Biochemical Pharmacology, Vol. 30, No. 12, pp. 1501–1505, 1981.
Printed in Great Britain.

0006-2952/81/121501-05 $02.00/0

EFFECT OF CURARE ON THE DEVELOPMENT OF CHICKEN EMBRYO SKELETAL MUSCLE *IN OVO*

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(*Received* 22 *September* 1980; *accepted* 11 *December* 1980)

Abstract—Injection of curare *in ovo* between days 7 and 14 of embryonic development delayed the differentiation of myoneural junctions, and greatly reduced the mass of pectoralis and leg muscles. Although the administration of curare was discontinued at 14 days of development, muscle paralysis persisted and prevented normal hatching at 21 days. The protein composition of muscles from curare-treated embryos was characterized by a decrease in myosin, Ca^{2+} transport ATPase, and ATP:creatine phosphotransferase. No significant differences were found in the total Ca^{2+} content of muscle, in the Ca^{2+} uptake measured in whole muscle homogenates, and in muscle ultrastructure as evident from electron microscopy and histochemical analysis.

Differentiation of muscle cells involves regulation of gene expression at various levels, some of which are under neural control. Cross-innervation of slow and fast muscles changes the Ca^{2+} transport activity of sarcoplasmic reticulum in a manner determined by the nerve [1, 2]. Several weeks after denervation of rat gastrocnemius muscle, the concentration of Ca^{2+} transport ATPase in isolated sarcoplasmic reticulum vesicles, determined by phosphorylation of the active site with [^{32}P]ATP, diminished from 2 nmoles/mg protein to 0.3 nmole/mg protein [3].

In cultured chicken pectoralis muscle cells grown in the absence of innervation, the concentration of Ca^{2+} ATPase after 10–12 days of growth remains about 0.04 nmoles/mg protein, i.e. about 10–20 per cent of that found in fully innervated adult chicken pectoralis muscle [4].

Striking effects of innervation on the expression of contractile proteins have also been reported [5, 6].

The purpose of these studies was to analyze the consequences of functional denervation of muscle cells by curare injection *in ovo* [7] upon the protein composition, Ca^{2+} content, Ca^{2+} uptake activity, and ultrastructure of leg and pectoralis muscles.

MATERIALS AND METHODS

Tubocurarine–HCl, deoxyribonuclease (beef pancreas), ribonuclease (bovine pancreas), phenylmethylsulfonylfluoride, dithiothreitol and iodoacetamide were purchased from the Sigma Chemical Co., St. Louis, MO. Benzamidine–HCl and *N,N,N′,N′*-tetramethylethylenediamine were obtained from Eastman Organic Chemicals, Rochester, NY. Arsenazo III was a product of the Aldrich Chemical Co., Milwaukee, WI and was purified before use according to Kendrick [8]. A23187 was a gift from Dr. Robert Hamill of Eli Lilly & Co., Indianapolis, IN.

D-*Tubocurarine–HCl injections.*

The chorioallantois sacs of fertilized chicken eggs were injected with 0.1 ml of *d*-tubocurarine–HCl (10 mg/ml in sterile saline) or 0.1 ml of sterile saline through a hole made with a sterile 25 gauge § inch needle in the blunt end of the eggs [7]. After injection, the hole was covered with a sterile tape. The eggs were injected twice daily from day 7 to day 11 of development and once daily from days 12 to 14. Samples of pectoralis and leg muscles were removed daily from 12–21 days of development, homogenized in a buffer containing 0.1 M KCl, 10 mM imidazole and 0.3 M sucrose (pH 7.3), and stored at −70° until further processing.

