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# ABNORMAL EXPRESSION OF THE CALMODULIN GENE IN MUSCLE FROM THE DYSTROPHIC CHICKEN

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SUMMARY. Compared to that of genetically-related normal chickens, pectoralis muscle from the dystrophic chicken contained increased calmodulin measured by radioimmunoassay. Determined by the dot blot procedure, expression of the calmodulin gene was enhanced in muscle from affected animals. The bioactivity of the gene product was normal. Together with previous studies reporting increased cell $Ca^{2+}$ content in dystrophic muscle, the current findings of increased sarcoplasmic calmodulin suggest the latter is a cellular response to defective $Ca^{2+}$ transport at the level of cell efflux or intracellular organelle (sarcoplasmic reticulum) uptake. 

The pathogenesis of the myopathy in the genetic model of muscular dystrophy in the chicken is presently unknown. Principal manifestations of the avian disease include: weakness and functional disability of involved muscles, elevated plasma levels of muscle enzymes, histopathological changes in muscle (1,2), reduced myofibrillar protein (3), and elevated proteolytic activity in muscle (4). With regard to the myopathogenesis, we (5) and others (6) have found abnormal sequestration of calcium in dystrophic chicken muscle. This calcium accumulation is coincident with reduced sarcoplasmic reticulum uptake of $Ca^{2+}$ (7), decreased $Ca^{2+}$-stimulable, $Mg^{2+}$-dependent adenosine triphosphatase ($Ca^{2+}$-ATPase) activity in sarcoplasmic reticulum (8) and elevated $Ca^{2+}$-activated neutral protease activity (9). These studies, together with clinical determinations of muscle $Ca^{2+}$ (10-14), provide a basis for relating impairment of control of intracellular $Ca^{2+}$ levels or of $Ca^{2+}$-dependent activities to dystrophic myopathogenesis. Critical to control of cytoplasmic $Ca^{2+}$ levels is calmodulin, an ubiquitous $Ca^{2+}$-binding protein.

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When complexed with calcium, calmodulin regulates plasma membrane $Ca^{2+}$-ATPase activity, cell phosphodiesterase activity, several protein kinases and other processes (15,16). We report here that the calmodulin content of avian dystrophic muscle is elevated, the result of increased expression of the calmodulin gene.

## MATERIALS AND METHODS

Chickens: Homozygous recessive, dystrophic (Line 413) and genetically-related normal (Line 412) chickens were obtained at one day of age from the Department of Avian Sciences, University of California, Davis, CA and maintained as previously described (3). At three months ex ovo, the birds were killed by decapitation and the affected breast muscle (pectoralis major) was quickly removed and freed of adherent connective tissue. Dystrophic chickens at this age express overt muscle weakness as determined by the widely-used flip test procedure (1-3).

Radioimmunoassay for Calmodulin: Muscle samples (100 mg each) were scissor-minced and homogenized in distilled water on ice using three short bursts of Tissumizer (Tekmar Co., Cincinnati, OH). The homogenates were adjusted to contain 10 mM Tris, pH 7.4 and 0.1% Triton X-100. Samples were assayed for protein (17) and for calmodulin, using a polyclonal monospecific antiserum as described previously (18). Purified rat testis calmodulin (CAABCO, Houston, TX) was used as a standard.

Calmodulin Bioactivity: Calmodulin bioactivity of muscle homogenates was determined in a human red blood cell membrane $Ca^{2+}$-ATPase assay, as described previously (18).

Calmodulin Messenger RNA: Calmodulin mRNA was quantified by the dot blot procedure using a calmodulin cDNA probe (pCM109), which contains sequences corresponding to approximately 38% of the peptide-coding region of the calmodulin structural gene sequence (19). We are grateful to Dr. Anthony R. Means, Baylor College of Medicine, Houston, Texas for providing the plasmid.

For RNA extraction, equal wet weight of both normal and dystrophic muscles were flash frozen, ground to a powder in liquid nitrogen and resuspended in 20 mM Tris-HCl pH 7.4 containing 0.5% SDS, 25mM EDTA and 75 mM NaCl. The RNA was extracted by the hot phenol method (20) and resuspended in 10 mM Tris-HCl, pH 7.4 containing 1 mM EDTA. The extracted RNA was serially diluted 2-fold and denatured in 20 mM sodium phosphate pH 7.0 containing 50% formamide and 6% formaldehyde. Samples were then dot blotted onto nitrocellulose paper, baked at 80°C for 2h and then hybridized with the radioactive plasmid probe, pCM109 (20). The plasmid was labelled with $^{32}$PdCTP using a nick translation system (Cooper Biomedical Inc., Malvern, PA). After hybridization, the dot blot was exposed to X-ray film at -70°C and the autoradiogram developed. Densitometer tracings of the autoradiogram corrected for background were performed by ISCO model UA-5 absorbance monitor with the gel scanner attachment (Model 1310).

