EFFECTS OF DRUG-INDUCED PARALYSIS AND DEPOLARISATION ON ACETYLCHOLINE RECEPTOR AND CYCLIC NUCLEOTIDE LEVELS OF CHICK MUSCLE CULTURES

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1. Introduction

Neuromuscular interactions play an important role in the development and maintenance of several myofiber properties including the regulation of its chemosensitivity. In adult innervated skeletal muscle, receptors for the neuromuscular transmitter, acetylcholine, are confined to the endplate region of the myofiber membrane. By contrast, non-innervated embryonic myotubes or denervated adult muscle fibers bear acetylcholine receptors (AChR) on their entire 'extrasynaptic' cell surface (reviewed [1,2]). Different investigators showed that the developmental localisation of AChR to the postsynaptic part of the sarcolemma involves:

(i) The aggregation of receptors under the nerve terminal [3,4];
(ii) The repression of the synthesis of extrasynaptic AChR [5–7].

Several lines of evidence suggest the latter process to be mediated by neurally-induced muscle activity:

(1) Electrical stimulation of adult denervated muscle prevents appearance of extrasynaptic AChR [8] and blocks the incorporation of radiolabelled amino acids into the receptor protein [9];
(2) Chronic paralysis of the motor endplate by cholinergic antagonists increases the synthesis of extrasynaptic AChR in adult and embryonic myofibers [6,10,11];
(3) In muscle cell cultures, the rate of AChR synthesis is decreased by electrical stimulation or depolarising agents [12,13]; under the same conditions, paralysis of the spontaneously contracting myotubes by tetrodoxin leads to an increase of newly synthesized receptor molecules.

At present, the molecular events underlying the regulation of extrasynaptic AChR by muscle activity are unknown. In experiments with chick myotubes in tissue culture, dibutyryl cGMP added to the medium was found to reduce the rate of AChR synthesis [13], whereas cAMP derivatives increased the latter [13,14]. Moreover, both cholinergic and electrical stimulation of adult muscle are known to induce transient increases in intracellular cGMP which depend on the presence of extracellular Ca^{2+} [15–17]. It, therefore, was postulated that Ca^{2+} and cGMP may constitute the intracellular messengers of the activity-mediated control of AChR [13]. In this letter, it is shown that there is no obvious correlation between the AChR and the cyclic nucleotide levels of chick muscle cell cultures under conditions of drug-induced paralysis and depolarisation.

2. Materials and methods

Muscle cell cultures were prepared from the hind limbs of 11-day-old chick embryos and maintained as in [13] with the following modifications:

(i) Dulbecco's modified Eagle's medium containing 10% horse serum (Gibco), 1% chick embryo extract, 100 U penicillin/ml and 100 μg streptomycin/ml was used for all experiments;
(ii) The culture medium was changed every second day only. The number of cell surface acetylcholine receptors was measured by the binding of ^{125}I-labelled α-bungarotoxin (NEN as detailed in [13].

For the determination of cyclic nucleotide levels, the medium was rapidly removed from the cultures,

and the dishes were covered with liquid nitrogen and stored at −70°C. After the addition of cold 5% (w/v) trichloroacetic acid, the cells were harvested and homogenized in a ground glass homogenizer. The precipitated material was removed by centrifugation, solubilised in 1 N NaOH, and its protein content measured according to [18] using bovine serum albumin as a standard. The supernatants were extracted 4 times with water-saturated ether, lyophilised and reconstituted with 50 mM sodium acetate (pH 6.2). Cyclic AMP and cyclic GMP were determined after acetylation [19] according to [20] using commercial kits of NEN. Nucleotide recovery was monitored in each sample by the addition of ~2000 cpm the respective tritiated cyclic nucleotide to the cell homogenate or the culture dish. In some experiments, the trichloroacetic acid supernatants were purified by chromatography on Dowex 50W-X8 [21] prior to the cGMP assays. All data were corrected for the amount of recovered marker added. The statistical significance of the results was checked by Student's *t*-test. Veratrin was obtained from Sigma, tetrodoxin from Boehringer (Mannheim) and sea anemone toxin II from Dr L. Béress (Kiel).

3. Results

The experiments were performed on differentiated, i.e., 7-day-old muscle cell cultures. As shown in table 1, these cultures contained spontaneously contracting myotubes whose acetylcholine receptor content could be modulated by paralysis and depolarisation. In accord with earlier findings [12,13], paralysis of the myotubes by tetrodotoxin, a blocker of the action potential sodium channel [22], increased the number of α-bungarotoxin binding sites, whereas the depolarising alkaloid veratrine, which permanently activates these same channels [23], decreased it to ~50% of that of controls. Sea anemone toxin II, a polypeptide which acts synergistically to veratrum alkaloids by preventing the closure of the activated voltage-dependent sodium ionophore [24], also reduced the acetylcholine receptor content of the cultures, suggesting that the observed losses of AChR were specifically mediated by membrane depolarisation. None of these agents significantly altered the protein content of the cultures (variation $\leq$9.5%). Thus presumably no major changes in cell number and total protein synthesis occurred during drug treatment.

