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Changes in Thiol Reactivity and Extractability of Myofibril Bound Cardiac Troponin C in Porcine Malignant Hyperthermia[1]

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We recently showed that a shift (~44%) in cardiac myosin isozyme from V3 to V1 occurs in the hearts of malignant hyperthermia (MH)–susceptible pigs and may be important in the disease. To follow up this finding, we investigated whether this myosin isozyme shift results in conformational changes in cardiac troponin C (cTnC). The two cysteine residues (Cys-35 and Cys-84) in the regulatory domain of cTnC make it possible to attach conformational probes to this region. Incorporation of a fluorescent probe, 7-diethylamino-3-(4′maleimidylphenyl)-4-methylcoumarin (CPM), into myofibril-bound cTnC was measured by alkaline urea gel electrophoresis, followed by quantification of the protein and the fluorescent label on the gels. The structural stability of cTnC incorporated into cardiac myofibrils was compared between normal and MH-susceptible pigs by selective cTnC extraction and re-incorporation. Changes were detected in both the reactivity of cTnC with CPM in rigor myofibrils and cTnC incorporation into myofibrils from the hearts of MH-susceptible pigs. These changes are very likely to be a consequence of the cardiac myosin isozyme shift in the hearts of MH-susceptible pigs, which may contribute to the changes in the myofilament response to Ca^{2+} binding and to the modulation of cardiac contractility seen in this disease.

Key words: conformational changes in cardiac troponin C (cTnC), cysteine residues (Cys-35 and Cys-84), fluorescent label, malignant hyperthermia (MH), myofibril incorporation.

Typical episodes of malignant hyperthermia (MH), which include hyperthermia, muscle rigidity and metabolic acidosis, can be triggered by anesthetics, such as halothane (*1, 2*). During MH crisis, tachycardia, increased myocardial contractility, and reduced energetic efficiency are seen, together with a general increase in blood catecholamine levels (*3*). However, it is still controversial whether the cardiac symptoms are due to a primary disorder in cardiac muscle (*4*) or to a secondary malfunction as a result of increased plasma catecholamine levels (*5*). The major defect in the affected skeletal muscle, an abnormal increase in myoplasmic Ca^{2+} levels, is attributed to a single base substitution (Hal-1843) in both alleles of the gene encoding the RyR1 form of the ryanodine receptor in the skeletal sarcoplasmic reticulum (SR) (*2*). In contrast, the cardiac SR membrane contains a different type of ryanodine receptor (RyR2) with quite different ryanodine binding properties from those of RyR1 (*6*). As shown by Ervasti *et al.* (*7*), SR vesicles isolated from the hearts of MH-susceptible and normal pigs have similar ryanodine binding properties and kinetic behavior. Thus, the changes seen in myocardial functions in MH seem to be indirect and secondary to the overall change in global metabolism caused by the RyR1 defect in the skeletal muscle of susceptible animals.

In a previous study (*8*), we showed that a shift (~44%) in the cardiac myosin isozyme from V3 to V1 occurs in the hearts of MH-susceptible pigs. This change in myosin isozyme distribution would be expected to affect the protein–protein interactions between other myofilament proteins that occur during cardiac Ca^{2+} activation. Myosin cross-bridge attachment to the thin filament can modify the structure and Ca^{2+} binding affinity of cardiac troponin C (cTnC) (*9*), a protein in which Ca^{2+} binding acts as a switch to permit actin-myosin interactions (*10*). We therefore postulated that a myosin isozyme shift might cause conformational differences in myofibril-bound cTnC in the hearts of MH-susceptible animals.

Two thiol groups, Cys-35 and Cys-84, are present in the regulatory region of cTnC (*11*), and the thiol reactivity of cTnC with Ca^{2+}-sensitive conformational probes indicates that Ca^{2+}-induced structural changes occur in the immediate vicinity of these groups (*12, 13*). In addition, studies using a divalent cation chelator to extract cTnC from myofibrils, in which the degree of cTnC extraction reflects the structural stability of cTnC incorporated into the thin filament, have provided information about the cooperative interactions between cTnC and other thin filament proteins in cardiac muscles (*14*). Since structural alterations in

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cTnC might affect the myofilament response to Ca^{2+} activation in the myocardium, which could lead to the increased myocardial contractility seen in MH crisis, we have used the thiol reactivity of cTnC with CPM and selective cTnC extraction from, and re-incorporation into cardiac myofibrils to examine whether these cTnC conformational changes associated with Ca^{2+} binding and protein-protein interactions occur in MH-susceptible hearts.

MATERIALS AND METHODS

Experimental Animals—All pigs used in the study (8 Landrace: 3 MH-positive and 5 normal; 6 Duroc: 2 MH-positive and 4 normal) were raised at the Taiwan Livestock Research Institute (Tainan, Taiwan). Two different breeds of pigs were used in order to increase the number of MH-susceptible animals, since the incidence of porcine MH is low. However, in all comparative studies, pigs of the same breed were used to exclude crossbreed difference.

MH susceptibility in Duroc and Landrace pigs was determined by having the animals inhale a mixture of 3% halothane, 2 liters/min of oxygen and 1 liter/min of nitrous oxide for 3 minutes, as described by Chang *et al.* (*15*). At the time of testing, animals were 8–12 weeks of age and weighed between 10 and 25 kg. Any piglet that developed muscle rigidity in any limb for 10 s during halothane inhalation was classified as MH-susceptible. After the test, all animals were maintained under the usual laboratory conditions recommended by the Animal Care and Use Committee of Taiwan Livestock Research Institute until sacrifice. A time period of 1–8 months elapsed between halothane exposure and experimental analysis.

Porcine MH has been linked to a single base substitution (Hal-1843) in both alleles of the gene encoding the skeletal muscle ryanodine receptor (*16*). The technique known as the mutagenically separated polymerase chain reaction (MS-PCR) was used to detect the genotype of the MH gene, as described by Lockley *et al.* (*17*).

