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Skeletal muscle sarcolemma in malignant hyperthermia: evidence for a defect in calcium regulation

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Sarcolemmal properties implicated in the skeletal muscle disorder, malignant hyperthermia (MH), were examined using sarcolemma-membrane vesicles isolated from normal and MH-susceptible (MHS) porcine skeletal muscle. MHS and normal sarcolemma did not differ in the distribution of the major proteins, cholesterol or phospholipid content, vesicle size and sidedness, ($Na^+ + K^+$)-ATPase activity, ouabain binding, or adenylate cyclase activity (total and isoproterenol sensitivity). The regulation of the initial rates of MHS and normal sarcolemmal ATP-dependent calcium transport (calcium uptake after 1 min) by Ca^{2+} ($K_{1/2}$ = 0.64–0.81 μM), calmodulin, and cAMP-dependent protein kinase were similar. However, when sarcolemmal calcium content was measured at either 2 or 20 min after the initiation of active calcium transport, a significant difference between MHS and normal sarcolemmal calcium uptake became apparent, with MHS sarcolemma accumulating approximately 25% less calcium than normal sarcolemma. Calcium transport by MHS and normal sarcolemma, at 2 or 20 min, had a similar calmodulin dependence ($C_{1/2}$ = 150 nM), and was stimulated to a similar extent by cAMP-dependent protein kinase or calmodulin. Halothane inhibited MHS and normal sarcolemmal active calcium uptake in a similar fashion (half-maximal inhibition at 10 mM halothane), while dantrolene (30 μM) and nitrendipine (1 μM) had little effect on either MHS or normal sarcolemmal calcium transport. After 20 min of ATP-supported calcium uptake, 2 mM EGTA plus 10 μM sodium orthovanadate were added to initiate sarcolemmal calcium efflux. Following an initial rapid phase of calcium release, an extended slow phase of calcium efflux (k = 0.012 min^{-1}) was similar for both MHS and normal sarcolemma vesicles. We conclude that although a number of sarcolemmal properties, including passive calcium permeability, are normal in MH, a small but significant defect in MHS sarcolemmal ATP-dependent calcium transport may contribute to the abnormal calcium homeostasis and altered contractile properties of MHS skeletal muscle.

Introduction

Malignant hyperthermia (MH) is an inherited muscle disorder thought to result from a primary defect in the skeletal muscle of susceptible individuals (for review see Refs. 1 and 2). The potentially fatal MH syndrome can be initiated by

Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; SDS, sodium dodecyl sulfate; Gpp(NH)p, 5′-guanylyl imidodiphosphate; MH, malignant hyperthermia; MHS, malignant hyperthermia-susceptible.

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volatile anesthetics, such as halothane, the depolarizing muscle-relaxant succinylcholine, and in certain breeds of swine by various forms of stress [1–3]. An MH episode is characterized by muscle rigidity, with markedly increased muscle metabolism, and rapidly rising body temperature [4]. Although a number of investigators have proposed that a defect in the control of sarcoplasmic calcium concentrations is responsible for MH, the primary defect, and the interrelationships among the many observed abnormalities in MH muscle, remain to be determined [1,2].

Abnormalities in the structure and function of the plasma membrane of skeletal muscle cells have been suggested in several neuromuscular disorders [5,6], and recent studies have indicated altered sarcolemmal properties in MH. These include reports of increased adenylate cyclase activity [7] and increased content of $(Na^+ + K^+)$-ATPase [8] in MH-susceptible (MHS) human muscle, altered $(Na^+ + K^+)$-ATPase, protein, and lipid distribution in a MHS porcine muscle sarcolemma fraction [9], indications of abnormal MHS muscle sarcolemmal calcium permeability [10–13], and a lower MHS muscle mechanical threshold [14]. Furthermore, increased MHS muscle acetylcholinesterase activity [15], and alterations in several properties of MHS erythrocyte membranes, have recently been demonstrated [16,17].

We have investigated the possible involvement of the sarcolemma in MH by examining properties of highly purified sarcolemma preparations, isolated from the longissimus dorsi muscle of genetically defined MHS and normal swine populations. Although there were no differences between the MHS and normal sarcolemma preparations in a number of properties, a 25% reduction in the ATP-dependent calcium transporting activity of MHS sarcolemma vesicles was observed. Interestingly, there was no difference in the rate of passive calcium efflux from MHS and normal sarcolemma vesicles which had been previously loaded with calcium. Our results indicate that although sarcolemmal passive calcium permeability appears normal in MH, a defect in MHS muscle plasma membrane active calcium transport may contribute, in part, to the reported elevation in sarcoplasmic Ca^{2+} concentration [18] and the abnormal contractile properties [19] of MHS skeletal muscle.

Materials and Methods

Materials

Experimental animals were obtained from the University of Minnesota Experimental Farm, where they were part of a MHS swine genetics herd maintained by Dr. William E. Rempel of the Department of Animal Science. The pigs were tested for susceptibility to MH by a halothane-challenge test (5 min exposure to 3% halothane) at least 3 weeks prior to use. MHS animals were of the Pietrain breed, which react positively to the halothane-challenge test by demonstrating limb muscle rigidity in virtually all cases [20]. Normal animals were of the Yorkshire and Minnesota Number One breeds, or of a Yorkshire-Pietrain cross, all of which were non-reactors during the halothane-challenge test.

Reagents of the highest purity available were obtained from Sigma Chemical Co., or prepared as described previously [21]. Halothane was from Halocarbon Laboratories, Inc., Hackensack, NJ. Dantrolene was a gift of Norwich-Eaton Pharmaceuticals, Norwich, NY, and nitrendipine was a gift of Miles Laboratories, Inc., New Haven, CT. Water was redistilled from glass. Radionuclides were from New England Nuclear.

Isolation of skeletal muscle sarcolemma

Animals were anesthetized by intravenous infusion of sodium thiamylal, placed on a respirator, and ventilated with room air. Further doses of thiamylal were then administered to maintain surgical anesthesia (usually longer than 30 min). During deep anesthesia, the longissimus dorsi muscle (approximately 80% fast twitch fibers [22,23]) was removed, and immediately placed in ice. This procedure ensured that an MH episode, with potentially damaging effects on membrane properties, had not been triggered before or during the muscle dissection. Skeletal muscle sarcolemma membranes were then isolated by a LiBr-extraction method adapted specifically for porcine muscle [21]. The final yield of sarcolemma membrane was 8–16 mg protein from 400 g muscle, and was independent of the breed or strain used. Enzymatic activities of the isolated membranes were not diminished by storage at $-70°C$ for up to 1 year.

