The ion channel of the nicotinic acetylcholine receptor is formed by the homologous helices M II of the receptor subunits

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A binding site for the channel-blocking noncompetitive antagonist [^{3}H]triphenylmethylphosphonium ([^{3}H]TPMP$^+$) was localized in the α-, β- and δ-chains of the nicotinic acetylcholine receptor (AChR) from *Torpedo marmorata* electric tissue. The photolabel was found in homologous positions of the highly conserved sequence helix II, α 248, β 254, and δ 262. The site of the photoreaction appears to not be affected by the functional state of the receptor. [^{3}H]TPMP$^+$ was found in position δ 262 independent of whether photolabeling was performed with the receptor in its resting, desensitized or antagonist state. A model of the AChR ion channel is proposed, according to which the channel is formed by the five helices II contributed by the five receptor subunits.

Acetylcholine receptor *Ion channel* *Noncompetitive antagonist* *Photoaffinity labeling*

1. INTRODUCTION

The nicotinic acetylcholine receptor (AChR) is a pentameric glycoprotein with the subunit composition $\alpha_2\beta\gamma\delta$. The primary structures of the polypeptide chains have been deduced from the precursor-cDNA sequences and various structural models of the protein complex in its membrane environment and of the ion channel formed by the receptor protein have been proposed (review [1]). The binding sites for agonists and competitive antagonists are located on the α-subunits, probably around position 192/193 of the primary structure [2,3]. The location and structure of the ion channel are unknown. Noncompetitive antagonists, a very heterogenous group of compounds, are thought to be tools for its elucidation because they block ion fluxes by sterically or allosterically interacting with the channel [4]. Recently a binding site for the noncompetitive antagonists chlorpromazine [5] and TPMP$^+$ [6] has been localized in the δ-subunit of AChR from *Torpedo marmorata* by photoaffinity labeling and microsequencing work. It was identified as serine 262 located in the membrane-spanning helix II (according to the five-helix model proposed by Finer-Moore and Stroud [7] and Guy [8]). Here we present evidence that our noncompetitive antagonist, [^{3}H]TPMP$^+$, reacts upon photoactivation with homologous sequences in α- and β-chains as well suggesting that the ion channel is formed by the hydrophobic helix II of each of the receptor subunits. We propose a channel model on the basis of this finding.

2. MATERIALS AND METHODS

2.1. *Photoaffinity labeling*

Photoaffinity labeling of the membrane-bound receptor was performed with receptor-rich membranes prepared from *T. marmorata* electric tissue [9] as described [10]. All photoaffinity labeling ex-

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periments with the noncompetitive antagonist [^{3}H]TPMP$^+$ were performed in the absence of any other cholinergic effector, agonist or antagonist. Specificity of the labeling was proven by histrionicotoxin (HTX) which prevented labeling completely [6].

2.2. *Purification of [^{3}H]TPMP$^+$-labeled subunits*

The procedure was essentially as described before [6]. The photolabeled receptor-rich membranes were dissolved in sample buffer 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 gel was 7.5% [11]. 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 reelectrophoresed after dialysis against water and lyophilization as above, but on 10% gel.

2.3. *CNBr cleavage*

CNBr cleavage of the labeled subunits was performed after lyophilization and reduction with β-mercaptoethanol according to Gross and Witkop [12].

2.4. *Tryptic digestion*

Tryptic digestion was performed for 5–20 h with 3–10% TPCK-trypsin at a protein concentration of 15 μg/ml.

2.5. *Reversed-phase chromatography (HPLC)*

Separation was performed on a pre-packed steel column (Knauer, Berlin), 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).

2.6. *Microsequencing*

Microsequencing was performed in a gas-phase sequenator from Applied Biosystems (model 470 A). 1/3 of each PTH-amino acid fraction was used for determining the radioactivity by liquid scintillation counting.

