INCREASE IN THE TRANSLATABLE mRNA FOR ACETYLCHOLINE RECEPTOR DURING EMBRYONIC DEVELOPMENT OF *TORPEDO OCELLATA* ELECTRIC ORGAN

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Received 8 January 1982

1. Introduction

The availability of large quantities of the nicotinic acetylcholine receptor (AChR) in the highly specialized electroplates of *Torpedo* permitted detailed studies of its structure [1–3], as well as preparation of specific antibodies against the receptor in its native and in its denatured forms [4–6]. Anti-AChR antibodies have also been used to immunoprecipitate all 4 nascent chains of the newly synthesized AChR, translated by *Torpedo* mRNA in vitro [7,8] and in ovo, in microinjected *Xenopus* oocytes [9]. The immunoprecipitation of newly synthesized AChR subunits with anti-AChR antibodies of various specificities provides a means to determine which of the receptor antigenic sites already exists in the nascent chains. Here, we used antibodies raised against the native and denatured AChR and have demonstrated that newly synthesized AChR subunits probably do not possess the final spatial conformation of the 'adult' receptor, but rather appear to be closer in structure to the denatured receptor.

Differentiation of electroplates within the embryonic electric organ is accompanied by a steep increase in cholinergic constituents such as AChR and acetylcholinesterase (AChE) [10]. Moreover, messenger RNA from the embryonic electric organ of *Torpedo ocellata* induces the production of catalytically active AChE in microinjected *Xenopus* oocytes [11]. Following their maturation, the electroplates lose most of their ribosomes [12] and their capacity for protein synthesis is hence greatly reduced.

Several mechanisms can explain the differentiation-induced accumulation of AChR in the electric organ. The elevation in AChR level might be preceded and/or accompanied by a selective increase in its specific mRNA species (AChR mRNA). Alternatively, AChR production could be a transient phenomenon, characteristic of electroplate maturation stage. In both cases, the regulation of AChR expression might involve variation in the rate of proteolytic degradation of the receptor itself.

In order to distinguish between these possibilities, we determined the level of AChR mRNA and compared it to the concentration of the receptor protein at 3 stages during electroplate differentiation: the electroblast stage (I); the stage when the cells acquire a columnar shape (II); and when morphological appearance of electroplates is displayed (III). Our findings indicate that the maturation stage of *Torpedo ocellata* electroplates is accompanied by a burst in AChR synthesis, and that following this stage, the mRNA species directing the synthesis of AChR represents a constant fraction out of total poly(A)-containing mRNA.

2. Materials and methods

2.1. *Preparation of samples*

Torpedo ocellata specimens were caught off the coast of Tel Aviv. Live gravid females were anesthetized by cooling on ice. Embryos, received from Dr D. Michaelson of Tel Aviv University, were excised, measured and pooled into 3 size groups representing the 3 developmental stages mentioned above (I, embryos 3.5–4.5 cm; II, embryos 5.5–6.0 cm; III, embryos 8.0 cm). These were stored at −70°C until used.

2.2. *Measurement of AChR level*

Electric organs were dissected on ice from pooled *Torpedo ocellata* embryos. Crude extracts were prepared in 30:1 (v/w) of 1% Triton X-100 (Sigma) containing 20 mM Hepes buffer (pH 7.6), 1 mM magne-

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$02.75

sium acetate and 133 mM potassium acetate. The level of AChR in electric organ extracts was assayed by measuring the amount of ^{125}I-labeled α-bungarotoxin (^{125}I-α-Bgt) which was coprecipitated with the receptor in 35% saturated ammonium sulfate solution [4].

2.3. *Protein determination*

Samples were dissolved in 1 M NaOH and protein estimated according to [13] with bovine serum albumin (Sigma) as standard.