Chemical determinations and enzymatic activities

Protein, total cholesterol, and phospholipids were determined as described previously [21].

$(Na^+ + K^+)$-ATPase activity was measured in 100 mM NaCl, 10 mM KCl, 40mM histidine, 0.1 mM EGTA (pH 7.0 at 37°C). The reactions were initiated by the addition of 5 mM MgATP and terminated after 15 min by the addition of 1% SDS. Mg^{2+}-ATPase activity was determined in the presence of 1 mM ouabain. Ouabain-inhibitable $(Na^+ + K^+)$-ATPase activity was defined as the difference between the $(Na^+ + K^+)$-ATPase and the Mg^{2+}-ATPase activities.

Ouabain binding was measured in 10 mM Tris (pH 7.2), 3 mM PO_4-Tris, 3 mM $MgSO_4$, 1 mM EGTA, 10% sucrose, 1 μM [^{3}H]ouabain, at 37°C by the method of Mitchell et al. [24].

To determine the latency of $(Na^+ + K^+)$-ATPase and ouabain-binding activities, sarcolemma membranes were preincubated with SDS for 20 min at room temperature [21]. Using the maximal activity, obtained at an optimal SDS concentration, the percent latency was calculated from the following formula: [[Maximal activity (in SDS) − Patent activity (no SDS)]/[Maximal activity (in SDS)]] × 100.

Adenylate cyclase activity was measured in 40 mM Tris (pH 7.5), 10 mM KCl, 5 mM $MgCl_2$ (or 0.5 mM $MgCl_2$), 0.2 mM ATP (containing 200 000 cpm [α-^{32}P]ATP), 1 mM isobutylmethylxanthine, 1 mM dithiothreitol, 1 mM cAMP, 2 mM creatine phosphate, 0.1 mg creatine kinase/ml, 25 μg myokinase/ml, 30 μg membrane protein, in a final volume of 100 μl, at 37°C. Basal and NaF-activated activities were linear with time, while Gpp(NH)p-activated activities increased with incubation time; therefore, a single time point of 30 min was chosen [21]. The [^{32}P]cAMP produced was quantified following chromatography on neutral alumina [25].

Sarcolemmal calcium binding and calcium transport were measured in 120 mM KCl, 40 mM histidine (pH 7.0), varying Ca^{2+} concentrations (containing 5 μCi/ml ^{45}Ca), and 0.1 mg protein/ml at 37°C [26]. Sarcolemmal active calcium accumulation was initiated by the addition of 5 mM MgATP (Na^+-free), and samples were taken at 1 min for initial rate measurements (at which point the rate of calcium accumulation is still linear), or 2 and 20 min for estimation of the total calcium accumulating capacity. The media for passive calcium binding, as well as 2 and 20 min active calcium uptake measurements, contained 0.1 mM total $CaCl_2$ (approximately 15 μM Ca^{2+}), while the media for initial rate measurements contained a defined Ca^{2+} concentration established with a Ca-EGTA buffer (1 mM total $CaCl_2$) [26]. Calcium accumulated by sarcolemma vesicles was determined by a Millipore filtration technique. Sarcolemmal calcium transport measurements were performed in the presence or absence of 1 μM calmodulin, or 5 μM cAMP plus 0.1 mg/ml cAMP-dependent protein kinase. $K_{1/2}$ for Ca^{2+} and V_{max} values were calculated for each sarcolemma preparation from the initial rate data of control, calmodulin, and cAMP-dependent protein kinase treatments [26].

In experiments where halothane was present, the calcium uptake medium was contained in 1 ml glass Reacti-vials (Pierce) with Teflon-lined septum and screw-on caps. Halothane from a saturated aqueous solution (20 mM) was added through the septum, by way of a Hamilton syringe, and allowed to incubate with the sarcolemma in the calcium uptake medium for 2 min. Calcium transport was then initiated by MgATP addition through the septum. Dantrolene and nitrendipine, if present during calcium uptake, were added by employing DMSO and PEG 500 respectively as solvent (each less than 1% final concentration).

To examine sarcolemmal calcium efflux, the sarcolemma vesicles were first loaded with ^{45}Ca in the presence of 0.1 mM $CaCl_2$ and 5 mM MgATP, as described above. Sarcolemmal calcium efflux was initiated after 20 min of calcium uptake by the addition of 0.01 vol. 120 mM KCl, 40 mM histidine, 200 mM EGTA-Tris (pH 7.0), 1 mM sodium orthovanadate. Calcium remaining in the sarcolemma vesicles following EGTA plus vanadate addition was determined by Millipore filtration, and was expressed relative to the sarcolemmal calcium content determined at 20 min (linear regression analysis of samples taken at 17, 18 and 19 min of calcium uptake.

Determination of vesicle size

Average sarcolemma vesicle diameter was determined by laser light scattering in a Langly Ford

Instruments Model LSA92 Photon Correlation Spectrometer.

Electrophoretic analysis

Sarcolemma membrane samples (50 μg protein in 100 μl 1% SDS) were heated in a 90°C water bath for 10 min, when 25 mM dithiothreitol (final) was added. These samples were electrophoresed on 5–20% gradient polyacrylamide gels, in the presence of 0.1% (w/v) SDS [27]. Gels were stained with Coomassie Blue, destained, and then analyzed in a gel-scanning densitometer at 540 nm (E-C Apparatus Corp.). The mean peak areas (with S.D.) of the major Coomassie-Blue staining bands of MHS and normal sarcolemma preparations were determined. M_r of sarcolemma proteins were determined with the use of molecular mass markers purchased from Sigma.

Statistical analysis

Data from the three different non-reactor (normal) strains were combined, after analysis with a one-sided Student's *t*-test demonstrated no significant difference in their means. The comparison of the mean of the MHS population with the combined mean of the normal population was also performed by use of the *t*-test. Due to variability of the data between preparations, and the small relative changes measured, the significance of the relative effects of calmodulin and cAMP-dependent protein kinase on sarcolemmal calcium transport was determined by paired comparisons of the data to the control value of each individual preparation.

Results

Protein and lipid composition of sarcolemma membranes

An electrophoretic analysis of the protein composition of sarcolemma membranes isolated from MHS and normal animals is shown in Fig. 1. Estimation of the relative amounts of the major Coomassie Blue-staining bands, by densitometric scan of the gels, revealed no significant differences in the quantity of these proteins between MHS and normal sarcolemma.