Sample preparation for gel electrophoresis

Method A. The muscle was homogenized in 0.1 M KCl 10 mM imidazole and 0.3 M sucrose (pH 7.3). The homogenate was suspended in equal volumes of 10 mM Tris–Cl buffer (pH 7.0) containing 5 mM $MgCl_2$, 50 μg/ml pancreatic RNase, 50 μg/ml phenylmethylsulfonylfluoride and 1 mM benzamidine–Cl. The suspension was sonicated and treated with 50 μg/ml DNase for 20 min. Trichloroacetic acid (final concentration 5%) was added and the mixture was incubated for 15 min in ice. The sample was centrifuged and the sediment was neutralized with a minimal amount of 2 M Tris. The sediment was solubilized in 1% sodium dodecylsulfate (SDS) and sonicated, and an aliquot was taken for protein determination according to the method of Lowry *et al.* [9], using bovine serum albumin as a standard. Dithiothreitol (10 mM) was added, and the samples

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were heated at 100° for 5 min. After cooling, iodoacetamide (12.5 mM), glycerol (3%), and bromphenol blue (0.1%) were added, 100 μg protein was loaded on a slab gradient gel (6–12%, 2 mm thick), and samples were subjected to electrophoresis according to the method of Laemmli [10].

Method B. The muscle was homogenized in 0.1 M KCl, 10 mM imidazole and 0.3 M sucrose (pH 7.3), and solubilized in 8 M urea, 5% SDS and 0.01 M Tris–Cl (pH 7.0). After the addition of 1% β-mercaptoethanol, the samples were heated at 100° for 5 min and cooled, and 100-μg protein aliquots were applied for electrophoresis as described above.

Methods A and B gave essentially identical results.

Calcium content

Chicken embryo pectoralis and leg muscles were homogenized in a buffer containing 0.1 M KCl, 0.3 M sucrose and 10 mM imidazole (pH 7.3), and diluted in the same buffer to 2 mg protein/ml. The muscle homogenate was suspended to a final concentration of 0.5 mg protein/ml in a solution containing 0.1 M KCl, 0.3 M sucrose, 10 mM imidazole (pH 7.3), 5 mM $MgCl_2$ and 50 μM arsenazo III. The absorbance of arsenazo III–Ca^{2+} complex was measured in an Aminco DW-2 spectrophotometer in the dual wavelength mode at 660 nm, using 685 nm as a reference wavelength. Ca^{2+} that was sequestered in intracellular organelles was released by adding 12.5 μM A23187; 5 min later 0.5 mM EGTA* was added. The free Ca^{2+} content of the muscle homogenates was calculated from the drop in absorbance after the addition of EGTA. The results are comparable to those obtained earlier [4] by atomic absorption spectrophotometry, since the amount of Ca^{2+} bound to proteins is relatively small.

Calcium uptake

Pectoralis and leg muscle homogenates in 0.1 M KCl, 0.3 M sucrose and 10 mM imidazole (pH 7.3) were suspended to a final concentration of 0.5 mg protein/ml in a solution containing 0.1 M KCl, 0.3 M sucrose, 10 mM imidazole (pH 7.3), 7.5 mM $MgCl_2$, 5 mM NaN_3, 5 mM K_2-oxalate and 50 μM arsenazo III. Ca^{2+} content of all samples was adjusted to 60 nmoles Ca^{2+}/mg protein by the addition of required amounts of $CaCl_2$ prior to the addition of 4 mM ATP. The change in absorbance after the addition of ATP was followed using an Aminco DW-2 spectrophotometer as described above. The rate of Ca^{2+} uptake was determined from the slope of the uptake curve 30 sec after the addition of ATP, which represented the linear phase of Ca^{2+} uptake.

Electron microscopy

Small pieces of leg muscles from 21-day-old chicken embryos were fixed in 4% glutaraldehyde in phosphate buffer for 2 hr followed by washing in cold buffer. After post-fixation in 2% osmium tetroxide for 1 hr, the samples were dehydrated and embedded in Epon–Araldite mixture. Sections were stained with uranyl acetate and lead citrate, and viewed in a Philips 300 electron microscope.

* EGTA, ethyleneglycol bis(β-aminoethyl ether) tetraacetate.

