Covalent Modification of a Critical Sulfhydryl Group in the Acetylcholine Receptor: Cysteine-222 of the α-Subunit†

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Received March 22, 1989; Revised Manuscript Received May 17, 1989

ABSTRACT: Chemical modification of the *Torpedo californica* acetylcholine receptor (AcChR) by the fluorescent agent *N*-(1-pyrenyl)maleimide (PM) under nonreducing conditions resulted in the labeling of cysteine residues in all subunits and marked inhibition of the AcChR ion channel opening [Clarke, J. H., & Martinez-Carrion, M. (1986) *J. Biol. Chem. 261*, 10063–10072]. The PM alkylation kinetics are not affected by the presence of agonists or a competitive antagonist. The PM-labeled α-subunit has been purified and digested with both CNBr and trypsin. The resulting fragments from both cleavages were fractionated by high-performance liquid chromatography. The amino acid analysis and sequencing data of the PM-labeled peptides identified cysteine-222 as the only residue labeled by PM on the α-subunit primary structure. Cysteine-222 is located in the middle of a hydrophobic domain designated M1, which contains a homologous class of cysteines (Cys-241 in the aligned sequences) conserved in the four subunits of the AcChR. Because of its reactivity and fluorescent properties of the bound probe, αCys-222 seems to be a free sulfhydryl group accessible through a hydrophobic pocket, and these properties should be incorporated into proposed folding models for the α-subunit.

The nicotinic acetylcholine receptor (AcChR)[1] from electric organ and muscle is a pentameric membrane glycoprotein that transiently opens a cation channel in response to ligand binding, thus initiating postsynaptic membrane depolarization (Conti-Tronconi & Raftery, 1982; Changeux et al., 1984; Hucho, 1986). The receptor protein from *Torpedo californica* has an apparent molecular weight of 270 000 (Martinez-Carrion et al., 1975) and consists of four homologous subunits with a relative stoichiometry $\alpha_2\beta\gamma\delta$ (Raftery et al., 1980). Its primary structure has been determined by sequencing the cDNAs (Noda et al., 1983; Claudio et al., 1983).

Recent work has used selective chemical modifications of the AcChR to ascertain the interrelationship between structure and function. Particularly, the role of the sulfhydryl groups has been the object of considerable interest due to the marked alterations that thiol reagents elicit in a number of the properties associated with the nicotinic receptor. The AcChR contains 10 free sulfhydryl groups of different accessibility and 6 disulfides per receptor (Chang & Bock, 1977). Sator et al. (1978) reported that in freshly isolated native receptor five sulfhydryl groups can readily react with DTNB, while about twice as many react after denaturation with 2% SDS.

Previous studies have shown that NEM does not impair AcChR function in native membranes unless they are previously treated with DTT (Karlin, 1969; Walker et al., 1981). Reduction with thiol reagents by itself causes a decrease in the affinity for acetylcholine analogues and an inhibition of the ion permeability response of the AcChR (Karlin & Bartels, 1966; Schiebler et al., 1977; Moore & Raftery, 1979; Barrantes, 1980; Walker et al., 1981). The disulfide bridge that links AcChR monomers through their δ-subunits is also susceptible to cleavage by DTT, but its implication in the functionality of the AcChR is not yet clear (Chang & Bock, 1977; Karlin, 1980; Anholt et al., 1980).

The α-subunit of the AcChR contains the agonist binding site. A readily reducible disulfide bond between Cys-192 and Cys-193 was demonstrated in the agonist binding site (Kao et al., 1984). After reduction with DTT, this site can be affinity labeled with the antagonist MBTA (Karlin, 1969; Weill et al., 1974; Kao et al., 1984) or with the agonist bromoacetylcholine (Walker et al., 1984; Chang et al., 1977; Damle & Karlin, 1978; Moore & Raftery, 1979). Shortly thereafter it was shown that Cys-128 forms a disulfide bond with Cys-142 in the α-subunit and this disulfide was more resistant to reduction than the agonist binding site disulfide (Kao & Karlin, 1986). Some of these conclusions are consistent with site-directed mutagenesis results of some of these residues (Mishina et al., 1985).

We have previously detailed the interaction of the AcChR with PM, a fluorescent, lipophilic, alkylating agent (Sator et al., 1978; Clarke & Martinez-Carrion, 1986). Preincubation of native AcChR with PM, under nonreducing conditions, resulted in the marked inhibition of the functional properties associated with AcChR-mediated cation translocation and ligand binding cooperativity, without perturbing agonist-induced affinity-state transitions. We also demonstrated that the PM adduct is formed with thiol groups in all the subunits of the AcChR, which led us to propose the existence of a homologous class of functionally sensitive cysteines within a similar hydrophobic domain in each subunit (Clarke & Martinez-Carrion, 1986).

Despite the substantial progress that has been made in the elucidation of the role of sulfhydryl groups in the AcChR, with the exception of the residues modified with MBTA, no other

† This work was supported by Grant GM-38341 from the National Institutes of Health.

