MODULATION OF CHOLINERGIC PROTEINS AND RNA BY OUABAIN IN CHICK MUSCLE CULTURES

P. M. WEIDOFF, jr, M. G. McNAMEE+ and B. W. WILSON
Department of Avian Sciences and +*Biochemistry and Biophysics, University of California, Davis, CA 95616, USA*

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1. Introduction

Two important molecules associated with neuromuscular transmission, acetylcholine receptor (ACHR) and acetylcholinesterase (ACHE, EC 3.1.1.7) are present in chick embryo muscle cells in culture [1,2]. Experiments have shown that muscle activity, acetylcholine analogs, and membrane-active drugs can alter levels of ACHE and ACHR [3–5]. One possible explanation for these effects is that ion balance regulates growth and differentiation of embryo muscle. The experiments reported here show that ouabain (strophanthin G), a specific inhibitor of Na^+/K^+ ATPase [6] increases ACHR levels, ACHE activity and RNA synthesis in cultured muscle cells.

2. Methods

2.1. *Muscle cultures*

Primary cultures were prepared by dissociating day 11 chick embryo pectoral muscle with 0.1% trypsin and, after preplating, inoculating them onto 35 mm collagen-coated dishes at 5×10^5 cells/dish in a medium of 88% (v/v) Eagles Minimal Essential Media (MEM) with Earles salts, 10% horse serum and 2% chick embryo extract. Horse serum and embryo extract were treated in advance with 10^{-5} M and 10^{-7} M diisopropyl phosphofluoridate (DFP), respectively, to reduce cholinesterase levels by ~85% in horse serum and 99% in embryo extract. Media were changed 72 h after inoculation and every 2 days thereafter. Cultures were maintained at 37°C and pH 7.2–7.5 in an atmosphere of humidified air and regulated CO_2.

Address reprint requests and correspondence to: B. W. Wilson

2.2. *ACHR assay*

Purified α-bungarotoxin, 200 μg (a gift from Dr M. Hanley, Chemical Biodynamics Lab., UC Berkeley) was iodinated by the iodine monochloride method [1]. The monoiodo derivative was isolated by ion-exchange chromatography on CM-52 cellulose as in [7]. The specific activity of the mono-[^{125}I]iodo-α-bungarotoxin (^{125}I-α-Bgt) was 2×10^5 Ci/mol.

The number of ACHR molecules on the surface of the muscle fibers was determined using a modification of the ^{125}I-α-Bgt binding assay [8] at 37°C and 20 nM α-Bgt final conc. Non-specific binding was determined by preincubation with cold α-Bgt, 10^{-7} M, followed by ^{125}I-α-Bgt, and usually averaged 5%.

2.3. *Enzyme and protein assays*

ACHE activity was determined on homogenized and sonicated tissue by the method in [9] using acetylthiocholine iodide (ACTC) as substrate. Non-specific cholinesterase (acylcholine acylhydrolase, EC 3.1.1.8) was selectively inhibited [10] with 0.1 mM iso-OMPA (tetraisopropyl pyrophosphoramide). Creatine kinase (CK, EC 2.7.3.2) activity was determined by the method in [11]. Cell protein was determined by the method in [12] using bovine serum albumin as standard.

2.4. *RNA and protein synthesis*

[5-^{3}H]Uridine at 3 μCi/dish was added to cells for 1 h to determine RNA synthesis. Incorporation of

radioactive leucine into protein was measured by adding 10 μCi/dish of L-[4,5-^{3}H]leucine to cells in leucine-free media for 1 h. For each assay trichloroacetic acid precipitates of cell homogenates were collected on glass filters, dissolved in protosol and a toluene-based fluor and counted in a liquid scintillation counter [13]. Non-specific binding of [^{3}H]-uridine to cell homogenates was determined and subtracted from total counts.

2.5. *Drugs and isotopes*

Ouabain, obtained from Sigma Chemical Co., was dissolved in MEM and filtered sterilely into complete media. Actinomycin D, obtained from Calbiochem, was dissolved sterilely in distilled water and diluted to 0.02 mg/ml final conc. in media. Carrier-free Na-^{125}I (5 mCi, 17 Ci/mg), [^{3}H]leucine (50 Ci/mmol) and [^{3}H]uridine (25 Ci/mmol) were obtained from New England Nuclear.

3. Results

ACHR increased by 75% after 24 h and by 270% after 48 h incubation in 10^{-5} M ouabain (table 1). Protein synthesis was only slightly increased (14%), and total cell protein was unchanged over the same time interval (table 1). No significant changes in either cellular ACHE or in the amount of enzyme released into the medium were noted after 24 h in the drug. Cell ACHE activity was increased by 99% after 48 h (table 2). There were no significant changes in cell or media CK activities (table 2).

