LABORATORY INVESTIGATIONS

Abnormal Action Potential Responses to Halothane in Heart Muscle Isolated from Malignant Hypertension-susceptible Pigs

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Background: During human and porcine malignant hypertthermia (MH), cardiac dysrhythmias and altered myocardial function can be observed. It is unknown whether a primary abnormality in cardiac muscle contributes to the cardiac symptoms during MH. An abnormal response to halothane has recently been demonstrated in action potentials (APs) from MH-susceptible (MHS) human skeletal muscles. We investigated the electrophysiologic properties in trabeculae isolated from the right ventricles of normal (MHN) and MHS pigs.

Methods: The experiments were performed on electrically stimulated (1 Hz) trabeculae isolated from the right ventricles of MHS and MHN pigs. Resting membrane potentials, APs, and tension were measured with and without the presence of 1% halothane. In addition, the halothane-equilibrated muscles were exposed to caffeine in increasing doses (1, 2, and 4 mM).

Results: In the absence of halothane, resting potential and AP characteristics in MHS and MHN muscles did not differ significantly. Halothane did not alter resting potential but produced different alterations in the APs in MHS and MHN muscles, whereas the decrease in twitch tension was identical. In contrast, the increase in AP amplitude and duration in MHS muscle, halothane produced an enlargement of the APs in MHS muscle. The addition of caffeine caused nearly identical prolongations of AP duration in MHS and MHN muscles.

Conclusions: This in vitro study demonstrates that halothane produces abnormal alterations in the dynamic electric properties of the ventricular excitable membrane from MHS pigs. These results suggest a latent defect in the myocardium of MHS pigs that becomes apparent in the presence of MH-triggering agents. (Key words: Anesthetics, volatile; halothane.

Hypertension: malignant. Heart: action potentials; resting potentials.)

MALIGNANT hypertthermia (MH) is a genetic disease triggered by volatile anesthetics or depolarizing muscle relaxants. This life-threatening condition is associated with cardiac disturbances such as tachycardia, other dysrhythmias, and increased myocardial O2 consumption, which often precede the increase in body temperature and muscle rigidity. It therefore has been speculated that MH is a disease of skeletal and cardiac muscle. However, it is unknown whether these symptoms are caused by a primary abnormality in cardiac muscle or by acidosis, hypercapnia, electrolyte imbalances, and an increase in plasma catecholamine concentration.

In swine the single defined genetic defect appears to be in the gene for the skeletal muscle Ca2+-release channel, identified as RYR1 in genetic cloning experiments. The expressed ryanodine receptor differs from that expressed in cardiac muscle, designated RYR2. Comparing MH-susceptible (MHS) and normal (MHN) heart muscle, a previous in vitro study did not reveal different effects of halothane on tension development. Another study revealed abnormal action potential (AP) changes by halothane in MHS skeletal muscle fibers.

It therefore was of interest to determine whether these observations made in skeletal muscle are accompanied by similar changes in heart muscle. The current study investigated the electrophysiologic effects of halothane in isolated heart preparations from MHS swine.

