Articles

Functional Role of the Cysteine 451 Thiol Group in the M4 Helix of the γ Subunit of *Torpedo californica* Acetylcholine Receptor†

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ABSTRACT: Previous chemical modification studies of the acetylcholine receptor [Yee, A. S., Corey, D. E., & McNamee, M. G. (1986) *Biochemistry 25*, 2110-2119] were extended by using fluorescent *N*-pyrenylmaleimide to alkylate purified *Torpedo californica* nicotinic acetylcholine receptor (AChR). Peptide sequencing of the tryptic fragments of the labeled AChR γ subunit identified cysteines 416, 420, and 451 as the modified residues. The functional role of Cys-451 in the M4 transmembrane domain of the AChR γ subunit was further investigated by studying the functional consequences of the site-specific mutation of this cysteine to either serine or tryptophan by using AChR mRNAs injected into *Xenopus laevis* oocytes. Both mutants displayed about 50% reduction in the normalized channel activity of the receptor measured as the ACh-induced conductance per femtomole of surface α-bungarotoxin binding sites. However, the mutations did not change other AChR functional properties such as agonist binding ability, the slow phase of desensitization, and blockade by competitive and noncompetitive antagonists. The significant reduction in AChR ion channel activity associated with the above point mutations, especially the simple change of the -SH group on Cys-451 to the -OH group, suggests that this thiol group in the M4 helix of γ subunit may play an important role in AChR ion channel function. Previous site-directed mutations of the Cys-416 and -420 residues showed a decreased response when both of these residues were changed to phenylalanine, but not when they were changed to serine [Pradier, L., Yee, A. S., & McNamee, M. G. (1989) *Biochemistry 28*, 6562-6571]. Since the M4 helix is believed to be at the lipid-protein interface, the large changes may indicate a strong link between ion channel activity and lipid-protein interactions. For both the mutant and wild-type receptors, the *normalized* channel activity of surface-expressed AChRs increased by 3- to 5-fold from day 2 to day 3 following microinjection of RNA transcripts into *Xenopus* oocytes. This result suggests that maturation of the AChR ion channel function may require some unknown posttranslational modifications or alterations in AChR-lipid interactions after the receptor is inserted into the plasma membrane.

The important roles of the thiol groups and disulfides in the assembly and function of nicotinic acetylcholine receptor (AChR)[1] have been extensively studied over the past 20 years [see Mosckovitz and Gershoni (1988) and Marquez et al. (1989) for two recent studies]. The amino acid sequences deduced from the cDNA sequences of the four subunits of *Torpedo californica* AChR (Noda et al., 1982, 1983a,b; Claudio et al., 1983) show that there are seven, five, eight, and six cysteines on each polypeptide of the α, β, γ, and δ subunits, respectively. Recent work by Kellaris and Ware (1989) showed there were 2, 1, 1, and 1 intramolecular cystines in each of the α, β, γ, and δ subunits in addition to the intermolecular disulfide link between two δ subunits in the native *T. calfornica* AChR dimer. The remaining cysteines existed as free sulfhydryls in the native receptor. Mishina et al. (1985) showed by site-directed mutagenesis that the pair of cysteines at positions 128 and 142 in the α subunit formed a disulfide bond which was required for the formation of the α-bungarotoxin (BGT) binding site and for receptor assembly. Kao and Karlin (1986) first established that a cystine connecting the adjacent Cys-192 and -193 in the α subunit exists near the acetylcholine binding site and is the site that is labeled by affinity alkylating agents after receptor reduction with dithiothreitol. Mosckovitz and Gershoni (1988) independently showed that two disulfide linkages, Cys-128 to -142 and Cys-192 to -193, existed in the α subunit of AChR and that Cys-412 and -418 did not exist as free sulfhydryls. They speculated that these latter two residues formed a disulfide linkage or were in the form of thioesters. Wennogle et al. (1981) inferred that Cys-500 in the δ subunit forms the intermolecular disulfide bond cross-linking the two δ subunits, and direct evidence supporting this inference has been obtained by DiPaola et al. (1989).

The free cysteines in *Torpedo* AChR have been shown to have functional significance by a series of chemical modification experiments which indicate that alkylation of unreduced receptor blocks the ion channel permeability (Huganir & Racker, 1982; Yee et al., 1986; Clarke & Martinez-Carrion, 1986). The exact locations of the cysteines involved in ion channel inactivation and their precise role are under investigation. Previous work in this laboratory (Yee et al., 1986;

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[1] Abbreviations, AChR, nicotinic acetylcholine receptor; BGT, α-bungarotoxin; MOPS, 3-(*N*-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; NPM, *N*-phenylmaleimide; NPyrM, *N*-pyrenylmaleimide; PMSF, phenylmethanesulfonyl fluoride; DOPC, dioleoylphosphatidylcholine; DMSO, dimethyl sulfoxide; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; PTH, phenylthiohydantoin.

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Schuchard et al., 1988) showed that under conditions which led to the full inhibition of AChR ion channel flux, the γ subunit was preferentially alkylated. Further peptide mapping of labeled γ subunit identified Cys-416, -420, and -451 as the possible modified residues (Pradier et al., 1989). Cys-416 and -420 are part of the amphipathic helix originally proposed by Finer-Moore and Stroud (1984) and Guy (1984) as a candidate for the ion channel domain of the receptor. Recently, Marquez et al. (1989) identified the Cys-222 in the M1 transmembrane domain as the only free sulfhydryl group on the native *Torpedo* AChR α subunit that is accessible to modification by the hydrophobic fluorescent agent *N*-(1-pyrenyl)maleimide (NPyrM). Site-directed mutagenesis experiments using AChR mRNAs injected into *Xenopus laevis* oocytes showed that replacement of Cys-416 and -420 in the γ subunit with a bulkier group (phenylalanine) led to partial inhibition of ion channel activity. However, replacement of the Cys residues with Ser had no effect on AChR function or expression (Pradier et al., 1989). These results indicate that the free thiol groups on these cysteines are not absolutely essential for receptor function.

The present study continues our efforts to probe and characterize the functional role of thiol groups on cysteines in *T. californica* acetylcholine receptor (Walker et al., 1984; Yee et al., 1986; Schuchard et al., 1988; Pradier et al., 1989). The chemical modification studies were extended by using the fluorescent *N*-pyrenylmaleimide to alkylate the *Torpedo* AChR. The labeled cysteine residues were unambiguously identified by peptide sequencing of the HPLC-separated tryptic fragments of the labeled γ subunit. The identified Cys-451 in the M4 helix of the γ subunit of AChR was then altered by site-directed mutagenesis to Ser (to investigate the significance of the free sulfhydryl group) or to Trp (to examine the effect of steric hindrance). The functional consequences of these mutations were studied by expressing the mutant AChRs in *Xenopus* oocytes and measuring their electrophysiological and pharmacological properties. In contrast to the Cys-416 and -420 mutations, the present results imply a significant role for the free cysteine at position 451 in ion channel activity. Preliminary results have been reported elsewhere in abstract form (Li et al., 1990).

