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The reaction site of a non-competitive antagonist in the δ-subunit of the nicotinic acetylcholine receptor

W.Oberthür, P.Muhn, H.Baumann, F.Lottspeich[1], B.Wittmann-Liebold[2] and F.Hucho

Institut für Biochemie, Arbeitsgruppe Neurochemie, Freie Universität Berlin, Thielallee 63, 1000 Berlin 33, [1]Max-Planck-Institut für Biochemie, 8033 Martinsried and [2]Max-Planck-Institut für Molekulare Genetik, Abteilung Wittmann Ihnestr. 63-73, 1000 Berlin 33, FRG

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A site in the primary structure of the nicotinic acetylcholine receptor from *Torpedo marmorata* covalently labeled with the non-competitive antagonist [^{3}H]triphenylmethylphosphonium (TPMP$^+$) was localized. The label was found in position 262 of the δ-polypeptide chain. This site is specifically labeled in the presence of the agonist carbamoylcholine. Labeling is prevented by the non-competitive antagonist histrionicotoxin. Position 262, probably a serine, is located in the highly conserved membrane-spanning helix M2 (according to the predicted folding scheme of Finer-Moore and Stroud (1984). The relationship of this site to the receptor's ion channel and its regulation is discussed.

Key words: acetylcholine receptor/non-competitive antagonist/photoaffinity labeling

Introduction

Non-competitive antagonists of the nicotinic acetylcholine receptor (nAChR) are believed to block cholinergic nerve impulse transmission by direct interaction with the receptor's ion channel or its gating mechanism (Changeux, 1981; Cohen *et al.*, 1974). Chemically they are a very heterogeneous group of compounds, comprising among others certain local anaesthetics, the frog-skin alkaloid toxin histrionicotoxin (HTX), detergents below their cmc, the hallucinogen phencyclidine (PCP), the neuroleptic chlorpromazin and the lipophilic cation triphenylmethylphosphonium (TPMP$^+$). There is one high affinity binding site for non-competitive antagonists per nAChR molecule (for agonists and competitive antagonists there are two binding sites, located on the two α-polypeptide chains of the heteropentameric receptor complex). Non-competitive antagonists may be useful tools to elucidate structural and functional details of the receptor's ion channel.

TPMP$^+$ has been shown to be a purely non-competitive antagonist, binding with high affinity specifically to one binding site of the receptor, thereby blocking ion flux through its ion channel without inhibiting binding of the agonist acetylcholine (Lauffer and Hucho, 1982). It can be incorporated covalently by u.v. irradiation of the nAChR–TPMP$^+$ complex (Muhn and Hucho, 1983). This photolabeling can be used for monitoring conformational transitions associated with receptor regulation (Fahr *et al.*, 1985). In the resting state, labeling occurs mainly in the α-polypeptide chain, after desensitization of the receptor by agonists enhanced labeling of the δ-subunit is observed. We have now localized a site of the photoreaction within the primary structure of the δ-subunit.

Results

Proof of specificity of the photoaffinity labeling with [^{3}H]TPMP$^+$

The yield of the photoreaction of [^{3}H]TPMP$^+$ with membrane-bound nAChR from *Torpedo marmorata* electric tissue is very low (only about 1%). We therefore had to prove that the site of the covalent reaction is the same as the site to which [^{3}H]-TPMP$^+$ binds reversibly when it blocks the ion channel. Figure 1 shows that HTX, the best characterized ion channel blocker (Albuquerque *et al.*, 1973; Kato and Changeux, 1976) competes with [^{3}H]TPMP$^+$ for a common binding site in the ligand binding assay; Figure 2A shows that HTX also prevents photolabeling of the δ-subunit. This effect occurs in the same concentration range as displacement of [^{3}H]TPMP$^+$ by HTX in the reversible binding assay (Figure 2B).

Labeling in presence of HTX and subsequent isolation of the δ-subunit by preparative SDS electrophoresis showed that no significant labeling of this chain was achieved (not shown).

