EVIDENCE FOR DEGRADATION OF THE ACETYLCHOLINE (NICOTINIC) RECEPTOR IN SKELETAL MUSCLE DURING THE DEVELOPMENT OF THE CHICK EMBRYO

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1. Introduction

As a first characteristic event in the development of skeletal muscle fibers myoblasts fuse into myotubes and the synthesis of acetylcholine receptors increases markedly. The newly synthesised receptor molecules become incorporated in the membrane of the developing fiber ([1,2], for review see [3,4]). But upon arrival of the exploratory nerve fibers some of them accumulate at discrete areas which underly some of the motor axons; in the adult innervated muscle fiber these 'junctional' receptors are almost the only ones to persist [3,4].

In order to investigate the mechanism of such 'localization' of the acetylcholine receptor protein in the membrane of the developing myotube, the pharmacological and biochemical properties of the extra- and subsynaptic receptors have been investigated in detail (for review see [3–5]). In particular, among other differences it has been reported that the isoelectric point of the subsynaptic receptor is more acidic than that of the extrasynaptic receptor [5,6] but that an interconversion between these two forms takes place in vitro [6]. Sodium fluoride, a known inhibitor of protein phosphatases, influences the interconversion in vitro suggesting that phosphorylation–dephosphorylation reactions [6,7] are involved in this process. Direct evidence for a phosphorylation of the acetylcholine receptor from fish electric-organ has been recently obtained in this laboratory [8] and in another one [9]. The hypothesis was thus proposed that the receptor protein becomes accumulated and stabilized in the subsynaptic membrane as a consequence of a 'stabilization reaction', for instance a covalent reaction [4], which would make it altogether immobile and resistant to degradation.

Evidence from electrophysiology and autoradiography indicates that during endplate formation functional receptor molecules disappear from extrasynaptic areas [3–5]. Also a decrease in the total number of receptor sites has been observed both in differenciating muscle cultures in vitro [10] and during reinnervation [11] or electrical stimulation [12] of denervated adult muscle. In this letter, it is shown that in ovo, during the establishment of the neuromuscular junction in chick embryo breast muscle, a major part of the acetylcholine receptor molecules synthesized during myogenesis indeed becomes degraded. The significance of this degradation process is discussed.

2. Materials and methods

Chick embryos were obtained by incubation of fertilized eggs of a local strain at 38°C. ^{125}I-Labelled α-bungarotoxin (initial spec. act. 250–300 Ci/mmol) was prepared according to Vogel et al. [2].

For biochemical studies breast muscles (*M. pectoralis major*) were excised from 8–20-day-old embryos, immediately frozen and stored at −80°C. With 8–12-day-old embryos, assays were routinely made on pools of 2–8 muscles. Some analyses were also performed on pools of *Anterior latissimus dorsi* (ALD) and *Posterior latissimus dorsi* (PLD) muscles. The tissue was homogenized in 10 mM Tris/Cl pH 7.4 containing 1 mM EDTA and 0.1 mM freshly added phenylmethylsulfonylfluoride using a PT-10-OD

Polytron Homogenizer (Lucern, Switzerland). Then 20% (v/v) Triton X-100 was added to a final concentration of 2% (v/v), and the homogenates were left at 4°C for 60–240 min, the longer extraction period being used for muscles from 14–20-day-old embryos.

Acetylcholinesterase was assayed with acetylthiocholine as substrate [13] in the presence of 0.1 mM *N,N'*-diisopropylphosphoryldiamide fluoride [14] after 10 min preincubation of the homogenates with the same concentration of inhibitor; 25–30% of the total acetylcholinesterase activity were inhibited under these conditions throughout embryonic development. Choline acetyltransferase was assayed by the method of Fonnum [15]. Proteins were determined after precipitation with 10 vol. cold (−20°C) acetone and dis-solution of the resulting precipitates in 1 N NaOH by the method of Lowry et al. [16], using bovine serum albumin as standard. To quantitate levels of acetylcholine receptor, aliquots of the homogenates were centrifuged for 4 min at 8000 × *g*. From the resulting Triton X-100 extracts, 5–100 μl were incubated with a final concentration of 4 nM ^{125}I-labelled α-bungarotoxin in total vol. 0.3 ml. After overnight incubation at room temperature, the amount of receptor-bound toxin was determined by the DEAE-filter method [17]. With the Triton extracts, unspecific toxin binding in the presence of 10 mM decamethonium [18] amounted to less than 10% total binding at all stages of differentitation, thus revealing ^{125}I-labelled α-bungarotoxin as a specific probe for the cholinergic receptor site. Throughout muscle development, 85–100% of the binding sites present in the homogenates were recovered in the Triton X-100 extracts.

