Pollock, J. S., Zapata, G. A., Weber, D. J., Berkowitz, P., Deerfield, D. W., II, Olson, D. L., Koehler, K. A., Pedersen, L. G., & Hiskey, R. G. (1988) in *Current Advances in Vitamin K Research* (Suttie, J. W., Ed.) pp 325–334, Elsevier Science, New York.
Prendergast, F. G., & Mann, K. G. (1977) *J. Biol. Chem. 252*, 840–850.
Resnick, R. M., & Nelsestuen, G. L. (1980) *Biochemistry 19*, 3028–3033.
Stenflo, J., & Suttie, J. W. (1977) *Annu. Rev. Biochem. 46*, 157–172.
Stenflo, J., Fernlund, P., Egan, W., & Roepstorff, P. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 2730–2733.
Thorsen, S., Clemmensen, I., Sottrup-Jensen, L., & Magnusson, S. (1981) *Biochim. Biophys. Acta 668*, 377–387.
Trexler, M., & Patthy, L. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 2457–2461.
Trexler, M., Zsofia, V., & Patthy, L. (1982) *J. Biol. Chem. 257*, 7401–7406.
Trexler, M., Banyai, L., Patthy, L., Pluck, N. D., & Williams, R. J. P. (1983) *FEBS Lett. 154*, 311–318.
Tulinsky, A., & Park, C. H. (1986) *Biochemistry 25*, 3977–3982.
Welsch, D. J., & Nelsestuen, G. L. (1988a) *Biochemistry 27*, 4939–4945.
Welsch, D. J., & Nelsestuen, G. L. (1988b) *Biochemistry 27*, 4946–4952.
Welsch, D. J., Pletcher, C. H., & Nelsestuen, G. L. (1988) *Biochemistry 27*, 4933–4938.
Wiman, B., & Collen, D. (1978) *Nature (London) 272*, 549–550.
Wright, S. F., Bourne, C. D., Hoke, R. A., Koehler, K. A., & Hiskey, R. G. (1984) *Anal. Biochem. 139*, 82–90.

Abnormal Response to Calmodulin in Vitro of Dystrophic Chicken Muscle Membrane Ca^{2+}-ATPase Activity†

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ABSTRACT: A skeletal muscle membrane fraction enriched in sarcoplasmic reticulum (SR) contained Ca^{2+}-ATPase activity which was stimulated in vitro in normal chickens (line 412) by 6 nM purified bovine calmodulin (33% increase over control, $P < 0.001$). In contrast, striated muscle from chickens (line 413) affected with an inherited form of muscular dystrophy, but otherwise genetically similar to line 412, contained SR-enriched Ca^{2+}-ATPase activity which was resistant to stimulation in vitro by calmodulin. Basal levels of Ca^{2+}-ATPase activity (no added calmodulin) were comparable in muscles of unaffected and affected animals, and the Ca^{2+} optima of the enzymes in normal and dystrophic muscle were identical. Purified SR vesicles, obtained by calcium phosphate loading and sucrose density gradient centrifugation, showed the same resistance of dystrophic Ca^{2+}-ATPase to exogenous calmodulin as the SR-enriched muscle membrane fraction. Dystrophic muscle had increased Ca^{2+} content compared to that of normal animals ($P < 0.04$) and has been previously shown to contain increased levels of immuno- and bioactive calmodulin and of calmodulin mRNA. The calmodulin resistance of the Ca^{2+}-ATPase in dystrophic muscle reflects a defect in regulation of cell Ca^{2+} metabolism associated with elevated cellular Ca^{2+} and calmodulin concentrations.

Biochemical changes described in genetically dystrophic chicken skeletal muscle include abnormal accumulation of calcium (Hudecki et al., 1983, 1984), increased total lipid content in dystrophic sarcoplasmic reticulum (SR)[1] (Scales et al., 1977; Tovar et al., 1983), decreased SR Mg^{2+}-dependent, Ca^{2+}-stimulatable ATPase (Ca^{2+}-ATPase) activity (Hanna & Baskin, 1978; Verjovski-Almeida & Inesi, 1979; Hanna et al., 1981), and increased Ca^{2+}-activated neutral protease activity (Sugita et al., 1982). The molecular mechanisms for these alterations are unknown.

We have recently reported that, compared to normal chickens (line 412), dystrophic chickens (line 413) have affected striated muscle that contains increased amounts of immunoassayable and bioactive calmodulin (Hudecki et al., 1986), a cytoplasmic Ca^{2+} binding protein which governs the

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[1] Abbreviations: SR, sarcoplasmic reticulum; Ca^{2+}-ATPase, Ca^{2+}-stimulatable, Mg^{2+}-dependent ATPase; Mg^{2+}-ATPase, Mg^{2+}-dependent ATPase; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; CAPP, 2-chloro-10-(aminopropyl)phenothiazine; Tris, tris(hydroxymethyl)aminomethane; MOPS, 3-(*N*-morpholino)propanesulfonic acid; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N′*-2-ethanesulfonic acid; ANOVA, analysis of variance; T-tubule, transverse tubule.