A comparison of the lipid content of MHS and normal sarcolemma membranes shows no significant differences in total cholesterol, total phospholipid, or cholesterol : phospholipid mole ratios between the MHS and normal sarcolemma (Table I).

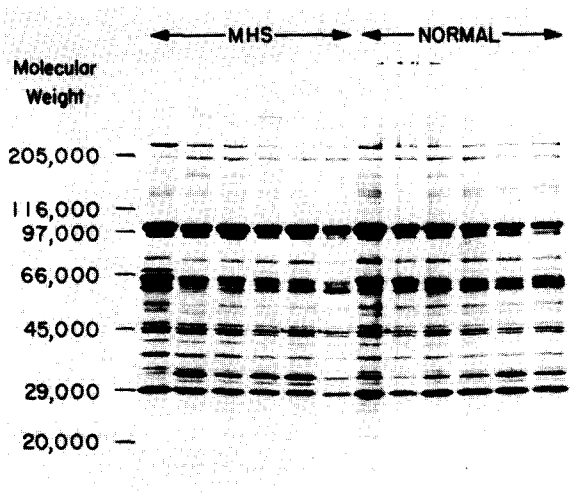

Fig. 1. Electrophoretic analysis of sarcolemmal proteins. Six different MHS and six different normal sarcolemma samples were prepared, electrophoresis was performed, and M_r were determined as described in Materials and Methods. The positions of molecular weight markers are given on the left.

TABLE I

LIPID CONTENT OF PORCINE MUSCLE SARCOLEMMA MEMBRANES

The values shown, expressed as means ± S.E., were determined as described in Materials and Methods.

	Cholesterol (μg/mg protein)	Phospholipid (μg P/mg protein)	Cholesterol : phospholipid mole ratio
MHS ($n = 9$)	243 ± 10	77.7 ± 1.8	0.247 ± 0.007
Normal ($n = 11$)	263 ± 10	76.7 ± 2.3	0.263 ± 0.008

$(Na^+ + K^+)$-ATPase activities, sidedness, and size of sarcolemma vesicles

A comparison of the patent $(Na^+ + K^+)$-ATPase activities (without SDS preincubation) of MHS and normal sarcolemma (Table II) shows that the ouabain-sensitive $(Na^+ + K^+)$-ATPase activities were 14.6 and 14.8 μmol P_i/mg per h for MHS and normal sarcolemma respectively. Preincubation of the sarcolemma membranes with SDS

TABLE II

$(Na^+ + K^+)$-ATPase ACTIVITIES OF PORCINE MUSCLE SARCOLEMMA MEMBRANES

Values are expressed as means ± S.E.

	Patent activity [a] (μmol P_i/mg per h at 37°C)			Total activity [b] (μmol P_i/mg per h at 37°C)			Percent latent [c]
	$(Na^+ + K^+)$-ATPase	Mg^{2+}-ATPase	ouabain-sensitive $(Na^+ + K^+)$-ATPase	$(Na^+ + K^+)$-ATPase	Mg^{2+}-ATPase	ouabain-sensitive $(Na^+ + K^+)$-ATPase	
MHS ($n = 9$)	18.5 ± 1.1	3.9 ± 0.4	14.6 ± 1.1	49.3 ± 3.2	3.6 ± 0.4	45.6 ± 3.0	67.8 ± 1.7
Normal ($n = 12$)	17.8 ± 1.0	3.0 ± 0.3	14.8 ± 1.1	48.4 ± 2.6	3.4 ± 0.3	46.5 ± 2.2	68.6 ± 2.2

[a] Determined without SDS preincubation.

[b] Determined after SDS preincubation as described in Materials and Methods; the maximal value expressed over a range of SDS concentrations.

[c] The percentage of the total ouabain-sensitive $(Na^+ + K^+)$-ATPase which was unmasked by SDS preincubation, calculated according to the formula described in Materials and Methods.

[21,24,28] led to the unmasking of $(Na^+ + K^+)$-ATPase activity (Table II). The optimal range of SDS concentrations for demonstrating the maximal enzyme latency, 0.40–0.55 mg SDS/mg protein, did not differ between MHS and normal sarcolemma. Table II shows that the total $(Na^+ + K^+)$-ATPase activity under these conditions was markedly inhibited by ouabain, resulting in ouabain-sensitive $(Na^+ + K^+)$-ATPase activities of 45.6 and 46.5 μmol P_i/mg per h for MHS and normal sarcolemma respectively. There was no significant difference in any aspect of $(Na^+ + K^+)$-ATPase activity between MHS and normal sarcolemma.

Based on the latency of K^+ – activated phosphatase, acetylcholinesterase, ouabain binding, and $(Na^+ + K^+)$-ATPase activities, we have previously concluded that sarcolemma membranes isolated from normal porcine muscle consist mainly of sealed inside-out vesicles (65–76%), with the remainder appearing to be leaky [21]. Since the latency of ouabain-sensitive $(Na^+ + K^+)$-ATPase activity was approximately 68% for both MHS and normal sarcolemma (Table II), it appears that the sidedness of the MHS and normal sarcolemma membrane preparations does not differ. This conclusion is also supported by the equal latency of the acetylcholinesterase activity of MHS and normal sarcolemma (65%) [15]. Measurements of average vesicle diameter for MHS sarcolemma ($n = 8$) and normal sarcolemma ($n = 8$) gave values of 0.33 ± 0.02 μm and 0.32 ± 0.01 μm (S.E.) respectively, which were also not significantly different.

The quantitation of $(Na^+ + K^+)$-ATPase can also be examined by determining total ouabain binding activity. Ouabain-binding capacity of SDS-treated sarcolemma membranes was measured following overnight incubation with 1 μM [^{3}H]ouabain, in the presence of P_i and Mg^{2+} to phosphorylate the $(Na^+ + K^+)$-ATPase [24]. It was found that MHS and normal sarcolemma bound similar amounts of ouabain, 70.0 ± 12.2 and 80.7 ± 13.4 pmol ouabain/mg, for MHS ($n = 10$) and normal ($n = 9$) membranes respectively (means ± S.E.).

