RAPID MODULATION OF ACETYLCHOLINE RECEPTOR SYNTHESIS

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Received 7 October 1981

1. Introduction

Electrical stimulation of adult denervated muscle leads to inhibition of acetylcholine receptor synthesis [1,2]. How information about the activity of the sarcolemma is conveyed to the protein-synthesizing apparatus is largely unknown. Some progress has been made with the use of myogenic cells in culture, which resemble denervated muscle fibers and respond to specific drug treatments with characteristic changes in receptor synthesis rate. For instance, pharmacological blockage [3], or activation [4], of sodium channels, result in acceleration or slow-down, respectively, of acetylcholine receptor synthesis. Dantrolene, which blocks release of Ca^{2+} from the sarcoplasmic reticulum (SR), stimulates receptor synthesis [5], while ryanodine, when used at the low concentration at which it is thought to deplete calcium from the SR, has the opposite effect (L. P., J. S., unpublished). The notion that calcium may play a messenger role in acetylcholine receptor regulation is supported by several other experiments [6,7].

Subsequent steps in the signaling pathway are not known. We have studied the effect of various drugs on receptor synthesis in chick myotubes at high temporal resolution, to determine if receptor synthesis is shut off at the transcriptional or post-transcriptional level. The findings suggest that pharmacological agents capable of turning off receptor synthesis act on the protein synthesizing apparatus rather than on the genome.

2. Experimental

2.1. *Cell culture*

Muscle cells for primary cultures were obtained from the legs of 12-day-old SPAFAS (Norwich CT) chick embryos by mechanical disruption as in [8]. Routinely, cells were plated at 5×10^5/35 mm gelatin-coated, tissue culture dish (Falcon). The cells were cultured in 88% Dulbecco's modified Eagle's medium (DME; GIBCO, Grand Island NY), 10% horse serum (GIBCO), and 2% embryo extract at 37°C, in a 95% air–5% CO_2 water-saturated atmosphere. Cells were treated with 10^{-5} M cytosine arabinoside from 48–96 h in culture to suppress proliferation of fibroblasts.

2.2. *Acetylcholine receptor synthesis rate assay*

Pre-existing cell surface receptors were blocked by incubation for 40 min in 10^{-7} M α-bungarotoxin (αButx) added to the medium. Cells were then freed of unbound αButx by 4 1-min washes with Hank's balanced salt solution containing 1 mg BSA/ml. The cultures were divided into two groups. Those to be assayed for receptor synthesis rate were fed with DME and incubated for another hour at 37°C. Drugs were included in the media for the desired period (before, during, and after treatment with αButx) such that the last hour of drug exposure coincided with the DME incubation. Afterwards, the cultures were washed twice for 1 min with 280 mM mannitol, 10 mM Hepes (pH 7.4) and incubated with 10^{-9} M ^{125}I-αButx for 30 min. The low ionic strength mannitol solution was based to reduce the time required for saturation of toxin-binding sites. Incubations were terminated by a 1-min wash with phosphate-buffered saline (PBS) containing 1 mg BSA/ml, followed by 3 1-min washes with PBS. Cultures were then solubilized in 0.1 N NaOH, 0.1% SDS and counted in a γ-counter. Cultures to be used as controls for background binding activity were incubated with 10^{-9} M ^{125}I-αButx in 280 mM mannitol, 10 mM Hepes (pH 7.4) immediately after washing out αButx. This binding activity (~60% of total) represents receptor

Published by Elsevier/North-Holland Biomedical Press
00145793/81/0000–0000/$02.75

not blocked by αButx during preincubation (due to protection by cations in the high-ionic strength medium); receptor inserted into the membrane during incubation with ^{125}I-αButx; and non-specific binding as defined by binding of ^{125}I-αButx in the presence of 10^{-5} M decamethonium. Non-specific binding was not routinely measured, as it was found to be identical for receptor appearance rate and control cultures. The difference between experimental and control values is considered as the amount of receptor appearing on the surface membrane per hour and culture.

Saturation studies showed that the binding reaction went to 90% completion in <30 min incubation with 10^{-9} M mono-^{125}I-αButx. In some experiments di-^{125}I-αButx was used. In this case incubation was for 45 min.

2.3. *Reagents*

Mono-^{125}I-αButx and di-^{125}I-αButx at spec. act. ~2.5 × 10^7 and 4.0 × 10^7 GBq/mol, respectively, were prepared as in [9]. Ryanodine was purchased from Penick (Lyndhurst NJ). Puromycin, actinomycin D, tetrodotoxin and veratridine were obtained from Sigma.

