Thiol-Group Modification of *Torpedo californica* Acetylcholine Receptor: Subunit Localization and Effects on Function†

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ABSTRACT: The effects of thiol-group modifications on acetylcholine receptor (ACHR) function were measured with purified ACHR reconstituted into asolectin vesicles. *N*-Phenylmaleimide (NPM) was used to modify sulfhydryl groups on ACHR in the absence of any prior reduction of dithiothreitol, so that only the functional relevance of free sulfhydryls was examined. Modification by NPM led to the inhibition of ion-channel activity without a detectable effect on ligand binding. The ion flux inhibition by NPM primarily affected channel activation, since the *initial rates* of activation were decreased over a wide range of carbamylcholine concentrations. The [^{3}H]NPM subunit labeling pattern of ACHR (a multisubunit membrane protein with $\alpha_2\beta\gamma\delta$ stoichiometry) revealed that there was preferential labeling of the γ subunit. At high NPM concentrations, the number of sulfhydryl groups on the γ subunit that could be modified with NPM was approximately two. Detergent was required during labeling for functionally relevant thiol-group modifications, and most of the label was protected from protease digestion in the reconstituted membranes. These results are consistent with the presence of the NPM modification in a bilayer and/or cytoplasmic domain.

The nicotinic acetylcholine receptor (ACHR)[1] from the electric organ of *Torpedo californica* is the best characterized neurotransmitter receptor. The ACHR is an example of a ligand-gated ion channel, in which the binding of acetylcholine triggers the opening of a cation channel within the protein. Upon prolonged exposure to agonist, the ACHR undergoes desensitization, which is characterized by a low- to high-affinity shift in ligand binding and a concomitant blockade of ion channel activity.

The relative ease with which *Torpedo* ACHR can be purified in high amounts allows for detailed biochemical studies. It is a five-subunit glycoprotein with a composition of $\alpha_2\beta\gamma\delta$. The apparent molecular weights are 40 000 (50 611), 50 000 (53 681), 60 000 (56 279), and 65 000 (57 565) for the α, β, γ, and δ subunits, respectively. The subunit molecular weights deduced from the primary sequence are indicated in parentheses. Several excellent reviews concerning the biochemistry of ACHR have recently appeared (Karlin, 1983; Stroud & Finer-Moore, 1985; Changeux et al., 1984).

A major advance in the study of ACHR is the elucidation of the primary sequence of each subunit as deduced from the cDNA sequence (Claudio et al., 1982; Noda et al., 1982, 1983a,b; Sumikawa et al., 1982; Devillers-Thiery et al., 1983; Hershey et al., 1983). Comparisons of the primary sequence of ACHRs from many species show significant homology, suggesting a remarkable conservation of function across species lines. The *Torpedo* ACHR can thus serve as a good general model for detailed studies of ACHR function.

Theoretical analyses of the primary sequence have led to interesting predictions concerning ACHR secondary structure, in particular the structure of the ion channel. Analysis of hydrophobicity predicts the existence of four transmembrane α helices, and Fourier-transform analysis predicts a novel amphipathic helix in each subunit. It is proposed that the amphipathic helices form the walls of the transmembrane ACHR ion channel (Finer-Moore & Stroud, 1984; Guy, 1984). Recent electron microscopic and immunochemical evidence support the existence of at least five transmembrane helices (Young et al., 1985; Lindstrom et al., 1984; Criado et al., 1985). In addition, deletion and point mutations in the putative amphipathic helix region of the α subunit demonstrate that ion channel activity is perturbed but ligand binding is unaffected—exactly what would be predicted if the region was involved in ion channel function (Mishina et al., 1985).

A current area of intense study is the elucidation of the molecular mechanisms of ligand binding and of channel opening. It is well established that the α subunit is the site of ligand binding. Using the affinity label MBTA [(4-maleimidobenzyl)trimethylammonium iodide], Kao et al. (1984) have shown that Cys-192 is half of the disulfide bridge, which is known to exist near the acetylcholine binding site. In addition, both site-specific mutation at Cys-192 and deletions in this region abolish ligand binding (Mishina et al., 1985). The combination of protein chemistry and recombinant DNA technology provides conclusive evidence for the role of Cys-192 in acetylcholine binding. Furthermore, these studies illustrate the importance of sulfhydryls and disulfides in ACHR function. Although detailed functional analysis of the subunit is under intense investigation, the function of the other subunits is largely unknown, except that all subunits are required for a functional, properly assembled ACHR (Mishina et al., 1984).

An advantage of studying *Torpedo* ACHR is the ease with which ACHR can be purified and reconstituted into artificial liposomes with retention of functional properties. The use of

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[1] Abbreviations: ACHR, acetylcholine receptor; BGT, α-bungarotoxin; CARB, carbamylcholine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MBTA, (4-maleimidobenzyl)trimethylammonium iodide; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; NPM, *N*-phenylmaleimide; PMSF, phenylmethanesulfonyl fluoride.

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purified and reconstituted functional ACHR allows for a relatively well-defined system for the detailed biochemical analysis of functional properties and also allows for a direct correlation of chemical modifications with functional effects. The development of methods for examining ion flux in the millisecond time range also aids in analysis of channel function by allowing the measurement of initial rates of channel activation (Walker et al., 1982, 1984).

One goal of our laboratory is to understand the role of free sulfhydryls and disulfides in ACHR function. Karlin first demonstrated that disulfide and sulfhydryls could play a role in function in electrophysiological studies of eel ACHR (Silman & Karlin, 1969). Chang & Bock (1979) have determined that there are 10 free sulfhydryls of varying accessibility and likely 6 disulfides per receptor, but neither the locations nor functional relevance of each sulfhydryl or disulfide is completely clear. Much of the study of thio groups has focused on disulfides and on effects of reduction on ACHR function. A disulfide exists between the δ subunits, which links two ACHRs together as a dimer, although the functional relevance of the dimer is unclear (Hamilton et al., 1979). It is well established that a disulfide bridge exists near or in the acetylcholine binding site and that reduction causes a decrease in the affinity for ligand binding (Moore & Raftery, 1979; Damle & Karlin, 1980; Walker et al., 1981). In addition, reduction of the disulfide followed by alkylation of the cysteines in the binding site can lead to inhibition of binding and, subsequently, of ion channel activity (Walker et al., 1981, 1984). In addition, some evidence has been presented for sulfhydryls relevant in function in sites distinct from the acetylcholine binding site (Raftery et al., 1983; Huganir & Racker, 1983; Walker et al., 1984).

In this paper, we have focused on the role of *free* sulfhydryls in ACHR ion channel activation using protein modification. The study described below is a first step to a more detailed characterization of functionally relevant regions or groups of ACHR. The next phase of our work will focus on the identification of the amino acid sequence and the location of these functionally important regions by peptide mapping and sequencing. Site-directed mutagenesis will then be used to make specific changes in these regions and also in the cysteine residue. As has been elegantly illustrated in the study of the acetylcholine binding site, this combined approach represents a powerful way to identify the location of important sites.

In this study, we have examined the modification of sulfhydryl groups in the absence of prior reduction. Previous studies have shown that modification of cysteines can lead to an inhibition of ion channel activity without affecting ligand binding in *native* membranes (Walker et al., 1984; Huganir & Racker, 1983). We have extended the chemical modification studies to *purified* ACHR using *N*-phenylmaleimide (NPM) in order to fully characterize the effects on function and to begin to localize the modification at the protein level. The current work demonstrates that sulfhydryl modifications lead to inhibition of ion flux activation. The results of the modifications in native and purified ACHR suggest the existence of specific sulfhydryls that may be important for ion channel activity. We also demonstrate that the NPM modifications occur preferentially on the γ subunit and present evidence for the existence of the functionally relevant sites in a bilayer or cytoplasmic region.