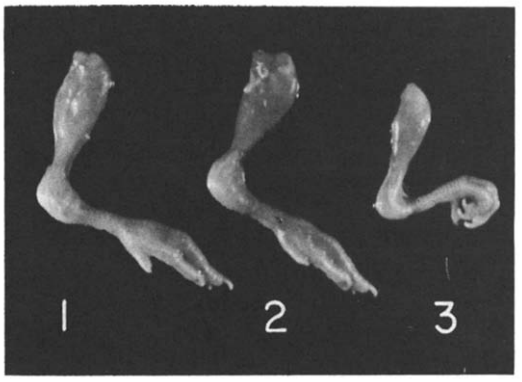

Fig. 1. Effect of curare on leg development in chicken embryo. The legs were excised from 21-day-old chicken embryos. Key: 1, control; 2, injected with saline; and 3, injected with curare, as described in Materials and Methods.

RESULTS

Injection of curare *in ovo* during 7–14 days of development of chicken embryos grossly affected muscle development (Fig. 1), in agreement with earlier observations by Drachman [11]. The total wet weight of leg and pectoralis muscles in curare-injected animals was smaller than that of uninjected

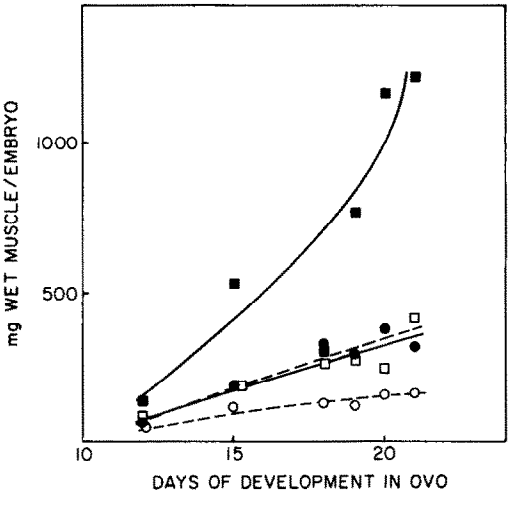

Fig. 2. Effect of curare on the wet weight of pectoralis and leg muscles during embryonic development. Pectoralis (●, ○) and leg (■, □) muscles were removed from control (●, ■) and curare-treated (○, □) embryos at various stages of development and their wet weights were determined.