Materials and Methods

Purification of AChR. All steps were carried out at 4 °C. Frozen electric organ from *T. californica* (300 g) was minced with a knife and homogenized for 5 min with a Brinkmann Polytron at maximum setting in 225 mL of homogenization buffer (20 mM MOPS, 5 mM EDTA, 5 mM EGTA, 0.02% sodium azide, 0.27 mM PMSF, and 14 mM iodoacetamide, pH 8.0). The homogenate was centrifuged at 5000*g* for 15 min, and the extract was filtered through cheesecloth and centrifuged at 70000*g* for 60 min. The membrane pellets were resuspended with 15 mL buffer A (10 mM MOPS, 0.1 mM EDTA, 100 mM NaCl, and 0.02% sodium azide, pH 7.4) and frozen in liquid nitrogen. The membranes were thawed and resuspended with stirring in 280 mL of membrane solubilization buffer (buffer A plus 1.2% cholate purified by recrystallization in methanol) for 20 min. The solubilized mixture was centrifuged at 93000*g* for 45 min. The supernatant was applied to a 15-mL affinity column equilibrated with buffer B (1% cholate in buffer A) at 1.5 mL/min. The affinity column was prepared by reacting cystamine with Affi-Gel 10 (Bio-Rad), reducing the cystamine with DTT, and then reacting bromoacetylcholine with the free SH groups. The column was washed with buffer C [1 mg/mL dioleoylphosphatidylcholine (DOPC) in buffer B], and the purified receptor was eluted with buffer D (10 mM carbamylcholine in buffer C). Fractions containing purified receptor were pooled and dialyzed against 2 L of buffer A for 48 h with three changes of buffer.

AChR Subunit Labeling with [^{14}C]NPyrM. [^{14}C]NPyrM (0.62 mCi/mmol) was synthesized from [2,3-^{14}C]maleic anhydride (Amersham) and aminopyrene according to Weltmann et al (1973). Labeling of AChR was performed in the presence of 1% cholate with a final [^{14}C]NPyrM concentration from 12 μM to 1.2 mM as described (Yee et al., 1986). The labeled AChR subunits were resolved on a SDS–PAGE gel, and the subunit region was excised and counted by following the same protocol as described previously (Yee et al., 1986).

Pyrenylmaleimide (NpyrM) Modification of AChR. The purified AChR was resolubilized in 1% cholate at a concentration of 1 mg/mL. Pyrenylmaleimide (200 mM in DMSO) was added to 5 mL of solubilized AChR at room temperature to give a final NPyrM concentration of 1 mM. The final DMSO concentration was less than 1%. The tube containing the reaction mixture was flushed with argon, sealed, and incubated at room temperature in the dark for 1 h. The modified NPyrM–AChR solution was centrifuged for 10 min to pellet NPyrM aggregates. The supernatant was treated in one of two different ways depending on the experiment. For assays designed to examine the labeling patterns, the samples were dialyzed overnight against 500 mL of SDS–PAGE sample buffer (0.0625 M Tris-HCl, 2% SDS, and 10% glycerol) without mercaptoethanol or tracking dye. For functional studies of either ligand binding or ion permeability, the modified, solubilized AChR was reconstituted in the presence of additional lipids (Asolectin) and detergent exactly as described by Yee et al. (1986). The rates of BGT binding and the carbamylcholine-stimulated influx of $^{86}Rb^+$ were measured on the reconstituted membranes as described by Yee et al. (1986) with minor modifications described in detail by McNamee et al. (1987).

Isolation of AChR Subunits. A 0.15 cm thick preparative SDS–PAGE gel appropriate for continuous elution was made by a modification of Laemmli (1970). A Hoeffer Scientific vertical slab gel apparatus was used with two 0.075-cm spacers. Each of the spacers was routed with a notch (about 2 cm from the end) so that when glued together the spacers would form a hole for a small plastic tube to slip through. A small hole was also drilled in each side clamp for the tube. The entire gel consisted of a base gel [24% acrylamide/0.64% bis(acrylamide)], a channel to elute the peptides, a resolving gel [8% acrylamide/0.21% bis(acrylamide)], and a stacking gel [3.75% acrylamide/0.1% bis(acrylamide)] with one large sample well. The elution buffer (0.1 M Tris-HCl and 0.05% SDS, pH 8.0) was pumped through the gel channel at 1.5 mL/min.

Mercaptoethanol and bromphenol blue were added to the NPyrM-modified AChR to final concentrations of 5% and 0.004%, respectively. The sample was loaded into the sample well, and the gel was run at 40 mA. The elution buffer from the gel was monitored by the absorbance at 280 nm, and 3-mL fractions were collected when the dye front started to elute. Pooled fractions containing the subunits were extensively dialyzed at 4 °C against 5 mM ammonium bicarbonate and 0.05% SDS. The dialyzed subunits were then dried under vacuum in a Savant RH 40-11 Speed-Vac concentrator.

Trypsin Digestion. SDS was removed from the subunits by acetone precipitation (Stone, 1988). SDS concentrations were monitored according to the method of Sharkey (1983). The purified NPyrM-labeled γ subunit polypeptide was re-

suspended in a 50-μL solution of 8 M urea, 0.4 M ammonium bicarbonate, and 5 μL of 45 mM DTT and incubated at 50 °C for 15 min. Then 5 μL of 100 mM iodoacetamide was added to the cooled mixture and incubated at room temperature for 15 min. Trypsin digestion was carried out by adding 5 μL of trypsin (1 mg/mL, sequence grade, Boehringer Mannheim) and incubating overnight (18 h) at 37 °C (Stone, 1988).