Adenylate cyclase activity of sarcolemma membranes

In the presence of 5 mM $MgCl_2$ the basal adenylate cyclase activities of MHS and normal porcine muscle sarcolemma were stimulated by both NaF (9-fold), and the GTP analog, Gpp(NH)p (approximately 3.5-fold) (Table III). Isoproterenol, in the presence of Gpp(NH)p, further stimulated both MHS and normal sarcolemmal adenylate cyclase activities approximately 3-fold over those activities in the presence of Gpp(NH)p alone. Maximal stimulation of adenylate cyclase was seen at 10^{-5}–10^{-6} M isoproterenol, while half-maximal stimulation was seen at $5 \cdot 10^{-8}$ M isoproterenol. There was no dif-

TABLE III

ADENYLATE CYCLASE ACTIVITY OF PORCINE MUSCLE SARCOLEMMA MEMBRANES DETERMINED AT TWO $MgCl_2$ CONCENTRATIONS

The values shown, expressed as means ± S.E., were determined as described in Materials and Methods

	Adenylate cyclase activity (nmol cAMP/mg per 30 min at 37°C)				
	basal	+50 μM Gpp(NH)p	+10μM isoproterenol	+50 μM Gpp(NH)p +10 μM isoproterenol	+10 mM NaF
5 mM $MgCl_2$					
MHS ($n = 7$)	3.4 ± 0.7	11.9 ± 1.5	5.0 ± 0.9	31.8 ± 3.2	32.4 ± 2.8
normal ($n = 9$)	4.0 ± 0.6	14.4 ± 1.3	5.6 ± 0.7	36.1 ± 3.0	35.0 ± 2.7
0.5 mM $MgCl_2$					
MHS ($n = 3$)	0.35 ± 0.08	–	–	17.5 ± 6.2	20.0 ± 4.5
normal ($n = 5$)	0.69 ± 0.28	–	–	21.4 ± 6.6	27.6 ± 7.1

ference in the isoproterenol sensitivities of MHS and normal sarcolemmal adenylate cyclase activities.

Since it has been reported that the adenylate cyclase activity of MHS muscle homogenates was greater than controls only at low Mg^{2+} concentrations [7], the adenylate cyclase activity of the porcine muscle sarcolemma was also measured in 0.5 mM $MgCl_2$. Under our conditions, the adenylate cyclase activity seen in the presence of 0.5 mM $MgCl_2$, Gpp(NH)p plus isoproterenol, or NaF, was less than the respective activities in 5 mM $MgCl_2$ (Table III). There was no significant difference in any of these aspects of adenylate cyclase activity between MHS and normal sarcolemma.

Initial rates of calcium transport by sarcolemma vesicles

MHS and normal sarcolemma membranes bound similar amounts of calcium in the absence of ATP, 4.0 ± 0.5 and 3.8 ± 0.4 nmol Ca/mg protein, respectively (means ± S.E. for six MHS and normal preparations). In the presence of ATP, sealed inside-out sarcolemma vesicles accumulate calcium, resulting in the formation of a transmembrane calcium gradient [26,29]. Sarcolemmal active calcium transport is distinguishable from that of sarcoplasmic reticulum by the lack of oxalate stimulation, the high sensitivity to vanadate inhibition, and by the phosphate donor requirement [26,29]. In addition, the LiBr procedure used to prepare sarcolemma for this study renders sarcoplasmic reticulum incapable of calcium uptake [26]. Thus, we have concluded that essentially all the ATP-dependent calcium uptake in our sarcolemma preparation is due to calcium transport by sarcolemma vesicles [26]. The sarcolemmal calcium transporting system is likely to be the in vitro equivalent of a sarcolemmal calcium pump, responsible for the in situ translocation of sarcoplasmic calcium to the extracellular space.

The Ca^{2+} dependence of the initial rates of MHS and normal sarcolemmal ATP-dependent calcium transport are presented in Fig. 2 (means for all normal and MHS sarcolemma preparations examined). The data demonstrate that both MHS and normal sarcolemmal calcium uptake were activated by Ca^{2+} concentrations in the range 0.1–10 μM. Both cAMP-dependent protein kinase and calmodulin appeared to lower the $K_{1/2}$ for Ca^{2+} and increase the V_{max} of calcium transport of the normal sarcolemma preparations, thus confirming our previous observations [26]. However, only calmodulin had this stimulatory effect on MHS sarcolemmal calcium uptake, as the MHS sarcolemma appeared relatively insensitive to the stimulatory effects of cAMP-dependent protein kinase (Fig. 2).

A statistical analysis of the means of the individual $K_{1/2}$ for Ca^{2+} values of sarcolemmal calcium transport, calculated for each of the six normal and five MHS sarcolemma preparations, is shown in Table IV. MHS sarcolemma exhibited a significantly lower $K_{1/2}$ for Ca^{2+} than normal sarcolemma in the absence of exogenous activa-

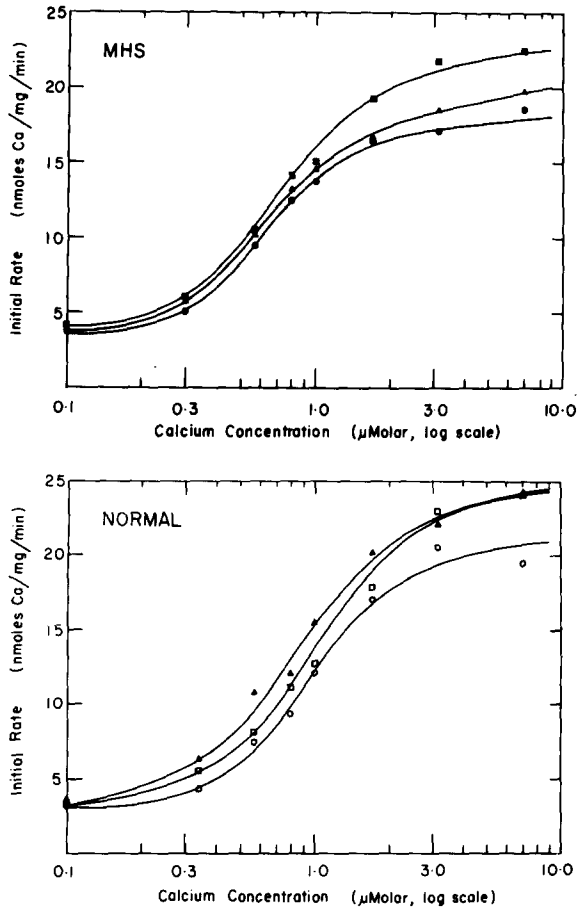

Fig. 2. Effects of calmodulin and cAMP-dependent protein kinase on the initial velocity of sarcolemmal calcium transport. MHS (top) and normal (bottom) sarcolemmal calcium transport, at varying ionized Ca^{2+} concentrations, was determined at 37°C as described under Materials and Methods. Data points are the means of five MHS or six normal sarcolemma preprations. ● and ○, no exogenous activator added; ■ and □, in the presence of 1 μM calmodulin; ▲ and △, in the presence of 5 μM cAMP plus 0.1 mg/ml cAMP-dependent protein kinase.