For the estimation of extrasynaptic acetylcholine receptor sites by optical autoradiography, PLD muscles were used. The freshly dissected muscles were pinned at their original lengths on a silicone support and incubated overnight at 4°C in 3×10^{-8} M ^{3}H-labelled α-toxin [19] from *Naja nigricollis* (15 Ci/mmol). After several washes with Tyrode solution, the muscles were fixed and processed to obtain smears of embryonic muscle fiber fragments on glass-slides (Bourgeois, J. P., Ryter, A. and Changeux, J. P., in preparation). Smears of fiber fragments were layered with K_5 Ilford emulsion and exposed for one month. All the PLD muscle fibers samples at different stages of development were labelled with the toxin and processed for autoradiography simultaneously (and thus rigorously under the same conditions). After development, the number of silver grains/μm myotube length were counted outside the high density spots. The diameter of the PLD muscle fibers did not vary significantly during embryonic development, and was not taken into account for the estimation of the grain density which is always expressed/unit length of myotube.

3. Results and discussion

The evolution during embryonic development of the total contents in acetylcholinesterase, nicotinic receptor sites and choline acetyltransferase of a single muscle of the chick, the breast muscle are compared, in fig.1. In agreement with the findings of Giacobini et al. [18] and others [20] the total activity of acetylcholinesterase and the total number of α-bungarotoxin sites increase continuously and in a rather parallel manner from the 8–14th day of incubation. However, at variance with these authors a significant difference appears in the late evolution of these two proteins. Starting on the 14th day the total number of nicotinic receptor sites (and not only their specific activity) decreases in a dramatic manner while the total activity of acetylcholinesterase/muscle stays more or less constant. The apparent half-life of receptor decay, from the 14–18th day, is approximately 3 days, and the number of α-bungarotoxin sites which remains on the 20th day of development corresponds to about 20% of the maximal number of sites found on the 14th day. The difference with the results of Giacobini et al. [18] presumably is due to the fact that these authors made their assays on a complex group of muscles rather than on a single one and that the mode of their innervation was not homogeneous and the evolution of the muscle fibers studied not synchronous.

On the other hand, and in agreement with the observations of Giacobini et al. [18], the total activity of choline acetyltransferase increases continuously up to the 20th day of incubation. In the last part of the embryonic development this rate of increase is even faster than that of protein accumulation. No evident correlation therefore seems to exist

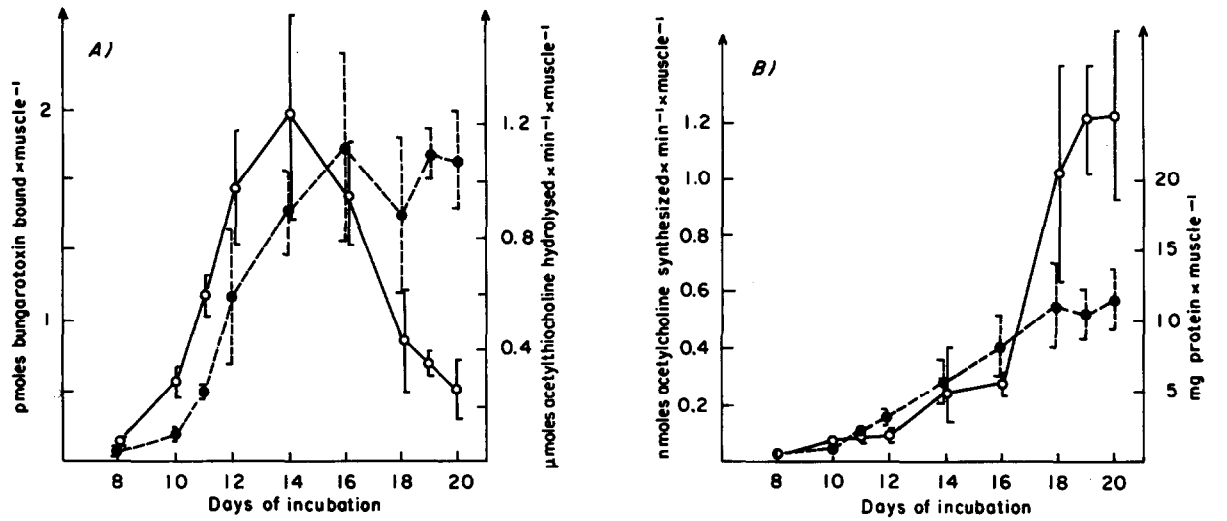

Fig.1. Total levels of acetylcholinesterase, acetylcholine receptor and choline acetyltransferase during breast muscle development. The various cholinergic proteins and total protein were determined as detailed under Materials and methods. Figure 1A: (o——o) Total level of ^{125}I-labelled α-bungarotoxin binding sites/breast muscle. (•- - -•) Total activity of acetylcholinesterase/breast muscle. Figure 1B: (o——o) Total activity of choline acetyltransferase/breast muscle. (•- - -•) Protein content/breast muscle. The bars indicate ± 1 SD.

in the evolution of the pre- and postsynaptic proteins studied.

