Synthesis of an antigenic site of native acetylcholine receptor peptide 159–169 of *Torpedo* acetylcholine receptor α -chain

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A region of the α -subunit of the nicotinic acetylcholine receptor (AChR) of the *Torpedo* electric organ, containing residues 161–166, has been proposed to be a major antigenic site in the native AChR protein. We report the synthesis of a peptide corresponding to residues 159–169, which contains the proposed antigenic region. In quantitative radiometric titrations, radiolabelled anti-(native AChR) antibodies from three different species, rabbit, rat and dog, exhibited considerable binding (approx. 15% relative to native AChR) to Sepharose-immobilized peptide 159–169, but did not bind significantly to Sepharose-immobilized unrelated proteins or peptides. Specificity was further confirmed by the finding that no rabbit anti-AChR antibodies bound to the peptide after absorption with native AChR. These data indicate that the region 159–169 contains an antigenic site that is readily accessible in solubilized native *Torpedo* AChR.

The nicotinic acetylcholine receptor (AChR) is a transmembrane glycoprotein located in the post-synaptic membrane of cholinergic synapses, including electroplaques and skeletal muscle. The receptor plays a central role in synaptic transmission because it opens a cation-permeable channel upon binding to acetylcholine (Changeux, 1981). In addition to its intrinsic interest to neurophysiologists, the AChR has been the subject of intensive immunochemical research, because it is the target antigen in the autoimmune disease myasthenia gravis (Fambrough *et al.*, 1973; Almon *et al.*, 1974; Lindstrom *et al.*, 1976). AChR of the *Torpedo* electric organ is a pentamer ($\alpha_2, \beta, \gamma, \delta$) of four different polypeptide chains (α , M_r 39 000–44 000; β , M_r 48 000–52 000; γ , M_r 58 000–62 000; δ , M_r 63 000–67 000) (Chang & Bock, 1977; Lindstrom *et al.*, 1978; Froehner & Rafto, 1979). Human AChR has the same basic subunit structure, with an additional co-purified peptide, M_r 61 000, that is not found in *Torpedo* AChR (Momoi & Lennon, 1982). Nucleic acid sequences of the genes encoding all four chains of AChR from *T.*

californica (Noda *et al.*, 1982, 1983a; Claudio *et al.*, 1983), the α -chains of *T. marmorata* (Devillers-Thiery *et al.*, 1983) and human and bovine muscle AChR (Noda *et al.*, 1983b) revealed marked homologies among the different species. It has been proposed (Noda *et al.*, 1982) that an antigenic site resides in the region of residues 161–166 of the α -chain of the *Torpedo* AChR. The present paper reports the synthesis and binding activity to antibodies raised in three different host species of a peptide containing the proposed antigenic region (i.e. peptide 159–169).

Materials and methods

Native AChR was solubilized from membranes of the electric organ of *T. californica* (Pacific Biomarine, Venice, CA, U.S.A.) in buffer containing 2% Triton X-100 and was purified by immunoaffinity adsorption on an adsorbent of a monoclonal anti-AChR antibody (Lennon *et al.*, 1980). Its α -bungarotoxin-binding activity (7.5 nmol/mg) was determined by an immunoprecipitation assay (Lennon *et al.*, 1980).

Antibodies to native AChR were prepared in New Zealand White rabbits, Lewis rats and a dog, by immunization with AChR emulsified in complete Freund's adjuvant, with *Bordetella pertussis* vaccine as a supplementary adjuvant (Lennon *et*

Abbreviations used: AChR, native acetylcholine receptor; PBS, 0.01 M-sodium phosphate, pH 7.2, containing 0.15 M-NaCl; BSA, bovine serum albumin; HbA, human haemoglobin A; Mb, myoglobin.

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al., 1976). IgG was prepared from rat antisera by precipitation with 40%-saturated $(\text{NH}_4)_2\text{SO}_4$, followed by chromatography on DE-52 DEAE-cellulose (Whatman). IgG was purified from rabbit antisera, and IgG, IgM and IgA were purified from the dog, by affinity adsorption on Protein A-Sepharose CL-4B (Pharmacia), followed by elution in 1M-acetic acid and dialysis against 0.01M-sodium phosphate buffer.

