Differences between embryonic and adult Torpedo acetylcholine receptor γ subunit

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Antibodies to a synthetic peptide corresponding to residues 346–359 of the Torpedo acetylcholine receptor (AChR) γ subunit, were employed to compare the adult and embryonic receptor. This peptide contains a consensus phosphorylation site for cAMP-dependent protein kinase (PKA). The anti-peptide antibodies discriminated between adult and embryonic AChRs, and reacted preferentially with the adult γ form. These observed immunological differences did not seem to stem from different phosphorylation states. Our results suggest that the embryonic Torpedo AChR may have a γ-like subunit that differs from the known adult form of this subunit, at least in the specific region that contains the phosphorylation site for PKA.

Adult and embryonic acetylcholine receptor; γ Subunit; Anti-peptide antibody; Phosphorylation

1. INTRODUCTION

The nicotinic acetylcholine receptor (AChR) is composed of four different subunits in the composition $\alpha_2\beta\gamma\delta$. In some mammalian species there exists a fifth subunit (ε), which is homologous to the γ subunit [1–3]. It was found that shortly before birth, the embryonic γ subunit is replaced by the adult ε subunit [2,4,5]. Functional assays have shown that this subunit substitution can account for the known differences between adult and embryonic AChR channel properties in mammalian muscle [6].

Based on the amino acid sequence, the γ subunit of Torpedo shows similar homology (55–57%) to both the ε and the γ subunits of mammalian muscles [1]. In some respects, however, it has typical characteristics of an ε (adult) subunit: the Torpedo γ subunit possesses two adjacent consensus sites for cAMP-dependent protein kinase (PKA) phosphorylation, one of which is present in all ε subunits described so far but is absent in their respective γ subunits (see Fig. 1). From this viewpoint, the Torpedo γ subunit might represent an adult form which has been defined as ε in mammalian species. It was therefore of interest to find out whether the Torpedo AChR has also an embryonic counterpart for the γ subunit.

In the following we report on differences between adult and embryonic Torpedo AChR which support the notion that the embryonic Torpedo AChR may have a

γ-like subunit that differs from the known adult form of this subunit, at least in one specific region that contains phosphorylation sites for PKA. It is possible that changes in this specific region play a functional role in the development and maturation of the neuromuscular junction.

2. MATERIALS AND METHODS

2.1. AChR preparations

Torpedo ocellata embryos at different stages of development were kindly provided by Prof. D. Michaelson from the Tel-Aviv University. Pregnant Torpedo females were dissected and their embryos were immediately frozen in liquid nitrogen. The embryos were divided into five age groups according to their overall length. The electric organs of embryos belonging to each age group were pooled for the purpose of AChR preparation. Triton extracts (1% Triton X-100) were prepared from the electric organs and the specific activity, in terms of α-bungarotoxin (α-BTX) binding sites of each preparation, was assessed as previously described [7].

2.2. Peptide conjugation

A fourteen amino-acid peptide corresponding to amino-acid residues 346–359 (K-P-Q-P-R-R-R-S-S-F-G-I-M-I; see Fig. 1) of the Torpedo γ subunit was synthesized by the solid-phase method of Merrifield [8] as previously described [9,10]. The peptide, following purification on a G-50 Sephadex column was conjugated to bovine serum albumin (BSA). For conjugation, the peptide (5 mg) was dissolved in 2 ml phosphate-buffered saline (PBS; 0.14 M NaCl, 0.01 M phosphate buffer, pH 7.4), and added to powdered BSA in a 40-fold molar excess. Following 10 min stirring at room temperature the coupling reagent 1-ethyl-3-(3'-dimethylaminopropyl)-carbodiimide hydrochloride (EDCI; Sigma), dissolved in 0.5 ml H_2O, was added in a 10-fold molar excess over the peptide. The mixture was stirred at room temperature for 18 h, dialyzed against PBS, aliquotted, and kept frozen at −20°C.

2.3. Immunological procedures

Polyclonal anti-peptide antibodies were elicited in rabbits by four

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        346                               359
Torpedo γ:  K  P  Q  P │R  R  R  S  S│ F  G  I  M  I

Calf    ε:  A  S  P  P │R  R  A  S  S│ L  G  L  L  L

Mouse   ε:  A  S  P  A │R  R  A  S  S│ V  G  I  L  L

Rat     ε:  A  S  P  A │R  R  A  S  S│ V  G  I  L  L

Calf    γ:  H  P  R  L │Q  N  G  S  S│ S  G  W  P  I

Mouse   γ:  R  F  R  L │Q  N  G  S  S│ S  G  W  P  I

Rat     γ:  R  L  R  L │Q  N  G  S  S│ S  G  W  P  I
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Fig. 1. Alignment of amino-acid residues 346–359 of the Torpedo AChR γ subunit [14], calf [1], mouse [3] and rat [15] muscle AChR ε subunits, and calf [16], mouse [17] and rat [15] muscle AChR γ subunits. The one-letter amino acid notation is used. Note that in the Torpedo γ subunit there are two adjacent serines which fulfill the substrate requirements for PKA.

