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Structure of the high-affinity binding site for noncompetitive blockers of the acetylcholine receptor: Serine-262 of the δ subunit is labeled by [^{3}H]chlorpromazine

(membrane protein/channel blocker/ionic channel/allosteric site/protein sequence)

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ABSTRACT **The membrane-bound acetylcholine receptor from *Torpedo marmorata* was photolabeled by the noncompetitive channel blocker [^{3}H]chlorpromazine under equilibrium conditions in the presence of agonist. Incorporation of radioactivity into all subunits occurred and was reduced by addition of phencyclidine, a specific ligand for the high-affinity site for noncompetitive blockers. The δ subunit was purified and digested with trypsin, and the resulting fragments were fractionated by reversed-phase HPLC. The labeled peptide could not be purified to homogeneity because of its marked hydrophobic character, but a combination of differential CNBr subcleavage and cosequencing of partially purified fragments enabled us to identify Ser-262 as being labeled by [^{3}H]chlorpromazine. The labeling of this particular residue was prevented by phencyclidine and thus took place at the level of, or in proximity to, the high-affinity site for noncompetitive blockers. Ser-262 is located in a hydrophobic and potentially transmembrane segment termed MII.**

The nicotinic acetylcholine receptor (AcChoR) from fish electric organ and vertebrate neuromuscular junction is a heterologous pentamer ($\alpha_2\beta\gamma\delta$) that both carries the acetylcholine (AcCho) binding sites at the level of the α chains and contains the agonist-gated ion channel (reviewed in refs. 1 and 2). The permeability response it mediates is blocked by a group of compounds referred to as noncompetitive blockers (NCBs) (1). Under equilibrium conditions, NCBs reversibly bind to a few categories of sites. The most prominent one is a high-affinity allosteric site distinct from, but strongly coupled to, the AcCho binding sites and present in one copy per AcChoR oligomer (3).

The complete primary structure of the AcChoR subunits has been established by DNA cloning and sequencing in *Torpedo californica* (α, β, γ, and δ) (4, 5), *T. marmorata* (α) (6), and various other species (reviewed in ref. 7). The subunits have a high degree of homology and in particular display two hydrophilic domains and four hydrophobic segments. All proposed models of the transmembrane organization of the AcChoR assume that the hydrophobic segments MI to MIV are transmembrane and that the subunits are symmetrically organized around the ion channel (4–6, 8, 9).

One approach to the understanding of AcChoR structure involves covalent labeling of functionally relevant binding sites and identification of the modified amino acids by protein-chemical techniques. In this respect, affinity reagents for the AcCho binding site have been shown to label the α subunit in a region located within the hydrophilic amino-terminal domain between Lys-179 and Met-207 (10, 11).

The high-affinity NCB site has been selectively labeled by a variety of NCBs (12–16). Depending on the ligand and on the species of *Torpedo* used, the α, β, or δ chains incorporate radioactive NCB preferentially but not exclusively. The NCB [^{3}H]chlorpromazine (CPZ) labels all four chains (3, 13). This suggests that all AcChoR subunits contribute to this unique high-affinity site which would then be located in the axis of 5-fold symmetry of the transmembrane oligomer.

We have analyzed the amino acid residues covalently labeled by [^{3}H]CPZ when bound to its high-affinity site and describe in this paper the results concerning the δ subunit. Our findings, obtained by a combination of differential cleavage and cosequencing of partially purified labeled fragments from the δ subunit, indicate that the hydrophobic segment MII contributes to the high-affinity NCB site.

MATERIALS AND METHODS

Materials. Phencyclidine was a gift from A. Jaganathen (Université Louis Pasteur, Strasbourg, France). [^{3}H]CPZ (20–25 Ci/mmol; 1 Ci = 37 GBq) was purchased from New England Nuclear; carbamoylcholine chloride and unlabeled CPZ, from Sigma; L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin, from Worthington; and cyanogen bromide, from Kodak. Live *T. marmorata* were provided by the Biological Station of Arcachon (France).

