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A modified nicotinic acetylcholine receptor lacking the 'ion channel amphipathic helices'

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Antibodies to a synthetic peptide from the 'amphipathic helix' of the α-chain of the nicotinic acetylcholine receptor (nAChR) bound both to detergent-solubilised and membrane-bound nAChR, indicating that this region, suggested as a component of the transmembrane ion channel in one model, is not buried in the membrane. Trypsinisation of membranes prior to affinity purification yielded preparations lacking the amphipathic helices of the α- and β-chains and probably also of the γ-and δ-chains. Such material should allow direct testing, by reconstitution experiments, of the importance of these regions for channel activity.

Acetylcholine receptor; Ion channel; Amphipathic helix; Synthetic peptide

1. INTRODUCTION

The nAChR from the electric ray, *Torpedo*, is a pentameric molecule, consisting of 4 different polypeptide chains ($\alpha_2, \beta, \gamma, \delta$), whose amino acid sequences have been determined from cDNA data (review [1]). A popular model for the insertion of the chains in the membrane suggests that each subunit has 4 hydrophobic transmembrane regions (M1-4) and that the intrinsic ion channel of the nAChR is formed from a transmembrane amphipathic helix (M5) donated by each of the 5 chains [2]. In the case of the α-chain, this region corresponds to residues 364-389. By trypsinisation of electric organ membranes before solubilisation and affinity purification, we have succeeded in obtaining preparations (tnAChR) lacking some or all of these suggested amphipathic helices. Such material should be useful in testing, by reconstitution experiments, the ion channel role of these regions.

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Abbreviations: DMF, dimethylformamide; KLH, Keyhole limpet haemocyanin; PBS, phosphate-buffered saline; DEAE, diethylaminoethyl; TFA, trifluoroacetic acid; PAGE, polyacrylamide gel electrophoresis; mAb, monoclonal antibody; MIR, main immunogenic region; αBTX, α-bungarotoxin; nAChR, nicotinic acetylcholine receptor; tnAChR, 'trypsinised' nAChR; RT, nAChR indirectly labelled with ^{125}I-αBTX

2. MATERIALS AND METHODS

2.1. *Protein purification, radiolabelling, radioimmunoassay methods and electrophoresis techniques*

nAChR and tnAChR were purified from electric organs of *T. marmorata* as described [3]. nAChR and αBTX were radiolabelled as in [4,5]. nAChR and tnAChR indirectly labelled with ^{125}I-αBTX were prepared as in [4]. Direct and competitive RIA were performed as described [6]. Electrophoresis on 12.5% SDS-PAGE, transfer and blotting were as described in [3].

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2.2. *Synthesis, purification and characterisation of peptides*

Peptides P1 and P3 (residues 151–169 and 426–437, respectively, of the α-chain of *T. californica*) were synthesised and characterised as described in [7,8].

Peptide P9 (residues 378–391 of the α-chain) was synthesised using Fmoc amino acids and the active ester solid-phase method [9]. Protected amino acids were purchased from Bachem (Switzerland); protecting groups were tertiary butyl ester (carboxyl groups), tertiary butyl ether (hydroxyl groups) and benzyloxybarbonyl (N-terminal glycine and ϵ-amino group of lysine).

The peptide was cleaved from the resin with simultaneous deblocking of side-groups using 55% TFA in DMF in the presence of 1 ml of anisol/g of resin, evaporated to dryness, dissolved in 1 M acetic acid and lyophilised. After gel filtration on Sephadex G25 in 10% formic acid, the main peak was lyophilised then fractionated by ion-exchange chromatography on DEAE-Sephadex A50, equilibrated in 50 mM ammonium acetate, pH 5.0. After washing, a linear gradient of ammonium acetate (50 mM, pH 5.0–1 M, pH 6.5) was applied. The major peak was lyophilised and analysed by reversed-phase HPLC, amino acid composition and amino acid sequencing. (i) Reversed-phase HPLC showed essentially a single peak. (ii) Amino acid analysis after hydrolysis in 5.5 M hydrochloric acid at 120°C for 15 h under vacuum was performed by precolumn derivatisation with 4-dimethylaminoazobenzene-4′-sulphonyl chloride [10]. The results for 3 analyses, each of 3 hydrolysates, were the following (expected values in brackets): Asp 0.94 (1), Glu 2.90 (3), Ser 1.08 (1), Gly 1.06 (1), Ala 1.00 (1), Val 0.97 (1), Met 0.88 (1), Lys 1.95 (2), His 1.03 (1), Tyr 0.97 (1). (iii) N-terminal sequence analysis was performed using an Applied Biosystems 477A protein sequencer. Released phenylthiohydantoin amino acid derivatives were identified by reversed-phase HPLC [11]. The unique sequence GVKYIAEHMKDSEE was found.