Experimental Procedures

Purification of ACHR. ACHR was purified from frozen *Torpedo californica* electric organ essentially as described previously (Walker et al., 1982). *Torpedo* were obtained from Bodega Bay Marine Laboratory and other local suppliers. All steps were carried out at 0–4 °C. Electric organ was homogenized in buffer A (10 mM phosphate, 5 mM EDTA, 5 mM EGTA, and 0.02% sodium azide, pH 7.5). Iodoacetamide and PMSF (Sigma) were added to final concentrations of 2 and 0.2 mM, respectively. Large fragments were removed by centrifugation at 5000*g*. The supernatants were then centrifuged at 55000*g* for 120 min in a Beckman Ty 19 rotor to pellet the crude membrane fraction. The membranes were resuspended in buffer B (10 mM MOPS, 100 mM NaCl, 0.1 mM EDTA, and 0.02% NaN_3, pH 7.5) and stored in liquid nitrogen for future use.

The crude membranes were solubilized in buffer C [buffer B plus 1% (w/v) sodium cholate]. After removal of the cholate-insoluble material by ultracentrifugation, the extract was applied to an affinity column prepared from Affi-Gel 401 (Bio-Rad) and (bromoacetyl)choline. The loaded affinity column was washed extensively with buffer C containing 1 mg/mL dioleoylphosphatidylcholine (Avanti Polar Lipids, Birmingham, AL). Purified ACHR was eluted in buffer C containing 10 mM CARB. The peak fractions were determined by absorbance at 280 nm and pooled. The specific activity of ACHR is expressed as picomoles of ^{125}I-BGT binding sites per milligram of protein, as determined by equilibrium toxin binding and Lowry assays, respectively (Walker et al., 1981; Lowry et al., 1951).

Chemical Modification of ACHR. Purified ACHR was modified in 1% cholate with *N*-phenylmaleimide (NPM) prior to reconstitution. The pH during modification was 7.5, and buffer C was used for all reactions. Typically, 1 mL of ACHR at approximately 1 mg/mL was incubated for 30 min at 25 °C with 10 μL of an NPM solution in Me_2SO for the preparation of NPM-modified ACHR membranes. Control membranes were treated with Me_2SO only. The final NPM concentration during the modification reactions ranged from 0 to 5 mM NPM, and the final Me_2SO concentration was 1% (w/v). At the end of the incubation period, asolectin and cholate were added for the reconstitution of NPM-modified and control ACHR, as described below.

Reconstitution of ACHR. For reconstitution, a solution of 4% cholate and 80 mg/mL asolectin (Associated Concentrates, Woodside, NY) was added such that the final lipid/protein ratio was 10 000 and the concentration of cholate was 1.5%. Reconstitution was achieved by cholate dialysis against at least 200 volumes of buffer with five changes over a 48-h period. The vesicle preparation was freeze-thawed 4 times prior to storage in liquid nitrogen.

Ligand Binding. The ligand binding properties were measured by an iodinated α-bungarotoxin (^{125}I-BGT) binding competition assay as described previously (Walker et al., 1981). The rates of ^{125}I-BGT binding to ACHR were measured in the absence and presence of CARB, which was added either simultaneously with toxin (coincubation curve) or prior to toxin addition (preincubation curve). In the latter case, CARB induces a time-dependent transition from low- to high-affinity state for ligand binding.

All assays were performed in buffer B containing 0.5 mg/mL bovine serum albumin (Sigma, fraction V). The concentrations of ACHR and toxin were 5 and 30 nM, respectively. If CARB was used, the concentration was 5 μM. For the preincubation curve, ACHR and CARB were incubated together for at least 30 min on ice prior to assay. Aliquots were withdrawn every 30 s, and the ACHR-bound toxin was separated from free toxin by vacuum filtration on DE-a81 filters (Whatman). The amount of bound toxin was determined by γ counting.

Ion Flux Measurements. The influx of $^{86}Rb^+$ into reconstituted membranes containing purified ACHR in the presence and absence of CARB was measured essentially as described (Walker et al., 1982). Buffer B was used in all experiments. The agonist-mediated ion flux response was measured in the second and subsecond time ranges as described below.

(*A*) *Thirty-Second Ion Flux Measurements.* Membranes and $^{86}Rb^+$ with or without CARB (final concentration of 10 mM) were incubated in buffer B for 30 s on ice. Typically, 50 μL of membranes and 15 μL of $^{86}Rb^+$ solution were used. A 50-μL aliquot was then applied to a 3-mL Dowex 50W-X8 column, which was equilibrated in 170 mM sucrose and 3 mg/mL bovine serum albumin (Sigma, fraction V). The vesicle-entrapped $^{86}Rb^+$ was eluted with 3 mL of 175 mM sucrose and counted without scintillation fluid. Free $^{86}Rb^+$ was retained on the column.

(*B*) *Millisecond Ion Flux Measurements.* Quench-flow techniques were used to measure the ion channel activation in the millisecond time range and have been described previously (Walker et al., 1982). For the influx curves, ion flux was allowed to proceed for times ranging from 15 to 1000 ms, and excess curare was used to quench the reaction. A CARB concentration of 1 mM was used to generate the influx curves. For the 15-, 25-, and 35-ms time points, the continuous mode was used with constant tubing length, but time was varied by varying push times. For the "longer" times, the pulse mode was used. To measure the ion flux response as a function of CARB concentration, a 25-ms time point was used since this time point provided a good estimate of the initial rates of channel activation (Walker et al., 1982).

Sulfhydryl Group Quantitation. The concentration of sulfhydryl groups was determined by reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). The concentration of DTNB in all reactions was 330 μM. Typically, 33 μL of 10 mM DTNB (in 0.1 M Tris, pH 8.0) was added to 1 mL of ACHR in cholate (10 μM, in buffer B), and the mixture was incubated for 30 min at 25 °C. In some cases, ACHR was modified first with NPM as described above, and then the concentration of SH groups was determined with DTNB. The presence of NPM did not affect color development. The absorbance at 412 nm was measured, and a standard curve of sulfhydryl concentrations from 0 to 100 μM, with 0–50 μM dithiothreitol, was used to determine the concentration of SH groups per ACHR.

ACHR Subunit Labeling with [^{3}H]NPM. All labelings with [^{3}H]NPM (Amersham Corp., 50–70 Ci/mmol, 1 μCi/μL) were performed in the presence of 1% cholate in buffer B—conditions identical with those used for the modification of ACHR for functional studies. Usually 10 μL of [^{3}H]NPM (1 μCi/μL) was used per 100 μL of ACHR (approximately 1 mg/mL). The radioactive NPM was first dried in vacuo and resuspended in 1 μL of Me_2SO; 100 μL of ACHR in cholate was added to initiate the labeling reaction. The final concentration of [^{3}H]NPM was 2.5 μM. The mixture was incubated at 25 °C for 30 min. NPM incorporation into ACHR was terminated with the addition of 4 μL of 14.3 M β-mercaptoethanol. An equal volume of SDS gel sample buffer (250 mM Tris, 6% SDS, 142 mM β-mercaptoethanol, and 10% (w/v) glycerol, pH 6.8) was then added to the reaction mixtures. The subunit labeling pattern was resolved by SDS–PAGE with 10% gels and fluorography with Enhance (New England Nuclear) (Laemmli, 1970; Bonner & Lanskey, 1974).

(*A*) *Subunit Labeling with Constant Specific Activity.* ACHR was first modified in 1% cholate with nonradioactive NPM at concentrations from 10 to 500 μM and then reconstituted as described above. Excess NPM was removed by dialysis. The membranes were resolubilized with cholate and then labeled with [^{3}H]NPM (10 μCi, 2.5 μM) exactly as described above. Usually, 50 μg of purified ACHR was used. The labeled subunits were resolved on a 10% gel and were visualized by fluorography. The band intensity in each subunit was quantitated by scanning with a laser densitometer attached to a peak integrator (Hewlett-Packard).

