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# Identification of a Novel Amino Acid $\alpha$ -Tyrosine 93 within the Cholinergic Ligands-binding Sites of the Acetylcholine Receptor by Photoaffinity Labeling

ADDITIONAL EVIDENCE FOR A THREE-LOOP MODEL OF THE CHOLINERGIC LIGANDS-BINDING SITES\*

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The native, membrane-bound, acetylcholine receptor from *Torpedo marmorata* was photolabeled by the competitive antagonist *p*-[<sup>3</sup>H]dimethylaminobenzene-diazonium fluoroborate (DDF) in the presence of the noncompetitive blocker phencyclidine and under energy transfer conditions. The isolated  $\alpha$ -subunits were treated with cyanogen bromide and fractionation of the resulting fragments yielded three radiolabeled peptides, at the level of which, incorporation of [<sup>3</sup>H]DDF (i) was equally inhibited by the agonist carbamoylcholine and the competitive antagonist  $\alpha$ -bungarotoxin and (ii) was insensitive to "scavenging" reagents. Subfragmentation of cyanogen bromide peptide III with *o*-iodosobenzoic acid or trypsin and sequence analysis of the fragments led to the identification of a novel amino acid  $\alpha$ -Tyr-93 (and possibly Trp-86) as labeled by [<sup>3</sup>H]DDF in a carbamoylcholine-sensitive manner.  $\alpha$ -Tyr-93 is conserved in the muscle and neuronal  $\alpha$ -subunits but not in the other subunits of muscle receptor. This result provides evidence for a site involving at least a third loop of the  $\alpha$ -subunit amino-terminal hydrophilic domain, in addition to the ones previously identified (Dennis, M., Giraudat, J., Kotz-yba-Hibert, F., Goeldner, M., Hirth, C., Chang, J. Y., Lazure, C., Chretien, M., and Changeux, J. P. (1988) *Biochemistry* 27, 2346-2357). Possible contribution of tyrosine side-chains to the complexation of the quaternary ammonium group of cholinergic ligands is discussed.

electric organ and vertebrate neuromuscular junction is a well-characterized transmembrane allosteric protein (Changeux *et al.*, 1984) composed of four polypeptide chains assembled into a heterologous  $\alpha_2\beta\gamma\delta$  pentamer (Reynolds and Karlin, 1978), which carries the acetylcholine (AcCho)-binding site and contains the cation-selective channel-forming elements (see Popot and Changeux, 1984; Hucho, 1986). Nicotinic agonists and competitive antagonists including snake venom  $\alpha$ -toxins bind reversibly and in a mutually exclusive manner to a class of primary AcCho-binding sites present as two distinct copies/receptor oligomer (Neubig and Cohen, 1979, 1980), which are located, at least in part, on the  $\alpha$ -subunits (Karlin 1969, 1983; Haggerty and Froehner 1981; Tzartos and Changeux, 1983, 1984; Gershoni *et al.*, 1983; Mishina *et al.*, 1984; Blount and Merlie 1988).

Several approaches to identifying the region(s) of the  $\alpha$ -subunit primary structure involved in the AcCho binding have been undertaken, including: (i) covalent affinity labeling of  $\alpha$ -subunit Cys-192 and -193 of the reduced membrane-bound receptor by 4-(*N*-maleimido)benzyltrimethylammonium iodide (Kao *et al.*, 1984), (ii) photolabeling with *d*-tubocurarine (Pedersen *et al.*, 1986), (iii) binding of snake  $\alpha$ -toxins to  $\alpha$ -subunit proteolytic fragments (Wilson *et al.*, 1984, 1985; Neumann *et al.*, 1985, 1986; Oblas *et al.*, 1986), synthetic peptides (Radding *et al.*, 1988; Wilson *et al.*, 1985; Mulac-Jericevic and Atassi, 1986; Ralston *et al.*, 1987), deletion mutants (Barkas *et al.*, 1987), or  $\alpha$ -subunit fragments expressed in *Escherichia coli* transformants (Gershoni, 1987). The convergent results of these studies pointed to the region containing Cys-192 and Cys-193 as a potential site of interaction with cholinergic ligands.

Other recent studies have further defined the region of the  $\alpha$ -subunit involved in AcCho binding. The cation *p*-[<sup>3</sup>H]dimethylaminobenzene-diazonium fluoroborate (DDF), which acts in the dark as a competitive antagonist, was recently shown to efficiently label the primary AcCho-binding sites on the native membrane-bound *Torpedo marmorata* AcChoR upon irradiation under conditions of energy transfer (Langenbuch-Cachat *et al.*, 1988). Cyanogen bromide (CNBr) cleavage of the purified  $\alpha$ -subunits yielded three distinct radioactive fragments (Dennis *et al.*, 1986) respectively labeled at the level of: (i) Trp-149 (and possibly Tyr-151), (ii) Tyr-190, Cys-192-Cys-193 (and possibly Tyr-198), and (iii) (an) additional unidentified labeled amino acid(s) in the 31-105 region (Den-

The nicotinic acetylcholine receptor (AcChoR)<sup>1</sup> from fish

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<sup>1</sup> The abbreviations used are: AcChoR, acetylcholine receptor; IBA, *o*-iodosobenzoic acid; NaDodSO<sub>4</sub>, sodium dodecyl sulfate; HPLC, high performance liquid chromatography; PTH, phenylthiohydantoin; DDF, dimethylaminobenzene-diazonium fluoroborate.

nis *et al.*, 1988). Furthermore, the competitive antagonist lophotoxin was recently found to label Tyr-190 of the native  $\alpha$ -subunit (Abramson *et al.*, 1989).

Using a monoclonal antibody (mAb 6) that specifically binds to the  $\alpha$ -subunit of AChR, Watters *et al.* (1983) observed competition of antibody binding to the receptor with agonists but not with all of the usual competitive antagonists (see also Mihovilovic and Richmann 1984). On this basis, they suggested that agonists and competitive antagonists could bind to locally overlapping or even separate sites. These observations raised the possibility that agonists and antagonists may block in a differential manner covalent labeling of the cholinergic ligands-binding sites, and in particular [ $^3$ H]DDF incorporation in the different  $\alpha$ -subunit fragments (or amino acids). In this report, we first explore this possibility by studying the inhibition of [ $^3$ H]DDF incorporation in the  $\alpha$ -subunit acetylcholine-binding site(s) by agonist and competitive antagonist ligands. Second, we identify (a) novel amino acid(s) labeled by [ $^3$ H]DDF in the 31–105 region of CNBr fragment III, thus supporting a minimal three-loop model of the cholinergic ligands-binding sites on the AChR (Dennis *et al.*, 1988).

