ANTIGENIC SIMILARITIES BETWEEN THE SUBUNITS OF ACETYLCHOLINE RECEPTOR FROM *TORPEDO MARMORATA*

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1. Introduction

The acetylcholine receptor (AChR) purified from the electric organ of *Torpedo* species is composed of 4 subunits with M_r ~40 000 (α), ~50 000 (β), ~60 000 (γ) and ~65 000 (δ), respectively, in a stoichiometry of $\alpha_2\beta\gamma\delta$ in the 9 S monomer [1,2]. With the exception of the α-polypeptide which binds acetylcholine [1–3], the function(s) of the others are not established; however, the δ-subunit can be labelled with a photoaffinity derivative of the local anaesthetic trimethisoquin [4], and is responsible for disulphide linkage between monomers [5]. Interestingly, primary amino acid sequence of ~50 residues in the N-terminal has revealed a high degree of homology between the subunits in *Torpedo californica* [2]. However, peptide maps of the subunits are distinct [6–8] and polyspecific antisera raised to isolated denatured chains show no appreciable cross-immunoreactivity [9–11] to heterologous subunits.

Here, antibodies were raised against isolated but renatured polypeptides of *Torpedo marmorata* and these were shown to recognise antigenic determinants on the heterologous chains to appreciable extents. Also, these antibodies cross-reacted with the oligomeric form of the receptor from avian and mammalian muscles. We conclude that the 4 chains of the fish receptors have extensive structural similarities and are related to those in the oligomeric form of muscle receptors, indicating possible evolutionary derivation from a parent precursor [2].

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2. Materials and methods

2.1. *Preparation of subunits*

AChR (3–5 mg), purified to homogeneity (spec. act. 4–6 nmol toxin binding sites/mg protein) by affinity chromatography on α-toxin–Sepharose [3], was subjected to preparative sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis (PAGE) [12] on slab gels of 10% acrylamide (120 × 130 × 3 mm) following denaturation of samples at room temperature for 5 min. After electrophoresis at 4°C for 16 h, the bands were located by Coomassie staining of strips cut from either side of the gel. Unstained sections of the gel containing each of the subunits were separated and macerated in Tris–glycine (pH 8.8)/1 mM dithiothreitol/0.1% Lubrol-PX/10% glycerol and equilibrated at 4°C overnight. The polypeptide chains were eluted and simultaneously renatured by electrophoresis [13] through a gel containing 6 M urea/0.25 M Tris–HCl (pH 8.8); myoglobin was used as a visual marker in a parallel tube. The resultant subunits were dialysed against 50 mM phosphate (pH 8.0)/0.1% Lubrol-PX. The total amount of polypeptides recovered was ~30% of the starting protein as measured in [14], using bovine serum albumin as standard. Purity of each preparation was checked by analytical SDS–PAGE [12]. The extent of renaturation of α subunit was assessed by measurement of its ability to bind α-bungarotoxin (α-BuTX) [15].

2.2. *Immunoreactivity studies*

Rabbits were immunised 4 times at bimonthly intervals with 50–100 μg of each subunit in Freund's adjuvant (complete for first inocalation and incomplete for subsequent injections), at multiple sites subcutaneously and intramuscularly.

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Immunoprecipitation experiments were performed with a constant concentration (0.15 pmol/assay tube) of either native AChR–^{125}I-α-BuTX complex or ^{125}I-labelled chains. Immune complexes were precipitated with sheep anti-rabbit immunoglobulin G (IgG) or inactivated *Staphylococcus aureus* cells containing protein-A. Competition assays were performed by reacting a fixed quantity of antiserum, capable of precipitating 0.1 or 0.2 pmol *Torpedo* native AChR, with varying amounts of purified receptor chains. The amount of anti-receptor antibodies remaining in the serum samples was then determined by measuring their capacity to precipitate *Torpedo* AChR–^{125}I-α-BuTX, as above.

2.3. *Radio-iodination of receptor chains*

Purified chains were labelled with ^{125}I by the chloramine-T reaction to spec. act. 900–1500 Ci/mmol, using 0.5 mCi ^{125}I and 10 μg protein. Free iodine was removed by gel filtration on Sephadex G-75. About 60–70% of the total radioactivity could be specifically precipitated with excess antiserum against that chain, the remainder probably being radioactive detergent or non-immunoreactive polypeptide.

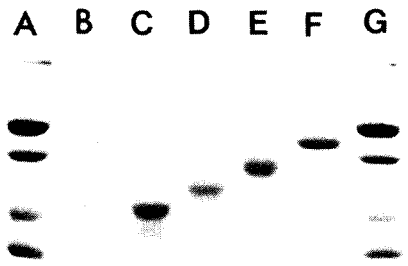

Fig.1. Analytical SDS–PAGE of purified AChR from *Torpedo* and its isolated subunits. Chains were purified by preparative electrophoresis as described: samples (~10 μg/band) were subjected to analytical SDS–PAGE under reducing conditions in 10% acrylamide slab gels [12], followed by staining with Coomassie brilliant blue R; (A,G) protein M_r standards (top to bottom), ferritin (half-unit) 220 000, bovine serum albumin 67 000, catalase 60 000, ovalbumin 43 000, lactate dehydrogenase 36 000; (B) *Torpedo* AChR; (C) α-chain; (D) β-chain; (E) γ-chain; (F) δ-chain. The small degree of heterogeneity seen in the samples is probably due to breakdown during the isolation procedure.