Microsequencing of ^{3}H-TPMP$^+$-labeled peptides

The [^{3}H]TPMP$^+$-δ-subunit (labeled in the presence of 10 μM carbamoylcholine) was separated from the nAChR complex by preparative SDS-polyacrylamide gel electrophoresis. Subsequently it was cleaved by cyanogen bromide (CNBr). The CNBr-peptides were separated by reversed-phase chromatography (h.p.l.c.). Only one major radioactive peak occurred which contained 50–60% of the total radioactivity of the labeled δ-chain (Figure 3). Microsequencing was performed by liquid-phase and

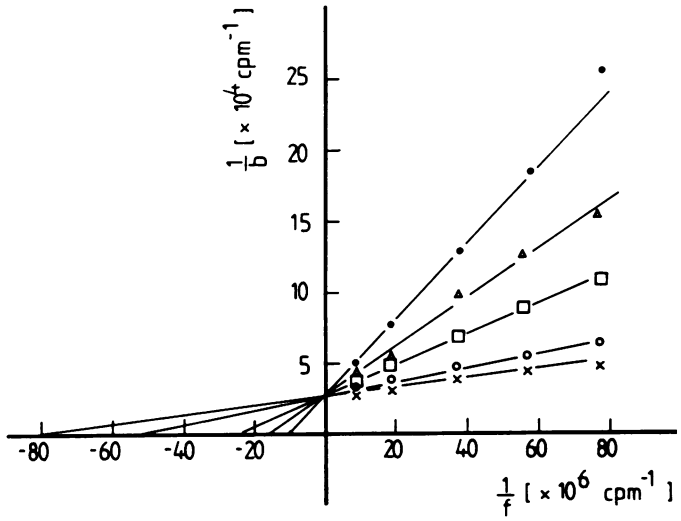

Fig. 1. Competition between [^{3}H]TPMP$^+$ and histrionicotoxin (HTX). Double reciprocal plot of binding data (ultrafiltration assay, Lauffer and Hucho, 1982) of TPMP$^+$ binding to nAChR-rich membranes in the presence of 10^{-6} M HTX and 10^{-5} M carbamoylcholine (○), 10^{-5} M HTX and 10^{-5} M carbamoylcholine (□), 4×10^{-5} M HTX and 10^{-5} M carbamoylcholine (△). Controls: [^{3}H]TPMP$^+$ binding in absence of carbamoylcholine (●) and in the presence of 10^{-5} M carbamoylcholine (x), both without HTX.