HPLC Separation of Tryptic Peptides of the γ Subunit and Amino Acid Sequence Analysis. A Perkin-Elmer Series 410 B10 LC pump equipped with an LC-235 array detector was used. The tryptic peptides of the subunit (1–5 nmol) were injected onto a Vydac C4 column (0.46 × 25 cm). The column was equilibrated with solvent A (0.07% TFA/5% acetonitrile). Elution was at 1 mL/min with a variable gradient of solvent B (0.1% TFA/99.9% acetonitrile), and 1-mL fractions were collected. The gradient system used is indicated in the legend to Figure 1. The eluate was monitored by absorbance at 210 and 280 nm to detect peptides and at 345 nm to detect the pyrenylmaleimide bound to peptides. Aliquots (0.3 mL) of each fraction with significant absorbance at these wavelengths were dried under vacuum on the Savant Speed-Vac concentrator along with an aliquot (0.02 mL) of the total tryptic mixture and analyzed by electrophoresis on a polyacrylamide [10% acrylamide/0.27% bis(acrylamide)] gel according to the method of Schagger and von Jagow (1987). The gel was washed for 15 min in a solution containing 40% methanol and 10% acetic acid. The fluorescently (pyrenylmaleimide) labeled peptides were visualized on a 300-nm UV light box and marked with a small cut in the gel. The gel was then silver stained (Bio-Rad). The fractions which contained a single fluorescently labeled peptide were subjected to automated Edman degradation in an AB1470 gas-phase sequenator (Applied Biosystems Inc.) to determine the first several amino acids at the N-terminal.

Site-Directed Mutagenesis. The mutation of Cys-451 to Ser (or Trp) was performed by the thionucleotide incorporation method (Amersham oligonucleotide-directed mutageneis system) using a 21-mer synthetic oligonucleotide [AATCCAGAACG(C)AAGCCTTGTC] as the primer and the recombinant M13mp9 (Pradier et al., 1989) containing an 800-bp insert of 3′-terminus fragment of the γ subunit cDNA as the template. After the mutations were confirmed by dideoxynucleotide sequencing (Sanger et al., 1977), the mutated inserts were subcloned back to pXγ (Pradier et al., 1989) to obtain pXγSer451 and pXγTrp451. The resulting pXγ mutants were checked to ensure that no unwanted changes in the codons had occurred by sequencing the double-stranded DNA according to the protocol for DNA sequencing with Sequenase from U.S. Biochemical Corp.

Expression in X. laevis Oocytes. RNA transcripts were synthesized in vitro from pα, pXβ, pXγ (both wild type and mutants) (Pradier et al., 1989) and SP64Tδ (Claudio, 1987) as described (Prader et al., 1989) except no radioactive nucleotide triphosphate was used. The integrity of the RNA transcripts was analyzed by electrophoresis on glyoxal agarose gels (Maniatis et al., 1982), and the yield was estimated by comparison to RNA standards from Bethesda Research Laboratories. The transcripts of different subunits were mixed and injected into *X. laevis* oocytes as described by Pradier et al. (1989).

Electrophysiology. Voltage-clamp experiments were performed 41–46 h (day 2) or 65–70 h (day 3) after injection by following the methods described by Pradier et al. (1989). Atropine (0.5 μM) was present in all agonist solutions. The standard assay for estimation of the channel activity of surface-expressed AChRs was the measurement of the whole-cell current response to 1 μM ACh at a holding potential of −80 mV. The channel activities induced by other agonists were assayed by measurement of the whole-cell current response to 0.5 μM suberyldicholine or 100 μM nicotine under the same conditions as above. The ACh concentrations used for the dose–response curves ranged from 1 to 10 μM for the oocytes with relatively low surface expression of AChRs or from 0.7 to 1.3 μM for the oocytes with higher levels of surface-expressed AChRs. The IC_{50} for antagonists was determined by measuring the current responses to 1 μM ACh in the presence of increasing concentrations of the antagonist (0–250 nM for *d*-tubocurarine, 0–5 μM for phencyclidine or tetracaine). The slow phase of desensitization of AChRs was measured by following the time course of the whole-cell current response in the presence of 1 μM ACh and fitting the rate of the current decay by a single exponential.

External Toxin Binding to Oocytes. Surface binding of ^{125}I-α-bungarotoxin (^{125}I-BGT) was measured on the same oocytes immediately following the electrophysiological recordings by incubating oocytes individually with 1 nM ^{125}I-BGT in MOR2 buffer solution with 0.5 mg/mL bovine serum albumin for 2 h at room temperature and then washing the oocytes and counting as described by Pradier et al. (1989).

Immunoblot. The expression level of individual AChR subunit polypeptides in the oocytes was estimated by immunoblot analysis of the proteins in the oocyte membrane fraction using the same protocol as described previously (Pradier et al., 1989).

Results

Identification of Labeled Cysteine Residues. The chemical modification studies of Yee et al. (1986) were extended by using a fluorescent maleimide, *N*-(1-pyrenyl)maleimide (NPyrM), to label the *T. californica* AChR. The fluorescent probe has the advantage that it can be visualized directly on SDS gels by using a UV transilluminator without staining or long-term exposure of radiolabeled derivatives using X-ray films. A disadvantage of the fluorescent probe is that it is not as suitable for quantitative analysis. To overcome this problem, [^{14}C]NPyrM, a radioactive derivative of the fluorescent probe, was synthesized and the relative rates and extent of incorporation of the label into the subunits were studied quantitatively under the same conditions as described by Yee et al. (1986) using [^{14}C]-*N*-phenylmaleimide (NPM). Results with NPyrM labeling correlated well with the NPM labeling studies, although a higher total number of free cysteines (8) were labeled at the highest concentrations (Schuchard et al., 1988). Complete inhibition of ion flux activity as measured by the carbamylcholine-stimulated influx of $^{86}Rb^+$ was obtained under conditions that preferentially labeled the γ subunit (1.2 mM NPyrM; two to three sites) with limited labeling of the α subunit (Schuchard et al., 1988). Our previous results (Yee et al., 1986) and the work of Clarke and Martinez-Carrion (1986) have shown that the alkylation by either NPM or NPyrM had no effect on the equilibrium binding of cholinergic activators and inhibitors nor on the interconversion between the high- and low-affinity binding states for activators.

To unambiguously identify the labeled cysteines on the γ subunit, the NPyrM-labeled γ subunit polypeptides were isolated by electroelution from preparative SDS–PAGE gels as described under Materials and Methods. The isolated γ subunit was shown to have no contaminants of other subunits by running an aliquot of the isolated polypeptides on SDS–PAGE gels followed by silver staining (data not shown). The

Table I: N-Terminal Amino Acid Sequence Analysis of the HPLC-Separated Tryptic Fragments of the Labeled AChR γ Subunit[a]

	fraction B		fraction C		fraction A	
cycle no.	amino acid	yield (pmol)	amino acid	yield (pmol)	amino acid	yield (pmol)
1	Val	32	Glu	25	Ser, Thr, Asp, Leu	20, 17, 15, 8
2	Ile	29	Gln	24	–,[b] Pro, Asp	–, 7 (5), 15 (0)
3	Asp	14	Asn	15	Val, Thr, Leu	10 (9), 9 (2), 5 (2)
4	Lys	9	Asp	8	Glu, Thr, –[b]	10 (9), 10 (2), –
5	Ala	12	Ser	8	Ala, Phe, Asn	10 (8), 5 (4), 2 (2)
6	–[b]	–	Gly	10		
7	Phe	8				