3. Results and discussion

AChR purified from *Torpedo marmorata* was shown by analytical SDS–PAGE to contain the expected 4 subunits with M_r (in our conditions, and as in [16]) of 44 500 (α), 50 000 (β), 55 000 (γ) and 63 000 (δ) (fig.1). After isolation of individual chains by preparative SDS–PAGE, followed by renaturation, preparations of each essentially free of impurities were obtained (fig.1). The extent of contamination was <2% of the total protein in the samples, as judged from scans of the stained bands; the larger part of these impurities was due to lighter proteolyzed forms, particularly in the case of the α-subunit. With a view to renaturing the receptor subunits, the samples were subjected to an elution technique shown to give effective removal of SDS and high recovery of biological activity with some soluble globular proteins [13]. All 4 subunits were treated similarly in this manner but ability to bind α-BuTX was found in the α-chain only. The value obtained for the latter using the ion-exchange disc assay for receptor (complexing for 16 h at 4°C with 50 nM mono-^{125}I-α-BuTX, 25 nM α-chain, washing 3 × 4 min), was 1160 pmol/mg protein. At least 90% of this binding was blocked by 10^{-4} M d-tubocurarine and all the activity was abolished by 10 μM α-BuTX. This index of renaturation should be treated with caution since the affinity of α-chain (thus prepared) for α-BuTX is expected to be low relative to that of native receptor necessitating use of impractical, high (~μM) levels of toxin to saturate the sites: this was found in [15] for another preparation of renatured α-chains when ~80% of the total sites were labelled.

High titres (0.5–1.0 μM) of antibodies to homologous chains and *Torpedo* AChR were obtained (table 1). However, the rabbits failed to exhibit obvious muscular weakness typical of experimental autoimmune myasthenia gravis, even after prolonged immunization with repeated injections of antigen. This accords with results of immunisation with denatured subunits in rabbits [9,10,17] but not in rats, which develop muscular weakness [10,11].

The occurrence of antigenic similarities between the subunits of *Torpedo* AChR is demonstrated by

Table 1
Relative titres of rabbit antisera to *Torpedo* AChR subunits against various preparations of the receptor

Preparation	Antibody titre (nmol antigen bound/L serum)[a]						
	^{125}I-Labelled chains				^{125}I-α-BuTX-labelled AChR[b]		
	α	β	γ	δ	*Torpedo*	Chick[c]	Cat[c]
Anti α {a	3400	250	235	75	1800	160	28
Anti α {b	1000	106	50	18	860	26	35
Anti β {a	333	1400	933	185	430	26	16
Anti β {b	280	720	533	120	520	6	4
Anti γ {a	27	300	300	183	370	53	30
Anti γ {b	13	143	350	80	400	6	2
Anti δ {a	38	216	300	1000	320	6	23
Anti δ {b	ND	55	133	850	800	3	0.5

[a] Titres were measured as in fig.2A, with 0.15 pmol antigen/assay tube (~1 nM) and are defined as nmol antigen precipitated/L serum under standard conditions; they were calculated from the initial slope of the immunoprecipitation curve. Estimated errors are ±10% of values given