## RESULTS

Pectoralis Muscle RNA and Calmodulin Content in Normal and Dystrophic Chickens. Dystrophic muscle showed a decrease in the amount of total protein per weight of tissue in dystrophic muscle (Table 1), as previously reported

Table 1. Properties of pectoralis muscle from normal and dystrophic chickens

| Properties | Normal Muscle | Dystrophic Muscle |
|---|---|---|
| Protein (mg/gm wet wt) | 178 ± 39 (3)[a] | 133 ± 17 (5) |
| Ribonucleic acid (μg/gm wet wt) | 333 (3) | 800 (5) |
| Calmodulin-immunoassay (μg/mg protein) | 1.0 ± 0.1 (8) | 1.4 ± 0.2 (10)[b] |
| Calmodulin-bioassay (μmoles $P_i$/mg/90 min) | 0.060 ± 0.009 (6) | 0.052 ± 0.013 (6)[c] |

[a]Results are mean ± SE of three or more determinations. In parentheses are the number of animals studied. For ribonucleic acid determination, the muscle samples were pooled.
[b]Difference between dystrophic and normal muscle is significant at $P<0.05$ (Student's *t* test).
[c]Calmodulin concentration in the assay was 100 ng/mg membrane protein. There is no significant difference in bioactivity between normal and dystrophic muscle calmodulin.

(3). There was a significant increase in the total amount of RNA in the dystrophic chicken muscle, as has been observed by others (21,22). Immunoactive calmodulin content of dystrophic muscle was also significantly increased, compared to that of normal muscle (Table 1). However, no significant difference was observed in the specific activity of the calmodulin from normal and dystrophic muscle as measured in a human red cell $Ca^{2+}$-ATPase assay.

Quantitation of Calmodulin mRNA Present in Normal and Dystrophic Pectoralis Major Muscle. The relative amount of calmodulin mRNA as determined by dot blot using pCM109 shows a 2-fold increase in the dystrophic muscle as compared to that present in normal muscle (Figure 1-A). This difference is most evident by the densitometer tracings of the dot blot at concentrations of RNA optimal for cDNA hybridization (Figure 1-B, wells 5-9). The elevated dystrophic calmodulin mRNA correlates with the increase in total RNA in the dystrophic muscle extracted per unit wet weight (Table 1).

## DISCUSSION

Studies from several laboratories have shown abnormally high intracellular calcium levels in dystrophic muscle (5,6,10-14). Although the mechanism

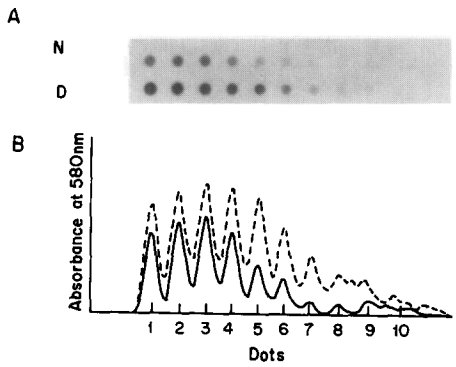


Figure 1. Calmodulin mRNA in pectoralis major muscle as measured by (A) dot blot hybridization and (B) densitometer tracings of the dot blot autoradiogram. The RNA was extracted from equal wet weight of normal (Lane N) and dystrophic (Lane D) muscle, bound to nitrocellulose paper and hybridized with radiolabelled calmodulin cDNA (pCM109) as described in the Materials and Methods. Spots from wells 1 through 10 represent a 2-fold serial dilution of the extracted RNA. The normal (solid line) and dystrophic (dashed line) dot blot autoradiograms were scanned at 580nm using an ISCO gel scanner and absorbance monitor.

of the abnormal increase in sarcoplasmic calcium content is incompletely understood, defective membrane $Ca^{2+}$-ATPase (calcium pump) activity has been implicated as a major factor contributing to elevated sarcoplasmic calcium levels. $Ca^{2+}$-ATPase activity in normal tissue is stimulated by increased cytoplasmic calcium concentrations and the formation of the calmodulin $Ca^{2+}$ complex that regulates $Ca^{2+}$-ATPase (23). The studies reported here (Table 1) and in a preliminary report elsewhere (24) show that calmodulin levels in dystrophic muscle are increased compared to that in normal muscle. The present results (Fig. 1) also suggest that this increase in calmodulin levels is due to enhanced expression of the calmodulin gene. Expressed per mg of immunoreactive calmodulin, the bioactivity of calmodulin in dystrophic muscle is normal, indicating that the gene product is normal. Although factors which regulate calmodulin gene expression are not known, the fact that both sarcoplasmic calmodulin levels and calcium content of dystrophic muscle are high suggest that increased gene expression is a cellular response to a defective calcium transport process ("calmodulin resistance") affecting either calcium efflux (sarcolemma) or sarcoplasmic reticulum uptake of calcium. Biochemical studies of the several $Ca^{2+}$-ATPases in dystrophic muscle are

currently addressing this possibility. Studies elsewhere which have shown $Ca^{2+}$-ATPase activity in dystrophic muscle to be decreased (25-27) have not dealt with the issue of calmodulin-responsiveness of the enzyme.

In addition to calmodulin, spectrin is increased in content in dystrophic muscle cytosol (28). Because spectrin is capable of binding calmodulin (29), it might be postulated that this interaction in cytoplasm contributes to decreased $Ca^{2+}$-ATPase activity in the chicken model of dystrophy by inhibiting calmodulin access to the membrane-associated enzyme. However, the normal bioactivity of calmodulin from dystrophic muscle (Table 1) makes this possibility unlikely.

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