenching by the aromatic ring of trimethisoquin.

Erabutoxin *b* could easily be resolved on 16% acrylamide gels, but, even when present in saturable amounts during the photolabeling experiment, never appeared labeled by either

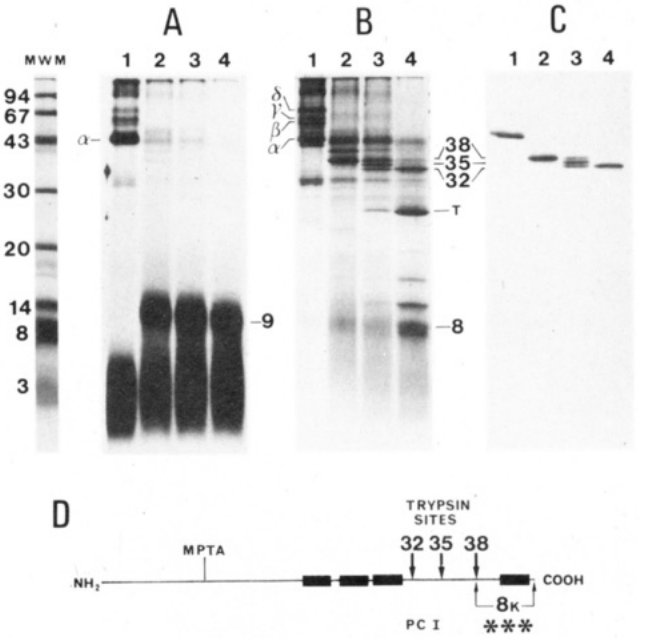

FIGURE 4: Controlled trypsin digestion of AcChR-rich membranes from *T. marmorata*. Membrane samples were solubilized in 1% Triton X-100 (2 mg/mL final protein concentration) and then incubated at 25 °C with increasing concentrations of trypsin: 0 mg/mL (lanes 1), 0.045 mg/mL (lanes 2), 0.45 mg/mL (lanes 3) for 15 min, and 5 mg/mL (lanes 4) for 30 min. After acetone precipitation the samples were electrophoresed on a 16% acrylamide gel. (B) Coomassie Blue staining pattern and (A) fluorogram obtained with PCI labeled membranes. The radioactivity present at the bottom of all lanes of panel A is due to remaining traces of PCI not associated with protein that had not been eliminated by acetone precipitation. (C) Fluorogram obtained in a parallel experiment with MPTA-labeled membranes. Apparent molecular weights are indicated in kilodaltons, and T indicates the position of trypsin. (D) Schematic drawing of the AcChR α-subunit. Dark boxes indicate the four hydrophobic stretches. The apparent molecular weights of trypsin fragments, defined by arrows, are given in kilodaltons. Asterisks symbolize the location of preferential PCI incorporation in *T. marmorata* α-subunit.

PCI or PCII. This suggests that neither the free nor the AcChR-bound toxin is exposed to lipids.

Identification of the Peptide Preferentially Labeled by PCI in the AcChR α-Subunit from T. marmorata. Limited trypsin proteolysis of AcChR-rich membranes in the presence of Triton X-100 was used to determine which portion of the α-subunit is responsible for its preferential labeling by PCI in *T. marmorata* AcChR. The same trypsin treatment was performed in parallel on AcChR-rich membranes previously labeled with PCI or MPTA, an affinity-labeling reagent of the acetylcholine binding site (Reiter et al., 1972). As shown in Figure 4B, all the AcChR subunits were progressively degraded by increasing concentrations of trypsin, and an identical protein pattern was observed with the two membrane samples (data not shown). The photolabeling conditions used with PCI thus did not modify the susceptibility of the AcChR to trypsin.

Peptides containing the N-terminal portion of the α-chain were easily identified in digests of the [^{3}H]MPTA-labeled membranes (Figure 4C). At low trypsin concentration (lane 2) only the cleavage site closest to the C-terminal end of the chain is known to be attacked (Wennogle & Changeux, 1980), thus generating a labeled 38 000-dalton fragment. In intermediate conditions (lane 3) partial cleavage occurred at two other sites, and finally (lane 4) the resistant N-terminal 32 000-dalton fragment (Wennogle & Changeux, 1980) was obtained.