2.4. *Cell-free protein synthesis*

mRNA was prepared as in [14]. Translation in vitro was carried out in nuclease-treated reticulocyte lysate cell-free system [15] with RNA from various stages of development of *Torpedo* electric organ. mRNA-directed incorporation of [^{35}S]methionine (730 Ci/mmol, Radiochemical Centre, Amersham) into trichloroacetic acid-insoluble polypeptides was determined as in [14].

2.5. *Immunoprecipitation of newly synthesized AChR*

Aliquots (15 μl) of reticulocyte lysate extracts were mixed with 50 μl 1% Triton X-100 solution, containing 0.01 M dithiothreitol (DTT), 0.2 M triethanolamine (pH 8.1), 5 mM L-methionine, 140 mM EDTA, 10 mM sodium phosphate buffer (pH 7.5) and 150 mM NaCl. Purified rabbit antibodies (4 μg) prepared against AChR or against denatured RCM-AChR [5] were then added and the reaction mixtures incubated for 2 h at 25°C. Equal amounts of normal rabbit immunoglobulins served as controls.

Antigen–antibody complexes were further purified by binding to solid-phase (immunobeads) goat anti-rabbit immunoglobulins. Immunoprecipitation reaction mixtures were added to samples of 0.5 mg immunobead reagent (BioRad), suspended in 200 μl 0.1% Triton X-100 containing 1 mM EDTA, 10 mM sodium phosphate buffer (pH 7.5), 150 mM NaCl, 0.5 mM sodium azide and 0.1% sodium dodecyl sulfate (SDS) and allowed to incubate in microfuge tubes for 30 min at 37°C, followed by incubation for 20 h at 4°C. Tubes were centrifuged for 1 min, supernatants were aspirated and precipitated resin samples washed 4 times with 1 ml 1% Triton X-100, containing 150 mM triethanolamine (pH 8.1), 5 mM EDTA, 0.1% SDS and 7.5% sucrose. Bound ^{35}S-labeled proteins were then dissociated from the washed beads by incubation for 1 h at 37°C in 30 μl of 0.625 M Tris–HCl buffer (pH 6.8) containing 2.3% SDS, 10% glycerol and 5% β-mercaptoethanol. Incubation was terminated by 1 min centrifugation and the supernatants were directly applied to 7.5–15% gradient SDS–polyacrylamide gels. Gel autoradiograms were densitometrically scanned in a Gilford 2400S spectrophotometer at 560 nm wavelength [14] and scanned peaks integrated by weight measurement.

3. Results

3.1. *Poly(A)-containing RNA from embryonic Torpedo electric organ directs the in vitro synthesis of immunoprecipitable AChR*

Translation in vitro of poly(A)-containing RNA from the electric organ of stage II embryos induced the incorporation of [^{35}S]methionine into polypeptides of heterogeneous M_r-values (fig.1(5)). Specific precipitation of AChR subunits produced in vitro was done with purified rabbit anti-AChR antibodies, and the antigen–antibody complexes were further purified by binding to solid-phase goat anti-rabbit immunoglobulins.

Polypeptide products comigrating with AChR subunits immunoprecipitated with anti-AChR antibodies (fig.1(1,2)) in agreement with [9]. Normal rabbit immunoglobulins failed to precipitate such polypeptides (fig.1(3)) and no reaction was observed when the lysate samples were incubated without anti-AChR antibodies (fig.1(4)). Rabbit antibodies elicited against a denatured AChR preparation, obtained by reduction and carboxymethylation of *Torpedo californica* AChR in 6 M guanidine–HCl (RCM-AChR, [8]) immunoprecipitated all 4 different AChR subunits (fig.1(1)). However, antibodies elicited against intact native *Torpedo californica* AChR immunoprecipitated mainly the α-subunit of the receptor (fig.1(2)). A split peak comigrating with the α-subunit of AChR was obtained with both antibodies (fig.1(1,2)) with a shoulder migrating faster than the α-subunit itself. This pattern, frequently observed following purification of AChR (D. B., S. F., unpublished) may be due to partial proteolysis of the α-subunit. In addition, immunoprecipitable high M_r products remained at the top of the gel (fig.1(1,2)). These are probably aggregates of AChR subunits, which formed complexes with the specific antibodies. The anti-denatured AChR antibodies also precipitated some low M_r polypeptides (fig.1(1)).