Preparation of Cardiac Troponin C (cTnC) and Myofibrils—The pigs (Landrace: body weight 46.6–88 kg, age 5–11 months; Duroc: body weight 23.6–32 kg, age: 3–4 months) were anesthetized by intravenous injection of thiopental sodium (15 mg/kg, Pentothal, Abbott Australasia, Sydney, Australia) and sacrificed by bleeding from the carotid artery. The hearts were then immediately removed, placed on ice, and delivered to the laboratory.

cTnC was prepared from the left ventricle of the hearts according to Szynkiewicz *et al.* (*18*) and stored freeze-dried in a freezer (–80°C). Before use, the freeze-dried cTnC was dissolved in 1 ml of 100 mM MOPS buffer, pH 7.0, containing 50 mM KCl and 2 mM EGTA, and its concentration was determined on the basis of an extinction coefficient (1 mg/ml, at 276 nm) of 0.3 in the presence of EGTA using an Hitachi U-2000 double beam spectrophotometer.

Cardiac myofibrils were prepared at 4°C according to Liou *et al.* (*19*). The isolated myofibrils were dissolved in 100 mM MOPS, pH 7.0, 90 mM KCl, 2 mM EGTA containing 50 % glycerol and stored in a freezer (–20°C) until use. The bicinchoninic acid reagent (Pierce, Rockford, IL) was used to determine myofibrillar protein concentrations, using bovine serum albumin as a standard.

Measurements of Myofibrillar ATPase Activity—The actomyosin ATPase activity was measured by suspending myofibrils (~0.2 mg) in 10 mM MOPS (pH 7.0), 90 mM KCl, 5 mM $MgCl_2$, 2 mM EGTA, and various additions as indicated. The reaction was initiated by the addition of 1 mM MgATP. The reaction mixtures were shaken in a water bath at controlled temperature for 10 minutes. ATP hydrolysis was stopped by the addition of malachite green reagent containing 33% malachite green, 16.7% polyvinyl alcohol, and 16.7% ammonium molybdate (*20*). The levels of inorganic phosphate released were determined by measuring the absorbance at 630 nm with a spectrophotometer (Hitachi U-2000). A calibration curve was constructed with KH_2PO_4 ranging from 20 to 200 nmol.

To test whether different Ca^{2+}-dependent regulation of the actin–myosin interaction occurs in the hearts of normal and MH-susceptible pigs, the steepness of the Ca^{2+}- ATPase activity relationship was measured in the two types of cardiac myofibrils. The measured ATPase activity at the actual pCa (–log $[Ca^{2+}]$) was subtracted from the activity at pCa 8.0. The subtracted activity (T_x) was normalized to the activity value (T_0) at saturating pCa. If normalized ATPase ($U = T_x/T_0$) is used, then a straight line is obtained with the expression of $\log[U/(1 - U)]$ versus the logarithm of the Ca^{2+} concentration. The data of this plot was fitted to the Hill equation:

$$\log[U/(1 - U)] = n(\log[Ca_x]) + \log k$$

where $[Ca_x]$ is the actual Ca^{2+} concentration, n (Hill coefficient) is the slope, and k is the x-axis intercept of the fitted line. The Hill coefficient is a measure of cooperativity of the Ca^{2+}-activated ATPase activity. By using the constants derived from the Hill equation, the curves of the normalized ATPase activity (T_x/T_0) versus pCa were fit by computer to the equation:

$$(T_x/T_0) = [Ca_x]^n/((EC_{50})^n + [Ca_x]^n)$$

where EC_{50} is the Ca^{2+} concentration that gives 50% activation of ATPase.

The pCa values were calculated by the computer program EQCAL (Biosoft, Cambridge, UK) with constants tabulated by Fabiato and Fabiato (*21*). Under our experimental conditions, the apparent stability constant for CaEGTA was taken to be 7.76×10^{10} M^{-1}.

CPM Labeling of Isolated and Myofibril-Bound cTnC—The two thiol groups of purified cTnC were first reduced by overnight incubation at 4°C with 2 mM dithiothreitol (DTT) in 8 M urea, 100 mM MOPS, pH 7.0, 50 mM KCl, 2 mM EGTA. Following exhaustive dialysis at 4°C, the reduced cTnC was reacted at room temperature with a 2- to 3-fold molar excess of 7-diethylamino-3-[4′-maleimidylphenyl]-4-methyl-coumarin (CPM), a compound that only emits fluorescence when covalently attached to an SH group, following the method of Fuchs *et al.* (*12*). Excess DTT, added to terminate the CPM labeling, was removed by solvent exchange using Centricon 10 (Amicon Corp., Danvers, MA) ultrafiltration cells. The protein was then dialyzed once against 25 mM Tris-HCl, pH 7.5, containing 6 M urea and twice against 50 mM Tris-HCl buffer, pH 7.5. The concentrations of labeled cTnC and the amount of CPM bound to cTnC were measured using, respectively, bicinchoninic acid with bovine serum albumin as a standard or an extinction coefficient at 387 nm of 28,000 M^{-1} cm^{-1}.

The labeling of the two thiol groups in the regulatory region of cTnC in myofibrils was measured by reacting the

two types of cardiac myofibrils with CPM under conditions in which the Ca^{2+} concentration and cross-bridge attachment could be experimentally controlled, *i.e.*, under conditions of rigor, relaxation and cross-bridge cycling. To induce rigor, myofibrils were suspended in a solution containing 100 mM MOPS, pH 7.0, 90 mM KCl, 5 mM $MgCl_2$, 2 mM EGTA, with or without 2.1 mM Ca^{2+}. To induce cycling of cross-bridges, 5 mM MgATP and various amounts of Ca^{2+} were added to the rigor solution, while, to induce relaxation, 5 mM MgATP and 1 mM sodium vanadate (V_i), a phosphate analogue which, in the presence of MgATP, suppresses cross-bridge interactions at all Ca^{2+} concentrations (*22*), were added to the rigor solution. In all cases, the myofibrils (~25 mg) were pre-treated overnight at 4°C with DTT (approximately 5 mM) to reduce all protein thiol groups. After three washes with 10 mM MOPS buffer, 90 mM KCl, 5 mM $MgCl_2$, 2 mM EGTA, pH 7, to remove DTT, the myofibrils were suspended in various solutions, then reacted for 30 minutes at 25°C with CPM (20–40 μM). CPM labeling was terminated by adding excess DTT (2 mM), after which the myofibrils were washed and dissolved in 8 M urea, 0.2 M KCl, 25 mM Tris-OH, pH 8.3. The extracted myofibrillar proteins were exhaustively dialyzed once against 25 mM Tris-HCl, pH 7.5, containing 6 M urea, then twice against 50 mM Tris buffer, pH 7.5, containing 0.2 M KCl. Non-specific fluorescence was removed by DEAE ion-exchange chromatography.

SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)—SDS-PAGE was performed as described by Laemmli (1970). Gels were stained with Coomassie Blue or silver stains. For the latter procedure, gels were first fixed with 12% trichloroacetic acid/50% methanol for 30 min, then with 10% ethanol/5% acetic acid for 20 min. After fixation, the gels were pre-treated with 0.057% (w/v) ammonium persulfate for 20 min, then stained with 0.1% (w/v) silver nitrate for 1 h, developed in 2% sodium carbonate (w/v)/0.05% formaldehyde (v/v), then soaked in 1% acetic acid.

To quantify fluorescence labeling, the unstained gels were photographed using a Kodak Digital Science DC 40 camera (Eastman Kodak Company, Rochester, NY) with UV transillumination and an appropriate cutoff. The gels were then stained with Coomassie Blue and photographed. A computer program with Scion Image Analysis software (Scion Corporation, Frederick, Maryland, USA) was used to analyze fluorescence and protein content; relative CPM incorporation was expressed as the fluorescence/protein ratio.

Alkaline Urea Gel Electrophoresis—Alkaline urea–polyacrylamide gel electrophoresis, as described by Blanchard and Solaro (*23*), was used to separate cTnC from other myofibrillar proteins. The slab gels consisted of 7.5% acrylamide (0.8% bis), 25 mM Tris-OH, pH 8.3, 192 mM glycine, 8 M urea. The protein samples, dialyzed against 25 mM Tris-OH, pH 8.3, containing 8 M urea, were mixed with 2 volumes of sample buffer (8 M urea, 2 mM EGTA, 10 mM β-mercaptoethanol, 2.5% glycerol, and 0.01% bromophenol blue) and electrophoresed toward the anode for 4–5 h at 25 mA (constant current) in the dark. The electrode buffer was 25 mM Tris-OH, pH 8.3, 192 mM glycine. Gel scanning was performed as described above.

Extraction of cTnC from, and Its Re-Incorporation into, Cardiac Myofibrils—The endogenous cTnC in porcine cardiac myofibrils was extracted with 40 mM Tris buffer, pH 8.4, containing 5 mM CDTA (trans-1,2-cyclohexanediamine-*N,N,N',N'*-tetraacetic acid), according to Morimoto and Ohtsuki (*24*), and the degree of extraction was determined by the SDS-PAGE and by the loss of Ca^{2+}-activated ATPase activity.

Exogenous cTnC was incubated with CDTA-extracted myofibrils at various amounts of cTnC (μg) per mg of myofibrillar protein. The myofibrils were suspended for 1 h at 25°C in 100 mM MOPS, pH 7.0, 90 mM KCl, 5 mM $MgCl_2$, 0.1 mM free Ca^{2+}, then centrifuged at 2,000 ×*g* to remove free cTnC; the pellet was resuspended in the same buffer and the centrifugation was repeated twice more. Alkaline urea-polyacrylamide gel electrophoresis and the restoration of Ca^{2+}-activated ATPase activity were used to quantify the degree of cTnC re-incorporation into extracted cardiac myofibrils.

Reagents—Unless otherwise specified, all reagents used were ACS grade and purchased from Sigma (Sigma, MO, USA). Electrophoresis reagents were purchased from Amersham Pharmacia Biotech Asia Pacific (Hong Kong). CPM was purchased from Molecular Probes (Eugene, OR, USA).

Statistics—Quantitative values are presented as the mean ± SEM. Statistical comparisons were made using non-paired Students *t* test, with a p value <0.05 regarded as significant.

RESULTS

Myofibrillar ATPase Activity—Recently, we have shown that a shift of cardiac myosin isozyme V3 to V1 (~44%) is associated with an increase in the cooperativity of the Ca^{2+}-activated myofibrillar ATPase with no change in the Ca^{2+} sensitivity (*8*). Table I summarizes the best-fit parameters (Hill coefficient, n; and Ca^{2+} sensitivity, pK_{ca}) for the Hill equation describing the relation of the normalized myofibrillar ATPase activity to pCa (–log [Ca^{2+}]) at temperatures from 20 to 30°C in two types of porcine hearts. Apparently, a greater cooperativity with no changes in Ca^{2+} sensitivity was observed in cardiac myofibrils of MH-susceptible pigs, as compared to normals.

Thiol Reactivity of Myofibril-Bound cTnC—The cardiac myofibrils of MH-susceptible pigs with substantial

TABLE I. **Parameters of the Hill equation relating relative myofibrillar ATPase to Ca^{2+} concentration in normal and MH-sensitive porcine hearts.** The measured myofibrillar ATPase activity at the actual Ca^{2+} concentration ([Ca_x]) was subtracted from the activity at 10^{-8} M Ca^{2+}. The subtracted activities (T_x) were normalized to the activity value (T_0) at saturating Ca^{2+}. The normalized ATPase activity (T_x/T_0) versus Ca^{2+} concentration was fitted to the Hill equation: $(T_x/T_0) = [Ca_x]^n/((EC_{50})^n + [Ca_x]^n)$, where, n is the Hill coefficient, and EC_{50} is the Ca^{2+} concentration giving 50% activation of ATPase ($pK_{ca} = \log[EC_{50}]$). Values are expressed as mean ± SEM Student's test was used to calculate p values. *Indicates a significant difference between the two myocardial preparations.