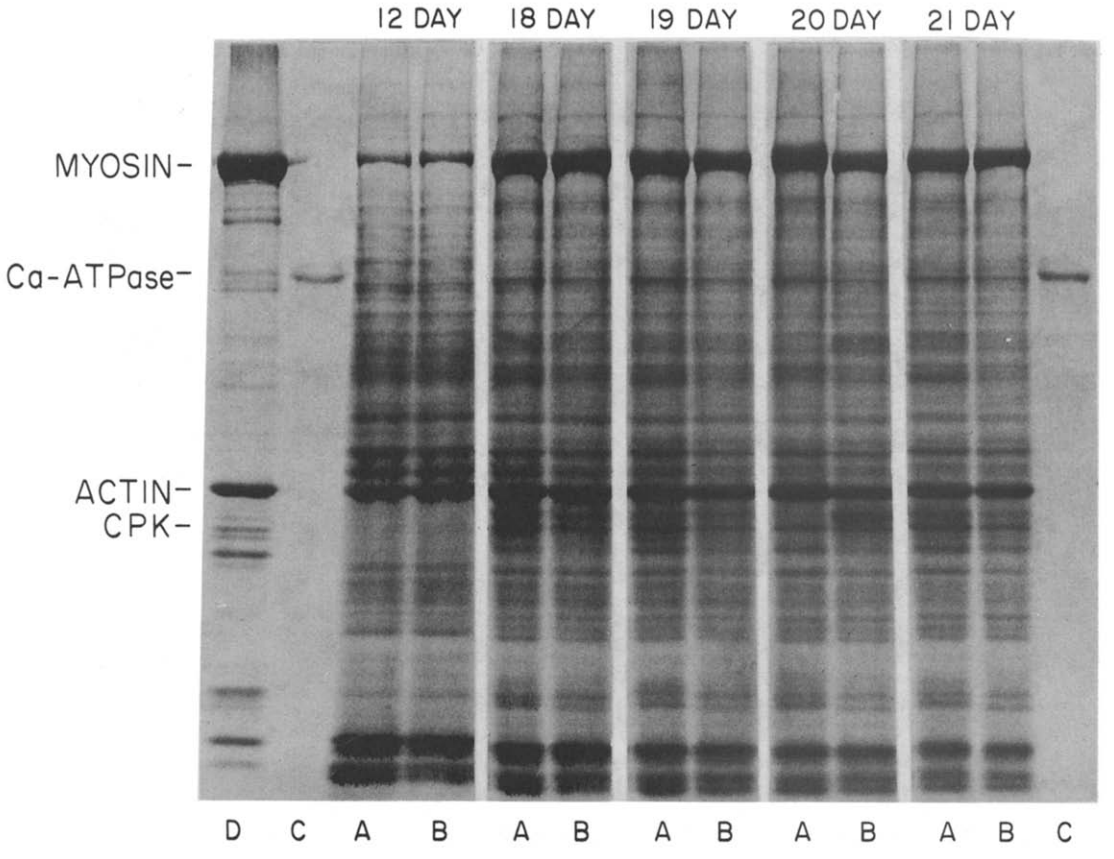

Fig. 3. Influence of curare on the protein composition of chicken embryo leg muscle. Leg muscles of control (A) and curare-injected (B) embryos were removed at days 12, 18, 19, 20 and 21 of development and the protein composition of the homogenate was analyzed by polyacrylamide gel electrophoresis (Method B in Materials and Methods). Purified sarcoplasmic reticulum Ca^{2+}-ATPase (C) and rabbit skeletal muscle actomyosin (D) were used as standards.

or saline-treated controls throughout late embryonic development and was less than half of control muscles at 20–21 days (Fig. 2).

Curare treatment produced characteristic changes in the protein composition of leg (Fig. 3) and pectoralis muscles (data not shown) which were most pronounced at later stages of development. In muscles of 12-day-old embryos the concentrations of myosin, sarcoplasmic reticulum Ca^{2+}-ATPase, and ATP:creatine phosphotransferase were small relative to muscles of older embryos, and the effects of curare injection were minor.

The increases in the myosin, Ca^{2+}-ATPase, actin, and ATP:creatine phosphotransferase content associated with normal development during 15–21 days were reduced significantly in curare-injected animals; the decrease in the myosin content of leg muscles based on densitography was 22–33 per cent, and in the Ca^{2+}-ATPase content 30–42 per cent after 21 days, compared with control animals. The changes in pectoralis muscles caused by curare injection were similar.

The reduction of muscle mass after curare treatment in 21-day-old embryos occurred without major changes in the ultrastructure of the muscle (Fig. 4). This is consistent with earlier histological studies [12] indicating a decrease in myofibril diameter in 16-day-old chicken embryo muscles exposed *in ovo* to curare, without loss of the organization of myofibril structure.

No significant effects of curare treatment were observed on the earlier reported [4] decrease in the Ca^{2+} content of muscle homogenates during development (Fig. 5) and on the ATPase (pH 9.4 and 4.2), esterase, and NADH dehydrogenase activities of pectoralis and leg muscles of 21-day-old chicken embryos assayed by enzyme histochemistry (data not shown).

The rate of ATP-mediated, oxalate-dependent Ca^{2+} transport measured in whole muscle homogenates increased 2- to 3-fold during development between 12 and 21 days, (Fig. 6), reflecting the accumulation of sarcoplasmic reticulum [4]. Curare treatment had no significant effect upon this change.

DISCUSSION

Treatment of chicken embryos with curare, *in ovo*, delays the differentiation of neuromuscular junctions [7]. Even though in our experiments the curare administration was stopped after 14 days, the effects of curare treatment persisted up to the 21 days of development, as indicated by muscle paralysis and the inability of the chicks to hatch normally.