Materials and Methods

The study was reviewed and approved by the institutional animal care and use committee. MHS (n = 14) and healthy MHN control (n = 14) homozygote Pietrain
swine were obtained from a pig breeding institute (Deutsche Pig Incorporation, Kiel, Germany) where they had been selectively bred for MH susceptibility or MH resistance by halothane challenge of at least five generations. The genotype of MH susceptibility was determined by analysis (polymerase chain reaction) of deoxyribonucleic acid from blood of the pigs used in this study. The anesthetized animals (metomidate HCl, 10 mg/kg intraperitoneally; Janssen, Neuss, Germany) were killed by a blow to the head and by bleeding from the carotid arteries. The heart and the musculus extensor digitorum longus were quickly excised and suspended in cold (4°C) prearated bathing solution (composition described below) and delivered to the laboratory within 10 min. Skeletal muscle was used for in vitro contracture testing according to the protocol of the European Malignant Hyperthermia Group,17 additionally performed to confirm MHS or MHN status. The experiments were performed on electrically stimulated trabeculae isolated from the right ventricle of the pig hearts. Trabeculae were dissected in aerated bathing solution (vide infra) at room temperature. Only thin (diameter <1.0 mm, length 3–10 mm) trabeculae were used to avoid diffusio hypoxia in the center of the muscle. The preparations were not injured except at their cut ends. Muscles were mounted in a single low-volume, high-flow recording chamber (0.8-ml volume, 8-ml/min flow) and superfused with Tyrode’s solution (millimolar concentrations: NaCl 140, KCl 4.7, CaCl2 2.5, MgCl2 1.0, NaH2PO4 0.42, NaHCO3 22.6, Na2EDTA 0.05, ascorbic acid 0.28, and glucose 5.0) at 35°C. This solution was circulated through the chamber from heated reservoirs through which 95% O2 and 5% CO2 was bubbled, maintaining pH at 7.4. The tendinous end of the muscle was connected to the arm of a mechanoelectric transducer (type SS 201, Collins, Long Beach, CA) for isometric measurements. Muscle length was adjusted to the least resting tension that produced maximum active tension. The preparations were electrically driven at 1 Hz by rectangular voltage pulses 4 ms in duration (stimulator SD 9, Grass, Quincy, MA) and about 10% above threshold voltage through one concentric bipolar platinum electrode close to the base of the muscle. Conventional glass microelectrodes filled with 3 M KCl (resistance 10–20 MΩ) connected to a preamplifier (microelectrode amplifier type 309, Hugo Sachs Elektronik, Freiburg, Germany) were used to impale the muscle fibers and monitor the intracellular potential. Bath potential, monitored through an agar-bridge Ag–AgCl electrode, was subtracted from the intracellular potential to give the membrane potential. The maximum rate of depolarization was determined with a differentiator exhibiting a linear response from 100 to 1,000 V/s and a sample and hold amplifier. APs and their time differential were displayed on a storage oscilloscope (D13, Tektronix, Beaverton, OR). In addition, the analog data were converted to digital form and analyzed on line on a microprocessor computer (International Business Machines, Armonk, NY). The values measured for each AP included the resting potential, AP amplitude, maximum rate of phase 0 repolarization, and AP duration measured at the 20%, 50%, and 90% repolarization levels (APD20, APD50, and APD90, respectively). These AP characteristics were sampled in groups of ten consecutive APs from a single cell and averaged at regular intervals during the course of experiments. The force of contraction was continuously recorded on a strip-chart recorder. Although in a previous investigation14 depression of twitch tension by halothane was found to be nearly equal in MHS and MHN preparations, twitch tension was used to monitor contractile activity throughout the experiments. All preparations were allowed to equilibrate in drug-free bathing solution (35°C) until complete electric and mechanical stabilization. Halothane (1 vol%) was equilibrated with solution in one reservoir by passing the carbogen (95% O2 and 5% CO2) through a calibrated vaporizer (Dräger, Lübeck, Germany) for at least 20 min before application to the organ bath. Halothane concentration in carbogen was measured with an anesthetic gas monitor (Normac, Datex, Helsinki, Finland). The equilibrium concentration of 1% halothane in the bath, determined directly by gas chromatography (n = 5), was 0.35 ± 0.05 mm. After control measurements, muscles were exposed for 20 min to the halothane-equilibrated solutions. Within this time, the reduction in twitch tension reached a steady state. The measurements with halothane were followed by a halothane washout period and recording of recovery responses. Drug washout was accomplished by a 30-min perfusion with halothane-free bathing solution. After the intervening washout period, the preparations were exposed again to 1% halothane for 20 min. Next, caffeine (Sigma Chemie, Deisenhofen, Germany) was applied cumulatively to the heart preparations (n = 10 muscles each group) with maintenance of the halothane concentration and with the reservoir solution preequilibrated with various caffeine concentrations.

