Temperature Perturbation Studies of Sarcoplasmic Reticulum from Malignant Hyperthermia Pig Muscle

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ABSTRACT The effects of varying temperatures from 25° to 37°C on calcium binding characteristics of sarcoplasmic reticulum from malignant hyperthermia-susceptible (MHS) and control pig muscle were examined. Two groups of MHS pigs were included: those with high susceptibility to malignant hyperthermia (MHS group) and a cross-bred, less susceptible group (MHX). At 25°C, calcium binding was lower for MHS than for controls and MHX. As temperature was increased by 2°C jumps, calcium binding decreased in all sarcoplasmic reticulum fractions. At 35°C a sharp decrease in calcium binding occurred in the MHS and MHX fractions. The sharp decrease in calcium binding at 35°C differentiated the MHS and MHX fractions from controls.

The initial velocity (Vi) for calcium binding was lower in MHS fractions between 25° and 35°C when compared with MHX and controls. All fractions had increased Vi values as temperature increased from 25° to 35°C. From 35° to 39° Vi for controls increased markedly. In contrast, Vi for the MHX fraction decreased as temperature exceeded 35°C.

These temperature effects on calcium binding characteristics of sarcoplasmic reticulum from MHS and MHX muscle may be indicative of a membrane transition that impairs calcium binding.

INTRODUCTION

Malignant hyperthermia is a genetic disease that predisposes affected individuals to certain anesthetic agents (1–3). The clinical syndrome is characterized by a rapid (0.5°C/min) increase in body temperature, severe metabolic acidosis, and skeletal muscle rigidity. Abnormalities in biochemical (4, 5) and pharmacologic (6–8) responsiveness of malignant hyperthermia-susceptible (MHS)[1] skeletal muscle have been described, and support the thesis that MHS skeletal muscle is the primary target tissue for anesthetic agents that trigger the syndrome.

The metabolic and contractile responsiveness of skeletal muscle are each influenced by the free calcium concentration of the myoplasm (9, 10). The free calcium ion concentration of the normal muscle cell is finely regulated by the sarcoplasmic reticulum. Under resting conditions myoplasmic free calcium concentration is estimated to be 0.5 μM or less, and during activation, calcium release from the sarcoplasmic reticulum increases the calcium concentration above 1 μM. Indirect evidence has been presented indicating that oxygen consumption of skeletal muscle increases with increasing myoplasmic calcium concentrations below the contraction threshold (9). It is conceivable, therefore, that a sustained increase in myoplasmic calcium could fulminate into the clinically serious sequellae that occur as malignant hyperthermia during general anesthesia.

Several reports have shown that volatile anesthetic agents can impair calcium uptake by fragmented sarcoplasmic reticulum from various species (11, 12). However, in these studies the concentration of agents that affect calcium uptake are in excess of the anesthetic concentrations, and the relevance to malignant hyperthermia is therefore unknown. Studies on the effects of halothane on sarcoplasmic reticulum from MHS patients and MHS pigs have produced conflicting results. Anesthetic concentrations of halothane impaired calcium uptake by MHS sarcoplasmic reticulum from affected human muscle and had little effect on normal sarcoplasmic reticulum calcium uptake (7). In contrast, a similar study showed that anesthetic concentrations of halothane equally impaired the calcium uptake by normal and MHS human muscle sarcoplasmic reticulum (13). Of four independent studies with MHS pig muscle, two demonstrated that halothane increased calcium uptake (5, 14); one showed no halothane effect (15); and one demonstrated a halothane depression of calcium uptake (16).

One characteristic of MHS muscle is the abnormal

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[1] *Abbreviations used in this paper*: MHS, malignant hyperthermia-susceptible; MHX, low susceptibility to malignant hyperthermia; T_{ss}, time to steady-state calcium binding; Vi, initial velocity.

contracture response to halothane in vitro (8). We have previously shown (17) that the abnormal halothane contracture of MHS pig muscle is temperature dependent, occurring at 37°C but not at 25°C. In the present experiment, we attempt to characterize the temperature effects on calcium binding by sarcoplasmic reticulum from MHS and control pig muscle.

