Identification of a Novel Amino Acid cr-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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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 \( \text{Torpedo marmorata} \) was photolabeled by the competitive antagonist \( p-[\text{H}]\text{dimethylaminobenzenediazonium fluoroborate} \) (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 \( [\text{H}] \text{DDF} \) 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 \( \text{o-iodosobenzoic acid} \) 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 \( \text{[H]} \text{DDF} \) 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) \( \text{Biochemistry} \) 27, 2346–2357). Possible contribution of tyrosine side-chains to the complexation of the quaternary ammonium group of cholinergic ligands is discussed.

The nicotinic acetylcholine receptor (AcChoR)\(^1\) from fish 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 \( \alpha_2\beta_2 \) pentamer (Reynolds and Karlin, 1978), which carries the acetylcholine (AcCho)-binding site and contains the cation-selective channel forming elements (see Popol 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, 1978, 1990), which are located, at least in part, on the \( \alpha \)-subunits (Karlin 1989, 1988; Haggerty and Froehner 1981; "Txarlos and Changeux, 1983, 1984; Gershoni et al., 1988; 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 \( (\text{Kao et al., 1984}) \), (ii) photoaffinity labeling with \( \text{D}-\text{tubocurarine} \) \( (\text{Pedersen et al., 1986}) \), (iii) binding of snake \( \alpha \)-toxins to \( \alpha \)-subunit proteolytic fragments \( (\text{Wilson et al., 1984}, 1985; \text{Neumann et al., 1985}, 1986; \text{Oblas et al., 1986}) \), synthetic peptides \( (\text{Radding et al., 1988}; \text{Wilson et al., 1985}; \text{Mulac-Jericevic and Atassi, 1986}; \text{Ralston et al., 1987}) \), deletion mutants \( (\text{Barkas et al., 1987}) \), or \( \alpha \)-subunit fragments expressed in \( \text{Escherichia coli} \) transformants \( (\text{Gershoni, 1987}) \). 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 \( p-[\text{H}]\text{dimethylaminobenzenediazonium fluoroborate} \) (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 \( \text{Torpedo marmorata} \) AcChoR upon irradiation under conditions of energy transfer \( (\text{Langen-buch-Cachat et al., 1988}) \). Cyanogen bromide (CNBr) cleavage of the purified \( \alpha \)-subunits yielded three distinct radioactive fragments \( (\text{Dennis et al., 1986}) \) 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-
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nis et al., 1988). Furthermore, the competitive antagonist lophotoxin was recently found to label Tyr 190 of the native α-subunit (Abramson et al., 1989).

Using a monoclonal antibody (mAb 6) that specifically binds to the α-subunit of AcChR, 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 [3H]DDF incorporation in the different α-subunit fragments (or amino acids). In this report, we first explore this possibility by studying the inhibition of [3H]DDF incorporation in the α-subunit acetylcholine-binding sites by agonist and competitive antagonist ligands. Second, we identify (a) novel amino acid(s) labeled by [3H]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

[3H]DDF precursor was tritiated by Dr. Van Hove and Rousseau (Commissariat à l’Energie Atomique, France) and was diazotized as described (Langeth-Cachat et al., 1988). Phencyclidine was generously provided by A. Jaganathen (Universite Louis Pasteur, Strasbourg, France). Carbamoylcholine and oxytocin were purchased from Sigma, CNBr from Eastman Kodak, o-iodosobenzene acid and ribonuclease from Pierce Chemical Co., p-cresol and 2-methyl-2-nitroso-propane from Aldrich, and 1,1-β-mercapto-2-phenylethyl chloro-methyl ketone-treated trypsin from Merck. Live T. marmorata were obtained from the Biological Station of Arcachon (France).

Covalent Labeling of the AChR by [3H]DDF—Preparative photo-labeling of large batches (100–200 nmol of α-bungarotoxin-binding sites) of purified (Saitoh et al., 1980) and alkali-treated (Neubig et al., 1985) AcChoR-rich membrane fragments was achieved by energy-transfer photolysis procedure (Langenbuch-Cachat et al., 1988). Analytical polyacrylamide gel electrophoresis of the labeled membranes confirmed that, as shown previously, the α-subunit carried the majority of the [3H]DDF incorporated within the AcChR oligomer (Dennis et al., 1988, 1986; Langenbuch-Cachat et al., 1988). The incorporation of [3H]DDF into the α-subunit was assayed in the presence of 100 μM phencyclidine to inhibit labeling of the high affinity site for competitive blockers by [3H]DDF (Kotys hypertension, 1986). Under these conditions, [3H]DDF labeling of the α-subunit decreased from 7800 dpm/μg to 4100 dpm/μg in the presence of 100 μM phencyclidine.

