Anesthesiology 2004; 101:1475–7

Inhibition of Sarcoplasmic Ca^{2+} Adenosine Triphosphatase in Porcine Skeletal Muscle Samples with Cyclopiazonic Acid Enables In Vitro *Malignant Hyperthermia Discrimination*

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IT is generally accepted that malignant hyperthermia (MH) susceptibility is caused by an abnormal Ca^{2+} metabolism within the skeletal muscle cell.[1] Ca^{2+} homeostasis in skeletal muscles is regulated by two main receptors, the ryanodine receptor type I and the dihydropyridine receptor, and by a variety of intracellular second messenger systems. They have a direct or indirect Ca^{2+} releasing potency from the sarcoplasmic reticulum in common. However, it is not known whether a passive accumulation of Ca^{2+} might also be a relevant mechanism in MH. Hence, the aim of this study was to examine whether the blockade of the Ca^{2+} reuptake in the sarcoplasmic reticulum is a potent method to induce *in vitro* contractures in MH susceptible (MHS) and MH normal (MHN) skeletal muscle specimens. To answer this question in porcine *in vitro* contracture tests we used cyclopiazonic acid (CPA). We examined the *in vitro* effects of CPA in a cumulative pattern in MHS and MHN porcine skeletal muscle specimens.

Materials and Methods

After approval by the animal care committee of the University Hospital Hamburg-Eppendorf (Hamburg, Germany) seven MHS swine (homozygous for the ryanodine receptor type I gene mutation, male and female Pietrain, weighing 28–37 kg, aged 3–4 months) and seven MHN swine (no mutation of the ryanodine receptor type I gene, male and female German Landrace, weighing 30–39 kg, aged 3–4 months) from a special breeding farm (test center Thalhausen, chair of stockbreeding, Technical University of Munich, Germany) were studied. Before the study genomic DNA was isolated from blood of all animals preserved in edetic acid to check for the presence of the ryanodine receptor type I Arg-615Cys point mutation on chromosome 6, indicating MH susceptibility.[2]

Swine were fasted overnight with free access to water. Premedication was performed with ketamine 15 mg/kg intramuscularly. After installation of a venous line into an ear vein, trigger-free general anesthesia was induced with bolus injections of fentanyl 10 μg/kg and propofol 5 mg/kg intravenously. After tracheotomy the lungs were mechanically ventilated with an inspired oxygen fraction of 0.3. End-tidal carbon dioxide was kept constant at 35–38 mmHg. Anesthesia was maintained with fentanyl 50 $\mu g \cdot kg^{-1} \cdot h^{-1}$ and propofol 10 $mg \cdot kg^{-1} \cdot h^{-1}$. Neuromuscular blocking agents were not administered.

Muscle biopsies and general *in vitro* contracture test procedures have been described elsewhere.[3]

Before the experiment a stock solution of CPA (minimum 98% thin layer chromatography powder) was prepared in dimethyl sulfoxide. Test solutions were freshly prepared from the stock solution by dilution with Krebs-Ringer's solution. All tests were performed within 5 h after biopsy.

Cumulative CPA organ bath concentrations were adjusted to 1.25, 3.75, 8.75, and 18.75 μmol/l. We defined a time frame of 10 min before administration of the next CPA concentration. Contracture development and muscle twitch responses were assessed.

Statistical Analysis

Statistical analysis was performed using a computer-based statistical program (SPSS Inc., Chicago, IL). Medians and ranges were calculated. Intergroup differences were evaluated with the nonparametric Mann-Whitney *U*-test. Intragroup differences were calculated with Wilcoxon's test. A value of $P \leq 0.05$ was accepted to indicate statistical significance.

Results

Contractures after cumulative administration of CPA were observed in all MHS samples. Original tracings of the *in vitro* contracture test with CPA in a skeletal muscle bundle of MHS and MHN swine are shown in Fig.

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Received from the Department of Anesthesiology, University Hospital Hamburg-Eppendorf, Hamburg, Germany. Submitted for publication April 30, 2004. Accepted for publication July 23, 2004. Support was provided solely from institutional and/or departmental sources. Presented in part at the Annual Meeting of the American Society of Anesthesiologists, Orlando, Florida, October 12–16, 2002.

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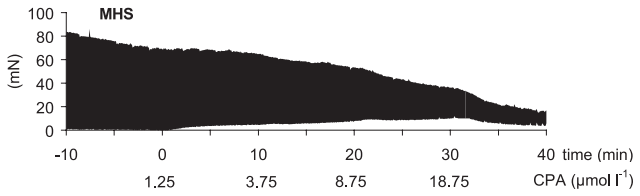

Fig.1a

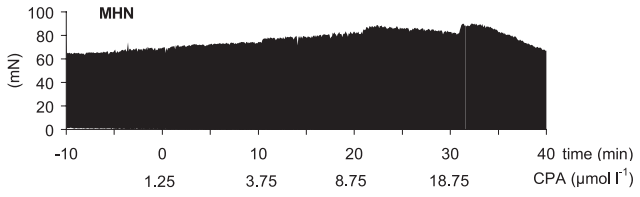

Fig.1b

Fig. 1. Original tracings of the *in vitro* contracture test with cyclopiazonic acid (CPA) in skeletal muscle specimen of a malignant hyperthermia susceptible (MHS, *top*) and normal (MHN, *bottom*) swine. After the baseline was stable in a range of 2 mN over 10 min, cyclopiazonic acid was added cumulatively to the tissue bath. The time period between concentration steps was 10 min. Although the malignant hyperthermia susceptible muscle displays a concentration-dependent increase in the baseline force and a decline in the twitch amplitude, the malignant hyperthermia susceptible muscle does not show any effect.