tors (0.70 vs. 0.90 μM). In the presence of calmodulin, MHS sarcolemma also had a significantly lower $K_{1/2}$ for Ca^{2+} than normal sarcolemma (0.64 vs. 0.81 μM), while in the presence of cAMP-dependent protein kinase, the $K_{1/2}$ for Ca^{2+} values of MHS and normal sarcolemma did not differ (0.73 vs. 0.68 μM). Due to the variability in the initial rates of calcium transport between sarcolemma preparations, and the small changes in the $K_{1/2}$ for Ca^{2+} induced by either cAMP-dependent protein kinase or calmodulin, the relative effect of calmodulin and cAMP-dependent protein kinase on the $K_{1/2}$ was determined for each individual preparation; the mean of these normalized effects was then calculated for the MHS and normal sarcolemma populations. Using this analysis it was found that for both MHS and normal sarcolemma, calmodulin significantly lowered the $K_{1/2}$ for Ca^{2+} ($P < 0.05$); however, in only the normal sarcolemma preparations did cAMP-dependent protein kinase addition result in a further decrease in the $K_{1/2}$ for Ca^{2+} (Table IV).

The V_{max} values for the initial rates of calcium transport were not significantly different for the MHS and normal sarcolemma preparations, regardless of the presence of cAMP-dependent protein kinase or calmodulin (Table IV). However, if the normalized data are examined (see above), then the relative increase in the V_{max} of individual normal sarcolemma preparations in response to cAMP-dependent protein kinase was approximately 2-fold greater than the relative increase in V_{max} observed in individual MHS sarcolemma preparations (30% ± 5 vs. 13% ± 2 respectively, $P <$ 0.05). The relative increase in the V_{max} of individual preparations of both normal and MHS sarcolemmal calcium transport in response to calmodulin was essentially identical (21% ± 3 vs. 25% ± 4 respectively).

Sarcolemmal calcium uptake 2 and 20 min after ATP addition

The rate of porcine muscle sarcolemmal calcium transport is linear with time for approximately 1.5 min, although net calcium accumulation continues for over 20 min [26]. We considered it possible that relatively small differences between MHS and normal sarcolemmal calcium transport could become more apparent after more calcium had been accumulated. Thus, MHS and normal sarcolemmal calcium transport was determined at increasing times after MgATP addition. Table V shows that when sarcolemmal calcium uptake was measured at either 2 or 20 min after the initiation of calcium transport, MHS sarcolemma had a significantly lower ability to accumulate calcium when compared with normal sarcolemma. Diminished MHS sarcolemmal calcium accumulating capacity was observed not only in the absence of exogenous activators, but also in the

TABLE IV

KINETIC PARAMETERS OF SARCOLEMMAL CALCIUM TRANSPORT

Parameters are expressed as the means of the respective values for each preparation ± S.E. Initial rates of calcium transport at varying ionized Ca^{2+} concentrations were determined, and $K_{1/2}$ and V_{max} values were calculated, as described in Materials and Methods. cAMP-PK, cAMP-dependent protein kinase.

	$K_{1/2}$ for Ca^{2+} (μM)			V_{max} (nmol Ca/mg per min at 37°C)		
	control	+1 μM calmodulin	+5 μM cAMP +0.1 mg/ml cAMP-PK	control	+1 μM calmodulin	+5 μM cAMP +0.1 mg/ml cAMP-PK
MHS ($n = 5$)	0.70 ± 0.03	0.64 ± 0.04 [a]	0.73 ± 0.05 [b]	18.7 ± 2.4	23.2 ± 2.8 [a]	20.2 ± 2.4 [a,b]
normal ($n = 6$)	0.90 ± 0.08 [c]	0.81 ± 0.08 [a,c]	0.68 ± 0.09 [a]	19.7 ± 3.3	23.8 ± 4.0 [a]	24.9 ± 4.9 [a]

[a] Different from control, paired comparisons of values for individual preparations within a group, $P < 0.05$.
[b] Different from calmodulin, paired comparisons of values for individual preparations within a group, $P < 0.05$.
[c] Different from MHS, $P < 0.05$.

presence of either calmodulin or cAMP-dependent protein kinase. The significance of the difference between MHS and normal sarcolemma was greater at the 20 min ($P < 0.025$–0.050) than at the 2 min ($P < 0.15$) time point. The average relative difference between the mean values of calcium uptake for the MHS and normal sarcolemma populations (all conditions at both time points of Table V) demonstrated that the calcium accumulating ability of MHS sarcolemma vesicles was reduced 23.7% ± 5.0 (S.D.) relative to normal sarcolemma vesicles.

Both calmodulin and cAMP-dependent protein kinase significantly stimulated MHS and normal sarcolemmal calcium accumulation above control values, at both the 2 and 20 min time points (Table V). The extent of the stimulation of both MHS and normal sarcolemmal calcium uptake by cAMP-dependent protein kinase (16–26%) was slightly less than that by calmodulin (25–32%). There was no significant difference between MHS and normal sarcolemma in the relative stimulation of sarcolemmal calcium transport by either cAMP-dependent protein kinase or calmodulin after either 2 or 20 min of calcium uptake (Table V).

TABLE V

EFFECT OF SAMPLE TIME ON SARCOLEMMAL CALCIUM TRANSPORT

Values are expressed as means ± S.E. Calcium transport in the presence of 0.1 mM $CaCl_2$ was determined at 2 and 20 min after MgATP addition, as described in Materials and Methods. cAMP-PK, cAMP-dependent protein kinase.

