Use of Chemical Modifications and Site-Directed Mutagenesis To Probe the Functional Role of Thiol Groups on the γ Subunit of *Torpedo californica* Acetylcholine Receptor†

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Received March 7, 1989; Revised Manuscript Received April 24, 1989

ABSTRACT: Alkylation of *Torpedo californica* purified nicotinic acetylcholine receptor (AChR) with *N*-phenylmaleimide (NPM) under nonreducing conditions led to ion flux inhibition without affecting ligand binding properties [Yee, A. S., Corey, D. E., & McNamee, M. G. (1986) *Biochemistry 25*, 2110-2119]. The γ subunit was shown to be preferentially labeled by [^{3}H]NPM with partial labeling of the α subunit at higher NPM concentrations. Alkylation occurs at cysteine residues as confirmed by amino acid analysis. Cyanogen bromide peptide mapping of the γ subunit indicates that at least two residues corresponding to Cys-416, -420, or -451 are labeled. Residues 416 and 420 are part of the proposed amphipathic helix, and the functional role of these two cysteines is further investigated by site-directed mutagenesis of *T. californica* AChR cDNAs and expression of the mutants in *Xenopus laevis* oocytes following injection of SP6 transcripts. Several features of SP6 transcripts are shown to be important for efficient translation in vivo. Mutations Cys → Serγ416,420 and Cys → Pheγ416 did not perturb either the receptor functional properties or its expression levels. The double mutant Cys → Pheγ416,420 displayed a 30% decrease of normalized AChR activity. The relatively small effect of large steric mutations in the amphipathic helix argues against its presence in the tightly packed transmembrane domain of the protein.

The nicotinic acetylcholine receptor (AChR)[1] from *Torpedo californica* has been the object of intensive investigation for many years [for reviews, see Hucho (1986), McCarthy et al. (1986), and Changeux et al. (1984)]. The nicotinic AChR is a multisubunit, ligand-gated cation channel that is directly involved in synaptic transmission. The ability to analyze the function of the receptor both at the protein structure level by biochemical and molecular biological techniques and at the cellular level by electrophysiological techniques has made the receptor an important prototype for similar analyses of other receptors. The cloning and sequencing of the genes for the four subunits of the *Torpedo* AChR have created new opportunities to correlate specific functional attributes of the receptor with specific domains of the polypeptide chain (Mishina et al., 1985; Imoto et al., 1988). The molecular biological techniques have also catalyzed the discovery of related receptors in the central nervous system [reviewed by Lindstrom et al. (1987)]. Despite the wealth of biochemical and physiological data available, the detailed molecular mechanism by which the binding of two acetylcholine molecules to the AChR is coupled to the opening of the ion channel is not known. In the absence of a three-dimensional structure for the protein, it remains necessary to apply a broad range of biochemical, immunological, and molecular biological techniques in order to deduce indirectly the overall structure and function of the receptor. Although conflicting models of the membrane topology of the protein have been proposed [for a review, see Guy and Hucho (1987)], most investigators believe that the ion channel exists as a central pore surrounded in a pseudosymmetric fashion by the five transmembrane subunits ($\alpha_2\beta\gamma\delta$). Acetylcholine binds to each of the two α subunits, and there is evidence that high-affinity noncompetitive blockers of ion channel activity can bind close to a transmembrane domain of each subunit (Oberthür et al., 1986; Giraudat et al., 1986).

The role of disulfide and sulfhydryl groups in receptor function has been of interest largely as a result of the pioneering chemical modification studies of Karlin and co-workers beginning in the 1960s [see Karlin (1983)]. It has now been clearly established that a disulfide bond between Cys-192 and Cys-193 on the α subunit is near the acetylcholine binding site (Kao & Karlin, 1986) and that this disulfide fortuitously makes it possible to affinity label and characterize the binding site following mild reduction with dithiothreitol. Recently, it has been shown that alkylation of free sulfhydryl groups with relatively nonpolar alkylating agents can block the ion channel conductance of AChR without affecting any of the ligand binding properties, including the ligand-induced transitions from low- to high-affinity binding (Huganir & Racker, 1982; Walker et al., 1984; Yee et al., 1986; Clark & Martinez-Carrion, 1986). In the experiments of Yee et al. (1986) using purified, reconstituted AChR, the labeling was enriched in the γ subunit with lesser labeling of the α subunit. Yee et al. (1986) further showed that the labeling was associated with either a membrane or a cytoplasmic domain based on trypsin

† Supported by Grant NS 22941 from the National Institute of Neurological and Communicative Disorders and Stroke. L.P. was a recipient of a Rhone-Poulenc Inc. fellowship. A preliminary account of this research has been presented (Pradier et al., 1987).

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[1] Abbreviations: AChR, acetylcholine receptor; ACh, acetylcholine; BGT, α-bungarotoxin; MOPS, 3-(*N*-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; NPM, *N*-phenylmaleimide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; NEM, *N*-ethylmaleimide.

0006-2960/89/0428-6562$01.50/0

hydrolysis experiments with intact membranes. Clark and Martinez-Carrion (1986) observed labeling in all four AChR subunits using native, alkaline-extracted membranes. In related studies, Mosckovitz and Gershoni (1988) characterized the seven sulfhydryl groups in isolated α subunits and concluded that Cys-222 was a free sulfhydryl while the other six were paired (128–142; 192–193; 412–418), although the evidence of the 412–418 pairing is very indirect.

In this paper, further characterization of the functional role of the free cysteines on the γ subunit is presented. Peptide mapping is used to identify more precisely which sulfhydryl groups are labeled, and site-directed mutagenesis is used to alter the sequence of this subunit. Mutations of identified cysteine residues into serine or phenylalanine residues are performed in order to determine what effect the free or modified sulfhydryls have on receptor function.

Materials and Methods

Purification of AChR and Chemical Modifications. AChR was purified from frozen *Torpedo californica* electric organ by affinity chromatography exactly as described by Yee et al. (1986). The AChR was labeled with 2 μM *N*-[^{3}H]phenylmaleimide ([^{3}H]NPM) in the presence of cholate as described by Yee et al. (1986), except that the scale of the reaction was larger. Typically, 100 μL of [^{3}H]NPM (40–60 Ci/mmol; 100 μCi; Amersham) was dried under argon and then resuspended in 10 μL of dimethyl sulfoxide (DMSO). One milliliter of purified AChR at 1–1.5 mg/mL protein in buffer A (100 mM NaCl, 10 mM MOPS, 0.1 mM EDTA, and 0.02% NaN_3, pH 7.4) containing 1% sodium cholate and 1 mg/mL dioleoylphosphatidylcholine was added, and the mixture was incubated for 30 min at 25 °C. The reaction mixture was then dialyzed against buffer A to remove unreacted NPM.

Purification of AChR Subunits. The individual AChR subunits were isolated by SDS gel electrophoresis followed by electroelution as described below. To 1 mL of NPM-labeled AChR were added 0.6 mL of 10% SDS (in H_2O), 0.25 mg of dithiothreitol (DTT), and then 20 mg of *N*-ethylmaleimide (NEM) to give 1 mM DDT and 100 mM NEM. To the reduced, alkylated AChR were added 250 μL of 1 M Tris-HCl, 200 μL of glycerol, 100 μL of β-mercaptoethanol, and 20 μL of 16 mg/mL bromophenol blue (Laemmli buffer). Subunits were resolved on a 5–10% gradient polyacrylamide slab gel (1.5 mm thick, full width loading lane) using the Laemmli buffers (Laemmli, 1970). Bands were visualized with 3 M sodium acetate (Higgins & Dahmus, 1979) and excised from the gels. The gel slices were rinsed with elution buffer (50 mM ammonium bicarbonate in 0.1% SDS), loaded into dialysis tubing, and attached to a Western blot apparatus. Electroelution was carried out for 12 h at 300 mA. The eluted samples were removed from the dialysis bags and lyophilized. In all steps, precautions were taken to avoid proteolysis; acid-washed glassware was used, and dialysis tubing was boiled in 2% bicarbonate, then in 1 mM EDTA, and finally in 1% SDS.