injections with 1 mg of the conjugated peptide in complete Freund's adjuvant (CFA; 1:1). Polyclonal anti-AChR antibodies were produced by immunizing rabbits twice or three times with purified Torpedo AChR in CFA. Anti-AChR monoclonal antibodies (mcAbs) specific to the δ subunit (mcAb 7F2) [11] were prepared as previously described. Monoclonal antibody 274D, specific for the Torpedo γ subunit [12] was a kind gift from Dr S.C. Froehner. Western blot analysis was performed as described by Neumann et al. [9].

2.4. Phosphorylation of AChR preparations

Triton extracts (20–30 μl) or synthetic peptides (1–10 μg) were phosphorylated by the catalytic subunit of cAMP-dependent protein kinase as previously described [11,13].

3. RESULTS

Torpedo embryos from different developmental stages were grouped according to their overall length. The protein concentration and the specific activity (pmol α-BTX bound per mg protein) for each group are given in Table I. In the early stages of development the number of α-BTX binding sites was very low and therefore in all further experiments only embryos longer than 5.0 cm were used. The quantities taken for all experiments from the different developmental stages were calculated to contain the same number of α-BTX binding sites as assessed also by blot analysis with α-BTX overlay (see Fig. 2).

Table I

Development of α-BTX binding activity in electric organs of Torpedo

Stage	Body length (cm)	Protein concentration (mg/ml)	Specific activity (pmol α-BTX/mg prot)
1	2	1.3	9
2	3.0–3.5	2.3	42
3	4.0–4.5	2.1	75
4	5.0–6.0	2.7	526
5	6.0–7.0	3.5	1130
6	adult	5.8	2000

In order to find out whether the adult Torpedo AChR γ subunit differs from the embryonic one, as was found in some mammalian AChRs, we focused on a specific amino acid sequence in the Torpedo γ subunit that is shared by all known ε subunits but is different in their γ subunit counterparts (Fig. 1). This particular sequence is of special interest since it contains the consensus phosphorylation sites (R-R-R-S-S) for PKA and may play a role in the regulation of AChR function and development. To this end we prepared antibodies to a synthetic peptide corresponding to residues 346–359 of the Torpedo γ subunit which includes the PKA phosphorylation sites, and employed them to find out whether there are immunological differences between the embryonic and the adult γ subunit of Torpedo.

Adult and embryonic AChR preparations containing the same number of α-BTX binding sites reacted differently in Western blot analysis with the anti-γ346–359 peptide antibodies. Adult Torpedo AChR bound strongly while embryonic AChR bound very poorly to these antibodies (Fig. 2). On the other hand, these AChR preparations reacted to the same extent with several other anti-AChR antibodies. These included polyclonal rabbit anti-AChR antibodies, polyclonal antibodies specific for peptide 126–143 of the Torpedo α subunit [9], monoclonal anti-AChR antibodies specific to the γ subunit (mcAb 274D) [12] and to the δ subunit (mcAb 7F2) [11] of the Torpedo AChR (Fig. 2) and several antibodies elicited against peptides corresponding to residues 1–20, 330–340 and 351–368 of the α subunit [18] and residues 354–367, 367–374 and 373–387 of the δ subunit [11] of the receptor (data not shown). It seems that the main transition in reactivity with the anti-γ346–359 peptide antibodies occurs in late stages of the embryonic development, since these antibodies react comparably with Torpedo AChR preparations derived from embryos 6–7 cm long or younger (data not shown).

It is of interest to note that the monoclonal antibody specific for the γ subunit (mcAb 274D), which is directed to a site different from peptide 346–359 [12], as well as polyclonal anti-AChR antibodies, reacted similarly with the adult and the embryonic Torpedo AChRs (Fig. 2). This suggests that the differences in reactivity with the anti-γ346–359 peptide antibodies do not reflect differences in the content of this subunit in the adult and embryonic Torpedo AChRs, but rather that the embryonic AChR contains a γ-like subunit which is cross reactive with the adult form of this subunit, and differs from it at least in an antigenic determinant recognized by the anti-γ346–359 peptide antibodies. The γ subunits present in the adult and in the embryo seem to have the same apparent molecular weights (Fig. 2).