Activators added	Calcium transport (nmol Ca/mg)			
	2 min		20 min	
	MHS ($n = 9$)	normal ($n = 11$)	MHS ($n = 9$)	normal ($n = 11$)
Control	23.3 ± 2.7	28.6 ± 3.4 [a]	83.1 ± 8.5	119.0 ± 11.8 [b]
1 μM Calmodulin	30.4 ± 3.3	37.6 ± 4.4 [a]	103.5 ± 10.9	149.8 ± 14.7 [b]
5 μM cAMP +0.1 mg/ml cAMP-PK	26.1 ± 3.1	33.2 ± 4.5 [a]	105.8 ± 12.3	140.2 ± 15.2 [c]

[a] Different from MHS, $P < 0.15$.
[b] Different from MHS, $P < 0.05$.
[c] Different from MHS, $P < 0.025$.

A similar calmodulin dependence for the stimulation of MHS and normal sarcolemmal calcium transport was observed (Fig. 3). Half-maximal stimulation of the 2 min sarcolemmal calcium uptake value was seen at approximately 150 nM calmodulin (Fig. 3). The stimulation of MHS and normal sarcolemmal calcium uptake after 20 min of calcium transport showed an identical calmodulin dependence to that of Fig. 3 (data not shown).

The effect of the MH triggering agent halothane (2 min preincubation) on sarcolemmal calcium uptake after 2 min of calcium transport is shown in Fig. 4. Below a concentration of 3 mM, halothane had no effect on either MHS or normal sarcolemmal calcium uptake, whereas at higher halothane concentrations calcium uptake by both MHS and normal sarcolemma was inhibited in a similar fashion (half-maximal inhibition of calcium uptake at approximately 10 mM halothane). The inhibition of MHS and normal sarcolemmal calcium uptake after 20 min of calcium transport showed an identical halothane dependence to that of Fig. 4 (data not shown). The effect of halothane on sarcolemmal calcium uptake was not altered by longer preincubation of the sarcolemma with halothane (up to 20 min) prior to MgATP addition, or by varying the sarcolemmal protein concentration (0.03–0.30 mg/ml) (data not shown). Neither dantrolene (10–30 μM) nor nitrendipine (0.1 nM–1.0 μM) had any effect on sarcolemmal calcium transport (data not shown).

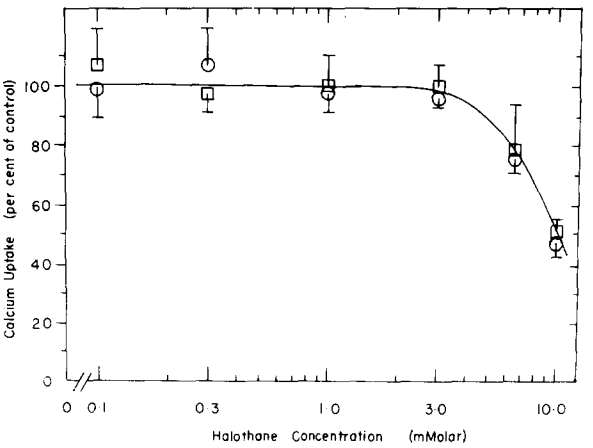

Fig. 4. Effect of halothane on sarcolemmal calcium transport. Sarcolemma membranes were preincubated for 2 min at 37°C with the indicated halothane concentration and the media described under Materials and Methods. MgATP was then added to initiate calcium transport, and the accumulated sarcolemmal calcium after 2 min of calcium uptake was determined. Data for the effect of halothane on sarcolemmal calcium uptake was expressed relative to each preparation's control value in the absence of halothane (100%). Points represent means ± S.E. for five preparations each of MHS (□) or normal (○) sarcolemma.

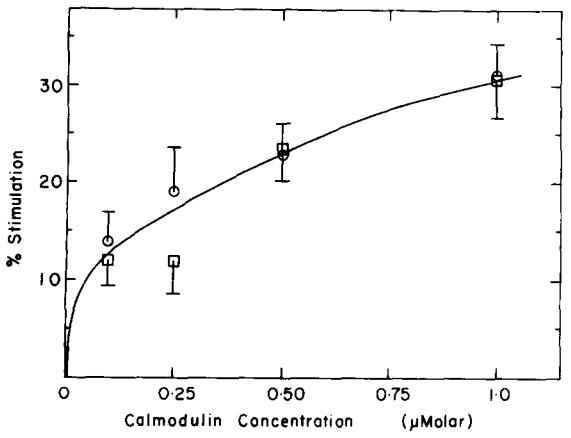

Fig. 3. Effect of calmodulin concentration on sarcolemmal calcium transport. Sarcolemmal calcium uptake, in the presence of varying concentrations of calmodulin, was determined 2 min after MgATP addition, as described under Materials and Methods. Data were expressed relative to each preparation's control value in the absence of calmodulin (zero percent stimulation). Data represent means ± S.E. for 9 MHS (□) or 12 normal (○) sarcolemma preparations.

Calcium efflux from sarcolemma vesicles

We initially chose to examine sarcolemmal calcium efflux utilizing sarcolemmal vesicles which had been passively loaded with ^{45}Ca, similar to the method used by Meissner for sarcoplasmic reticulum calcium loading [30]. However, this technique proved inappropriate because of the small amount of calcium which could be passively loaded, as well as the very slow rate of calcium release from the sarcolemma vesicles. Thus, to examine sarcolemmal calcium permeability and calcium efflux, sarcolemma vesicles were actively loaded with ^{45}Ca for 20 min at 37°C in the presence of MgATP, at which time various additions were made to the reaction mixture. Fig. 5 shows a representative experiment, demonstrating that under these conditions sarcolemmal calcium

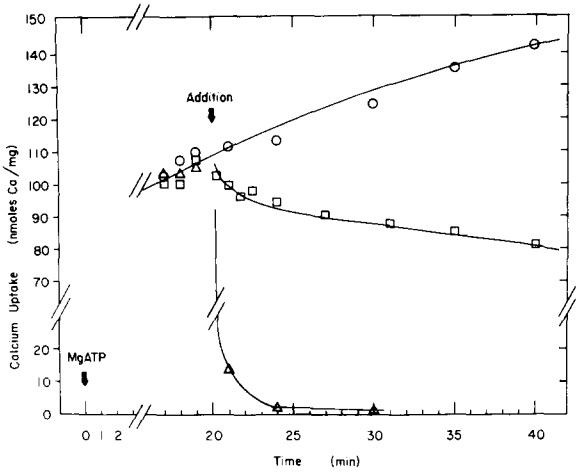

Fig. 5. Sarcolemmal calcium efflux. Sarcolemmal calcium uptake was initiated at zero time by the addition of MgATP, and the accumulated calcium at 17, 18, and 19 min was determined by Millipore filtration as described under Materials and Methods. At 20 min, 0.01 vol. of a calcium release solution (final concentration indicated below), in 120 mM KCl, 40 mM histidine (pH 7.0, 37°C) was added, and the sarcolemmal calcium content at times thereafter was determined. Points are the means of replicates for a representative experiment. ○, control; □, 2 mM EGTA, 10 μM sodium orthovanadate; △, 5 μM A23187.