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Caffeine was administered only in the MH group. Only imipramine was administered to both MH groups throughout experiments; other protocols were identical.

The data were analyzed by analysis of variance on unpaired observations and were evaluated using either multiple-range or Student’s t-test with a level of significance of less than 0.05.

Results

Original fast twitch tension (A0) was significantly lower in the MH group than in the MHS group (2.9 ± 0.5 mm, and 8.1 ± 0.5 mm, respectively). Baseline values (APD90) were significantly (p < 0.05) decreased by caffeine exposure. Baseline values (APD90) were significantly (p < 0.05) increased after halothane exposure. Although caffeine increased APD50 and APD90 in both groups, the percentage increase was significantly (p < 0.05) greater in the MHS group. Halothane did not significantly alter twitch tension in both groups. Halothane increased twitch tension in both groups, but APD90 was significantly greater in the MHS group. Maximum rates of calcium inactivation were significantly (p < 0.05) higher in all variables of the MH group. Halothane was removed from the solution, and the final values (3.3 ± 0.5 mm) in the MHS group. The addition of caffeine (4 mm) to the medium produced a dose-dependent effect in each group. Twitch tension was significantly increased.
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Caffeine was present for 5 min at each concentration. Only impalements of single cells that were maintained throughout control, experimental, and recovery periods were included in the study.

The data presented are means ± SEM. Statistical significance was estimated with Student's t test for paired or unpaired observations. In addition, the AP characteristics were evaluated by analysis of variance, and Duncan's multiple-range test was used to compare mean values obtained in various conditions. A probability (P) value less than 0.05 was considered statistically significant.

Results

Original fast APs of MHN and MHS heart muscle are shown in figures 1A and 1B, respectively. The mean values of the AP variables in the absence and presence of halothane are summarized in table 1. In drug-free Tyrode's solution, the AP parameters in MHN muscle were not significantly different from those in MHS muscles. In the presence of halothane, the AP configuration in MHN and MHS muscles differed. In MHN muscle, the twitch tension significantly (P < 0.05) decreased, from 3.9 ± 0.4 to 2.6 ± 0.5 mN, and the AP amplitude decreased by 4% from baseline value and narrowed in a way that the plateau (APD90) was shortened (fig. 1A). APD20, APD50, and APD90 decreased by approximately 10%, 12%, and 11% from baseline values, respectively (table 1). No significant changes were noted in the resting membrane potential and maximum rate of phase 0 repolarization. In MHS muscle, however, the AP was slightly prolonged and slightly increased in amplitude (fig. 1B), whereas the twitch tension depression (from 3.8 ± 0.8 to 2.5 ± 0.9 mN) did not differ significantly from the MHN group. Halothane increased the amplitude by approximately 5%, and the APD20, APD50, and APD90 by approximately 9%, 4%, and 6%, respectively (table 1). With these changes, maximum rate of phase 0 repolarization did not increase significantly, and mean resting potential continued identical to control (−91 ± 2 mV). In MHN and MHS muscles, all variables returned nearly to control values when halothane was removed (table 1). In the drug-free Tyrode's solution, the depressed twitch tension increased back to 3.5 ± 0.5 mN in the MHN and to 3.4 ± 0.9 mN in the MHS group.