3. RESULTS

Fig.1a–c shows the HPLC chromatogramms of tryptic digests of the [^{3}H]TPMP$^+$-labeled α-, β- and δ-chains, respectively. The UV absorption patterns are fairly different, as one would expect considering the differences in size and sequence of these subunits. The distribution of radioactivity on the other hand is surprisingly similar. Three peaks of low radioactivity are followed by a broad peak containing the vast majority of the label.

Microsequencing of the center fractions of the latter released in each case radioactivity exclusively in the sixth Edman-degradation step (fig.2). This indicates that the label is located six amino acids downstream from a lysine or arginine, the tryptic cleavage sites. Treatment of the same peptide with cyanogen bromide and subsequent microsequencing yielded the radioactivity in the fifth Edman cycle, indicating that the label is located five amino acids downstream from a methionine. Both experiments together prove that labeling occurred in a sequence starting with Lys (or Arg) Met. This sequence occurs only once at position 263/264 (numbering of the aligned sequences). In the primary structure of receptor from *T. californica* six, respectively five amino acids downstream is located a serine residue (Ser 262 in δ, Ser 254 in β, and Ser 248 in α). Very little radioactive label was incorporated into the γ-chain and therefore could not be localized by the method described. The corresponding position in this chain is Ser 257.

The peptide fractions used for microsequencing were not pure. The amino acids identified by Edman degradation indicated a mixture of sequences. The sequence of the tryptic peptide T 21 (and the CNBr peptide CB 5) were clearly identified among others. The localization through the radioactive PTH-amino acid was unequivocal.

The conclusion from these experiments is that the noncompetitive blocker [^{3}H]TPMP$^+$ binds to a site near the helix II of at least three of the five polypeptide chains of the receptor and upon irradiation with UV light it reacts covalently with serine residues in homologous positions within this helix.