METHODS

A total of 11 pigs from two different litters were used in this experiment. Seven of the pigs were from a litter in which a MHS pure-bred Poland-China female was crossed to a negative reactor pure-bred Hampshire male. After muscle biopsy for sarcoplasmic reticulum under sodium thiopental anesthesia, each animal was challenged with 2% halothane anesthesia for 1.5 h. None of the seven cross-bred pigs developed malignant hyperthermia during this halothane challenge. After 1.5 h of halothane anesthesia, each pig was administered a 100-mg dose of succinylcholine which resulted in the development of malignant hyperthermia in four of the seven cross-bred pigs. The positive reactors were classified as MHX and the three negative reactors were considered as resistant controls. A second litter of four pure-bred Poland-China pigs were the offspring of MHS positive parents. Each of these four pigs developed malignant hyperthermia during less than 1 h of halothane anesthesia. Each of these four pigs were classified as MHS.

Approximately 80 g of longissimus dorsi was biopsied from each pig under thiopental anesthesia. Immediately after biopsy, the muscle was placed into cold homogenization solution and minced into thin sections with scissors. The composition of the homogenization solution in millimoles was: sucrose, 220; KCl, 80; $MgCl_2$, 5; EGTA, 5; and Hepes, 5. The pH was 6.8. The minced muscle was suspended in 5 vol of homogenizing solution and homogenized by 3 × 30-s periods of full speed with a Tekmar Tissuemizer (Tekmar Co., Cincinnati, Ohio). During homogenization, the homogenate was maintained at 4°C. The homogenized muscle was transferred to 50-ml centrifuge tubes and a 18,000–48,000 *g* pellet was fractioned in the following manner: an SS 34 head in a Sorvall RC-5 (Dupont Co., Instrument Products Div., Wilmington, Del.) refrigerated was centrifuged at a temperature of 4°C; 20 min 1,000 *g* pellet discarded; 20 min 4,000 *g* pellet discarded; 30 min 18,000 *g* pellet discarded; and 60 min 48,000 *g* pellet recovered. The 18,000–48,000 *g* pellet was extracted for 1 h with 0.6 M KCl, 10 mM Hepes. After extraction, the pellet was recovered by centrifuging the extract for 60 min at 48,000 *g*. The resultant pellet was then suspended in 20 mM Hepes, 50 mM KCl, and 10 mM $MgCl_2$ at pH 6.8. All solutions were maintained at 4°C throughout. Sarcoplasmic reticulum protein was determined by the Folin method using crystalline bovine serum albumin as standard.

Calcium binding was measured in an Aminco-Morrow stopped-flow apparatus (American Instrument Co., Travenol Laboratories Inc., Silver Spring, Md.). Calcium concentration changes were determined by using murexide as indicator (18). The final mixed solution contained the following: Hepes, 20 mM; KCl, 50 mM; $MgCl_2$, 10 mM; ATP, 1.2 mM; murexide, 100 μM; calcium, 140 μM; and sarcoplasmic reticulum 0.50 mg/ml. The mixing of reactants was accomplished by loading two drive syringes that contained the following: one syringe contained twice the final sarcoplasmic reticulum concentration plus all other components except for ATP and calcium; and the other syringe contained twice the final ATP and calcium concentrations plus all components, except no sarcoplasmic reticulum was present. The final pH of 6.8 was not significantly altered by reagent mixing or by temperature changes. The time of mixing included a dead time of 4 ms and a transport time of 20 ms. The photometric component of the system consisted of an optical beam splitter, two narrow band (one-half bandwidth = 10 nm) interference filters (500 and 540 nm), and two photomultiplier tubes. The photomultiplier output voltages were balanced to within 2 mV and the measured output signal (Δ absorbance$_{540}$ − Δ absorbance$_{500}$) was produced by a differential amplifier. Output voltage from the differential amplifier was simultaneously recorded on a storage oscilloscope and by a conventional recorder. The fast reaction component of calcium binding was estimated from photographs of the oscilloscope trace and total calcium binding was estimated from the conventional recorder records.