By use of PCI-labeled membranes, the β-, γ-, and δ-subunits (and nonreceptor proteins) contributed only faint bands to the

overall pattern of radioactivity, allowing the behavior of heavily labeled peptides from the α-subunit to be followed on these gels. As shown in Figure 4A, the vast majority of the PCI radioactivity cross-linked to the α-chain in *T. marmorata* migrated, after trypsin treatment, as a band of apparent M_r 9000. In agreement with previous observations (Bisson et al., 1979b; Hoppe et al., 1983a; Montecucco et al., 1983), peptides cross-linked to a PCI molecule had on $NaDodSO_4$ gels an apparent molecular weight about 1000 higher than their nonlabeled counterpart. The radioactive 9000-dalton band thus most likely corresponds to the 8000-dalton peptide band observed in Figure 4B. This small labeled fragment was generated even at the lowest trypsin concentration (Figure 4A, lane 2). It therefore originates from the most C-terminal portion of the α-subunit (see Figure 4D).

We then considered the possibility that the large difference of PCI labeling observed between *T. marmorata* and *T. californica* α-subunits resulted from the different reactivity of amino acid residues exposed to the lipid bilayer. Interestingly, inspection of the complete sequence shows that in the region corresponding to the C-terminal 8000-dalton fragment a single amino acid difference exists between the cDNA sequences of *T. marmorata* and *T. californica* α-subunits (Devillers-Thiéry et al., 1983). Cysteine-424 is substituted by a serine in *T. californica*. This mutation, located in the fourth hydrophobic segment of the mature α-chain, is sufficient to explain the different labeling patterns observed. Indeed, the lateral SH group of cysteine has been shown to be by far the most reactive amino acid side chain, acting as a sort of trap for the photogenerated nitrene (Staros et al., 1978; Montecucco et al., 1983; Hoppe et al., 1983a). These results thus are consistent with the proposal that the fourth hydrophobic stretch of the α-subunit is exposed to the lipid bilayer (Devillers-Thiéry et al., 1983; Noda et al., 1983b) and that residue 424 occurs at the level of the polar head groups of the lipids where the photoreactive group of PCI probe is localized.

Discussion

Convergent pieces of evidence from selective proteolysis (Wennogle & Changeux, 1980; Strader & Raftery, 1980), radioiodination (Hartig & Raftery, 1979), or antibody-labeling experiments (Froehner, 1981) indicate that, in agreement with its function as an ion channel, the AcChR protein spans the postsynaptic membrane and that the same conclusion holds for each of its four subunits. Attempts to identify the domains of the protein that interact with the membrane lipid phase have been made by using hydrophobic probes carrying photogenerated nitrenes or carbenes that are able to bind covalently even to the poorly reactive aliphatic amino acid side chains of the transmembrane segments. The two phosphatidylcholine analogues used in the present study carry the photoreactive group either at the level of the polar head group (superficial probe PCI) or at the fatty acid methyl terminus of the aliphatic chain (deep probe PCII) and thus are able to probe different levels of the lipid–protein boundary of the receptor (Bisson & Montecucco, 1981). Both of them label all four subunits of the receptor in the two species of *Torpedo* studied, as demonstrated by analysis of the labeling profiles of AcChR-rich membrane fragments and affinity-purified AcChR previously labeled in its membrane environment.

As commonly found in hydrophobic photolabeling experiments (Bisson & Montecucco, 1985a,b), the yield of protein labeling with PCI and PCII was low because of the competing reactions of the photoactivated intermediates with lipids and with themselves. However, the photolabeled AcChR bound to, and was eluted from, the bromoacetylcholine affinity column, without alteration of the radioactive profile. The labeled protein thus did not represent a denatured subfraction of the AcChR pool, and the covalent cross-linking of the PC probes did not cause a significant loss of acetylcholine binding capacity.

In previous studies with *T.californica* receptor, only the α-subunit was found to be labeled by a lipophilic probe, [^{125}I]iodonaphthyl azide (Tarrab-Hazdai et al., 1980), or, alternatively, only the β- and γ-subunits by a pyrenesulfonyl azide (Sator et al., 1979). On the other hand, in agreement with the present findings, Middlemas & Raftery (1983) found the four subunits of *T. californica* AcChR to be labeled by a small lipophilic reagent, adamantanediazirine. The above differences might originate from the low reactivity of the naphthalene nitrene (Hoppe et al., 1983b) or from a restricted access of the large pyrene ring.