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[1] Abbreviations: AcChR, acetylcholine receptor; PM, *N*-(1-pyrenyl)maleimide; CbCh, carbamylcholine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Me_2SO, dimethyl sulfoxide; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; NPM, *N*-phenylmaleimide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; NEM, *N*-ethylmaleimide; MBTA, [4-(*N*-maleimido)-benzyl]trimethylammonium iodide; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; kDa, kilodalton(s); EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride.

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selectively labeled sulfhydryl groups have been located on the AcChR primary structure. Yet, assignment of a role for the thiol groups labeled with PM in the modulations of cation translocation or ligand binding cooperativities largely depends on its location in the protein structure. In this paper, we describe the identification in the α-subunit of the AcChR, of a strategic sulfhydryl group different from those directly implicated in the agonist binding region.

Experimental Procedures

Frozen *T. californica* electroplax was purchased from Pacific Biomarine Laboratories, Inc. (Venice, CA), and was stored in a −70 °C freezer until use. *Bungarus multicinctus* venom was obtained from Miami Serpentarium. α-Bungarotoxin was purified (Mebs et al., 1972) and radiolabeled with ^{14}C as previously described (Calvo-Fernandez & Martinez-Carrion, 1981). PM was obtained from Molecular Probes (Junction City, OR) and carbamylcholine chloride from Aldrich (Milwaukee, WI). Reagents for SDS–polyacrylamide gel electrophoresis and electroelution were products of Bio-Rad.

AcChR-Enriched Membranes. AcChR-enriched membranes from *Torpedo californica* electroplax were prepared as previously described (Soler et al., 1984), including the alkaline extraction treatment (Neubig et al., 1979; Elliot et al., 1980; Lindstrom et al., 1980). The alkaline-extracted membrane fragments were resuspended in HEPES buffer (10 mM HEPES/100 mM $NaNO_3$, pH 7.6) to a final protein concentration of 5–10 mg/mL. Protein concentrations were determined by the method of Lowry et al. (1951), with bovine serum albumin as a standard. Specific α-bungarotoxin binding activities were determined by the DEAE filter disk assay (Schmidt & Raftery, 1973) and typically ranged from 1 to 2 nmol of α-bungarotoxin bound per milligram of protein.

Incorporation of PM into AcChR-Enriched Membranes. Alkaline-extracted membranes (15 mL, 2.5 mg of protein/mL) were incubated with 480 μM PM from a concentrated Me_2SO solution at room temperature in the dark for 90 min with stirring. The final Me_2SO concentration was less than 0.9%. The reaction was then quenched with 60 mM β-mercaptoethanol. The PM-labeled membranes were diluted to 120 mL with distilled water and centrifuged for 30 min at 14000 rpm in a Sorvall SS-34 rotor. The pellets were solubilized in sample loading buffer (Laemmli, 1970) and submitted to preparative SDS–PAGE.

SDS–Polyacrylamide Gel Electrophoresis and Electroelution. SDS–PAGE was performed as described by Laemmli (1970) using 10% isocratic polyacrylamide gels with a 3% polyacrylamide stacking gel. The catodic buffer contained 0.1 mM sodium thioglycolate to prevent oxidation of proteins during electrophoresis (Hunkapiller et al., 1983). The protein band corresponding to the α-subunit was visualized from its associated fluorescence under a UV lamp and excised from the gel. The isolated α–PM subunit was electroeluted, using an electroelution buffer composed of 25 mM Tris, 0.19 M glycine, and 0.1% SDS, pH 8.3, and an ISCO Model 1750 electrophoretic concentrator for 24 h at room temperature with three changes of buffer. The amount of α-subunit labeled with PM was measured by determining the absorbance of covalently attached PM at 342 nm ($\epsilon = 4 \times 10^4\ M^{-1}\ cm^{-1}$) (Wu et al., 1976). Typically, about 300–400 μg of pure α-subunit could be recovered from 12–15 mg of AcChR-enriched membranes. The final material migrated as a single band in a polyacrylamide gel.

S-Carboxymethylation of the α–PM Subunit. The electroeluted α–PM subunit in Tris–Gly buffer at a final concentration of 0.5 mg/mL was treated with 20 mM DTT and incubated for 60 min, at room temperature, under an argon atmosphere. Solid iodoacetamide was added to a final concentration of 50 mM, and the sample was incubated under argon in the dark for 30 min. The reaction was then quenched by the addition of excess β-mercaptoethanol. After dialysis against 0.1% SDS and concentration by vacuum dialysis, the α–PM subunit was acetone-precipitated to remove the bound SDS with 9 volumes of cold acetone and kept at −20 °C for 2 h. The precipitated protein was then collected by centrifugation at 14000 rpm in a Sorvall SS-34 rotor. The supernatant was removed and the pellet dried under a gentle stream of argon.

