Volume 253, number 1,2, 190–198 FEB 07498 August 1989

# The noncompetitive blocker [$^3$H]chlorpromazine labels segment M2 but not segment M1 of the nicotinic acetylcholine receptor α-subunit

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Received 16 June 1989

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 the agonist carbamoylcholine. The radioactivity incorporated into the AChR subunits was reduced by addition of phencyclidine, a specific ligand for the high-affinity site for noncompetitive blockers. The α-subunit was purified and digested with trypsin and/or CNBr and the resulting fragments fractionated by HPLC. Sequence analysis resulted in the identification of Ser-248 as a major residue labeled by [$^3$H]chlorpromazine in a phencyclidine-sensitive manner. This residue is located in the hydrophobic and putative transmembrane segment M2 of the α-subunit, a region homologous to that containing the chlorpromazine-labeled Ser-262 in the δ-chain [1] and the Ser-254 and Leu-257 in the β-chain [2]. Extended sequence analysis of the hydrophobic segment M1 further showed that no labeling occurred in this region.

Acetylcholine receptor; Ion channel; Noncompetitive blocker

## 1. INTRODUCTION

The nicotinic acetylcholine receptor (AChR) from vertebrate muscle and fish electric organ is a heterologous pentamer ($\alpha_2\beta\gamma\delta$) which carries the acetylcholine-binding sites and contains the cation-selective channel forming elements [3,4]. The noncompetitive blockers (NCBs) are compounds that block the permeability response by interfering directly and/or indirectly with the functioning of the ion channel [5]. Under equilibrium conditions, NCBs reversibly interact with a few categories of sites present on the membrane-bound AChR. The most prominent one is a high-affinity site that binds histrionicotoxin and phencyclidine, is distinct from, but allosterically coupled to, the acetylcholine-binding sites and is present as a single copy per AChR oligomer [6,7]. Covalent labeling of this high-affinity NCB site has been achieved by using a variety of NCBs under equilibrium conditions [6,8–11]. Depending on the ligand and on the species of *Torpedo* used, the α-, β- and/or δ-chains are selectively labeled, leading to the proposal that this unique allosteric site is common to all subunits and located in the axis of quasi-symmetry of the AChR protein [6,12].

Under equilibrium conditions and in the presence of agonist, [$^3$H]chlorpromazine (CPZ) bound to its high-affinity site [6,8] selectively photolabels all four chains of *Torpedo marmorata* AChR. The residues labeled under these conditions

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*Abbreviations:* AChR, acetylcholine receptor; NCB, noncompetitive blocker; CPZ, chlorpromazine; rp HPLC, reverse-phase HPLC; PTH, phenylthiohydantoin



*Published by Elsevier Science Publishers B.V. (Biomedical Division)*
00145793/89/$3.50 

belong to the hydrophobic segment M2 of the $\beta$- and $\delta$-chains (residues $\beta$-Ser-254 and $\beta$-Leu-257, $\delta$-Ser-262) [1,2]. Also, $\delta$-Ser-262 [13] and homologous residues of the $\alpha$- and $\beta$-chains [12] are labeled by the NCB [$^3$H]triphenylmethylphosphonium. These data thus support the proposal that the unique high-affinity NCB site is delimited by homologous regions of the five subunits, which include segment M2, where all the labeled residues identified so far are located.

Karlin et al. [14] have mentioned data (still unpublished) according to which labeling of *T. californica* $\alpha$-chain by the NCB [$^3$H]quinacrine azide would be located 'in a cyanogen bromide fragment containing the segment M1'. Since the $\alpha$-subunits already carry the ACh-binding sites, the possibility exists that their contribution to the high-affinity site for NCB differs from that of the other subunits. In this report, we analyze the labeling of the $\alpha$-chain by [$^3$H]CPZ and, in particular, look for the possible occurrence of labeled residues in segment M1.

## 2. MATERIALS AND METHODS

### 2.1. *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; dimethyl sulfide from Fluka; L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin from Merck (Darmstadt); and cyanogen bromide from Eastman-Kodak. Live *T. marmorata* were provided by the Station Biologique d'Arcachon (France).

### 2.2. *Covalent labeling of the AChR by [$^3$H]CPZ*

Large batches (200 nmol of $\alpha$-toxin-binding sites) of purified [15] and alkali-treated [16] AChR-rich membrane fragments were labeled according to Giraudat et al. [2] with [$^3$H]CPZ [2–3 Ci/mmol (preparation 1) or 12–14 Ci/mmol (preparation 2)].

The purification of the labeled $\alpha$-subunit, subsequent carboxymethylation, and acetone precipitation were performed as previously described [2].

The specific radioactivity of the purified $\alpha$-chain did not differ significantly from that just after irradiation (typically 1700 dpm/$\mu$g of $\alpha$-chain vs 650 dpm/$\mu$g for the phencyclidine-protected batch in preparation 1, and 12200 dpm/$\mu$g of $\alpha$-chain vs 8200 dpm/$\mu$g for the phencyclidine-protected batch in preparation 2).