The effect of temperature on calcium binding was investigated in the following manner. the components of each drive syringe were preincubated for 10 min at the temperature to be tested. The temperature of the stopped-flow apparatus was maintained by a circulating water jacket. Calcium binding was measured at 2°C temperature jumps from 25° to 39°C. Fresh solutions were prepared for each temperature study and the absorbance change (540–500 nm) for calcium-murexide standards was determined at each temperature. Tests for each sarcoplasmic reticulum preparation were completed within 24 h of isolation.

RESULTS

Characterization of calcium-murexide response and of calcium binding kinetics for pig sarcoplasmic reticulum. The absorbance change for calcium-murexide and the specificity for calcium is illustrated in Fig. 1. Addition of 5 μM increments of calcium produce a linear change in absorbance (Δ 540–500 nm), and this absorbance change is reversed by incremental additions of EGTA, a chelating agent with high specificity for calcium (Fig. 1). As shown in Fig. 2, the absorbance changes linearly with changes in calcium concentrations exceeding those measured in the present experiment.

The kinetics for calcium binding by the 18,000–48,000 *g* sarcoplasmic reticulum fraction of pig muscle are qualitatively similar to those reported for a rabbit sarcoplasmic reticulum fraction utilizing comparable techniques (18). At 25°C, initial calcium binding is characterized by a linear rate lasting ≅400 ms (Fig. 3). Thereafter, calcium binding progressively declines with time until steady state levels are reached (Fig. 3). The time to steady-state calcium binding (T_{ss}) and the total calcium bound were lower for the MHS fractions when compared with control and MHX fractions (Table I). The larger quantity of sarcoplasmic reticulum recovered from MHS muscle and its lower calcium binding capacity at 25°C compared with MHX, and controls (Table I) agree with a previous report (19).

Effect of temperature on total calcium bound. At 25°C control and MHX calcium binding values did not differ, but each was greater than the MHS value ($P < 0.05$; Fig. 4). As temperature increased above 29°C,

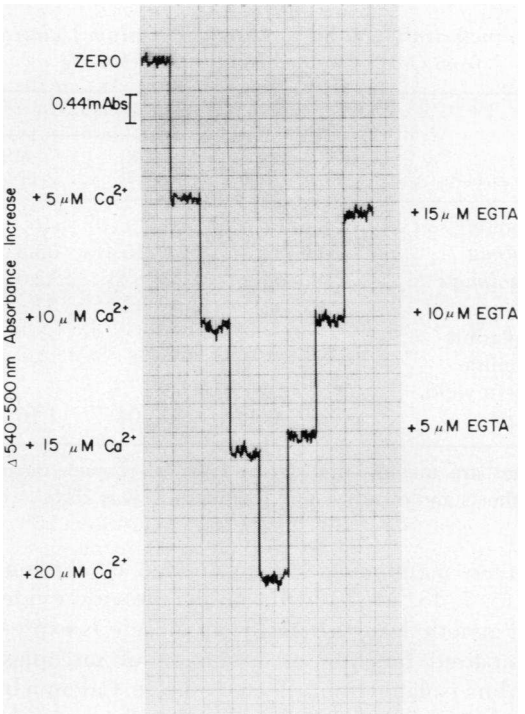

FIGURE 1 Absorbance (Abs) change for murexide with varying concentration of calcium. Downward deflections on left side of figure represent absorbance decrease with calcium addition. Upward deflections on right side of figure represent stoichiometric absorbance increase when calcium is chelated by EGTA. Before additions of calcium and EGTA, concentrations are: HEPES, 20 mM; KCl, 50 mM; $MgCl_2$, 10 mM; murexide, 100 μM; and Ca^{2+}, 140 μM.