Cyanogen Bromide and Trypsin Fractionation. Dry α–PM subunit (600 μg) was dissolved in 1.2 mL of argon-deaerated 70% formic acid, and CNBr was added to a final concentration of 0.2 M. After 40 h at room temperature in the dark under argon, the reaction mixture was diluted 8-fold with water, and excess reagents were removed by vacuum centrifugation. The mixture of CNBr peptides was dissolved in 88% formic acid, and insoluble material was removed by centrifugation for 10 min (Eppendorf 5414 microfuge).

Purified and carboxymethylated α–PM subunit was resuspended (1 mg/mL) in 2 M urea, 0.1 mM $CaCl_2$, and 50 mM NH_4HCO_3, pH 8.0, and incubated at 37 °C with TPCK-trypsin (Sigma Chemical). Trypsin was added periodically up to a total 1:10 (w/w) enzyme:substrate ratio during the 24-h incubation period under argon. The digest was dried under vacuum and dissolved in a minimal volume of 88% formic acid.

Both the CNBr and trypsin digests were separated by reversed-phase HPLC in a Beckman 332 system using a Vydac protein and peptide C_{18} (0.46 × 25 cm) column. Solvent A was 0.1% TFA in 20% acetonitrile, and solvent B was 0.1% TFA in 60% acetonitrile/40% 2-propanol. The gradient systems used are indicated in the legends to the figures. The elution of peptides was monitored by the absorbance at 214 nm. Fractions were collected in HNO_3-washed siliconized tubes for further analysis.

Amino Acid Composition and Sequence Analysis. The amino acid composition of the isolated PM-labeled peptides was performed on a Beckman HPLC 338 equipped with a Model 230 post-column reactor. Following acid hydrolysis in 6 N HCl, containing 0.1% phenol, at 110 °C in a vacuum for 24, 48, and 72 h, the amino acids were resolved on a Beckman Spherogel column (0.46 × 25 cm) and identified by fluorescence detection of their OPA derivatives.

Automated Edman degradation of the PM-peptides was performed in a gas-phase sequencer (Applied Biosystems, Inc., Model 470-A) with the assistance of Ben Madden at the Mayo Clinic, Rochester, MN.

Spectroscopic Methods. Fluorescence spectra and intensity measurements were performed at 25 °C with an SLM 8000C Aminco spectrofluorometer (SLM Instruments) interfaced to an IBM PC/XT computer for control of the instrument and for data storage and manipulation. The samples were continuously stirred, and usually 4-nm slits were used for both excitation and emission. Absorption spectra were obtained by using a Hewlett-Packard 8452A diode array spectrophotometer.

Results

Labeling of the AcChR by PM: Effect of Agonist. The labeling conditions were similar to those previously published (Clarke & Martinez-Carrion, 1986) except that labeling at room temperature during 90 min was chosen in order to in-

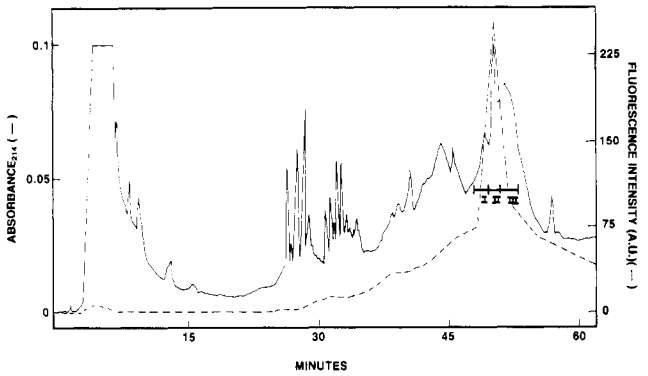

FIGURE 1: HPLC separation of the fragments resulting from CNBr cleavage of the PM-labeled α-subunit. The separations in a Vydac C_{18} column were monitored by measuring the absorbance at 214 nm (—) and by measuring the fluorescence intensity of each fraction (---) (excitation, 345 nm; emission, 382 nm). The flow rate was maintained at 1.0 mL/min, and 2-mL fractions were collected. After an initial isocratic period of 10 min at 100% solvent A, peptides were eluted with a linear gradient from 0% to 100% solvent B in 84 min. The relevant fractions fluorescently labeled and subsequently rechromatographed are indicated by horizontal bars (I, II, and III). The composition of solvents A and B was as indicated under Experimental Procedures.