Cyanogen Bromide. The isolated γ subunit was dissolved in 400 μL of buffer A and treated again with 1 mM DTT and 20 mM NEM, and the excess reagents were removed by dialysis against 0.1% SDS in water. All subsequent reactions were carried out in sealed microcentrifuge tubes. For CNBr reactions, 50 μL of subunit ($\sim$10 μg) was added to 200 μL of 87.5% formic acid (Fluka; high purity), and 25 μL of 2 M CNBr in 70% formic acid was then added. To a control sample was added 25 μL of 70% formic acid without CNBr. Both samples were flushed with argon and incubated in the dark at room temperature for 48 h. The reaction mixture was diluted into 8 mL of water and lyophilized to remove formic acid. The lyophilization was repeated 3 times, and the CNBr digestion products were resolved by SDS–PAGE on 15% gels.

Cyanogen bromide cleavage of the subunit at tryptophan residues rather than methionine was carried out by using the procedure of Huang et al. (1983). Briefly, 50 μL of purified subunit (10 μg) was lyophilized, and 4.9 μL of oxidation mixture was added (prepared by mixing 300 μL of glacial acetic acid, 150 μL of 9 N HCl, and 40 μL of DMSO). The mixture was incubated for 30 min at room temperature and then chilled on ice; 44 μL of cold concentrated ammonia was added, and then 40 μL of 60% formic acid containing 0.3 mg/mL cyanogen bromide was added. The mixture was incubated for 24 h at room temperature and then treated as above for normal cyanogen bromide reactions.

Amino Acid Analysis. Purified AChR was labeled with NPM at two different concentrations (0.1 and 1 mM), and the γ subunit was separated and isolated as described above. The samples were dialyzed extensively against 0.1% SDS in water and then lyophilized in hydrolysis tubes. The concentration of the subunit was estimated by comparing the intensity of Coomassie Blue staining of the isolated subunit with an AChR preparation of known concentration following SDS–PAGE. Amino acid analysis, including cysteic acid quantitation, was carried out by the Protein Structure Laboratory at UC Davis. The number of Cys residues per subunit was calculated by using the known composition and Val as a reference amino acid. Similar results were obtained using Phe.

SP6 Constructs. The full-length cDNAs for the four *T. californica* AChR subunits were generous gifts from Dr. N. Davidson, California Institute of Technology (α, γ, and δ), and T. Claudio, Yale University (β). All four were subcloned downstream to the specific *Salmonella* phage SP6 promoter. For both α and δ (White et al., 1985), the *Pst*I–*Xho*I restriction fragment containing the subunit cDNA was inserted into the *Pst*I–*Sal*I sites of the plasmid pSP64 (Melton et al., 1984) to give the constructs pαL and pδL. Plasmid pSS2-β (Claudio, 1987) was cleaved with *Eco*RI and the β-subunit cDNA fragment subcloned in the *Eco*RI site of the vector pSP65 (construct pβ). The 3.2-kb *Nco*I–*Hin*cII restriction fragment of plasmid pSP64-Xβm (Krieg & Melton, 1984) was ligated to the γ-subunit cDNA (Ballivet et al., 1982) cleaved at *Nco*I–*Pvu*II sites (construct pXγ).

Several alternate constructs were also designed. Plasmid pαL was partially digested with *Bgl*II. It was further cleaved with *Bam*HI and the complete α-subunit coding sequence inserted in the *Bam*HI site of pSP64 (construct pα). Alternatively, the *Bgl*II site was filled in with DNA polymerase I (Klenow fragment) and the plasmid further cleaved with *Eco*RI. This fragment was inserted in the *Bal*I–*Eco*RI site of plasmid pSP64-Xβm (construct pXα). Plasmid pβ was linearized with *Eco*RI, and ends were filled in with DNA polymerase I (Klenow fragment). The fragment generated by a second *Eco*RI cleavage was then inserted into plasmid pSP64-Xβm at the *Bal*I–*Eco*RI site (construct pXβ). Finally, the *Sac*I restriction fragment of clone pδL was inserted in the *Sac*I site of pSP65 (construct pδ).

In Vitro Transcription. The SP6 constructs were linearized at the 3′ end of the insert with *Sma*I except for pXβ (*Sac*II). In vitro transcription was carried out as described (White et al., 1985) in the presence of the cap analogue diguanosine triphosphate, m^{7}G(5′)ppp(5′)G, for efficient capping of the transcripts. Reactions (volume 100 μL) usually included 5–10 μg of linearized plasmid and 400 units/mL SP6 polymerase (Promega) and were incubated for 1 h at 37 °C. Nucleotide

concentrations were the following: ATP, UTP, and CTP, 500 μM; GTP, 100 μM; G(5′)ppp(5′)G, 500 μM. Trace amounts, 10 μCi/mL, of [^{3}H]UTP (40–60 Ci/mmol, Amersham) were included to monitor transcription yields. DNA templates were removed by a 10-min incubation with 2 units/mL RNase-free DNase (grade DPRF, Worthington). Reactions were phenol extracted twice, and unincorporated nucleotides were removed by the spun-column method (5Prime-3Prime, Inc.) followed by two successive ethanol precipitations. Aliquots were electrophoresed on a formaldehyde-containing agarose gel and treated for fluorography to ensure transcript integrity. Aliquots were also quantitated by liquid scintillation counting.

In Vitro Translation. A mRNA-dependent rabbit reticulocyte lysate (gift from Dr. J. Hershey, UC. Davis) was used for analysis of in vitro translation as directed. Generally, 0.2–0.4 μg of individual transcripts was incubated for 1 h at 30 °C in 25 μL of lysate in the presence of [^{35}S]methionine (5 μCi, >600 Ci/mmol, Amersham). Translation efficiency was assayed by measuring levels of incorporated acid-precipitable counts. Aliquots of 3 μL were diluted in 1 mL of water and incubated for 10 min at 37 °C in 0.3 M KOH. Incorporated counts were then precipitated on ice in 8% trichloroacetic acid. Precipitates were recovered by filtration on glass disks (GF/C, Whatman) which were then counted in scintillation fluid. Reaction products were also analyzed on a 10% SDS–acrylamide gel followed by fluorography.

Site-Directed Mutagenesis. The *Sal*I–*Sma*I 800 base pair 3′-terminus fragment of the γ-subunit cDNA was subcloned in the vector M13 mp9 (Messing, 1983). The 21-mer oligonucleotides corresponding to mutations Cys-416 to Ser (or Phe) [AGCCTCGACG*G*(*A*)AGGATTTTAT] and Cys-420 to Ser (or Phe) [AATAAAGTTG*G*(*A*)AAGCCTCGAC] were obtained from the Protein Structure Research Laboratory at UC, Davis. They were purified on a 20% acrylamide/8 M urea gel as described (Lloyd et al., 1986) and phosphorylated using T4 polynucleotide kinase. The first mutation from Cys-416 to Ser was performed in vitro using the double-primer extension method (Zoller & Smith, 1983). Putative mutants were identified by differential hybridization. All other mutations were prepared by the thionucleotide incorporation method (Taylor et al., 1985) using the oligonucleotide-directed in vitro mutagenesis system (Amersham). Double mutations were obtained by performing a second mutagenesis round on single-mutant templates. Mutations were confirmed by dideoxynucleotide sequencing (Sanger et al., 1977). As a control, the complete 800-base insert was sequenced to ensure that no other mutation had been introduced. The mutated inserts were then subcloned back in the full-length pXγ construct to give clones pXγSer416 (serine-416 mutant), pXγSS (double-serine mutant), pXγF416 (phenylalanine-416 mutant), and pXγFF (double-phenylalanine mutant).