### 2.3. *Trypsin cleavage*

The purified, carboxymethylated $\alpha$-chain (preparation 1) was resuspended (2 mg of protein/ml) in 2 M urea/0.1 mM $CaCl_2$/50 mM $NH_4CO_3$, pH 8.2. Trypsin was added to a 1:20 (w/w) enzyme/substrate ratio. After a 3-h incubation at 37°C, the same amount of enzyme was added and incubation prolonged overnight.

### 2.4. *CNBr cleavage*

The dry sample was resuspended (0.5–1 mg of protein/ml) in 5.7 N HCl and incubated for 2 h at room temperature in the presence of 0.5 M dimethyl sulfide to reduce methionine sulfoxides [17]. The reaction mixture was then lyophilized. The sample was redissolved (0.5–1 mg of protein/ml) in 70% heptafluorobutyric acid (preparation 1) or formic acid (preparation 2), CNBr was added to an estimated 2000-fold molar excess over methionine residues, and the mixture incubated for 24 h at room temperature under nitrogen in the dark.

### 2.5. *High-pressure liquid chromatography*

The HPLC system used was as described [1]. Peptides were chromatographed on a Nucleosil C4 (1000 Å–7 $\mu$m) reverse-phase column (4.6 × 250 mm) (Société Française Chromato Colonne), or on a $\mu$Bondapack C18 (10 $\mu$m) reverse-phase column (3.9 × 300 mm) (Waters).

### 2.6. *Sequence analyses*

Automated Edman degradation was carried out in a Beckman 890 C spinning cup sequenator with 0.1 M quadrol in the presence of polybrene as described [2,18]. The sequenator output was dissolved in 20% acetonitrile and aliquots used for radioactivity measurements and for identification and quantification of phenylthiohydantoin-amino acids by HPLC [18] on a Microbore column with a solvent system similar to that used with the Applied Biosystems 120A PTH analyzer. A few degradations were carried out with an Applied Biosystems 470A sequencer, with on-line PTH-amino acid analysis in the 120A analyzer using the standard programs provided by the supplier.

## 3. RESULTS

### 3.1. *Labeling of the $\alpha$-subunit by [$^3$H]CPZ*

Large batches of AChR-rich membrane fragments (200 nmol of $\alpha$-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 confirmed that, as shown previously, all AChR chains were labeled [6]. In the presence of 200 $\mu$M phencyclidine, a specific ligand for the high-affinity NCB-binding site, the incorporation of [$^3$H]CPZ into all receptor chains was decreased.

The AChR $\alpha$-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 section 2).

Analytical polyacrylamide gel electrophoresis of the purified material indicated that approx. 2% of the $\alpha$-subunits were labeled by [$^3$H]CPZ in a phencyclidine-sensitive manner.

### 3.2. *Analysis of $\alpha$-subunit tryptic fragments*

Purified $\alpha$-subunit (preparation 1) was cleaved with trypsin, and the digest fractionated by rp HPLC (see legend to fig.1A). Approx. 15% of the injected radioactivity was associated with unbound material (data not shown): the amount of radioactivity in this fraction was not lower in the phencyclidine-protected sample. This material was not further analyzed.

All the phencyclidine-sensitive labeling was associated with the broad $A_{230}$ peak shown in fig.1A, which contained 65% of the injected radioactivity (80% decrease in the protected sample). When this material (pool T1 in fig.1A) was subjected to automated sequence analysis, three amino-terminal sequences could be identified (table 1) using the known primary structure of *T. marmorata* $\alpha$-subunit m19). The major one corresponded to a tryptic fragment extending from Ile-210 (no.1, cleavage at Arg-209), and the region sequenced included the entire hydrophobic segment M1 (Pro-211–Thr-237) (fig.2). The other two sequences corresponded to overlapping fragments extending from Ser-388 (no.2, cleavage at Lys-387) and Tyr-401 (no.3, cleavage at Lys-400) which derived from the carboxyl-terminal portion of the $\alpha$-subunit.

At each cycle, on the recordings from the PTH analyzer, a certain number of variable background peaks were present, which could not be ascribed to any specific sequence or mixture of sequences arising from tryptic cleavage at the three positions mentioned above. But neither the sequences of M2, beginning after Lys-242, nor that of M3, after Lys-276, could be detected. As several sequences were present and overlap problems increase with the number of cycles, the course of the Edman degradation could no longer be followed at the PTH-amino acid level after about 28 cycles.

Results of radioactivity measurements for this degradation are given in table 1. A small increase over background release was detected at cycle 6 which, however, by correcting for the repetitive yield (88%) calculated from the PTH data, would account for only $(1213-500)/(0.88)^6 = 1535$ dpm loaded. This small amount of radioactivity could a priori reflect either a minor site of [$^3$H]CPZ incorporation on one of the identified peptides or the presence of [$^3$H]CPZ on a minor peptide that escaped identification at the PTH-amino acid level.