Temperatures	Hill coefficient (n)		pK_{ca}	
	MH	Normal	MH	Normal
20°C	1.96 ± 0.15	1.41 ± 0.16	6.10 ± 0.07	6.11 ± 0.11
	(*p = 0.03)		(p = 0.93)	
25°C	2.96 ± 0.28	2.31 ± 0.12	6.32 ± 0.04	6.33 ± 0.06
	(*p = 0.03)		(p = 0.86)	
30°C	2.24 ± 0.16	1.85 ± 0.10	6.56 ± 0.07	6.46 ± 0.07
	(*p = 0.05)		(p = 0.31)	

amounts of V1 myosin might cause changes in myofilament protein-protein interactions and produce a greater cooperativity of the contractile response to Ca^{2+}-binding. Since cTnC is essential for turning on Ca^{2+}-activated cyclic interactions of actin and myosin, we further determined whether a conformational change in myofibril-bound cTnC occurs in the hearts of MH-susceptible pigs. Experiments were performed to see whether skinned cardiac myofibrils from control and MH-susceptible pigs showed a difference in the reactivity of cTnC with a fluorescence probe, CPM. To check whether there is a linear relationship between fluorescence intensity and the number of CPM-labeled molecules and between protein staining and the amount of cTnC applied, various amounts of CPM-labeled cTnC were electrophoresed in urea gels. Figure 1A shows that both CPM labeling and cTnC staining in the gel were proportional to the amount of CPM-labeled cTnC loaded on the gel. A positive correlation was obtained between the staining intensity and the amount of cTnC (correlation coefficient: $R^2 = 0.75$), and between the CPM fluorescence and the amount of cTnC ($R^2 = 0.74$). Figure 1B shows that more CPM incorporation into isolated cTnC can be seen in the presence of 2.1 mM Ca^{2+} (pCa 4) than in its absence (pCa 8), demonstrating that the method detects a Ca^{2+}-dependent conformational change in cTnC.

A typical profile for the separation of CPM-labeled cTnC from other myofibrillar proteins in urea gels is illustrated in Fig. 2. The fluorescent and stained protein bands are shown in the upper sections of Fig. 2, A and B, respectively; the lower sections show densitometric scans of the same gels. The CPM labeling ratio was calculated by dividing the area of the fluorescence peak by the area of the protein peak.

To determine the stoichiometry of labeling, the myofibril protein was run in parallel with CPM-labeled cTnC standards with a labeling ratio of between 0.8 and 1.2 (mole CPM/mole cTnC). The quantitative results are shown in Fig. 3. At pCa 8, CPM incorporation into myofibril-bound cTnC in the rigor state was greater in MH-susceptible pigs (1.28 ± 0.19 mol CPM/mol cTnC) than in normal animals (0.76 ± 0.12 mol CPM/mol cTnC) (*p = 0.0002). Taking into account that both the V1 (~44%) and V3 (~56%) myosin isozymes are found in the hearts of MH-susceptible pigs, whereas only V3 myosin occurs in normal hearts (*8*), the

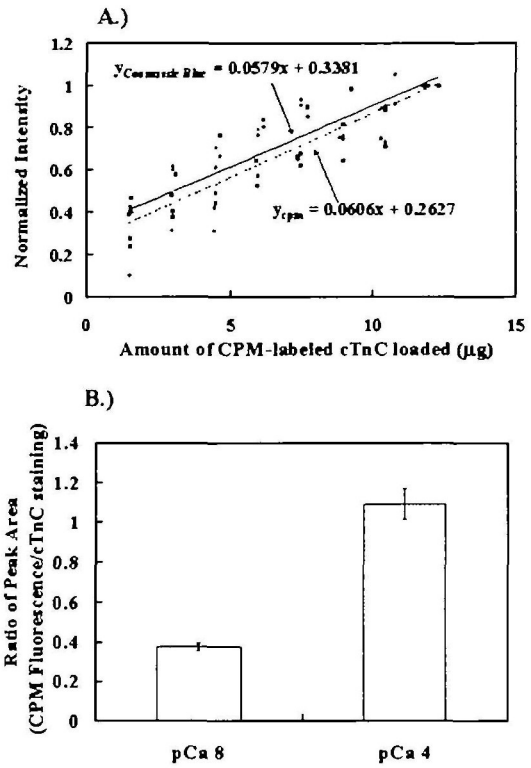

Fig. 1. **Quantitative analysis of CPM labeling of isolated cTnC by alkaline urea gel electrophoresis.** (A) Regression lines showing the quantitative relationship between protein staining (—) or CPM fluorescence labeling (---) and the amount of CPM-labeled cTnC loaded on the gel; the equation for each line is also given. Labeled cTnC was prepared as described in "MATERIALS AND METHODS" at a CPM/cTnC labeling ratio of 1.6–2.0. The gels were scanned for fluorescence (open squares) and protein staining (closeds diamonds) and the values correlated with the CPM and cTnC added to the gel. (B) Urea gel analysis of the Ca^{2+}-dependency of CPM labeling at pCa 4 and pCa 8. Each histogram shows the mean ± SEM of eight measurements.

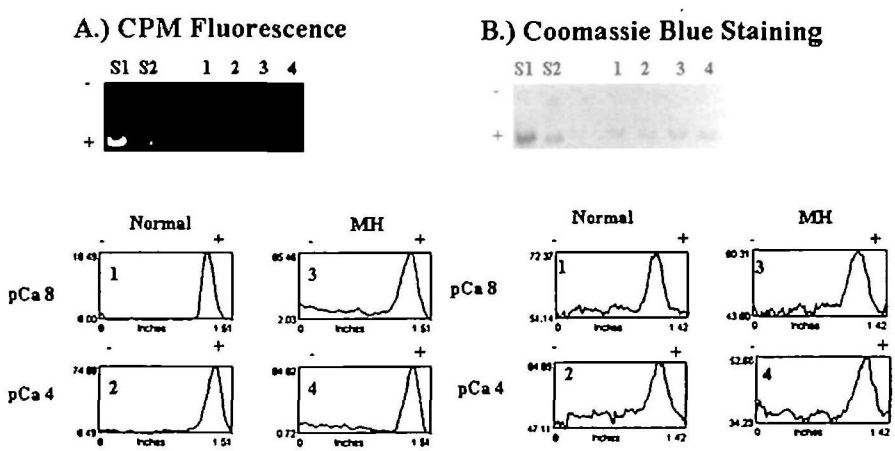

Fig. 2. **Effects of Ca^{2+} on CPM incorporation into myofibril-bound cTnC from normal and MH-susceptible pigs.** (A) A typical urea gel, showing the CPM-labeled cTnC in the two types of cardiac myofibrils at pCa 8 and pCa 4; the fluorescence scan is shown in the lower panel (note the difference in the scales of the y axes). (B) The same gel stained with Coomassie Blue and scanned to estimate the cTnC content. The samples are normal myofibrils labeled at pCa8 (lane 1) or pCa4 (lane 2) and MH-sensitive myofibrils labeled at pCa 8 (lane 3) or pCa 4 (lane 4). S1 and S2 are standards of CPM-bound cTnC at a labeling ratio of 0.85 (S1: 3.6 μg, S2: 1.8 μg) used to determine the stoichiometry of labeling. The reaction was carried out in rigor solution consisting of 100 mM MOPS, pH 7.0, 90 mM KCl, 5 mM $MgCl_2$, 2 mM EGTA, with (pCa 4) or without (pCa 8) 2.1 mM $CaCl_2$. CPM-labeled myofibrillar proteins (150 μg) were loaded on the gel and electrophoresed from the cathode (–) to the anode (+).