Purified Ig fractions (50 μg) of each antiserum were labelled with ^{125}I (1.0mCi) by the chloramine-T method of Hunter & Greenwood (1962). Free label was removed by gel filtration on Sephadex G-25 (Pharmacia) in PBS containing 0.2% BSA, followed by dialysis against the same solvent. The specific radioactivity of labelled antibody preparations ranged from 8.5 to 12.6 $\mu\text{Ci}/\mu\text{g}$ of protein. A control preparation of IgG was similarly prepared from rabbit antiserum to HbA and was radioiodinated.

The peptide Ser-Pro-Glu-Ser-Asp-Arg-Pro-Asp-Leu-Ser-Thr-(Gly) was synthesized with t-butyloxycarbonyl-L-amino acid derivatives (Vega Biochemicals, Tucson, AZ, U.S.A.) on Merrifield (1963) resin esterified to t-butyloxycarbonyl-L-glycine (substitution 0.71mmol of glycine/g of resin), in accordance with established procedures (Koketsu & Atassi, 1973, 1974a). The side-chain-protecting groups used were: γ - and β -benzyl esters for glutamic acid and aspartic acid; *guan*-nitro for arginine; and *O*-benzyl for serine and threonine. The completed peptide was cleaved (-20°C , 30min) from the resin by anhydrous HF (distilled in CoF_3). It was then dissolved in trifluoroacetic acid, precipitated three times in cold diethyl ether, redissolved in water and freeze-dried. The peptide was purified by gel filtration on Sephadex G-25 in 0.05M- NH_4HCO_3 , followed by chromatography on DEAE-Sepharcel with a pyridine/acetate buffer, pH4.5, gradient (0.05–1.0M) (Atassi & Pai, 1975). Homogeneity of the product was tested by high-voltage paper electrophoresis in pyridine/acetate buffer, pH6.65. Amino acid analysis was performed on acid hydrolysates (constant-boiling HCl, 110°C , 22h) in triplicate on a Beckman model 6300 amino acid analyser. The peptide structure was confirmed by sequence analysis on a Beckman 890 M automatic sequencer. The control proteins, HbA and BSA, peptide 159–169, and control synthetic peptides, Mb-(56–62)-peptide (Atassi, 1975), HbA- β -chain-(1–15)-peptide (N. Yoshioka & M. Z. Atassi, unpublished work) and HbA- α -chain-(121–135)-peptide (Kazim & Atassi, 1982), were coupled to CNBr-activated Sepharose CL-4B (March *et al.*, 1974) with the use of optimal conditions for protein and peptide coupling (Twining & Atassi, 1979), AChR was coupled in a similar manner to that used for HbA and BSA,

except that the coupling buffer was 0.2M- NaHCO_3 buffer, pH9.5, containing 0.1M-NaCl and 0.2% Triton X-100. The amounts of proteins and peptides coupled on the adsorbents were determined by amino acid analysis. Protein adsorbents contained (per ml of packed volume) 1.3mg (HbA and BSA) and 0.35mg (AChR), and peptide adsorbents had 0.5–0.7mg.

Quantitative radioimmuno-adsorbent titrations with fixed amounts of ^{125}I -labelled antibodies and increasing amounts of adsorbents were carried out by the method described by Twining & Atassi (1979). Non-specific antibody binding was determined by titrating equivalent volumes of control protein and peptide adsorbents, in parallel assays.

Triplicate samples of ^{125}I -labelled rabbit anti-AChR IgG (100000c.p.m. in 50 μl) were absorbed with adsorbents of native AChR or BSA, for 6h at 24°C in PBS containing 0.2% BSA. The supernatants from each adsorbent were pooled separately and 50 μl samples were tested in duplicate for binding activity to adsorbents (50 μl of packed volume) of AChR, peptide 159–169 or BSA. Each experiment was performed at least twice.

Results

After purification, the major fraction of peptide 159–169 that was eluted from DEAE-Sepharcel with pyridine/acetate buffer, pH4.5, at an ionic strength of 0.25–0.3M, was homogeneous by high-voltage electrophoresis in heavily loaded maps. Its amino acid composition was in excellent agreement with that expected from its structure: Asp 2.11 (2); Thr 1.04 (1); Ser 3.10 (3); Glu 1.06 (1); Pro 2.05 (2); Gly 1.07 (1); Leu 1.08 (1); Arg 0.92 (1). Finally, its amino acid sequence coincided completely with the aforementioned structure.