The addition of caffeine in cumulative doses (1, 2, and 4 mM) to the muscles reequilibrated with halothane produced a dose-dependent alteration of the AP shape for each group. In MHN preparations, the AP was slightly depressed in amplitude, in association with an increase in the AP duration (fig. 1A). Exposure to 4 mM caffeine increased APD20, APD50, and APD90 by approximately 26%, 17%, and 12%, respectively, from initial control values (table 1). In the muscles of the MHS pigs, the AP was increased in amplitude and markedly widened (fig. 1B) as expressed by the marked prolongation of APD20, APD50, and APD90 (+52%, +44%, and +42%, respectively, at 4 mM). Compared with the AP duration in the presence of halothane alone, the relative changes in AP duration were nearly identical in MHN (+40%, +33%, and +25%) and MHS (+39%, +38%, and +34%). In both muscle preparations caffeine induced small but significant decreases in maximum rate of phase 0 repolarization and in resting membrane potential. An increase in baseline tension was not observed in any of the experiments, either with halothane or with the addition of caffeine.

Discussion

This in vitro study shows an abnormal response of the fast AP in cardiac muscle of MHS pigs exposed to
Table 1. Effects of Halothane (1%) Alone and in the Presence of Caffeine (1–4 μM) on the Transmembrane Potential in Trabeculae Isolated from the Right Ventricles of Normal (MHN) and MH-Susceptible (MHS) Pigs (n = 10 Each Group)

<table>
<thead>
<tr>
<th>Action Potential Characteristics</th>
<th>RP (mV)</th>
<th>Amp (mV)</th>
<th>V_{max} (V/s)</th>
<th>APD_{90} (ms)</th>
<th>APD_{50} (ms)</th>
<th>APD_{0} (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>MHN</td>
<td>−91 ± 1</td>
<td>130 ± 1</td>
<td>179 ± 9</td>
<td>84 ± 6</td>
<td>177 ± 6</td>
</tr>
<tr>
<td></td>
<td>MHS</td>
<td>−91 ± 2</td>
<td>129 ± 2</td>
<td>177 ± 8</td>
<td>91 ± 7</td>
<td>181 ± 8</td>
</tr>
<tr>
<td>Halothane (1%)</td>
<td>MHN</td>
<td>−91 ± 1</td>
<td>125 ± 1†</td>
<td>176 ± 8</td>
<td>76 ± 7*</td>
<td>156 ± 8*</td>
</tr>
<tr>
<td></td>
<td>MHS</td>
<td>−91 ± 2</td>
<td>134 ± 2†</td>
<td>188 ± 9</td>
<td>99 ± 8*†</td>
<td>189 ± 7†</td>
</tr>
<tr>
<td>Control (R)</td>
<td>MHN</td>
<td>−91 ± 1</td>
<td>129 ± 2</td>
<td>181 ± 7</td>
<td>86 ± 8</td>
<td>173 ± 6</td>
</tr>
<tr>
<td></td>
<td>MHS</td>
<td>91 ± 2</td>
<td>131 ± 2</td>
<td>180 ± 8</td>
<td>92 ± 6</td>
<td>184 ± 7</td>
</tr>
<tr>
<td>Halothane (1%) + 1 mm caffeine</td>
<td>MHN</td>
<td>−91 ± 1</td>
<td>126 ± 2</td>
<td>175 ± 7</td>
<td>94 ± 10*</td>
<td>188 ± 8*</td>
</tr>
<tr>
<td></td>
<td>MHS</td>
<td>−91 ± 1</td>
<td>137 ± 2†</td>
<td>186 ± 6</td>
<td>113 ± 9</td>
<td>217 ± 10</td>
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<td>MHN</td>
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<td>MHS</td>
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<td>139 ± 2†</td>
<td>178 ± 8†</td>
<td>126 ± 8††</td>
<td>242 ± 12††</td>
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<td>4 mm caffeine</td>
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<td>−87 ± 2</td>
<td>119 ± 1†</td>
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<td></td>
<td>MHS</td>
<td>−88 ± 2</td>
<td>141 ± 2††</td>
<td>173 ± 6††</td>
<td>138 ± 11†‡</td>
<td>261 ± 14†‡</td>
</tr>
</tbody>
</table>

Values are mean ± SEM.