#### EXPERIMENTAL PROCEDURES

[ $^3$ H]DDF precursor was tritiated by Drs. Van Hove and Rousseau (Commissariat à l'Energie Atomique, France) and was diazotized as described (Langenbuch-Cachat *et al.*, 1988). Phencyclidine was generously provided by A. Jaganathan (Université Louis Pasteur, Strasbourg, France). Carbamoylcholine and oxytocin were purchased from Sigma, CNBr from Eastman Kodak, *o*-iodosobenzoic acid and ribonuclease from Pierce Chemical Co., *p*-cresol and 2-methyl-2-nitrosopropane from Aldrich, and L-1-*p*-tosylamino-2-phenylethyl chloromethyl ketone-treated trypsin from Merck. Live *T. marmorata* were obtained from the Biological Station of Arcachon (France).

**Covalent Labeling of the AChR by [ $^3$ H]DDF**—Preparative photolabeling of large batches (100–200 nmol of  $\alpha$ -bungarotoxin-binding sites) of purified (Saitoh *et al.*, 1980) and alkali-treated (Neubig *et al.*, 1979) AChR-rich membrane fragments was achieved by energy transfer ( $\lambda = 290$  nm) as described (Langenbuch-Cachat *et al.*, 1988), except that the total volume for each irradiation was 2.0 ml. Final concentrations during irradiation were 1–2  $\mu$ M  $\alpha$ -bungarotoxin-binding sites at approximately 1 nmol of sites/mg of protein, 100  $\mu$ M phencyclidine, 200  $\mu$ M [ $^3$ H]DDF and, when indicated, 100  $\mu$ M carbamoylcholine.

Following irradiation, dithiothreitol was added to a final concentration of 10 mM to destroy unreacted [ $^3$ H]DDF, and aliquots of the membrane suspension were removed for titration of  $\alpha$ -bungarotoxin-binding sites (Weber and Changeux, 1974); the remainder was centrifuged and the pellets solubilized in NaDodSO<sub>4</sub> sample buffer (Laemmli, 1970). Aliquots of the solubilized membranes were analyzed on NaDodSO<sub>4</sub>-polyacrylamide gels, and the incorporation of radioactivity into the various AChR subunits was quantified as previously described (Dennis *et al.*, 1986). The amount of each polypeptide was quantified by densitometric scanning of the Coomassie Blue-stained gel using bovine serum albumin as standard.

**Purification of the  $\alpha$ -Subunit**—Solubilized membranes were subjected to preparative NaDodSO<sub>4</sub>-polyacrylamide gel electrophoresis and the separated AChR subunits eluted by diffusion (Giraudat *et al.*, 1986). The eluates were dialyzed against 0.1% NaDodSO<sub>4</sub>, 0.01%  $\beta$ -mercaptoethanol, then against water, and lyophilized. The purified  $\alpha$ -subunit was carboxymethylated and precipitated twice with acetone (Giraudat *et al.*, 1986).

The purified  $\alpha$ -subunit migrated as a single band on a polyacrylamide gel, and its specific radioactivity (see "Results") was not significantly different from that estimated just after irradiation.

**CNBr Cleavage of the  $\alpha$ -Subunit**—The purified, carboxymethylated  $\alpha$ -subunit was dissolved in 70% formic acid (2 mg of protein/ml) and tryptophan (Trp) added in a 5-fold molar excess (4 mM) over methionine (Met) residues to prevent oxidation of endogenous Trp. CNBr was added to a final concentration of 60 mM (80-fold excess over Met), and the mixture was incubated for 24 h at room temperature under nitrogen in the dark. Samples were then diluted with three volumes of water and lyophilized. Fractionation of the CNBr frag-

ments was performed as described (Dennis *et al.*, 1988).

***O*-Iodosobenzoic Acid (IBA) Subcleavage of CNBr Fragments**—Purified CNBr fragment III was resuspended (approximately 1 mg/ml) in 20% acetic acid in 4 M guanidinium chloride. *p*-Cresol was added to a final concentration of 15 mM (150-fold excess over Tyr) to prevent tyrosine oxidation and *o*-iodosobenzoic acid to a final concentration of 1 mM (15-fold excess over Trp) (Fontana *et al.*, 1983). The mixture was incubated overnight at room temperature under nitrogen in the dark. The crude reaction mixture was subjected to reversed-phase high performance liquid chromatography (HPLC) on a Brownlee RP-300 aquapore column (4.6  $\times$  250 mm) equilibrated with 90% solvent A (0.1% aqueous trifluoroacetic acid), 10% solvent B (60% 1 propanol, 30% acetonitrile, 0.1% trifluoroacetic acid). After injection, development at 10% solvent B for 10 min was followed by a linear gradient from 10 to 50% solvent B over 90 min.

**Trypsin Subcleavage of CNBr Fragments**—Purified CNBr fragments were resuspended (approximately 1 mg/ml) in 2 M urea, 0.1 mM CaCl<sub>2</sub>, 50 mM NH<sub>4</sub>CO<sub>3</sub>, pH 8.5. Trypsin was added periodically up to a total 1:10 (w/w) enzyme/substrate ratio during the 24-h incubation at 37 °C under nitrogen. After a 5-fold dilution with solvent A (0.1% aqueous trifluoroacetic acid), the mixture was loaded onto the RP 300 column and eluted as indicated for IBA fragments.

**Sequence Analyses**—Peptides were dissolved in 50% propanol, 50% acetonitrile for loading. Automated Edman degradation was carried out on an Applied Biosystems gas-phase automated Sequencer (ABI 470 A) or a pulsed liquid-phase sequencer (ABI 477 A) using standard cycle programs provided by the manufacturer. Aliquots of the PTH-amino acids released from the sequencer at each cycle were analyzed on-line using the ABI 120 A reverse-phase HPLC system. The remaining sequencer output from each cycle was used for determination of radioactivity.