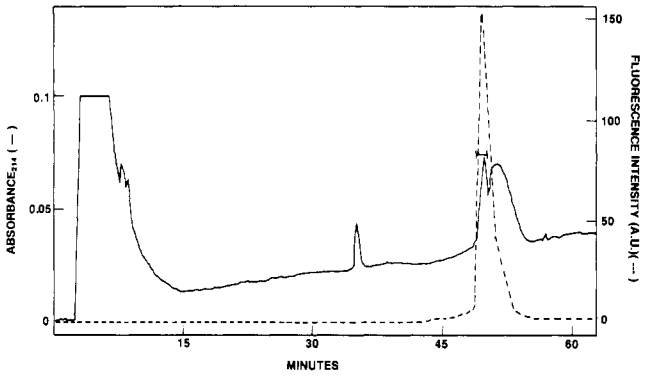

FIGURE 2: Rechromatography of PM-labeled peak II isolated in the first HPLC separation of the CNBr digest of the α-subunit. The separations in a Vydac C_{18} column were monitored by measuring the absorbance at 214 nm (—) and the fluorescence intensity (---) (excitation, 345 nm; emission, 382 nm). The flow rate was maintained at 1.0 mL/min, and 1.5-mL fractions were collected. The elution gradient started at 100% solvent A. Five minutes after sample injection, the concentration of solvent B was increased to 20% over 15 min, followed by an isocratic period of 5 min at 20% solvent B and a linear gradient from 20% to 50% solvent B in 35 min. The fraction containing the fluorescence peak is indicated by a horizontal bar.

crease the amount of probe incorporated. The PM-labeled membranes were resolved by preparative gel electrophoresis, and the subunit receptor bands were visualized by its associated fluorescence. All the receptor subunits, as well as the 95-kDa band, showed fluorescence labeling. The increase in fluorescence with time reflects adduct formation, since free PM does not fluoresce when free in solution (Weltman et al., 1973). Preincubation of the AChR-enriched membranes with CbCh has no effect on either the alkylation kinetics or the final fluorescence intensity over a wide range of CbCh concentrations. Moreover, the fluorescence emission spectra of the samples treated with CbCh were identical with the untreated control ones. Similar results were obtained with the agonist decamethonium and the competitive antagonist hexamethonium.

HPLC Separation of Peptides. The strongly hydrophobic character of the peptides generated by the CNBr treatment of the α-subunit makes their separation by reverse-phase HPLC extremely difficult. The chromatographic profile obtained for the CNBr peptides of the α-subunit is shown in Figure 1, along with the fluorescence intensity of the different fractions collected. Theoretically, 16 peptides can be generated in the CNBr cleavage of the α-subunit. The chromatogram exhibited eight/nine peptides eluting at the beginning of the gradient, followed by several more hydrophobic peptides with a broader shape eluting at higher organic phase concentrations. The fluorescence profile showed a strong peak with the maximum intensity associated with peak II. This fraction II was analyzed by using a different gradient system (see legend to Figure 2 for details), and the absorbance profile showed a peak eluting at about 50 min, containing a main fluorescent peak (Figure 2). Residual fluorescence was found in the next broad peak eluting at 52 min, which represents 10% of the total fluorescence intensity.

Peaks I and III from the first chromatographic step (Figure 1) were also repurified in a similar manner. They appear to contain a small amount of the 50-min peptide (accounting for most of the fluorescence) and some other components eluting at longer retention times that had only minimum fluorescence intensity associated with them (data not shown). It is concluded that the fluorescence found in peaks I and III in the first chromatography is due to its partial contamination with

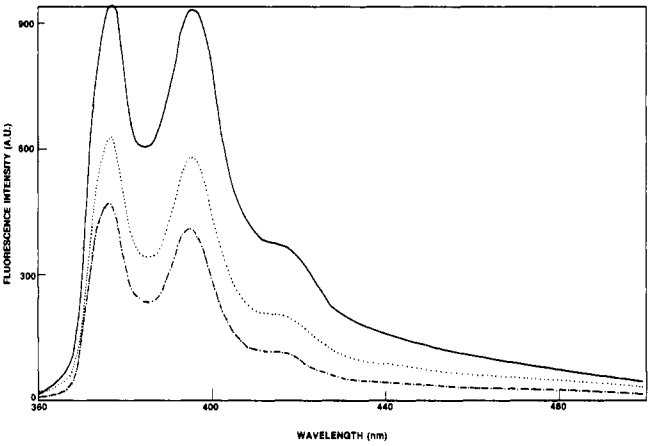

FIGURE 3: Normalized and corrected fluorescence emission spectra of bound PM. AcChR-enriched membranes in 10 mM HEPES buffer, pH 7.6 (—), isolated α-subunit in 70% formic acid (···), and purified CNBr peptide of the α-subunit in 0.1% TFA, 36% acetonitrile, and 16% 2-propanol (-·-). Excitation was at 345 nm.

peak II and/or partially cleaved products, and not to the presence of other PM-labeled peptides.

The characteristic fluorescence emission spectra of PM-labeled AChR membranes, isolated α-subunit, and purified CNBr peptides are compared in Figure 3. The three spectra were similar without significant shifts in the emission bands appearing at 377 and 395 nm or in the intensity ratios of the emission maxima.