We have also carried out PKA phosphorylation experiments with the adult and embryonic preparations of Torpedo AChR. These experiments have indicated that
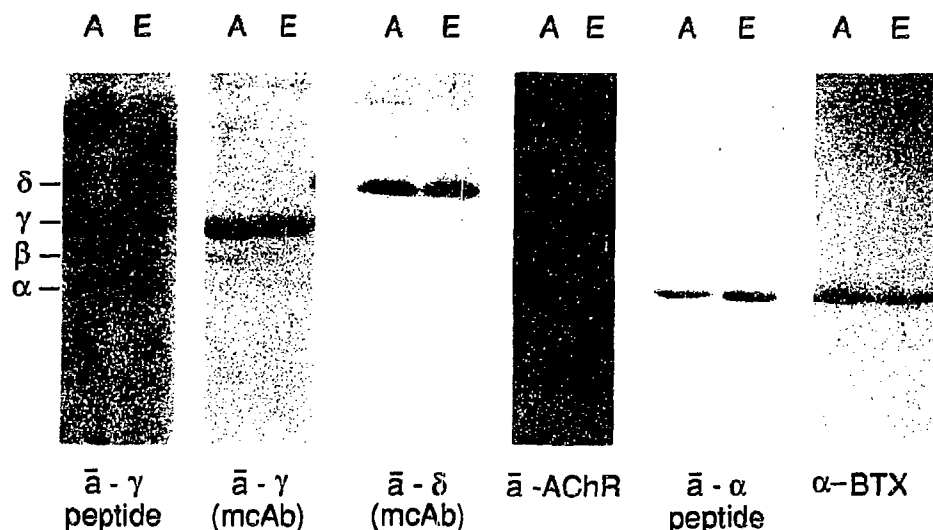
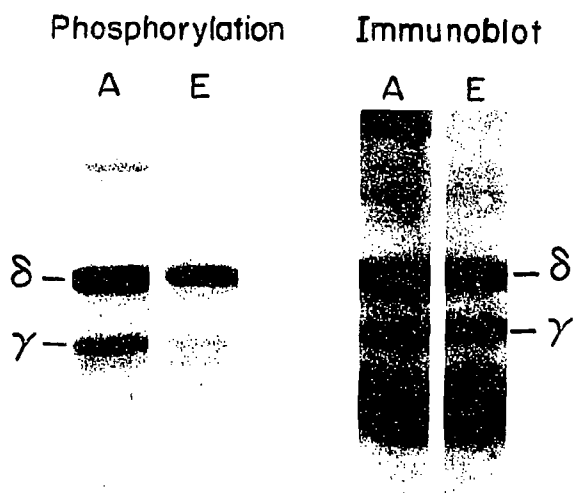Fig. 2. Blot analysis of adult and embryonic Torpedo AChR. Triton extracts of adult (A) and embryonic (E) Torpedo electric organs were separated in polyacrylamide gel electrophoresis and then blotted onto a nitrocellulose membrane filter. Different strips of the filter were overlayed with antibodies to peptide 346-359 derived from the γ-subunit (ā-γ peptide), polyclonal anti-AChR antibodies (ā-AChR), antibodies to a synthetic peptide derived from the α-subunit (ē-α peptide), monoclonal AChR-specific antibodies directed at the γ (mcAb 274D) and δ (mcAb 7F2) subunits (ā-γ, ā-δ and with α-bungarotoxin (α-BTX).

the adult Torpedo AChR is a better substrate for exogenous PKA than the embryonic AChR (Fig. 3, left). Differences in the extent of the δ subunit phosphorylation were also observed. It was difficult for us to determine whether the adult and embryonic AChR differ in their initial state of phosphorylation. However, we could rule out the possibility that the different phosphorylation profiles of the embryonic and adult AChRs

Fig. 3. Phosphorylation of adult and embryonic Torpedo AChR by PKA. Triton extracts of adult (A) and embryonic (E) Torpedo electric organs, containing each 0.1 nmol of α-BTX binding sites, were phosphorylated by the catalytic subunit of PKA in the presence of [γ-^{32}P]ATP (left) or unlabeled ATP (right). The phosphorylated receptor preparations were electrophoresed and exposed for autoradiography (left) or blotted and overlayed with polyclonal anti-AChR antibodies (right).
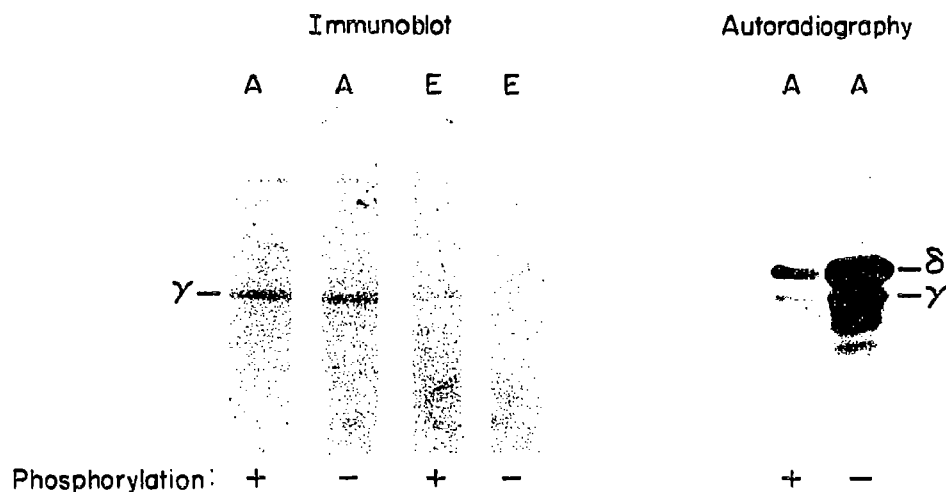
could be due to dissimilarities in the content of the γ and δ subunits, since both AChR preparations reacted to the same extent in immunoblot analysis with anti-AChR antibodies (Fig. 3, right).