Fig.1. Reverse-phase HPLC of $\alpha$-subunit fragments. (A) Purified labeled $\alpha$-subunit (50 nmol of preparation 1) was incubated with trypsin (see section 2). The dried digest was dissolved in formic acid, then diluted with 9 vols of 25% solvent A (0.1% trifluoroacetic acid in $H_2O$)/75% solvent B (0.1% trifluoroacetic acid, 90% *n*-propanol) and filtered through a Sep-pak $C_{18}$ cartridge (Waters) equilibrated at 75% solvent B. The flowthrough was diluted to 40% solvent B by addition of solvent A, and one third loaded onto the column by multiple injections under isocratic conditions (data not shown) at 40% solvent B. The column was then eluted at 0.5 ml/min with the gradient of solvent B indicated, and the eluate was monitored at 230 nm (solid trace). Aliquots of the fractions collected at 2-min intervals were subjected to liquid scintillation counting (●). Parallel treatment of $\alpha$-subunit from the phencyclidine-protected sample produced a similar $A_{230}$ profile (not shown) and the radioactivity profile shown here (▲). The horizontal bar indicates the material pooled (pool T1) for subsequent characterization. (B) Purified labeled $\alpha$-chain (20 nmol of preparation 2) was treated with CNBr in 70% formic acid (see section 2). The incubation mixture was then dried, and hydrophilic peptides were extracted in 4 M guanidinium chloride, 6% *n*-propanol, 3% acetonitrile, 0.1% trifluoroacetic acid. The remaining insoluble material (90% of original radioactivity) was dissolved in pure formic acid, diluted in 70% solvent A (10% formic acid in $H_2O$)/30% solvent B (10% formic acid, 90% *n*-propanol). Samples of this material were then loaded onto a $C_{18}$ $\mu$Bondapack column equilibrated at 30% solvent B. The column was eluted at 1 ml/min with the indicated gradient. Eluate was monitored at 280 nm. Even at the highest injected amount, no absorption peak was detected. Fractions were collected at 1-min intervals and aliquots of non-protected sample (●) and of equal amounts of phencyclidine-protected digest (▲) were subjected to liquid scintillation counting. The horizontal bars indicate the material pooled (pools $B_F$I and II) for subsequent characterization. (C) Tryptic peptides contained in pool T1 of A (corresponding to 27 nmol of $\alpha$-starting chain) were dried and subcleaved with CNBr in 70% heptafluorobutyric acid. The incubation mixture was then diluted with 25% solvent A (0.13% heptafluorobutyric acid in $H_2O$)/75% solvent B (0.13% heptafluorobutyric acid, 90% *n*-propanol), filtered through a Sep-pak cartridge and loaded onto the nucleosil $C_4$ column equilibrated at 40% solvent B. With one third of the sample, elution of the column produced the $A_{230}$ (solid line) and radioactivity (●) profiles shown here. Total recovery of injected radioactivity was 60%. The horizontal bar indicates the material pooled (pool $T+B_{H1}$) for sequence analysis. (D) The unbound material in the above chromatography (C) was collected, dried, solubilized in formic acid and loaded on the column equilibrated at 5% solvent B (0.1% trifluoroacetic acid, 90% *n*-propanol). Only the portion of the $A_{230}$ (solid line) and radioactivity (●) elution profiles containing the peak of radioactive material is shown. Total recovery of injected radioactivity was 75%. The bar indicates the material pooled (pool $T+B_{H2}$) for sequence analysis.