difference in the thiol reactivity of myofibril-bound cTnC can be attributed to the presence of different cardiac myosin isozymes in the hearts of normal and MH-susceptible animals. At pCa 4, CPM incorporation was slightly less than at pCa 8 in MH-susceptible pigs (pCa 4: 1.06 ± 0.14 mol CPM/mol cTnC; pCa 8: 1.28 ± 0.19 mol CPM/mol cTnC, $p = 0.11$), but significantly increased in normal animals (pCa 4: 1.06 ± 0.12 mol CPM/mol cTnC; pCa 4: 0.76 ± 0.12 mol CPM/mol cTnC, $*p = 0.007$). This finding suggests that, in the rigor state, in the presence of V3 myosin, Ca^{2+} binding and cross-bridge attachment have a cooperative effect on CPM incorporation into myofibril-bound cTnC, whereas, in the presence of V1 myosin, they have opposing effects. Consistent with this interpretation, the Ca^{2+}-activated response in rigor is greater in normal animals (~1.5) containing only V3 myosin than in MH-susceptible animals (~0.8) expressing both V1 and V3 myosin (Table II). However, this difference between the two types of cardiac myofibrils in the Ca^{2+}-mediated effect on CPM incorporation was not seen in the cycling or V_1-relaxed states. For normal animals, no significant difference was observed between rigor cross-bridges and cycling cross-bridges with respect to the effect of Ca^{2+} on CPM incorporation ($p = 0.46$). In contrast, in MH-susceptible animals, a greater Ca^{2+} effect was seen for cycling cross-bridges than for rigor cross-bridges ($*p = 0.03$). When myofibrils were treated with V_1 to maintain the cross-bridges of V1 and V3 myosin in the weak binding state, Ca^{2+} had no effect on CPM incorporation in either normal or MH-susceptible animals. All these data are consistent with the notion that the different cardiac myosin isozymes, V1 and V3, may have different cross-bridge effects on cTnC conformation in the cardiac myofibrils of normal and MH-susceptible animals.

CTnC Extraction from Cardiac Myofibrils—To further characterize myofilament protein-protein interactions between cTnC and other thin filament proteins in the hearts of normal and MH-susceptible pigs, we measured the CDTA extractability of cTnC from the two types of porcine cardiac myofibrils. Using purified myofilament proteins, we identified cTnC and cardiac myosin light chains (MLC1

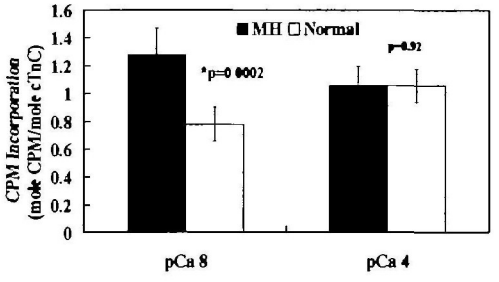

Fig. 3. **Quantitative analysis of CPM incorporation into myofibril-bound cTnC in normal and MH-susceptible pigs at pCa 8 and pCa 4 in rigor solution.** CPM was reacted with cardiac myofibrils as described in the legend to Fig. 2 and the extent of labeling determined from the fluorescence/protein molar ratio. Each histogram shows the mean ± SEM of 12 measurements. Students *t* test was used to calculate the *p* values. * indicates a significant difference between the two types of myofibrils at the same Ca^{2+} concentration; the p values comparing the same preparation at different pCas are given in the text.

TABLE II. **Effects of cross-bridge activity on Ca^{2+} activation of CPM incorporation into myofibril-bound cTnC in normal and MH-susceptible pigs.** The Ca^{2+}-activated response was measured as the ratio of CPM incorporation into myofibril-bound cTnC at pCa 4 compared to that at pCa 8. CPM incorporation was measured as described in "MATERIALS AND METHODS." The number of measurements (*n*) is indicated. Students *t* test was used to calculate *p* values. The symbol "*" indicates a significant difference between the rigor and cycling states, whereas the symbol "**" indicates a significant difference between the two types of myocardial preparations.

	Normal	MH
Cycling (5 mM MgATP)	1.33 ± 0.11	*1.13 ± 0.17 ($n = 6$)
Rigor (No ATP)	**1.51 ± 0.05	*,**0.83 ± 0.05 ($n = 29$)
Relaxed (1 mM V_i, 5 mM MgATP)	1.05 ± 0.09	0.96 ± 0.06 ($n = 4$)