Covalent Labeling of the AcChoR by [^{3}H]CPZ. Labeling by [^{3}H]CPZ was performed essentially as reported (13). Purified (17) and alkali-treated (18) AcChoR-rich membrane fragments were resuspended to a final concentration of 10 μM α-toxin binding sites in *Torpedo* physiological solution supplemented with carbamoylcholine at a final concentration of 2 mM and, when indicated, phencyclidine at a final concentration of 400 μM. The suspension was mixed with an equal volume of a solution of isotopically diluted [^{3}H]CPZ (2–3 Ci/mmol) at a concentration of 5 μM. Samples of 5 ml were placed in a cylindrical chamber (3.5 cm internal diameter), flushed with nitrogen for 15 min, and irradiated for 5 min with a Mineralight shortwave UV lamp (254 nm).

After illumination, the membranes were centrifuged and the pellets were solubilized in sample loading buffer (19) and submitted to $NaDodSO_4$/polyacrylamide [10% acrylamide/0.13% *N*,*N*′-methylenebis(acrylamide)] gel electrophoresis. Appropriate aliquots of the solubilized membranes

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Abbreviations: AcCho, acetylcholine; AcChoR, acetylcholine receptor; DABTH, dimethylaminoazobenzene thiohydantoin; NCB, noncompetitive blocker; CPZ, chlorpromazine; rp HPLC, reversed-phase HPLC.