A representative example of the binding of rabbit anti-AChR IgG to native AChR, and to peptide 159–169, is illustrated in Fig. 1. Table 1 summarizes the binding results with antisera raised in three different species (rabbit, rat and dog). Each of the antisera exhibited considerable binding of antibodies to peptide 159–169 (approx. 15% relative to native AChR), whereas the binding to unrelated control proteins and peptides was negligible (usually less than 3%; Table 1). Conversely, the binding of ^{125}I -labelled rabbit anti-HbA IgG to adsorbents of native AChR and peptide 159–169 was negligible (less than 4%) compared with the reactivity of anti-HbA IgG with HbA (38760c.p.m.; results not shown). Finally, radio-labelled rabbit anti-AChR antibodies did not bind to peptide 159–169 after absorption of the antibodies with native AChR. On the other hand, similar absorption with BSA adsorbents did not

Table 1. Binding of anti-AChR antibodies to synthetic peptide 159–169 of the sequence of AChR α -chain of *T. californica* and to other molecules conjugated to Sepharose

Binding was quantified by radioimmunoassay titration assays. The mean plateau binding values of duplicate tubes measured in two separate binding assays are summarized as mean c.p.m. $\pm$ S.D. ($n = 4$). Examples of titration curves are shown in Fig. 1. Total amounts of ^{125}I -labelled IgG (from rabbit, rat and dog antisera) added to each tube were 315000, 319000 and 314000 c.p.m. respectively. All values shown have been corrected for mean non-specific binding to BSA–Sepharose (rabbit, 6010 c.p.m.; rat, 3490 c.p.m.; dog, 5180 c.p.m.) at plateau binding values.

^{125}I -labelled anti-(native AChR) antibodies bound

Sepharose adsorbent	Rabbit		Rat		Dog	
	Mean c.p.m.	Relative reaction (%)	Mean c.p.m.	Relative reaction (%)	Mean c.p.m.	Relative reaction (%)
Native AChR	104090 $\pm$ 1440	100	81950 $\pm$ 7550	100	136500 $\pm$ 4320	100
Peptide 159–169	15460 $\pm$ 1840	15	13160 $\pm$ 2100	16	23720 $\pm$ 1350	17
HbA	920 $\pm$ 310	1	1220 $\pm$ 410	1	6440 $\pm$ 590	5
Mb-(56–62)-peptide	1600 $\pm$ 60	1	1470 $\pm$ 120	2	2850 $\pm$ 100	2
HbA- β -chain-(1–15)-peptide	1010 $\pm$ 120	1	1000 $\pm$ 50	1	4280 $\pm$ 110	3
HbA- α -chain-(121–135)-peptide	1160 $\pm$ 130	1	1960 $\pm$ 180	2	2460 $\pm$ 120	2

Fig. 1. Radioimmunoassay titration of ^{125}I -labelled rabbit anti-(native AChR) antibodies

Various amounts of Sepharose adsorbents coupled to native AChR (●), synthetic peptide 159–169 (□) and control peptides Mb-(56–62)-peptide and HbA- α -chain-(121–135)-peptide (▲) were gently mixed with a fixed amount of ^{125}I -labelled rabbit anti-AChR IgG (315000 c.p.m.) for 18 h at 22°C before being washed and counting of their γ -emission. The value of non-specific binding to BSA–Sepharose (6010 c.p.m.) was subtracted from all values plotted. Each point represents the mean value obtained for each adsorbent volume (done in duplicate) in two separate binding assays.

remove antibody-binding activity to native AChR or to peptide 159–169.

Discussion

The region consisting of residues 161–166 (Glu-Ser-Asp-Arg-Pro-Asp) was produced by Noda *et*

al. (1982, 1983b) as the most likely candidate for an antigenic site on the α -subunit of the *Torpedo* AChR. The present paper demonstrates that a synthetic peptide, residues 159–169, which includes the proposed antigenic region, specifically binds antibodies raised against the native *Torpedo* AChR. Lack of antibody binding to unrelated synthetic peptides [Mb-(56–62)-peptide, HbA- β -chain-(1–15)-peptide and HbA- α -chain-(121–135)-peptide], representing antigenically reactive regions in the proteins Mb and HbA respectively, affirms our conclusion that anti-AChR antibodies bind specifically to the peptide 159–169, and that this region represents an antigenic site on the solubilized native AChR molecule of *T. californica*. It is noteworthy, in view of suggestions to the contrary (Benjamin *et al.*, 1984), that the antigenic site reported here is recognized similarly by three different species, rabbits, rat and dog.