In three independent experiments, the cyclic nucleotide levels of muscle cultures were determined after 1 and 2 days of exposure to tetrodotoxin or veratrin, respectively. All gave the same result: neither drug produced significant changes in the cAMP or cGMP content of the cultures. In table 2, the combined results of two experiments performed under identical conditions are presented. One of these experiments involved parallel cultures to those used for the experiment shown in table 1. Another one in which only the cyclic nucleotide levels/dish were determined also gave no indication for alterations of either nucleotide under any of the culture conditions used.

By contrast to these observations with *chronically*

Table 1
Acetylcholine receptor levels in paralysed and depolarized muscle cell cultures

Culture condition	Exposure to drug (days)	
	1	2
	α-Bungarotoxin bound (fmol/dish) (±SD)	
Control	85.1 ± 6.4	84.8 ± 5.0
+ Tetrodotoxin	99.2 ± 1.7	120.5 ± 9.0
+ Veratrin	58.5 ± 13.0	49.8 ± 2.9
+ Sea anemone toxin II	51.6 ± 5.3	60.3 ± 4.1

Myotubes were seeded at 7.5×10^5 cells/dish in a large number of 35 mm culture dishes. After 7 days in vitro, tetrodotoxin (10^{-6} M), veratrin (17 μg/ml) and sea anemone toxin II (10^{-6} M) were added to several independent dishes. One and two days later, the binding of ^{125}I-labelled α-bungarotoxin to the intact cells was determined ($n = 4$)

Table 2
Cyclic nucleotide levels in tetrodotoxin- and veratrin-treated muscle cell cultures

Culture conditions	Exposure to drug (days)			
	1	2	1	2
	(pmol cAMP/mg protein)		(pmol cGMP/mg protein)	
Control	3.66 ± 1.03	5.18 ± 1.29	0.155 ± 0.066	0.180 ± 0.075
+ Tetrodotoxin	3.93 ± 1.13	4.31 ± 1.27	0.151 ± 0.053	0.182 ± 0.017
+ Veratrin	3.52 ± 0.83	4.66 ± 1.18	0.232 ± 0.060	0.168 ± 0.058

The cells were seeded at 2–2.5 × 10^6 cells/dish in a large set of 60 mm culture dishes. After 7 days, the drugs were added to the culture medium at concentrations identical to those described in table 1. The data are pooled from 2 separate experiments which gave similar results (n = 7–8). None of the values is significantly different from the others ($p > 0.01$)

treated cultures, and in accord with similar findings [15–17,25], short-time exposure of 7-day-old cultures to veratrin (59 μg/ml), or carbachol (10^{-4} M), led to a highly significant increase in cGMP formation when followed by a modification of the [^{3}H]guanosine prelabelling technique detailed in ([26,27], H. B., unpublished). After 1 or 2 min of exposure, ^{3}H-radioactivity in cGMP was increased by 163 ± 13% with veratrin, and 179 ± 16% with carbachol, respectively. This increase, was, however, lost after 5–10 min.

4. Discussion

These results show that the continuous paralysis or depolarisation by tetrodotoxin, or veratrin, respectively, or chick muscle cells did not significantly change the intracellular cyclic nucleotide levels. Both conditions efficiently modulated AChR synthesis. These findings contrast with the previously noted effects of cyclic nucleotide derivates on AChR metabolism and question the hypothesis that Ca^{2+} and cGMP might be involved in the control of AChR by muscle activity [13]. One should, however, consider that both here and in [15–17,25] muscle cells and neurons were found to respond to depolarisation with a rapid transient increase in cGMP. It therefore cannot be ruled out that such transient alterations in cGMP levels cause repression of AChR synthesis by some kind of 'priming' mechanism. With adrenal medullary cells, temporary changes in cAMP are known to suffice for eliciting tyrosine hydroxylase induction [28]. In accord with such a model of regulation are the observations [8,29] that rather rare electrical stimulation (10 Hz for 10 s every 5.5 h) suffices to reduce the extrasynaptic acetylcholine sensitivity of denervated rat muscle, provided the stimuli are given in trains of high frequency.

Alternatively, the increase in cGMP upon muscle activation may not be related to AChR regulation. In this case, the repressive action of dibutyryl cGMP on myotube AChR [13] has to be attributed to other effects of the drug, as, for example, on Ca^{2+} permeability or membrane potential.

Cyclic GMP and its derivatives were shown to modulate neuronal excitability and cardiac contractions in [30]. Future experiments dissociating the effects of dibutyryl cGMP and depolarising drugs on AChR synthesis should distinguish between these possibilities.

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