total calcium binding decreased ($P < 0.05$) for control, MHX, and MHS fractions of sarcoplasmic reticulum (Fig. 4). Between temperatures of 25° and 35°C no difference was found among calcium bound by control and MHX fractions. As the temperature jump increased from 35°C to 37°C, a significant ($P < 0.05$) deflection occurred in the calcium binding vs. temperature curve for the MHX fraction (Fig. 4). At 37°C the MHX fraction bound less calcium than did controls. Differences in total calcium bound were observed between MHX and MHS fractions at 37° and 39°C, but these were not statistically significant. Compared with controls, a greater drop in calcium binding was observed for the MHS fraction when the temperature jump increased from 35° to 37°C.

When represented by Arrhenius plots (Fig. 5), temperature effects on calcium binding were different in MHX and MHS fractions when compared to controls. As temperature increased from 25° to 27°C, increased calcium binding was apparent in control and MHX fractions (Fig. 5). As temperature increased above 29°C, calcium binding was decreased in all fractions. A transition temperature (35°C) evident in the MHS and MHX Arrhenius plots was not observed in the controls (Fig. 5). At 27°C, a transition temperature was observed in controls and MHX, but absent in MHS Arrhenius plots (Fig. 5).

Effect of temperature on initial velocity (Vi) of calcium binding. As temperature was increased from 25° to 35°C, a progressive increase in Vi for calcium binding occurred in each sarcoplasmic reticulum fraction (Fig. 6). The Vi values of MHS fraction were less than Vi for the MHX control fractions between 25° and 35°C. As temperature increased from 35° to 37°C and then to 39°C, the Vi for control sarcoplasmic reticulum continued to increase with a sharp upward increase as temperature was changed from 37° to 39°C (Fig. 6). In contrast, Vi for the MHX fraction decreased as temperature exceeded 35°C, although Vi at 39°C was greater than Vi at 25°C (Fig. 6). For the MHS fraction, Vi increased in a linear fashion with increasing temperature (Fig. 6). Compared in another manner, Vi increased with temperature changes from 25° to 37° by factors of 2.0, 2.2, and 1.8 for control, MHX, and MHS values, respectively. Comparing Vi changes from 25° to 39°C, controls, MHX, and MHS increased by factors of 3.6, 1.7, and 2.0, respectively. The proportion of change in Vi with temperature was comparable for all fractions until temperature exceeded 35°C, when control values continued to increase sharply, MHX values declined, and MHS increases remained linear.

Effect of temperature on T_{ss} levels. The T_{ss} was longer for MHX and control sarcoplasmic reticulum when compared to the MHS fraction at each temperature except at 39°C ($P < 0.05$; Fig. 7). At 39°C no significant difference was detected for T_{ss} among the three different fractions. The T_{ss} values decreased in each

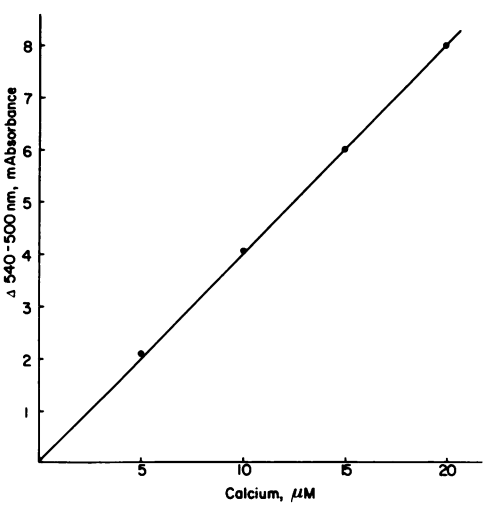

FIGURE 2 Standard curve for absorbance changes for calcium-murexide.