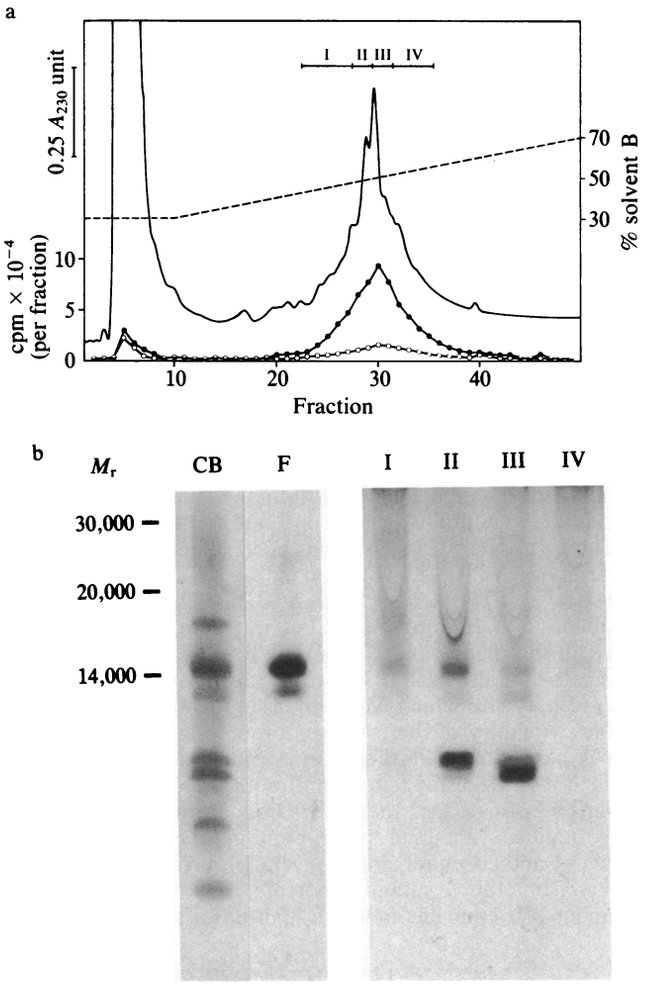

FIG. 1. Analysis of the tryptic digest of [^{3}H]CPZ-labeled δ subunit. (*a*) Reversed-phase HPLC. Purified labeled δ subunit (9 nmol) was incubated with trypsin. The dried digest was dissolved in formic acid and applied to a C_{18} column equilibrated in 0.1% trifluoroacetic acid/27% 1-propanol. Elution was at 0.5 ml/min with a linear gradient of solvent B (90% 1-propanol/0.1% trifluoroacetic acid) as indicated by the broken line. The eluate was monitored by absorbance at 230 nm (solid line). Aliquots (5 μl) of the fractions collected at 2-min intervals were subjected to liquid scintillation counting (●). Parallel treatment of δ subunit labeled by [^{3}H]CPZ in the presence of phencyclidine produced a similar A_{230} profile (not shown) and the radioactivity profile shown here (○). (*b*) Polyacrylamide gel electrophoresis. The tryptic digest of 400 pmol of [^{3}H]CPZ-labeled δ subunit was analyzed by electrophoresis in a polyacrylamide [10% acrylamide/1% *N,N'*-methylenebis(acrylamide)] gel in the presence of $NaDodSO_4$ and urea (25), followed by Coomassie blue staining (lane CB) and fluorography (lane F). Similar material was subjected to HPLC and fractions were pooled, as indicated in *a*. The pools (I–IV) were dried and analyzed by electrophoresis under the same conditions (lanes I–IV, respectively); peptides were visualized by Coomassie blue staining.

were loaded on analytical gels, and radioactivity incorporated into the various AcChoR subunits was measured (13).

Purification of the δ Subunit. Individual AcChoR subunits were eluted from appropriate sections of preparative gels (20) by agitation in 10 mM Tris Cl, pH 7.5/0.1% $NaDodSO_4$/0.5 mM EDTA, under nitrogen. After dialysis against water and lyophilization, purified subunit was dissolved at 1 mg of protein/ml in 1% $NaDodSO_4$, treated with dithiothreitol, and reacted with iodoacetic acid (21). After desalting on a PD-10 column (Pharmacia) run in 0.1% $NaDodSO_4$, the δ subunit was precipitated twice with acetone.

The final material migrated as a single band in a polyacrylamide gel. The amount of protein was quantified by densitometric scanning of the Coomassie blue-stained gel, using bovine serum albumin as a standard. The overall yield of purification was ≈50% and the specific radioactivity of the purified δ subunit did not differ significantly from that just after irradiation (typically 1300–1700 ^{3}H cpm/μg of δ chain vs. 300–400 cpm/μg of δ chain for the phencyclidine-protected batch).

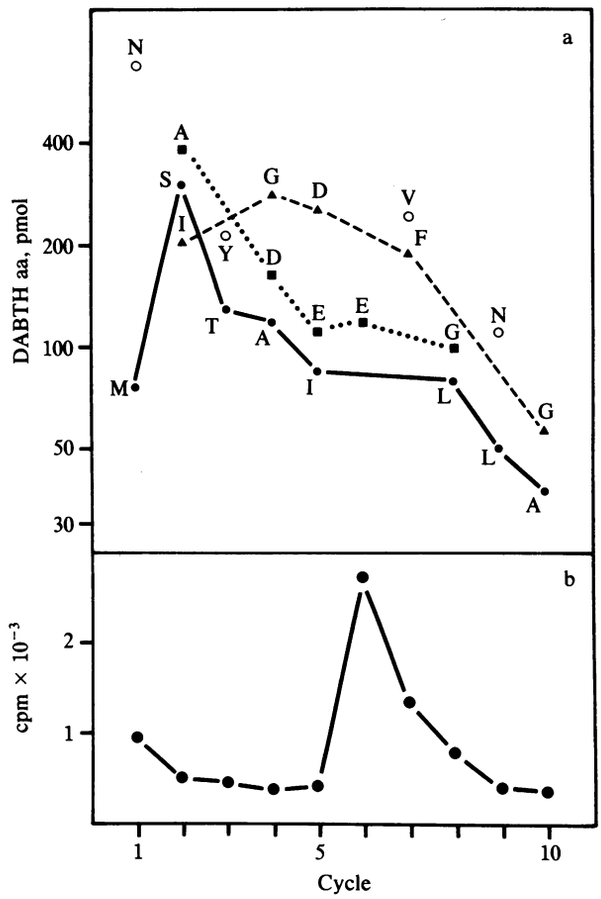

FIG. 2. Sequence analysis of tryptic peptides. Fractions corresponding to pools I–IV of Fig. 1*a* were mixed and dried, and the peptide contents were characterized by manual Edman degradation. (*a*) DABTH derivatives in each cycle were identified and quantified by HPLC. Residues assigned to the same fragment of δ subunit have been connected (●—●, ▲—▲, and ■·····■). Open circles indicate residues present in two different peptides at the same position. Residues that could not be confidently quantified are not represented. The standard one-letter amino acid abbreviations are used. (*b*) Radioactivity released in sequence cycles.