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activity of a wide variety of enzymes in many tissues (Scharff, 1981). We also found that dystrophic chicken muscle contained a 2-fold increase in calmodulin mRNA (Hudecki et al., 1986). Animal skeletal muscle sarcoplasmic reticulum contains calmodulin, which is reported to activate protein kinases (Chiesi & Carafoli, 1982; Campbell & MacLennan, 1982) and to slow Ca^{2+} release (Meissner, 1986), but is reported not to stimulate mammalian skeletal muscle SR Ca^{2+}-ATPase activity (Chiesi & Carafoli, 1982). In a variety of mammalian tissues other than striated muscle, calmodulin stimulates plasma membrane Ca^{2+}-ATPase and other intracellular Ca^{2+}-dependent enzymes (Itano & Penniston, 1985). We report here that normal chicken skeletal muscle SR-enriched Ca^{2+}-ATPase, in contrast to that described in the rabbit (Chiesi & Carafoli, 1982), is stimulated in vitro by bovine and avian calmodulins but that dystrophic chicken muscle SR-enriched Ca^{2+}-ATPase is calmodulin resistant.

Experimental Procedures

Materials. Na_2ATP, CNBr-activated Sepharose, and reagents for 5′-nucleotidase assay were purchased from Sigma Chemical Co. (St. Louis, MO). Anti-calmodulin antibody and ^{125}I-calmodulin for radioimmunoassay were obtained from Amersham (Arlington Heights, IL). 2-Chloro-10-(aminopropyl)phenothiazine (CAPP) was kindly provided by Dr. Stephen Kennedy, National Institute of Mental Health, NIH (Bethesda, MD).

Preparation of SR-Enriched Membranes. Normal (line 412) and dystrophic (line 413) chickens were obtained from the Department of Avian Sciences, University of California, Davis, CA, and maintained as previously described (Hudecki et al., 1979). Animals were sacrificed at 50–90 days of age, and 2–10 g of pectoralis major muscle was immediately dissected, minced, and placed in ice-cold buffer containing 50 mM TES/0.25 M sucrose, pH 7.0. Crude SR-enriched vesicles were prepared according to the method of Hanna et al. (1981) from a muscle homogenate in 50 mM TES/0.25 M sucrose, with final suspension in 10.0 mM TES/1.0 mM KCl, and stored at −70 °C for Ca^{2+}-ATPase assays within 1–21 days of preparation. Aliquots of minced muscle and SR-enriched membranes were also frozen for calmodulin extraction and measurement, and for protein assay by the Lowry method (1951), with bovine serum albumin as standard. Membrane protein concentrations in the final suspensions ranged from 5 to 12 mg/mL.

This method results in 27- and 21-fold enhancement of Ca^{2+}-ATPase activity in normal and dystrophic tissue, respectively. Mg^{2+}-dependent ATPase (Mg^{2+}-ATPase) activity (see below) was increased 12–15-fold and 5′-nucleotidase activity (Arkesteijn, 1976) 10–12-fold in the membrane preparation. To define a possible contribution of transverse tubule (T-tubule) Ca^{2+}-ATPase (Malouf & Meissner, 1979) to the ATPase activity measured in the current studies, ATP hydrolysis was also measured at 1–4 mM Ca^{2+}, in addition to the micromolar Ca^{2+} concentration which is optimal for sarcoplasmic reticulum Ca^{2+}-ATPase. No Ca^{2+}-stimulatable ATPase activity was obtained at 1–4 mM Ca^{2+}.

Further purification of the crude SR-enriched vesicles was carried out by calcium phosphate loading, discontinuous sucrose density gradient centrifugation, and calcium phosphate unloading, as described by Hanna et al. (1981). The normal and dystrophic vesicles capable of calcium loading (henceforth called purified vesicles) were assayed for Ca^{2+}-ATPase and Na,K-ATPase activity, the latter by measurement of enzyme inhibition by 1 mM ouabain, as we have previously described (Davis & Bernardis, 1984). The results of these studies are shown in Table I, where it is evident that loss of sarcolemmal membranes, indicated by Na,K-ATPase activity, is virtually complete after this purification step.

Table I: Comparison of Ca^{2+}-ATPase and Na,K-ATPase Activities in Crude and Calcium Phosphate Loaded SR Vesicles from Normal and Dystrophic Chickens

		ATPase act.[a] (μmol of P_i mg^{-1} min^{-1})	
preparation	tissue source	Ca^{2+}	Na,K
crude	normal	0.692 ± 0.140	0.332 ± 0.108
Ca^{2+} loaded	normal	0.918 ± 0.230	−0.005 ± 0.015
crude	dystrophic	0.732 ± 0.073	0.345 ± 0.093
Ca^{2+} loaded	dystrophic	0.345 ± 0.148	0.012 ± 0.001

[a] Results are mean ± SE of three experiments performed in duplicate on two sets of paired normal and dystrophic membrane preparations as described under Experimental Procedures. The source of crude and Ca^{2+}-loaded vesicles, normal or dystrophic, was the same within each experiment. Ca^{2+}-ATPase was measured as activity stimulatable by 12 μM free Ca^{2+}, while Na,K-ATPase was that inhibited by 1 mM ouabain.