The α-subunit labeled either in the presence or in the absence of carbamoylcholine and α-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 1), 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 α-bungarotoxin similarly inhibited 70–90% of [3H]DDF incorporation in the CNBr fragments. Protection against [3H]DDF incorporation in these fragments did not differ when carbamoylcholine or α-bungarotoxin were used in the absence of phencyclidine. Inhibition of [3H]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 α-bungarotoxin.

The high reactivity of the photogenerated species of DDF
(Scainano 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 arylcation, the half-life of which has been estimated, in water, to be about or slightly shorter than 500 ps (Scainano 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 AcChoR, 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 AcChoR by [3H]DDF was performed in the absence and presence of ribonuclease A (1 ng/ml), Tris-hydroxymethylaminomethane (10 mM) (Kuo et al., 1984), tyrosine (1 mM), oxytocin (1 mM), a nonapeptide-containing tyrosine and cystine residues, and the radical scavenger 2-methyl 2-nitropropane (Johnstone et al., 1971). The covalent incorporation of [3H]DDF in the α-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 radioactivity 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 α-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 a-subunit is equally insensitive to the presence of scavenging reagents. When bound to its site [3H]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 [3H]DDF-labeled Amino Acid(s) in the 31-105 Region

Large batches of CNBr peak III, obtained from cleavage of a-subunit labeled by [3H]DDF (0.6 Ci/mmol) either in the absence (26,000 dpm/µg of a-subunit) or in the presence of 100 µM carbamoylcholine (9,500 dpm/µg of a-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 a-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 95% 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 [3H]DDF-labeled material eluted from the reversed-phase column between 26 and 35% solvent B as three discrete radioactive peaks associated with UV absorption peaks. Approximately 40% of the starting material was recovered in uncleaved form (40-45% 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 a-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 a-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 (α-Met-105).

Upon sequencing of the corresponding carbamoylcholine-protected batch (pool A'), the same unique sequence extending from a-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' (data not shown), demonstrates unambiguously that photolabeling of a-Tyr-93 by [3H]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 α-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 a-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 [3H]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 (Fontans et al., 1983), respectively, present at cycles 18 and 19 in the sequence extending from α-Leu-87, can be among possible explanations for such differences.

Trypsin Digestion of CNBr Fragment III—In order to establish whether α-Trp-67 and Gly-74 represented sites of specific incorporation of [3H]DDF in the region 1-105 of a-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 biobrenetreated 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...
### TABLE I

Yields of PTH amino acids upon sequence analysis of IBA fragments

| Cycle | PTH amino acids (pmol) in pool |
|-------|-------------------------------|
|       | A | R | C | A* |
| 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 | 287 | (R) | G 78 | (L) | D 50 | 41 | L 5.4 |
| 7     | 749 | Y 8 | 5949 | Y 192 | 1177 | (W) | G 60 | Y 40* | Y 40* | 43 | Y 3.1 |
| 8     | 199 | N 11.5 | 1784 | N 184 | 679 | N 82* | 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* | A 16* | A 2.5 |
| 11    | 63 | D 11 | 278 | D 57 | 271 | D 13* | D 13* | D 13* | D 1.9 |
| 12    | 77 | G 10 | 195 | G 75 | 287 | Y 10 | G 22 | G 2 | G 2 |
| 13    | 60 | D 6 | 193 | D 49 | 44 | D 1.8 | 1.1 |

[| [H]DDPF loaded (pmol) | 6 | 32 | 65 |

Initial peptide amount (pmol) | 31 | 275 | 10 |

Repetitive yield (%) | 86 | 90 | 87 |

Starting position | 87 | 87 | 87 |

Fig. 5. Radioactivity released upon sequence analysis of IBA subfragments (pool A and A*) of [H]DDPF-labeled CNBr fragment III. The sample loaded (pool A) contained approximately 7.5 x 10^7 dpm. The amount of radioactivity present in the PTH fraction at each cycle is shown (O). Radioactivity released upon sequencing of similar material derived from carbamoylcholine-protected α-subunit (O) was corrected for equal amounts of protein.