[a] Fractions A, B, and C from HPLC (Figure 1) were subjected to automated Edman degradation in an AB1470 gas-phase sequenator (Applied Biosystems Inc.). The PTH-amino acid observed on each cycle is given in the absolute yield. The number in parentheses indicates the yield (pmol) increased from the previous cycle (i.e., it was obtained by subtraction of the background of the previous cycle from the absolute yield). [b] A dash (–) indicates that nothing could be detected at the retention times corresponding to the standard PTH-amino acids.

isolated γ subunit of the NPyrM-labeled receptor was then subjected to trypsin digestion, and the resulting tryptic peptides were separated by reverse-phase HPLC on a Vydac C4 column. The chromatographic profile is shown in Figure 1. All the A_{210} peaks were spectroscopically analyzed by measuring A_{280} and A_{345}, and nine fractions containing coincident A_{210}, A_{280}, and A_{345} peaks were saved. Aliquots of each of these fractions were analyzed along with an aliquot of the total trypsin digestion mixture by electrophoresis on SDS–PAGE gels. Only three fractions (A–C, Figure 1) had fluorescently labeled bands as visualized on a 300-nm UV light box (data not shown). Following silver staining, fractions B and C appeared to contain a single peptide corresponding to fluorescent bands with apparent molecular weights of 2100 and 3600. The migration of the fluorescent band in fraction A corresponded to a peptide with a molecular weight of 1000. All peptides below M_r 1500 eluted from the gel during the silver staining process. The control lane of the digestion mixture showed that the trypsin digestion was substantially complete since no bands migrated at the molecular weight of the intact γ subunit, and only three fluorescent bands existed, each corresponding to the molecular weight of the isolated fluorescent peptides.

The above three purified NPyrM-labeled fragments were submitted to N-terminal amino acid sequence analysis. The results showed that fraction B was a homogeneous sequence, indicating a single peptide with the following N-terminal sequence: Val-Ile-Asp-Lys-Ala-X-Phe- (see Table I). The amino acid (X) at cycle 6 would be an NPyrM-labeled Cys residue since it did not match the retention times of all the standard PTH-amino acids. The above N-terminal sequence corresponds to positions 446–452 of the γ subunit immediately following Lys-445 (Noda et al., 1983b; Claudio et al., 1983) and is consistent with the specific cleavage of the peptide bond on the carboxyl side of a Lys or Arg residue by trypsin. Assuming an average amino acid molecular weight of 110, a molecular weight of 2100 would contain approximately 19 amino acids and the C-terminal of this peptide would be at position 464. The labeled cysteine residue in this peptide would be 451 in the M4 transmembrane domain of the γ subunit. Fraction C also contained a homogeneous N-terminal sequence: Glu-Gln-Asn-Asp-Ser-Gly- (Table I), which corresponds to positions 429–434 of the γ subunit immediately following Lys-428. A molecular weight of 3600 would contain approximately 33 amino acids, and the C-terminal would be at position 461, which is close to the C-terminal of peptide B. The labeled cysteine residue in this peptide is again 451. Peptide B thus appears to be a more completely digested fragment of peptide C.

The N-terminal sequence of fraction A was not homogeneous (Table I), which suggested that fraction A was a mixture of several peptides. Analysis of the yield of each cycle (Table I) showed that Val-Glu-Ala appeared to be the only definite sequence. Examination of the cycle location of each of the amino acids in fraction A (Table I) following a Lys or Arg residue suggested that there were three possible peptides with at most one mismatch. Of the three possibilities, only the peptide with the N-terminal sequence 415–419 (Ser-Cys-Val-Glu-Ala-) contains cysteine residues within the molecular weight range of 1000. They are cysteines 416 and 420 in the MA helix of the γ subunit. Cys-416 at cycle 2 would be modified since it did not match the retention times of all the standard PTH-amino acids. Comparison of the intensity of the A_{345} peak of fraction A with that of fraction B or of fraction C on the HPLC profile (Figure 1) suggests that most likely both cysteines are labeled.

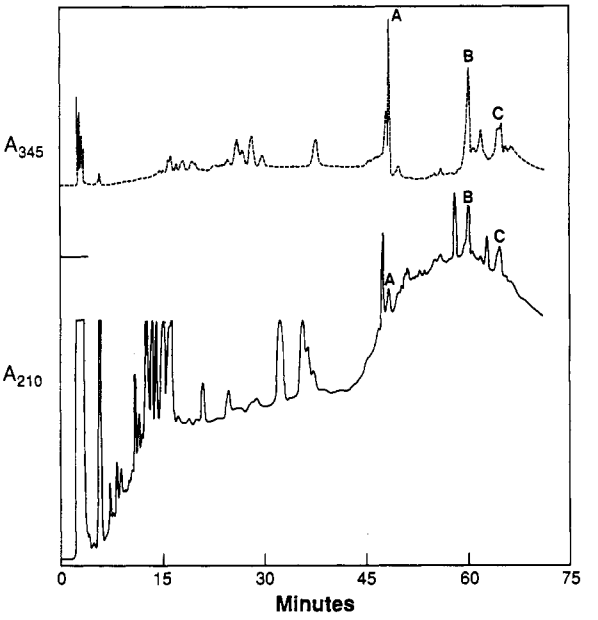

FIGURE 1: HPLC chromatographic profile of the separation of the tryptic fragments of NPyrM-labeled AChR γ subunit on a Vydac C4 column (0.46 × 25 cm). The column was eluted with the following linear gradients between solvent A (0.07% TFA/5% acetonitrile) and solvent B (0.1% TFA/99.9% acetonitrile): 0–3 min, 0–5% B; 3–13 min, 5–25; B; 13–43 min, 25–32% B; 43–63 min, 32–75% B; 63–83 min, 75–85% B. The flow rate was at 1 mL/min, and 1-mL fractions were collected. The eluate was monitored by absorbance at 210 nm (—, 0.2 absorbance unit full scale) and at 345 nm (---, 0.02 absorbance unit full scale). Fractions 51 (A), 63 (B), and 67–68 (C) were confirmed to contain a fluorescent peptide by SDS–PAGE gel electrophoresis.