Ca^{2+}-ATPase Assay. Enzyme activity was assayed according to our previously published method (Davis & Blas, 1981) and measured as the difference in ATP hydrolysis in the presence and absence of 12 μM free Ca^{2+} (measured by calcium electrode). The assay buffer contained 25 mM Tris, pH 7.45, 75 mM NaCl, 25 mM KCl, 1 mM $MgCl_2$, 1 mM Na_2ATP, and 0.1 mM EGTA, with or without 0.15 mM $CaCl_2$. For measurement of Na,K-ATPase activity, ATP hydrolysis was measured in the presence and absence of 1 mM ouabain, without Ca^{2+}. Mg^{2+}-ATPase activity was that found in the presence of ouabain and Mg^{2+} but without Ca^{2+}. In each experiment, all samples contained either 2 or 5 μg/mL of membrane protein. Enzyme activity was linear for 90 min, and 60 min was selected as the assay time. Ca^{2+}-ATPase activity was expressed as micromoles of P_i liberated per milligram of membrane protein per minute of assay period. Each group of membrane samples, obtained from pairs of normal and dystrophic animals, was assayed simultaneously in duplicate with the results expressed as mean ± SE of enzyme activity in at least three experiments. Intra- and interassay coefficients of variation were 1% and 31%, respectively.

In selected studies, Ca^{2+}-ATPase activity was assayed by using selected buffers other than Tris, including MOPS, TES, HEPES, and imidazole buffers. The concentration of each buffer was 25 mM, and the amounts of other additives were the same as in the Tris-based medium. Assays were conducted with each buffer at pH 7.0 and pH 7.45.

Calmodulin Preparation. Purified bovine calmodulin was prepared according to the method of Charbonneau et al. (1983), using CAPP–Sepharose affinity chromatography. Calmodulin was extracted from normal and dystrophic chicken muscle after tissue homogenization in 125 mM borate/1 mM EGTA/75 mM NaCl, pH 8.4, followed by heating at 90 °C for 5 min and centrifugation of the resultant mixture at 20000*g* for 30 min. Supernatant protein concentration was then determined (Lowry), and aliquots were analyzed for calmodulin by immunoassay.

Calmodulin Immunoassay. The calmodulin extracted from normal and dystrophic muscle, and that present in the SR membrane preparations, was quantitated by radioimmunoassay as reported by Chafouleas et al. (1979).

Muscle Calcium Concentration. Skeletal muscle Ca^{2+} levels were measured by the technique of Ettienne and Singer (1978), in which muscle aliquots are treated with lanthanum chloride in order to remove extracellular Ca^{2+} prior to acid-ashing. Ashed samples were suspended in $HCl/LaCl_3$ and assayed by

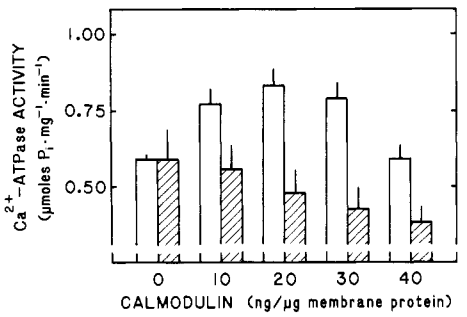

FIGURE 1: Response of normal and dystrophic SR Ca^{2+}-ATPase activity to a range of exogenous purified bovine brain calmodulin concentrations. Results shown are mean ± SE of activities from three normal and three dystrophic animals with three assays conducted in duplicate. There is a parabolic response to calmodulin in normal membranes (open bars, $P = 0.001$, ANOVA), whereas there is a reduction in enzyme activity (not significant overall by ANOVA) in dystrophic membranes (hatched bars) with the addition of calmodulin.

atomic absorption spectroscopy.

Statistical Analysis. Basal and calmodulin-stimulated Ca^{2+}-ATPase activity levels were compared by the Student's paired *t* test. Normal and dystrophic basal activities, calmodulin and Ca^{2+} dose response curves, and tissue calmodulin and Ca^{2+} concentrations were compared by analysis of variance (ANOVA).

Results

Basal Ca^{2+}-ATPase Activity and Response of Ca^{2+}-ATPase in Vitro to Calmodulin. Basal Ca^{2+}-ATPase activities (without added calmodulin) of six pairs of normal and dystrophic crude SR-enriched membrane preparations were 0.678 ± 0.037 and 0.602 ± 0.043 μmol of P_i (mg of membrane protein)$^{-1}$ min^{-1}, respectively, in 30 experiments. These levels are not significantly different by ANOVA. Purified bovine brain calmodulin, 6 nM (20 ng/μg of membrane protein), stimulated enzyme activity in normal vesicles by 33.0 ± 2.9% ($P < 0.001$), whereas in dystrophic vesicles the enzyme was inhibited by exogenous calmodulin (−8.5 ± 2.6%, $P < 0.001$).