Fig. 6. Reversed-phase HPLC of CNBr fragment III subcleaved with trypsin. Purified CNBr fragment III from unprotected and carbamoylcholine-protected α-subunit CNBr digests (~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 (O) and protected (x) samples were subjected to liquid scintillation counting. Recovery in radioactivity was 85%. 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-
subjected to automated Edman degradation. Details are as given in Table I.

The marked increase in tritium release observed at cycles 14 and 16 of peptides degradation corresponded to the expected release of \([^{3}H]DDF\)-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 \([^{3}H]DDF\) 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 Layten, 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 photoactive 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) \([^{3}H]DDF\) 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) \([^{3}H]DDF\) 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-

### Table II

| Cycle | PTH amino acids |     |     |
|-------|-----------------|-----|-----|
|       | pmol | pmol |
| 1     | 1.355 | 0.97 |
| 2     | 1.244 | 0.59 |
| 3     | 1.156 | 0.40 |
| 4     | 0.97  | 0.22 |
| 5     | 0.73  | 0.54 |
| 6     | 0.61  | 0.34 |
| 7     | 0.53  | 0.10 |
| 8     | 0.44  | 0.02 |
| 9     | 0.36  | 0.01 |
| 10    | 0.22  | 0.00 |
| 11    | 0.10  | 0.00 |
| 12    | 0.07  | 0.00 |
| 13    | 0.05  | 0.00 |
| 14    | 0.02  | 0.00 |
| 15    | 0.01  | 0.00 |
| 16    | 0.01  | 0.00 |
| 17    | 0.00  | 0.00 |
| 18    | 0.00  | 0.00 |
| 19    | 0.00  | 0.00 |

\([^{3}H]DDF\) loaded (pmol) | 98 | 1.22 |

Initial peptide amount (pmol) | 478 | 277 | 265 | 86 |

Repetitive yield (%) | 78 | 78 | 78 | 78 |

Starting position | 78 | 80 | 78 | 80 |

FIG. 7. Radioactivity released upon sequence analysis of trypsin subfragments (pool T and T* in Table II) of \([^{3}H]DDF\)-labeled CNBr fragment III. The sample loaded (pool T (**) contained 1.3 10^6 dpm. Radioactivity released upon sequencing of similar material derived from carbamoylcholine-protected \(\alpha\)-subunit (C) was corrected for equal amount of protein. For comparison, raw values of PTH-tyrosine (x) release in pool T are shown.
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 ligand binding. In this respect, it is noteworthy that these [3H]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. 5). In contrast, in agreement with the known role of α-subunit in the binding of agonists, these [3H] 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, however, present at homologous positions in the chick non-α (Nef et al., 1988) and rat β, δ, and non-α subunit amino acid sequences of neuronal nicotinic AcChoR (Deneris et al., 1988, 1989; Isenberg and Meyer, 1990).

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 [3H] subunit radioactive fragments. This was mainly due to the 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) 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 cholineergic ligands to the AcChoR. Indeed, carbachol-sensitive [3H]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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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., 1994; Lefin 1986; see also Kieffer et al., 1986), which could be both provided by non-carboxylate containing entities such as macrocyclic polyether derivatives (Schmidinger 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.

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, in Karlin, 1983; Culver et al., 1984, Dowding and Hall, 1987; Jackson, 1988).

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The location of Gln-138, Trp-152, Arg-159, Asp-192, and Lys-210 was supported by the labeling of two fragments containing these residues. The former can be present only if Tyr-93 is present, since the labeling at Tyr-93 has been observed in the corresponding cycles of the sequencer. However, the latter can be present only if Gln-138 is present, since the labeling at Gln-138 has been observed in the corresponding cycles of the sequencer. Therefore, it is possible that the labeling at Tyr-93 and Gln-138 is due to the presence of Tyr-93 and Gln-138, respectively. However, the labeling at Trp-152, Arg-159, Asp-192, and Lys-210 is not due to the presence of these residues, since the labeling at these residues has not been observed in the corresponding cycles of the sequencer. Therefore, it is possible that the labeling at Trp-152, Arg-159, Asp-192, and Lys-210 is due to the absence of these residues, or to the presence of other residues that are not labeled at the corresponding cycles of the sequencer.
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