Amino Acid Analysis. The purified PM-labeled CNBr peptide was submitted to amino acid analysis to compare its primary structure with one of the theoretically expected CNBr peptides of the α-subunit. Due to the strong hydrophobic character of the isolated peptide, it showed resistance to acid hydrolysis, requiring long hydrolysis time and extrapolation of several residues to infinite hydrolysis time to obtain qualitative results. The amino acid composition (Table I) corresponds to that predicted for a CNBr peptide of the α-subunit (α208–243) that contains Cys-222. No other CNBr peptide, containing Cys or not, can account for the experimentally determined amino acid composition.

Sequence Analysis. The PM-labeled peptide was submitted to Edman degradation. When approximately 1 nmol of pep-

Table I: Amino Acid Composition of the PM-Labeled α-Subunit CNBr Peptide Determined by Acid Hydrolysis

amino acid	α-CNBr peptide	
	predicted[a]	obtained[b]
Asp	2	2.2
Thr	2	1.1[c]
Ser	2	1.9[c]
Glu	2	2.0
Gly	2	2.1
Ala	0	0.3
Val	4	4.2[d]
Met	0	0
Ile	3	3.0
Leu	6	4.7[d]
Tyr	2	0.7
Phe	4	1.9
His	0	0.3
Lys	1	1.1
Arg	1	1.0
S-(carboxymethyl)-Cys	0	0

[a] From amino acid sequence (Claudio et al., 1983; Noda et al., 1983). [b] Values normalized for Glu content and average of three determinations; values between runs differ by less than 10%. [c] Ser and Thr determined in the 24- and 48-h samples. [d] Values extrapolated to infinite hydrolysis time for timed hydrolysis of 24, 48, and 72 h.

tide was first analyzed, no PTH derivative was detected. This result suggested that the peptide was probably blocked at the amino-terminal end and was not contaminated with any significant amount of other peptides. The presence of glutamine as the amino-terminal residue of peptide α208–243 and the use of concentrated formic acid for the CNBr treatment of the sample suggested that the amino-terminal group was probably blocked as pyroglutamic acid. It is well-known that glutamine-terminating peptides are specially liable to cyclize under acid conditions (Smyth et al., 1962). Various attempts either to unblock the peptide or to hydrolyze it further were mostly unsuccessful. These involved incubation with pyroglutamate aminopeptidase and partial acid hydrolysis in 2% formic acid under vacuum for 16 h at 110 °C. Under the latter conditions, the aspartic acid (Asp-238) and asparagine (Asn-217) should be released (Inglis, 1983). However, when this material was subjected to Edman degradation, it yielded Asp, Ser, and Gly in the first cycle with no other amino acids detected in the second cycle. It was concluded that the bonds Ser-239/Gly-240 and Gly-240/Glu-241, along with those involving the Asp-238, were cleaved with the prolonged digestion, as previously reported for similar peptide bonds (McDowall & Smith, 1965). In contrast, the Asn-217 residue, located between two valines, remains unaltered, attesting to the resistance of the peptide to acid hydrolysis. The above results are consistent with the predicted COOH-terminal region of the peptide primary structure as Asp^{238}-Ser-Gly-Glu-Lys-Met.

In order to obtain supporting evidence, we subjected the α-subunit to a different hydrolytic treatment using trypsin. Peptides generated by digestion of the purified α–PM-labeled subunit with trypsin were resolved by HPLC (Figure 4) under the same conditions as those used for the chromatography of the CNBr fragments. The fluorescent fraction T1, after being rechromatographed, was also analyzed by Edman degradation. Although this peak containing most of the fluorescence intensity was isolated in very low yield, probably due to solubility problems, it still could be partially sequenced for its first residues (IPL) which correspond to that of the tryptic fragment extending from Ile-210 to Lys-242. Thus, the amino acid sequence of the fluorescent peptides obtained from CNBr and tryptic digestion corresponds to a segment in the primary structure containing Cys-222.

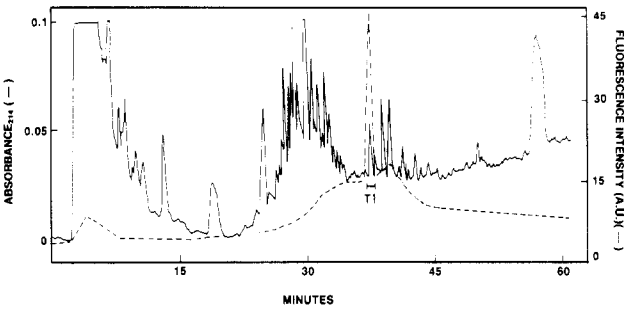

FIGURE 4: HPLC separation of tryptic peptides from PM-labeled α-subunit. Absorbance monitored at 214 nm (—) and the fluorescence intensity of the 1.5-mL fractions collected were measured (---) with excitation at 345 nm and emission at 382 nm. The fraction containing the fluorescence peak is marked T1. The elution conditions were as in Figure 1.