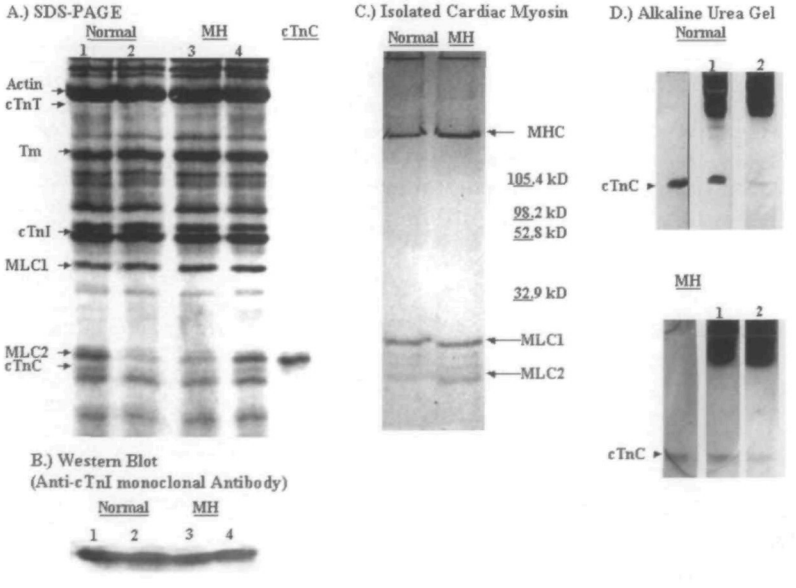

Fig. 4. **Composition analyses of myofibril proteins in untreated and CDTA-extracted cardiac myofibrils from normal and MH-susceptible pigs.** (A) SDS gels showing the main constituents of myofibril proteins in untreated (lanes 1 and 4) and CDTA-extracted (lanes 2 and 3) myofibrils from normal (lanes 1 and 2) and MH-susceptible pigs (lanes 3 and 4). Fifty micrograms of myofibrillar proteins or 0.5 μg of cTnC standard (S) was loaded on the gel, and silver staining was used to visualize the proteins. (B) Western blot analyses of cTnI in untreated (lanes 1 and 3) and CDTA-extracted (lanes 2 and 4) myofibrils from normal (lanes 1 and 2) and MH-susceptible pigs (lanes 3 and 4). cTnI was recognized using a mouse specific monoclonal primary antibody against TnI (Mab 1691, Chemicon; 1: 10,000 dilution) followed by a second anti-mouse antibody conjugated with alkaline phosphatase (Bio-Rad, 1.5:10,000 dilution) and colorimetric detection by BCIP-NBT substrate reaction. (C) An SDS gel resolving MLC1 and MLC2 from the corresponding MHC in isolated cardiac myosin from the hearts of normal and MH-susceptible pigs. (D) Alkaline urea gels showing endogenous cTnC in untreated (lane 1) and CDTA-extracted (lane 2) myofibrils from normal (above) and MH-susceptible pigs (bottom).

and MLC2) in cardiac myofibrils of normal and MH-susceptible pigs. Tropomyosin (Tm), cTnT, and cTnI were detected by western analyses applying specific monoclonal antibodies against Tm (monoclonal CH1, Sigma; 1:10,000 dilution), cTnT (monoclonal Ab JLT-12, Sigma; 1:500 dilution), and cTnI (monoclonal Ab1691, Chemicon; 1:10,000 dilution). For untreated myofibrils, MH caused a shift in cardiac myosin isozyme from V3 to V1, as shown previously (*8*). However, there was no detectable change in the normal ventricular isoform expression pattern of cTnT, Tm, cTnI, MLC1, MLC2, and cTnC (Fig. 4, A and C). In addition, densitometric analyses of these two groups of myofibril samples showed no significant alteration in the stoichiometry of the main constituent proteins as assessed by the ratio of the respective protein peak area to the actin peak (Table III). Taken together, these results suggest that only the MHC isoform is altered in the hearts of MH-susceptible pigs.

On the other hand, CDTA treatment removed significant amounts of cTnC and MLC2 (Fig. 4, A and D), but did not seem to extract other proteins such as cTm, cTnT,cTnI, and MLC1 (Fig. 4, A and B). This finding is in agreement with the previous work by Morimoto and Ohtsuki (*24*). Since CDTA treatment causes the extraction of MLC2 in addition to cTnC, it might be important to determine whether MLC2 extraction contributes to conformational changes of cTnC in MH-sensitive porcine hearts. Figure 5 A shows the densitometer traces for the myofibril preparation before and after extraction in the two types of porcine hearts. The extent of extraction of MLC2 and cTnC is quantified in Fig. 5B. Using 5 mM CDTA at pH 8.4, ~90% of the cTnC was extracted from cardiac myofibrils of normal pigs, but only ~60% could be extracted from those of MH-susceptible animals. However, CDTA treatment causes almost the same extraction of MLC2 from cardiac myofibrils of normal (~53.1%) and MH-susceptible pigs (~54.6%) ($p > 0.90$). Thus, it is unlikely that changes in MLC2 contribute to the observed differences in the two types of porcine hearts. To achieve better separation, the extent of cTnC extraction was also determined by alkaline urea gel electrophoresis, in which cTnC migrates far ahead of other myofibrillar proteins; the results confirmed that cTnC in MH-susceptible cardiac myofibrils is more resistant to CDTA extraction (Fig. 4D).

CTnC Re-Incorporation into CDTA-Extracted Cardiac Myofibrils—To quantify the degree of cTnC re-incorporation into extracted cardiac myofibrils, the myofibrillar cTnC content was analysed by urea gel electrophoresis and by restoration of Ca^{2+}-activated ATPase activity in both types of cardiac myofibrils. The results show that when 7.5 μg or more of cTnC added to 1 mg of myofibrils, exogenous cTnC inserted into the extracted myofibrils at the same level as in control non-extracted myofibrils in normal pigs (Fig. 6B), but at a lower level (70%) in MH-susceptible pigs (Fig. 6A). In myofibrils from normal pigs, full recovery of Ca^{2+}-activated ATPase activity was obtained when 10 μg or more of cTnC was added per mg of extracted myofibrillar proteins, while, in the case of MH-sensitive pigs, a maximum of only ~75% recovery was seen using amounts of cTnC ranging from 5 to 25 μg per mg of myofibrillar proteins. The lower re-incorporation of cTnC in MH-sensitive myofibrils is very likely due to the already greater amount left after extraction. Thus lower cTnC re-incorporation would be expected

TABLE III. **Relative contents of cTnT, Tm, cTnI, MLC1, MLC2, and cTnC in cardiac myofibrils of normal and MH-sensitive porcine hearts.** SDS-PAGE was performed as described by Laemmli (1970). Gels were stained with Coomassie Blue, photographed with a digital camera, and analysed for protein content using a computer program with image analysis software. The relative contents of cTnT, Tm, cTnI, MLC1, MLC2, and cTnC were obtained by normalizing the peak areas of these proteins to the area under the actin peak. Seven different gels were analysed ($n = 7$). Values are expressed as mean ± SEM. Student's test was used to calculate p values between the two experimental groups.