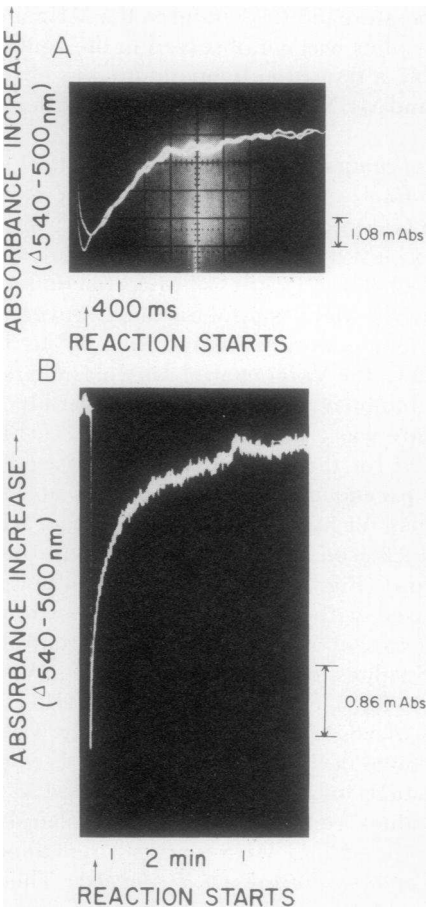

FIGURE 3 Kinetics for calcium binding by sarcoplasmic reticulum from pig skeletal muscle. (A) Duplicate runs of a fast (oscilloscope) sweep for the Vi of calcium binding. The Vi component is linear for only 600 ms. (B) A slower recorder trace of the same reaction illustrating overall kinetics of calcium binding. Arrow indicating "reaction starts" is time at which contents of two syringes were mixed. Upward deflection of traces represents calcium uptake. Abs, absorbance.

sarcoplasmic reticulum fraction as temperature increased (Fig. 7). For the MHS and control sarcoplasmic reticulum, the T_{ss} values decreased linearly with increasing temperature. A sharp deflection in T_{ss} was observed for the MHX fraction when temperature increased above 33°C, whereas the control sarcoplasmic reticulum value declined sharply at 37°C (Fig. 7).

DISCUSSION

Skeletal muscle from MHS pigs and man is characterized by abnormal contracture response to halothane (6, 8) or to caffeine (7, 8). These observations provide the basis for a thesis that calcium regulation is impaired in MHS muscle (6). A logical calcium regulatory target for these pharmacologic effects is the sarcoplasmic reticulum, but previous studies of sarcoplasmic

TABLE I
Characterization of Sarcoplasmic Reticulum Isolated from Control and Susceptible Pig Muscle

Variable	Pig groups		
	Control (n = 4)	MHX (n = 3)	MHS (n = 4)
Ca^{2+} bound, *nmol/mg*	54.5 (9.8)*	62.2 (15.4)	33.2 (3.2)
Vi, *nmol/mg* s^{-1}	24.3 (5.8)	23.7 (8.8)	12.0 (1.4)
T_{ss}, *s*	155 (32)	146 (12)	37 (9)
Sarcoplasmic reticulum protein yield, *mg/gm*	0.89 (.09)	0.81 (.04)	1.16 (.10)

* Values are means with standard errors of each mean in parenthesis and experimental temperature was 25°C.

reticulum membranes have provided conflicting results (5, 7, 13, 14, 16). Our studies provide evidence that a genetic lesion of MHS pig muscle is expressed by impaired function of a fraction of sarcoplasmic reticulum isolated from affected muscle. Calcium binding capacity of sarcoplasmic reticulum from pure-bred, highly susceptible MHS pigs is lower than controls at each temperature tested from 25° to 39°C. In contrast, calcium binding capacity of sarcoplasmic reticulum from less susceptible cross-bred MHX pigs was lower than controls only when tested at 37° or 39°C. For the MHS membranes, the defect is expressed to an extent