Discussion

Understanding the interrelationship between structure and function in the AcChR is one of the basic challenges when trying to characterize the mechanism of action of the nicotinic receptor. One experimental design we have used to approach this subject is to study the interaction between chemical probes and the AcChR at various functional levels and elucidate which regions of the membrane protein have been modified by the probe. This should allow for the preparation of maps of functionally sensitive domains of the AcChR primary structure, allowing for some physiological and biochemical data to be correlated.

The modification of alkaline-extracted AcChR-enriched membranes with the hydrophobic fluorescent agent PM, in the absence of reducing agents, has been shown to impair the AcChR cation translocation process by reaction with sensitive cysteines embedded in a hydrophobic environment on the AcChR, and it follows a labeling stoichiometry of 2:1:1:1 in accordance with the $\alpha_2\beta\gamma\delta$ subunit stoichiometry (Clarke & Martinez-Carrion, 1986). Even though all the subunits are labeled, we have directed our attention to detect first the alkylation target for the α-subunit, since this subunit is best characterized and contains all the identified cholinergic ligand binding sites. Several observations of the alkylation behavior are of interest to the properties of the modified cysteine residues. First, the overall rate of alkylation of AcChR-enriched membranes by PM is not affected by preincubation with agonists such as CbCh even in the millimolar range, and the final fluorescence intensity is unaffected by the presence of CbCh. Yet, the inactivation of the ion flux by PM modification seems uncoupled with the ligand binding ability (Clarke & Martinez-Carrion, 1986), and PM shows the same affinity for the receptor in the resting and desensitized states. Second, another agonist, decamethonium, and the competitive antagonist hexamethonium were also ineffective in altering the rate or extent of overall alkylation. Finally, the fluorescence emission spectra of PM membranes control and of those preincubated with CbCh were similar, without intensity ratio changes or spectral shifts of the emission bands. Two possible interpretations can be given to the above observations. First, it could be that the probe's environment in all subunits remains unaltered in the transition between resting and desensitized states. Second, and more likely, the lack of detection of perturbation in the PM fluorescent emission spectra during possible ligand-induced conformational change in the AcChR seems to reflect that the probe is relatively insensitive to microenvironmental changes, as has been noticed with a variety of proteins (Weltman et al., 1973). This is supported by the similarity of the PM emission spectra of AcChR-enriched

membranes (with all subunits labeled), purified α-subunit, and isolated CNBr peptide of the α-subunit, where structural differences in the molecules providing the possible hydrophobic pocket PM environments and several solvents used do not affect their emission spectra (Figure 3).

The chromatographic analysis of the CNBr-cleaved α-subunit (Figure 1) shows only one peak containing most of the fluorescence (Figure 2), and this is consistent with one site of PM labeling, and the presence of one labeled cysteinyl residue per α-subunit. The amino acid analysis of this fraction (Table I) displayed the marked hydrophobic character of the peptide, matching reasonably well only with a CNBr peptide for the α-subunit containing the Cys-222 (α208–243). The amino acid sequence of this possible peptide (QRIPLYFVVNVIIPCLLFSFLTGLVFYLPTDSGEKM) contains several vicinal bonds between very hydrophobic amino acids.

The partial results obtained with the sequence studies of this peptide were consistent with its predicted primary structure deduced from the amino acid analysis. That is, the sample was homogeneous and seems blocked in the amino terminus, as can be expected if glutamine is the NH_2-terminal residue. Moreover, this peptide seems to contain Ser and Gly vicinal to the Asp residue. To circumvent the problems arising in the sequence of the CNBr peptide (which was resistant to one attempt of treatment with pyroglutamic aminopeptidase), we cleaved the PM-labeled α-subunit with trypsin and isolated fluorescently labeled peptides under the same HPLC conditions as those used for the CNBr peptides (Figure 4). Trypsin digest generated peptide containing Cys-222 should start at Ile-210 (α210–242) with only three amino acid residues less than the CNBr-produced peptide. The sequence analysis of the isolated tryptic peptide, which contains most of the fluorescence label, supports this hypothesis. Thus, all results of the trypsin digest provide independent and convergent evidence that the residue labeled by PM in the α-subunit is Cys-222.

The sequence of each subunit shows that there is a highly conserved cysteine residue in position 241 of the aligned sequences (Noda et al., 1983) which in the α-subunit is Cys-222. All secondary structure predictions imply that this cysteine is located in a hydrophobic α-helix segment that could be a transmembranous segment (called M1). The first model proposed for the topology of the sequence of all four types of subunits of the AChR stated that the transmembrane domain of each subunit was comprised of four hydrophobic α-helices, M1, M2, M3, and M4 (Claudio et al., 1983; Devillers-Thiery et al., 1983; Noda et al., 1983). An additional amphipathic helix, M5, was included in the five-helix model (Guy, 1984; Finer-Moore & Stroud, 1984), suggesting that the highly charged groups of this helix could be lining the channel, with helices 2 and 3 bundled together forming an inner layer around it and helices 1 and 4 stacking to form the outer layer. In building this late model, assumptions concerning the state of sulfhydryl groups of the different AChR subunits were necessarily made (Guy, 1984). So, in the different arrangements of the outer layer proposed between helices 1 and 4, Cys-222 of the α-subunit was always paired, forming a disulfide bridge with other α-cysteines. Our results indicate that Cys-222 is unpaired and can be easily labeled with PM under nonreducing conditions. The position of Cys-222 in the primary structure of the α-subunit is represented in Figure 5. Our conclusion also agrees with the recent suggestion that Cys-222 may be the only free cysteine in the α-subunit (Mosckovitz & Gershoni, 1988).