The region recognized by the anti-γ346-359 antibodies contains consensus phosphorylation sites for PKA. It was therefore important to find out whether changes in this region, resulting from phosphorylation reactions, govern the differential reactivity of the antibody with adult and embryonic AChR. Anti-γ346-359 antibodies were shown to react similarly with phosphorylated and non-phosphorylated adult γ subunit (Fig. 4, first two lanes). This rules out the possibility that the anti-γ346-359 antibodies discriminate between the adult and the embryonic AChRs because of differences in their degree of phosphorylation. Moreover, following complete phosphorylation with unlabeled ATP, the anti-γ346-359 antibodies still discriminated between adult and embryonic Torpedo γ subunit (Fig. 4). Taken together, these experiments suggest that the immunological differences between the adult and the embryonic forms of the γ subunit do not result from differences in their initial state of phosphorylation, but rather reflect dissimilarities in their amino acid composition within the phosphorylation site or its close vicinity.

4. DISCUSSION

The immunological analysis and phosphorylation experiments carried out in this study indicate that the γ subunits of the adult and embryonic Torpedo AChR

Fig. 4. Effect of phosphorylation on the reactivity of anti-γ346-359 antibodies with adult and embryonic Torpedo AChR. Triton extracts of adult (A) and embryonic (E) Torpedo electric organs were phosphorylated by the catalytic subunit of PKA in the presence of unlabeled ATP. The phosphorylated receptor (in the presence or absence of enzyme as indicated) was separated in polyacrylamide gel electrophoresis, and blotted onto a nitrocellulose membrane filter which was then overlayed with antibodies to the γ-peptide (left), or subjected to further phosphorylation in the presence of labeled ATP (right), in order to indicate that the phosphorylation of the AChR prior to the antibody binding assay was indeed complete.

may differ from each other at least in a region that contains PKA phosphorylation sites. Since phosphorylation sites are present in this region in all ε subunits but are missing in their γ counterparts, it is possible that they are involved in the maturation of the neuromuscular junction, or alternatively, play a functional role in the adult AChR, for instance in the process of desensitization [19]. The Torpedo electric organ is a muscle analogue with no contractile muscle activity. Therefore, it may provide a natural model system for elucidating the factors that govern the developmental transition from the embryonic AChR to its adult form.

Differences between the adult and immature Torpedo AChR were reported previously [20]. It has been demonstrated that in vivo the adult Torpedo AChR has a more acidic isoelectric point than does the immature AChR. This would be in agreement with our suggestion that the adult Torpedo AChR possesses phosphorylation sites that are absent, or altered, in the immature receptor. Yee and Huganir [21] have demonstrated that serine-353 is the site for PKA phosphorylation on the Torpedo AChR γ subunit. It is thus possible that a change in this residue and/or its close vicinity may take place during embryonic development.

The replacement of the adult γ subunit by an embryonic ε subunit has been so far reported only for mammalian species. However there is some evidence that such a replacement may exist also in the chicken [22]. In the Torpedo, the known γ subunit possesses some characteristics of an adult ε subunit. It lacks, however, a deletion (amino-acid residues 395-409 of the mammalian γ subunit) that the three described ε subunits seem to have in comparison to their respective γ subunits. Interestingly, this stretch of amino acids in

the Torpedo γ subunit (amino acids 388-405) is completely different from that of the corresponding sequence in the mammalian γ subunits. This may raise the possibility that the dissimilarities between the embryonic and adult Torpedo γ subunits are not necessarily identical to those observed in mammalian species.

Our study suggests that in the Torpedo there may be structural differences between the adult and embryonic AChR γ subunits. It should still be assessed whether the differences between the adult and embryonic forms of the Torpedo AChR γ subunits reflect the existence of two different genes, as has been demonstrated for the bovine and murine muscle receptor γ and ε subunits.

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