	MH ($n = 7$)	Normal ($n = 7$)	
cTnT	0.20 ± 0.03	0.19 ± 0.03	$p = 0.92$
Tm	0.61 ± 0.11	0.45 ± 0.06	$p = 0.38$
cTnI	0.27 ± 0.09	0.26 ± 0.08	$p = 0.93$
MLC1	0.36 ± 0.06	0.33 ± 0.04	$p = 0.66$
MLC2	0.21 ± 0.08	0.24 ± 0.07	$p = 0.73$
cTnC	0.04 ± 0.003	0.04 ± 0.005	$p = 0.75$

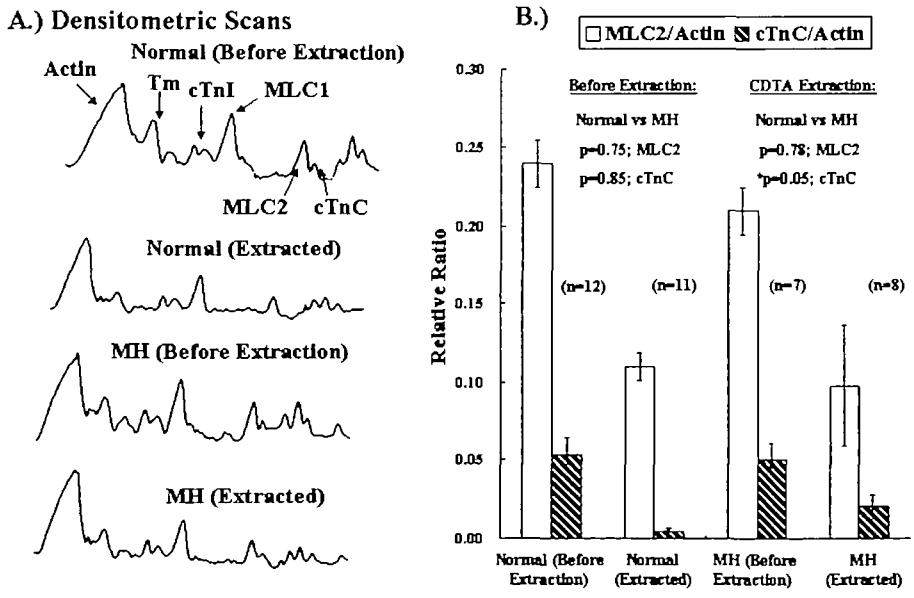

Fig. 5. **CDTA extraction of cTnC and MLC2 from the cardiac myofibrils of normal and MH-susceptible pigs.** (A) Densitometer scans showing the degree of cTnC, and MLC2 extraction from the two types of myofibrils. (B) Quantitative analysis of cTnC and MLC2 content in MH-susceptible and normal myofibrils with or without CDTA extraction. Gels were processed as described in the legend Fig. 4. Myofibril-bound cTnC, and MLC2 was estimated by normalizing the ratio of the area under the peaks of cTnC or MLC2 to that under the actin peak. Each column is shown as the mean ± SEM of at least 7 gel scans. p values are indicated and * represents a significant difference between the two experimental groups after CDTA extraction.

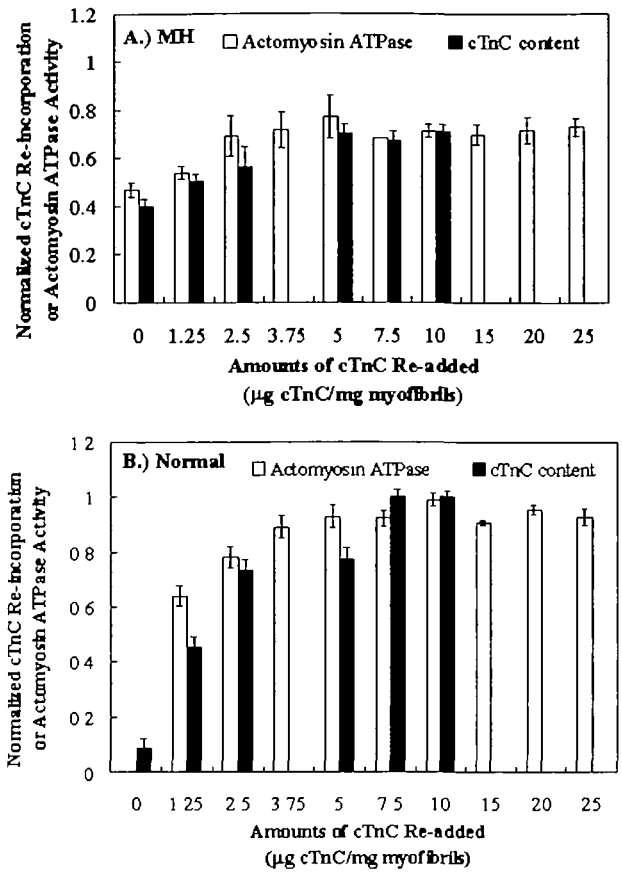

Fig. 6. **Incorporation of exogenous cTnC into CDTA-extracted myofibrils.** Exogenous cTnC (1.25–25 μg) was added to 1 mg of CDTA-extracted myofibrils from MH-sensitive (A) and control (B) pigs and the degree of cTnC re-incorporation was determined by both alkaline urea-polyacrylamide gel electrophoresis and the restoration of Ca^{2+}-activated ATPase activity. The relative increase in the latter activity was calculated by dividing the increase of the Ca^{2+}-activated cardiac myofibrillar ATPase (activity at pCa4–Activity at pCa 8) to the cardiac myofibrillar ATPase at pCa 8. The results for the cTnC-reincorporated myofibrils were compared and normalized to those for untreated myofibrils from the same type of pig (susceptible/non-susceptible). One hundred and fifty micrograms of myofibrils was loaded on the gels. At least three different gels were analyzed. Each point is the mean ± SEM of 7 measurements.

on the basis of the law of mass action. These results suggest that the myofibrils of normal and MH-susceptible pigs differ with respect to cTnC extractability.

DISCUSSION

As shown by Gronert *et al.* (*3, 5*), MH is characterized by an increase in circulating catecholamines. Studies of the effects of isoproterenol on force generation of trabeculae isolated from the right ventricles of normal (Yorkshire) and MH-susceptible (Pietrain) pigs showed increased β-adrenergic activation in MH-susceptible hearts (*25*). Levels of the second messenger, IP_3, are also increased in the hearts of MH-susceptible pigs (*4*). Increased β-adrenergic activation might cause an enhanced intracellular Ca^{2+} transient and an increase in myocardial contractility, as well as affect pacemaker cells and alter the heart rate. In this study, we found changes in the conformation of cTnC and in its incorporation into cardiac myofibrils of MH-susceptible pigs. Conformational changes in cTnC might affect the Ca^{2+} dependence of myocardial contractility during MH crisis.