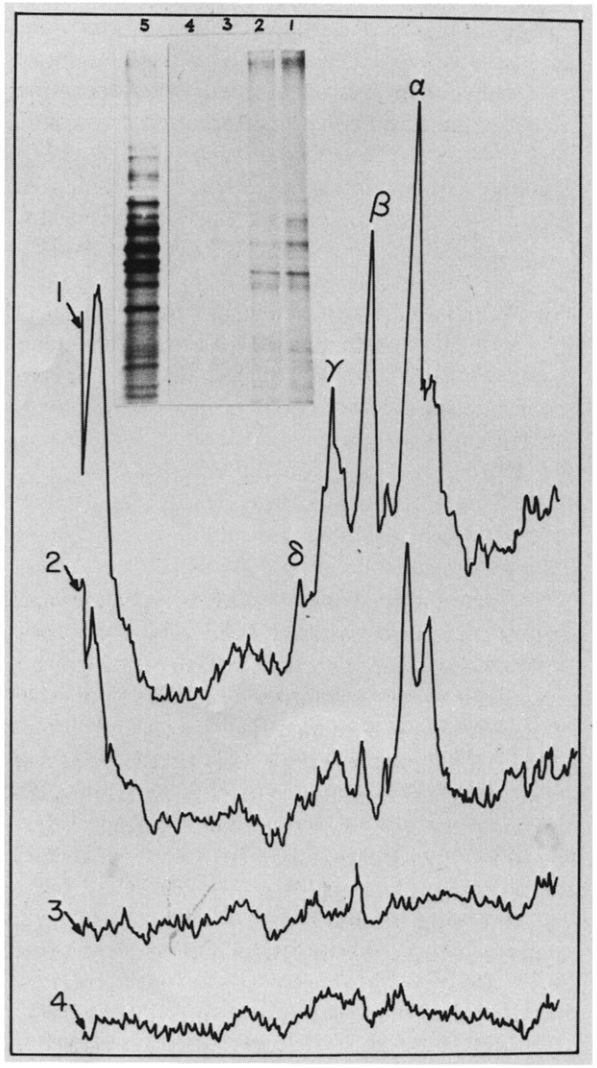

Fig.1. Gel electrophoresis and densitometric scanning of the in vitro synthesized AChR as purified by immunoprecipitation. Translation in vitro was carried out with mRNA from electric organs of stage II embryos. Each immunoprecipitation mixture contained 3.7×10^5 cpm, produced by 1.5 μg poly(A)-containing mRNA. Immunoprecipitation and gel conditions were as in section 2. Exposure was for 14 and 3 days for lanes 1–4 and 5, respectively. Lanes 1–4 represent translation products immunoprecipitated by anti-RCM-AChR antibodies (1), anti-AChR antibodies (2), normal rabbit immunoglobulins (3) and control buffer (4). Lane 5, total translation products. Samples of the radioiodinated isolated receptor subunits (α,β,γ,δ) were run in parallel lanes and their migration indicted on the scan. A photograph of the original gel is inserted.