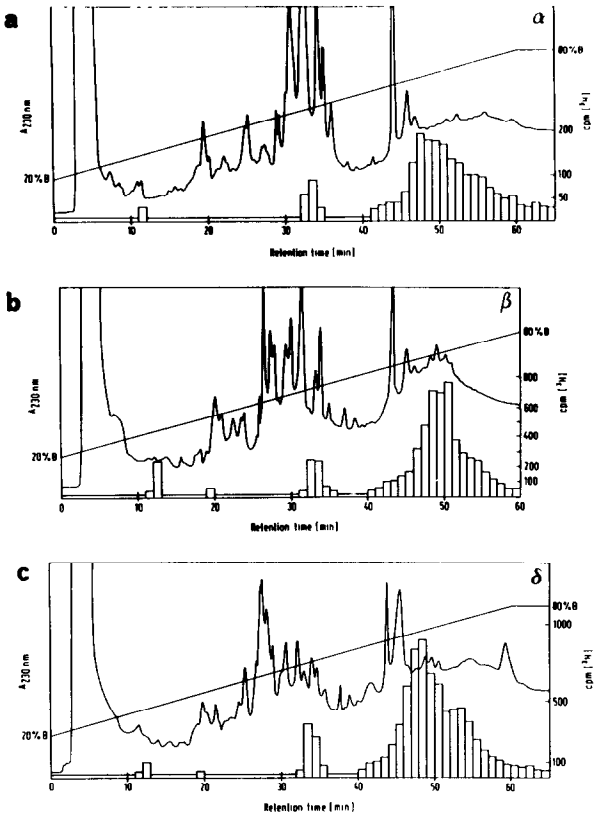

Fig.1. Purification of peptides obtained by tryptic digestion of [^{3}H]TPMP$^+$-labeled subunits of acetylcholine receptor. (a) α-Subunit, (b) β-subunit, (c) δ-subunit. Solid line, UV-absorption; bars, radioactivity in 50 μl aliquots of 1 ml fractions. Elution profiles are different for the different subunits but radioactivity distribution is similar indicating that homologous regions are labeled by [^{3}H]TPMP$^+$. Column, HP-Gel-RP-7 (Knauer, Berlin), pore size 300 Å, particle size 7 μm, column dimensions 250×4.00 mm. Elution buffer A, 0.1% aqueous TFA; buffer B, 0.03% TFA in 2-propanol/acetonitrile (70:30); gradient, from 20% B to 80% B in 60 min. Absorbance measured at 230 nm. Photoaffinity labeling of membrane-bound acetylcholine receptor from *T. marmorata* electric tissue was performed with [^{3}H]TPMP$^+$ without agonist or competitive antagonist, according to [10]. Separation of labeled subunit from the receptor complex by preparative SDS-polyacrylamide gel electrophoresis was described before [6]. Tryptic digestion was performed by incubating the separated subunits with 10% (w/w) TPCK-trypsin (Worthington) for 24 h. The hydrolysate was concentrated in a vacuum concentrator in the presence of 1 mM dithioerythritol (Merck). Precipitated protein was redissolved by adding 90% formic acid. This solution was subjected to HPLC chromatography.

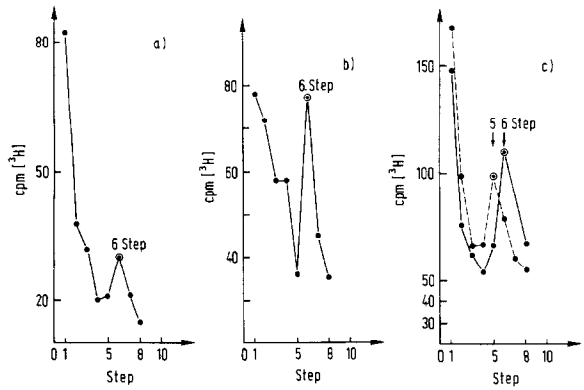

Fig.2. Localization of the [^{3}H]TPMP$^+$-labeled position in the primary structure by microsequencing. The main radioactive fractions of the tryptic hydrolysate (retention time 47–51 min, fig.1) obtained from the α-, β- and δ-subunits (a,b,c, respectively) were automatically microsequenced in a gas-phase sequenator (Applied Biosystems model 470A). The phenylthiohydantoin (PTH) amino acids were identified on an isocratic recycling HPLC. Aliquots of the PTH amino acids were removed for determination of radioactivity. Maximum radioactivity was always found in step 6. Treatment of the peptide fraction with CNBr before microsequencing shifted radioactivity to the fifth Edman-degradation cycle (dashed line in c).

The primary structure of receptor from *T. marmorata* is known only for the α-chain [13]. For the other chains we only postulate the serine in this position. Because of its modification by [^{3}H]TPMP$^+$ we are not able to identify the amino acid; there appear to be very few exchanges between the receptors from the two species, even fewer appear to occur in the highly conserved helix II.

This result is obtained irrespective of the functional state of the receptor protein: Labeling in this presence of agonist, i.e. with the receptor in its desensitized state, causes preferential labeling of the δ-subunit and to a lesser degree of β; labeling in the absence of effector (i.e. with the receptor in its resting state) causes incorporation of radioactivity into the α-, β- and δ-polypeptide chains. Even on photolabeling in the presence of an antagonist which was shown to cause a conformational change in the receptor protein as well [14], a similar pattern of radioactivity in the HPLC chromatogram was obtained (not shown). The conformational changes appear to affect the yield

of the photolabeling but not the site in the sequence.

We have not shown that homologous sites in the γ-chain and in both α-chains (which have identical sequences but are not functionally equivalent [15,16]) are photolabeled by the noncompetitive antagonist. Nevertheless based on positive evidence for three of the five subunits and on the results from electron microscopy which had shown that the ion channel has a 5-fold axis of symmetry [17], we propose a model of the channel (fig.3) according to which it is formed by the five homologous helices II contributed from the five receptor subunits.