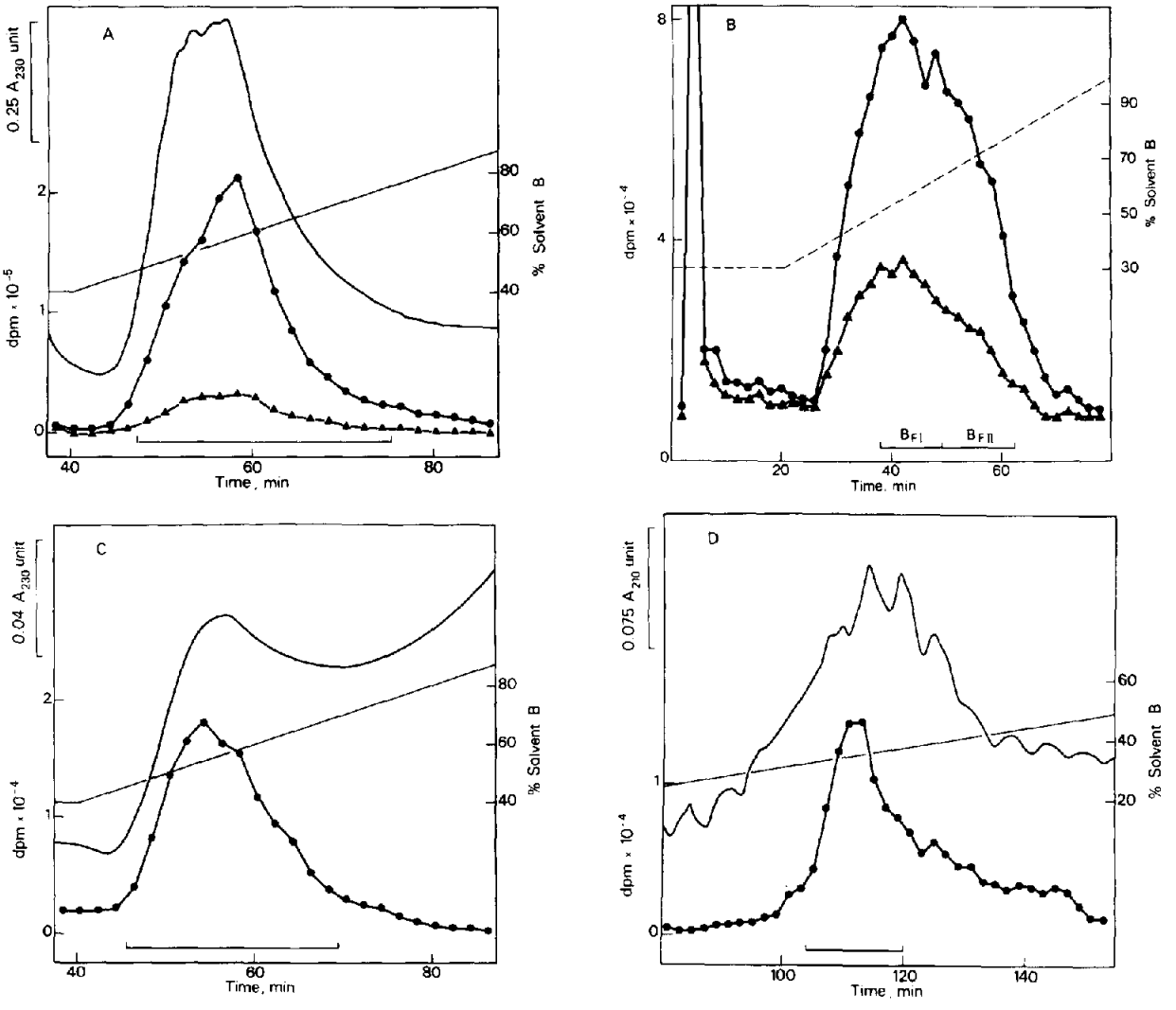
At cycles 2, 12 and 27, where proline residues were expected in fragment no.1, the acid cleavage reaction was extended from 2 to 7 min to improve the Edman degradation yield [20]. Although unexpected, the small increase in radioactivity release observed at these cycles (table 1) most probably resulted from this prolonged incubation in acid.

Most interestingly a marked release of radioactivity was found between cycles 38 and 41 with a peak at cycle 39 (table 1). Correction for the repetitive yield indicates that this release must have represented (a) major site(s) of [$^3$H]CPZ labeling of the material loaded.

Among the three identified sequences, sequence no.3 starting at Tyr-401 could, at most, extend down to the ultimate $\alpha$-chain residue (Gly-437) and thus could not carry the radioactivity detected here. Furthermore, since sequence no.2 precedes sequence no.3 by 13 residues, the radioactivity which reached a peak at cycle 39 could not belong to Phe-426, otherwise one would have obtained a peak also at cycle 39 − 13 = 26. The remaining candidates thus appear to belong to sequence no.1 (Ser-248 and possibly Ile-247 within segment M2).

### 3.3. *Analysis of α-subunit cyanogen bromide fragments*

Purified $\alpha$-subunit (preparation 2) was cleaved with cyanogen bromide in 70% formic acid. The



total digest was submitted to reverse-phase HPLC (see legend to fig.1B). The amount of loaded material (3 nmol) was too low to permit detection of the eluted peptides at 280 nm. Furthermore, the presence of 10% formic acid in solvent A and B precluded detection at 210 nm. Approx. 60% of the recovered radioactivity was eluted between 40 and 80% solvent B (fig.1B). This material was divided into pool $B_FI$ (F 38–49) and pool $B_FII$ (F 50–65) and subjected to automated Edman degradation in an ABI 470 A sequencer. No program modification was attempted at cycles where proline was expected.