3.2. *Developmental variations in the level of AChR and its translatable mRNA species*

The estimation of the content of AChR mRNA was first attempted by determining the amount of [^{35}S]-methionine incorporated into the complex obtained with specific anti-AChR antibodies. However, we found this way of measurement inaccurate since non-specific precipitation was considerable. To reduce the non-specific background, we estimated the percentage of total translation products which was immunoprecipitated with anti-AChR antibodies and migrated as AChR. Thus, the fraction of translatable AChR mRNA out of total mRNA was determined by integration of the densitometrically scanned peaks of immunoprecipitable radioactivity (within M_r 30 000–70 000) as compared with the integrated scan of an equally exposed gel containing the total translation products with a measured amount of radioactivity. This minimal estimation is based on the assumptions that all mRNA species within the known mRNA amounts analyzed are translated with similar efficiency and that the AChR subunits produced are quantitatively precipitated and reliably detected. It was found that the fraction which AChR mRNA represents out of total translatable mRNA increases by ⩾10-fold during the transfer from stage I embryos, with electroblast morphology, to stage II embryos, in which the elec-

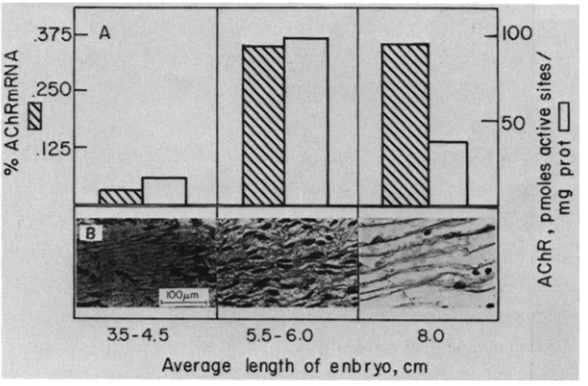

Fig.2. Modulation of AChR expression during electric organ development. (A) Messenger RNA from electric organ of stage I, II and III embryos was translated in vitro and translation products immunoprecipitated with anti-RCM-AChR antibodies. The percent of AChR mRNA was calculated by integration of scanned peaks and the concentration of AChR was determined by binding of ^{125}I-α-Bgt (details in section 2). (B) Transverse sections, 4 μm thick, of electric organs of representative specimens from each group were stained with hematoxilin–eosin and photomicrographed.

tric organ cells acquire a columnar shape, and remains as high in stage III embryos, in which electroplate morphology is displayed (fig.2A,B). The fraction of AChR mRNA in the electric organ of *Torpedo ocellata* embryos reaches a maximal level of 0.4%.

The level of the receptor protein also increases with electric organ development, from ~15 pmol active sites/mg protein in stage I up to 100 pmol/mg protein in stage II electric organ, in agreement with [9]. However, at stage III [AChR] decreases to ~40 pmol/mg protein, in contradistinction with the percentage of AChR mRNA which remains similar at stages II and III (fig.2A).

3.3. *AChR expression is selectively modulated during electric organ development*

The content of AChR mRNA (ng/g tissue) was derived from the yield of unfractionated poly(A)-containing RNA and the fraction which AChR mRNA represents out of total translatable mRNA in each of the developmental stages examined. The content of AChR mRNA appears to be maximal in the electric organ of stage II embryos, and much higher than earlier or later in development (fig.3B). The peak in AChR mRNA content is specific to this mRNA species, since the content of unfractionated poly(A)-containing RNA remains apparently unchanged in stages I and II, and only in stage III embryos it decreases by ~10-fold (fig.3A). The content of the receptor protein in pmol active sites/g tissue increases at stage II and remains apparently similar following this stage (fig.3D). This is also a pattern different from that observed with total protein content, which increases only at stage III (fig.3C). The expression of AChR is therefore modulated differently than that of the bulk of major constituents of the electric organ, both at the level of mRNA and protein.