Trypsin Cleavage. Purified δ subunit was resuspended (≈2 mg/ml) in 2 M urea/0.1 mM $CaCl_2$/50 mM NH_4CO_3, pH 8.0. Trypsin was added periodically up to a total 1:20 (wt/wt) enzyme/substrate ratio during the 24-hr incubation at 37°C under nitrogen.

CNBr Cleavage. Dry sample was dissolved (≈1 mg /ml) in 70% formic acid and CNBr was added to a final concentration of 0.07 M. After 24 hr at room temperature under nitrogen in the dark, the reaction mixture was lyophilized.

HPLC of Peptides. A Waters HPLC system with a Valco injector and an Anacomp 220 (Kontron) data module was used. Dried samples were solubilized in pure formic acid and injected onto a μBondapak C_{18} column (3.9 × 300 mm).

Amino Acid Sequence Analyses. Automated Edman degradation was performed in a gas-phase sequenator (Applied Biosystems, Foster City, CA) (22). Glass filters were loaded

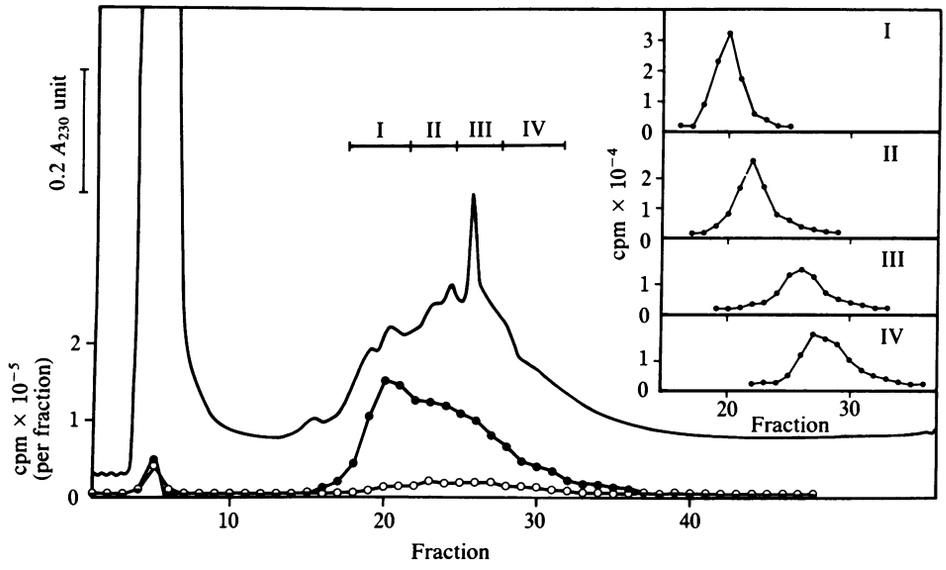

FIG. 3. Fractionation of peptides generated by CNBr subcleavage of the specifically labeled tryptic pool. Pooled tryptic peptides, corresponding to ≈18 nmol of δ subunit, were dried and cleaved with CNBr. The total digest was analyzed by rp HPLC as in Fig. 1*a*. Solid line represents A_{230}. Radioactivity profiles obtained with δ subunit labeled in the absence (●) or presence (○) of phencyclidine are shown. (*Inset*) Each of the pools I–IV was subjected to two additional HPLC steps as above, but using 0.13% heptafluorobutyric acid and then 0.2 M triethylamine phosphate (pH 4.5) as counterions. Finally, analyses under the same conditions as in Fig. 1*a* were performed; the resulting radioactivity profiles are displayed here.

with 5 mg of Polybrene and precycled 5 times before peptides (in 50% formic acid) were loaded. Phenylthiohydantoin derivatives of amino acids were identified by HPLC (23). Manual Edman degradation was done by the 4-(dimethylamino)azobenzene 4′-isothiocyanate/phenyl isothiocyanate double-coupling method (24). Dimethylaminoazobenzene thiohydantoin (DABTH) amino acid derivatives were identified by HPLC on a Brownlee reversed-phase C_{18} (5 μm) column, using a modification of published conditions (24).