Control (R) = recovery recordings after 30 min halothane washout; RP = resting membrane potential; Amp = amplitude; V_{max} = maximum rate of phase 0 depolarization; APD_{90}, APD_{50}, and APD_{0} = action potential duration at 20%, 50%, and 90% repolarization, respectively.

* P = 0.05 versus initial control.
† P = 0.05 versus control.
‡ P = 0.05 versus halothane alone.

Halothane. In contrast to the reduction in the AP amplitude and duration in MHN muscle, 1% halothane prolonged the AP duration in MHS muscle. These alterations were accompanied by similar negative inotropic effects in both types of muscle. Furthermore, in both MHS and MHN muscle, the resting potential and the maximum rate of depolarization in phase 0 were not significantly affected. The administration of caffeine caused a nearly identical prolongation of the AP duration in MHS and MHN muscle. In the absence of any drug, resting potential and AP characteristics in MHS and MHS muscle did not differ significantly.

The observed effects of halothane on ventricular muscle from MHN pigs in this study are consistent with findings in cardiac muscle of MHN sheep, guinea pigs, and dogs. However, reduction in the AP duration and amplitude during exposure to halothane is dose-dependent and varies among species and heart regions. The degree to which externally derived or internally released Ca²⁺ participates in contractile activation of the muscle may be one reason for the differences among species in the response of AP duration to halothane. In certain regions of the heart, halothane may affect the AP duration by inhibition of the plateau phase inward Na⁺ current. In the heart muscle of our MHS pigs, the depression of contractility by 1% halothane was associated with a slight increase in the AP amplitude, an enhancement of phase 2 (plateau phase) and a prolongation of phase 3 repolarization. After the halothane washout phase, the AP changes in MHS muscle were as reversible as those in MHN muscle. The delayed repolarization could be the result of (1) decreased inactivation of the inward Na⁺ current, in particular, of its plateau component, (2) increased inward Ca²⁺ current, (3) increased Na⁺--Ca²⁺ exchange rate to reduce abnormally high intracellular Ca²⁺, or (4) inhibition of the outward K⁺ current.²⁰,²¹

Because the current study of AP responses cannot discriminate among these complex ionic mechanisms of AP prolongation, we can only suggest in a general way the potential changes in ionic currents responsible for the observed AP responses. The increase in amplitude and duration of the AP plateau by halothane may reflect a contribution by an increased slow inward Ca²⁺ current to the electrogenesis of the plateau.²² The simultaneous contractile depression by halothane rather contradicts the possibility of an increased inward Ca²⁺ current. Furthermore, it is well established that the depression of the inward Ca²⁺ current is a major mechanism by which halothane exerts its myocardial-depressant effect.²³ However, differential effects on Ca²⁺ current kinetics may permit similar depression but different ef-
effects on the AP, because the initial Ca$^{2+}$ entry mediated by the Ca$^{2+}$ channel and, probably, by Na$^+$-Ca$^{2+}$ exchange-activated Ca$^{2+}$ release from the sarcoplasmic reticulum (SR), are responsible for tension development. In this situation, because halothane, which seems to have an effect similar to that of a small dose of caffeine, causes increased Ca$^{2+}$ release from the myocadiovascular SR, the modest lengthening of the AP that is observed in MHS may be anticipated.

A prolonged AP duration also may be induced by a reduction in intracellular Ca$^{2+}$ release from the SR, assuming that intracellular Ca$^{2+}$ concentrations modulate changes in K$^+$ conductance, which in large part determines AP duration. This possibility is unlikely at the onset of AP changes if a ryanodine receptor defect causes an increase in intracellular Ca$^{2+}$. Studies at the subcellular level and contractile studies suggest that halothane alters the uptake and release of Ca$^{2+}$ by SR. If an increase in intracellular activated Ca$^{2+}$ occurs late in the contractile response, for instance because of a delayed or depressed Ca$^{2+}$ release, the increment in K$^+$ conductance may also be delayed. Such mechanisms have been suggested for isoflurane$^{11}$ and dantrolene.$^{25,26}$ It is conceivable that such an effect contributes to the delayed repolarization and prolongation of the AP observed with halothane in MHS cardiac muscle. However, the effect of Ca$^{2+}$ regulation on K$^+$ channels is dependent on species and on the expression of the transient outward currents, only one of which is Ca$^{2+}$ activated. Thus, it is unclear whether Ca$^{2+}$ activated K$^+$ channels have a major effect in swine.