(*B*) *Subunit Labeling with Constant Label.* ACHR was modified in the presence of 1% cholate with NPM at concentrations from 10 to 500 μM. A fixed concentration of [^{3}H]NPM was included in all reaction mixtures. A typical 1-mL reaction mixture contained 1 mg of ACHR and approximately 2.2×10^7 dpm. The mixture was incubated at 25 °C for 30 min. The ACHR subunits were resolved on a 10% gel and stained with Coomassie blue. Typically, 80 μg of labeled ACHR was analyzed.

The labeling per subunit was quantitated by solubilizing and counting the radioactivity in 1-mm slices through the subunit region (M_r 40000–66000). Because purified ACHR was used, no labeled bands of other molecular weights were detected (see Figure 5). The radioactivity in each slice was solubilized by incubation for 16 h at room temperature with a 9:1 solution of Protosol (New England Nuclear) and water. A 10-mL volume of toluene–POPOP–PPO scintillation fluid was added to each vial. The picomoles of [^{3}H]NPM incorporated per subunit was calculated by determining the counts incorporated into each subunit and then was normalized for the differing specific activities. The amount of ACHR was determined by equilibrium toxin binding, and the concentration of each subunit was calculated by assuming a stoichiometric ratio of $\alpha_2\beta\gamma\delta$.

Protease Digestion Experiments. In all experiments, sealed, reconstituted vesicles containing purified ACHR reconstituted into the correct orientation were used. For these experiments, purified ACHR was labeled with [^{3}H]NPM prior to reconstitution under conditions identical with those used in the functional studies. Buffer B was used in the digestions with trypsin, and buffer B containing 2 mM each of $MgCl_2$, $ZnSO_4$, and $CaCl_2$ was used for Pronase digestions. The digestion products were resolved by SDS–PAGE with 10–15% gradient gels.

Trypsin Digestion. Purified ACHR in vesicles was digested with trypsin (TPCK treated; Sigma) in the absence and presence of 1% cholate. The ACHR to trypsin ratio (w/w) was either 20/1 or 100/1. The incubations were at 37 °C for 3 h, and a fresh aliquot of trypsin was added at the same ratio after the initial incubation. The digestion was allowed to proceed for an additional 3 h. The trypsin digestion was terminated with the addition of PMSF to a concentration of 40 mM. A typical reaction volume was 100 μL. An equal volume of SDS sample buffer (see above) was added to each mixture to stop the reaction.

Pronase Digestions. The procedure was identical with that used for trypsin except that the Pronase (Calbiochem) reactions were performed in buffer B with the salts indicated above and were terminated with sample buffer, which had been heated to 100 °C.

RESULTS

Inhibition of Ion Flux Response by N-Phenylmaleimide. The role of free sulfhydryls in ACHR function was explored with *N*-phenylmaleimide (NPM) to modify purified ACHR in reconstituted membranes. Reconstitution of ACHR into asolectin vesicles at a high lipid to protein ratio gives functional

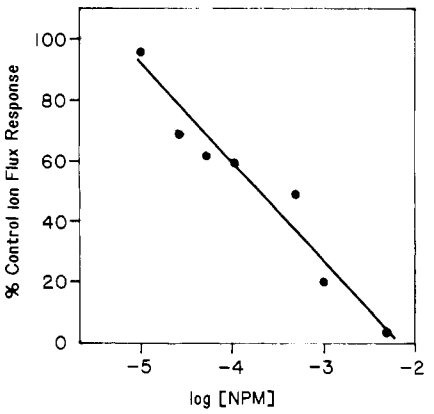

FIGURE 1: Effect of NPM moficiation on ion flux response. Purified ACHR (2.5 mg) was modified with NPM at different concentrations and reconstituted into asolectin membranes as described under Experimental Procedures. The ion flux response to CARB was determined by the manual flux assay with $^{86}Rb^+$ as the tracer ion as described under Experimental Procedures. All data are expressed as a percentage of the control ion flux. Each point represents the mean of triplicate measurements, and the solid line is a least-squares fit of the data.

ACHR-containing vesicles in which agonist-mediated cation influxes can be conveniently measured in both the second and millisecond time domains (Walker et al., 1982). The conditions were optimized to ensure that effects of thiol-group modifications on function could be correlated with *covalent* modification of ACHR. In contrast to most previous studies with maleimides, the treatments here do not include prior reduction of the receptor with dithiothreitol. All reactions were performed in the presence of cholate, and unreacted or degraded maleimide and cholate were removed by dialysis prior to any functional measurements.

To explore the effects of NPM on the CARB-mediated ion flux response, purified ACHR was modified with various concentrations of maleimide ranging from 10 μM to 1 mM prior to reconstitution into asolectin by cholate dialysis. The ion flux response was measured initially in a manual assay with 10 mM CARB, which measures the integrated ion flux response and allows a qualitative determination of the maximal response. All data are expressed as percentage of the response in control membranes, which have been treated with the same concentration of Me_2SO (1%) used in all reactions involving NPM. In the manual flux assays, a Me_2SO concentration of 1% (w/v) does not markedly affect the ion flux response to CARB. In two separate experiments, the degree of inhibition was 10 and 0% (data not shown). As shown in Figure 1, NPM leads to a progressive inhibition of the ion flux response to CARB with complete inhibition at 5 mM NPM.

Kinetics of Ion Flux Responses. To further explore the ion flux inhibition, the kinetics of channel activation were examined by measuring the ion flux response in the millisecond time domain with quench-flow techniques (Walker et al., 1982). NPM at a concentration of 100 μM was chosen to modify ACHR since the manual flux measurements demonstrated that this concentration resulted in 40% inhibition of control ion flux levels. The concentration of CARB was 1 mM since this concentration gives close to the maximal initial rate of activation (Walker et al., 1982). As shown in Figure 2, the influx curves for control and modified membranes demonstrate that the modifications by NPM result in a decrease in the initial rate of ion flux activation by CARB, confirming the results of the manual flux measurements described in Figure 1.

To examine in more detail the kinetics of activation following chemical modification, the 25-ms influx measurements

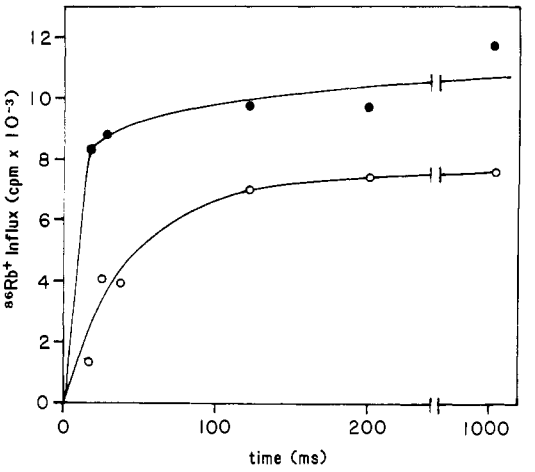

FIGURE 2: Effect of NPM modification on ion channel activation. The influx of $^{86}Rb^+$ into reconstituted vesicles containing either NPM-modified (○) or control ACHR (●) was measured by quench-flow techniques (see Experimental Procedures). The concentration of NPM during modification was 100 μM, and conditions for modification of ACHR are described in under Experimental Procedures. The concentrations of CARB and ACHR were 1 mM and 8 μM, respectively. Time points from 15 to 1000 ms were taken. After being quenched with curare at the given time, vesicle-entrapped $^{86}Rb^+$ was quantitated by ion-exchange chromatography and counting. Each point represents the mean of duplicate points, which differ by less than 5%.