#### RESULTS

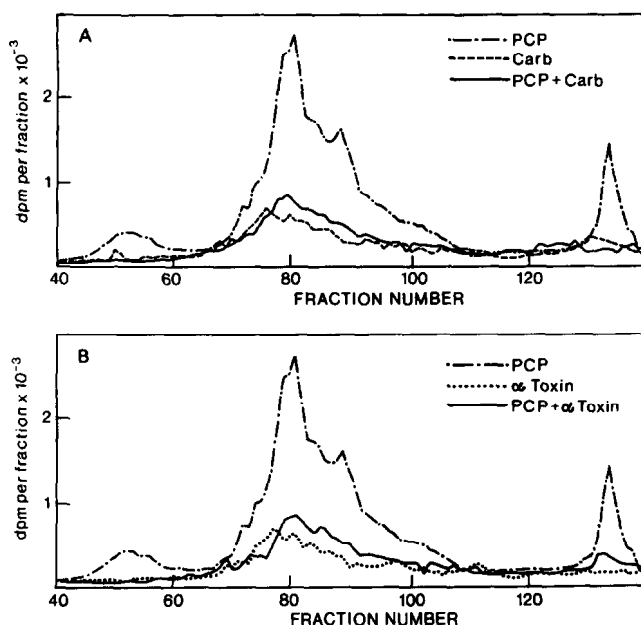
##### *Specificity of [ $^3$ H]DDF Incorporation into the Three Peptide Domains of the $\alpha$ -Subunit*

Alkali-treated AChR-rich membranes (30 nmol/batch) were photolabeled with [ $^3$ H]DDF (0.3 Ci/nmol) in the presence of 100  $\mu$ M phencyclidine using the previously described energy-transfer photolysis procedure (Langenbuch-Cachat *et al.*, 1988). Analytical polyacrylamide gel electrophoresis of the labeled membranes confirmed that, as shown previously, the  $\alpha$ -subunit carried the majority of the [ $^3$ H]DDF incorporated within the AChR oligomer (Dennis *et al.*, 1986, 1988; Langenbuch-Cachat *et al.*, 1988). The incorporation of [ $^3$ H]DDF into the  $\alpha$ -subunit was assayed in the presence of 100  $\mu$ M phencyclidine to inhibit labeling of the high affinity site for noncompetitive blockers by [ $^3$ H]DDF (Kotzyba-Hibert *et al.*, 1985). Under these conditions, [ $^3$ H]DDF labeling of the  $\alpha$ -subunit decreased from 7980 dpm/ $\mu$ g to 4100 dpm/ $\mu$ g in the presence of 100  $\mu$ M carbamoylcholine and to 3700 dpm/ $\mu$ g in the presence of 10  $\mu$ M  $\alpha$ -bungarotoxin.

The  $\alpha$ -subunit labeled either in the presence or in the absence of carbamoylcholine and  $\alpha$ -bungarotoxin was then purified by preparative polyacrylamide gel electrophoresis, carboxymethylated, and treated with CNBr. Fractionation of the CNBr fragments performed as described (Dennis *et al.*, 1988) yielded the three expected radioactive peaks eluting in fractions 42–65 (peak I), 71–105 (peak II), and 130–140 (peak III) (Fig. 1). Injection of equal protein amounts of each CNBr digest followed by measurement of the radioactivity associated with each peak revealed that carbamoylcholine and  $\alpha$ -bungarotoxin similarly inhibited 70–90% of [ $^3$ H]DDF incorporation in the CNBr fragments. Protection against [ $^3$ H]DDF incorporation in these fragments did not differ when carbamoylcholine or  $\alpha$ -bungarotoxin were used in the absence of phencyclidine. Inhibition of [ $^3$ H]DDF labeling, at the level of the CNBr fragments, can thus be simply accounted for by a common blocking mechanism by the agonist carbamoylcholine and the competitive antagonist  $\alpha$ -bungarotoxin.

The high reactivity of the photogenerated species of DDF





**FIG. 1. Effects of various ligands of the AChR on  $[^3\text{H}]\text{DDF}$  labeling.** Semipreparative batches of alkali-treated AChR-rich membrane fragments were photolabeled with  $[^3\text{H}]\text{DDF}$  in the presence of: *panel A*, 100  $\mu\text{M}$  phencyclidine (PCP) (●—●), 100  $\mu\text{M}$  carbamoylcholine (— — —), 100  $\mu\text{M}$  phencyclidine + 100  $\mu\text{M}$  carbamoylcholine (—); *Panel B*, 100  $\mu\text{M}$  phencyclidine (●—●), 10  $\mu\text{M}$   $\alpha$  bungarotoxin (— — —), 100  $\mu\text{M}$  phencyclidine + 10  $\mu\text{M}$   $\alpha$  bungarotoxin (—); and the labeled  $\alpha$ -subunits were purified and treated with CNBr (see "Experimental Procedures"). 15  $\mu\text{g}$  of each  $\alpha$ -subunit digest was loaded onto a Brownlee RP 300 column equilibrated with 90% solvent A (0.1% trifluoroacetic acid in  $\text{H}_2\text{O}$ ), 10% solvent B (60% 1-propanol, 30% acetonitrile, 0.1% trifluoroacetic acid in  $\text{H}_2\text{O}$ ). After 10 min of elution, at 1 ml/min, with the same solvent composition, a linear gradient was developed from 10% B to 60% solvent B during 100 min. Total eluate was collected (2 fractions/min) and submitted to scintillation counting.

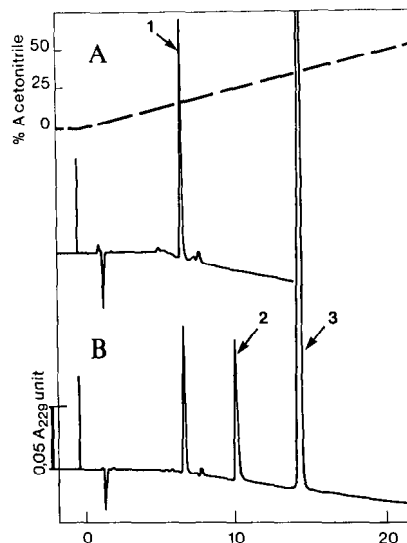
(Scaiano and Nguyen, 1983) renders it unlikely that one of these peptides is labeled after diffusion of the reactive intermediate away from the DDF-binding site. This possibility was nevertheless tested by analyzing the effects of "scavenging" reagents.

**Effect of Water**—When irradiated under conditions of direct illumination, DDF gives rise to an arylation, the half-life of which has been estimated, in water, to be as about or slightly shorter than 500 ps (Scaiano and Nguyen, 1983) before reacting with the solvent to yield over 95% of the corresponding phenol (Kieffer *et al.*, 1981).