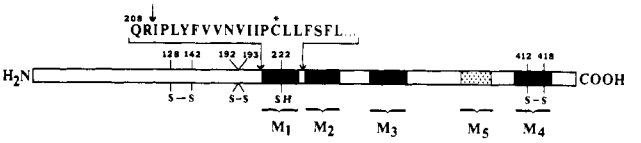

FIGURE 5: Location of the PM-labeled cysteine within the α-subunit primary structure. In this structural map of the AcChR α-subunit, black boxes indicate the four hydrophobic segments (M1–M4) and the stippled box the amphipathic helix M5. The cysteine labeled by PM is indicated by an asterisk and included into the isolated CNBr peptide (α208–243). The arrow indicates the amino-terminal position of the sequenced trypsin peptide. The disulfide bonding arrangement is taken from Kao and Karlin (1986) and from Mosckovitz and Gershoni (1988).

Another difficult question to answer is the mechanism of inhibition of the ion channel activity by PM. While site-directed mutagenesis studies showed that the four transmembrane domains 1–4 seem to be essential in the AcChR ion gating activity (Mishina et al., 1985), some recent experimental evidence using membrane-impermeant reagents has argued against the transmembrane orientation of segment M5 (McCrea et al., 1988; Dwyer, 1988). The transmembrane orientation of segment M1 could be consistent with the fact that a lipophilic probe such as PM labels Cys-222 while other more hydrophilic maleimide derivatives are poor alkylating reagents or even poor competitors of PM (Clarke & Martinez-Carrion, 1986). Yet, the probe seems to label the AcChR-enriched membranes at the same rate and extent in the desensitized state, showing no conformational preference for either protein conformation of the resting or desensitized states. Uncoupling between ion gating and agonist-induced desensitization, characteristic of the PM–AChR interaction, has been noted in other studies (Soler et al., 1984; Donnelly et al., 1984; Yee et al., 1986). The latter authors labeled solubilized AcChR with *N*-phenylmaleimide (NPM), and this required the presence of detergent. Under these conditions, the γ-subunit was preferentially labeled. The requirement of detergent, at least for this solubilized preparation, can be explained by the fact that NPM is less hydrophobic than PM, and solubilized receptor requires detergent to make the residues accessible to NPM. On the other hand, for in situ preparations of AcChR (AcChR-enriched membranes), NPM provides protection to PM labeling of all subunits (Clarke & Martinez-Carrion, 1986) which could imply a similar sulfhydryl environment as target for both reagents.

Lately, attention has been focused on segment M2 as a plausible channel-forming candidate because two different noncompetitive blockers (chlorpromazine and triphenylmethylphosphonium) have been associated with homologous binding sites in the α-, β-, and δ-subunits (Giraudat et al., 1986; Oberthur et al., 1986). Yet, the possible significance of segment M1 in the cation channel remains open. This relationship was first stressed by Numa's laboratory pointing to the highly conserved primary structure of M1 along the four subunits (Numa et al., 1983). M1 contains three proline residues and one cysteine residue [position 222 of the α-subunit and position 241 of the aligned (Noda et al., 1983) primary sequences] which are conserved in all four subunits. Interestingly, Cys-222 is adjacent to one of the conserved proline residues that probably breaks the helix in two portions: helix 1a and helix 1b (Guy, 1984). Since the fluorescent properties of bound PM are identical for intact receptor (with all subunits labeled) and for peptide fragments, consideration must be given to the assignment of a hydrophobic environment near Cys-222 at the channel itself or a nearby membrane sequence providing a hydrophobic pocket which accounts for the hydrophobicity

required for the chemical alkylating reagent to be effective. This could situate Cys-222, and probably the homologous cysteines in the other subunits, in a unique position interacting directly with other protein transmembrane segments and connecting the two helix 1 segments, or near the channel itself. In fact, it has been proposed (Giraudat et al., 1985) that Cys-222 does not face directly the lipid phase. Thus, assuming that the agonist-induced conformational change to open the channel is due to the tilting of the transmembrane helices (Guy, 1984; Davis et al., 1983), labeling of Cys-222 (and those in the homologous positions in the other subunits) with PM could either block the channel or block (or induce) a conformational rearrangement needed for opening the channel. The latter conformation is not the same as that in the desensitized state because PM does not perturb the agonist-induced desensitization (Clarke & Martinez-Carrion, 1986). These mechanisms seem more plausible taking into account that the effect caused by PM could not be assigned to the intrinsic need of a free cysteine because the mutation of αCys-222 to Ser yielded a functional receptor (Mishina et al., 1985).