The prediction (Noda *et al.*, 1982, 1983b) that this region of the α -chain may contain a major antigenic site of native AChR was based on the proposed transmembrane topology of the subunits and the fact that this region coincides with a major hydrophilic maximum of the α -chain. However, it is necessary to caution that it has been shown (Atassi & Webster, 1983; Atassi, 1984) that antigenic sites cannot be predicted on the basis of hydrophilicity profiles proposed by Hopp & Woods (1981). For example, in our laboratory the definition of antigenic sites in several proteins has revealed very little correlation between hydrophilic sequence maxima and antigenicity (Atassi, 1975, 1984; Sakata & Atassi, 1980; Kazim & Atassi, 1982; Atassi & Webster, 1983).

The synthesis of a peptide corresponding to the residues 151–169 of the *Torpedo* AChR, i.e. encompassing the peptide described here, was

reported by Juillerat *et al.* (1984). In contrast with our data, those authors were not able (using an immunoprecipitation technique) to demonstrate binding of anti-(native AChR) antibodies to peptide, as did antibodies raised in rabbits against the peptide itself. In our study, the binding of anti-(native AChR) antibodies to peptide 159–169 accounted for about 15% of the total binding to native AChR. The reason why binding of anti-(native AChR) antibodies to peptide 152–169 was not demonstrable in the study by Juillerat *et al.* (1984) is at present unclear. Our differing data may reflect the different assay methods used to detect the binding of antibodies. For example, the effective affinity of antibody binding in solid-phase assays such as those employed in our study is generally higher. Alternatively, the failure of peptide 151–169 to react with anti-(native AChR) antibodies may have resulted from a steric hindrance imposed by bulky side chains in the additional residues 151–158 of their peptide (see 'Note added in proof'). It has been shown in this laboratory that the addition of hydrophobic side-chain residues to synthetic peptides can obstruct the binding of antibodies to reactive residues in antigenic regions (Koketsu & Atassi, 1974*b*; Atassi & Koketsu, 1975; Atassi & Pai, 1975).

The present work demonstrates unequivocally that AChR-specific antibodies in antisera prepared in three different species against native *Torpedo* AChR bind to a synthetic peptide corresponding to residues 159–169 of the α -subunit of the *Torpedo* AChR. However, the relatively low proportion of anti-(native AChR) antibodies that bind to this site suggests that the region 159–169 is not a major antigenic site of the AChR molecule. It remains to be determined whether or not the synthetic peptide 159–169 is capable of inducing experimental autoimmune myasthenia gravis. In all likelihood, the native AChR molecules in both *Torpedo* electric organ and human skeletal muscle have several antigenic sites of relevance to the immunopathogenesis of myasthenia gravis.

Note added in proof (received 27 November 1984)

After this paper was submitted for publication, and on the suggestion of the Editor, peptide 151–169 [Tyr-Asp-Gly-Thr-Lys-Val-Ser-Ile-Ser-Pro-Glu-Ser-Asp-Arg-Pro-Asp-Leu-Ser-Thr(-Gly)] was synthesized and purified by procedures described in the Materials and methods section. The amino acid composition of the pure peptide, in comparison with the values expected from its structure (given in parentheses), was: Asp 3.10 (3); Ser 4.01 (4); Thr 2.09 (2); Glu 0.98 (1); Pro 1.96 (2); Gly 2.10 (2); Val 1.03 (1); Leu 1.05 (1); Ile 0.97 (1); Tyr 1.02 (1); Lys 1.08 (1); Arg 0.96 (1). In quantitative

radiometric titrations, with fixed amounts of ^{125}I -labelled antibodies and various amounts of peptide adsorbents, peptide 151–169 bound the following amounts of antibodies (relative to native AChR as 100%): rabbit antibodies, 4.6%; rat antibodies, 3.9%; dog antibodies, 4.9%. Comparison with the binding to the shorter peptide 159–169 (Table 1) indicates that the extension 151–158 exerts some steric interference on the binding region of the peptide (see the Discussion section).

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