There were no changes in ACHR after 24 h or 48 h in the presence of 10^{-6} M ouabain (table 1). However, protein synthesis, cell protein, and cellular ACHE activity increased by 33%, 12% and 42%, respectively (tables 1, 2).

Cytotoxicity was evident as early as 24 h after addition of 10^{-4} M ouabain. Myotubes were smaller than controls, and there was much floating cell debris. By 48 h, ACHR, protein synthesis and cell protein decreased by 27%, 85% and 36%, respectively (table 1). Large decreases in cell and media ACHE activity also occurred (table 2). There were many degenerating myotubes and much floating cell debris.

At 10^{-5} M, there was some flattening of myotubes, some floating cell debris was observed, and spontaneous contractions were absent. At 10^{-6} M, the cells looked normal and spontaneous contractions were observed.

[^{3}H]Uridine incorporation was increased 3.8-fold by 10^{-5} M ouabain, and 13.8-fold by 5×10^{-6} M ouabain after 24 h (table 3). Myotubes appeared normal but spontaneous contractions were not observed at either concentration.

Actinomycin D at 0.02 μg/dish blocked [^{3}H]uridine incorporation in control and ouabain-treated cells without gross cytotoxic effects. Myotubes were intact and retained cross-striations. Actinomycin D abolished spontaneous contractions regardless of the presence or absence of ouabain. [^{3}H]Uridine incorporation was 22% of untreated in controls, 3.3% of untreated at 10^{-5} M ouabain, and 0.6% of untreated at 5×10^{-6} M ouabain (table 3). Higher actinomycin D levels caused extensive destruction of the myotubes.

Table 1
Changes in ACHR, [^{3}H]leucine incorporation, and cell protein in chick muscle cultures in response to ouabain

	ACHR	[^{3}H]Leucine incorporation	Cell protein
0 h			
Control	170 ± 24	136 ± 16	1.03 ± 0.005
24 h			
Control	203 ± 8	–	1.16 ± 0.075
Ouabain			
10^{-4} M	168 ± 8[b]	–	0.98 ± 0.103[a]
10^{-5} M	355 ± 32[b]	–	1.08 ± 0.137
10^{-6} M	201 ± 28	–	1.13 ± 0.035
48 h			
Control	211 ± 10	172 ± 10	1.21 ± 0.044
Ouabain			
10^{-4} M	153 ± 12[b]	25 ± 1.5[c]	0.78 ± 0.078[c]
10^{-5} M	783 ± 45[c]	196 ± 0.8[a]	1.17 ± 0.163
10^{-6} M	197 ± 18	229 ± 8.0[b]	1.35 ± 0.005[b]

[a] $p \leq 0.02$, [b] $p \leq 0.01$, [c] $p \leq 0.001$, statistically different from controls

Day 8 pectoral muscle cultures were incubated for 24 h and 48 h with 10^{-4} M, 10^{-5} M and 10^{-6} M ouabain. ACHR in fmol ^{125}I-α-BGT bound/dish; [^{3}H]leucine incorporation into protein in cpm/dish × 10^3; cell protein in mg/dish. Means ± SD, 3 samples/value

Table 2
Changes in enzyme activities in chick muscle cultures in response to ouabain

	ACHE		CK	
	Cell	Media	Cell	Media
0 h				
Control	19.5 ± 6.6	–	242 ± 29	–
24 h				
Control	30.2 ± 4.9	125 ± 23	500 ± 19	4.65 ± 0.38
Ouabain				
10^{-4} M	7.2 ± 0.1[c]	23 ± 8[b]	–	–
10^{-5} M	30.4 ± 2.2	79 ± 21	536 ± 51	1.54 ± 0.66[b]
10^{-6} M	35.0 ± 1.6	117 ± 16	476 ± 70	3.79 ± 0.20[a]
48 h				
Control	27.6 ± 2.0	221 ± 3		
Ouabain				
10^{-4} M	4.9 ± 0.7[c]	16 ± 2[c]		
10^{-5} M	55.0 ± 3.0[c]	283 ± 53		
10^{-6} M	39.1 ± 3.2[b]	229 ± 10		

[a] $p \leq 0.02$, [b] $p \leq 0.01$, [c] $p \leq 0.001$, statistically different from controls

Day 8 pectoral muscle cultures incubated for 24 h and 48 h in 10^{-4} M, 10^{-5} M, 10^{-6} M ouabain. ACHE in nmol ACTC hydrolyzed/min/dish; CK in μmol $NADPH_2$ formed/min/dish × 10^{-2}. Means ± SD, 3 samples/value