It is shown that Ca$^{2+}$ release from the SR, which may reflect either a decreased Ca$^{2+}$ current or an increase in amplitude of the Ca$^{2+}$ current, may reflect either a decreased Ca$^{2+}$ current or an increase in amplitude of the Ca$^{2+}$ current, or both. The major problem is whether such an effect contributes to the delayed repolarization and prolongation of the AP observed with halothane in MHS cardiac muscle.

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consonance with the current findings, the different effects of halothane on AP in MHS cardiac muscle affected mainly the repolarization phase.\textsuperscript{15} The time for 90% repolarization was dose-dependently increased in MHS than in MHN skeletal fibers.\textsuperscript{15}

Extrapolation of these experimental results cannot be made directly to human MH, because the pig is more closely inbred, and genetic studies indicate differences between human and porcine MHS subjects with respect to the genetic MH locus.\textsuperscript{12,13} The ryanodine receptor, which is a Ca\textsuperscript{2+}-release channel of the SR in skeletal muscle, has been proposed as the candidate structure for the MH defect, with pigs of various breeds demonstrating the same mutation in the skeletal muscle ryanodine receptor gene \textit{RYR1}.\textsuperscript{5,10} That single point mutation that is expressed primarily in skeletal muscle is presumed to be responsible for all aspects of the MH syndrome in pigs.\textsuperscript{12} However, this defect accounts for a far smaller fraction (perhaps only 5%) of the genetic alterations observed in MHS humans.\textsuperscript{11,14} Additional mutations in the human \textit{RYR1} gene have been observed.\textsuperscript{15} Although an additional genetic defect in pigs appears unlikely, its complete absence has not yet been proven. In this context, it has to be considered that different genes encode Ca\textsuperscript{2+}-release channels of the SR: \textit{RYR1} encodes the RYR1 isoform of slow- and fast-twitch skeletal muscle, and \textit{RYR2} encodes a short RYR2 isoform that is expressed in cardiac muscle.\textsuperscript{15} Expression of an \textit{RyR3} has been identified in some nonstriated muscle cells and other tissues.\textsuperscript{15}

When there is no mutation in \textit{RYR1} or \textit{RYR2} in cardiac muscle there are then two possibilities to fit this study’s results in the current understanding of the pig MH pathogenesis. First, if a small fraction of skeletal muscle type \textit{RYR1} or \textit{RYR2} was expressed in myocardium this would explain the exquisite sensitivity to halothane. To our knowledge, no studies using immunohistochemistry or \textit{in situ} hybridization have demonstrated the complete absence of the skeletal muscle ryanodine receptor in heart muscle, in MHN or in MHS. However, a recent study found identical ryanodine binding kinetics and channel activity from three regions of the dog hearts,\textsuperscript{12} arguing against the speculation that multiple ryanodine receptor types may exist in the heart. In the case that there is no skeletal muscle ryanodine receptor in cardiac muscle, the changes in MHS porcine myocardium may be the result of ongoing metabolic or sympathetic changes in MHS pigs that result in secondary changes in the behavior of the myocardium.

In conclusion, the current \textit{in vitro} study demonstrates that halothane produces abnormal alterations in APs of the ventricular muscle fiber characterized by marked increases in AP duration. In the absence of halothane, the electrophysiologic behavior of MHS and MHN heart cells is identical. These results suggest a latent defect in the myocardium of MHS pigs that becomes apparent in the presence of MH-triggering agents.

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