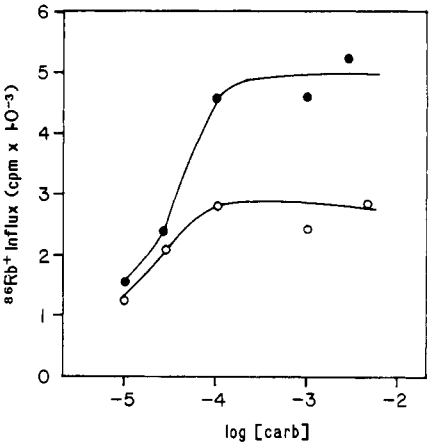

FIGURE 3: Effect of NPM modification on initial rates of activation as a function of CARB concentration. The influx of $^{86}Rb^+$ into reconstituted vesicles containing NPM-modified (○) or control ACHR (●) was measured at 25 ms by quench-flow techniques. The concentration of CARB was varied from 10 μM to 3 mM. The concentration of NPM and ACHR was 100 and 8 μM, respectively. The conditions for ACHR modification are described under Experimental Procedures. Each point represents the mean of duplicate values, which differ by less than 5%.

were obtained as a function of CARB concentration. The 25-ms time point is in the initial rate region of channel activation and can be used to approximate the actual rate. If modification resulted in an increase in the concentration of CARB required for channel opening, the concentration curves would be shifted to higher CARB but at high concentrations should reach the same ion channel activity. If, however, modification resulted in ion channel inactivation, the overall initial rates would be lower at all concentrations, within experimental error, but the EC_{50} for activation would be unchanged with modification.

The dose–response curves for control and NPM-treated purified ACHR in reconstituted membranes are shown in Figure 3. The CARB concentration was varied from 10 μM to 3 mM, and all influx measurements were performed at 25

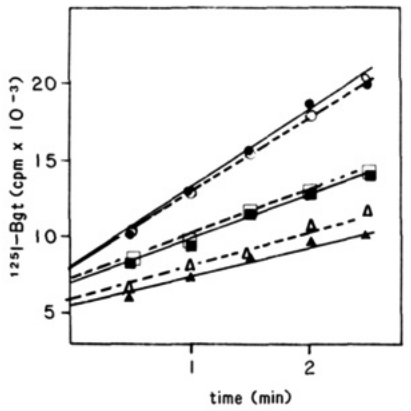

FIGURE 4: Effect of NPM modification on ligand binding. The ligand binding properties of modified (closed symbols, solid lines) and control (open symbols, dashed lines) ACHR-containing membranes were measured with the toxin competition assay as described under Experimental Procedures. The initial rates of ^{125}I-labeled bungarotoxin binding were measured in the absence of CARB (○, ●), by coincubating with 5 μM CARB (□, ■) or by preincubating with 5 μM CARB (△, ▲). The latter two sets of curves represent the binding of ligand to the resting and desensitized states of ACHR, respectively. The concentration of NPM used during modification was 5 mM. The concentrations of ACHR and toxin were 5 and 35 nM, respectively.

ms. NPM treatment resulted in a decreased initial rate of channel activation at CARB concentrations over a 300-fold range (Figure 3). Significantly, the EC_{50} for CARB with NPM modification was comparable to that observed with control membranes. The observation that the EC_{50} was unaltered with modification suggests that the nature of the decrease in ion flux is due to the inhibition of ion channel activation.

Ligand Binding Studies. In addition to ion flux studies, the ligand binding properties of ACHR modified with 5 mM NPM were measured. This concentration gave maximal inhibition of ion flux (see Figure 1), so any effect on ligand binding should be apparent at these concentrations of NPM. The ^{125}I-labeled bungarotoxin competition assay was used for all measurements (see Experimental Procedures). The curve obtained in the absence of CARB represents the maximal rate of toxin binding. The coincubation curve examines the binding of the resting state of ACHR, by virtue of the competition of CARB and toxin. The preincubation curve examines the binding primarily to the desensitized state, a high-affinity binding state. Preincubation with ligand shifts ACHR to the desensitized state, and results in the minimal rate of toxin binding.

As shown in Figure 4, modification by 5 mM NPM did *not* result in a significant alteration in the ability of ACHR to bind toxin or ligand, nor on the ability to undergo the low- to high-affinity shift, which is correlated with desensitization. At concentrations of maleimide that result in near complete inhibition of ion flux, the ligand binding properties were not affected. In agreement with work on native membranes, modification by NPM leads to a selective inhibition of ion flux but not of ligand binding with *purified* ACHR in reconstituted membranes (Walker et al., 1984).

It is important to emphasize that this inhibition is a result of covalent modification by NPM. In other experiments, purified, reconstituted ACHR was modified in the absence of detergent, and the ion flux response was measured in four different preparations. Under these conditions, inhibition of ion flux by NPM was observed but could be reversed by dialysis. With 10 mM NPM, the ion flux response was 1.4 ± 0.6% of the control membranes prior to dialysis. After dialysis, the ion flux response was 97 ± 4% of the control

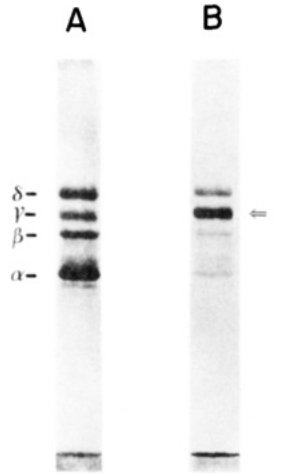

FIGURE 5: ACHR subunit labeling by [^{3}H]NPM. Fifty micrograms of purified ACHR was labeled with 10 μCi or [^{3}H]NPM (2.5 μM), and the subunits were resolved on a 10% gel as described under Experimental Procedures. Lanes A and B are the Coomassie-stained dried gel and the corresponding autoradiogram, respectively. Lane A had been treated with Enhance (NEN) and dried prior to photography. The positions of the ACHR subunits are indicated.

membranes. The inhibition in this case was not a result of covalent modification. Thus, modifications that lead to irreversible inhibition require the presence of detergent, suggesting that these sites might be in the membrane or in the cytoplasmic region.

Similar effects on function were obtained with modification of ACHR by *N*-ethylmaleimide (NEM) in the presence of detergent under identical conditions. The modification by NEM led to an inhibition of the ion flux response at the level of ion channel activation, but no effects on ligand binding were detected, even when 5 mM NEM was used (data not shown). It is likely that, in the presence of detergent, NEM and NPM modify the same class of functional sites.

Subunit Labeling. The next phase of study was to determine if a particular subunit was modified preferentially and if the subunit labeling pattern could be correlated with ACHR function. Reconstituted, purified ACHR was modified with [^{3}H]NPM in the presence of 1% cholate, and the labeled subunits were resolved by SDS–PAGE and fluorography (see Experimental Procedures).

The subunit labeling pattern obtained with [^{3}H]NPM is shown in Figure 5. A photograph of a Coomassie blue stained and fluorographed dried gel of purified ACHR is shown in panel A. All four subunit types are clearly evident. The corresponding autoradiogram in panel B indicates that the labeling of the γ subunit by [^{3}H]NPM is prominent. There is also some labeling of the δ subunit and lesser amounts of labeling in the β and α subunits.

To determine if the labeling of any subunit could be directly correlated with the inhibition of ion channel activity, purified ACHR was labeled by two different, but related approaches. The first approach used constant specific activity, and the second used constant label, but varying unlabeled NPM concentrations. SDS–PAGE was used to resolve labeled subunits, and radioactivity was quantitated by fluorography and densitometry or by direct counting, respectively (see Experimental Procedures).