Table 3
RNA synthesis in chick muscle cultures in the presence of ouabain and actinomycin D

	Untreated	OUA/C	Actinomycin D	ACT/U
0 h				
Control	12.7 ± 6.3	–	–	–
24 h				
Control	20.0 ± 3.4	–	4.48 ± 3.31	0.220[a]
Ouabain				
10^{-5} M	75.2 ± 19.4	3.8[a]	2.46 ± 0.44	0.033[a]
5×10^{-6} M	276.0 ± 18.0	13.8[a]	1.58 ± 0.68	0.006[a]

[a] $p \leq 0.05$, statistically different from controls

Pectoral muscle cultures, 8 days in vitro, incubated for 24 h with 10^{-5} M or 5×10^{-6} M ouabain and 0.04 μg/dish actinomycin D. Values expressed as incorporation of [^{3}H]uridine into RNA, cpm/dish × 10^{-3}. Means ± SD from triplicate dishes. OUA, ouabain treated; C, control; ACT, actinomycin D treated; U, untreated

4. Discussion

The results demonstrate that ouabain increases ACHR levels, ACHE activity and [^{3}H]uridine incorporation into RNA of cultured muscle in a dose-dependent manner. The increases were accompanied by a modest increase in protein synthesis and the activity of a muscle specific enzyme, creatine kinase, did not increase at all.

The increase in cellular but not media ACHE activity after ouabain treatment suggests that ouabain may have affected release of ACHE. ACHE is normally released in large quantities by chick muscle and nerve cultures [2,14]. Its release can be blocked by verapamil [15], and has been reported to be affected by specific ionophores [16].

The mechanism by which ouabain exerts the effects reported here is not known. The major known action of the drug is to inhibit the Na^+/K^+ ATPase and thus active Na^+ extrusion, although changes in ATP pools, adenyl cyclase, or cyclic nucleotides may also occur [17].

Ouabain and other drugs affecting ACHE and ACHR levels have been observed to inhibit spontaneous contractions of cultured muscle, supporting the idea that contractile activity per se may be a part of the system regulating muscle proteins. Evidence against this theory is that regulation of ACHE has been found in cultured nerve cells with at least one agent (acetyl-β-methylcholine) that acts upon muscle [14]. Ouabain also affects tissues other than muscle. It increases RNA, DNA and protein synthesis in cultured chick spinal neurons [18,19] and hemoglobin synthesis in Friend erythroleukemia mouse cells [20]. Ouabain decreases 35 S-methionine incorporation into cultured rat lens protein [21], growth of HeLa cells [22], and RNA, DNA and protein synthesis in lymphocytes after mitogen activation [17].

There are several similarities between the actions of ouabain on cultured muscle and the effect of denervation on muscle in situ. Denervation of skeletal muscle increases ACHR levels and sensitivity to acetylcholine in all species studied [23], ACHE levels in at least the chicken and rabbit [24,25] and RNA synthesis in the frog [26]. Ouabain depolarizes skeletal muscle to the same extent as denervation [27] and does not produce any further depolarization of denervated muscle [28,29].

A possible basis for these actions of ouabain is through alteration of ionic concentrations and fluxes. Serum has been shown [30] to regulate DNA synthesis in fibroblasts through an action on the Na^+/K^+ pump by altering the availability of Na^+ and K^+. Additional evidence suggesting that ion regulation is involved in muscle development are the findings that agents such as tetrodotoxin, lidocaine, and D-600 increase ACHR, ACHE and turnover of myosin heavy chains in chick muscle cultures [4,5,31,32]. Intracellular K^+ concentrations have been shown to regulate protein, RNA and DNA synthesis of cultured human fibroblasts [33].

General stimulation in RNA synthesis is suggested by the large increase in [^{3}H]uridine incorporation by ouabain that can be blocked by actinomycin D. The possibility that some of the increase could be due to altered nucleotide pool sizes is being investigated. Induction of mRNA synthesis is possible, but whether increases in ACHR and ACHE stimulated by ouabain can also be blocked by actinomycin D is not known. Actinomycin D blocks increases in ACHR stimulated by tetrodotoxin, possibly indicating specific induction of muscle genes [5], but does not block increases in ACHE activity stimulated by acetyl-β-methylcholine [34].

The specificity of ouabain's effect on ACHR and to a lesser extent on ACHE and general protein synthesis may be the result of either induction of specific messages or regulation of post-transcriptional events. Cell-free experiments indicate that specific mRNAs are preferentially translated depending upon the Na^+ and K^+ concentrations [35–37].

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