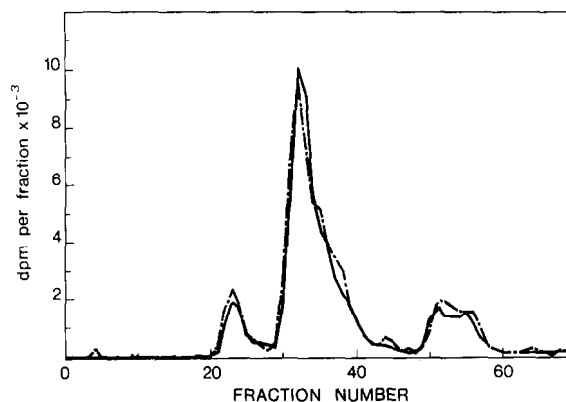
Fig. 2 shows that under the energy transfer irradiation conditions used to photolabel the AChR, DDF yields the same phenol derivative as under conditions of direct illumination (380 or 435 nm). No by-product is detected. These results indicate that DDF reacts efficiently with water under the present experimental conditions.

**Effect of Other Reagents**—Photolabeling of the AChR by  $[^3\text{H}]\text{DDF}$  was performed in the absence and presence of ribonuclease A (1 mg/ml), Tris-hydroxymethylaminomethane (10 mM) (Ruoho *et al.*, 1984), tyrosine (1 mM), oxytocin (1 mM), a nonapeptide-containing tyrosine and cystine residues, and the radical scavenger 2-methyl-2-nitrosopropane (Johnstone *et al.*, 1971). The covalent incorporation of  $[^3\text{H}]\text{DDF}$  in the  $\alpha$ -subunit was measured as described previously in the presence and absence of saturating concentration of carbamoylcholine ( $10^{-4}$  M).

Ribonuclease A did not affect the labeling (not shown). The other reagents decreased the carbamoylcholine-sensitive ra-



**FIG. 2. A**, a 2-ml aqueous solution of DDF ( $2.10^{-4}$  M) was irradiated at 380 nm until complete photodecomposition, and a 50- $\mu\text{l}$  aliquot was analyzed on a  $\mu\text{Bondapak C}_{18}$  HPLC column ( $300 \times 3.9$  mm). The elution was performed at 1 ml/min using a linear gradient from 100% solvent A (0.1% trifluoroacetic acid in  $\text{H}_2\text{O}$ ) to 100% acetonitrile in 40 min. **B**, a 2-ml aqueous solution of DDF ( $2.10^{-4}$  M) and *N*-acetyltryptophanamide ( $2.10^{-4}$  M) was irradiated under the conditions of energy transfer (Goeldner and Hirth, 1980) at the same energy and during the same time used to photolabel the AChR ( $E = 60$   $\mu\text{V}$ ;  $t = 30$  min). A 100- $\mu\text{l}$  aliquot was analyzed as in A. 1, *p*-(*N,N*-dimethylamino)phenol; 2, DDF; 3, *N*-acetyl tryptophanamide.



**FIG. 3. Labeling by  $[^3\text{H}]\text{DDF}$  in the presence of oxytocin.** Semipreparative batches of alkali-treated AChR-rich membrane fragments were photolabeled by  $[^3\text{H}]\text{DDF}$  in the absence (●—●) and presence (—) of 1 mM oxytocin. The covalent incorporation of radioactivity in the  $\alpha$ -subunits (dpm/ $\mu\text{g}$  of chain) was measured as described in the absence (4900 versus 3800 in the presence of 1 mM oxytocin) and in the presence (1800 versus 890 in the presence of 1 mM oxytocin) of saturating concentration ( $10^{-4}$  M) of carbamoylcholine. 30  $\mu\text{g}$  of each  $\alpha$ -subunit CNBr digest was loaded onto a Brownlee RP 300 column equilibrated with 100% solvent A (0.1% trifluoroacetic acid in  $\text{H}_2\text{O}$ ) and eluted at 1 ml/min with a linear gradient from 100% solvent A to 50% solvent A, 50% solvent B (60% *n*-propanol, 30% acetonitrile, 0.1% trifluoroacetic acid) in 70 min. Total eluate was collected (1 fraction/min) and submitted to scintillation counting. Each point represents the specific radioactivity contained in a given fraction, i.e. the difference between the radioactivity eluted from a sample initially labeled in the absence and that of a sample initially labeled in the presence of carbamoylcholine. The recovery of injected radioactivity was 80% for each of the sample initially irradiated in the absence of carbamoylcholine.

dioactivity incorporation by less than 5–10%, a value standing within the limits of experimental error. As illustrated in Fig. 3, the distribution of radioactivity among the three CNBr peptides of  $\alpha$ -subunit did not differ significantly when the

labeling was performed in the absence or presence of 1 mM oxytocin.

In conclusion, the labeling of the three CNBr fragments of  $\alpha$ -subunit is equally insensitive to the presence of scavenging reagents. When bound to its site [ $^3\text{H}$ ]DDF would thus be expected, upon photolysis, to label amino acids in its vicinity without significant diffusion of the reactive intermediate, the aqueous solvent most probably serving as scavenger.

#### Identification of [ $^3\text{H}$ ]DDF-labeled Amino Acid(s) in the 31–105 Region

Large batches of CNBr peak III, obtained from cleavage of  $\alpha$ -subunit labeled by [ $^3\text{H}$ ]DDF (0.6 Ci/mmol) either in the absence (26,000 dpm/ $\mu\text{g}$  of  $\alpha$ -subunit) or in the presence of 100  $\mu\text{M}$  carbamoylcholine (9,500 dpm/ $\mu\text{g}$  of  $\alpha$ -subunit), were purified.

CNBr peak III contained the peptide-extending from residue 1 to 105 and/or 117 (estimated molecular weight: 12,600) and another peptide starting at residue 106 (Dennis *et al.*, 1988). The corresponding fractions were pooled, concentrated, and used without further purification.

**IBA Digestion of CNBr Fragment III**—The material contained in peak III of  $\alpha$ -subunit CNBr digests of either the unprotected or the carbamoylcholine protected batches was incubated with *o*-iodosobenzoic acid (see "Experimental Procedures") in the presence of excess *p*-cresol to prevent tyrosine oxidation. When the crude reaction mixture was subjected to reversed-phase HPLC, approximately 25% of the injected radioactivity was associated with unretained material (not shown). The amount of radioactivity in this fraction was the same as in the carbamoylcholine-protected sample. This material was not further analyzed.