The normal operation of the heart involves a cooperative process in which myosin cross-bridges and protein-protein interactions along thin filaments modify the structure and Ca^{2+}-binding affinity of cTnC (*26*). In our studies, the cooperativity of Ca^{2+}-activated myofibrillar ATPase was increased in MH, although the Ca^{2+} sensitivity was unchanged (Table I). This increased cooperativity is very likely due to a shift in the cardiac myosin isozyme and a change in thin filament protein-protein interactions. Using electron microscopy and optical diffraction, Weisberg and Winegrad (*27*) have shown that relatively rigid cross-bridges exist in thick filaments containing V1 myosin, while cross-bridges in V3 myosin-expressing filaments are more flexible. This difference in flexibility would affect the kinetics of attachment and detachment of cross-bridges and the co-operative behavior of cardiac muscles. In an earlier study, Bremel and Weber (*28*) reported that the attachment of rigor cross-bridges to actin turns on a tropomyosin (TM)-dependent potentiated state of the thin filament, as measured by the ATPase activity. The thin filament can be viewed as being made up of regulatory units, each composed of seven actin monomers, one TM molecule, and one troponin (Tn) complex (*29*). Based on the three-state model of thin filament activation, regulatory units can exist in the blocked, closed, and open states (*30, 31*). At physiological MgATP concentrations and in the absence of Ca^{2+}, most of the regulatory units are in a blocked state in which TM prevents cross-bridges from interacting with actin. The binding of Ca^{2+} to TnC causes a partial movement of TM into the grooves of the actin filament, thus allowing myosin to form a weak bond with actin (closed state). With Ca^{2+} still bound to TnC, the cross-bridge undergoes isomerisation to the strong-binding force-generating state (open state), in which TM is pushed further into the groove of the actin filament, allowing the attachment of a greater number of strong-binding cross-bridges. Thus, the attachment of strong-binding cross-bridges shifts most of the thin filament regulatory units to the open state and causes an increased affinity of cTnC for Ca^{2+} (*32*). In addition, studies with fluorophore-labeled cTnC inserted into myofibrils (*19*) and skinned fibers (*33*) have shown conformational changes in cTnC associated with the attachment of strong-binding cross-bridges.

Recent NMR studies have resolved the three-dimensional structures of human (*34, 35*) and chicken (*36*) cTnC and shown that the regulatory domain of cTnC exists in a "closed conformation" in both the apoprotein (absence of metal binding) and Ca^{2+}-bound states. This finding suggests that, in addition to the binding of Ca^{2+} to cTnC, Ca^{2+} activation of cardiac muscle might involve a strong interaction between cTnC and other thin filament proteins. Consistent with this interpretation is the observation reported in this study that the conformational state of myofibril-bound cTnC is altered in the hearts of MH-susceptible pigs, which contain substantial amounts of V1 myosin.

It should be noted that with native cTnC labeling can occur at both Cys-35 and Cys-84, although in solution, Ca^{2+} induces the preferential labeling of Cys-84 (*12, 13*). Cys-35 is located in the inactive loop of site I (residues 28–40),

whereas Cys-84 is located at the N-terminal end of the central helix, near Ca^{2+} binding site II. Studies with monocysteine mutants both in solution and filament-bound indicate that the Ca^{2+}-induced conformational change in the regulatory region of cTnC occurs only in the region of Cys-84 (*37, 38*). Thus, it is unlikely that our present results reflect a confounding influence of opposite effects on the two Cys residues. The data therefore suggest that different conformational states of myofibril-bound cTnC exist in normal and MH-susceptible pigs.

Our understanding of Ca^{2+}-mediated myofilament protein-protein interactions has been facilitated by the technique of selective extraction of native TnC from skinned fibers or myofibrils using a low ionic strength solution containing the Ca^{2+}/Mg^{2+} chelator, EDTA (*14*). The proposed mechanism for TnC extraction from muscle bundles or myofibrils is based on the removal of the divalent cation, Ca^{2+} or Mg^{2+}, from the high affinity sites of TnC (*39*), which are thought to play a structural role in stabilizing the whole Tn complex on the thin filament. Removal of divalent metals from these two sites would, therefore, weaken the TnC-TnI interaction, resulting in the preferential extraction of TnC. In addition, the use of a protocol for TnC isolation in the absence of 6 M urea described by Cox *et al.* (*40*) suggested that TnC extraction is reduced by an effect of rigor cross-bridges on TnC binding to the regulated thin filament. More recently, greater extraction of TnC was seen in regions without filament overlap, suggested that strong binding of myosin to actin stabilizes TnC incorporation into the thin filament (*41, 42*). In this study, our results show that cTnC binds more strongly to thin filaments in MH cardiac myofibrils than to those in normal myofibrils. This stronger binding, in parallel with an increase in interactions between myofilament proteins, could account for the changes in thiol reactivity of myofibril-bound cTnC seen in the hearts of MH-susceptible animals.

Cardiac myosin is composed of two heavy chains and four light chains: two essential MLC1 molecules, and two regulatory MLC2 molecules. In both skeletal and cardiac muscle, the phosphorylation of MLC2 causes some modulation of contractility *via* increased Ca^{2+} sensitivity for force production. In skeletal muscle thick filaments, MLC2 phosphorylation causes a disordering of the cross-bridge helical array that is thought to reflect increased mobility of the cross-bridges and a greater potential for actin–myosin interaction (*43*). In addition, it has been shown that the removal of MLC2 significantly decreases the velocity of actin movement on skeletal myosin, and suggests that MLC2 has the physiological role in producing the mechanical effect on the shortening velocity of the actin–myosin interaction (*44*). However, it remains to be determined if these results are applicable to cardiac muscle. In our study, no difference was detected in the isoform expression pattern of myofibrils or in the stoichiometry of MLC1 and MLC2 for isolated cardiac myosin from the hearts of normal and MH-susceptible pigs as analyzed by SDS-PAGE (Fig. 4, A and C). Although the possibility can not be ruled out that small changes in the stoichiometry of the contractile apparatus or in isoform expression of other contractile proteins, which may have gone undetected in the SDS-PAGE analyses, could contribute to the conformational change in myofibril-bound cTnC, it is likely that changes in the thiol reactivity and extractability of cTnC in myofibrils of MH-susceptible pigs are attributed to altered MHC isoform expression.

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