RESULTS

Specific Labeling of the δ Subunit by [^{3}H]CPZ. Large batches of AcChoR-rich membrane fragments (200 nmol of α-toxin binding sites) were photolabeled by [^{3}H]CPZ under equilibrium conditions in the presence of 1 mM carbamoylcholine. Analytical polyacrylamide gel electrophoresis of [^{3}H]CPZ-labeled membranes demonstrated that, as shown previously (13), all AcChoR subunits were labeled. In the presence of phencyclidine, a specific ligand for the high-affinity NCB binding site, the incorporation of [^{3}H]CPZ into all receptor subunits was decreased (by 75–80% in the case of the δ subunit) (3, 13).

The AcChoR δ subunits derived from membranes labeled in the absence or presence of phencyclidine were purified by preparative polyacrylamide gel electrophoresis, with no detectable loss of covalently bound [^{3}H]CPZ (see *Materials and Methods*). Analytical polyacrylamide gel electrophoresis of the purified material indicated that ≈5% of the δ-subunit molecules were labeled by [^{3}H]CPZ in a phencyclidine-protectable way.

Analysis of δ-Subunit Tryptic Fragments. The tryptic digest of purified δ subunit was analyzed by $NaDodSO_4$/urea polyacrylamide gel electrophoresis (Fig. 1*b*). Two fragments, of apparent molecular weight 14,000 and 12,000, were found to contain essentially all the bound [^{3}H]CPZ.

Fractionation of the total tryptic digest by reversed-phase (rp) HPLC produced the chromatogram shown in Fig. 1*a*. Approximately 10% of the injected radioactivity was recovered in the unbound material, while 75% of injected radioactivity, including all the phencyclidine-inhibitable labeling, was associated with a broad A_{230} peak eluted between 40% and 50% 1-propanol. Analysis of four pools from this peak (I–IV) by $NaDodSO_4$/urea polyacrylamide gel electrophoresis (Fig. 1*b*, lanes I–IV) revealed that the M_r 14,000 and 12,000 species were distributed throughout this peak. Scintillation counting of gel slices showed that these fragments were radiolabeled (data not shown). Two unlabeled fragments, of apparent molecular weight 9000 and 8000, were concentrated in the two sharp peaks resolved on top of the absorbance profile (II and III).

Attempts to purify the radiolabeled species by a variety of chromatographic procedures (26, 27) proved unsuccessful; however, the two unlabeled components (M_r 9000 and 8000) were obtained in ≈75% purity (data not shown) by rechromatography in the original rp HPLC system described for Fig. 1*a*. Upon sequence analysis (data not shown), these fragments exhibited the same major amino-terminal sequence, Asn-Ala-Tyr-Asp-Glu-Glu-. Comparison with the known primary structure of AcChoR δ subunit from *T. californica* (28) indicates that this sequence corresponds to a unique tryptic fragment derived from the carboxyl-terminal portion of the δ subunit extending from Asn-437 (see Fig. 5).

When the entire peak of radioactive material obtained after rp HPLC (pools I–IV in Fig. 1*a*) was subjected to manual sequence analysis, two amino-terminal sequences, in addition to that ascribed to the unlabeled components, were observed (Fig. 2). The first (Asn-Ile-Tyr-Gly-Asp-Xaa-Phe-) corresponded to a tryptic fragment extending from Asn-200 (cleavage at Lys-199), and the second (Met-Ser-Thr-Ala-Ile-Xaa-Leu-) extended from Met-257 of the δ subunit (cleavage at Lys-256) (see Fig. 5). The presence at position 203 of a glycine residue, found here, instead of a proline, as deduced from *T. californica* cDNA sequence (28), probably reflects a species-specific mutation, as previously noted for the AcChoR α subunit from the two *Torpedo* species (6).