As shown in Fig. 4, carbamoylcholine-protectable [ $^3\text{H}$ ]DDF-labeled material eluted from the reversed-phase column between 26 and 35% solvent B as three discrete radioactive peaks associated with UV<sub>210</sub> absorption peaks. Approximately 40% of the starting material was recovered in uncleaved form (40–43% solvent B, not shown).

Automated sequence analysis of material contained in pools A and B (Fig. 4) revealed identical unique amino-terminal

sequence corresponding to the  $\alpha$ -subunit IBA fragment extending from Leu-87 (Table I). The results of radioactivity measurements on the sequencer output (Table I and Fig. 5) revealed a release of tritium at cycle 7 which corresponded to labeling of  $\alpha$ -Tyr-93 in the sequence of the fragment extending from Leu-87. No further release of radioactivity above background was observed in up to 19 cycles of sequence, which corresponded to the next cyanogen bromide cleavage site ( $\alpha$ -Met-105).

Upon sequencing of the corresponding carbamoylcholine-protected batch (pool A<sup>+</sup>), the same unique sequence extending from  $\alpha$ -Leu-87 was detected, but the radioactivity associated with cycle 7 was reduced to background level (Fig. 5). This result, together with those of pool B<sup>+</sup> (data not shown), demonstrates unambiguously that photolabeling of  $\alpha$ -Tyr-93 by [ $^3\text{H}$ ]DDF was inhibited by the agonist carbamoylcholine.

When material contained in pool C was submitted to Edman degradation, four amino-terminal sequences were identified. Three of them corresponded to IBA fragments (cleavage after  $\alpha$ -Trp-60, -67, and -86) extending from Ile-61, Asn-68, and Leu-87. The remaining sequence was identified as the CNBr fragment extending from Thr-106 which was present in the starting material. Results of radioactivity measurements for this degradation are given in Table I. A radioactivity release which was markedly decreased in the carbamoylcholine-protected batch (1127 to 128 dpm for equal amounts of protein) was observed at cycle 7 which could at least partly be attributed to  $\alpha$ -Tyr-93. Among the three other identified sequences, the one starting at Thr-106 could not account for radioactivity release at cycle 7 since it has been previously shown that [ $^3\text{H}$ ]DDF was not incorporated in this fragment (Dennis *et al.*, 1988). Yet, additional labeling of Trp-67 and/or Gly-74 could not be ruled out at this stage.

The separation of three radioactive peaks corresponding to identical amino-terminal sequences remains unclear in view of our results. Carboxyl-terminal heterogeneity of the CNBr fragments associated with partial chemical modification by IBA, of histidine and methionine residues (Fontana *et al.*, 1983), respectively, present at cycles 18 and 19 in the sequence extending from  $\alpha$ -Leu-87, can be among possible explanations for such differences.

**Trypsin Digestion of CNBr Fragment III**—In order to establish whether  $\alpha$ -Trp-67 and Gly-74 represented sites of specific incorporation of [ $^3\text{H}$ ]DDF in the region 1–105 of  $\alpha$ -subunit, CNBr peak III was subcleaved with trypsin and the radiolabeled fragments purified by reversed-phase HPLC.

Approximately 40% of the radioactivity recovered from the HPLC column eluted with unbound material (Fig. 6). The labeling of this material was decreased by 50% in the carbamoylcholine-protected samples. Repeated Edman degradations performed on this sample never led to any release of detectable PTH amino acids nor of radioactivity in up to 20 cycles of sequence. Moreover, no radioactivity could be detected on the filter disc after sequencing, suggesting that this radioactive material did not strongly adsorb to the biobrene pretreated filter disc and probably eluted from the filter during the various solvent wash steps. Initial adsorption of the sample on the filter disc could not be improved by desalting (Bio-Gel P-10 or reversed-phase C18 HPLC).

A second peak of carbamoylcholine-sensitive radioactivity was eluted from the column between 34 and 41% solvent B. The purified material was pooled (fractions 65–74) and subjected to automated Edman degradation. Two overlapping amino-terminal sequences corresponding to trypsin cleavage after Lys-77 and Arg-79 were present (Table II). Quantitative analysis of the sequence data revealed significant carry-over

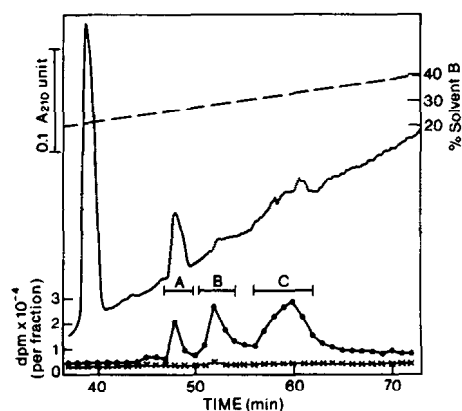


FIG. 4. Reversed-phase HPLC of CNBr fragment III subcleaved with IBA. Purified  $\alpha$ -subunit labeled by [ $^3\text{H}$ ]DDF in the absence and in the presence of 100  $\mu\text{M}$  carbamoylcholine was cleaved with CNBr, and fragment III was purified. Radioactivity associated with this fragment was decreased by 75–80% in the carbamoylcholine-protected sample. Purified CNBr fragment III ( $\sim 10$  nmol) was treated with IBA and submitted to reversed-phase HPLC (see "Experimental Procedures"). UV absorbance was monitored at 210 nm, and aliquots of the 1-ml fractions of unprotected ( $\bullet$ ) and protected ( $\times$ ) samples were subjected to liquid scintillation counting. Recovery in radioactivity was 85%. Horizontal bars indicate the fractions pooled (A, B, and C) for subsequent characterization.

TABLE I

Yields of PTH amino acids upon sequence analysis of IBA fragments

Samples of material contained in pool A, B, C, and A\* (fractions corresponding to pool A in the carbamoylcholine-protected sample) were subjected to automated sequence analysis. The amount of covalently bound [ $^3\text{H}$ ]DDF loaded onto the sequencer was calculated from the specific activity of [ $^3\text{H}$ ]DDF used for photolabeling. The PTH amino acids identified at each cycle are designated by the conventional one letter code. Yields are corrected for background. The initial amount and average repetitive yields were calculated by linear regression analysis. Due to the high number of cycles where the same residue (\*) could be attributed to different sequences (pool C) no average repetitive yield or initial amount were calculated. Residues in parentheses are those expected according to the deduced sequence when they could not be identified or when their corresponding PTH peak, although present, did not rise above the background. The starting position denotes the position of the amino-terminal residue in the complete *T. marmorata*  $\alpha$ -subunit sequence. Radioactivity released at each cycle of the Edman degradation is indicated.