Site-Directed Mutagenesis of Identified Cysteines. Cys-451 in the M4 helix of *Torpedo* AChR γ subunit was altered site specifically to either Ser or Trp by oligonucleotide-directed mutagenesis as described under Materials and Methods. The wild-type and mutant injection mixes made of pα, pXβ, pXγ,

Table II: Functional Consequences of Cys-451 Mutations on the AChR γ Subunit[a]

day	AChR	whole-cell current[b] (nA)	^{125}I-BGT binding[c] (fmol/oocyte)	normalized act.[d] (μS/fmol)
2	wild type	268 ± 77 (11)	6.1 ± 1.1 (11)	0.63 ± 0.11 (11)
	Ser-451	155 ± 42 (11)	4.5 ± 0.7 (10)	0.34 ± 0.06 (10)
	Trp-451	91 ± 25 (12)	4.4 ± 0.9 (11)	0.21 ± 0.04 (11)
3	wild type	631 ± 72 (35)	5.1 ± 0.3 (35)	1.86 ± 0.10 (28)
	Ser-451	358 ± 43 (11)	4.1 ± 0.5 (11)	1.10 ± 0.09 (11)
	Trp-451	373 ± 44 (33)	5.3 ± 0.4 (32)	1.01 ± 0.05 (25)

[a] Data are pooled from five separate experiments and given as means ± SEM. Numbers in parentheses indicate the number of oocytes tested. [b] Whole-cell currents are responses to 1 μM ACh (V_h = −80 mV) measured 41–46 (day 2) or 65–70 (day 3) h after injection of transcripts. [c] ^{125}I-BGT binding was measured on the same oocytes immediately following the electrophysiological recordings by incubating oocytes individually in 1 nM ^{125}I-BGT in MOR2 solutions. [d] Normalized activity is expressed in 1 μM ACh-induced conductance per femtomole of surface BGT binding sites.

and SP64Tδ transcripts in the ratio 2:1:1:1 were injected in parallel into oocytes obtained from the same ovary in each experiment. All measurements of functional properties were carried out at the same time for the wild type and the mutants. The whole-cell current response to acetylcholine was measured by voltage-clamp techniques, and the binding of BGT was measured directly on the same oocytes by using ^{125}I-BGT. The ACh-induced conductance (μS) was obtained from the measured current response (nA), reversal potential (0 to −10 mV), and the holding potential (−80 mV). The pooled data from five separate experiments are shown in Table II. Although the levels of expression vary among oocytes and among frogs, all five experiments gave very similar results, which is in agreement with the observation (Yoshii et al., 1987) that the normalized channel activity (expressed as μS/fmol of BGT binding sites) varied little among oocytes from different frogs.

Functional Effects of Cys-451 Mutations. The surface BGT binding data showed that the mutation of Cys-451 to Ser or Trp gave rise to surface AChR expression levels similar to that of the wild-type receptor (Table II). Western blot analysis of the oocyte membrane preparation revealed that the amounts of the four subunit polypeptides synthesized were about the same for both mutants and wild-type receptors (Figure 2). Therefore, the mutations did not affect the AChR biosynthesis, assembly, and transport to the cell surface.

Over a widespread range of expression levels (0.5–11.5 fmol of surface BGT binding sites per oocyte), the normalized ion channel activity measured as μS/fmol of BGT sites (Table II) of the Ser-451 mutant receptor was 47% lower on day 2 and 41% lower on day 3 than the wild-type AChR. The steric hindrance caused by the change of Cys to Trp did not further alter this decrease in the normalized channel activity significantly; the Trp-451 mutant receptor gave 66% reduction on day 2 and 46% reduction on day 3 in the normalized channel activity (Table II). In addition, both mutations also decreased

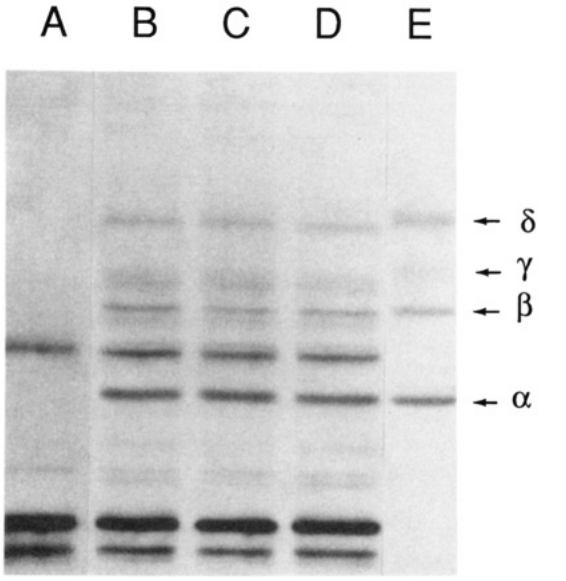

FIGURE 2: Western blot analysis of mutant AChRs synthesized in the injected *Xenopus* oocytes. Aliquots of total cell membrane fractions corresponding to five oocytes were analyzed as described (Pradier et al., 1989). (A) noninjected oocytes; (B) oocytes injected with wild-type AChR transcripts; (C) oocytes injected with Ser-451 mutant AChR transcripts; (D) oocytes injected with Trp-451 mutant AChR transcripts; (E) 50 ng of AChR purified from *T. californica* electroplax.

the normalized channel activities induced by other agonists such as suberyldicholine (SCh) and nicotine to about 50% (data not shown).

The Ser-451 and Trp-451 mutants displayed the same agonist *binding* properties as the wild-type AChR. Their ACh dose–response curves all had a Hill coefficient close to 2 (Table III) over the range of 0.7–1.3 μM ligand, showing the strong cooperativity between the two ACh binding sites. At the higher ACh concentrations (1–10 μM), the Hill coefficient (Table III) for the wild type and mutants decreased to about 1.4. This decrease in Hill coefficients, also noticed by Yoshii et al. (1987), may be due to the effect of desensitization at higher ACh concentrations.

The mutations did not affect other pharmacological properties of the *Torpedo* AChR (Table III). The agonist-induced current responses were atropine-resistant and *d*-tubocurarine-sensitive, specifying nicotinic properties. The fact that the current responses could also be induced by nicotine further assured that the responses were from the activation of the injected *Torpedo* nicotinic AChRs instead of the muscarinic AChRs in the follicle cells, which may still remain attached to the oocytes even after the 2-h collagenase digestion. The IC_{50} values for *d*-tubocurarine were very close to the ranges (70–160 nM) usually found for the *Torpedo* AChRs expressed on the plasma membrane of *Xenopus* oocytes (Yoshii et al., 1987; Pradier et al., 1989). Therefore, the mutations did not affect the blockade of AChR ion channels by the

Table III: Cys-451 Mutations Did Not Affect Other Functional Properties of AChR[a]