2.3. *Preparation of antibodies*

Antisera to nAChR and tnAChR were raised in rabbits as in [12]. Antisera to P9 conjugated to KLH were raised as described for antisera to P1 and P3 [7,8]. Anti-P9 antibodies were purified by affinity chromatography on P9 conjugated to Affigel 10 (Biorad).

The production and characterisation of mAbs to the nAChR and its subunits have been described [13–16].

2.4. *ELISA assays*

nAChR or tnAChR (2.5 μg/ml in 0.1 M sodium bicarbonate, 50 μl/well) was used to coat microtitre plates overnight at 4°C. All further manipulations were at 22°C. The plates were washed with distilled water and blocked with PBS containing 0.5% BSA, 5% Tween 20 (150 μl/well, 20 min). After 3 washes with PBS, 0.05% Tween 20 (buffer A), sequential incubations with suitable dilutions of primary antibody (50 μl/well, 1 h), second antibody coupled to alkaline phosphatase (50 μl/well, 1 h) and *p*-nitrophenylphosphate (1 mg/ml in 1 M diethanolamine, 0.5 mM magnesium chloride, pH 9.8, 100 μl/well, 30 min) were performed. 3 washes with buffer A (150 μl/well) were carried out between steps. 25 μl of 2 M NaOH was added and the optical density at 405 nm measured.

In blocking experiments, dilutions of primary antibody were chosen so as to be in the non-plateau region of the curve. The diluted antibody was then preincubated with an equal volume of test antigen at different concentrations for 1 h before addition to the plates.

2.5. *Precipitation of ^{125}I-αBTX trace-labelled* Torpedo *membranes*

Crude membranes (16.9 mg/ml, 0.9 nmol αBTX binding sites/mg protein) from the electric organ of *T. marmorata* were prepared as in [3] and stored at −70°C. Before use, the closed vesicles were opened by 3 cycles of freeze-thaw and 2 sonication steps; the effectiveness of this process was monitored by the increased sensitivity of the α-chain to trypsin degradation [8]. An aliquot (30 μl) was mixed with an equal volume of ^{125}I-αBTX (52 nM) and incubated for 30 min. 3 ml of PBS, 1% BSA (buffer B) was added. 100 μl aliquots were incubated in duplicate with different volumes of affinity-purified anti-P9 antibodies or control rabbit IgG (approx. conc. of 0.25 mg/ml) for 1 h, before the addition of 50 μl of 10% (v/v) *Staphylococcus aureus* Cowan strain I in buffer B. After 30 min, 1 ml of buffer B was added and the tubes centrifuged at 840 × *g*, before 2 more washes in the