A clear release of radioactivity was detected at cycle 6 of the sequence, with a slight tailing on cycles 7 and 8 compatible with the extent of carry-over associated with the sequence. As calculated from the phencyclidine-sensitive labeling of the uncut δ subunit used here (≈55,000 cpm/nmol of δ chain), the radioactivity recovered at cycle 6 corresponded to at least 45 pmol of released amino acid, which is well above the detection limit for DABTH amino acids. The labeled peptide was thus among the identified sequences. Since we can exclude the sequence derived from the unlabeled fragments, this corresponds to CPZ labeling of Lys-205 and/or Ser-262 of the δ chain.

Identification of a CPZ-Labeled Residue by Differential Cleavage and Microsequencing. In view of the intrinsic difficulty in isolating the [^{3}H]CPZ-labeled tryptic fragments, we adopted a strategy that led us to distinguish which of the two candidate peptides carried [^{3}H]CPZ linked to the sixth

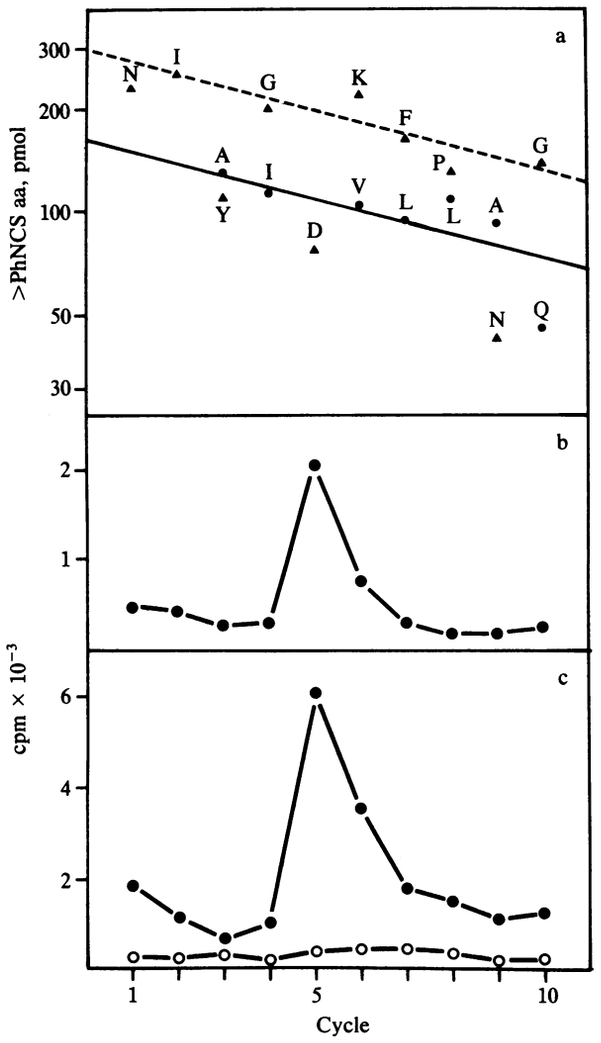

FIG. 4. Sequence analysis of CNBr subfragments. Repurified peptides contained in pool I of Fig. 3 *Inset* were subjected to automated gas-phase sequencing. Only the results of the first 10 cycles are shown here. (*a*) Yield of phenylthiohydantoin derivatives (>PhNCS aa) released in each cycle. Amino acids (one-letter abbreviations are used) assigned to the same δ-subunit fragment are represented by the same symbol (● or ▲). Release of serine was detected at cycles 1 and 5, and release of threonine at cycle 2, but these residues could not be quantified and are not represented here. For both sequences, the apparent repetitive yield was 93% (solid and dashed lines). (*b*) Radioactivity released in sequence cycles of the same experiment. (*c*) In a separate experiment, unfractionated peptides contained in pools I–IV of Fig. 3 were subjected to manual Edman degradation. Radioactivity released upon sequencing of similar samples originating from [^{3}H]CPZ-labeled δ subunit (●) and phencyclidine-protected δ subunit (○) is shown. Data have been normalized to the amount of DABTH derivatives released at cycles 3 and 4 in each sample (in the range of 150 pmol). For the same amount of identified amino acid sequence, a lower yield of released radioactivity was observed upon gas-phase versus manual sequencing and was attributed to uncontrollable losses of [^{3}H]CPZ-derivatized amino acid in the automated sequenator (29).