The effect of 6 nM avian calmodulin, extracted from normal and dystrophic skeletal muscle, on SR Ca^{2+}-ATPase activity was compared with that of bovine brain calmodulin. Both preparations from skeletal muscle stimulated the normal and inhibited the dystrophic enzyme (results not shown), in a manner comparable to the effect of 6 nM bovine calmodulin indicated above.

The Ca^{2+}-ATPase activity of SR-enriched membranes from normal and dystrophic animals, in response to increasing in vitro concentrations of purified bovine calmodulin, is shown in Figure 1. Exogenous calmodulin concentrations ranged from 10 to 40 ng/μg of membrane protein (3–12 nM). Ca^{2+}-ATPase from normal SR increased significantly in a parabolic response to calmodulin ($P = 0.001$) with maximal effect seen at a calmodulin concentration of 20 ng/μg of membrane protein (6 nM calmodulin). Calmodulin inhibited Ca^{2+}-ATPase in dystrophic SR at all concentrations, although the overall effect was not significant by ANOVA.

Effect of Assay Buffer and pH on Calmodulin–Ca^{2+}-ATPase Interaction. Calmodulin-responsive Ca^{2+}-ATPase activity of normal SR-enriched membranes assayed in our 25 mM Tris-based assay medium was compared with activity obtained in media containing no Tris, but rather 25 mM MOPS, TES, HEPES, or imidazole buffers, at pH 7.0 and 7.45. The ionic strength of these buffer media was otherwise similar. As shown in Table II, calmodulin stimulated enzyme activity in the presence of Tris at both pH levels, but in the presence of MOPS and TES, there was calmodulin stimulation only at pH 7.45. Enzyme stimulation was seen with HEPES, more at pH 7.0 than at pH 7.45. In the presence of imidazole buffer at either pH, there was enzyme inhibition by calmodulin. Dystrophic SR Ca^{2+}-ATPase was not stimulated by calmodulin in any of the five buffers at either pH (results not shown).

Table II: Effect of Assay Buffer and pH on Response of Normal Skeletal Muscle SR Ca^{2+}-ATPase Activity to Purified Bovine Brain Calmodulin

			Ca^{2+}-ATPase act. (μmol of P_i mg^{-1} min^{-1})	
buffer	pH	p*K*	basal	change with calmodulin[b]
Tris	7.0	8.1	0.423 ± 0.030	+0.140 ± 0.017 (+33)[c]
	7.45		0.448 ± 0.017	+0.180 ± 0.025 (+40)[c]
MOPS	7.0	7.2	0.240 ± 0.025	−0.038 ± 0.010 (−16)[d]
	7.45		0.303 ± 0.025	+0.157 ± 0.007 (+52)[c]
TES	7.0	7.5	0.423 ± 0.018	−0.022 ± 0.003 (−5)[e]
	7.45		0.340 ± 0.025	+0.112 ± 0.025 (+33)[e]
HEPES	7.0	7.6	0.390 ± 0.027	+0.127 ± 0.012 (+33)[c]
	7.45		0.385 ± 0.030	+0.065 ± 0.015 (+17)[d]
imidazole	7.0	7.0	0.297 ± 0.017	−0.028 ± 0.008 (−9)[d]
	7.45		0.425 ± 0.037	−0.103 ± 0.028 (−24)[d]

[a] Results are mean ± SE of enzyme activity in three experiments performed in duplicate. The concentration of each buffer was 25 mM, and the ionic strength of each assay medium was the same. Basal activity was that without exogenous calmodulin. [b] Results show change in enzyme activity with addition of 6 nM exogenous calmodulin (20 ng/μg of membrane protein). Figures in parentheses show percent change from basal activity. [c] $P < 0.001$, calculated by paired *t* test, comparing activity with and without calmodulin. [d] $P < 0.02$, paired *t* test. [e] $P < 0.01$, paired *t* test.

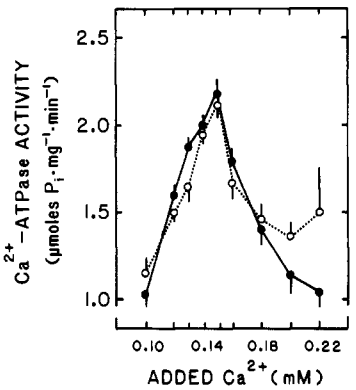

FIGURE 2: Calcium dose–response of normal and dystrophic chicken muscle SR Ca^{2+}-ATPase activity. Data from normal and dystrophic membranes are indicated by closed and open circles, respectively. Maximal response was seen with both enzymes at an added calcium concentration of 0.15 mM (12 μM free calcium by calcium electrode). The free calcium concentration, calculated by computer program, ranged from 1.2 to 46.6 μM in the samples shown in this figure. The EGTA concentration was 0.1 mM in all samples.