The consistent labeling of the four subunits found in at least two studies is supported by the information given by their complete sequence. The four subunits display striking sequence homologies (up to 60% identity) and similar clustering of hydrophilic and hydrophobic amino acids along the chains, suggesting that all of them possess a similar tertiary structure (Noda et al., 1983b). In particular, the four subunits disclose four highly hydrophobic segments about 20 amino acid long, interpreted as representing transmembrane α-helices. The proposal has been made that the pentameric AcChR oligomer is organized in a quasi-symmetrical manner with a 5-fold axis of pseudo-symmetry perpendicular to the plane of the membrane, the five subunits being arranged like staves in a barrel (Changeux, 1981; Conti-Tronconi & Raftery, 1982; Karlin, 1983; Stroud, 1983). In this model the subunits are all exposed to the lipid phase, and our results using the lipid probes support this view.

Previous studies by ESR spectroscopy suggested the possibility that the acetylcholine and α-toxin binding sites lie close to the lipid bilayer (Bienvenue et al., 1977). On the other hand, recent X-ray diffraction data indicate a more apical localization of the bound α-toxin toward the synaptic cleft (Fairclough et al., 1983). The observation that erabutoxin *b* was not labeled by the photoreactive phospholipid analogues, in particular the "superficial" probe PCI, indicates that its binding site is located above or, at the most, at the polar head-group level.

Cholinergic ligands that elicit conformational transitions of the membrane-bound receptor have been shown to modify the fluorescence of the hydrophobic probe pyrenesulfonyl azide previously cross-linked to the AcChR (Gonzalez-Ros et al., 1983). With both PCI and PCII no significant change in the pattern of labeling was noticed, suggesting that these conformational rearrangements do not involve major reorientation of the residues interacting with lipids such as shielding or exposure of large surfaces which might have been detected in the present study.

In the native membrane the AcChR-associated 43 000-dalton ν_1 protein was not labeled even by the superficial PCI probe. A short stretch of poorly reactive residues embedded in the membrane might, however, not have been detected by hydrophobic photolabeling. Nevertheless, this result supports the view that the ν_1 protein, which faces the cytoplasm (Wennogle & Changeux, 1980; St John et al., 1982; Nghiem et al., 1983; Sealock et al., 1984), is bound to the outside surface of the cytoplasmic membrane. In the native membrane, the ν_1 protein can be strongly labeled with iodoacetic acid (Sobel et al., 1978). Several free SH groups from this protein are thus exposed to water. The lack of labeling of these

residues or of large amounts of free erabutoxin when present, despite the incomplete partition of the PC probes into the membrane, provides internal controls that the photoreactive phosphatidylcholines used here label the receptor protein only via the membrane phase.

Detailed models of the transmembrane organization of the *Torpedo* receptor subunits have been proposed on the basis of their complete amino acid sequence (Claudio et al., 1983; Devillers-Thiéry et al., 1983; Noda et al., 1983b; Finer-Moore & Stroud, 1984; Guy, 1984). A common feature of these models is the assignment of four hydrophobic segments, in each subunits, assumed to make α-helices that traverse the 45 Å thick lipid bilayer. Some authors (Finer-Moore & Stroud, 1984; Guy, 1984) have suggested that additional transmembrane segments contribute to the formation of the ion channel. One way of testing these models is to label specifically the lipid-exposed protein segments with probes such as PCI or PCII (Brunner, 1981; Bayley, 1983; Bisson & Montecucco 1985a,b) and then to identify these peptides by biochemical methods.

In the present study, as a first step, we have localized one of the PCI cross-linking sites in the α-subunit sequence without using peptide purification methods. We took advantage of the few amino acid differences noticed between the AcChR α-subunit sequences of the *Torpedo* species: *T. marmorata* and *T. californica*. Eleven mutations occur in the cDNA coding sequence but only six of them introduce changes in the amino acid sequence (Devillers-Thiéry et al., 1983). This number is sufficiently small to utilize them as intrinsic probes for the functional domains of the receptor molecule that show characteristic species-specific differences. Interestingly, analysis of the labeling profiles of the AcChR proteins after affinity purification revealed that incorporation of the PCI "superficial" probe into the α-subunit was much larger in *T. marmorata* than in *T. californica* while the PCII-labeling patterns were similar. Controlled trypsin digestion indicates that the vast majority of the PCI label cross-linked to the *T. marmorata* α-subunit was actually incorporated into the 8000-dalton C-terminal fragment. The exact borders of this 8000-dalton fragment are not known, but analysis of the trypsin degradation pattern and of the peptides apparent molecular weights suggests that its N-terminal end is located at least C-terminal from α-subunit residue 350. The corresponding fragments in *T. marmorata* and *T. californica* α-subunits have an identical primary structure except at position 424 where a serine in *T. californica* is replaced by a cysteine in *T. marmorata* (Devillers-Thiéry et al., 1983). Since cysteine residues react much more efficiently than serines with nitrenes (Staros et al., 1978; Montecucco et al., 1983; Hoppe et al., 1983a), the striking difference in the PCI-labeling pattern between the two species can be assigned to this particular mutation. The same large difference of PCI incorporation is observed between *T. marmorata* α-subunit and *T. californica* β-, γ-, and δ-subunits. Alignment of the known sequences of the four *T. californica* subunits (Noda et al., 1983b) reveals that none of them contain a cysteine at that amino acid position. It can thus be concluded that in *T. marmorata* α-subunit, the majority of PCI is cross-linked to Cys-424. The fourth hydrophobic stretch to which it belongs is thus most likely embedded in the membrane and exposed to lipids.