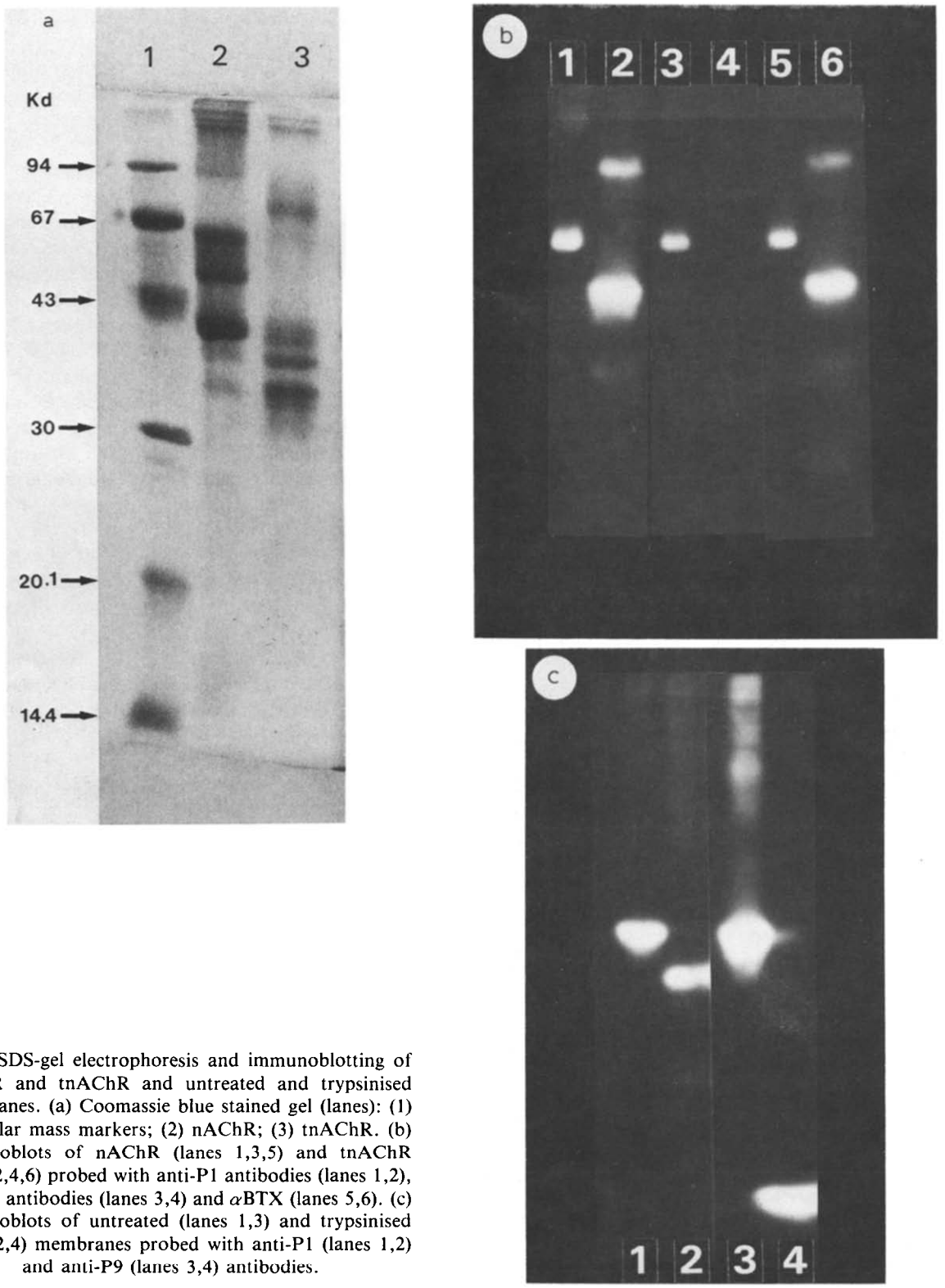
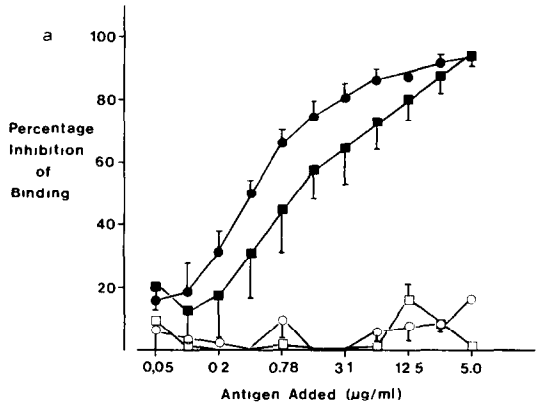
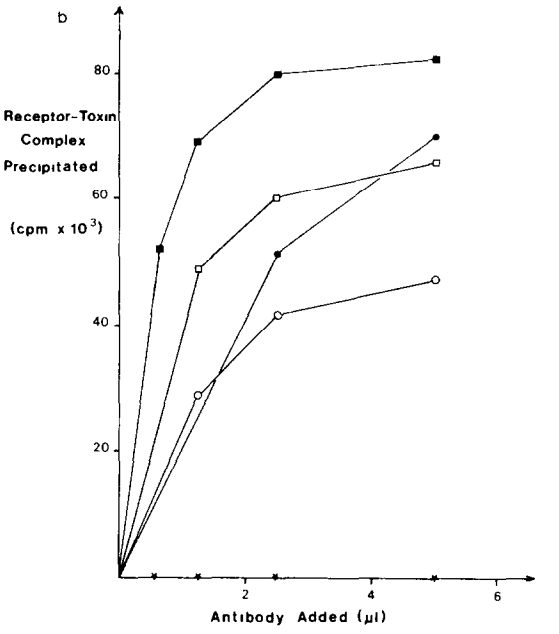