Ca^{2+} Optima of Ca^{2+}-ATPases. The possibility that the known increase in dystrophic cytoplasmic Ca^{2+} concentration may alter Ca^{2+}-ATPase responsiveness to exogenous Ca^{2+} was investigated. Figure 2 depicts the effect of a range of in vitro Ca^{2+} concentrations on enzyme activity in crude normal and dystrophic SR-enriched membranes. The optimal Ca^{2+} concentration for both normal and dystrophic Ca^{2+}-ATPase activities was 12 μM free Ca^{2+} [measured by calcium electrode and confirmed by a computer program adapted from Scharff (1979)], achieved with an added Ca^{2+} concentration of 0.15 mM. Calcium dose response studies were also conducted with normal and dystrophic SR membranes in the presence and absence of exogenous calmodulin, 6 nM. The addition of exogenous calmodulin did not alter the Ca^{2+} optimum in either normal or dystrophic membranes (results not shown).

Table III: Effect of Exogenous Calmodulin on Ca^{2+}-ATPase Activity of Crude and Purified Muscle SR Vesicles from Normal and Dystrophic Chickens[a]

	Ca^{2+}-ATPase act. (μmol of P_i mg^{-1} min^{-1})	
	basal	change with calmodulin
normal crude	0.802 ± 0.097	+0.250 ± 0.030[c]
normal Ca^{2+} loaded	1.097 ± 0.118	+0.217 ± 0.052[d]
dystrophic crude	1.013 ± 0.187	−0.112 ± 0.025[d]
dystrophic Ca^{2+} loaded	0.345 ± 0.070	−0.017 ± 0.013

[a] Crude membranes and purified vesicles were prepared by the method of Hanna et al. (1981); purified SR vesicles were those capable of taking up calcium phosphate in the presence of ATP (Ca^{2+} loaded). [b] Results shown are mean ± SE of four experiments conducted in duplicate, utilizing tissue from two pairs of normal and dystrophic chickens. Basal activity is that without added purified calmodulin. Calmodulin concentration was 6 nM, or 20 ng/μg of membrane protein. [c] $P < 0.001$, calculated by paired t test, comparing activity with and without exogenous calmodulin. There was no significant difference between normal crude and Ca^{2+}-loaded membranes in the percent change in activity with calmodulin addition. [d] $P < 0.01$, paired t test.

Response of Purified SR Vesicles to Exogenous Calmodulin. Normal and dystrophic purified SR vesicles capable of Ca^{2+} loading were assayed for calmodulin-stimulatable Ca^{2+}-ATPase activity. The response to exogenous calmodulin in these membranes, shown by Na,K-ATPase assay to be virtually devoid of sarcolemmal vesicles (Table I), is similar to the responses of the more crude membrane preparations, and is shown in Table III. The normal enzyme is calmodulin-stimulatable, while the dystrophic enzyme is unaffected or inhibited by calmodulin. We have also determined (results not shown) that the concentrations of added Ca^{2+} (0.15 mM), Mg^{2+} (1 mM), and ATP (1 mM) in our assay are optimal concentrations for these purified vesicles as well as for the crude vesicle preparations.

Muscle Ca^{2+} Concentrations. Intracellular Ca^{2+} concentrations in pectoralis major muscle from four normal and four dystrophic animals were measured. Dystrophic tissue contained 6.4 ± 0.7 and normal muscle 4.1 ± 0.5 μmol of Ca^{2+}/g of Lowry protein ($P < 0.04$, ANOVA).

Muscle Calmodulin Concentrations. Calmodulin concentrations in pectoralis major muscle from six normal and six dystrophic animals contained 0.97 ± 0.15 and 1.77 ± 0.23 μg of calmodulin/mg of protein, respectively ($P < 0.02$, ANOVA). These figures confirm our earlier studies (Hudecki et al., 1986).

Discussion

It is generally acknowledged that skeletal muscle SR membranes contain calmodulin and calmodulin-responsive enzymes [protein kinases (Chiesi & Carafoli, 1982, 1983; Campbell & MacLennan, 1982); phosphatase (Chiesi & Carafoli, 1983)]. Normal mammalian skeletal muscle SR Ca^{2+}-ATPase, however, is regarded as unresponsive to the calmodulin–Ca^{2+} complex (Chiesi & Carafoli, 1982), and an important aspect of the present experiments was to establish securely that normal avian skeletal muscle SR Ca^{2+}-ATPase was calmodulin-stimulatable. Because assay buffer composition can importantly affect the behavior of Ca^{2+}-ATPase activity (Davis et al., 1983), we compared normal chicken SR Ca^{2+}-ATPase response in vitro to calmodulin in a variety of buffers used in Ca^{2+}-ATPase assays. Buffer pK's ranged from 7.0 to 8.1. In MOPS buffer, pH 7.0, used in studies of mammalian SR by Chiesi and Carafoli (1982), we found Ca^{2+}-ATPase activity in avian muscle to be unresponsive to calmodulin, whereas the enzyme was calmodulin-stimulatable in MOPS buffer at pH 7.45. A variety of other buffers (Table II) had selective pH-dependent effects on the response to calmodulin of avian muscle SR Ca^{2+}-ATPase. The mechanism by which Tris, and to a lesser extent HEPES, permits expression of the calmodulin response of Ca^{2+}-ATPase in avian striated muscle SR membranes at both pH 7.0 and pH 7.45 is unknown. It has been suggested that Tris may determine the oligomeric state of the SR enzyme[2] and alter access of the Ca^{2+}–calmodulin complex to the Ca^{2+}-ATPase. Regardless of the mechanism, calmodulin responsiveness of Ca^{2+}-ATPase in normal avian SR was demonstrable under a variety of experimental conditions.