AChR	Hill coefficient 0.7–1.3 μM	Hill coefficient 1–10 μM	tubocurarine IC_{50} (nM)	phencyclidine IC_{50} (μM)	tetracaine IC_{50} (μM)	desensitization τ_{decay} (min)
wild type	1.8 ± 0.1 (2)	1.4 ± 0.1 (7)	109 ± 37 (4)	1.1 ± 0.2 (5)	1.4 ± 0.1 (5)	12 ± 5 (4)
Ser-451	1.7 ± 0.1 (3)	1.3 ± 0.1 (7)	53 ± 7 (4)	1.3 ± 0.2 (5)	1.7 ± 0.3 (5)	21 ± 8 (3)
Trp-451	1.9 ± 0.1 (2)	1.3 ± 0.1 (7)	92 ± 20 (5)	1.2 ± 0.3 (5)	1.4 ± 0.1 (5)	15 ± 3 (3)

[a] Data are given as means ± SEM, and the number in parentheses is the number of oocytes tested. ACh concentrations used for dose–response curve ranged from 1 to 10 μM for the oocytes with relatively low surface expression of AChRs or from 0.7 to 1.3 μM for the oocytes with higher level of surface-expressed AChRs. The antagonist concentrations used for IC_{50} determination ranged from 0 to 250 nM for tubocurarine and from 0 to 5 μM for both phencyclidine and tetracine. τ_{decay} is the decay time constant of 1 μM ACh-activated current responses obtained by single exponential curve fitting.

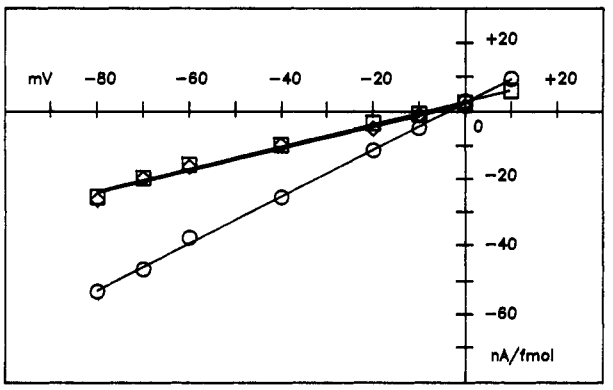

FIGURE 3: Current–voltage relation for representative responses from the wild type (○), Ser-451 (□), and Trp-451 (◇). Whole-cell current responses to 1 μM ACh were measured by two-electrode voltage clamp (Pradier et al., 1989) and normalized to nA/fmol of surface BGT binding sites.

competitive antagonist *d*-tubocurarine.

In addition, the mutants were inhibited by noncompetitive antagonists, such as phencyclidine and tetracaine, in the same manner as wild-type AChR channels. The IC_{50} values for phencyclidine and tetracaine (Table III), obtained from the electrophysiological studies of the blockade by 0–5 μM phencyclidine or tetracaine, were comparable to the ranges (2–10 μM) found for the muscle AChRs in BC_3H-1 cells by single-channel studies (Papke & Oswald, 1989). These noncompetitive blockers have been shown to react with sites on the M2 segments of the AChR subunits (Giraudat et al., 1986; Oberthur et al., 1986) which probably line the ion channel pore of the receptor (Imoto et al., 1988; Leonard et al., 1988). Furthermore, they have been shown to be able to block the open channel by decreasing the channel opening frequencies and duration of single-channel currents (Changeux et al., 1986; Papke & Oswald, 1989). Therefore, the above results tend to rule out the possibility that the mutations decreased the normalized channel activity by physically blocking or altering the ion permeation pathway.

Both mutants displayed a linear current–voltage relationship with a reversal potential or approximately −5 mV similar to that of the wild-type receptors over the range of −80 to 10 mV (Figure 3), which suggests that the mutations did not change the selective permeability of the AChR channel for cations. However, the normalized channel activities of the mutants were consistently about 50% lower than that of wild-type AChR at all voltages tested.

Measurements of the slow phase of desensitization of AChRs by monitoring the current decay constant in voltage-clamp experiments showed that the mutations did not change the time course of the slow phase of desensitization (Table III). The desensitization rates of both mutant and wild-type AChRs increased with the increasing concentrations of noncompetitive blockers (data not shown). For example, the presence of 5 μM tetracaine increased the desensitization rates of both mutant and wild-type receptors by more than 10-fold.

Time Dependence of AChR Expression in X. laevis Oocytes. In the above studies 5–10 ng of AChR mRNA transcripts was injected per oocyte and the oocytes were cultured in supplemented modified Barth's saline (Colman, 1984; Pradier et al., 1989) at 19 °C. Under these conditions, the injected RNAs were translated and the synthesized AChRs were expressed on the oocyte plasma membrane. Surface ^{125}I-BGT binding assays showed that the expression level reached a relatively high level on day 2 for the wild type and the two mutants and that there was little change during the following 24 h (Table II). This steady state of surface AChR expression is consistent with the results obtained with injected globin mRNAs (Gurdon et al., 1973), where it was shown that the oocytes reached a steady translational and processing state by 24 h after injection, which can last up to 7 days in the culture conditions.

In contrast to the toxin binding levels, the expressed AChRs were slower to acquire their ion channel activity. Voltage-clamp experiments at holding potentials of −80 mV showed that the mean of the whole-cell current response to 1 μM ACh increased by 2–4-fold from day 2 to day 3 (Table II). When the same individual oocytes were traced over time for their current responses to 1 μM ACh, they displayed a 10–33-fold increase from day 1 to day 2 and another 2–4-fold increase from day 2 to day 3. The large variations (SEM in Table II) in the numbers of the surface receptors and the current responses among oocytes reflect the intrinsic variability of translation and processing efficiency among the oocytes, which has been observed in many laboratories (Tobimatsu et al., 1987; Yoshii et al., 1987; Pradier et al., 1989).

To minimize the variation caused by changes of AChR expression with time (Olson et al., 1983), the same oocytes were individually subjected to the surface ^{125}I-BGT binding assay immediately following the electrophysiological recordings. The finding that the *normalized* channel activity on day 2 was 3–5-fold lower than that on day 3 was somewhat surprising. Since the oocytes displayed the same resting potentials (−40 to −80 mV), reversal potentials (0 to −10 mV), and expression levels (Table II) on day 2 as those on day 3, the lower normalized channel activity could imply that some unknown posttranslational modifications or AChR–lipid interactions are required for the assembled AChR to acquire its full ion channel function after its transport to the cell surface. In addition, the discrepancy between the normalized channel activity of day 2 and of day 3 oocytes obtained from the same frog and injected at the same time may explain the variations of the reported normalized channel activity of the wild-type *Torpedo* AChR expressed on oocyte surface among the different laboratories or even within the same laboratory (Yoshii et al., 1987; Pradier et al., 1989).