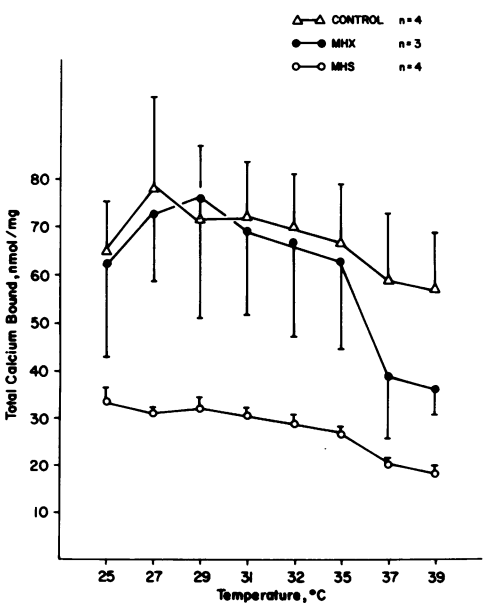

FIGURE 4 Effect of temperature on total calcium bound by sarcoplasmic reticulum from control, MHX, and MHS pig skeletal muscle.

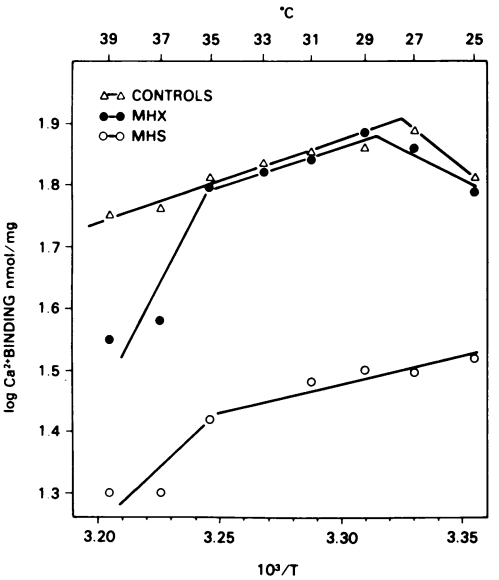

FIGURE 5 Arrhenius plots of calcium binding by sarcoplasmic reticulum from control, MHX, and MHS muscle.

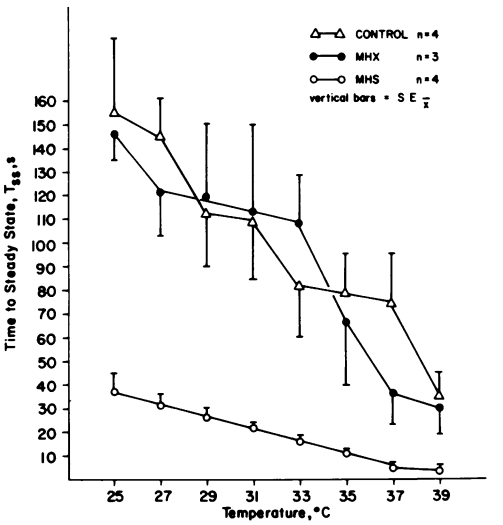

FIGURE 7 Effect of temperature on T_{ss}, in sarcoplasmic reticulum from control, MHX, and MHS pig muscle.

that sarcoplasmic reticulum function in vitro differs from controls at each temperature studied. However, the observed deflection of the Arrhenius plot at 35°C for MHS membrane function but not for controls demonstrates yet another abnormality. A deflection of the Arrhenius plot observed for the MHX membrane fraction at 35°C suggests a similar abnormality for the MHS and MHX membranes. The observed difference between MHX and control sarcoplasmic reticulum function only at the higher temperatures tested may relate to the temperature dependence observed for abnormal halothane contracture in susceptible muscle (17). The temperature-dependent halothane contracture of susceptible muscle is of central importance