When we proceeded to study dystrophic Ca^{2+}-ATPase activity, however, the latter was found to be unresponsive to calmodulin. Thus, an important attribute of the enzyme in dystrophic muscle was absent. It was next established that the Ca^{2+}-ATPase which was studied in the SR-enriched muscle membrane preparation from normal and dystrophic animals was indeed of SR origin.

The SR preparation of Hanna et al. (1981) used in the present studies has been partially characterized in terms of enzyme markers and includes sarcolemmal and T-tubule membranes that contain Ca^{2+}-ATPase activity. T-tubule Ca^{2+}-ATPase activity, however, is minimal (Michalak et al., 1981) and is stimulatable by millimolar concentrations of Ca^{2+}, in contrast to the ATPase activity reported in our studies which is maximal at micromolar levels of Ca^{2+}, consistent with predominantly sarcoplasmic reticulum origin of enzyme activity (Malouf & Meissner, 1979). Enzyme markers monitored in our crude membrane samples suggest that our preparations are nonetheless heterogeneous, with a 2-fold enhancement in Ca^{2+}-ATPase specific activity relative to Mg^{2+}-ATPase or 5′-nucleotidase activities. For that reason, we further purified the membranes by Ca^{2+} loading, resulting in exclusion of Na,K-ATPase activity, and thus of sarcolemma. The purified normal and dystrophic SR vesicles, capable of Ca^{2+} loading in the presence of ATP, exhibited the disparate Ca^{2+}-ATPase responses of crude membrane preparations to exogenous calmodulin.

We also investigated the possibility that the Ca^{2+}-ATPases in normal and dystrophic avian muscle responded differently to calmodulins which originated in different species. The responses of normal and dystrophic Ca^{2+}-ATPase to calmodulin from bovine brain, and normal or dystrophic avian muscle, were similar and supported our previous finding in the human red cell Ca^{2+}-ATPase model (Hudecki et al., 1986) that the calmodulins from both normal and dystrophic muscle are equally active in a calmodulin bioassay system.

We have found dystrophic SR membrane calmodulin content to be 80% increased over that of normal membranes. Endogenous calmodulin concentrations are calculated to be approximately 0.04 and 0.07 nM in normal and dystrophic enzyme assay samples, respectively.[3] Addition of up to 30 nM calmodulin to normal SR membranes has not caused significant inhibition of Ca^{2+}-ATPase activity. Thus, the difference in response to exogenous calmodulin cannot be explained by endogenous membrane calmodulin concentration. Calmodulin is not the sole Ca^{2+} binding protein to be increased in concentration in dystrophic muscle; spectrin (Repasky et al., 1986), which binds Ca^{2+} and calmodulin, and Ca^{2+}-activated neutral protease (Sugita et al., 1982), which contains two calmodulin domains (Suzuki et al., 1987), are both in-

[2] J. Froehlich, personal communication.

[3] J. Galindo and M. Schoenl, unpublished observations.

creased in avian dystrophic muscle.

The pathogenesis of the avian model of muscular dystrophy is not established. In the Duchenne form of human dystrophy, a protein, dystrophin, important to excitation–contraction coupling is reduced in amount in affected muscle (Hoffman et al., 1987). Avian dystrophy, however, is not regarded as a model of Duchenne dystrophy, and affected chicken muscle contains normal amounts of dystrophin.[4] The present studies suggest that the pathogenesis of avian dystrophy includes loss of regulation by calmodulin of SR Ca^{2+} handling in affected muscle.

The physiologic relevance of the resistance of dystrophic avian muscle Ca^{2+}-ATPase to calmodulin is supported by sensitive studies of muscle strength, based on strain gauge measurements,[5] which indicate decreased function in dystrophic muscle by 18 days post-hatch; recent studies in our laboratories have shown that the calmodulin sensitivity of Ca^{2+}-ATPase is decreased and the calmodulin mRNA content of affected muscle significantly increased by 13 days ex ovo (Thacore et al., 1988). These changes occur well in advance of loss of the righting reflex and increases in serum creatine phosphokinase activity which are observed at 30–40 days in dystrophic animals (Cosmos et al., 1980; Hudecki et al., 1984).

The relationship of poorly regulatable SR Ca^{2+}-ATPase in dystrophy to the accumulation of cell Ca^{2+} and the recently demonstrated increase in expression of the calmodulin gene is speculative. Increased calmodulin mRNA in affected muscle of line 413 chickens may be due to increased cell Ca^{2+} concentration or to cell efforts to "compensate" for calmodulin unresponsiveness of Ca^{2+}-ATPase, or perhaps of kinases involved in contraction (Chiesi & Carafoli, 1983).