Discussion

Extension of the chemical modification studies on Yee et al. (1986) using fluorescent *N*-pyrenylmaleimide to label the solubilized AChR enabled us to directly identify the modified cysteines on the γ subunit by peptide sequencing. The identification of Cys-416, -420, and-451 as the NPyrM-labeled residues is consistent with the peptide mapping results of the *N*-phenylmaleimide-labeled γ subunit (Pradier et al., 1989). The labeling of the same sites by both NPyrM and NPM explains the similar labeling pattern and functional consequences obtained from the alkylation of AChR by either one of these two reagents. Recently, Marquez et al. (1989) have identified Cys-222 in the M1 transmembrane domain of the α subunit as a residue modified by NPyrM in native membrane prepared from *T. californica* electroplax. The contrast between the preferential labeling of γ subunit observed in our work and the uniform labeling of all subunits observed by others (Clarke & Martinez-Carrion, 1986) may reflect the different accessibilities of the cysteine residues to the alkylating reagent in different environments (solubilized state vs native membrane). At the highest labeling concentrations of NPyrM we observed labeling of the α subunit (Schuchard et al., 1988) and predict that Cys-222 is the site since Mosckovitz and Gershoni (1988) demonstrated that it is the only free cysteine on the α subunit.

Coupling of site-directed mutagenesis and electrophysio-

logical measurements of responses from the receptors incorporated into *Xenopus* oocytes has proved to be a powerful tool for study of the molecular mechanism of AChR ion channel function (Mishina et al., 1985; Imoto et al., 1988; Leonard et al., 1988). The normalized channel activity, expressed as the ACh-induced conductance per femtomols of surface α-bungarotoxin binding sites, has been shown to be a useful parameter which distinguishes the effects of mutations on the ion channel function from the perturbations of receptor assembly and expression (Yoshii et al., 1987; Pradier et al., 1989). Since the normalized channel activity is obtained from a dual assay in which both whole-cell ACh-induced current responses and cell surface α-bungarotoxin binding levels are measured on the same oocyte, it is important to synchronize the two measurements with each other as closely as possible. The present study showed that the reduction of the time lag between the two measurements to less than 5 h significantly increased the reproducibility of the normalized channel activity among the different oocytes.

Single mutants of Cys-451 to either Ser or Trp reduced the normalized channel activity of AChR by about 50%. This macroscopic inhibition of ion channel conductance may reflect the change of channel opening frequency, open time, or conductance at the single-channel level. Indeed, our preliminary experiments using patch-clamp recordings (A. Palma, P. Pappone, and M. G. McNamee, unpublished results) show that both mutations appear to decrease the channel opening frequency and change the channel open duration. The change of only one amino acid in a protein as large as AChR, especially the small change of the –SH on Cys-451 to the –OH group, strongly argues for the direct involvement of this free sulfhydryl group on Cys-451 in AChR channel function. Our previous mutagenesis experiments (Pradier et al., 1989) focused on Cys-416 and -420 since these two sulfhydryl groups are located (Figure 4) within the amphipathic domain that was postulated by several groups to form the ion channel structure (Finer-Moore & Stroud, 1984; Guy, 1984). Although double mutations from Cys to Phe gave a 30% reduction in normalized channel activity, mutations from Cys to Ser had no effect. We concluded that the amphipathic helix had at most an indirect effect on ion channel structure and function.

Cys-451 is located in the M4 transmembrane domain of the γ subunit, close to the cytoplasmic side, assuming the four channel crossing model is correct (Figure 4). This cysteine is conserved in the γ and ϵ subunits of all known nicotinic AChRs for which sequences are available and is also conserved in the δ subunit of all species except *Torpedo*. The membrane buried-helix parameter (Rao & Argos, 1986) of Ser (0.97) or of Trp (1.00) is close to that of Cys (1.27), whereas the side-chain volume (Richards, 1974; Finney, 1975) of Trp is significantly larger than that of Cys (44.9 Å^3) or of Ser (37.9 Å^3). The tolerance of Cys-451 for replacement by Ser and Trp is consistent with its predicted location at the edge of M4 transmembrane domain (Figure 4) where it may not be tightly packed against other transmembrane helices.

Although the M4 transmembrane segment of AChR has been shown to be accessible to the lipid bilayer (Giraudat et al., 1985), its functional role remains unknown. Tobimatsu et al. (1987) demonstrated that the M4 helix of the *Torpedo* AChR α subunit could be either partially deleted or completely replaced by foreign transmembrane sequences without loss of the channel activity. However, some of the altered AChRs resulted in more than a 20-fold increase in channel activity, which might imply that the M4 helix can modulate channel

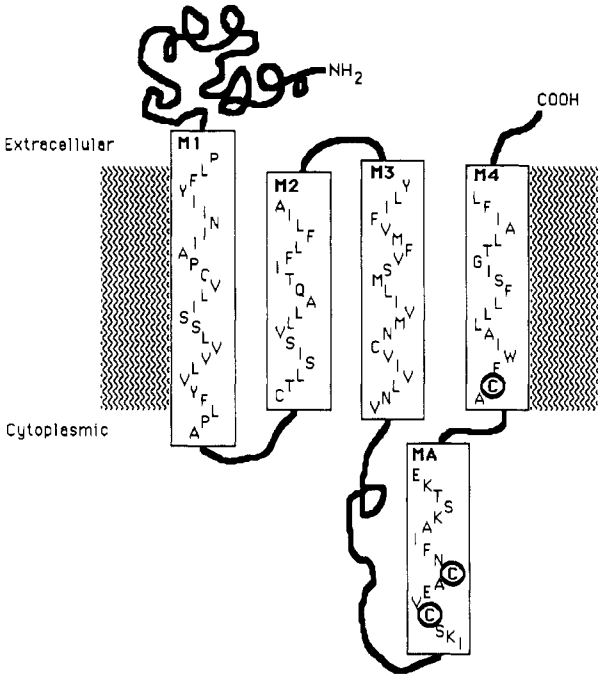

FIGURE 4: Locations of the NPyrM-labeled Cys-416, -420, and -451 (circled) in the AChR γ subunit according to the four-helix model (Claudio et al., 1983; Noda et al., 1983b). In the five-helix model (Guy, 1984; Finer-Moore & Stroud, 1984), MA forms additional membrane-spanning domain and the orientation of M4 transmembrane domain has to be reversed. Cys-416 and -420 in the MA domain have been studied by site-directed mutagenesis (Pradier et al., 1989). The functional consequences of point mutations at Cys-451 in the M4 transmembrane domain are discussed in the text.

function. Our results of the mutation of Cys-451 in the M4 transmembrane helix of AChR γ subunit seem to favor the hypothesis that the M4 helix is important in channel function, possibly in channel gating.