residue. We took advantage of the presence of a methionine residue at the amino terminus of one of the tryptic fragments (Met-Ser-Thr-Ala-Ile-Ser-). Treatment of the mixture of tryptic peptides with CNBr should shorten this peptide by one residue and result, if Ser-262 were labeled, in the shifting of radioactivity from cycle 6 to cycle 5, whereas the second candidate fragment (Asn-Ile-Tyr-Gly-Asp-Lys-) would be unaffected by such treatment.

The pool of tryptic peptides containing specifically labeled material was treated with CNBr and the total digest was analyzed by rp HPLC as above (Fig. 3). Approximately 5% of the injected radioactivity was recovered in the unbound material, while 85% was eluted between 30 and 45% 1-propanol in association with a broad A_{230} peak. This second radioactive peak was decreased 85% in material labeled in the presence of phencyclidine and therefore was characterized.

Rechromatography of pools from the asymmetrical radioactive peak using various solvent systems (details in the legend to Fig. 3) yielded four symmetrical and distinct peaks of radioactivity (Fig. 3 *Inset*) and optical density (not shown). Upon gas-phase sequence analysis, however, the same mixture of two amino-terminal sequences was observed with all four samples without any apparent purification. The results of sequence analysis of one of the samples, which is representative for all four analyses, are shown in Fig. 4*a*.

Two amino-terminal sequences were observed, one corresponding to the unaltered tryptic fragment (Asn-Ile-Tyr-Gly-Asp-Lys-) and the other derived from CNBr subcleavage at Met-257 (Fig. 5). As expected from the distribution of Met residues in the carboxyl-terminal region of the δ subunit (28), the unlabeled tryptic fragments (M_r 8000 and 9000) had undergone CNBr cleavage to give small, more polar peptides that were eluted with unbound material on rp HPLC.

A release of radioactivity was observed at cycle 5, with some tailing on cycles 6 and 7, for all four samples (Fig. 4*b*). No other radioactive peaks over background were observed up to 33 cycles, where quantifiable phenylthiohydantoin amino acids were identified. As discussed above, the shift of radioactivity from cycle 6 to cycle 5 following CNBr cleavage of the tryptic fragments clearly points to Ser-262 as a site of incorporation of [^{3}H]CPZ into the δ chain.

Labeling of this particular residue was protected by phencyclidine, as shown by manual sequencing of CNBr peptides purified in parallel from δ-subunit batches labeled in the absence or presence of phencyclidine. Whereas the same two amino-terminal sequences described above were observed with both samples, the peak of radioactivity released at cycle 5 was completely abolished in the phencyclidine-protected material (Fig. 4*c*). As for the tryptic peptides, we can here again exclude the possibility that the radioactivity released from the unprotected sample was contributed by a peptide that was below the sequence detection level. Indeed, upon