Fig.1. SDS-gel electrophoresis and immunoblotting of nAChR and tnAChR and untreated and trypsinised membranes. (a) Coomassie blue stained gel (lanes): (1) molecular mass markers; (2) nAChR; (3) tnAChR. (b) Immunoblots of nAChR (lanes 1,3,5) and tnAChR (lanes 2,4,6) probed with anti-P1 antibodies (lanes 1,2), anti-P9 antibodies (lanes 3,4) and αBTX (lanes 5,6). (c) Immunoblots of untreated (lanes 1,3) and trypsinised (lanes 2,4) membranes probed with anti-P1 (lanes 1,2) and anti-P9 (lanes 3,4) antibodies.





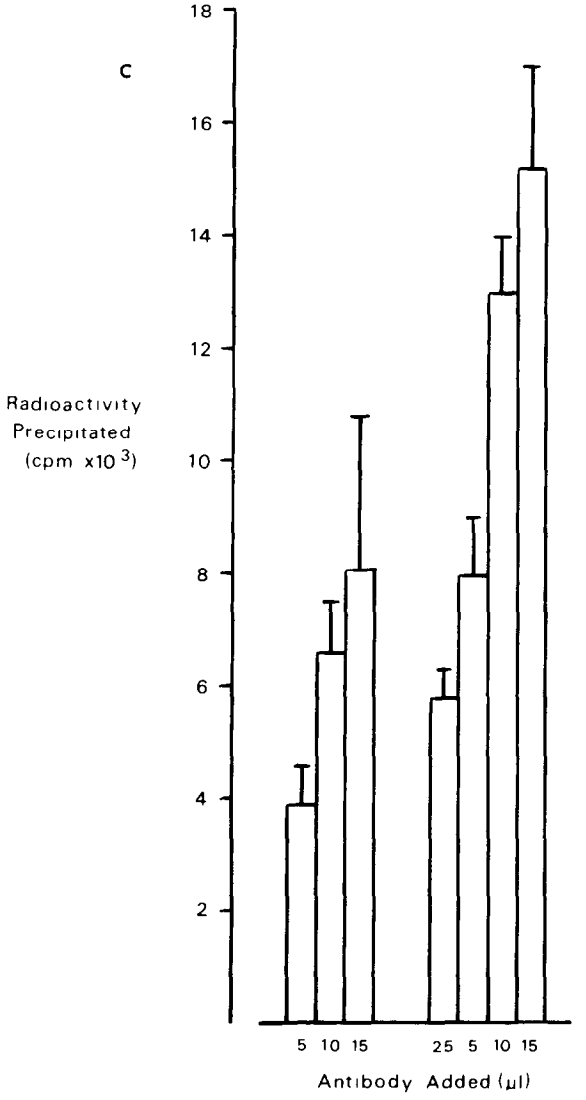


Fig.2. Reactivity of anti-P9 antibodies with nAChR. (a) ELISA assay using nAChR-coated plates. Affinity-purified anti-P9 antibodies from rabbit 1 (■,□) or rabbit 2 (●,○) were preincubated with buffer alone or different concentrations of nAChR (■,●) or tnAChR (□,○), then tested for binding to nAChR-coated plates as described in section 2. Results are means ± SE of 3 tests. (b) RIA using ^{125}I-α-BTX trace-labelled nAChR or tnAChR. Trace-labelled nAChR (■,□,●,○) or tnAChR (*), 138×10^3 cpm, was incubated with different volumes of anti-P9 antiserum (□,○,★) or affinity-purified antibodies (■,●) from rabbit 1 (■,□,*) or rabbit 2 (●,○,*) before precipitation with *S. aureus*. Results of blanks using normal rabbit serum or IgG have been subtracted. A polyclonal anti-nAChR antiserum precipitated 104×10^3 cpm (nAChR) and 84×10^3 cpm (tnAChR). (c) Precipitation of ^{125}I-αBTX trace-labelled *Torpedo* membranes. Crude membranes vesicles were opened by freeze-thaw and sonication steps then trace-labelled with ^{125}I-αBTX and reacted with affinity-purified anti-P9 antibodies as described in section 2. Results shown are means ± SE of 2–5 determinations. Antibodies are from rabbit 1 (left) and rabbit 2 (right). Blanks using normal rabbit IgG have been subtracted. Polyclonal anti-nAChR antisera precipitated 15 000 cpm.

same buffer. All incubations and all centrifugation steps were at 22°C and 4°C, respectively. Under these centrifugation conditions, precipitation of membranes in the absence of antibody was low.

3. RESULTS

3.1. *Characterisation of anti-P9 antisera*

Immunoblotting experiments using purified nAChR (fig.1b) or crude membranes (fig.1c) showed that affinity-purified anti-P9 antibodies reacted specifically with the α-chain of the nAChR. Immunoblots of trypsinised membranes (fig.1c) showed that the anti-P9 antibodies bound to the same 10 kDa fragment previously described as containing the C-terminal peptide, P3 [8].