Another interpretation of our mutation results could be that the sulfhydryl group on Cys-451 is posttranslationally modified by fatty acid acylation and this acylation is important for the correct folding and assembly of the AChR receptor. The mutation of the Cys-451 to Ser or Trp would abolish its ability to form a thioester linkage with fatty acid, and the altered receptor may have impaired ion channel function even though it appears normally on the cell surface and retains all its ligand binding properties. It has been known that the thiol group on cysteine residues may play an important role in protein structure and function by forming a disulfide bond, or a thioester linkage with fatty acids (Creighton, 1984; Towler & Gordon, 1988; Schultz et al., 1988; Schmidt, 1989). These disulfide bonds and thioester bonds have been implicated in several physiological functions, including the maintenance of intracellular stability of proteins, correct folding and assembly of proteins, receptor binding, and biological activity (Schmidt, 1989; Malbon et al., 1987; Olson & Lane, 1989; Matsumura et al., 1989; Schultz et al., 1988). Cys-451 lies just within or at the cytoplasmic edge of the M4 transmembrane domain (Figure 4), a typical location for fatty acid attachment as has been found with the human transferrin receptor and the glycoprotein of vesicular stomatitis virus (Schultz et al., 1988). The dynamic nature of the fatty acylation (Schultz et al., 1988) may explain why Cys-451 is available to be labeled in our chemical modification experiments. Further experiments will be directed to investigate the possibility of fatty acylation of Cys-451 and other cysteine residues in the M4 transmembrane domain and the functional role of the M4 helix in AChR ion channel properties.

The improvements in obtaining the normalized channel activity within a short time-frame also led to a surprising finding: the *normalized* channel activity of surface-expressed AChRs increased by 3- to 5-fold from day 2 to day 3. Since the surface-expressed AChR level assayed by the surface ^{125}I-BGT binding was similar on day 2 and day 3, this result seemed to suggest that the expressed AChRs were slower in acquiring their ion channel activity than in obtaining their BGT binding ability. The delay in acquiring ion channel activity could be due to (1) the requirement of some unknown posttranslational modifications or AChR–lipid interactions for the assembled AChR to acquire its full ion channel function after its transport to the cell surface or (2) the insertion of the partially assembled AChR, e.g., lacking one of the subunits, into the membrane with the subsequent completion of AChR assembly leading to development of the full ion channel activity. Kurosaki et al. (1987) showed that the incomplete AChRs lacking one or several subunits were capable of binding the α-bungarotoxin with lower affinity, and the ability of such incompletely assembled AChRs to be expressed on the cell surface was much lower. The relatively high level of surface toxin binding sites detected by our assays performed with 1 nM toxin argues against the second possibility.

Although the biosynthesis and assembly of muscle AChRs have been studied extensively (Merlie & Smith, 1986), the time and the events required for assembled AChR to acquire its ion channel activity have not been fully explored. Recent work by Higgins and Berg (1988) showed that the neuronal AChRs on bovine adrenal chromaffin cells "mature" in the plasma membrane with respect to function and regulation by secondary messenger systems and the "maturation" took 20 h after insertion into plasma membrane to reach the steady state. It will be interesting to explore further the time-dependent maturation of *Torpedo* AChRs in the plasma membrane of injected oocytes with respect to ion channel function.

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Divalent Metal Ion Binding to the CheY Protein and Its Significance to Phosphotransfer in Bacterial Chemotaxis†

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ABSTRACT: Signal transduction in bacterial chemotaxis involves transfer of a phosphoryl group between the cytoplasmic proteins CheA and CheY. In addition to the established metal ion requirement for autophosphorylation of CheA, divalent magnesium ions are necessary for the transfer of phosphate from CheA to CheY. The work described here demonstrates via fluorescence studies that CheY contains a magnesium ion binding site. This site is a strong candidate for the metal ion site required to facilitate phosphotransfer from phospho-CheA to CheY. The diminished magnesium ion interaction with CheY mutant D13N and the lack of metal ion binding to D57N along with significant reduction in phosphotransfer to these two mutants are in direct contrast to the behavior of wild-type CheY. This supports the hypothesis that the acidic pocket formed by Asp13 and Asp57 is essential to metal binding and phosphotransfer activity. Metal ion is also required for the dephosphorylation reaction, raising the possibility that the phosphotransfer and hydrolysis reactions occur by a common metal-phosphoprotein transition-state intermediate. The highly conserved nature of the proposed metal ion binding site and site of phosphorylation within the large family of phosphorylated regulatory proteins that are homologous to CheY supports the hypothesis that all these proteins function by a similar catalytic mechanism.

Phosphoryl group transfer between regulatory proteins plays a central role in the control of gene expression and cell motility in bacteria [for a recent review, see Stock et al. (1989a)]. Two families of homologous regulatory proteins are involved. Generally, a histidine protein kinase (HPK) catalyzes the transfer of ATP γ-phosphoryl groups to an imidazole nitrogen in one of its own histidine side chains; this group is then transferred to an aspartic acid side chain in a response regulator (RR) protein. Finally, this phosphoaspartyl mixed anhydride is hydrolyzed to restore the response regulator to its dephosphorylated state:

$$\text{ATP} + \text{HPK-His} \rightleftharpoons \text{ADP} + \text{HPK-His-P}$$

$$\text{HPK-His-P} + \text{RR-Asp} \rightleftharpoons \text{HPK-His} + \text{RR-Asp-P}$$

$$\text{RR-Asp-P} + \text{H}_2\text{O} \rightarrow \text{RR-Asp} + \text{P}_i$$

The phosphorylated response regulators control the activities of effector components such as the flagellar motor apparatus or RNA polymerase. Environmental signals elicit responses by regulating the rate of autophosphorylation, phosphotransfer, and/or dephosphorylation.

Here we report the effects of divalent cations such as Mg^{2+} on the phosphorylation and dephosphorylation of CheY, the response regulator that controls motor behavior during chemotaxis in *Escherichia coli* and *Salmonella typhimurium*. The histidine kinase of the chemotaxis system, CheA, has been purified as a soluble homomultimer of approximately 250000 molecular weight composed of 73000 molecular weight subunits (Stock et al., 1988b). When incubated with $MgATP^{2-}$, CheA is phosphorylated at a histidine residue to levels approaching one phosphoryl group per monomer (Hess et al., 1988a; Stock et al., 1988a). Phosphoryl-CheA, isolated free of nucleotide, will act as a phosphodonor for CheY (Hess et al., 1988b; Wylie et al., 1988). CheY has been purified as a 128-residue monomeric protein. Its three-dimensional structure has been determined by X-ray crystallography to be

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