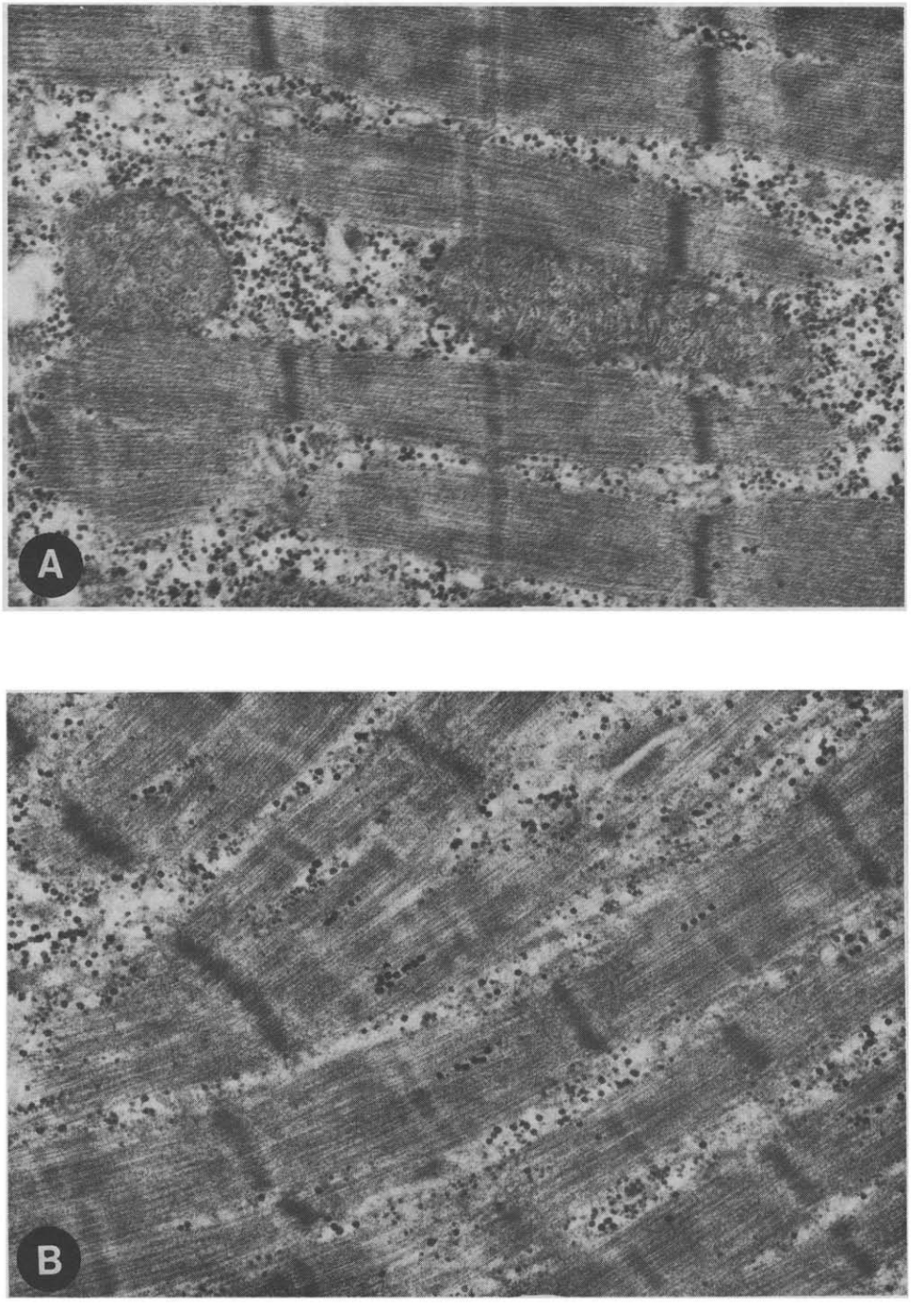

Fig. 4. Electron micrographs of control and curare-treated leg muscles. Chicken embryos were treated with curare as described in Materials and Methods. After 21 days of development, the leg muscles were excised and processed for electron microscopy. Panel A, control muscle; panel B, curare-treated muscle.