To directly compare the band intensity between different samples, constant specific activity of [^{3}H]NPM during the labeling reaction is required. ACHR was first modified with nonradioactive NPM and then reconstituted as in the previous sections. The ion flux response of ACHR-modified samples was determined by the manual flux assay (see Figure 1). The

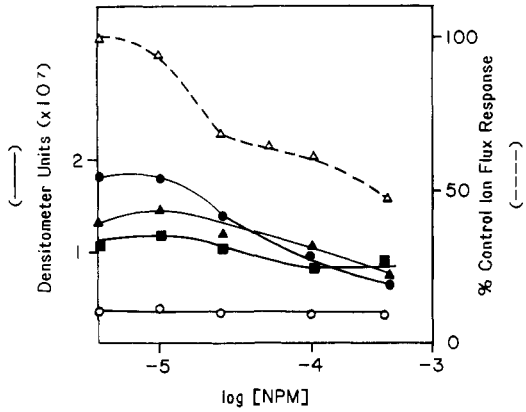

FIGURE 6: ACHR subunit labeling with [^{3}H]NPM under constant specific activity. Fifty micrograms of purified ACHR, which had been modified with nonradioactive NPM in cholate at concentrations from 0 to 500 μM, was then modified with 10 μCi of [^{3}H]NPM (2.5 μM) in the presence of 1% cholate as described under Experimental Procedures. The ACHR subunits were resolved on a 10% gel and were visualized by fluorography. Each lane was scanned with a laser densitometer, and the density of each peak was quantitated with a Hewlet-Packard integrator. The labeling within each subunit is plotted [α (○), β (■), γ (●), and δ (▲)]. The ion flux response (△) was determined as described in Figure 1.

membranes were solubilized with cholate, and the remaining NPM-susceptible groups were modified with [^{3}H]NPM. The specific activity was constant during labeling, since all nonradioactive NPM had been removed during the dialysis step of the reconstitution. If groups that were involved in ion flux had been modified by nonradioactive NPM, they should be protected from labeling with [^{3}H]NPM. The labeling should be higher with the unmodified sample and lower with the 500 μM NPM sample. If the labeling by [^{3}H]NPM in a given subunit was correlated with ion channel inhibition, then the labeled band intensity and ion channel activity should both *decrease* in parallel.

As shown in Figure 6, the band intensity of the γ subunit over a 50-fold NPM concentration range correlated reasonably well with the inhibition of ion channel activity. The labeling of the other subunits remained relatively unchanged over the same concentration range. The data of Figure 6 would suggest that the modification of groups on the γ subunit by NPM results in the inhibition of ion channel activity.

The second approach was to vary the [^{3}H]NPM concentration by varying the nonradioactive NPM over a 50-fold range and holding the amount of label constant. To quantitate the subunit labeling, each band was excised from the gel, and the incorporated radioactivity was solubilized and counted. The picomoles of NPM incorporated per subunit was calculated by normalizing for the specific activity of the [^{3}H]NPM concentration used. The ACHR concentration was determined by equilibrium toxin binding, and the subunit concentration was calculated with the known subunit stoichiometry of $\alpha_2\beta\gamma\delta$.

As shown in Figure 7A, the labeling of the γ subunit increased with increasing [^{3}H]NPM. At 500 μM, at least two sulfhydryls on the γ subunit were modified. In addition, at this NPM concentration, one sulfhydryl on the α subunit was also modified. In contrast, the labeling of the β and δ subunits did not vary appreciably over the 50-fold NPM concentration range and accounted for much less than one sulfhydryl per subunit. It should be noted that all subunits contain free sulfhydryl groups and at high NPM concentrations the groups on the other subunits can also be modified.

In good agreement with previous studies (Chang & Bock, 1979), DTNB quantitation demonstrated that there were four

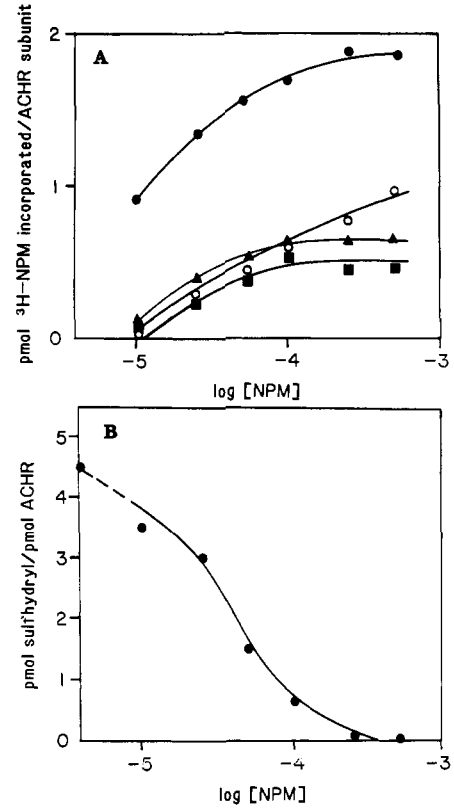

FIGURE 7: ACHR subunit labeling with [^{3}H]NPM under constant label and sulfhydryl group quantitation as a function of NPM modification. (A) Eighty micrograms of purified ACHR was modified in 1% cholate with [^{3}H]NPM at concentrations varying from 10 to 500 μM. The amount of labeled NPM in each case was 10 μCi. The ACHR subunits were resolved on a 10% SDS gel, and the subunit region was excised into 1-mm slices and counted as described under Experimental Procedures. The counts in each subunit were determined by totaling the counts in each slice through the subunit region. The picomoles of NPM incorporated into each subunit was calculated after normalizing for the varying specific activity [α (○), β (■), γ (●), and δ (▲)]. The picomoles of NPM incorporated per picomole of subunit was calculated assuming a stoichiometry of $\alpha_2\beta\gamma\delta$ and two toxin sites per ACHR. The concentration of ACHR was determined by equilibrium toxin binding, and the specific activity of this preparation was 8000 pmol of binding sites/mg of protein. (B) The sulfhydryl group concentration was determined by assay with DTNB in cholate as described under Experimental Procedures. The ACHR concentration was 10 μM, corresponding to a sulfhydryl concentration of 45 μM in the absence of prior modification with NPM. The picomoles of ACHR was determined by equilibrium toxin binding, and the specific activity was also 8000 pmol of binding sites/mg of protein.

to five free sulfhydryls per ACHR in cholate. As expected, with increasing NPM and modification, the sulfhydryl content was decreased (see Figure 7B), and at 500 μM NPM, all four sulfhydryls (in cholate) were modified. The data of Figure 7A are consistent with two and one sulfhydryl modified for each γ and α subunit, respectively.

Domain Localization of NPM Modification. The detergent requirement for covalent NPM modifications that lead to functional inhibition suggested that the relevant sites may be located in a bilayer and/or cytoplasmic domain of ACHR, since modification of extracellular sites by NPM did not give functional impairment. In order to determine if there were sites in the bilayer and/or cytoplasmic region that could be modified by NPM, two complementary approaches were used. The first approach used the accessibility of reconstituted, [^{3}H]NPM-labeled ACHR to proteases as a criteria for the existence of bilayer and/or cytoplasmic sites. The second was selective labeling of "internal" sites by [^{3}H]NPM with ACHR

that had been first modified extracellularly with nonradioactive NPM in the absence of detergent.

In all cases, purified ACHR in sealed vesicles and reconstituted in the correct orientation (extracellular outside) were used. Previous studies have shown that the ACHR in reconstitued vesicles were oriented with greater than 90% of the extracellular domain outside, as determined by equilibrium ^{125}I-BGT binding in the presence and absence of detergent (Ochoa et al., 1983). By a similar procedure, over 90% of the toxin binding sites were oriented correctly in the vesicles used in these experiments (data not shown).