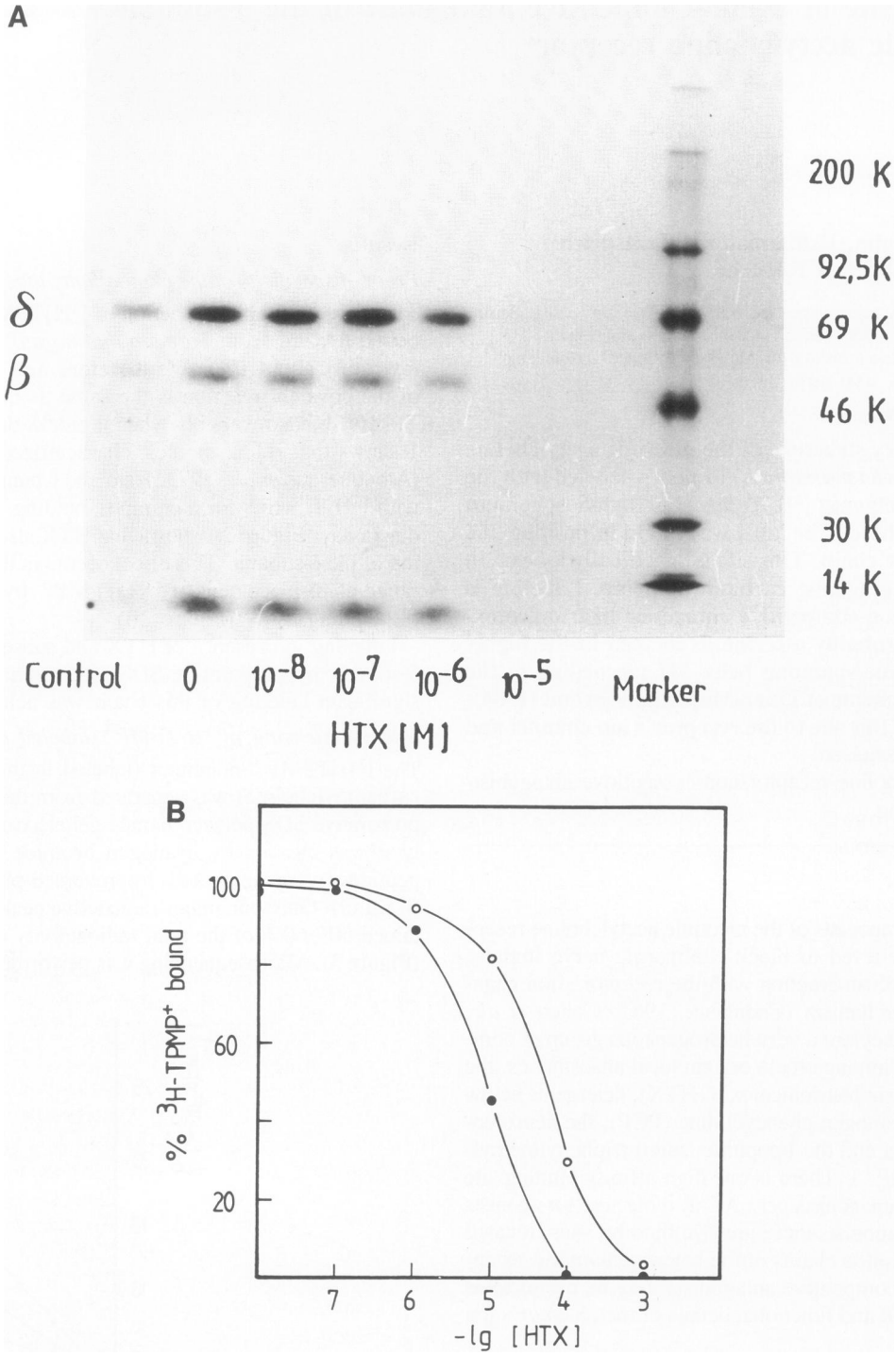

Fig. 2. Inhibition of photoaffinity labeling of nAChR by HTX. **A.** Autoradiograph of an SDS-polyacrylamide electrophoresis gel of AChR photolabeled with [^{3}H]TPMP$^+$. Control: labeling in absence of carbamoylcholine or HTX. All other traces: labeling in the presence of 10^{-5} M carbamoylcholine and HTX as indicated. **B.** Displacement of [^{3}H]TPMP$^+$ by the authentic non-competitive blocker HTX. Binding of [^{3}H]TPMP$^+$ was determined by an ultrafiltration assay (Lauffer and Hucho, 1982). ○—○—○: reversible binding determined in absence and ●—●—●: in presence of 10^{-5} M carbamoylcholine and HTX as indicated. Almost total inhibition of photolabeling was obtained with 10^{-5} M HTX.

gas-phase sequencing of three different preparations. In the gas-phase sequenator it yielded predominantly the amino acid sequence of the CNBr peptide CB 5 (position 258–298, according to the sequence deduced from the cDNA sequence of the *Torpedo californica* δ-chain precursor by Noda *et al.*, 1983). In the liquid phase runs the identification of the released phenylthiohydantoin (PTH)-amino acid was not possible, due to the small starting sample amounts and the sequence of the RNase added as carrier. The maximum radioactivity was found in all three runs in the fifth step of the automatic Edman degradation (Figure 4).

The CNBr peptide sequenced was not pure. Several peptides in addition to CB 5 could be identified. This made unambiguous localization of [^{3}H]TPMP$^+$ in the sequence impossible at this stage. Therefore we subsequently attempted the localization by tryptic digestion of the ^{3}H-TPMP$^+$-labeled δ-chain. Again one