Other sites of the α-subunit are labeled by the deep and superficial probes but none, however, with the same efficiency as is *T. marmorata* Cys-424 by PCI. No other cysteine residue of the α-subunit seems therefore to be exposed to the PC probes. In particular Cys-412 and Cys-418, which are present

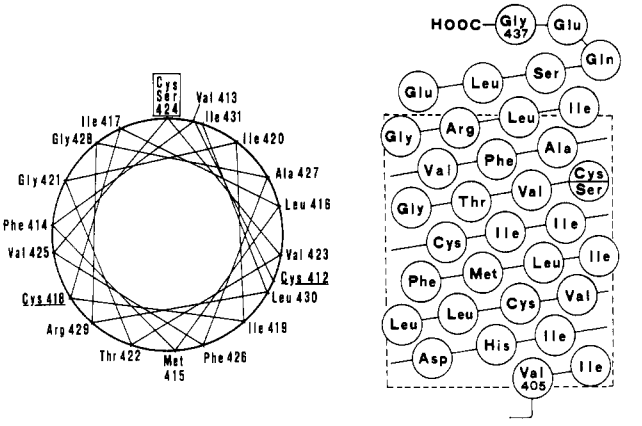

FIGURE 5: Current model of the C-terminal portion of the AcChR α-subunit in the membrane. Axial (left panel) and vertical (right panel) projection of the C-terminal part of the α-subunit arranged in an α-helical structure. At position 424, the cysteine in *T. marmorata* is replaced by a serine in *T. californica*. This model is independent of the orientation of the α-subunit COOH terminus toward the cytoplasmic vs. synaptic side of the membrane.

in the fourth hydrophobic stretch of the α-subunit from both *Torpedo* species, most likely do not face the lipid phase and are possibly involved in protein–protein contacts within the α-subunit and/or with other subunits. In the case of Cys-418, this last assumption is supported by the fact that this residue is conserved in all the species so far investigated (*Torpedo*, calf, and human) (Noda et al., 1983c) as opposed to Cys-412 and -424. As shown in Figure 5, an α-helical folding of the fourth hydrophobic segment will place cysteines-412 and -418 on different sides of the helix from the lipid-exposed Cys-424. The difference in labeling efficiency of the α-subunits was observed by using the superficial probe PCI but not the deep one PCII, indicating that Cys-424 is not significantly labeled by PCII and also that at variance with other systems (Brunner & Richards, 1980) no "kinking" of the aliphatic chain of that probe occurs in these membranes. In the α-helical arrangement shown in Figure 5, residue 424 has been positioned near the membrane surface at a position easily accessible to the superficial reactive group of PCI but not to that of PCII, which is more deeply buried in the bilayer. This α-helical arrangement, postulated in all models, is thus consistent with the experimental data reported here.

Popot & Changeux (1984) have listed in detail a number of published observations that tend to suggest that the fourth transmembrane helix is located at the periphery of the AcChR molecule and thus directly contacts the lipid phase. Furthermore, on the basis of the chemical properties of the amino acids present, these authors delimited a highly hydrophobic surface on this helix which according to them should interact with the lipid phase. Interestingly, this surface contains residues 424 but not 412 or 418 in the case of α-subunit. The present findings thus bring support to their suggestion.

Cys-222 is present in the first hydrophobic segment of AcChR α-subunit from all species investigated (Noda et al., 1983c). The absence of selective labeling of this residue by PCI or PCII indicates that if it is indeed part of a transmembrane segment, it is not exposed to lipids, as with Cys-418.

Further work is needed to localize the other cross-linking sites of PC probes, i.e., the non-cysteinyl ones that appear less efficiently though significantly labeled. In any case, the combination of the molecular genetics approach and that of the in situ labeling by specific probes should offer original insights into the transmembrane topology of the acetylcholine receptor subunits.

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