uptake was unaffected by the addition of 0.01 vol. of 120 mM KCl plus 40 mM histidine at 20 min. Sarcolemmal calcium efflux was initiated, however, when in addition to KCl plus histidine, EGTA and sodium orthovanadate were also added (final concentrations of 2 mM and 10 μM respectively). EGTA lowered the ionized calcium concentration to less than 10 nM, thus forming a calcium gradient across the sarcolemma-membrane vesicles favoring calcium efflux. In addition, this Ca^{2+} concentration is far below that at which the sarcolemmal calcium pump is effective in accumulating calcium [26,29]. Orthovanadate was also included to inhibit the sarcolemmal calcium pump [26,29], thus further inhibiting calcium uptake. Sarcolemmal calcium efflux induced by EGTA plus orthovanadate addition appeared to exhibit an initial rapid phase of calcium release, immediately followed by a long slow phase (Fig. 5). In contrast, the addition of the calcium ionophore A23187 after 20 min of calcium uptake caused a very rapid release of all the accumulated calcium.

When the sarcolemmal calcium content at various times after EGTA plus orthovanadate addition was normalized to the sarcolemmal calcium content determined at the initiation of calcium efflux, the release of calcium appeared biphasic (Fig. 6, average of triplicate determinations on six preparations each of MHS or normal sarcolemma). The calcium efflux data for individual preparations, however, could not be satisfactorily fit to a double exponential form with a non-linear least-squares fitting program. For both MHS and normal sarcolemma, approximately 10% of the initial calcium load was released rapidly, in the first 1–2 min. Linear correlation analysis of the log percent calcium retained versus time plot, for each sarcolemma preparation, demonstrated that the slow phase of calcium efflux was approxi-

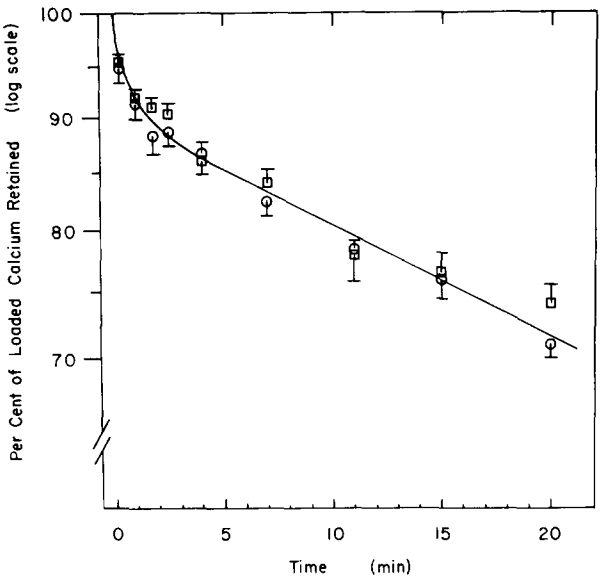

Fig. 6. Time course of calcium efflux from MHS and normal sarcolemma. ATP-dependent sarcolemmal calcium uptake proceeded at 37°C for 20 min, as described in Materials and Methods. Sarcolemmal calcium content at 20 min was estimated by linear regression analysis of the samples assayed at 17, 18, and 19 min following MgATP addition. At 20 min sarcolemmal calcium efflux was initiated by the addition of 2 mM EGTA plus 10 μM orthovanadate, as described in the legend to Fig.5. At the indicated times after addition of EGTA plus orthovanadate, the calcium remaining with the sarcolemma was determined, and expressed relative to the initial calcium load after 20 min of calcium uptake (100%). Points represent the means ± S.E. for triplicate determinations on each of six MHS (○) or normal (□) sarcolemma preparations.

mately linear from 1.75 to 20 min after the addition of EGTA plus orthovanadate (average r value of -0.984). Thus, first-order rate constants of 0.0126 ± 0.0011 min^{-1} and 0.0114 ± 0.0010 min^{-1} could be obtained for the slow phase of MHS and normal sarcolemmal calcium efflux, respectively (means $\pm$ S.E. for six preparations each). MHS and normal sarcolemmal calcium efflux values were not significantly different.

Discussion

A number of studies have indicated that some aspect of the structure or function of the skeletal muscle surface membrane is abnormal in MHS pigs and humans [7–15]. In this report we have compared various compositional and functional characteristics of a highly purified sarcolemma preparation isolated from the muscle of normal and MHS pigs. MHS and normal sarcolemma membranes of identical yield, protein distribution (Fig. 1), total cholesterol and phospholipid content (Table I), and vesicle size and sidedness (Table II) were obtained, indicating that our sarcolemma isolation technique was appropriate for both MHS and normal porcine muscle. These similarities between sarcolemma fractions are an important prerequisite, as muscle from MHS animals often undergoes rapid post-mortem changes [2], which can lead to damage of isolated membrane fractions [31].

In the only previous report examining properties of isolated sarcolemma membrane fractions in MH [9], $(Na^+ + K^+)$-ATPase activities were approximately 10-times less than those reported here (Table II), and the cholesterol/protein ratio was approximately 5-fold lower than this study (Table I). Furthermore, in that report the degree of vesiculation, membrane sidedness, and enzyme latency were not determined. The low content of sarcolemmal markers in that study makes it difficult to evaluate the author's contention that alterations in sarcolemmal properties occur after initiation of MH in vivo [9]. Their results could also be explained by different degrees of contamination by non-sarcolemmal membranes as a result of MH onset, or sarcolemmal damage due to acidification or proteolysis in the muscle or in the muscle homogenates.

Previous studies have indicated both an increased adenylate cyclase activity [7] and an increased $(Na^+ + K^+)$-ATPase content [8] in MHS human muscle. In addition to possible human versus porcine differences, a likely explanation for the discrepancy between those reports and our own data is that different systems were used for the examination of sarcolemmal activities; we have utilized purified sarcolemma membranes, while the other workers have used muscle homogenates. An isolated membrane fraction offers advantages for the study of sarcolemmal properties, as enzymatic specific activities are higher, the membrane orientation and substrate accessibility can be determined, and assay conditions can be accurately controlled.