Expression in Oocytes. Xenopus laevis frogs were maintained in the laboratory of Dr. R. Nuccitelli, Department of Zoology, UC, Davis. Ovarian lobes were obtained from anesthesized *X. laevis* frogs and dissected into clumps of 20–40 oocytes. Follicle cell layers were removed by incubation in Ca^{2+}-free Oocyte Ringer (Ca^{2+}-free OR2; Wallace et al., 1973) containing 2 mg/mL collagenase (type 1A, Sigma) at room temperature with shaking for approximately 2 h. Oocytes were then washed thoroughly in OR2, and stage V and VI oocytes were transferred to modified Barth saline (MB; Colman, 1984) supplemented with 2.5 mM pyruvate, 10 μg/mL streptomycin, 100 μg/mL gentamycin, 0.5 mM theophylline, and 0.25 mg/mL BSA (fraction V, Miles Scientific) and sterile-filtered.

Transcripts of the different subunits were mixed at a α:β:γ:δ ratio of 2:1:1:1 at a total RNA final concentration of 200 ng/μL. Injections were performed using a 10-μL micropipetor (Drummond Inc.) and glass injection needles of tip diameter 25–30 μm. Approximately 10 ng was injected per cell, and 35–50 oocytes were injected for each transcript mix. Cells were incubated at 20 °C in supplemented MB and the media changed daily.

Priming of *Xenopus* frogs with human chorionic gonadotropin hormone (50–100 units, Sigma) or pregnant mare serum (50 units, Sigma) 2 days before surgery yielded oocytes enriched in stages V and VI and giving rise to significantly larger responses (Stith & Maller, 1985).

Electrophysiology. ACh-induced whole cell current responses were measured by using an Axoclamp 2A two-electrode voltage-clamp (Axon Instruments). Electrodes were filled with 3 M KCl and had a tip resistance of 0.5–5 MΩ. All measurements were performed 2 days after injection. In all experiments, both wild-type and a given mutant were tested, and recordings were performed in a staggered fashion, five to eight oocytes of each type consecutively. The recording chamber (volume approximately 1 mL) was continuously perfused with modified OR2 (MOR2: 82 mM NaCl, 2.5 mM KCl, 1 mM Na_2HPO_4, 5 mM $MgCl_2$, 0.2 mM $CaCl_2$, and 5 mM HEPES, pH 7.4) at a rate of 15–20 mL/min. A system of valves and reservoirs was used to superfuse agonists and antagonists. Atropine (0.5 μM) was present in all agonist solutions. The holding potential in all experiments was −80 mV, and the ACh concentrations used for the dose–response curves ranged from 0.2 to 1 μM. Tubocurarine IC_{50} (White, 1987) was determined by measuring current responses to 1 μM ACh in the presence of increasing *d*-tubocurarine concentrations (5–400 nM). A recovery period of 10 min was allowed between agonist applications.

External Toxin Binding. Levels of cell surface acetylcholine receptors were measured on the day following recordings. Healthy oocytes were incubated individually with 2 nM ^{125}I-BGT (>200 Ci/mmol, Amersham) in MOR2 solution containing 2 mg/mL BSA for 2 h at room temperature. Cells were then washed 3 times in MOR2 and transferred to new tubes, and levels of bound bungarotoxin were measured in a γ counter. Nonspecific binding was estimated by using noninjected oocytes.

Oocyte Membrane Isolation. Oocyte membrane fractions were prepared by homogenizing 20–30 oocytes in 800 μL of ice-cold homogenization buffer (83 mM NaCl, 1 mM $MgCl_2$, and 10 mM HEPES, pH 7.9; Blondeau & Baulieu, 1985) with 5 mM EDTA, 5 mM EGTA, and 1 mM PMSF in a Dounce homogenizer. Homogenates were centrifuged at 800*g* for 10 min at 4 °C to remove cortical granules and pigments. This step was repeated and the supernatant loaded onto a 15% sucrose layer (150 μL) and centrifuged at 160000*g* for 1.5 h at 4 °C. Membrane pellets were resuspended in NMT-100 buffer (100 mM NaCl, 10 mM MOPS, and 0.2% Triton X-100) at 8 μL per oocyte and kept in liquid nitrogen. Total protein yield was measured by a Lowry assay using BSA as a standard to ensure that all preparations had similar yields (~15 μg of protein/oocyte). Levels of α subunit in these extracts were measured by incubation of a membrane aliquot (corresponding to 1.5 oocyte) in 2 nM ^{125}I-BGT in NMT-100 for 1 h at room temperature and filtration on DE 81 paper (Walker et al., 1981). To account for time-dependent inactivation of the labeled toxin (Weber & Changeux, 1974), the actual specific activity of ^{125}I-BGT was normalized in all experiments using a *T. californica* AChR standard. Both a

Table I: Cyanogen Bromide Peptide Fragments of the *Torpedo* AChR γ Subunit[a]

Cleavage at Methionine[b]				
Met position	no. of amino acids in peptide	estd mol wt	no. of Cys residues	positions of Cys residues
116	116	14.5	0	
291	176	22.0	4	128, 142, 230, 252
295	5	0.6	0	
299	5	0.6	0	
335	37	4.6	1	301
358	24	3.0	0	
374	17	2.1	0	
390	17	2.1	0	
489[c]	100	12.5	3	416, 420, 451
Cleavage at Tryptophan[d]				
Trp position	no. of amino acids in peptide	estd mol wt	no. of Cys residues	positions of Cys residues
55	55	6.9	0	
60	6	0.8	0	
67	8	1.0	0	
118	52	6.5	0	
139	22	2.8	1	128
170	32	4.0	1	142
184	15	1.9	0	
197	14	1.8	0	
440	244	30.5	5	230, 252, 301, 416, 420
453	14	1.8	1	451
489[c]	37	4.6	0	

[a] Sequence of *Torpedo* γ subunit obtained from Numa et al. (1983). [b] Cyanogen bromide cleavage at methionine residues carried out as described under Materials and Methods. Length of peptides and estimated molecular weight calculated ($\times 10^{-3}$) from sequence. [c] Amino acid 489 represents the C term. [d] Cyanogen bromide cleavage at tryptophan residues carried out as described under Materials and Methods.

standard curve and saturating binding levels were used, and specific activities of 50–65% of manufacturer's values were obtained.

Immunoblot. Rabbit antisera were raised against SDS-denatured AChR (A. Yee, unpublished data). The sera were further affinity purified by using diazophenyl thioether paper bound AChR subunits (Kim & Dahmus, 1986). Oocyte membrane aliquots (corresponding to five cells) were run on a 10% acrylamide SDS–PAGE gel and transferred onto an Immobilon membrane (Millipore). All further steps were performed according to Dahmus et al. (1988). Blots were probed with the purified antibodies, and alkaline phosphatase linked anti-rabbit antibodies (Promega) were used as secondary antibodies. Color reaction was performed according to manufacturer's directions.