Cycle	PTH amino acids (pmol) in pool										
	A		B		C				A*		
	dpm		dpm		dpm					dpm	
1	70	L 27	292	L 100	453	I 93	N 403	L 115	T 43	48	L 13
2	78	P 21	152	P 282	311	(D)	P 312*	P 312*	K	47	P 10
3	74	D 11.5	472	(D)	447	V 67	A 181	(D)	L 48	43	D 3.6
4	80	L 17	249	L 211	303	R	D 106	L 70*	L 70*	49	L 5.3
5	90	V 6.5	277	V 222	350	L 76*	Y 52	V 37	L 76*	40	V 3.4
6	77	L 13	267	L 135	267	(R)	G 78	(L)	D 50	41	L 5.4
7	749	Y 8	5242	Y 193	1127	(W)	G 62	Y 40*	Y 40*	43	Y 3.1
8	199	N 11.5	1784	N 184	679	N 52*	I 10	N 52*	T 24	39	N 2.9
9	83	N 8.5	580	N 205	468	P 30		N 42	G 42	44	N 4.6
10	60	A 10	350	A 151	315	A 16*		A 16*		40	A 2.5
11	63	D 11	278	D 57	271	D 13*		D 13*		44	D 1.9
12	77	G 10	195	G 75	287	Y 10		G 22		42	G 2
13	60	D 6	193	D 43						44	D 1.8
[ <sup>3</sup> H]DDF loaded (pmol)		6		32				65			1.1
Initial peptide amount (pmol)		31		275							10
Repetitive yield (%)		86		90							87
Starting position		87		87		61	68	87	106		87

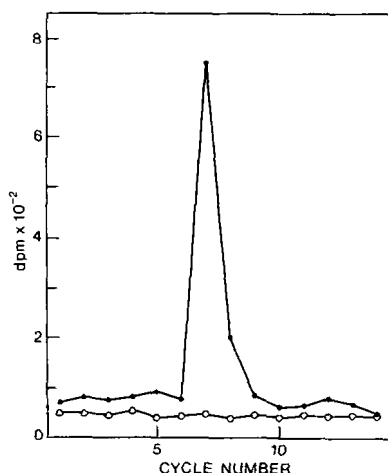


FIG. 5. Radioactivity released upon sequence analysis of IBA subfragments (pool A and A\*) of [ $^3\text{H}$ ]DDF-labeled CNBr fragment III. The sample loaded (pool A) contained approximately  $7.5 \times 10^3$  dpm. The amount of radioactivity present in the PTH fraction at each cycle is shown (●). Radioactivity released upon sequencing of similar material derived from carbamoylcholine-protected  $\alpha$ -subunit (○) was corrected for equal amounts of protein.

of the PTH amino acids and quite low repetitive yields. In an attempt to overcome these problems, known to occur when proline residues are present in the amino acid sequence (Tarr, 1977), we tried an extension of the acid cleavage reaction at the corresponding cycles of peptide degradation. In the present analysis, this treatment carried out on pool T but not pool

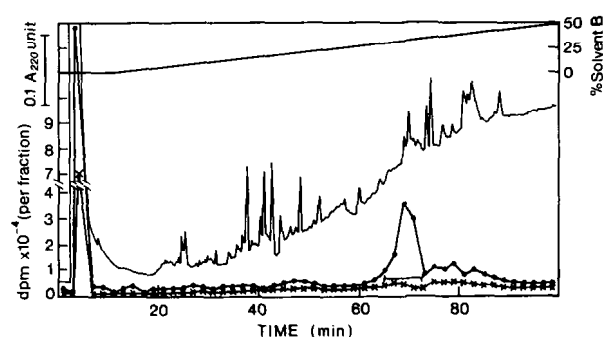


FIG. 6. Reversed-phase HPLC of CNBr fragment III sub-cleaved with trypsin. Purified CNBr fragment III from unprotected and carbamoylcholine-protected  $\alpha$ -subunit CNBr digests ( $\sim 10$  nmol) were treated with trypsin and submitted to reversed-phase HPLC (see "Experimental Procedures"), and the UV absorbance was monitored at 220 nm. Aliquots of 1-ml fractions of unprotected (●) and protected (x) samples were subjected to liquid scintillation counting. Recovery in radioactivity was 65%. Horizontal bar indicates material pooled (pool T for fractions corresponding to unprotected batch and T\* for fractions corresponding to carbamoylcholine-protected batch) for further characterization.

T\* did not improve significantly the repetitive yield of Edman degradation (see Table II).

The results of radioactivity measurements on the sequencer output are shown in Fig. 7. Although not always in the same relative amount (as compared with cycles 14–16), some tritium release was observed at cycle 7 and 9 in each Edman degradation of independently prepared samples and was not ob-



TABLE II

Yields of PTH amino acids upon sequence analysis of tryptic fragments

Samples of purified trypsin digest (pools T and T<sup>+</sup> in Fig. 3) were subjected to automated Edman degradation. Details are as given in Table I.

Cycle	PTH amino acids			
	T		T <sup>+</sup>	
	pmol			
1	I 435	L 255	I 155	L 97
2	R 198	P 244	R 171	P 59
3	L 228	S 126	L 136	S 40
4	P 288	D 97	P 84	D 22
5	S 131	D 126	S 63	D 34
6	D 187	V 16.5	D 53	V 10
7	D 180	(W)	D 78	(W)
8	V 23.4	L 16	V 32	L 25
9	(W)	P 19	(W)	P 8
10	L 29	(D)	L 22	(D)
11	P 40	(L)	P 10	(L)
12	(D)	V 4.7	(D)	(V)
13	L 19 *	L 19*	L 11*	L 11*
14	V 6.4	Y 2.9	V 5	Y 3
15	L 21	N 3.6	(L)	(N)
16	Y 5.7	N 7.2	Y 4	(N)
17	N 12	A 2.5	N 3	
18	N 13		N 5	
19	A 3.6		A 1	