The mechanism by which calmodulin responsiveness of SR Ca^{2+}-ATPase is suppressed is not known. Membrane lipid composition is altered in dystrophic muscle (Kawamoto & Baskin, 1983; Hsu & Kaldor, 1971), and studies of mammalian skeletal muscle membrane Ca^{2+}-ATPase and of plasma membrane Ca^{2+}-ATPase from red cells have emphasized the importance of the lipid microdomain in control of ATPase activity (Kawamoto & Baskin, 1986; Niggli et al., 1981; Cheng & Hui, 1986). Recently, Kawamoto and Baskin (1986) have demonstrated an increase in free fatty acids and triglycerides in purified SR vesicles from dystrophic muscle, as compared to normal, as well as dissociation of Ca^{2+}-ATPase activity and Ca^{2+} transport. We have also shown that calmodulin responsiveness of human red cell Ca^{2+}-ATPase is modulated by fatty acids (Davis et al., 1987). Thus, altered calmodulin effect in dystrophic muscle Ca^{2+}-ATPase may result from primary changes in the lipid environment of the enzyme. We were unable to demonstrate a difference in the Ca^{2+} concentration response of normal and dystrophic SR Ca^{2+}-ATPase activities, indicating that altered Ca^{2+} kinetics of the enzyme do not contribute to the change in behavior of the dystrophic muscle enzyme.

We have preliminarily examined the possibility that unresponsiveness to calmodulin in dystrophic avian muscle exists in other enzyme systems. Sarcoplasmic protein kinase activity is enhanced by calmodulin in normal muscle in vitro, but phosphorylation in dystrophic muscle is not stimulated by calmodulin.[6] Thus, the Ca^{2+}-ATPase observations reported here may reflect a generalized phenomenon of calmodulin resistance in avian dystrophic muscle.

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REFERENCES

Arkesteijn, C. L. M. (1976) *J. Clin. Chem. Clin. Biochem. 14*, 155–158.

Campbell, K. P., & MacLennan, D. H. (1982) *J. Biol. Chem. 257*, 1238–1246.

Chafouleas, J. G., Dedman, J. R., Munjaal, R. P., & Means, A. R. (1979) *J. Biol. Chem. 254*, 10262–10267.

Charbonneau, H., Hice, R., Hart, R. C., & Cormier, M. J. (1983) *Methods Enzymol. 102*, 17–39.

Cheng, K. H., & Hui, S. W. (1986) *Arch. Biochem. Biophys. 244*, 382–386.

Chiesi, M., & Carafoli, E. (1982) *J. Biol. Chem. 257*, 984–991.

Chiesi, M., & Carafoli, E. (1983) *Biochemistry 22*, 985–993.

Cosmos, E., Butler, J., Mazliah, J., & Allard, E. P. (1980) *Muscle Nerve 3*, 252–262.

Davis, F. B., Davis, P. J., & Blas, S. D. (1983) *J. Clin. Invest. 71*, 579–586.

Davis, F. B., Davis, P. J., Blas, S. D., & Schoenl, M. (1987) *Biochem. J. 248*, 511–516.

Davis, P. J., & Blas, S. D. (1981) *Biochem. Biophys. Res. Commun. 99*, 1073–1080.

Davis, P. J., & Bernardis, L. L. (1984) *Metab., Clin. Exp. 33*, 591–595.

Ettienne, E. M., & Singer, R. H. (1978) *J. Membr. Biol. 44*, 195–210.

Hanna, S. D., & Baskin, R. J. (1978) *Biochim. Biophys. Acta 540*, 144–150.

Hanna, S. D., Kawamoto, R., McNamee, M., & Baskin, R. J. (1981) *Biochim. Biophys. Acta 643*, 41–54.

Hoffman, E. P., Brown, R. H., Jr., & Kunkel, L. M. (1987) *Cell (Cambridge, Mass.) 51*, 919–928.

Hsu, Q.-S., & Kaldor, G. (1971) *Proc. Soc. Exp. Biol. Med. 138*, 733–737.

Hudecki, M. S., Pollina, C. M., Bhargava, A. K., Hudecki, R. S., & Heffner, R. R. (1979) *Muscle Nerve 2*, 57–67.

Hudecki, M. S., Pollina, C. M., & Heffner, R. R. (1981) *J. Clin. Invest. 671*, 969–974.

Hudecki, M. S., Pollina, C. M., & Heffner, R. R. (1983) *J. Neurol. Sci. 60*, 55–66.

Hudecki, M. S., Pollina, C. M., & Heffner, R. R. (1984) *Exp. Neurol. 84*, 512–523.

Hudecki, M. S., Kibler, P. K., Pollina, C. M., Thacore, H. R., Davis, P. J., & Davis, F. B. (1986) *Biochem. Biophys. Res. Commun. 137*, 507–512.

Itano, T., & Penniston, J. T. (1985) in *Calmodulin Antagonists and Cellular Physiology* (Hidaka, H., & Hartshorne, D. J., Eds.) pp 335–344, Academic, San Diego.

Kawamoto, R. M., & Baskin, R. J. (1983) *Biochim. Biophys. Acta 732*, 620–626.

Kawamoto, R. M., & Baskin, R. J. (1986) *Muscle Nerve 9*, 248–256.

Lowry, P., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Malouf, N. N., & Meissner, G. (1979) *Exp. Cell Res. 122*, 233–250.