Results

The experiments described here are designed to identify the free cysteine residues on the γ subunit that are labeled by *N*-phenylmaleimide under conditions that lead to inhibition of ion flux activity and then to modify these sulfhydryl groups using site-directed mutagenesis. The functional role of the groups is then analyzed by expressing the altered receptors in *Xenopus laevis* oocytes and measuring ligand binding and ion channel properties.

Localization of Labeled Sulfhydryl Groups. The *Torpedo* acetylcholine receptor γ subunit has eight Cys residues based on the deduced amino acid sequence of the cDNA. If one or more of these groups are specifically labeled by *N*-[^{3}H]-phenylmaleimide, peptide mapping can reveal the location of the labeled group since the location of cleavage sites is known from the protein sequence. Table I shows how cyanogen bromide fragmentation under two different sets of reaction conditions can localize the labeled groups. Although nine peptide fragments are expected using cleavage at methionine, only three of them contain Cys residues, and the three have very different molecular weights. In order to ensure that labeling with *N*-phenylmaleimide was restricted to cysteine residues, as expected, amino acid analysis of labeled AChR γ subunit was carried out after the AChR had been reacted with NPM at two different concentrations. Under labeling at 1 mM NPM, the number of Cys groups per subunit was reduced to 5.2 from a control value of 8.4 while the number of lysine groups was unaffected. The results are consistent with the labeling of two to three Cys groups at the highest NPM concentration. Previously, Yee et al. (1986) showed by quantitative analysis of labeled SDS gel slices that approximately two Cys groups were labeled at 500 μM NPM, conditions that inhibited ion channel activity.

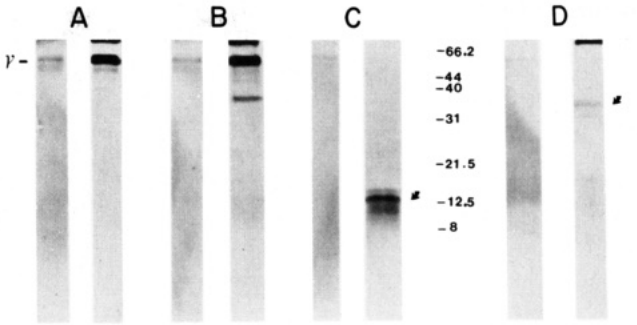

FIGURE 1: Cyanogen bromide digestion of NPM-labeled γ subunit. The [^{3}H]NPM-labeled *Torpedo* AChR subunits were separated by SDS–PAGE electrophoresis, and the γ subunit was isolated by electroelution. A 10-μg aliquot was digested with cyanogen bromide either at methionine residues or at tryptophan residues as described under Materials and Methods. The digestion products were separated on a 15% polyacrylamide gel and visualized by Coomassie Blue staining to show protein bands and by fluorography after 3-days exposure to show NPM-labeled bands. For each pair of gel photographs, the one on the left is the Coomassie-stained gel, and the one on the right is the corresponding fluorograph. (A) Untreated γ subunit; (B) formic acid treatment only; (C) cyanogen bromide digestion at methionine; (D) cyanogen bromide digestion at tryptophan. The molecular weight markers were run in a separate lane, and the arrows in (C) and (D) represent the major labeled bands.

Cyanogen bromide fragmentation of the isolated, labeled γ subunit revealed the presence of a radioactively labeled 12-kDa band on SDS gels (Figure 1). No labeling was detected at 22 and 4.6 kDa, the other two fragment sizes that should contain Cys residues. This simple analysis identified Cys-416, -420, and -451 as possible target sites for labeling. Treatment with cyanogen bromide under conditions that should result in selective cleavage at tryptophan residues gave only limited proteolysis as judged by SDS gel electrophoresis. A major band is present at 34–35 kDa. Fainter bands at 36 and 31 kDa have also been consistently noticed and correspond to the pattern expected from partial cleavage of a region including the large 30.5-kDa fragment (Table I). The small peptide including Cys-451 could be undetected under the gel conditions used, but no labeling was present in the 6.4-kDa region corresponding to partial cleavage at position 440. This analysis confirms the localization of the NPM-derivatized residues to the C-terminus but cannot conclusively resolve labeling of Cys-416 and -420 from Cys-451. Since at least two of those groups were probably labeled, no further effort to resolve them was carried out. Cys-416 and -420 are located in a region of the polypeptide chain that has been postulated

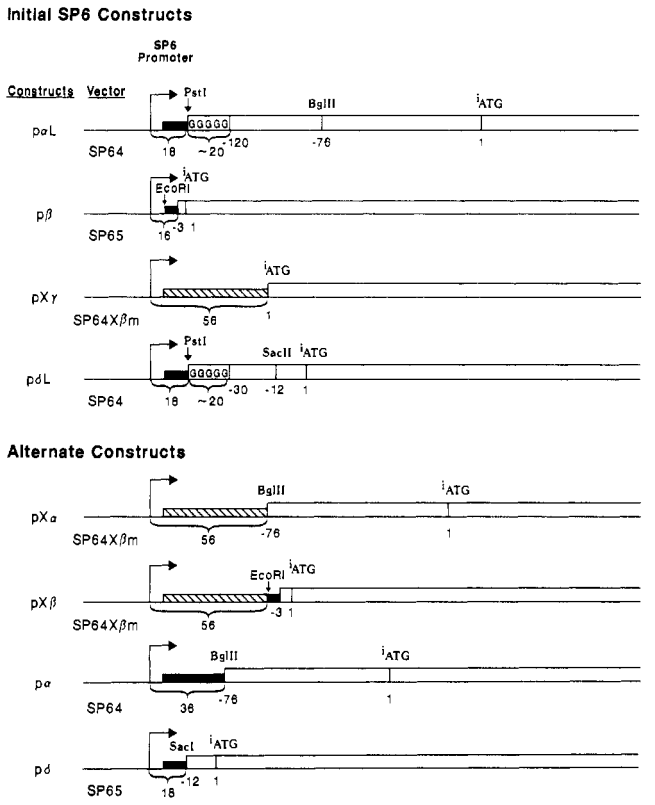

FIGURE 2: 5′-Noncoding region of SP6 constructs of the four *T. californica* AChR subunits. The cDNAs coding for the four AChR subunits were subcloned downstream to the SP6 promoter (see Materials and Methods) in different vectors as indicated. The SP6 promoter and the initiation codon are shown as well as relevant restriction sites. Positions are given with reference to the initial ATG, and the length of extraneous sequences added during cloning is indicated. The hatched segments represent the *Xenopus* globin 5′-noncoding region from plasmid SP64-Xβm. Remains of the multiple cloning site are shown in solid segments.

to form an amphipathic helix. In one of the models for AChR structure, this region is believed to form part of the ion channel (Finer-Moore & Stroud, 1984). In all models, this domain is either within the membrane or in the cytoplasmic region, consistent with the previous proteolysis data obtained by Yee et al. (1986). On the basis of the possible relevance of one or both of these groups to ion channel function, the initial experiments described below were designed to modify these groups by site-directed mutagenesis and to examine the functional effects of the modifications on AChR function.

SP6 Constructs. The cDNAs coding for the four subunits of AChR were subcloned downstream to the *Salmonella* phage SP6 promoter using convenient restriction sites (see Figure 2). The α- and δ-subunit coding sequences were obtained in the Okayama–Berg cloning vector (Okayama & Berg, 1983). The cDNAs, including a significant portion of the 5′-noncoding region and flanked by two homopolymeric tails, were subcloned 3′ to the SP6 promoter. The γ-subunit cDNA (Ballivet et al., 1982) starting at the initial ATG was subcloned downstream to the complete 5′-noncoding region of the *X. laevis* β-globin gene as recommended by Krieg and Melton (1984). The β-subunit cDNA (pSS2β; Claudio, 1987) including three bases upstream to the initial ATG was subcloned into plasmid SP65. The 5′-noncoding region of all constructs is described in Figure 2.