In the first half-dozen cycles, it was clear that several peptides were being degraded, but most of them could not be identified with certainty. The only sequence which could be followed down to cycle 13 corresponded to the fragment beginning at Gln 208 (table 2). No peptide sequence resulting from cleavage at the Met-243–Thr-244 bond (beginning of the hydrophobic segment M2) could be detected at the PTH-amino acid level. Results of radioactivity measurements on the sequencer output are given in table 2: no significant radioactivity release was observed. This showed that the peak observed at cycle 6 upon sequencing of the tryptic fragments (table 1) did not correspond to the labeling of Val-215 in segment M1. This amino acid was released at cycle 8 and, if it had indeed been labeled, the corresponding radioactivity should have been approximately 300 dpm over background (for details, see footnote), instead of the 35 dpm observed. Analysis of pool $B_FI$ (not shown) did not provide further information.

### 3.4. *Analysis of CNBr subfragments of the tryptic digest*

In order to establish with more confidence the identity of the residue(s) found labeled at cycles 38–41 of the tryptic fragments sequence analysis, the tryptic peptides of pool $T_1$ were subcleaved by CNBr.

However, as mentioned above, after treatment of the $\alpha$-chain by CNBr in 70% formic acid, we could not detect any sequence resulting from cleavage of the $\alpha$-Met-243–Thr-244 bond. Cleavage of Met–Thr bonds by CNBr has indeed been reported to occur in low yields [21] under these conditions (70% formic acid). We thus tested various solvents in order to achieve cleavage of the $\alpha$-Met-243–Thr-244 bond. Heptafluorobutyric acid and high concentrations of CNBr (see section 2) proved to be satisfactory [22] and were used here (see legend to fig.1C).

The digest was loaded on the rp HPLC column equilibrated at 40% solvent B. Approx. 33% of the radioactivity recovered from the HPLC column was eluted between 45 and 70% solvent B in association with a broad $A_{230}$ peak (fig.1C). This material was pooled (pool $T + B_{H1}$) for subsequent sequence analysis. The unbound material, which contained the rest of the radioactivity, was dried and reanalyzed using a gradient starting at 5% solvent B. Approx. 65% of the recovered radioactivity was eluted between 30 and 40% solvent B (fig.1D). This material (pool $T + B_{H2}$) was also characterized. The unadsorbed radioactive material was not analyzed further.

When pool $T + B_{H1}$ was submitted to Edman degradation in a Beckman 890C sequencer, several sequences were present. Their identification was facilitated by the previous analysis of the starting mixture of tryptic peptides. On the basis of the increase and the decrease of a few characteristic amino acids at selected cycles, six sequences were identified (table 3). Two of them (nos 1 and 2) were present in the starting mixture, while sequence 3 could not be observed; instead, fragments arising from cleavage at Met-404 (no.3 bis), before segment M4, and at Met-415, in segment M4 (no.3 ter), were present. Moreover sequences beginning after Met-243 (no.4) and after Met-282 (no.5) were also detected (see fig.2).

An important phencyclidine-sensitive release of radioactivity was observed at cycle 5 (fig.3a). Taking into account the $\alpha$-chain specific radioactivity (42000 dpm/nmol) and an assumed average repetitive yield of 88%, this must have corresponded to at least 235 pmol of labeled peptide at this cy-

Upon sequencing of tryptic peptides (preparation 1; 68 dpm/pmol of $\alpha$-chain) 439 pmol of Val-215 together with 713 dpm were released at cycle 6 (table 1). Upon sequencing of CNBr fragments (preparation 2; 488 dpm/pmol of $\alpha$-chain) 29 pmol of Val-215 were released at cycle 8 (table 2). After correction for the different specific radioactivity of $\alpha$-chain preparations used, radioactivity associated with 29 pmol of Val-215 should have been approx. $713 \times (29/439) \times (488/68) = 338$ dpm