To determine if the NPM label was present in a bilayer or cytoplasmic domain, the reconstituted ACHR in sealed membrane vesicles was digested with proteases in the presence and absence of detergent. Accessibility to protease has been used extensively to determine the membrane protein topology with reference to extracellular, bilayer, and cytoplasmic domains (Wennogle & Changeux, 1980; Strader & Raftery, 1980). This approach has been applied successfully to localize the N-terminal of δ subunit in the extracellular domain (Anderson et al., 1983). In the presence of a membrane and in the *absence* of detergent, the extracellular portion of the ACHR should be accessible to protease digestion, but the bilayer and cytoplasmic portions should be protected. In the absence of a membrane or in the *presence* of detergent, all portions of ACHR should be accessible to protease digestion. SDS–PAGE and fluorography of [^{3}H]NPM-labeled purified ACHR in reconstituted membranes digested with proteases in the presence and absence of detergent should show distinct patterns. If a portion of the NPM label is indeed present in the bilayer or cytoplasmic region, there should be a band(s) that is (are) present in the absence of detergent but disappear(s) in the presence of detergent.

In our experiments, both trypsin and Pronase were used in the presence and absence of 1% cholate. A 5-fold range in protease concentrations was used, as well as exhaustive digestion conditions (see Experimental Procedures). Lanes 1 and 2 and lanes 5 and 6 in Figure 8 show digestion by trypsin and Pronase in the presence of detergent, respectively. Lanes 3 and 4 and 7 and 8 show the digestion in the absence of detergent.

A comparison of these two sets shows a prominently labeled band with a molecular weight of approximately 30000, which is protected from digestion in the presence of a membrane but becomes accessible to digestion when the membrane is disrupted by cholate. The digestion pattern of the M_r 30000 band is consistent with what would be expected for a bilayer or cytoplasmic protein domain. The nature of the relatively protease-resistant band of M_r 40000 is not entirely clear but has been observed by others (Lindstrom et al., 1980; Wennogle & Changeux, 1980). It is possible that the labeled band of M_r 40000 represents a tightly associated ACHR structure comprised of the hydrophobic, bilayer regions of the subunits, since cholate does not lead to subunit dissociation. Even after extensive protease digestion, the ACHR subunits still remain tightly associated (Lindstrom et al., 1980). The experiments described here indicate the existence of at least one membrane-protected domain that can be labeled by NPM.

In addition to the protease accessibility experiments, selective labeling of bilayer and cytoplasmic domains was accomplished by first modifying purified ACHR in *sealed* vesicles with nonradioactive NPM at a concentration of 1 mM in the absence of detergent to modify extracellular sites. Excess and degraded NPM were removed by exhaustive dialysis. Control membranes were treated with 1% Me_2SO and were also dialyzed. Both control and NPM-treated reconstituted ACHR were then labeled with [^{3}H]NPM in the presence of 1% cholate to modify bilayer, cytoplasmic sites, or other protected sites. ACHR subunits were resolved by SDS–PAGE and fluorography. The control membranes should show labeling of all three domains—extracellular, bilayer, and cytoplasmic. In contrast, the NPM-pretreated ACHR should show labeling of bilayer and cytoplasmic regions only.

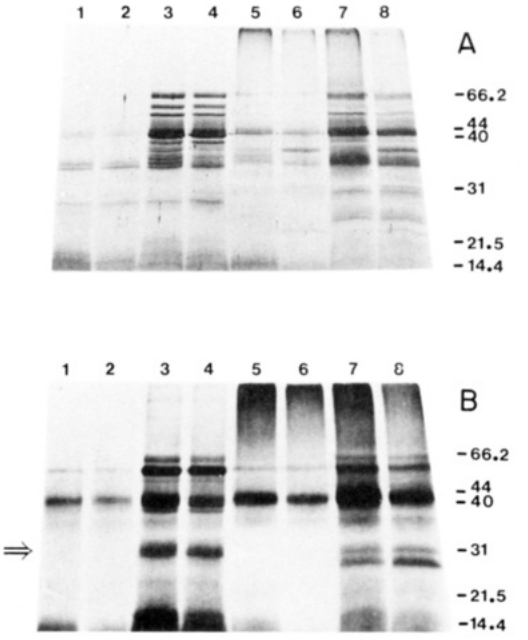

FIGURE 8: Trypsin and Pronase digestion of [^{3}H]NPM labeled, reconstituted ACHR in the presence and absence of cholate. Reconstituted, purified [^{3}H]ACHR (90 μg) was digested with trypsin or Pronase in the presence and absence of 1% cholate. The conditions are described under Experimental Procedures. The ratio (w/w) of protease to ACHR was either 1:20 or 1:100. Each aliquot of protease was added twice at the indicated concentrations in order to achieve more complete digestion. All protease reactions were terminated prior to electrophoresis. For the trypsin reactions, PMSF was added to a final concentration of 40 μM. For the Pronase reactions, the tubes were boiled immediately after the addition of SDS gel sample buffer. These treatments ensure that no further digestion occurred prior to electrophoresis. The digestion products were resolved on a 10–15% gradient gel and visualized by fluorography. Panel A represents the Coomassie-stained pattern, and Panel B is the corresponding autoradiogram. The molecular weight markers are indicated in both panels. (Lanes 1 and 2) These lanes represent the trypsin digestion pattern in the presence of cholate at trypsin to ACHR ratios (w/w) of 1:100 and 1:20, respectively. (Lanes 3 and 4) These lanes are identical with lanes 1 and 2, except that trypsin digestions were performed in the absence of cholate. (Lanes 5 and 6) These lanes represent the Pronase digestion pattern in the presence of cholate at Pronase to ACHR ratios (w/w) of 1:100 and 1:20, respectively. (Lanes 7 and 8) These lanes are identical with lanes 5 and 6 except that Pronase digestions were performed in the absence of cholate.

As shown in Figure 9, [^{3}H]NPM resulted in the preferential labeling of the γ subunit in the presence of detergent and when the extracellular sites were premodified with nonradioactive NPM. There was much less γ labeling in the absence of detergent. In control membranes, both γ and δ subunits were labeled in the absence of detergent, but the δ subunit modification decreased markedly with extracellular modification, suggesting that the sulfhydryl on δ was on the outside and is probably not functionally relevant. No α labeling was detected.

The results in Figure 8 suggest that at least a portion of the NPM label is in the bilayer or cytoplasmic region. This result was observed with proteases of different and broad specificity, suggesting that it is not due to the digestion by a particular protease. In other experiments, saponin was used to gain

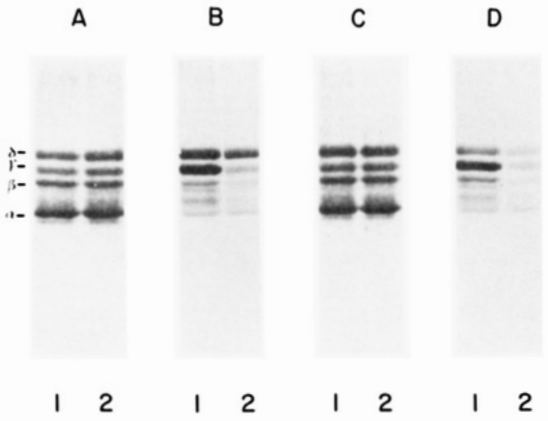

FIGURE 9: ACHR subunit labeling in the presence and absence of extracellular modification with NPM. Reconstituted ACHR (80 μg) was treated with 1 mM NPM in the absence of detergent to modify extracellular sites. Control membranes were treated with 1% Me_2SO. The excess NPM was removed by exhaustive dialysis in buffer B. The control and externally modified ACHR were then modified with 10 μCi of [^{3}H]NPM (2.5 μM final concentration) in the presence and absence of 1% cholate. The labeled ACHR subunits were resolved on a 10% gel and visualized by fluorography. (Panel A) Lanes 1 and 2 represent the Coomassie-stained pattern of [^{3}H]NPM-labeled ACHR subunits without prior modification of external sites in the presence and absence at 1% cholate, respectively. (Panel B) Lanes 1 and 2 represent the fluorograph of lanes 1A and 2A. (Panel C) Lanes 1 and 2 represent the Coomassie-stained pattern of [^{3}H]-NPM-labeled subunits with prior modification of external sites by 1 mM NPM. Lanes 1 and 2 shows the labeling pattern obtained in the presence and absence of 1% cholate during radioactive modification, respectively. (Panel D) Lanes 1 and 2 represent the fluorograph of lanes 1C and 2C, respectively.