Added in Proof

After this paper was submitted, Kellavis et al. (1989) reported location of the cysteine pairings in all four subunits of *Torpedo californica* AcChR. This report indicates that Cys-241 (Cys-222 in the α-subunit) probably exists as a free sulfhydryl group.

Acknowledgments

We thank Bernardo Perez and Drs. M. A. Chinchetru and J. C. Garcia-Borron for helpful suggestions. We also thank Carol McArthur for typing the manuscript.

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Effects of Diacylglycerols on the Structure of Phosphatidylcholine Bilayers: A ^2H and ^{31}P NMR Study†

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ABSTRACT: The interaction of four diacylglycerols (DAGs) with multilamellar phospholipid bilayers consisting either of dipalmitoylphosphatidylcholine (DPPC) or of a mixture of DPPC and bovine liver phosphatidylcholine (BL-PC) extracts was investigated by a combination of ^{31}P and ^2H NMR spectrometry. We found that saturated and unsaturated long-chain DAGs induce different types of perturbations into the bilayer structure. The saturated DAGs dipalmitin and distearin induce lateral phase separation of the lipids into (i) DAG-enriched gellike domains and (ii) relatively DAG-free regions in the liquid-crystalline phase. In the latter regions, the order parameters along the fatty acyl chains of DPPC are practically identical with the control. This phase separation effect was observed in both model systems studied, and its extent is dependent upon DAG concentration and temperature. Only bilayer phases were present upon addition of dipalmitin or distearin at all concentrations and temperatures studied. The unsaturated DAGs diolein and DAG derived from egg PC (egg-DAG) affect PC bilayers in the following two ways: (i) by increasing the order parameters of the side chains, as observed for both DPPC and BL-PC model systems; (ii) by inducing nonbilayer lipid phases, as observed for BL-PC, but not DPPC. At a concentration of 25 mol % of an unsaturated DAG in mixed PC bilayers, a peak corresponding to isotropic lipid conformation appeared and increased in intensity with increase in temperature, while at 32 mol % hexagonal and bilayer phases coexisted. Previous studies showed that DAGs containing unsaturated chains can activate a variety of enzymes, such as phospholipases and protein kinase C. Our work indicates that this enzyme-activating ability correlates with the induction of nonbilayer lipid phases by diolein or egg-DAG and is not simply due to the lateral phase separation of the lipids into regions of different fluidities, with corresponding defects in the bilayer, as exhibited by dipalmitin or distearin.

One of the mechanisms of transmembrane signal transduction involves increased metabolism of phosphadidylinositol lipids, and a product of this process is diacylglycerol (DAG)[1] (Michell et al., 1981; Berridge et al., 1983). DAG is produced in vivo from phosphatidylinositols by stripping the polar group of these phospholipids and is a very hydrophobic molecule, acting at the membrane level. Numerous studies demonstrated that DAG and related molecules can directly influence cell behavior. Cellular processes affected by DAG are, for example, activation of cell secretion and induction of morphological changes in platelets (Hokin & Hokin, 1953; Rink et al., 1983) and erythrocytes (Allan & Michell, 1975), exocytosis in adrenal medulla chromaffin granules (Knight et al., 1982) and in sea urchin egg plasma membrane (Whitaker & Aitchison, 1985), the fusion of myoblasts (Wakelam, 1985), and changes in intracellular pH (Moolenaar et al., 1984).

Physicochemical studies of the effects of DAGs on the phospholipid bilayer structure consistently report that DAGs induce destabilization of the bilayers. Observed effects include the appearance of ^{31}P NMR signals characteristic of isotropic or hexagonal lipid phases (Dawson et al., 1984), changes in Ca^{2+}-induced lateral phase separation of lipid mixtures (Ohki et al., 1981), and changes in the bilayer to hexagonal phase transition temperature of phosphatidylethanolamine (Epand,

† This work was supported by grants from the International Union against Cancer and NFWO (to H.D.B.) and from the American Cancer Society (BC-590) (to R.Z.). The ^2H NMR work was done at the NSF Southern California Regional NMR Facility (NSF CHE-791634 and CHE 844-137) located at the California Institute of Technology. The ^{31}P NMR was done with a GN 500 spectrometer (NSF DMB 840491 and NIH BRSG 2 507).
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[1] Abbreviations: BL, bovine liver; DAG, diacylglycerol; DO, diolein; DP, dipalmitin; DPPC, dipalmitoylphosphatidylcholine; DPPC-d_{62}, bis(perdeuteriopalmitoyl)phosphatidylcholine; DS, distearin; PC, phosphatidylcholine; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

0006-2960/89/0428-7439$01.50/0