Table 1

Sequence analysis of α-subunit tryptic fragments in pool T1

| Cycle | dpm | PTH-amino acids (pmol) in sequence | | | Cycle | dpm | PTH-amino acids (pmol) in sequence | | |
|---|---|---|---|---|---|---|---|---|---|
| | | No.1 | No.2 | No.3 | | | No.1 | No.2 | No.3 |
| 1 | 196 | I 835 | S + | Y 472 | 23 | 247 | V 37[a] | L 85 | V 37[a] |
| 2 | 550 | P 373 | D 302 | V 276 | 24 | 302 | F 95 | L 114 | (C) |
| 3 | 435 | L 1068 | E 554 | A 528 | 25 | 290 | Y 73 | (C) | (V) |
| 4 | 494 | Y 823 | E 878 | M 310 | 26 | 329 | L 103 | V 108 | F 21 |
| 5 | 565 | F 548 | S + | V 244 | 27 | 802 | P 30 | F 27 | A 13 |
| 6 | 1213 | V 439 | S + | I 235 | 28 | 245 | (T) | M 33 | G 18 |
| 7 | 707 | V 790 | N 155 | (D) | 29 | 258 | (D) | | |
| 8 | 709 | N 308 | A 454 | (H) | 30 | 257 | (S) | | |
| 9 | 641 | V 652 | A 506 | I 197 | 31 | 228 | G 17 | | |
| 10 | 572 | I 346 | E 322 | L 243 | 32 | 254 | E 12 | | |
| 11 | 357 | I 296 | E 461 | L 233 | 33 | 251 | | | |
| 12 | 721 | P 256 | (W) | (C) | 34 | 219 | | | |
| 13 | 492 | (C) | (K) | V 195 | 35 | 222 | | | |
| 14 | 547 | L 164 | Y 220 | F 42 | 36 | 215 | | | |
| 15 | 605 | L 216 | V 158 | M 100 | 37 | 288 | | | |
| 16 | 445 | F 108 | A 171 | L 71 | 38 | 478 | | | |
| 17 | 526 | (S) | M 79 | I 116 | 39 | 772 | | | |
| 18 | 413 | F 88 | V 127 | (C) | 40 | 662 | | | |
| 19 | 551 | L 100 | I 268[a] | I 268[a] | 41 | 547 | | | |
| 20 | 432 | (T) | (D) | I 77 | Starting position | | α210 | α388 | α401 |
| 21 | 183 | V 79 | (R) | G 43 | Initial amount (pmol) | | 1032 | 691 | 549 |
| 22 | 160 | L 134 | I 71 | (T) | Repetitive yield | | 88% | 90% | 88% |

A sample (488000 dpm) of material contained in pool T1 (fig.1A) was analyzed. The PTH-amino acids identified at each cycle are designated by the conventional one-letter code. Yields are corrected for background. For lack of a reliable calibration standard, PTH-Ser could not be quantified. Residues ([a]) can be attributed to two different fragments at the particular cycle. 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 chosen background. The start position indicates the location of the amino-terminal residue in the complete sequence; the initial amount of peptide undergoing degradation and repetitive yields were calculated by linear regression analysis

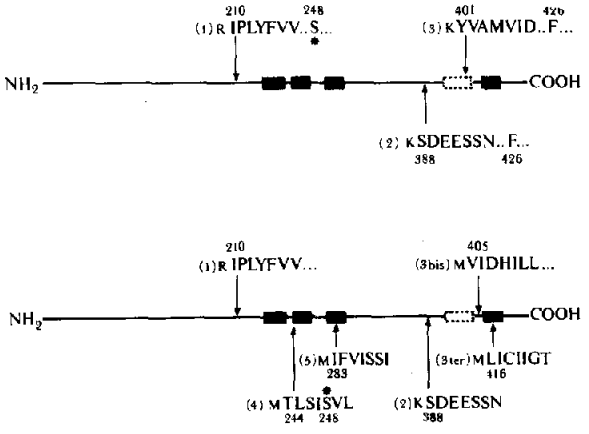



Fig.2. Location of sequenced peptides within the α-subunit primary structure. Schematic drawing of the α-subunit. Black boxes indicate the four hydrophobic segments (M1–M4), and the open box indicates the putative amphipathic helix A. Identified tryptic (upper part) and CNBr (lower part) peptides from α-subunit digests are localized along the sequence of *Torpedo marmorata* α-subunit. [$^3$H]CPZ-labeled α-Ser-248 is marked with an asterisk.

cle. This value was above the detection limit for PTH-amino acids, therefore the sequence corresponding to the labeled peptide must have been among the six identified ones. Since the analysis of the tryptic peptide mixture had ruled out sequences nos 1, 2, 3 bis and 3 ter, the labeled residue was either Ser-287 or Ser-248.

Table 2

Yields of PTH-amino acids upon sequence analysis of pool $B_{FII}$ peptides

| Cycle | dpm | $\alpha$-subunit sequence extending from Gln-208 | pmol of PTH-amino acid |
|---|---|---|---|
| 1 | 889 | Q | 60 |
| 2 | 454 | R | 50 |
| 3 | 324 | I | 90 |
| 4 | 245 | P | 25 |
| 5 | 276 | L | 25 |
| 6 | 305 | Y | 44 |
| 7 | 314 | F | 28 |
| 8 | 350 | V | 29 |
| 9 | 268 | V | 54 |
| 10 | 276 | N | 18 |
| 11 | 232 | V | 15 |
| 12 | 220 | I | 15 |
| 13 | 192 | I | 30 |
| Initial amount | | | 65 pmol |
| Repetitive yield | | | 90% |

A sample (280000 dpm) of material contained in pool $B_{FII}$ (fig.1B) was subjected to automated Edman degradation. Details are as given in table 1

The idea that Ser-248 was the labeled residue is supported by its tentative identification at cycle 39 of the tryptic peptide degradation. It is also supported by the consideration that the small radioactivity increase at cycle 6 of the tryptic peptides degradation may have arisen from a small amount of tryptic cleavage at the Lys-243–Met-244 bond, which would have yielded an amount of peptide undetectable at the PTH level, yet degradable in the sequencer.