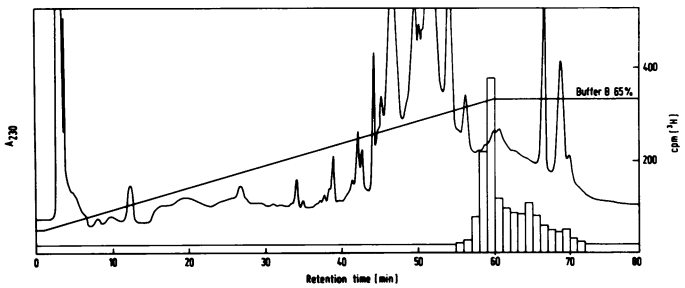

Fig. 3 Purification of a ^{3}H-TPMP$^+$-labeled CNBr-peptide. Chromatography of peptides after 24 h cleavage of ^{3}H-TPMP$^+$-labeled δ-chain from *T. marmorata* by CNBr on Organogen-HP-Gel-RP-7, 250 × 4.0 ID column. Ca 150 μg protein-hydrolysate containing ~200 000 c.p.m. in 50 μl 70% formic acid was injected. Buffer A was 0.1% aqueous TFA and buffer B 0.03% TFA in 2-propanol/acetonitrile 70:30. The gradient applied was from 10% B (starting conditions) increasing in 60 min to 65% B. The eluate was monitored at 230 nm; sensitivity 1.28, flow-rate was 1.0 ml/min; column temperature was 60°C and recorder speed 5 mm/min. Fractions of 1 ml were collected and aliquots of 10 μl counted in a liquid scintillation counter in 5 ml of Supertron. Yield of the injected activity was about 70–80% and the main peak (fractions 58–61) contains 50–60% of the eluted activity.

major radioactive peak was obtained when we separated the tryptic peptides by reversed-phase h.p.l.c. It contained >70% of the radioactivity (Figure 5). Among the peptides present in the peak fractions we identified by gas-phase sequencing the tryptic peptide T-21 starting with methionine (position 257–277 according to the sequence by Noda *et al.*, 1983). Maximum radioactivity was found in the sixth step (Figure 6). Treatment of the labeled peptides with CNBr before microsequencing shifted the peak of radioactivity from the sixth step to the fifth step, indicating that the N-terminal amino acid was methionine.

We cannot exclude that low affinity binding of [^{3}H]TPMP$^+$ to the nAChR occurs and that photolabeling takes place at one or several of the low affinity binding sites. This 'unspecific' labeling would be <10% of the HTX-displaceable labeling. We would not detect such small amounts of radioactivity in the gas phase sequencer because of the low yield of the sequencing procedure (1–3%). These are some of the possible explanations for this low yield. (1) The 'initial yield' may be low because only part of the peptide is accessible to sequencing. (2) A repetetive yield of, in our case, 93% reduces the yield to 62% in the fifth and 56% in the sixth step. (3) The washout is high, as usually observed with sequencing with pmole amounts, especially with hydrophobic peptides. Washout is demonstrated with the blank cycles shown in Figure 4a. (4) Extraction of the labeled PTH-amino acid is incomplete. (5) There are losses by transfer (adsorption to vial, tubings, apparatus). (6) Sequence termination because of N-O-acylshift.

Discussion

We conclude that one site of photolabeling with [^{3}H]TPMP$^+$ in the δ-polypeptide chain of nAChR from *T. marmorata* is position 262 according to the known homologous sequence of the δ-subunit from *T. californica*. This conclusion is based on the following observations: (i) Edman degradation of the labeled CNBr-peptide released the maximum of radioactivity in the fifth step. (ii) A radioactive tryptic peptide was identified as peptide T-21 (position 257–277). The peak of radioactivity in this peptide was found in step 6, corresponding to position 262. (iii) Removal of the N-terminal methionine from T-21 shifted the peak of radioactivity to step 5. Table I summarizes the CNBr- and tryptic peptides of *T. californica* indicating that there is on-