All constructs were linearized and transcripts synthesized in vitro as described under Materials and Methods. Transcripts of the correct size were obtained as judged by gel electrophoresis (data not shown).

In Vitro Translation. Translation efficiency of the transcripts was tested in a rabbit reticulocyte lysate translation system. Individual transcripts (0.2–0.4 μg) were incubated for 1 h in lysate in the presence of [^{35}S]methionine. Incorporated acid-precipitable counts were measured, and fluorographic analysis of an SDS–PAGE gel of the reaction products was performed.

Translation levels of transcripts pαL, pδL, and pβ were very low, less than twice background levels of incorporated counts, even if peptides of the correct molecular weight were synthesized as judged by fluorography (data not shown). The pXγ transcripts were, however, very efficiently translated up to 20 times background levels.

Expression in Oocytes. Despite their low translation efficiency in vitro, these transcripts were tested in *Xenopus* oocytes (see Materials and Methods) since in vivo translation requirements could be different. Under whole cell voltage-clamp conditions, no ACh-induced current response was observed. In contrast, large ACh responses were elicited from oocytes injected with poly(A+) RNA isolated from *T. californica*. On the basis of the in vitro experiments, it appeared likely that translation of the SP6 transcripts was inhibited. More specifically, the initiation step and the 5′-noncoding region seemed involved since polypeptides of the correct molecular weights were synthesized. A second set of SP6 clones was designed to further analyze translation requirements. The poly(G) sequence as well as part of the 5′-distal noncoding region was deleted from pαL and pδL to construct clones pα and pδ (Figure 2). Secondly, the *X. laevis* globin translation signal was inserted upstream from the shortened α and β cDNAs to construct clones pXα and pXβ.

In vitro translation levels of transcripts pα and pδ were up 36 and 17 times background values, respectively. Thus, deletion of the poly(G) sequence led to a very large increase in translation efficiency. Similar results have recently been reported (Jobling & Gehrke, 1987) using the cDNA sequences of human interleukin 1 and barley α-amylase genes.

The pXβ transcripts were also translated at a much higher efficiency, 11-fold enhancement over pβ. Thus, insertion of the globin translation signal right upstream to the coding sequence also increased translation levels. However, replacement of that signal by part of the SP64 vector multiple cloning site led to a significant increase in translation efficiency of the α subunit (pXα and pα, 1.7-fold enhancement), arguing against any specific role of the globin 5′-noncoding sequence.

Injection of the new transcripts (pα or pXα, pXβ, pXγ, pδ) in *Xenopus* oocytes led to expression of functional AChR as measured under voltage-clamp conditions. ACh current responses on the order of 100 nA were obtained (1 μM ACh, V_h = −80 mV). The relative expression of the different subunits was measured indirectly by Western blot analysis. The δ-subunit levels appeared much lower than its counterparts. Recently, functional "deltaless" receptors have been successfully expressed in oocytes (Kurosaki et al., 1987; White, 1987) and δ-limiting conditions might have led to the expression of a heterogeneous population of wild-type and "deltaless" receptors. When the construct pSP64Tδ (Claudio, 1987) was used, much higher ACh current responses were obtained (800 nA) correlating with higher δ-subunit synthesis levels comparable to the other subunits as judged on Western blot (Figure 6). The pSP64Tδ plasmid contains both the 5′- and 3′-nontranslated region of the *X. laevis* globin gene, the latter possibly important for efficient translation in vivo (Drummond et al., 1985). Despite the discrepancy between in vitro and in vivo translation efficiency for the pδ construct, the rabbit

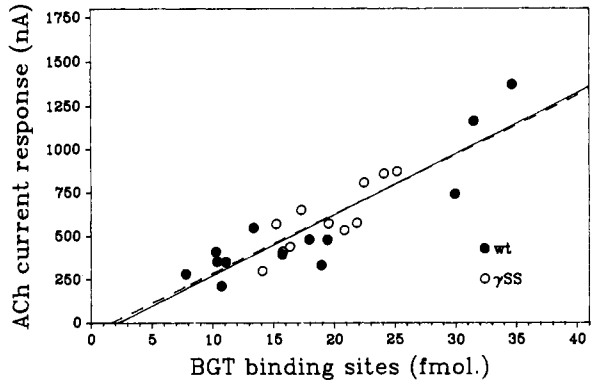

FIGURE 3: Correlation of ACh-induced current response with α-bungarotoxin binding sites in oocytes expressing wild-type or mutant Cys → Serγ416,420 (γSS) AChR. Oocytes were injected with transcripts coding either for wild-type or for mutant γSS AChR (see Materials and Methods). ACh-induced whole cell currents were recorded under voltage-clamp on day 3 after injection, and cell surface BGT binding was measured on the same individual oocytes the next day using ^{125}I-BGT: (●) wild type; (○) mutant γSS. The normalized activities are the slopes of the least-squares fit regression lines: wt, solid line, s = 35 nA/fmol. BGT binding site, r = 0.90. γSS, dashed line, s = 34nA/fmol. BGT binding site, r = 0.92.

reticulocyte translation system has been a useful tool to predict in vivo translation efficiency of all other SP6 constructs.

All further experiments were performed with injection mixes made of pα or pXα, pXβ, pXγ (wild-type or mutant), and SP64Tδ transcripts in the ratio 2:1:1:1. Whole cell functional responses were recorded 2 days after injection using a two-electrode voltage-clamp. In contrast to earlier reports (Mishina et al., 1985; White et al., 1985), ACh responses were shown to be strongly desensitizing even at 1 μM ACh (peak response in 15–20 s). When slower rates of agonist superfusion were used, leveling-off responses could be obtained with a much slower time course (40–60 s) as previously published. However, amplitudes of the slow responses were only 60–80% of peak responses and probably reflected a concomitant desensitization. Only responses with a rise to peak within 20 s were selected for further analysis. The mean ACh current response was 800 nA, which is noticeably higher than previously reported (Tobimatsu et al., 1987; Buller & White, 1988).

The ACh-induced current response expressed in oocytes displayed the same pharmacological properties as reported by others (Mishina et al., 1985; White et al., 1985). Dose–response curves had a Hill coefficient of 1.7 (n = 3), showing strong cooperativity between the two ACh binding sites. The ACh response was atropine resistant and d-tubocurarine sensitive, specifying nicotinic properties. The tubocurarine IC_{50}, obtained on the electrophysiological response, was 80 nM, as reported by White (1987) and Yoshii et al. (1987). Low Ca^{2+} concentrations (0.2 mM) were used to minimize desensitization and to prevent activation of an endogenous Ca^{2+}-activated Cl^- conductance (Mishina et al., 1985). Under these conditions, a linear I/V curve was obtained with a reversal potential of approximately −5 mV as expected from a nonspecific cationic channel. A slight inward rectification was noticed at potentials more negative than −100 mV.

Levels of AChR expression on the cell surface were measured by using the bungarotoxin binding assay on intact oocytes as modified from Mixter Mayne et al. (1987). Functional responses were measured on day 2 after injection. Each oocyte was separately saved, and the cell surface binding assay was performed the next day on individual oocytes. Only cells which appeared healthy were used for this assay. Such an assay allowed for the correlation of the functional ion channel response to levels of surface receptor for individual oocytes.