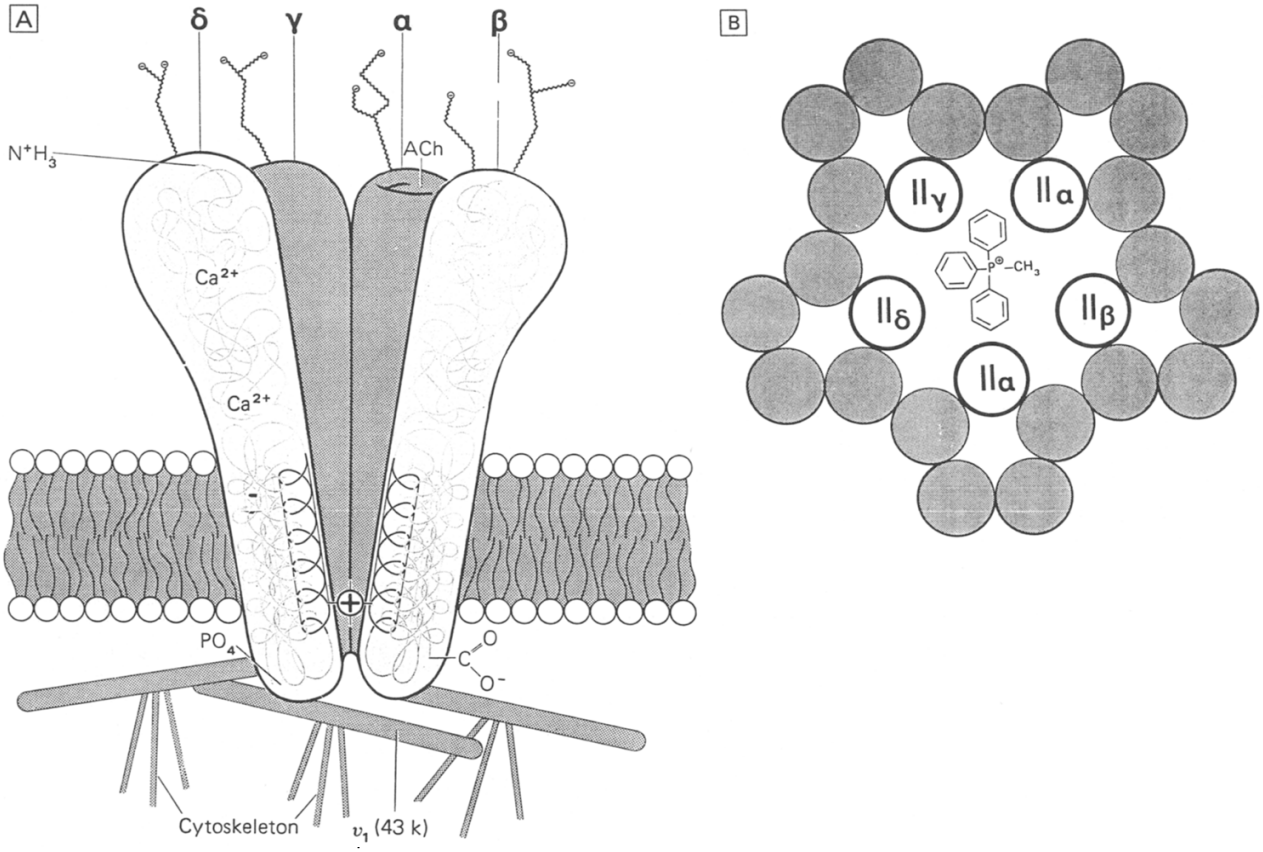

Fig.3. Model of the nicotinic acetylcholine receptor and its ion channel. (A) Section through the receptor complex perpendicular to the membrane plane. Extracellular space: top. About 50% of the receptor protrudes from the outer surface, 30–35% is immersed in the lipid bilayer. The five polypeptide chains (one of the two α-chains is removed) span the membrane with each five hydrophobic helices, the amino terminus being located extracellularly, the carboxy terminus intracellularly. The entrance of the ion channel is depicted large enough to let hydrated cations and the channel blocker [^{3}H]TPMP$^+$ into a site close to the inner surface. The ion channel is formed by each one of the five membrane-spanning helices contributed by the receptor subunits. The proposed helices comprise amino acids 260–287 of the aligned sequences [1]. The site of covalent attachment of [^{3}H]TPMP$^+$ is in position 269 in the aligned sequences (or position 262, 254, and 248 in α, β and δ, respectively. Note that the five helices in this model are parallel, with the N-terminal end (and thereby the positive pole of the helix-dipole) oriented towards the cytoplasm. The carbohydrate residues (zig-zag lines on top), as well as sialic acids (negative charges), Ca^{2+}, phosphate groups, cytoskeleton and 43 kDa protein are neither drawn to scale nor do they represent the correct stoichiometry. (B) Cross-section through the receptor complex in the membrane plane. Depicted are the membrane-spanning helices (shaded circles and open circles representing helix II). The helices II (according to the numbering of Finer-Moore and Stroud, [7]) form the wall of the ion channel, giving it its 5-fold symmetry axis. The other helices are arranged arbitrarily. Each five helices (circles) represent one of the five receptor subunits.