3. Results and discussion

Use of a receptor appearance rate assay of high temporal resolution minimizes distortion caused by receptor turnover (~47%/day; 2.6%/hour in our cultures). Fig.1 shows receptor appearance rate as a function of culture age. Receptor production peaks at the time of myoblast fusion, and then gradually declines to ~5 fmol/h and culture. Little change in synthesis rate is seen after day 5, the age used for these pharmacological experiments.

The pathway linking membrane activity and acetylcholine receptor regulation differs from other signaling pathways in muscle (e.g., excitation–contraction coupling (ECC) or regulation of glucose mobilization) in that it affects macromolecular synthesis. To obtain more information on its distal limb we have compared drugs that specifically affect acetylcholine receptor synthesis with general RNA and protein synthesis inhibitors. Reduction of receptor synthesis rate was observed as early as 1 h after addition of the protein synthesis blocker puromycin (fig.2). A reduction to half the initial value was seen after ~3 h, the average time required for newly made membrane proteins

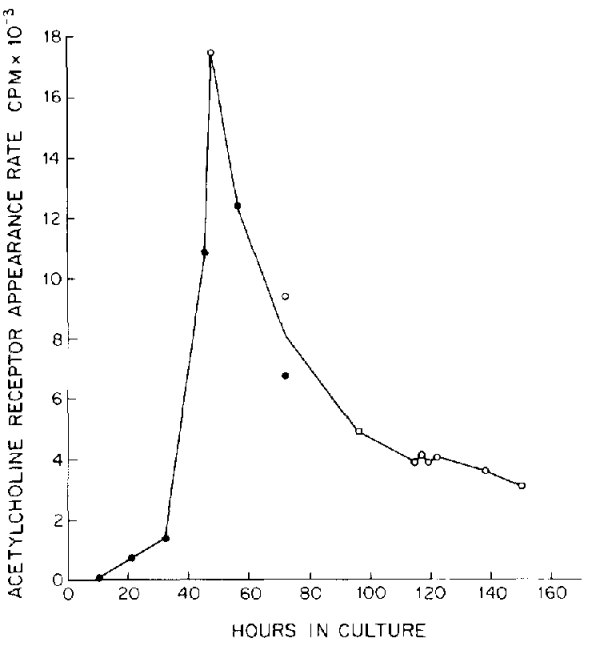

Fig.1. Acetylcholine receptor appearance rate as a function of culture age. Myogenic cells were prepared and cultured, and hourly receptor appearance rates measured at the indicated times after plating, as in section 2. Different symbols refer to separate experiments.

in myogenic cells to traverse the rough endoplasmic reticulum (RER) and Golgi apparatus and emerge on the cell surface [10]. After ~6 h cells began to deteriorate rapidly and by 8 h receptor proliferation ceased. The gradual course of the puromycin effect probably arises from a range of transport times.

The specific inhibitory effect of electrical stimulation on receptor synthesis can be mimicked with compounds that activate sodium channels such as veratridine [4], or with ryanodine, a drug that shifts Ca^{2+} from the SR to the cytosol [11]. As seen in fig.3, both drugs bring about a rapid inhibition of receptor proliferation. The latency periods are somewhat longer which may be related to their action on the protein synthesis machinery being less direct than that of puromycin. With ryanodine, a fairly rapid partial recovery is seen, and veratridine never produces more than partial inhibition. Perhaps this indicates that compensatory activities are triggered.

About 8 h after actinomycin D, at a dose sufficient to terminate RNA synthesis, is applied to the cultures, a gradual decline of the health and viability (as judged morphologically) of the myotubes, and concomitant reduction in receptor appearance rate

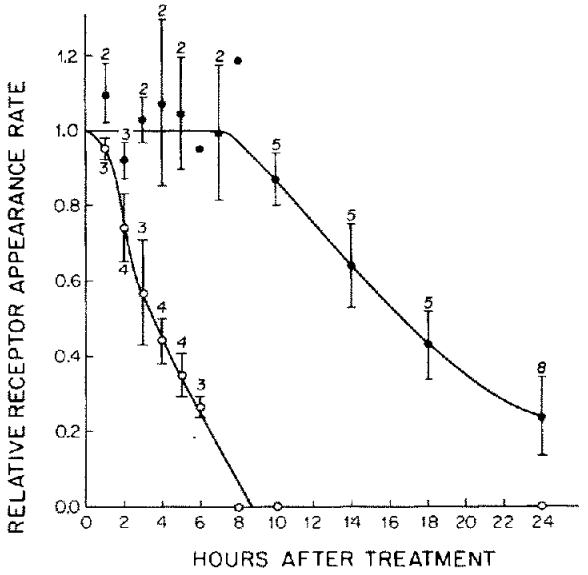

Fig.2. Effect of puromycin and actinomycin D on receptor appearance rate. Receptor appearance rates were measured, as in section 2, after exposure to 4×10^{-5} M puromycin (○) or 8×10^{-6} M actinomycin D (●) for the indicated periods. Data from several independent experiments are presented as fractions of drug-free controls ± SE. Water:ethylene glycol (1:1) was used as a solubilizer for actinomycin D. Control experiments showed that ethylene glycol has no effect on receptor appearance rate at the final concentration used (0.5% v/v).