Anti-P9 antibodies bound to purified nAChR coated on microtitre plates; the binding was completely abolished by competition with an excess of soluble nAChR (fig.2a). Anti-P9 antisera and affinity-purified antibodies bound both to RT complex (fig.2b) and open but intact membranes (fig.2c).

3.2. *Characterisation of nAChR prepared from trypsinised membranes (tnAChR)*

On SDS-PAGE, tnAChR and nAChR showed an overall similar pattern of protein bands. Their apparent molecular masses ranged from 36 to 40 kDa for tnAChR compared with from 41 to 68 kDa for nAChR (fig.1a). The band at 36 kDa reacted with polyclonal antibodies to peptide P1 (α-chain residues 151–169), with mAbs to the MIR and with αBTX, as described [8]. In addition, a high molecular mass band was always present which reacted with these same reagents (fig.1b).

The specific activity of tnAChR was higher than that of nAChR (8123 ± 996, 6122 ± 394 nmol toxin binding/mg, respectively, mean and SE of 3 determinations), in agreement with the decrease in apparent molecular masses of the different chains.

In terms of antigenicity, tnAChR competed almost as effectively as nAChR with RT for polyclonal antibodies to nAChR (not shown). The anti-nAChR antibody titres and onset of experimental myasthenia gravis in rabbits were similar in animals immunised with tnAChR or nAChR (not shown).

3.3. *Absence of regions containing the 'amphipathic helix'*

3.3.1. Lack of reaction of tnAChR with anti-P9 antibodies

As shown in fig.2b, anti-P9 antibodies did not bind to tnAChR labelled with ^{125}I-αBTX, nor did tnAChR inhibit the binding of anti-P9 antibodies either to soluble RT (not shown) or to immobilised nAChR (fig.2a).

On immunoblots, no binding of anti-P9 antibodies to tnAChR was observed (fig.1b). Similar results were obtained using polyclonal antibodies to the C-terminus of the α-chain [3].

Table 1

Reactivity of anti-nAChR mAbs with nAChR or tnAChR in ELISA tests

mAb	Epitope	Direct ELISA		Competitive ELISA
		nAChR	tnAChR	
198	α 37–85	+	+	100%
149,187	α339–347	+	–	ND
142	α349–365	+	+	1–2%
147	α360–379	+	+	1–2%
8	α360–379	+	–	ND
153,155,164	α371–386	+	–	ND
169	β335–375	+	+	12.5%
111,148	β370–405	+	–	ND
125	β430–445	+	–	ND
163,170	β460–470	+	+	6%
172	β460–470	+	–	ND
97, 99,109	β ?	+	–	ND
118,151	β ?	+	–	ND
11	β ?	+	+	1–2%
132,165,168	γ ?	+	–	ND
141,166	δ 315–415	+	–	ND
134,150	δ ?	+	–	ND