accessibility to the cytoplasmic portion of ACHR without leading to solubilization (Froehner, 1981). The protease digestion experiments with saponin indicated a decrease in intensity of the M_r 30 000 band, indicating also that its origin is bilayer or cytoplasmic (data not shown). In addition, the results of Figure 9 suggest that the γ subunit contains bilayer and/or cytoplasmic site, suggesting, though not proving, that the origin of the M_r 30 000 band could be the γ subunit.

Discussion

The chemical modification results provide evidence for the involvement of cysteines in ACHR ion channel activity. All studies were performed with unreduced ACHR, so the groups modified by *N*-phenylmaleimide (NPM) exist as free cysteines in ACHR. Previous functional and labeling studies by our laboratory and others have focused on thio-group modifications with ACHR, which had been previously reduced with dithiothreitol (Moore et al., 1979; Damle & Karlin, 1980; Delegeane & McNamee, 1980; Walker et al., 1981; Otero & Hamilton, 1984). A well-studied disulfide is the one near the acetylcholine binding site, which, after reduction, can be affinity labeled by MBTA and which has recently been identified as Cys-192 on the α subunit (Kao et al., 1984). However, little is known about the possible role of the free sulfhydryls in ACHR function.

The present studies use purified ACHR reconstituted into lipid vesicles as the system for characterizing the functional effects of modifications. Quantitative measurements in the millisecond time range of ion flux activation provide evidence that NPM modification leads to a decrease in the initial rates of ion channel activation at CARB concentrations ranging from 10 μM to 3 mM. With NPM modification, the EC_{50} for CARB activation was not significantly different from that of control membranes, suggesting that the decrease in initial rates is due to the inhibition of ion channel activation.

Interestingly, the ligand binding properties of the receptor were not affected by NPM modifications even at high concentration of NPM. The modified ACHR could still bind ligand normally and, more importantly, could still undergo the conformational change associated with desensitization, as shown by the presence of the binding affinity shift in curve c of Figure 4A,B. Thus, binding to the resting and desensitized states of ACHR was unaltered by modification at NPM concentrations that gave complete ion channel inhibition.

The use of purified ACHR permitted a direct correlation of the effects of NPM modification with function and with the labeling of individual subunits. With NPM, the γ subunit was preferentially modified under conditions identical with those required for functional inhibition. All functionally relevant modifications required the presence of detergent, so direct effects of agonists on the labeling process are difficult to discern.

Quantitative analysis of the [^{3}H]NPM labeling under several different labeling protocols (see Results) revealed that at least two groups per γ subunit, as well as one on each α could be modified by NPM. The degree of label incorporation into the γ subunit correlated well with the degree of inhibition observed at each concentration of NPM. The ion flux inhibition is likely to be a direct consequence of γ labeling but could involve a combination of modification with groups on both γ and α.

The requirement for detergent to give covalent modifications that lead to functional alteration suggests that the relevant sulfhydryls might be in a bilayer or cytoplasmic domain. Modifications in the absence of detergent give labeling primarily of the δ subunit. Modification of extracellular cysteines with nonradioactive NPM prior to radioactive labeling abolished the subunit labeling, suggesting that the location is extracellular. However, the γ subunit is preferentially labeled in the presence of detergent, even with prior modification of external sites. The protease accessibility experiments support the existence of at least a portion of the NPM label in a bilayer and/or cytoplasmic domain of ACHR. The strong preferential labeling of γ subunit in detergent suggests that at least a portion of the functionally relevant groups are in this subunit.

The data of Figures 1–4 indicate that NPM modification leads to a selective inhibition of channel activity without affecting the binding of ligand. The inhibition is unlikely due to a gross alteration of ACHR structure since only ion channel function is affected. It is also important to note that not all modifications by NPM lead to functional perturbation. Only the modification of specific, detergent-accessible groups can lead to irreversible channel inhibition. Modifications in the absence of detergent did not result in an irreversible inhibition of channel activity, even at high NPM concentrations. Thus, NPM likely modifies sulfhydryls in a region of the protein that is involved in the ion permeation process, perhaps in activation and/or in the coupling of ligand binding to ion flux. The data of Figure 5–9 suggest that the γ subunit may contain these sites. These data indicate the existence of cysteines on the γ subunit, whose reactivity increases markedly in the presence of detergent.

Previous work with native membranes has identified the existence of a highly reactive sulfhydryl on the β subunit (Hamilton et al., 1979). In our work with purified ACHR, such β selectivity was not observed. A possible explanation is that, during the purification, precautions were taken to ensure that ACHR was in dimeric form, which requires treatment with iodoacetamide during tissue homogenization (Chang & Bock, 1977). It is possible that the β-reactive

sulfhydryl is already modified by iodoacetamide prior to ACHR purification. However, the purified ACHR used in all our experiments is completely functional. In addition, previous work with native membranes indicated that iodoacetamide treatments did not lead to an inhibition of ion channel activity, so it is likely that the modifications by iodoacetamide are not functionally important. Experiments are under way to quantitate ion channel activity and the number of sulfhydryl groups with ACHR that had been purified and reconstituted in the absence of iodoacetamide during tissue homogenization.

Despite recent advancements in the elucidation of ACHR primary sequence and structure, relatively little is known concerning the role of individual subunits. The α subunit has been long established as the site of ligand binding, and at least a portion of the acetylcholine binding site has been elucidated (Kao et al., 1984). The most definitive evidence for a functional role of the other subunits is that all are required for the formation of a functional channel, as elegantly demonstrated by Numa and his co-workers by expressing various combinations of ACHR subunits in *Xenopus* oocytes (Mishina et al., 1984).

Work with local anesthetics and other noncompetitive blockers has, in some cases, revealed the labeling of sites other than those for acetylcholine binding. Changeux and coworkers have shown that the δ subunit is selectively labeled by trimethisoquin and phencyclidine, whereas chlorpromazine, another anesthetic, leads to labeling of all subunits (Oswald & Changeux, 1981; Heidmann & Changeux, 1984). Karlin and co-workers have demonstrated that quinacrine mustard and quinacrine azide give labeling of the α and β subunits (Kaldany & Karlin, 1983; Cox et al., 1985). The modifications with chlorpromazine and quinacrine azide were performed on the millisecond time scale and could represent a modification of a functional state of ACHR.

The approach that we and others have employed represents a complementary approach to the study of ACHR structure and function and should also allow the identification of functional groups. Recent work using a phospholipid photoaffinity label and peptide mapping has identified portions of ACHR in contact with the lipid bilayer (Giraudet et al., 1985). In this work, modification with NPM clearly results in a selective perturbation of channel function. Of course, the results do not distinguish whether it is the loss of the free sulfhydryl or the presence of the modified sulfhydryl that gives rise to the functional effects.