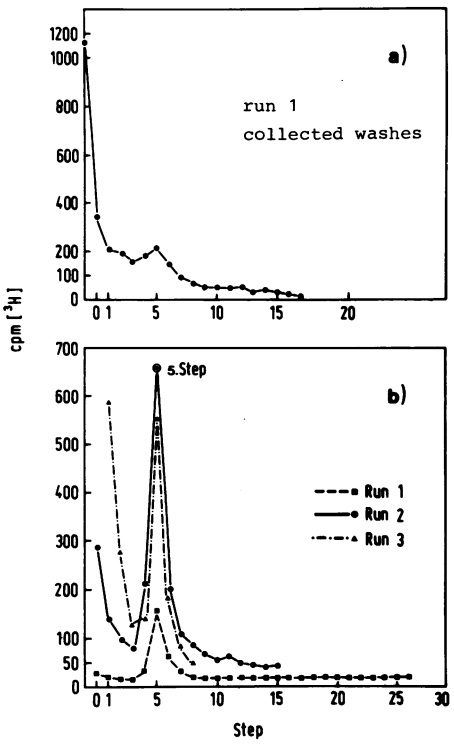

Fig. 4. Edman degradation of the ^{3}H-TPMP$^+$-labeled CNBr-peptide. Radioactivity of the PTH-amino acid fractions after automatic sequencing of the main radioactive fractions from the h.p.l.c. separation (see Figure 1) of CNBr-peptides from labeled δ-chain (*T. marmorata*). Run 1: liquid-phase sequencing under the addition of 2.5 nmol RNase but without polybrene in the Berlin Sequenator (Wittmann-Liebold, 1983), 26 steps. Run was started by washing and with one cycle without phenylisothiocyanate, all the washes of each step were collected and counted. (**a**), Radioactivity of the collected washes of each step indicating the wash-out rate of the protein. One-third of the PTH-amino acid fractions were counted. (**b**), Radioactivity in the cup after 26 steps was lower than 1% of the starting material. Run 2: the same as in run 1, starting without washing; new preparation, 15 steps. Run 3: gas-phase sequencing from a third preparation without O-cycle, 8 steps. Maximum of radioactivity was found in all runs in the fifth step. The main sequence was identified as CB 5 (see text).

ly one CNBr-peptide having a trypsin-cleavage site one amino acid upstream of the CNBr-cleavage site which would fit the pattern of radioactivity released with Edman degradation. This is the peptide CB 5, the predominant peptide identified in the radioactive component isolated from the CNBr digest of ^{3}H-TPMP$^+$-labeled δ-chain. T-21 overlaps this peptide at the N-terminus by one amino acid (Table I).

The amino acid in position 262 in the primary structure of the δ-chain of nAChR from *T. californica* is a serine. The amino acid sequence of the corresponding subunit from the *T. marmorata* receptor is unknown and the amino acid carrying the photolabel was not identified. Because of the significant homology of nAChR from different species and because of the highly conserved primary structure of this part of the amino acid chain it might be concluded that the receptor of *T. marmorata* possesses a serine residue in position 262 of its δ-chain as well. A serine was also identified in this position by microsequencing of the radioactive peptide CB 5; but since this peptide was not homogeneous we can only tentatively assign a serine to be located at this position 262 in CB 5. We cannot exclude that other sites in the primary structure are labeled too, because our argument is based on experiments with a mixture of labeled peptides. Other labeled peptide fractions obtained by h.p.l.c. of δ-chain digests have not been investigated. Therefore at present we can only state

that one of the sites of labeling is position 262.

To summarize we assign the labeling to position 262 with the following restrictions. (1) We may have localized only one of several labeling sites. (2) We may have 'lost' other labeling sites because of the low yield of the procedure. (3) Our localization is based on the assumption that we localized the 'HTX-displaceable' label. This assumption is unproven, though probably correct.

Concerning the functional role of the site localized with the help of a non-competitive antagonist we can assume that this site is not necessarily the ion channel itself: first of all, this site (on the δ-chain) becomes accessible to the photolabel much slower than receptor activation and channel blocking takes place (Fahr *et al.*, 1985). Furthermore, position 262 (or 269 in the aligned sequences of all four polypeptide chains) is located in the region M2, predicted to be a membrane-transversing stretch of the sequence apart from the channel-forming helices (Finer-Moore and Stroud, 1984).

The part of the nAChR labeled by [^{3}H]TPMP$^+$ is probably an allosteric site involved in regulating receptor activity. This conclusion is based on several observations: (i) accessibility to photolabeling increases after addition of agonist with a time scale related to regulatory phenomena (desensitization) but not to channel opening and closing (Fahr *et al.*, 1985); (ii) non-competitive antagonists themselves promote regulatory phenomena (Changeux, 1981); (iii) the δ-polypeptide chain has been shown to determine the regulation of the channel gating kinetics rather than the conductivity of the open channel (Sakmann *et al.*, 1985).