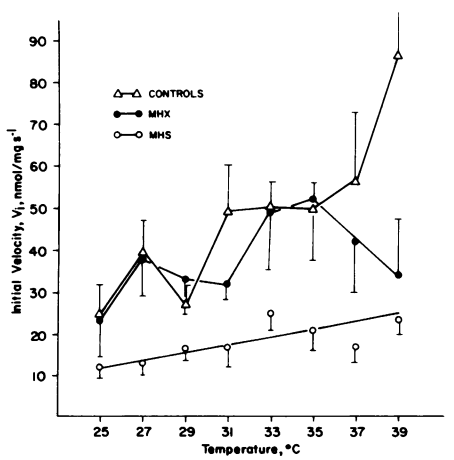

FIGURE 6 Effect of temperature on Vi of calcium binding for control, MHX, and MHS sarcoplasmic reticulum.

because this abnormal contracture response provides a diagnostic test for susceptibility to malignant hyperthermia in man (20).

These temperature-dependent abnormalities of susceptible muscle and isolated sarcoplasmic reticulum may also provide new insight to the etiologic factors involved in the pathopharmacology of malignant hyperthermia. The abnormal halothane contracture of MHS pig muscle occurs only after exposure at 31°C to halothane and the contracture response increases with temperature up to 37°C (unpublished data). The observed temperature-dependent halothane contracture and impaired sarcoplasmic reticulum membrane function may be associated with a temperature-induced transition in membrane structure/function relationships. The observed deflections in Arrhenius plots for susceptible membranes but not in controls lends support to this. Other studies (19) on similar, if not identical (21), MHS type pig muscle, have also demonstrated impaired sarcoplasmic reticulum function and that ATPase from susceptible membranes had altered temperature dependence in the temperature range of 33°–45°C. Also, it has been postulated that for normal sarcoplasmic reticulum, the ATPase coupled to calcium transport exists in two functional states and the transition from a "basic" Ca^{2+}-independent state to the other "extra" calcium-dependent state involves conformational changes in the protein and/or its membrane environment (22, 23). A separate study (24) demonstrated a higher transition temperature for Ca^{2+} stimulated respiration of MHS muscle mitochondria when compared with controls.

As demonstrated in the present and in other (18) experiments, the Vi of calcium binding is linear only for about 400 ms, after which the rate of calcium binding

progressively decreases as a consequence of internal calcium inhibition (18). The Vi for MHS fractions was lower than controls at each temperature tested. A similar relationship was observed between MHS and control sarcoplasmic reticulum for the time required to reach steady-state levels of calcium binding. The total calcium bound by sarcoplasmic reticulum under conditions of substrate (Mg-ATP and Ca^{2+}) saturation may be described as a function of several variables. The rate of net calcium accumulation is a function of membrane permeability and concentration gradients. It is not possible to conclude from our studies if the impaired calcium binding by sarcoplasmic reticulum from MHS muscle is the result of decreased active inward pumping, increased passive diffusion, or both, since passive diffusion was not measured. However, similar studies (19) showed no enhancement of membrane permeability to calcium in sarcoplasmic reticulum from affected muscle. More definitive studies are required to assess the lesion of isolated sarcoplasmic reticulum from MHS muscle.

In conclusion, abnormal function in calcium binding has been demonstrated for a fraction of sarcoplasmic reticulum from two groups of pigs with varying susceptibility to malignant hyperthermia. In the less susceptible group, abnormal sarcoplasmic reticulum function was evident only at higher (37°–39°C) temperatures. This may explain why previous studies performed on susceptible membranes at lower temperatures failed to demonstrate impaired function. The similar temperature dependence between abnormal halothane contracture in MHS muscle strips and that of impaired calcium binding by sarcoplasmic reticulum draws speculation to a defect in sarcoplasmic reticulum function as an etiologic basis for malignant hyperthermia.

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