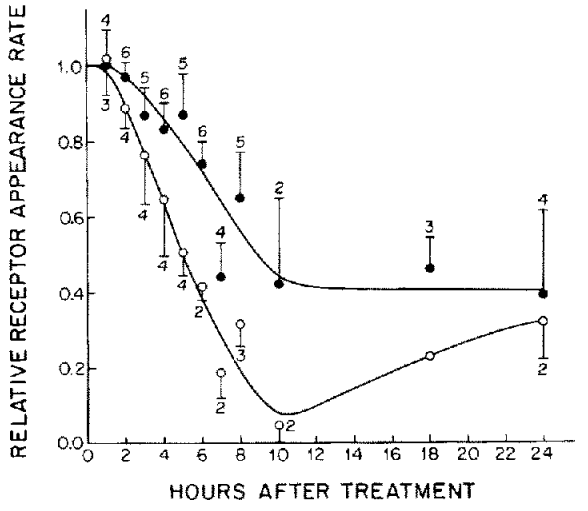

Fig.3. Effect of veratridine and ryanodine. Receptor appearance rates were measured as in section 2 after exposure to 3.0×10^{-6} M veratridine (●) and 3.0×10^{-7} M ryanodine (○) for the indicated periods. Ryanodine was made up fresh at 3×10^{-5} M in Hank's balanced salt solution, 10 mM Hepes (pH 7.4), 1 mg BSA/ml. Results are expressed as fraction of drug free controls ± SE.

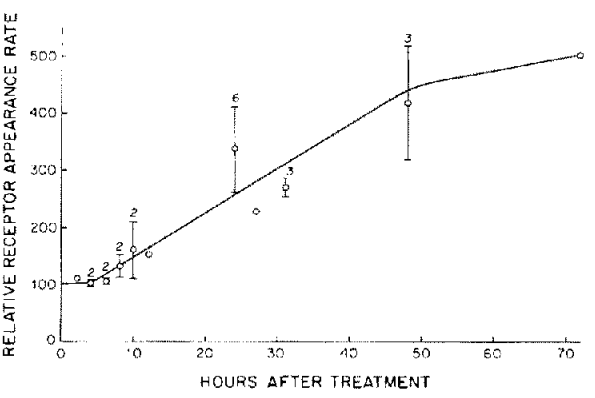

Fig.4. Effect of tetrodotoxin. Receptor appearance rates upon exposure to 1.0×10^{-6} M tetrodotoxin for the indicated periods were measured as described and results are presented as in fig.2.

are observed (fig.2). Appearance rate is reduced to 50% of control values between 8–16 h incubation, i.e., over 8 h. This represents a lower limit of the half-life of receptor-specific message.

Another estimate of this message half-life can be derived from the tetrodotoxin experiment (fig.4). Here, as above, receptor synthesis rate is assumed to reflect the level of translatable receptor message. Tetrodotoxin causes an increase in receptor synthesis rate which probably involves a stimulation of receptor message synthesis, as is suggested by the actinomycin D sensitivity of this effect [6]. Thus the drug switches myotubes from a state, characterized by a specific amount of receptor specific mRNA, to a new state in which the level of mRNA is elevated, and the half-life of receptor mRNA is revealed by the time constant with which the new steady state is being approached [12]. Both the actinomycin D and tetrodotoxin experiments indicate that receptor mRNA is fairly stable (minimal half-lives of ~10 h and 20 h, respectively).

There have been several attempts to measure the kinetics of the reduction of receptor synthesis; results have been difficult to interpret because of variability and insufficient temporal resolution of the measurements (review [13]). This work clearly shows that termination of receptor synthesis upon treatment of myotubes with activators of sodium channels and SR is faster than would be expected from cessation of receptor mRNA synthesis and is thus mediated by some post-transcriptional event.

Acknowledgements

The expert technical assistance of Mrs Anna Maria Vidal is gratefully acknowledged. This study was supported by a grant from the Muscular Dystrophy Association.

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