By contrast the synthesis of the two postsynaptic proteins acetylcholinesterase and nicotinic receptor appears to be coordinated during the first part of development. When the amount of acetylcholinesterase is calculated from the currently available data on the enzyme of *Electrophorus electricus*, assuming approx. mol. wt 100 000/active site and spec. act. 6 mmol acetylthiocholine/min/mg protein [21], approx. 1.5 pmol esteratic sites are found at the 20th day of incubation, a value which is in the same range as that found for the total number of α-toxin binding sites present at this time*.

Since, however, the behaviour of esterase and receptor markedly diverges after the 14th day, the synthesis and/or stability of these two proteins are presumably submitted to different regulatory mechanisms in the late part of development. A similar conclusion was reached independently from denervation experiments of the adult muscle where after section of the motor nerve the total content in acetylcholine receptor sites was found to increase markedly while that of acetylcholinesterase tended to decrease [23,24].

In order to investigate further the evolution of the acetylcholine receptor site in the developing muscle, fragmented myotubes or myofibers were isolated from the *Posterior latissimus dorsi* (PLD) muscle after labelling with the tritiated α-toxin from *Naja nigricollis* and studied by quantitative optical autoradiography. Throughout development, the silver grains are distributed evenly over the surface of the fragmented muscle fibers with the only exception of some dense accumulations of receptor sites which most likely correspond to postsynaptic 'localizations' of acetylcholine receptor molecules under the nerve terminal. Outside these regions, i.e. in the extrasynaptic areas, the density of silver grains increases about threefold from the 4th day, reaches a peak between the 10th and 11th day, but then decreases down to virtually the background level after the 18th day of incubation (fig.2). The disappearance of extrasynaptic receptor sites after the 11th day proceeds with an apparent half-life of about 3 days which

*In the adult motor endplate, autoradiographic studies give a 1:1 stoichiometric distribution of the α-toxin binding sites and esterase catalytic sites [22]

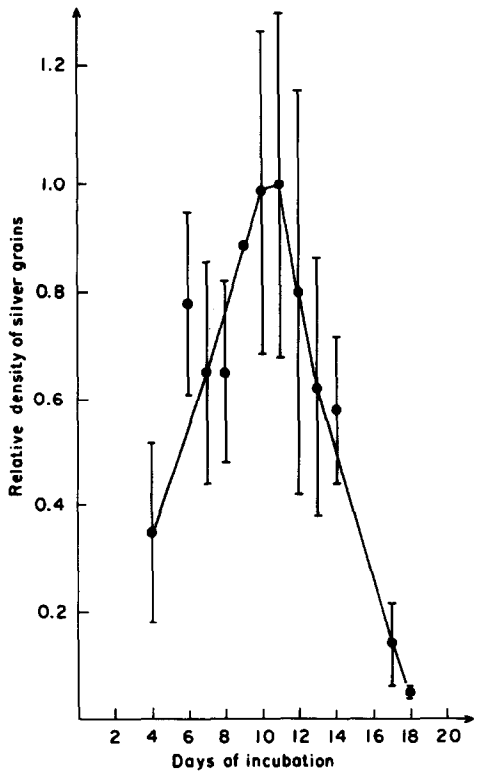

Fig.2. Relative density of extrasynaptic silver grains on PLD muscle fibers labelled with ^{3}H-labelled α-toxin from *Naja nigricollis* during chick embryo development. Extrasynaptic receptor sites were determined on PLD muscle fibers fragments by optical autoradiography as detailed under Materials and methods. With 4–8-day-old embryos, the determinations were performed on muscle pieces excised from the presumptive PLD region. All values were normalized to the maximum value found at the 11th day. The background represented only 4% of the maximum value and was constant in all the autoradiograms. The bars indicate ± 1 SD.

corresponds to that found when the total content in receptor sites was followed (fig.1A).

The similarity of the rates of decay of extrasynaptic and total receptor sites strongly suggests that the decrease in total receptor number after the 14th day is primarily due to the degradation of extrasynaptic receptor molecules. Accordingly, the synthesis of acetylcholine receptor should stop in the muscle cell around the 14th day at the time where typical and most likely functional motor endplates staining positively for acetylcholinesterase become apparent [25]. This result is consistent with the in vitro observation that the electrical stimulation of either denervated adult rat diaphragm [31] or non-innervated chick embryonic myotubes in tissue culture [27] shuts off the synthesis of acetylcholine receptor.