[4] E. Hoffman, L. Kunkel, and M. Hudecki, unpublished observations.

[5] M. Hudecki and C. Pollina, unpublished observations.

[6] M. Schoenl, P. Davis, W. Lawrence, and F. Davis, unpublished observations.

Meissner, G. (1986) *Biochemistry 25*, 244-251.
Michalak, M., Famulski, K., & Carafoli, E. (1984) *J. Biol. Chem. 259*, 15540-15547.
Niggli, V., Adunyah, E. S., & Carafoli, E. (1981) *J. Biol. Chem. 256*, 8588-8592.
Repasky, E. A., Pollina, C. M., Menold, M. M., & Hudecki, M. S. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 802-806.
Scales, D., Sabbadini, R., & Inesi, G. (1977) *Biochim. Biophys. Acta 465*, 535-549.
Scharff, O. (1979) *Anal. Chim. Acta 109*, 291-305.
Scharff, O. (1981) *Cell Calcium 2*, 1-27.
Stryer, L., & Bourne, H. R. (1986) *Annu. Rev. Cell Biol. 2*, 391-419.
Sugita, H., Kimura, M., Tarumoto, Y., Tamai, M., Hanada, K., Ishiura, S., Nonaka, I., Ohzeki, M., & Imahori, K. (1982) *Muscle Nerve 5*, 738-744.
Suzuki, K. (1987) *Trends Biochem. Sci. (Pers. Ed.) 12*, 103-105.
Thacore, H. R., Kibler, P. K., Hudecki, M. S., Galindo, J., Jr., Davis, F. B., Davis, P. J., Pollina, C. M., Blas, S. D., & Schoenl, M. (1988) *Biochem. Biophys. Res. Commun. 151*, 1434-1440.
Tovar, A. M., & Verjovski-Almeida, S. (1983) *Mol. Cell. Biochem. 56*, 39-48.
Verjovski-Almeida, S., & Inesi, G. (1979) *Biochim. Biophys. Acta 558*, 119-125.

Molecular Mechanisms Responsible for the Drug-Induced Posttranscriptional Modulation of Ribonucleotide Reductase Levels in a Hydroxyurea-Resistant Mouse L Cell Line†

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ABSTRACT: Ribonucleotide reductase, which catalyzes the formation of deoxyribonucleotides from ribonucleoside diphosphate precursors, is the rate-limiting enzyme in DNA synthesis. The enzyme consists of two nonidentical subunits called M1 and M2, both of which are required for activity. Hydroxyurea, a specific inhibitor of DNA synthesis, acts by destroying the unique tyrosyl free radical of protein M2. Previously, we have described a mouse L cell line which exhibited a stable resistance to high concentrations of hydroxyurea. This mutant cell line contains elevated quantities of both proteins M1 and M2 as a result of corresponding increases in the levels of mRNAs for both subunits. Interestingly, both M1 and M2 protein levels were further elevated when mutant cells were cultured in the presence of hydroxyurea, and this elevation was not accompanied by increases in their corresponding mRNAs. These results indicated that hydroxyurea can modulate ribonucleotide reductase expression posttranscriptionally. In this report, we show that the level of both subunits of ribonucleotide reductase responds to hydroxyurea in a drug concentration dependent manner. Furthermore, results from kinetic studies indicate that protein M2 levels rise much more rapidly than protein M1. Pulse–chase experiments indicated that the half-lives of both the M1 and M2 polypeptides are increased by approximately 2-fold when the mutant cells are cultured in the presence of hydroxyurea. We also present evidence indicating that exposure of these cells to hydroxyurea leads to a relatively slow but specific increase in the rate of biosynthesis of both proteins M1 and M2, as assayed by pulse labeling. Therefore, we conclude that both components of ribonucleotide reductase are synthesized at an increased rate and turn over at a slower rate when these mutant cells are grown in the presence of hydroxyurea. In addition, experiments were performed to examine the effects of exogenously added iron on the biosynthesis of proteins M1 and M2. Interestingly, the results suggested a role for iron in regulating the level of M2 protein when cells are cultured in hydroxyurea-supplemented medium.

Mammalian ribonucleotide reductase is a highly regulated enzyme that is responsible for the conversion of ribonucleotides to their corresponding deoxyribonucleotides, the precursors of DNA synthesis (Thelander & Reichard, 1979; Wright, 1988). This reaction is a rate-limiting step in DNA synthesis, and, therefore, the enzyme plays an important role in the regulation of cell division. In mammalian cells, the enzyme consists of two nonidentical subunits called M1 and M2, both of which have been purified to homogeneity (Thelander et al., 1980, 1985). The M1 protein is a dimer of molecular weight 170 000, and it contains the binding sites for nucleoside triphosphates which act as allosteric effectors (Thelander et al., 1980). The M2 protein is a dimer of molecular weight 88 000, and it contains stoichiometric amounts of a non-heme iron center and a tyrosyl free radical essential for activity (Thelander et al., 1985). The two subunits are differentially regulated during the cell cycle with reductase activity being controlled by the S-phase-dependent synthesis of protein M2

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