mAbs, produced and characterised as in [13–16], were tested for direct binding to nAChR or tnAChR coated on microtitre plates. mAbs showing significant reaction with tnAChR (more than 12% of the binding of anti-MIR mAb 198) were retested in a competitive ELISA. mAbs were preincubated with different concentrations of nAChR or tnAChR, then free mAb was estimated using nAChR-coated plates. The efficiency of tnAChR relative to nAChR in blocking the mAb was estimated from the amounts required for equivalent effects. (?) The epitope recognised by the mAb has not yet been localised. ND, not determined

3.3.2. Lack of reaction of tnAChR with mAbs to defined sites near the 'helical' regions

mAbs whose binding sites have been characterised [13–16] were tested by a direct ELISA for their ability to recognise nAChR or tnAChR. As shown in table 1, almost all mAbs tested, including 8/8 reactive with residues 339–386 of the α-chain, 7/7 reactive with residues 335–470 of the β-chain (N- and C-terminal to the 'helical' region) and 2/2 reactive with residues 315–415 of the δ-chain, showed little or no reactivity with tnAChR. Of the above antibodies, only 142, 147, 163, 169 and 170 showed a significant reaction. Using a competitive ELISA in which tnAChR or nAChR were competed with immobilised nAChR for mAb, the inhibitory activity of tnAChR relative to that of nAChR for these 5 antibodies was estimated as less than 2% for mAbs 142 and 147, 6% for 163 and 170 and 12.5% for mAb 169. Of a further 11 mAbs (6 anti-β, 3 anti-γ and 2 anti-δ) whose binding sites are not characterised, only mAb 11 bound to tnAChR in solution or on microtitre plates.

4. DISCUSSION

Our results using anti-peptide antibodies specific for the putative amphipathic helix of the α-chain clearly show that this region is absent from the tnAChR. The lack of reactivity of mAbs known to bind on either side of the corresponding segment of the β-chain [14] strongly suggest that the amphipathic helix of this subunit is also absent (the low residual reactivity of a few mAbs presumably reflects a low degree of cross-reactivity with a second site on the tnAChR). Owing to the lack of mAbs specific for this region of the γ- and δ-chains, we cannot completely rule out the possibility that the amphipathic helices of these subunits might still be present on the tnAChR. However, given the similarity in the structure of the 4 subunits, the availability of suitable trypsin-susceptible sites, the decrease in apparent molecular mass of all chains and the loss of the binding sites recognised by 7/7 mAbs specific for the γ- and δ-chains, this would seem unlikely.

The step in the preparation at which the amphipathic helices are lost is not yet clear. Preliminary data suggest that extensive degradation occurs at the trypsinisation step, the remainder of the helical region being lost on affinity chromatography. Wennogle et al. [17] have previously reported that trypsinisation of purified nAChR causes release of a 16 kDa fragment from the δ-chain. Anderson et al. [18] showed that the β- and δ-chains of *Torpedo* nAChR, during in vitro synthesis in dog pancreatic rough ribosomal membranes, were cleaved by trypsin, yielding fragments of 7 and 12 kDa, respectively; in this case, the fragments were partially water-soluble at pH 10 and were degraded by higher trypsin concentrations, similar to those used in our experiments. Our results using mAbs 125 and 169 (anti-β) and 166 (anti-δ) are in agreement with those of Anderson et al. [18].

Our experiments showing that anti-P9 antibodies bind both to membranes and to detergent-solubilised nAChR suggest that the amphipathic helix of the α-chain is not a transmembrane ion-channel, in agreement with the results of Ratnam et al. [14,16] which showed that mAbs binding near the amphipathic helices bind to the cytoplasmic face of the membrane, and with the results of other groups which showed that the second putative hydrophobic transmembrane region (M2) of the δ-chain is involved in ion-flux regulation [19] and is labelled by reactive channel blockers [20,21]. Reconstitution experiments using tnAChR should help to resolve this point.

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