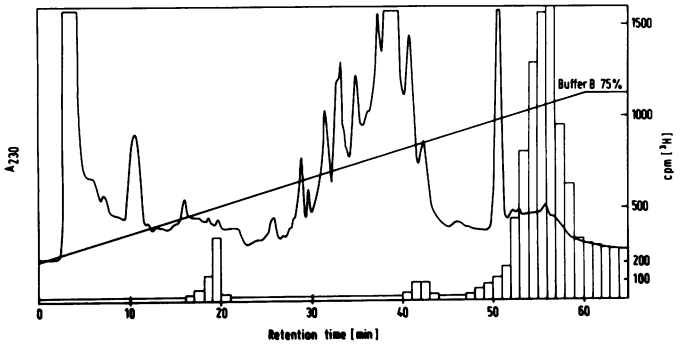

Fig. 5. Purification of a ^{3}H-TPMP$^+$-labeled tryptic peptide. Chromatography of the tryptic peptides after 15 h digestion of ^{3}H-TPMP$^+$-labeled δ-chain of *T. marmorata*. About 90 μg hydrolysate in 185 μl 70% formic acid were injected. Gradient was from 15% B (starting conditions) to 75% B in 60 min, monitoring at 230 nm, sensitivity 0.64. Column buffers and other conditions as in Figure 3. Aliquots of 100 μl were counted; 70–80% of the injected activity was recovered from the column, >70% was found in fractions 53–59.

Materials and methods

Preparation of nAChR-rich membranes

Membranes rich in nAChR were prepared from *T. marmorata* electric tissue as described previously (Schiebler and Hucho, 1978). Specific receptor activity was 1500–2500 nmol/g [^{125}I]α-bungarotoxin binding sites. The electric fish was kindly

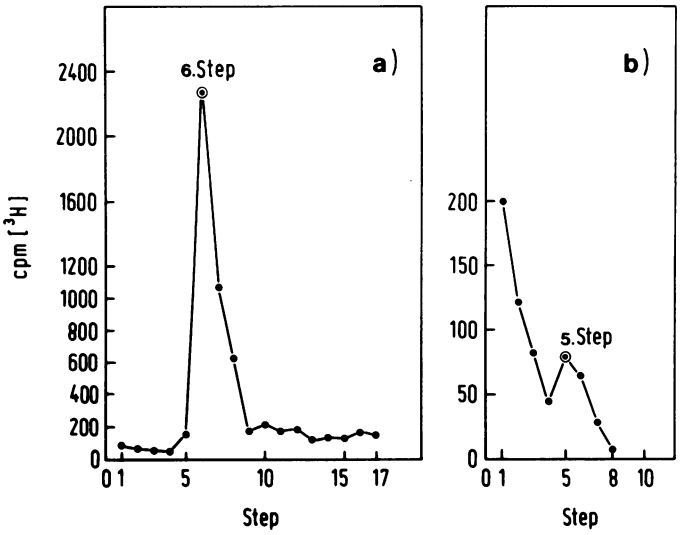

Fig. 6. Edman degradation of the [^{3}H]TPMP$^+$-labeled tryptic peptide. Radioactivity of the PTH-amino acid fractions after automatic gas-phase sequencing of the main active fractions from the h.p.l.c. separation of tryptic peptides (see Figure 5) before (**a**) and after treatment with CNBr (**b**). In (**a**) 2/3 of the total tryptic peptide was applied. In step 1–4 9/10 of the released PTH-amino acid were used for identification and 1/10 for counting; from step 5 2/3 for identificaiton and 1/3 for counting. In (**b**) the rest of 1/3 tryptic peptide was cleaved with CNBr and rechromatographed by h.p.l.c. The active fractions were subjected to microsequencing (2/3 of the PTH-amino acid were used for identificaton and 1/3 for counting). The N-terminal sequence of the tryptic peptide T-21 (positions 257–277) was identified and the maximum of activity was found in the sixth step. After removing the N-terminal Met (position 257) by CNBr treatment, the maximum radioactivity was shifted from the sixth to the fifth step.

Table I. CNBr peptides and the corresponding overlapping tryptic peptides of the δ-chain from *Torpedo californica* according to the sequence of Noda *et al.* (1983). Sequencing results show that the labeled amino acid must be in position 262 (radioactivity in CNBr-peptide in the fifth and in tryptic peptide in the sixth step)

CNBr peptide	CB 1	CB 2	CB 3	CB 4	CB 5	CB 6	CB 7	CB 8	CB 9	CB 10	CB 11	CB12
Position	1–58	59–160	161–163	164–257	258–293	294–296	297–340	341–382	383–458	459–465	466–475	476–501
Number of amino acids	58	102	3	95	36	3	44	42	76	7	10	26
Amino acid in fifth step and position in sequence	Glu5	Tyr63	–	Asp168	Ser262	–	Gly301	Glu345	Ser387	Pro463	Ile470	His480
Corresponding tryptic peptide	T 1	T 7	–	T 12	T 21	–	T 23	T 29	T 36	T 44	T 44	T 44
Position	1–6	47–66	–	148–170	257–277	–	291–315	343–356	386–389	455–484	455–484	455–484
Radioactivity must be found in tryptic peptide in step	5	17	–	21	6	–	11	3	2	8	15	25

supplied by V.P.Whittaker, who obtained it from Arcachon, France. The electric tissue was taken from fish killed without anaesthesia, frozen in liquid nitrogen and stored at −75°C.