A major discrepancy between our results and the in vitro data, however, remains the difference between the relatively long apparent half-life for receptor disappearance in ovo as compared to the protein half-life determined for the degradation of the extrasynaptic acetylcholine receptor in different systems. Values of 16–18 h for intrinsic half-lives have been found with either denervated adult muscle [28] or developing non-innervated myotubes in vitro after labeling of the acetylcholine receptor molecule with α-bungarotoxin [26,29,30] or incorporation of labelled amino acids [26,30].

Several interpretations may be proposed for this difference:

(i) The slower apparent half-life observed in vivo may be due to the progressive decrease in the rate of receptor synthesis, some new receptor molecules being still incorporated in the extrasynaptic areas even after the 14th day. The total number of receptor molecules determined at any given time both in extracts or by autoradiography in situ therefore decreases more slowly than the intrinsic degradation of the receptor molecule.

(ii) The evolution of the myotubes in the whole muscle may be asynchronous.

(iii) Muscle activity may affect the intrinsic rate of receptor degradation. Indeed the half-life of 16–18 h was determined in vitro mostly with 'silent' myotubes; on the other hand, electrical stimulation of denervated rat diaphragm in vitro significantly slows down the rate of degradation of the extrasynaptic receptor [31]. As in ovo chick embryo moves as a consequence of a neurogenic activation of its skeletal muscles as early as the 4th day of embryonic development [32], the decay of acetylcholine receptor after the 14th day should take place under conditions where the skeletal muscles are active and therefore may be slower than that found in vitro.

Actual measurements of the intrinsic rate of receptor degradation in ovo, for instance after labelling with ^{125}I-labelled α-bungarotoxin, should lead to a distinction between these various hypotheses.

When one closely compares the evolution of the total number of receptor sites per muscle with the density of sites in extrasynaptic areas it becomes apparent that even if the two curves (figs 1 and 2) have a rather similar shape they differ in two important respects.

(i) It is evident that at the time where the density of extrasynaptic sites has returned to background level, i.e., after the 18th day of incubation, a significant fraction (about 20%) of the total population of sites present on the 11th day persists. This fraction most likely consists of subsynaptic sites concentrated at functional motor endplates.

(ii) The two curves also differ by the time at which they reach their maximum: the peak takes place at the 11th day of incubation in the case of extrasynaptic site density but 3 days later in the case of the total number of sites. Such difference presumably does not result from the fact that the experiments compared are done on different muscles since preliminary experiments also reveal a peak of total receptor number in the PLD muscle at the 13–14th day. Since the subsynaptic receptors are counted in the estimate of the total number of sites but not in the grain density measurements, the time-lag between the two curves most likely reflects the synthesis of presumptive subsynaptic receptor molecules and their localized accumulation under the developing nerve terminal. In agreement with this interpretation, optical autoradiography has shown the number of dense accumulations of acetylcholine receptor/PLD muscle to increase dramatically from the 11th day of development even if single accumulations on isolated embryonic myotubes can be demonstrated as early as the 4th day (Bourgeois, Ryter and Changeux, in preparation).

Interestingly, and relevant to this point, preliminary investigations indicate that the evolution of the total number of sites is rather different in a slow muscle the *Anterior latissimus dorsi* (ALD) which being multiply innervated possesses the highest yet known number of endplates/muscle fiber [33]. Table I shows that in contrast with what happens in PLD a typical focally innervated fast muscle, the total number of α -toxin sites in ALD does not fall after the 14th day. It continues to increase up to the 20th day of development despite the fact that, as in PLD, receptors disappear from extrasynaptic areas (Bourgeois, Ryter and Changeux, unpublished). This evolution is not unexpected since the formation of multiple synapses requires a much higher accumulation of receptor molecules than in a focally innervated fiber of approximately the same size.

In any case, the currently available data on the evolution of the pool of acetylcholine receptor in developing skeletal muscle in ovo are consistent with the idea that the localization of the receptor protein coincides with

(i) A shut-off of the synthesis of a labile species of receptor which becomes degraded in extrasynaptic areas

(ii) a stabilization and immobilization of the receptor protein under the nerve terminals.

In other words as recently discussed [4] the subsynaptic 'localization' of the acetylcholine receptor may be viewed as a management of a limited pool of receptor molecules via a lateral diffusion in the cytoplasmic membrane and a stabilisation reaction under the nerve terminals.

Table 1

Total acetylcholine receptor levels in a focally innervated fast muscle, PLD and in a multiply innervated slow muscle, ALD, at the 14th and 20th day of chick embryo development

	fmol α-Bungarotoxin bound/muscle	
	14th Day	20th Day
PLD	117.0 ± 5.6	55.8 ± 9.0
ALD	90.7 ± 5.4	134.0 ± 4.7

Acetylcholine receptor sites were determined in the PLD and ALD muscle samples as detailed under Materials and methods.

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