Table II: Characterization of Cys-416 and -420 Mutations on the AChR γ Subunit[a]

	normalized act.[b] (nA/fmol)	total BGT binding sites[c] (fmol/oocyte)	dose–response Hill coeff[d]	tubocurarine IC_{50}[e] (nM)
WT	32 ± 2 (6)	76 ± 10 (5)	1.7	80
γSS	34 (1)	58	1.7	ND[f]
γF416	26 (3)	57 ± 23 (2)	1.5	70
γFF	22.8 (2)	93 ± 18 (2)	1.7	100

[a] Functional properties of γ-subunit AChR mutants on Cys-416 and -420 expressed in oocytes were studied. [b] Normalized activities were obtained as slopes of the first-order regression for the ACh current response vs BGT binding site plots; number of separate experiments indicated in parentheses. [c] Oocyte total membranes were prepared by homogenization (20–30 cells) and differential centrifugation. Levels of BGT binding were measured by incubating a membrane aliquot (1.5 oocyte) in 2 nM ^{125}I-BGT as described. Samples were filtered on DE81 paper (Whatman). All assays were performed in duplicate. [d] Whole cell ACh current response were measured under voltage-clamp conditions (V_h = −80 mV). ACh concentrations ranged from 0.2 to 1 μM. [e] ACh current responses were measured in the presence of increasing concentrations of d-tubocurarine (0–400 nM). Recovery periods of 10 min were allowed between applications, which was sufficient for complete recovery of the response. [f] ND, not determined.

As shown in Figures 3, 4, and 5, the two properties correlated reasonably well ($0.86 < r < 0.94$) provided a large number (>20) of oocytes was used. It was important to obtain data over a significant range of expression levels among oocytes. The intrinsic expression variability encoutered with oocytes was an advantage for the analysis. The range was further extended by injection of subsets of oocytes with RNAs diluted 10-fold. Those subsets displayed responses 3–6-fold lower than the normal average, providing data for low expression levels. Most of the regression lines calculated from the plots intercept axes close to but not at the origin. The small discrepancy is likely due to synthesis of new receptors in the lag time separating functional recordings from toxin binding assays (Buller & White, 1988). Reproducible normalized activities of channel response per BGT binding site were obtained from the slope of the first-order regression lines. For wild-type AChR, the value was 32 ± 2 nA/fmol of BGT binding site (six experiments) at V_h = −80 mV, 1 μM ACh. Fluctuations of 10% were observed but were mainly due to variations in the x-axis intercept. Comparable values can be calculated from published data: 11.2 (Kurosaki et al., 1987), 29 (Tobimatsu et al., 1987), and 80 nA/fmol (Yoshii et al., 1987). While these values are similar, there are significant variations even within the same laboratory. Since current response and expression level standard deviations are quite large in all cases, care has to be taken to calculate the normalized activity only on cells for which both whole cell current responses and cell surface toxin binding could be measured.

To study total expression levels, oocyte membrane fractions were prepared and subjected to Western blot analysis. Oocyte-synthesized receptor displayed bands corresponding to the four AChR subunits (Figure 6, lane D). α and β subunits migrated identically with the *T. californica* polypeptides. As observed by others (Kurosaki et al., 1985), the γ subunit seem to give rise to a doublet with the upper band migrating slightly faster than the *T. californica* peptide and of variable relative intensity (data not shown). The lower band is likely a proteolytic product. The oocyte-synthesized δ subunit also appeared to have a slightly lower molecular weight than its *T. californica* counterpart.

Protein synthesis levels were analyzed by comparing band intensities on Western blot to standards (Figure 6). For the

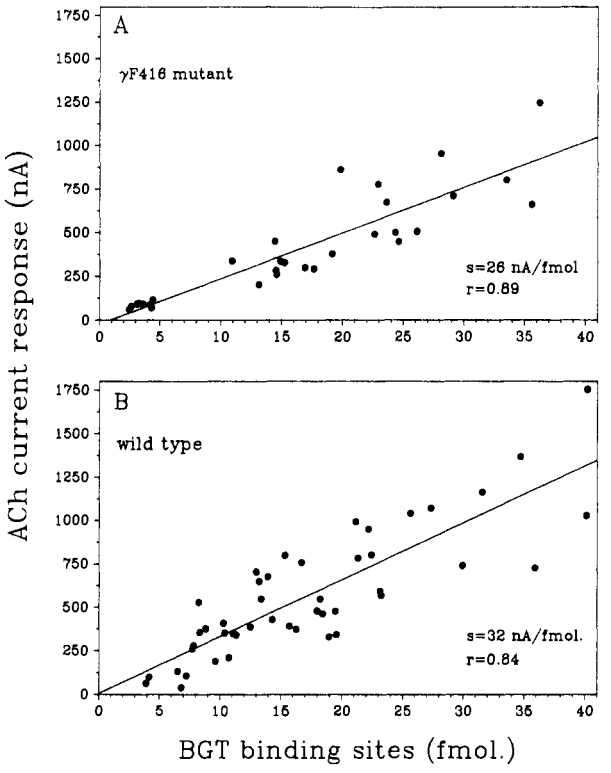

FIGURE 4: Comparison of normalized activity for wild-type and mutant Cys → Pheγ416 (γF416) AChR. Cell surface BGT binding site levels and ACh whole cell currents were measured on the same cells as described in Figure 3. (A) Cells expressing γF416 AChR mutant; normalized activity s = 26 nA/fmol. BGT binding site, r = 0.89. (B) Cells expressing wild-type receptor; normalized activity s = 32 nA/fmol. BGT binding site, r = 0.84. In both (A) and (B), results from three experiments were pooled.

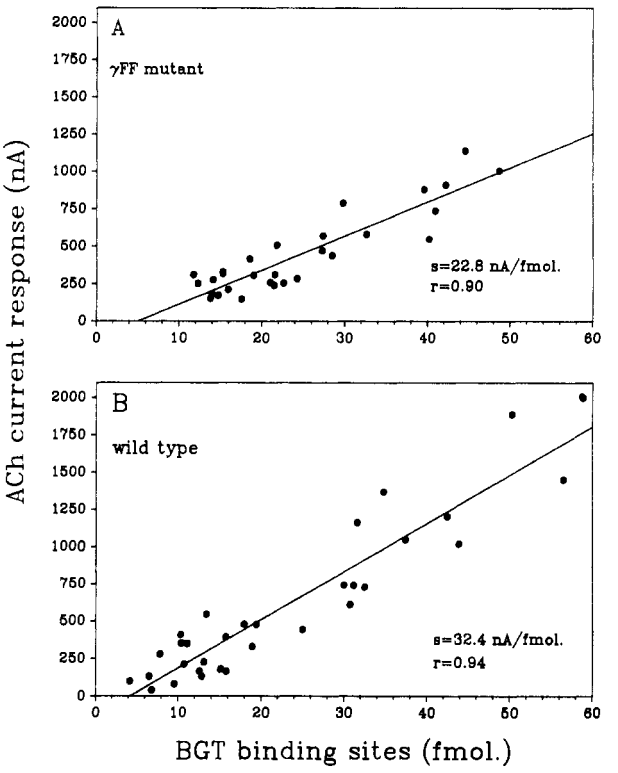

FIGURE 5: Comparison of normalized activity of the double-mutant Cys → Pheγ416,420 (γFF) and wild-type AChR. Cell surface BGT binding site levels and ACh whole cell currents were measured on the same cells as described. (A) Cells expressing γFF AChR mutant; normalized activity s = 22.8 nA/fmol. BGT binding site, r = 0.90. (B) Cells expression wild-type receptor; normalized activity s = 32.4 nA/fmol. BGT binding site, r = 0.94. Results from two experiments were pooled, but both gave identical normalized activities, and only slight changes in the x-axis intercept were observed.

equivalent of five oocytes, synthesis levels of the different subunits ranged from 100 to 400 fmol which is in agreement with the value of 80 fmol of BGT binding site/oocyte obtained by direct ^{125}I-BGT binding on the same membrane fractions (Table II).