[ <sup>3</sup> H]DDF loaded (pmol)	98		1.22	
Initial peptide amount (pmol)	478	277	265	86
Repetitive yield (%)	78	74	77	78
Starting position	78	80	78	80

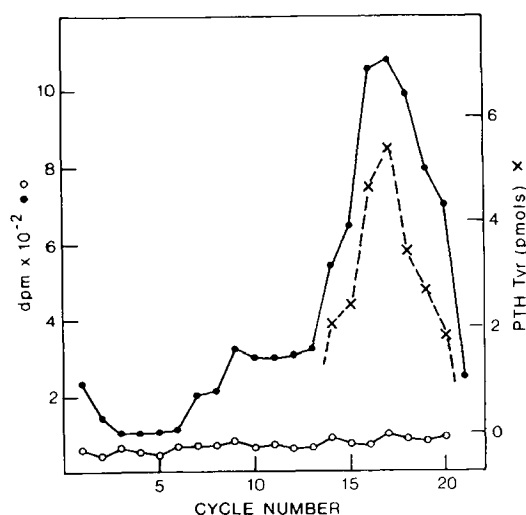


FIG. 7. Radioactivity released upon sequence analysis of trypsin subfragments (pool T and T<sup>+</sup> in Table II) of [<sup>3</sup>H]DDF-labeled CNBr fragment III. The sample loaded (pool T (●)) contained 1.3 10<sup>3</sup> dpm. Radioactivity released upon sequencing of similar material derived from carbamoylcholine-protected  $\alpha$  subunit (○) was corrected for equal amount of protein. For comparison, raw values of PTH-tyrosine (x) release in pool T are shown.

served in the protected batch (pool T<sup>+</sup>). As deduced from *T. marmorata* electric organ  $\alpha$ -subunit sequence (Devillers-Thiery *et al.*, 1983), the residue expected at cycle 7 in the trypsin fragment extending from Leu-80 was Trp-86. Its labeling could, however, not be established with certainty. Indeed, PTH-Trp release could not be quantitatively corre-

lated to the radioactivity profile for cycles 7 to 14 of the tryptic peptides degradation, since under the standard HPLC amino acid analysis conditions used (Hunkapiller, 1985), it coeluted with diphenyl urea.

The marked increase in tritium release observed at cycles 14 and 16 of peptides degradation corresponded to the expected release of [<sup>3</sup>H]DDF-labeled Tyr-93. Minor increase of radioactivity was also observed at cycles 15 and 17. It probably did not reflect labeling of Asn-94 since, in the IBA fragments, a clear decrease could be observed at cycle 8 (Asn-94) in each of the sequenced samples (Table I and Fig. 5). In fact, the radioactivity profile between cycles 14 and 20 was found to correlate with PTH-Tyr release from the sequencer (Fig. 7) illustrating the carry-over problem discussed above.

No other carbamoylcholine-sensitive radioactive peak was present in the fractionation of the tryptic fragments (Fig. 6). Possible specific incorporation of the label in Trp-67 and/or Gly-74, raised by the sequence analysis of pool C of IBA fragments, did not receive further experimental support. Thus, Tyr-93 and probably Trp-86 are most likely the only labeled amino acid(s) in the 1–105 region of the  $\alpha$ -subunit.

#### DISCUSSION

Since the elucidation of the complete primary structure of the AcChoR (Noda *et al.*, 1982, 1983; Claudio *et al.*, 1983; Devillers-Thiery *et al.*, 1983), several attempts have been made to localize ligands-binding sites for agonists and competitive antagonists (review in Stroud and Finer-Moore, 1985).

Our approach has consisted in the covalent labeling of the functionally significant AcCho-binding sites by [<sup>3</sup>H]DDF and the identification of the modified amino acids by protein chemical techniques.

We now show by sequence analysis of radioactive subfragments of CNBr peptides in peak III, that the labeled amino acid in the region 1–105 of  $\alpha$ -subunit is Tyr-93 (and possibly Trp-86). Tyr-93 and Trp-86 are located approximately 100 residues away from the pair of cysteines 192 and 193 in the large hydrophilic amino-terminal domain of the  $\alpha$ -subunit. Their photolabeling was not suspected on the basis of  $\alpha$ -toxin and acetylcholine binding to proteolytic fragments (or corresponding synthetic peptides) of AcChoR  $\alpha$ -subunit (review in Luyten, 1986; references in Gotti *et al.*, 1988) or affinity and photoaffinity labeling of the AcCho-binding sites using ligands other than DDF (see Introduction). It brings additional evidence in favor of a multiple-loop-site involving at least three segments of the  $\alpha$ -subunit hydrophilic amino-terminal domain.

Different arguments suggest that the three radioactive peptides (CNBr fragments I–III), which contain all the identified labeled residues, are indeed related to the binding sites for cholinergic ligands. (i) The photoreactive species of DDF is generated by energy transfer (Langenbuch-Cachat *et al.*, 1988) from an aromatic amino acid which belongs to the receptor and is thus initially produced in the close vicinity of the ligand-binding site. (ii) [<sup>3</sup>H]DDF incorporation in the three CNBr fragments is not sensitive to aqueous photochemical scavengers and thus does not occur after significant diffusion of the reactive intermediate. (iii) [<sup>3</sup>H]DDF incorporation in the CNBr fragments is abolished by 100  $\mu$ M carbamoylcholine, a concentration at which primary AcCho-binding sites are selectively occupied (Cohen and Strnad 1987). (iv) Labeling of the three CNBr fragments of  $\alpha$ -subunit is identically inhibited by carbamoylcholine and  $\alpha$ -bungarotoxin (see "Results"), showing thus that the two competitive antagonists (DDF and  $\alpha$ -bungarotoxin) and the agonist (carbamoylcholine) all inter-