[b] AChR in Triton extracts of tissues was complexed with ^{125}I-α-BuTX

[c] Denervated muscle used

ND, not detectable

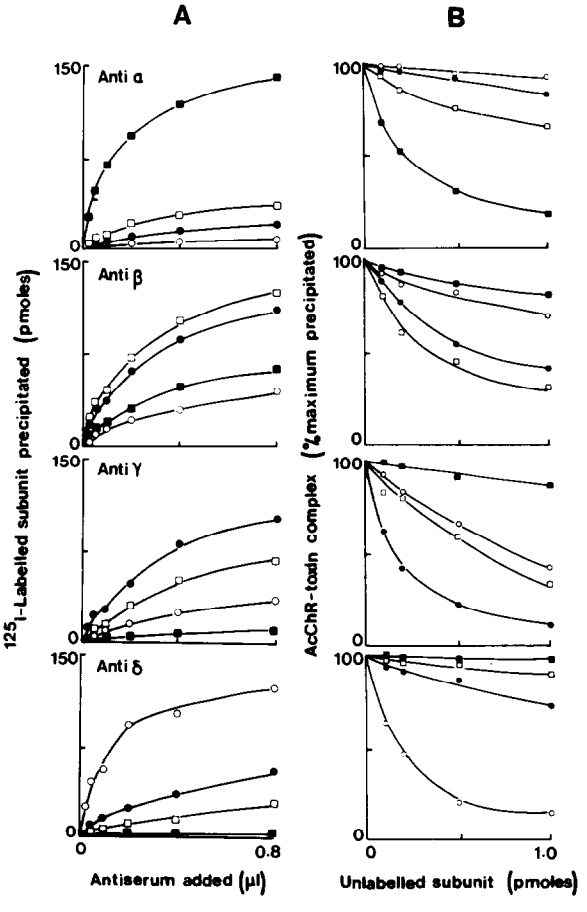

Fig.2. Immunoprecipitation of *Torpedo* receptor and its subunits by antibodies raised against the individual polypeptides. (A) Each ^{125}I-labelled chain (α■,β□,γ●,δ○, 0.15 pmol, ~1 nM final) was incubated with varying quantities of antiserum and an amount of normal serum to reach the same final volume. After reaction for 3–4 h at room temperature, immune complexes were precipitated by sheep anti-rabbit IgG (F_c), centrifuged, washed twice with phosphate-buffered saline pH 7.4/0.2% Triton X-100, and counted in a γ-counter. Non-specific binding measured similarly by use of an equal volume of non-immune serum instead of antibody, was subtracted from all values shown which are the mean of duplicate determinations. As the specific activity of iodinated chains could only be estimated to an accuracy of ±10%, the absolute value of titres are reliable to within the same approximate limits. (B) In these competition experiments, various amounts of subunits (α,β,γ,δ) were incubated with a quantity of antiserum against a given chain capable of precipitating 0.1 pmol *Torpedo* native receptor–toxin complex. After reaction at room temperature for 3–4 h, the remaining antibodies in the serum samples were reacted with *Torpedo* native receptor (0.2 pmol) complexed with ^{125}I-α-BuTX, by overnight incubation at 4°C. Finally the immune complexes were precipitated and counted after washing as in (A). Comparable curves were obtained when ^{125}I-labelled chains were used, instead of *Torpedo* receptor–^{125}I-α-BuTX complex, to determine the activity remaining in the antisera towards the homologous antigen. Antisera used were (top to bottom) anti-α, anti-β, anti-γ, and anti-δ; similar results were obtained with a different set of antisera (table 1a) against the subunits.

direct immunoprecipitation of heterologous chains with antisera to each of the chains (fig.2A). Antibodies against the β or γ chains seem to exhibit a greater degree of cross-reactivity to heterologous chains than do antisera to α or δ subunits. Competition experiments between the subunits and native receptor for the antibodies (fig.2B) support the above conclusion and indicate that the antibodies do not recognise all heterologous chains through the same number and/or type of antigenic determinants. Also, the β and γ chains show greater immunological resemblance than any other pairs of chains. This may be due to a more pronounced similarity in overall structure; alternatively, of the many possible determinants on these polypeptides only a small number are common to both chains and these could be largely responsible for the immunogenicity of these molecules. This immunochemical demonstration of structural similarities in all 4 subunits accords with the extensive homology in amino acid sequence known to exist between them [2]. The very minor contaminants in the immunogens in some cases cannot give rise to the nature of the inter-subunit cross-reactivity actually observed. For example, the β-polypeptide is appreciably more immunogenic than γ, and the level of contamination required to account for the cross-reactivity manifested would be 50% or more of the total protein in the sample. An auto-immune reaction (producing antibodies to host muscle receptor), is excluded as a potential source of the cross-reactivities since:

(i) Clinical signs thereof were absent in all the animals;
(ii) The extent of reactions of these antisera with muscle AChR were orders of magnitude lower than their titres against the *Torpedo* AChR subunits.

The failure of other investigators to show a good degree of antigenic similarity between the *Torpedo* polypeptides [9–11] is likely to be due to the use of receptor chains as immunogens which were denatured in SDS but not adequately renatured; this could minimise the number of structural features typical of the native polypeptides. It is interesting that cross-reactivity between single determinants on 2 pairs of chains, namely α–β and γ–δ (but not other pairs), was demonstrated using monoclonal antibodies where the chance of selection is increased greatly [18].

The reaction of native receptor from mammalian and avian muscles with antisera against the chains was studied. They were all found to recognise these receptors to varying degrees, but the extent of cross-reactivity was low in all cases (table 1), in agreement with [10,11]. In itself, this does not necessarily indicate the presence in these receptors of 4 subunits of different size and analogous to those of *Torpedo* receptor, as speculated [11]. For example, receptor from chick and cat denervated muscles, which contains 3 [19] or less polypeptides [3], gave similar levels of cross-reactivities. Immunological reactions of AChR oligomer with any antiserum to a given chain could possibly be a reflection of common determinants on multiple forms of a single size of subunit. Alternatively, a receptor could contain more than one size of subunit which could be structurally related, as in *Torpedo*, but not exclusively 4 in number or of the same sizes as in the latter, since for example they differ in chick [19] and human muscle [20].

It is concluded that the component polypeptide chains of *Torpedo* AChR are antigenically similar and that the receptors from muscle, irrespective of their apparent subunit composition, have some antigenic determinants related to those in the fish receptor. This is in agreement with current views regarding the genealogy of the AChR molecule [2].

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