Site-Directed Mutagenesis. The functional role of cysteine residues 416 and 420 on the AChR γ subunit was investigated by mutagenesis. Ion flux inhibition by NPM alkylation, observed in the chemical modification experiments, could result either from the block of the free sulfhydryl groups or from steric hindrance by the phenylmaleimide moiety. Both hypotheses were studied by oligonucleotide-directed mutagenesis. Mutations coding for serine residues at one (mutant γSer416) or both positions (mutant γSS) were generated to probe the role of the free sulfhydryls. Similar mutations to phenylalanines (mutant γF416 and γFF) were also synthesized to partially mimic the phenylmaleimide steric hindrance.

The last three mutations were synthesized as described under Materials and Methods using the thionucleotide incorporation method (Taylor et al., 1985). Mutation efficiency was above 90% as compared to 4% for the double primer extension method. Direct identification of mutants by sequencing was then possible, greatly facilitating the mutagenesis step.

Replacement of both cysteines by serine residues (γSS) or of Cys-416 to phenylalanine (γF416) did not affect the ionic properties of the channel as measured with our macroscopic assay (Figures 3 and 4). The normalized activities for γSS and γF416 were respectively 34 and 26 nA/fmol, not significantly different from wild type. The I/V curves for both mutants were linear (data not shown), and their ligand binding properties were not affected as measured by the Hill coefficient of the dose/response curve and the tubocurarine IC_{50} (Table II). Total expression levels in oocytes were also comparable to wild type both on the membrane extract BGT binding assay (Table II) and by Western blot analysis (data not shown). In preliminary analysis, the γSer416 mutant also displayed properties identical with wild-type AChR.

The γFF double mutant normalized activity was 22.8 nA/fmol (Figure 5, r = 0.90, 29 cells). This value is 30% lower than for the wild-type receptor (32 nA/fmol) at comparable expression levels. This difference is clearly significant ($P <$ 0.01, two-tail Student's t test). None of the other properties of the whole cell ACh response (linear I/V curves, n_H = 1.7, tubocurarine IC_{50} = 100 nM) differed from wild-type receptor, and BGT binding site levels were not affected by the mutation either on the cell-surface or on membrane fractions (Figure 5 and Table II). Polypeptide synthesis on those fractions as measured by Western blot analysis was also not affected (Figure 6, lane E). In conjunction with the normal ligand binding properties of the whole cell response, these results tend to rule out any effect on the receptor BGT binding affinity which could have resulted in an altered normalized activity. The decrease in ACh current response is thus specific to the ion channel properties of the receptor as observed with the NPM chemical modification, but the degree of inhibition is lower.

Discussion

The approach developed here is to use site-directed mutagenesis to selectively alter amino acid residues on the AChR that are believed to play a direct role in the function of the protein. Chemical modification analyses carried out by Yee et al. (1986) established a correlation between the labeling of free cysteine residues on the protein and the inhibition of ion

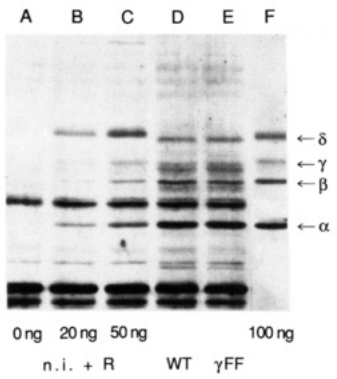

FIGURE 6: Western blot analysis of AChR double-phenylalanine mutant (γFF) synthesized in *Xenopus* oocytes. Aliquots of oocyte total membrane fractions (see Materials and Methods) corresponding to five cells were run on SDS–PAGE and transferred onto Immobilon membranes. Lanes A–C: Noninjected oocyte membranes with increasing amounts of *Torpedo*-purified AChR added after the membrane preparation; 0 ng (lane A), 20 ng (lane B), and 50 ng (lane C). Lane D: Oocyte injected with wild-type AChR transcripts. Lane E: Oocyte injected with the mutant γFF AChR. Lane F: 100 ng of purified AChR.

channel activity. An interesting aspect of the labeling was the apparent uncoupling of the channel inhibition effects from the ligand binding properties, which were unaffected, suggesting that the labeling was physically close to the ion channel itself. The labeling with *N*-phenylmaleimide was primarily associated with the γ subunit. This subunit has recently been shown to be associated with receptor desensitization (Sumikawa & Miledi, 1989).

In order to focus the mutagenesis experiments on the most functionally relevant sites, further efforts to localize the labeled sulfhydryl groups were carried out. By taking advantage of the known sequence of the receptor subunits and the chemical specificity of the maleimides for sulfhydryl groups, it was possible to deduce the location of the labeled cysteine residues using cyanogen bromide cleavage of labeled γ subunit followed by analysis of the peptides by SDS gel electrophoresis. The results obtained here are consistent with the labeling of Cys-416, -420, and -451. Since both the amino acid analysis results and the previous analysis of labeled subunits indicated that more than one cysteine was modified under conditions that gave maximal flux inhibition, it is likely that at least one of the two Cys-416 and -420 was labeled. A possible functional role for Cys-451 has not been ruled out, but in an initial attempt, we focused our attention on the first two cysteines which are particularly interesting since they are located in the amphipathic helix region of the γ subunit. Each subunit has such an amphipathic domain, and Finer-Moore and Stroud (1984) and Guy (1984) have speculated that these helices could assemble to form the ion channel. Only the γ subunit has cysteine residues associated with this domain, which may explain its selective labeling. Interestingly, the cysteine residues in the amphipathic domain of the γ subunit are conserved in all species for which sequences are available. It has been very difficult to obtain direct experimental evidence to support the amphipathic helix model of the ion channel. In fact, photoaffinity labeling studies with noncompetitive blockers and amino acid sequence comparison of AChR to other ion channel proteins suggest that transmembrane domain M2 is a more likely candidate for the ion channel region (Oberthür et al., 1986; Giraudat et al., 1986). Recent mutagenesis experiments (Imoto et al., 1986, 1988; Leonard et al., 1988) directly identified the permeability pathway to the M2 domain. To obtain additional information about the possible functional role of the amphipathic helix domain in receptor function, Cys-416 and -420 were modified by site-directed mutagenesis.

AChR expression was obtained by in vitro synthesis of SP6 RNAs coupled to injection in *X. laevis* oocytes. This system has now been largely used for synthesis of exogenous proteins (Krieg & Melton, 1984). However, little is known about the features of these artificial mRNAs which are necessary for efficient expression.

We have shown that removal of poly(G) sequences (15–20 bases long) at the 5′ end of transcripts led to a large increase in their translation efficiency in rabbit reticulocyte lysate. These results confirm a recent report by Jobling and Gehrke (1987) and are of practical importance since poly(G) tails are inherent to cDNA clones obtained by using the Okayama and Berg method.

The complete native 5′-untranslated region of the *Xenopus* β-globin gene did not specifically increase the SP6 transcripts' translation efficiency as similarly reported with the rabbit α-globin untranslated leader (Jobling & Gehrke, 1987). However, higher expression levels in vivo were obtained in a construct containing both the 5′- and 3′-untranslated regions of the *Xenopus* β-globin gene, and the latter could be a key element for high translation levels as proposed (Drummond et al., 1985). Further analysis of the requirements for efficient translation of SP6 transcripts will be reported elsewhere.