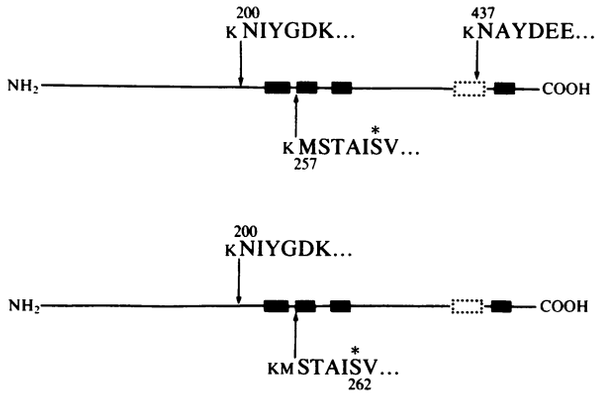

FIG. 5. Locations of sequenced peptides within the δ-subunit primary structure. Schematic drawing of the AcChoR δ subunit. Black boxes indicate the four hydrophobic segments, and the open box the amphipathic helix A (8, 9). Identified tryptic (*Upper*) and CNBr (*Lower*) peptides are localized along the sequence of δ subunit from *T. californica*. The amino-terminal sequences of the peptides are shown in larger type. One-letter amino acid abbreviations are used. Numbers indicate residue positions within the δ-subunit sequence. Ser-262 is marked with an asterisk.

manual sequencing, where the fate of radioactivity could be followed at all steps, the radioactivity recovered at cycle 5 in this last experiment (Fig. 4*c*) corresponded to at least 90 pmol of released amino acid (55,000 cpm/nmol of uncut δ chain).

We can therefore conclude that Ser-262 of the AcChoR δ subunit from *T. marmorata* is labeled by [^{3}H]CPZ in a phencyclidine-protectable way. This residue, which is located within the hydrophobic segment MII, thus lies within or in close proximity to the single high-affinity NCB binding site.

DISCUSSION

Several lines of evidence support the view that the AcChoR possesses a single high-affinity site for NCBs (3, 30) which can be defined as an allosteric site distinct from the AcCho binding sites. Cholinergic ligands enhance the interaction of NCBs with this site (3, 31, 32), whereas the binding of NCBs accelerates the desensitization process and stabilizes a state with high affinity for cholinergic agonists (3, 31, 33–35). Previous studies (3, 13) have shown that the covalent attachment of [^{3}H]CPZ to the AcChoR subunits is inhibited by phencyclidine, a highly selective ligand for this site, and that the concentration dependence of this inhibition correlates with the reversible dissociation of [^{3}H]CPZ from this site. The phencyclidine-sensitive labeling of the δ subunit analyzed in the present work thus represents covalent labeling by [^{3}H]CPZ of the high-affinity site for NCBs.

Biochemical characterization of the δ-subunit fragments labeled by [^{3}H]CPZ was hindered by their marked hydrophobicity. The combination of differential cleavage and cosequencing of partially purified δ-chain fragments, however, permitted us to identify Ser-262 as a site of specific incorporation of [^{3}H]CPZ.

We cannot rule out the possibility that other residues of the δ chain were also specifically labeled. However, the high recoveries of radioactivity observed upon HPLC fractionation exclude the selective loss of a labeled peptide at these stages. Under the conditions used here for rp HPLC, hydrophilic peptides were probably eluted with unbound material, which contained only small amounts of radioactivity. Any additional labeled residues, if they exist, could therefore only be in the unsequenced part of the two peptides detected in the radioactive peak after CNBr cleavage; i.e., between Ile-233 and Lys-256 or between Gly-299 and (approximately) Arg-325.

No phencyclidine-sensitive incorporation of [^{3}H]CPZ was detected in the region of the δ chain homologous to that labeled on the α chain by affinity reagents for the AcCho binding sites (10, 11). The NCB site thus does not simply derive from regions of the non-α subunits that are homologous to the AcCho binding site.

The functional characterization of this NCB site has been achieved by electrophysiological and biochemical experiments. On the basis of voltage jump and single-channel recording studies using procaine, lidocaine derivatives, and other compounds (reviewed in ref. 36), it has been proposed that NCBs could, among other effects, enter the ion channel in its open configuration and sterically inhibit ion flux. Recent studies employing patch-clamp analyses of single AcCho-activated channels on mouse C2 myotubes have shown that low concentrations of CPZ and phencyclidine reduce the mean channel-open time, though in a voltage-insensitive manner (37). Also, rapid-mixing photolabeling experiments have shown that CPZ labeling of the high-affinity site occurs several orders of magnitude faster under conditions where the channel is open than when it is closed (38). Accordingly, the hydrophobic segment MII of the δ subunit (which contains Ser-262) could be related to the ion channel and might even be one of its components.

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