Ile-247 was clearly not found labeled in the degradation of the CNBr peptides. The reason why radioactivity was already observed, in the tryptic peptide degradation, at cycle 38 (which could have corresponded to Ile-247), remains unexplained, as sequence no.1 does not contain a histidine which could have given rise to a certain amount of preview [23].

No radioactivity was released at cycle 5 upon sequence analysis of material purified in parallel from the $\alpha$-subunit labeled in the presence of phencyclidine (fig.3A). Analysis of pool $T + B_{H2}$ indicated a somewhat different relative amount of the peptides compared to pool $T + B_{H1}$ (not shown) but radioactivity was also released at cycle 5 (fig.3B). This result strengthened the idea derived from the degradation of pool $T + B_{H1}$ that Ser-248 is the amino acid labeled by chlorpromazine in the $\alpha$-subunit.

Table 3

Yields of PTH-amino acids upon sequence analysis of pool $T + B_{H1}$ peptides

| Cycle | dpm | PTH-amino acids (pmol) in sequence | | | | | |
|---|---|---|---|---|---|---|---|
| | | No.1 | No.4 | No.5 | No.2 | No.3 bis | No.3 ter |
| 1 | 3234 | I 1206[b] | T + | I 1206[b] | S + | V 727 | L 1486 |
| 2 | 1228 | P 464 | L 897 | F 534 | D 174 | I 557[b] | I 557[b] |
| 3 | 1494 | L 544 | S 994[a] | V 160 | E 280 | D 67 | (C) |
| 4 | 834 | Y 421 | I 855[b] | I 855[b] | E 576 | (H) | I 855[b] |
| 5 | 11856 | F 504 | S 1455[a,b] | S 1455[a,b] | S 1455[a,b] | I 1177[b] | I 1177[b] |
| 6 | 3956 | V 513[b] | V 513[b] | S 1024[a,b] | S 1024[a,b] | L 566 | G 382 |
| 7 | 1420 | V 396 | L 719[b] | I 476 | N 101 | L 719[b] | (T) |
| 8 | 1574 | N 158 | L 650 | I 471 | A 338 | | V 702 |
| 9 | 1086 | V 770[b] | S 360[a,b] | V 770 | A 385 | V 770 | S 360[a,b] |
| 10 | 738 | I 113 | L 141 | | (E) | F 150 | V 416 |
| 11 | 704 | I 304 | (T) | | E 188 | | F 380 |
| Starting position | | $\alpha$210 | $\alpha$244 | $\alpha$283 | $\alpha$388 | $\alpha$405 | $\alpha$416 |

[a] These amounts of identified PTH-Ser are given on an arbitrary scale (see table 1), only to illustrate the relative amounts released at various cycles

A sample (331000 dpm) of material contained in pool $T + B_{H1}$ (fig.1C) was subjected to automated Edman degradation. Details are as given in table 1. Due to the high number of cycles where the same residue ([b]) can be attributed to different peptides, no average repetitive yield could be calculated

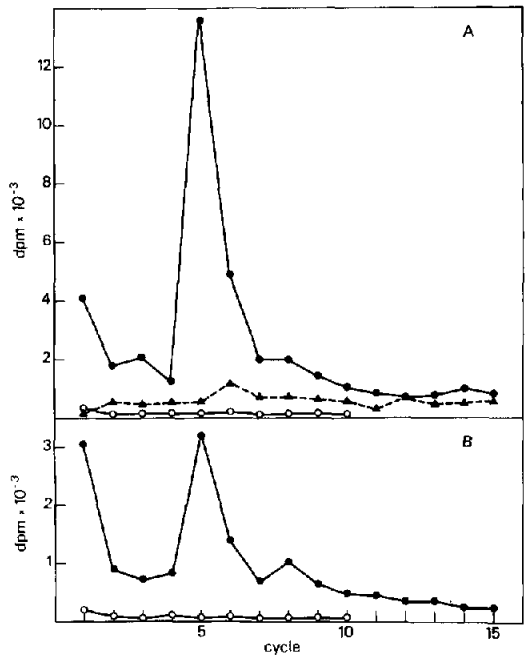



Fig.3. Radioactivity released upon sequence analysis of CNBr subfragments from [$^3$H]CPZ-labeled $\alpha$-chain. (A) A sample (331000 dpm) of pool T + $B_{H1}$ was subjected to automated sequence analysis. The amount of radioactivity present in the PTH fraction at each cycle is shown (●). Radioactivity released upon sequencing of similar material (19000 dpm, corresponding to 4–5 times less protein as judged from PTH-amino acids quantification) derived from phencyclidine-protected $\alpha$-chain is also shown (○). For comparison, radioactivity released upon sequencing of material (488000 dpm) in pool T1 is shown at the same scale (▲). All these samples were derived from preparation 1 of $\alpha$-chain. (B) Same as in (A) for a sample (106000 dpm) of pool T + $B_{H2}$ (●), and for the similar phencyclidine-protected material (10500 dpm) (○).