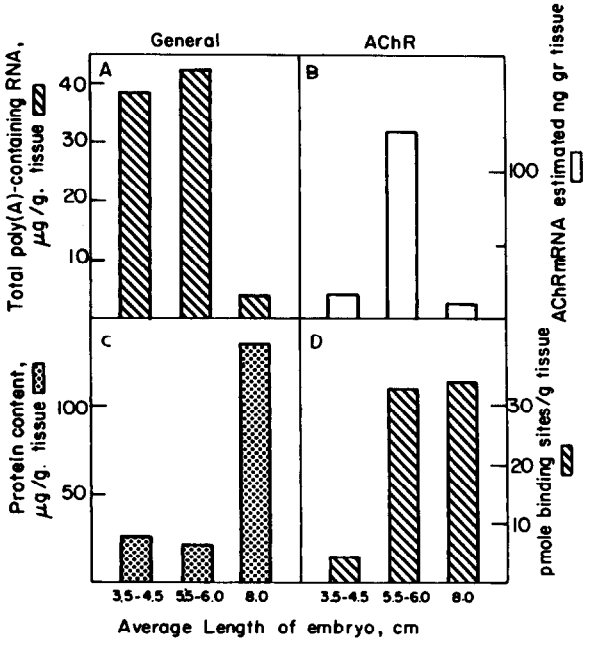

Fig.3. Accumulation of acetylcholine receptor parallels and exceeds the increase in AChR mRNA content. Poly(A)-containing RNA yields (A) were obtained from 15, 7 and 11 pooled embryos for stages I, II and III, respectively. The content of AChR mRNA (ng/g tissue) (B), was then calculated, based on the general yields of poly(A)-containing RNA presented in (A) and the fractions of AChR mRNA presented in fig.2. Protein content (C) was determined according to [13] and AChR content (D) was determined by binding of ^{125}I-α-Bgt. See text for details.

4. Discussion

Immunoprecipitation of AChR polypeptide chains, synthesized in vitro by mRNA from the electric organ of *Torpedo ocellata* embryos at various developmental stages, allows one to follow the modulation of AChR expression in the developing electric organ. We found that AChR production is precisely timed, increasing at stage II, at the onset of synaptogenesis, similarly to the appearance of characteristic morphological and biochemical properties in the maturing electroplates at this stage [10,12,16]. Messenger RNA prepared from this stage embryos was also shown to induce the synthesis of catalytically active AChE in microinjected *Xenopus* oocytes [11].

Increase in the concentration of mRNA species required for the functioning of highly specialized cells has been recently followed in pure cell populations, such as differentiating erythroleukemic cells [17] and skeletal muscle cell cultures [18,19]. The increase in AChR mRNA level, observed by immunoprecipitation of reticulocyte lysate products, is therefore compatible with these phenomena. The transient peak in AChR biosynthesis may also be related to the innervation process, as it has been reported that AChR production is regulated at the mRNA level by synaptic transmission in vertebrate skeletal muscle [20] and in *Electrophorus electroplax* [21].

Newly synthesized AChR subunits react with antibodies raised against AChR in its denatured, random-coiled structure, better than they react with antibodies

raised against the receptor in its intact, native form. This difference may reflect quantitative and/or qualitative variations between the 2 antibodies used. One possible interpretation is that the nascent AChR chains obtained by in vitro translation have not yet acquired their final 3-dimensional conformation. The better reactivity of the in vitro produced *Torpedo ocellata* AChR with antibodies to the denatured *Torpedo californica* receptor, may also be explained by a higher cross-reactivity between the denatured as compared with the native forms of AChR in these 2 subspecies.

The availability of antibodies directed against specific antigenic sites in AChR and its isolated subunits, together with the application of the mRNA translation in vitro and in microinjected *Xenopus* oocytes, may allow the detailed comparative analysis of the biogenesis of the individual receptor subunits and their final assembly to yield a physiologically active entity.

Acknowledgements

We are grateful to Dr D. Michaelson for *Torpedo ocellata* embryos, to Professors I. Silman and U. Z. Littauer for helpful discussions and to Ms R. Zisling for excellent technical assistance. This research was supported by grants from the Muscular Dystrophy Association of America and the Los Angeles Chapter of the Myasthenia Gravis Foundation (to S. F.) and from the Israeli Commission for Basic Research (to H. S.). H. S. is a recipient of a Charles Revson Career Development Chair.

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