Expression in oocytes is the system of choice for the study of ion channels since ion flux activity can be detected by using electrophysiological techniques (Snutch, 1988). This system has been extensively used for the detailed characterization of AChR [see, e.g., Mishina et al. (1985), White et al. (1985), Imoto et al. (1986, 1988), and Leonard et al. (1988)].

Functional properties of oocyte-expressed AChR were analyzed by whole cell voltage-clamping. Large variations in ACh current responses were observed from cell to cell which may due to biological variability in expression. Characterization of the receptor activity therefore required normalization of the whole cell responses to the receptor cell surface expression levels. Both properties could be measured on the same cell by using ^{125}I-BGT binding to individual intact cells after electrophysiological recordings, and are reported in Figures 3–5. The two parameters correlated fairly well provided large number of cells were used and expression levels were spread over a large range. The normalized activity for wild-type AChR was 32 ± 2 nA/fmol of BGT binding sites at a holding potential V_h = −80 mV in response to 1 μM ACh (6 experiments, >90 cells). The normalized activity is a useful parameter which allows specific functional effects of mutations on AChR to be distinguished from perturbations of assembly and expression. Functionally relevant mutations can further be characterized by single-channel recordings, but this technique will not provide any direct information on mutations affecting expression levels (Imoto et al., 1988). Such mutations are important, however, since subunit folding and assembly into the pentameric complex may be involved and the mutations could provide new insights into the role of specific residues in the overall structure of the receptor.

Using chemical modification data to identify possible functional sites on AChR, we investigated the functional role of Cys-416 and -420 on the γ subunit by coupling site-directed mutagenesis to expression in oocytes. The requirement for free sulfhydryl groups at those positions was tested by mutagenesis to serine residues, and possible steric hindrance of the NPM moiety was analyzed by mutagenesis to phenylalanines.

Mutagenesis to serine at both positions or to phenylalanine at position 416 did not alter any of the receptor properties: normalized activity, expression levels, or binding properties.

Free sulfhydryl groups at positions 416 and 420 on the γ subunit are thus not necessary for normal function or expression.

The normalized activity of the double-phenylalanine mutant was decreased by 30% while neither expression levels nor binding properties were affected. Preliminary experiments using patch-clamp recordings did not reveal any difference in the single-channel conductance in 1 mM Ca^{2+} (data not shown). In one of the models proposed for AChR structure (Finer-Moore & Stroud, 1984; Guy, 1984), Cys-416 and -420 of the γ subunit are part of an amphipathic helix contributing to the receptor ion channel. Interestingly, both residues are one turn apart on the hydrophobic side of that helix, facing the other transmembrane spanning regions of the protein. Large steric mutations in the hydrophobic interior of a protein are expected to have large effects on stability (Terwilliger et al., 1988), and the small alterations of the double-mutant Cys $\rightarrow$ Pheγ416,420 properties argue against the presence of the putative amphipathic helix in a transmembrane helix bundle. This report complements results by Imoto et al. (1988) which indicated that mutations of charged groups on the amphipathic segment did not affect the single-channel conductance.

The limited decrease in mutant γFF ion channel function is also in contrast to the full inhibition observed under labeling at high NPM concentrations. Sterically, phenylalanine does not fully mimic the NPM-derivatized cysteine groups, which could explain the partial inhibition. On the other hand, other NPM alkylation sites could contribute to the full effect of chemical modification. Indeed, Cys-451, part of the M4 transmembrane domain, was shown to be a possible candidate, and recently, the α subunit has also been shown to be labeled by an analogous compound, *N*-pyrenylmaleimide (Schuchard et al., 1988). Further studies will focus on such sites.

ACKNOWLEDGMENTS

We are grateful to Drs. Norman Davidson (California Institute of Technology), Toni Claudio (Yale University), and Doug Melton (Harvard University) for generously providing cDNAs and plasmids. We thank Drs. Richard Nuccitelli and Pamela Pappone for the use of their facilities and Dr. John Hershey for the gift of the rabbit reticulocyte lysate. We also thank Drs. Eric Paulson and James Fergusson for their advice on molecular biology techniques and oocyte handling, respectively. We are also grateful to Drs. Michael White (University of Pennsylvania) and Terry Snutch (California Institute of Technology) for helpful discussions.

Registry No. L-Cys, 52-90-4; tubocurarine, 57-94-3.

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Induction of Conductance Heterogeneity in Gramicidin Channels†

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Received February 3, 1989; Revised Manuscript Received May 4, 1989

ABSTRACT: In previous work from our laboratory, 5–10% of the channels formed by [Val^1]gramicidin A have conductances that fall outside the narrow range that conventionally has defined the standard gramicidin channel [e.g., see Russell et al. (1986) *Biophys. J. 49*, 673]. Reports from other laboratories, however, show that up to *50%* of [Val^1]gramicidin channels have conductances that fall outside the range for standard channels [e.g., see Prasad et al. (1986) *Biochemistry 25*, 456]. This laboratory-to-laboratory variation in the distribution of gramicidin single-channel conductances suggests that the conductance variants are induced by some environmental factor(s) [Busath et al. (1987) *Biophys. J. 51*, 79]. In order to test whether extrinsic agents can induce such conductance heterogeneity, we examined the effects of nonionic or zwitterionic detergents upon gramicidin channel behavior. In phospholipid bilayers, detergent addition induces many changes in gramicidin channel behavior: all detergents tested increase the channel appearance rate and average duration; most detergents decrease the conductance of the standard channel; and all but one of the detergents increase the conductance heterogeneity. These results show that the conductance heterogeneity can result from environmental perturbations, thus providing a possible explanation for the laboratory-to-laboratory variation in the heterogeneity of gramicidin channels. In addition, the differential detergent effects suggest possible mechanisms by which detergents can induce the conformational perturbations that result in gramicidin single-channel conductance variations.

An important problem pertaining to understanding membrane protein function is how function depends on the membrane environment in which a protein resides. This is a difficult problem to address in studies on populations of membrane proteins that reside within a sometimes rather poorly controlled native lipid environment. Single-channel studies on membrane-spanning channels in planar lipid bilayers, however, allow the functional consequences of lipid environment modulations to be studied in detail. Single-channel experiments are particularly useful to examine questions pertaining to the possible heterogeneity among membrane proteins, because one by definition studies single molecular assemblies (cf. Figure 1). The cation-selective channels formed by the linear gramicidins are in fact exquisitely sensitive to subtle perturbations of the host bilayer.

The linear gramicidins are pentadecapeptides that have the sequences (Gross & Witkop, 1965) HCO-L-Xxx---Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp-D-Leu-L-Yyy-D-Leu-L-Trp-D-Leu-L-Trp-$NHCH_2CH_2OH$, where Xxx can be Val or Ile and Yyy can be Trp (in gramicidin A), Phe (in gramicidin B), and Tyr (in gramicidin C). The predominant form is [Val^1]gramicidin A. The sequences are extremely hydrophobic (Segrest & Feldman, 1974). In lipid bilayers, they form $\beta^{6.3}$-helical dimers that act as cation-selective ion channels that have measurable conductance and lifetime (Figure 1) [for a recent review, see Andersen et al. (1988)]. Most gramicidin channels have conductances that fall within a narrow, approximately Gaussian distribution. Occasionally, a channel has a conductance that is significantly different from that of the standard channels (Busath & Szabo, 1981). Most

† This work supported by USPHS Grants GM 21342 and GM 34968. D.B.S. was supported by the M.D./Ph.D. student fellowship program at Cornell University Medical College.
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0006-2960/89/0428-6571$01.50/0