Skeletal muscle sarcolemma contains an ATP-dependent calcium transporter, stimulable by cAMP-dependent protein kinase and calmodulin [26,29], which is similar to the plasmalemmal calcium pumps of other tissues [32]. That the values for the $K_{1/2}$ for Ca^{2+} in the presence of calmodulin or cAMP-dependent protein kinase (0.64–0.81 μM, Table IV), the $C_{1/2}$ for calmodulin stimulation (150 nM, Fig. 3), and the relative stimulation of calcium uptake by cAMP-dependent protein kinase or calmodulin (2 and 20 min values, Table V) are similar for both MHS and normal sarcolemma suggests that the control of the sarcolemmal calcium pump is not abnormal in MH. The explanation for the minimal effect of cAMP-dependent protein kinase on the $K_{1/2}$ for Ca^{2+} and V_{max} values for MHS sarcolemmal calcium transport is unknown at this time (Table IV).

Although the regulation of MHS sarcolemmal active calcium transport appeared normal, net sarcolemmal calcium uptake, when monitored at either 2 or 20 min after the initiation of calcium transport, was significantly lower in MHS than normal sarcolemma (Table V). This 25% reduction in calcium uptake was observed either in the absence or presence of the exogenous activators calmodulin and cAMP-dependent protein kinase. Because measurements of vesicle size and sidedness demonstrated no difference between MHS and normal sarcolemma preparations (Table II; [15]), diminished calcium accumulating capacity cannot be attributed to a decreased vesicle volume,

or different percentage of inside-out vesicles in the MHS sarcolemma-membrane fraction. This suggests that MHS sarcolemma vesicles were unable to generate as large a calcium gradient as normal sarcolemma vesicles. One explanation for this difference is the possibility of less calcium pump protein in the MHS sarcolemma preparations. However, measurements of the V_{max} of sarcolemmal calcium transport did not support this possibility, as no significant difference was detected between MHS and normal sarcolemma preparations (Table IV). It is possible, though, that due to the variability between preparations, and the relatively small difference between MHS and normal sarcolemma, our techniques could not detect a potential difference in V_{max} values of sarcolemmal calcium uptake. V_{max} values were calculated from the initial rate data, obtained during the first min of calcium uptake, on a limited number of preparations. That the statistical significance of the difference between MHS and normal sarcolemmal calcium uptake activities became greater with increasing time and amount of calcium uptake (2 versus 20 min, Table V), and the evaluation of more preparations (Table V), would support this conclusion. Thus, the hypothesis that MHS sarcolemma vesicles have less calcium pump protein than normal sarcolemma remains a possibility.

An alternative explanation for the difference between MHS and normal sarcolemmal calcium transport is that MHS sarcolemma vesicles may be 'leakier' to calcium, or have an abnormal membrane environment, such that they are unable to maintain as large a transmembrane calcium gradient as normal sarcolemma. The possibility that the diminished calcium accumulating capacity of the MHS sarcolemma was due to a greater than normal leakiness of the MHS vesicles to calcium was investigated (Figs. 5 and 6). After the addition of EGTA plus orthovanadate to the calcium-loaded sarcolemma vesicles, both MHS and normal sarcolemma exhibited an initial rapid phase of calcium release (approximately 10% of the calcium released in the first 2 min). Depending upon the sarcolemma preparation examined, passive calcium binding (4 nmol Ca/mg) represents 2–5% of the total sarcolemmal calcium content before the initiation of calcium efflux. Thus, it is likely that calcium release from these membrane sites contributes, in part, to the rapid phase of sarcolemmal calcium release. However, this rapid calcium release is probably complex, and its precise nature is unknown at this time. An extended slow phase of sarcolemmal calcium efflux, which was approximately first-order ($k = 0.012$ min^{-1}), immediately followed the rapid calcium release. This slow phase of sarcolemmal calcium release (corresponding to 0.5–1.5 nmol Ca/mg per min), which may represent calcium efflux from the interior of the vesicles, did not differ between MHS and normal sarcolemma. Therefore, the proposal that an increased leakiness of the MHS sarcolemma vesicles to calcium may be responsible for the diminished MHS sarcolemmal calcium uptake was not supported. We would also conclude from this data that the passive permeability of MHS sarcolemma to calcium is not abnormal in MH. The very low permeability of the isolated sarcolemma vesicles to calcium, approximately 10 000-fold slower than the rate of sarcoplasmic reticulum calcium release [33], would be expected in view of the sarcolemma's role in maintaining the surface membrane calcium gradient in vivo. The possibility that MHS sarcolemma might have an abnormal membrane environment, which might influence calcium transport, is currently under investigation. In this regard, a decreased MHS surface membrane lipid bilayer fluidity, both in intact red blood cells and in erythrocyte ghost membranes, has recently been found in this laboratory [17]. Therefore, further work is required to clarify the nature of the difference in calcium accumulating capacity between MHS and normal sarcolemma vesicles.

Our evidence of a defect in MHS sarcolemmal calcium transport supports hypotheses of abnormal calcium regulation in MH (for review, see Ref. 1), and may help to explain the elevated resting sarcoplasmic Ca^{2+} concentration reported in MHS muscle [18]. It appears unlikely, however, that the sites of action of the MH initiator, halothane, or the therapeutic agent, dantrolene, involve sarcolemmal calcium transport or calcium permeability. Halothane affected MHS and normal sarcolemma in a similar fashion, and inhibition of calcium uptake was observed only at non-clinically relevant concentrations (Fig. 4); dantro-

lene had no effect at all on sarcolemmal calcium uptake. Rather, halothane and dantrolene appear to primarily affect sarcoplasmic reticulum calcium release [34,35].

It is possible that defective MHS sarcolemmal calcium transport is also an indication of abnormal MHS T-tubule calcium regulation, as T-tubules possess a calcium transporting mechanism [36,37] very similar to that of the sarcolemma [26,29]. Such a generalized MHS muscle surface membrane defect could contribute, in part, to the abnormal contractile properties characteristic of MHS muscle [19]. Thus, we conclude that altered MHS sarcolemmal calcium regulation, in concert with an abnormally sensitive sarcoplasmic reticulum calcium release mechanism [14,38–42], probably plays a significant role in the MH syndrome.

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