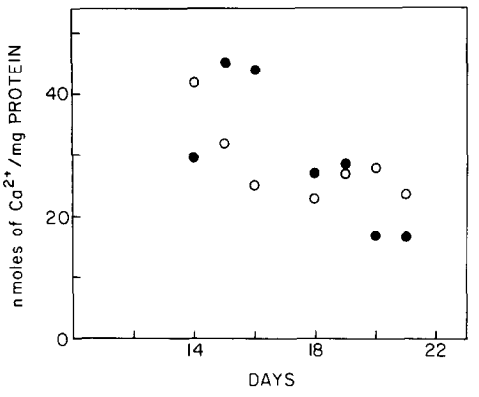

Fig. 5. Ca^{2+} content of muscle during development. Control (●) and curare-treated (○) chicken embryo leg muscles were excised after 12–21 days of development and analyzed for Ca^{2+} content as described in Materials and Methods.

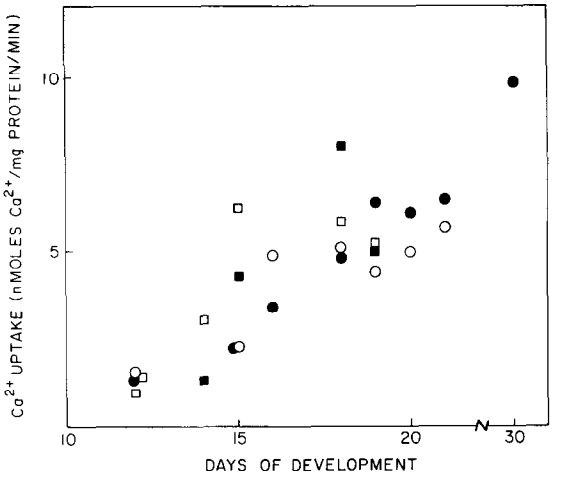

Fig. 6. Ca^{2+} uptake of muscle during development. Control (●, ■) and curare-treated (○, □) chicken embryo leg (□, ■) and pectoralis (○, ●) muscles were excised after 12–21 days of development and analyzed for Ca^{2+} uptake as described in Materials and Methods.

In view of the well-known effects of innervation upon muscle growth and differentiation [13], we expected gross differences in the biochemical and ultrastructural characteristics of muscles from curarized animals.

While curare inhibited the increase in muscle mass associated with normal development and decreased the content of myosin, Ca^{2+}-ATPase, and ATP:creatine phosphotransferase, compared with normal muscle, the changes in protein composition were relatively modest. Nevertheless, these changes indicate that curare selectively interferes with the accumulation of certain muscle-specific proteins during development.

The reduction of myosin content in curarized muscle did not lead to well-defined changes in myofibrillar structure, and the decrease in the Ca^{2+}-transport ATPase content of sarcoplasmic reticulum was not reflected in significant differences in the total Ca^{2+} content or oxalate-dependent Ca^{2+} uptake activity of the muscle, measured in muscle homogenates. The effect of curare on Ca^{2+} uptake activity may have been obscured by the relatively larger scatter of the data.

The influence of innervation upon late muscle differentiation and the expression of muscle-specific protein synthesis may, in part, be related to Ca^{2+} influx into muscle cells during nerve activity and the resulting increase in free cytoplasmic Ca^{2+} concentration [14]. Curare, while it reduces the responsiveness of skeletal muscle fibers to nerve stimulation as a competitive antagonist to acetylcholine, also evokes depolarization of embryonic, but not adult, rat muscle fibers by direct action on acetylcholine receptors [15]. If the same effect occurs in chicken muscle, the increase in membrane permeability caused by curare might mimic nerve activity in promoting synthetic processes within muscle cells.

Acknowledgements—This work was supported by research grants AM 18117 from the National Institute of Arthritis, Metabolism and Digestive Diseases, PCM 7600707 from the National Science Foundation, and a grant-in-aid from the Muscular Dystrophy Association, Inc. This work was performed by Dikla Roufa as part of the requirement for a Ph.D. degree.

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