The availability of the primary sequence is a distinct advantage in identifying the sites of chemical modifications. An analysis of the structure model proposed by Finer-Moore and Stroud (1984) reveals the presence of cysteine residues in the hydrophobic portion of the amphipathic helix of the γ subunit. If these cysteines exist as free sulfhydryls, modification of these groups by NPM could potentially lead to a perturbation of ion channel function. Work by Numa and co-workers (Mishina et al., 1985) has revealed that a site-specific change in the amphipathic helix region can lead to a perturbation of ion flux but not ligand binding—effectively uncoupling the two functional properties as has been observed in our experiments.

In addition, recent work by Lindstrom and co-workers has used monoclonal antibodies against γ and found that the functional properties were altered (Wan & Lindstrom, 1985). Interestingly, like our NPM modifications, the antibody inhibition was only apparent when the antibody was added in the presence of detergent. In their case, the epitope is cytoplasmic, but both ligand binding and ion flux were affected. It is possible that the mAb site is near or identical with the NPM-modified site on γ. The inhibition of both functional properties in the antibody work could be a consequence of attaching a large and bulky probe, particularly if the sites for coupling and/or opening are relatively close together.

It would be interesting to compare the amino acid sequence of the NPM sites and the location with reference to the membrane. A possible strategy for distinguishing whether the modification is bilayer or cytoplasmic is to generate antibodies against a synthetic peptide of the NPM-modified regions and then to use the antibodies to localize the site with reference to the bilayer. This approach has been applied successfully to the cytoplasmic localization of the C-terminal of the ACHR subunits and of sequences on the γ subunit (Lindstrom et al., 1984; Young et al., 1985; LaRochelle et al., 1985). Recent estimates by Stroud's group on the distribution of the protein indicate that the fraction in the extracellular, bilayer, and cytoplasmic domains is 61, 27, and 12%, respectively (Finer-Moore & Stroud, 1983).

Future efforts will be directed at determining the amino acid sequence of the NPM sites. Site-directed mutagenesis will be used as an independent test of the functional relevance of the NPM sites identified by protein chemistry approaches. A localization of these groups and a direct correlation of particular groups with ACHR ion channel function would represent an advancement in the definition of structure–function relationships.

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REFERENCES

Anderson, D., Blobel, G., Tzartos, S., Gullick, W., & Lindstrom, J. (1983) *J. Neurosci. 3*, 1773–1784.

Bonner, W. M., & Laskey, R. A. (1974) *Eur. J. Biochem. 46*, 83–88.

Chang, H. W., & Bock, E. (1977) *Biochemistry 16*, 4513–4519.

Chang, H. W., & Bock, E. (1979) *Biochemistry 18*, 172–179.

Changeux, J. P., Devillers-Thiery, A., & Chemoulli, P. (1984) *Science (Washington, D.C.) 225*, 1335–1345.

Claudio, T., Ballivet, M., Patrick, J., & Heinemann, S. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1111–1115.

Cox, R. N., Kaldany, R.-R. J., DiPaola, M., & Karlin, A. (1984) *J. Biol. Chem. 260*, 7186–7193.

Criado, M., Hochschwender, S., Sarin, V., Fox, J. L., & Lindstrom, J. L. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 2004–2008.

Damle, V., & Karlin, A. (1980) *Biochemistry 19*, 3924–3932.

Devillers-Thiery, A., Giraudet, J., Bentaboulet, M., & Changeux, J. P. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 2067–2071.

Finer-Moore, J., & Stroud, R. M. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 155–159.

Froehner, S. C. (1981) *Biochemistry 20*, 4905–4915.

Giraudet, J., Montecucco, C., Bisson, R., & Changeux, J. P. (1985) *Biochemistry 24*, 3121–3127.

Guy, R. (1984) *Biophys. J. 45*, 249–261.

Hamilton, S. L., McLaughlin, M., & Karlin, A. (1979) *Biochemistry 18*, 155–163.

Heidmann, T., & Changeux, J. P. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1897–1901.
Hershey, N. D., Noonan, D. J., Mixter, K. S., Claudio, T., & Davidson, N. (1983) *Cold Spring Harbor Symp. Quant. Biol. 48*, 79–82.
Huganir, R., & Racker, E. (1983) *J. Biol. Chem. 257*, 9372–9378.
Kaldany, R.-R. J., & Karlin, A. (1983) *J. Biol. Chem. 258*, 6232–6242.
Kao, P. N., Dwork, A. J., Kaldany, R.-R. J., Silver, M. L., Wideman, J., Stein, S., & Karlin, A. (1984) *J. Biol. Chem. 259*, 11662–11665.
Karlin, A. (1983) *Neurosci. Comment. 1*, 111–123.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Larochelle, W. J., Wray, B. E., Sealock, R., & Froehner, S. C. (1985) *J. Cell Biol. 100*, 684–691.
Lindstrom, J., Criado, M., Hochscwender, S., Fox, J. L., & Sarin, V. (1984) *Nature (London) 311*, 573–575.
Lowry, O. H., Rosenborough, N. L., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Mishina, M., Kurosaki, T., Tobimatsu, T., Morimoto, Y., Noda, M., Terao, M., Lindstrom, J., Takahashi, T., Kuno, M., & Numa, S. (1984) *Nature (London) 307*, 604–608.
Mishina, M., Tobimatsu, T., Imoto, K., Tanaka, K.-I., Fujita, Y., Fukuda, K., Kurasaki, M., Hideo, T., Morimoto, Y., Hirose, Inayama, S., Takahashi, T., Kuno, M., & Numa, S. (1985) *Nature (London) 313*, 364–369.
Moore, H. P., & Raftery, M. A. (1979) *Biochemistry 18*, 1907–1911.
Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Furutani, Y., Hirose, T., Asai, M., Inayama, S., Miyata, T., & Numa, S. (1982) *Nature (London) 299*, 793–797.
Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Kikyotani, S., Hirose, T., Asai, M., Takashima, H., Miyata, T., & Numa, S. (1983a) *Nature (London) 301*, 281–285.
Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Kikyotani, S., Furutani, Y., Hirose, T., Takashima, H., Inayama, S., Miyata, T., & Numa, S. (1983b) *Nature (London) 302*, 528–532.
Ochoa, E. L. M., Dalziel, A. W., & McNamee, M. G. (1983) *Biochim. Biophys. Acta 727*, 151–162.
Oswald, R. E., & Changeux, J. P. (1981) *Biochemistry 20*, 7166–7174.
Otero, A., & Hamilton, S. L. (1984) *Biochemistry 23*, 2321–2326.
Raftery, M. A., Dunn, S. M. J., Conti-Tronconi, B. M., Middleman, D. S., & Crawford, R. D. (1983) *Cold Spring Harbor Symp. Quant. Biol. 48*, 21–34.
Silman, I., & Karlin, A. (1969) *Science (Washington, D.C.) 164*, 1420–1421.
Strader, C. D., & Raftery, M. A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5807–5811.
Stroud, R. M., & Finer-Moore (1985) *Annu. Rev. Cell Biol. 1*, 369–403.
Sumikawa, K., Houghton, M., Smith, J. C., Bell, L., Richards, B. M., & Barnard, E. A. (1982) *Nucleic Acids Res. 10*, 5809–5821.
Walker, J. W., Lukas, R. J., & McNamee, M. G. (1981) *Biochemistry 20*, 2191–2199.
Walker, J. W., Takeyasu, K., & McNamee, M. G. (1982) *Biochemistry 21*, 5384–5389.
Walker, J. W., Richardson, C. A., & McNamee, M. G. (1984) *Biochemistry 23*, 2329–2338.
Wan, K.-K., & Lindstrom, J. (1985) *Biochemistry 24*, 1212–1221.
Wennogle, L. P., & Changeux, J. P. (1980) *Eur. J. Biochem. 106*, 381–393.
Young, E. F., Ralston, E., Blake, J., Ramachadran, J., Hall, Z. W., & Stroud, R. M. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 626–630.