## 4. DISCUSSION

The high-affinity site for NCB [24,25] is present as a single copy per AChR and appears delimited by all five subunits [7,26]. The degree of symmetry of the AChR molecule inferred from morphological and primary structure data, can thus be experimentally tested at this level. Furthermore, electrophysiological (review in [27–29]) as well as biochemical (review in [6,30]) evidence supports the view that the high-affinity NCB site is located within the ion channel. Photoactivatable NCBs are thus useful tools for direct identification of potential channel-forming elements within each of the AChR subunits [1,2,12,13].

Due to the marked hydrophobic character of the $\alpha$-subunit fragments studied here, their biochemical characterization faced strong technical limitations (discussed in [2]), also encountered by others [12] with several AChR subunits. It turned out even more difficult than for $\beta$- and $\delta$-chains [1,2] to separate from one another the various fragments containing one or multiple hydrophobic segments, possibly owing to aggregation. Sequence analysis of partially purified tryptic peptides and CNBr subfragments enabled us, however, to propose Ser-248 as a major site of phencyclidine-sensitive incorporation of [$^3$H]CPZ into the $\alpha$-subunit [additional labeling of $\alpha$-Ser-287 cannot be ruled out at this stage (see section 3)].

Residue $\alpha$-Ser-248 is found in segment M2 at a position homologous to $\beta$-Ser-254 and $\delta$-Ser-262, which are also specifically labeled by [$^3$H]CPZ [1,2]. The present finding thus brings additional evidence that homologous regions from each subunit contribute to the single phencyclidine-protectable CPZ-binding site. Accordingly, the transmembrane segment M2 of each subunit would lie close to the central axis of symmetry of the AChR molecule and would be oriented so as to render the above residues accessible to CPZ when bound to its high-affinity site [2]. This conclusion can also be derived from the observation that [$^3$H]triphenylmethylphosphonium labels segment M2 of the $\alpha$-, $\beta$- and $\delta$-chains [12].

Our extended sequence analysis on the tryptic pool T1 and the CNBr fragments of pool $B_{FII}$ conclusively showed the absence of [$^3$H]CPZ incorporation on any of the residues composing segment M1. The report by Karlin et al. [14] that "the site on $\alpha$ which is specifically labeled by [$^3$H]quinacrine azide is within a CNBr fragment containing M1" thus appears challenging. Indeed, [$^3$H]quinacrine azide specific labeling is inhibited by histrionicotoxin and thus is expected to take place at the level of a site overlapping, if not identical, to the high-affinity NCB site studied here [31]. In the absence of published experimental data, the possibility that the residue(s) labeled by [$^3$H]quinacrine azide belong to segment M2 rather than to M1 cannot be excluded since, in our hands, the Met-243–Thr-244 bond (preceding segment M2) resisted CNBr treatment of the $\alpha$-subunit under standard conditions, whereas the Met-207–Glu-208 bond (preceding M1) was normally cleaved.

The results of covalent labeling with CPZ and triphenylmethylphosphonium provide convergent evidence that segments M2 are potential components of the ion translocation device. Consistent with this view, site-directed mutagenesis experiments have shown, following the original observation of Giraudat et al. [1] and Oberthür et al. [13], that a region of the $\delta$-subunit comprising segment M2 and the portion between segments M2 and M3 contribute to the regulation of ion transport [32]. More recent works from the same groups have shown that clusters of charged residues neighbouring both ends of segment M2 of the $\alpha$-, $\beta$-, $\gamma$- and $\delta$-subunits are major determinants of the rate of ion transport [33]. Furthermore, mutation of the serines homologous to $\delta$-Ser-262 [1] into alanines on several subunits of the AChR from mouse $BC3H_1$ cells [34] caused a decrease in outward $K^+$ currents and reduced residence time of the NCB QX222 thought to bind within the open channel [28]. Also, synthetic peptides corresponding to segment M2, but not to segment M1 of $\delta$-subunit from *Torpedo* AChR, form discrete ion channels in artificial bilayers [35].

The present data on the $\alpha$-subunit pointing at Ser-248 as a major residue labeled by [$^3$H]CPZ thus bring additional support to the contribution of segment M2 to the structure of the ion channel [1,2,12,33].

*Acknowledgements:* We thank Dr A. Jaganathen for phencyclidine. This work was supported by research grants from the Muscular Dystrophy Association of America, the Association Française contre les Myopathies, the Collège de France, the Centre National de la Recherche Scientifique, the Ministère de la Recherche, INSERM (contract no.872 004) and the DRET (contract no.87 211), and by fellowships from Fondation pour la Formation par la Recherche à l'Interface Chimie-Biologie (F2RCB), and from the Fondation Roux.

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