4. DISCUSSION

[^{3}H]TPMP reacts after photoactivation with homologous positions in the primary structures of the α-, β- and δ-polypeptide chains. This indicates that it is bound in the center of the receptor complex, from where covalent reaction with either of the subunits is possible. It is tempting to assume that the central [^{3}H]TPMP$^+$-binding site represents a part of the ion channel. Evidence in favor of this assumption is provided by the following observations: [^{3}H]TPMP$^+$ binds reversibly with high affinity and noncompetitively with respect to acetylcholine; there is only one high-affinity binding site for [^{3}H]TPMP$^+$ for each two acetylcholine-binding sites of the membrane-bound receptor [18]. Proven channel blockers such as HTX or phencyclidine compete with [^{3}H]TPMP$^+$ for a common binding site; HTX prevents photolabeling of AChR with [^{3}H]TPMP$^+$ [6,18]. [^{3}H]TPMP$^+$ inhibits AChR-regulated cation flux [18] and has been shown to block neuromuscular transmission in a voltage-dependent manner [19,20]. In single channel analysis it shortens the channel lifetime [19].

On the other hand, TPMP$^+$ has all the characteristics of an allosteric effector: its binding site is affected allosterically by agonists [10,18] and it enhances desensitization [19]. It binds to AChR in its resting or desensitized state, indicating that channel activation is not necessary for binding and that the binding site is probably not located within the open channel.

The model proposed in fig.3 takes these observations into account. The photolabel [^{3}H]TPMP$^+$ has a larger diameter [21] than the largest cations able to permeate through the channel (6.4 Å according to [22]). It may therefore bind to a wider part of the channel entrance where it may block it sterically and allosterically at the same time. At this site it may not even sense whether or not the channel is open. This would explain why the same site is labeled (though to a different extent) when photolabeling is performed in the presence or absence of cholinergic effectors.

We postulate that the ion channel of the AChR is lined by helix II, predicted to be one of five membrane-spanning helices [7,8]. The five helices II (each one from the five receptor subunits according to this model) contribute to the architecture of a membrane-spanning, cation-selective, agonist gated pore. This does not preclude that other parts of the receptor protein not being labeled by [^{3}H]TPMP$^+$ are involved as well. Comparing the known sequences of AChR subunits from a variety of organisms it is obvious that helix II is the best conserved [21], much better conserved than the amphipathic helix MA predicted by others [7] to form the channel.

Helix II may look at first sight too hydrophobic to form a water-filled pore [23] transporting charged particles. But we would like to point out that it is hydrophobic only in the upper part where the pore appears wide enough to let the large photolabel enter. From the reaction site on the photolabel (Ser 262 in δ) several polar residues occur in all subunits. Since [^{3}H]TPMP$^+$ does not enter this part of the pore it might be too narrow, and it might form the selectivity filter of the ion channel.

Gramicidin and even more so alamethicin may be seen as examples of channels formed by peptides without charged side chains. Charges within a channel would pose problems because strong ionic interactions would inhibit ion flux, especially in a domain contributing to the selectivity filter. At the wide channel entrance charges may be located much more favourably. There they would attract and concentrate ions as has been shown with a pyromellit-derivatized gramicidin channel [24]. Note that charged amino acids are located at the start of helix II.

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