Photolabeling with [^{3}H]TPMP$^+$

The procedure followed the protocol described before (Muhn and Hucho, 1983). It should be pointed out that labeling was performed after 5 min pre-incubation with and in the presence of the 10 μM carbamoylcholine by 3 min irradiation. Under these conditions the receptor is fully equilibrated and in its desensitized state. All labeling experiments were performed with membrane-bound receptor.

Purification of the ^{3}H-TPMP$^+$-labeled δ-chain

The photolabeled receptor-rich membranes were dissolved in sample buffer (Laemmli, 1970) and the polypeptide chains were separated in two steps by preparative SDS-polyacrylamide gel electrophoresis, using the apparatus from BRL, Bethesda. In the first run the upper gel was 3% and the lower 7.5% (Laemmli, 1970). About 4 mg protein were applied. Electrophoresis was performed at 6 mA/150 V. The elution buffer contained 0.1% SDS in 0.576 M glycine and 0.075 M Tris/HCl, pH 8.3. Elution rate was 15 ml/h.

Purity of the chains in the fractions was assessed by analytical SDS-polyacrylamide gel electrophoresis. Fractions containing predominantly δ-chains were pooled and re-electrophoresed after dialysis against water and lyophilization as above, but on 10% gel.

CNBr cleavage

Lyophilized chains collected from the preparative electrophoresis were treated after reduction with β-mercaptoethanol with an excess of CNBr in 70% formic acid 7−24 h according to Gross and Witkop (1961).

Tryptic digestion

Dialyzed chains were digested for 5−20 h with 3−10% L1-tosylamide-2-phenylethylchloromethyl (TPCK)-trypsin (Worthington, USA) in a large volume (5−20 ml) of water adjusted to pH 9 with ammonia. The fragmentation was stopped by adding formic acid. The volume of the hydrolysate was reduced in a vacuum concentrator before h.p.l.c. Protein concentration during digestion was 15 μg/ml.

Reversed-phase chromatography (h.p.l.c.)

The gradient liquid chromatograph was composed of two h.p.l.c.-pumps, model 64.00, controlled by a 50B microprocessor programmer and a variable-wavelength u.v.-detector, model 87.00 (all from Knauer, Berlin, FRG), an automatic sampler Wisp 710 B from Waters Assoc., USA, a data processor Chromatopac C-R3A from Shimadzu, Japan, a recorder, model 2210 from LKB, Munich, FRG, a degasser model ERC-3320 from ERC, Regensburg, FRG, and a fraction collector microcol TCD 80 from Abimed, Düsseldorf, FRG.

The separation was performed on a pre-packed steel column purchased from Knauer, 250 × 4.0 mm i.d., filled with Organogen HP-Gel-RP-7, pore size 300 Å, particle size 7 μm.

Proteins were eluted at 60°C with gradients from buffer A (0.1% trifluoroacetic acid (TFA) in water) and buffer B (0.03% TFA in 2-propanol/acetonitrile 70:30).

Microsequencing

Sequencing was performed automatically in the Berlin Sequenator by liquid-phase sequencing (Wittmann-Liebold, 1983) and in a gas-phase sequenator from Applied Biosystems, model 470 A (USA). Identification of the released PTH-amino acids was made on-line, employing isocratic, recycling h.p.l.c. (Ashman and Wittmann-Liebold, 1985) or by injecting in a separate isocratic system (Lottspeich, 1985). One-third of each PTH-amino acid fraction was counted in 5 ml Supertron (Kontron) in a liquid scintillation counter.

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Note added in proof

During the reviewing process of this paper we localized the [^{3}H]TPMP$^+$ in the α- and β-chains as well. It reacted with positions homologous to δ 262, i.e. β 254, α 248. This result was obtained regardless of whether photolabeling was performed with the receptor in its resting, desensitized, or antagonist state. From these observations we conclude that we actually label the AChR-ion channel and that this channel is formed by the homologous helices II of the five receptor subunits.