α1	Torpedo	V <u>W</u> L <u>P</u> D <u>L</u> V <u>L</u> YNN... GI <u>W</u> T <u>Y</u> DG... VY <u>Y</u> T <u>C</u> CPD-TP <u>X</u> LD	85	95	147	153	188	200
α1	Human...	I <u>W</u> R <u>P</u> D <u>L</u> V <u>L</u> YNN... GT <u>W</u> T <u>Y</u> DG... VT <u>Y</u> S <u>C</u> CPD-TP <u>X</u> LD						
α2	Rat....	I <u>W</u> I <u>P</u> D <u>I</u> V <u>L</u> YNN... GS <u>W</u> T <u>Y</u> DK... KK <u>Y</u> D <u>C</u> C-AEI- <u>Y</u> PD						
α3	Rat....	I <u>W</u> K <u>P</u> D <u>I</u> V <u>L</u> YNN... GS <u>W</u> S <u>Y</u> DK... IK <u>Y</u> N <u>C</u> C-EEI- <u>X</u> QD						
α4	Rat....	I <u>W</u> R <u>P</u> D <u>I</u> V <u>L</u> YNN... GS <u>W</u> T <u>Y</u> DK... RK <u>Y</u> E <u>C</u> C-AEI- <u>Y</u> PD						
β	Torpedo	V <u>W</u> Q <u>P</u> D <u>I</u> V <u>L</u> MNN... KS <u>Y</u> T <u>Y</u> DT... ----RSDD <u>P</u> S <u>Y</u> ED						
γ	Torpedo	L <u>W</u> L <u>P</u> D <u>V</u> V <u>L</u> ENN... RSQT <u>Y</u> NA... NWQLTK-DDTDFQE						
δ	Torpedo	V <u>W</u> I <u>P</u> D <u>I</u> V <u>L</u> QNN... TALN <u>Y</u> DA... -Y <u>P</u> DK <u>F</u> PN <u>G</u> T <u>N</u> YQD						
β2	Rat....	I <u>W</u> L <u>P</u> D <u>V</u> V <u>L</u> YNN... RS <u>W</u> T <u>Y</u> DR... RRNEN-PDDST <u>Y</u> VD						

FIG. 8. Comparison of the amino acid sequences containing the [<sup>3</sup>H]DDF-labeled amino acids of *Torpedo* AcChoR α-subunits (Devillers-Thiery *et al.*, 1983) with the corresponding fragments of human muscular α<sub>1</sub> (Noda *et al.*, 1983), neuronal rat-α<sub>2</sub> (Wada *et al.*, 1988), -α<sub>3</sub> (Boulter *et al.*, 1986), -α<sub>4</sub> (Goldman *et al.*, 1987), -β<sub>2</sub> (Deneris *et al.*, 1988), and electric organ α-, β-, and δ- (Noda *et al.*, 1983) subunits of nicotinic AcChoR. The amino acids unambiguously labeled (Dennis *et al.*, 1988, this report) are indicated by capital bold letters and those for which suggestive evidence for labeling was obtained are underlined.

act with binding areas which contain common regions represented by the three radioactive peptides.

The present findings along with previous results (Kao *et al.*, 1984; Dennis *et al.*, 1988; Abramson *et al.*, 1988, 1989) suggest that Tyr-93, Trp-149, Tyr-190, Cys-192, and Cys-193 lie within, or in close proximity to, the AcCho-binding sites and may contribute to cholinergic ligands binding. In this respect, it is noteworthy that these [<sup>3</sup>H]DDF-labeled amino acids are highly conserved, throughout evolution, in the α-subunit primary structure of AcChoR, where they are found at homologous positions in all muscle and neuronal α-subunits sequenced to date (Fig. 8). In contrast, in agreement with the known role of α-subunit in the binding of agonists, these [<sup>3</sup>H]DDF-labeled amino acids are absent on the corresponding portions of the β-, γ-, and δ-subunits from fish electric organ and muscle AcChoR. Some of them are, however, present at homologous positions in the chick non-α (Nef *et al.*, 1988) and rat β<sub>2</sub>, β<sub>3</sub>, and non-α<sub>2</sub> amino acid sequences of neuronal nicotinic AcChoR (Deneris *et al.*, 1988, 1989; Isenberg and Meyer 1989).

The labeling of three other amino acids Trp-86 (this report), Tyr-151, and Tyr-198 (Dennis *et al.*, 1988) could not be established with certainty upon sequence analysis of the α-subunit radioactive fragments. This was mainly due to the low amount of radioactivity released from the sequencer at the corresponding cycles, which probably reflected weak [<sup>3</sup>H]DDF incorporation in these amino acids. Possible explanations for this low incorporation are (i) the immobilization of the probe in a definite orientation in the binding site and (ii) that these amino acids are not located immediately within the binding site of DDF. (iii) Finally, it cannot be excluded that labeling by [<sup>3</sup>H]DDF reflects, to some extent, the pharmacological nonequivalence of the two AcCho-binding sites (review in Karlin, 1983; Culver *et al.*, 1984; Dowding and Hall, 1987; Jackson, 1988).

The proposal that binding of the choline moiety of acetylcholine would involve electrostatic interactions between negatively charged amino acid(s) and the quaternary ammonium group led to models of the AcCho-binding site where aspartic or glutamic acid formed the negative subsite for AcCho (review in Luyten, 1986). These assumptions were indirectly supported by crystallographic analyses of phosphocholine-specific antibody M 603 (Padlan *et al.*, 1976, see, however,

Satow *et al.*, 1986), and by molecular recognition studies showing that macrocyclic compounds substituted with carboxylate groups (Behr *et al.*, 1976, 1982) bound unsubstituted ammonium ions in aqueous medium with high affinities. However, binding of methyl-substituted ammonium groups was shown to require not only electrostatic but also hydrophobic interactions (Dhaenens *et al.*, 1984; Lehn 1985; see also Kieffer *et al.*, 1986), which could be both provided by non-carboxylate containing entities such as macrobicyclic polyphenoxides (Schneider *et al.*, 1986). Complexation of the quaternary ammonium group of choline by such bowl-shaped cavities, delimited by the aromatic rings of ionized phenols, occurs with micromolar affinity constants in aqueous medium. An interesting possibility is that such a mechanism of interaction could account for the binding of cholinergic ligands to the AcChoR. Indeed, carbamoylcholine-sensitive [<sup>3</sup>H]DDF incorporation in aromatic amino acids of the AcChoR α-subunit and in particular at the level of tyrosine residues 93 and 190 (and possibly 151 and 198) makes plausible that, as in the case of macrocyclic compounds, their aromatic ring may directly contribute to quaternary ammonium binding. In agreement with this hypothesis, expression studies in oocytes using the recently isolated neuronal nicotinic AcChoR α-5 clone (Boulter *et al.*, 1990), which lacks the tyrosine residues homologous to *Torpedo* α-Tyr-93 and -190, do not lead to any depolarizing response to acetylcholine.

Crystallographic studies of the liganded receptor protein combined with site-directed mutagenesis experiments may lead to direct tests of this hypothesis.

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