1. The MHS specimen developed a maximal contracture of 4.7 mN after administration of 1.25 μmol/l CPA. After the cumulative administration up to 18.75 μmol/l CPA contracture increased to 10.5 mN. The muscle twitch amplitudes declined throughout the experiment. In the MHN muscle no contracture baseline elevation was recorded. The twitch amplitudes increased continuously up to the 18.75 μmol/l CPA administration, whereafter the twitch amplitudes decreased.

All seven MHS muscle specimens showed a baseline contracture starting at a concentration of 1.25 μmol/l with 4.6 mN (0.4–15.8 mN), at a concentration of 3.75 μmol/l with 8.3 mN (1.2–21.3 mN), and at a concentration of 8.75 μmol/l with 10.2 mN (2.2–21.3 mN) (Fig. 2). The highest contracture development was recorded at 18.75 μmol/l CPA with 10.5 mN (4.1–20.4 mN). The specimens of MHN swine did not exhibit any contractures throughout the experiment. No overlapping single values between the groups were observed.

The initial muscle twitch amplitudes with 34.8 mN (10.1–111.8 mN) in the MHS group were comparable to the MHN group with 40.7 mN (11.3–81.6 mN) (Fig. 3). Both diagnostic groups demonstrated a decline in twitch amplitudes throughout the experiment. The intragroup analysis showed a significant difference in the MHS muscles after 8.75 and 18.75 μmol/l CPA.

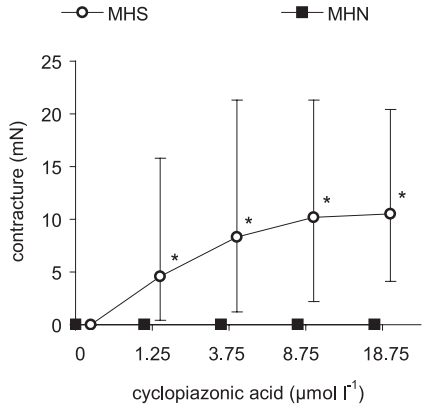

Fig. 2. Contracture development following cumulative administration of cyclopiazonic acid in concentrations of 1.25, 3.75, 8.75, and 18.75 μmol/l in skeletal muscle specimen of seven malignant hyperthermia susceptible (MHS) and seven normal (MHN) swine. The value "0" refers to the last contracture before the application of 1.25 μmol/l cyclopiazonic acid. Dots respectively squares indicate medians; error bars illustrate ranges. Intergroup difference: *$P \leq 0.05$

Discussion

CPA is a toxic metabolite ubiquitously produced by distinct mycetes (aspergillus flavus, penicillium cyclopium). CPA is a selective inhibitor of the sarcoplasmic reticulum Ca^{2+}-adenosine triphosphatase,[4] exerting no effect on the actomyosin adenosine triphosphatase.[5] Yet, in very high concentrations it is able to weakly stimulate the ryanodine receptor type I.[6] Various studies in frog muscles were able to demonstrate the concentration-dependent prolongation of the relaxation time and an increase in contracture development and muscle twitch amplitudes with CPA.[7–9] CPA should have identical inhibitory effects on the sarcoplasmic reticulum pumps of MHS and MHN muscles because the Ca^{2+}-

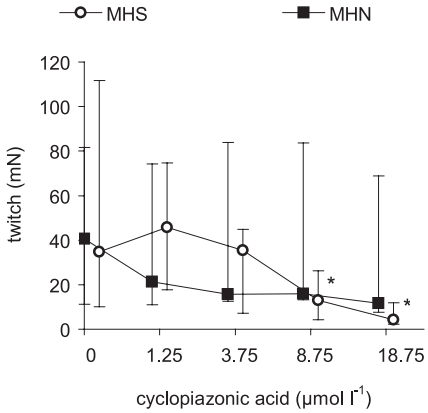

Fig. 3. Muscle twitch responses following cumulative administration of cyclopiazonic acid in concentrations of 1.25, 3.75, 8.75, and 18.75 μmol/l in skeletal muscle specimen of seven malignant hyperthermia susceptible (MHS) and seven normal (MHN) swine. The value "0" refers to the last twitch amplitude before the application of 1.25 μmol/l cyclopiazonic acid. Dots respectively squares indicate medians; error bars illustrate ranges. Intragroup difference: *$P \leq 0.05$

adenosine triphosphatase does not differ between both muscle types.[1]

In the present study we were able to show that cumulative CPA administration (1.25–18.75 μmol/l CPA) induced marked concentration-dependent contractures in each MHS muscle preparation. In contrast, no contractures were recorded in MHN samples. A clear differentiation without overlap between the two diagnostic groups was obtained.

The twitch amplitudes of the MHS muscles declined significantly throughout the experiment. During a contracture, twitch amplitudes commonly decrease (during caffeine-induced or halothane-induced contracture of MHS muscle), likely owing to the fact that the maximal force production capabilities of the myofilaments have occurred.

In conclusion, the cumulative *in vitro* contracture test with CPA might be an improvement for presymptomatic MH diagnostics. This hypothesis should be studied in human skeletal muscle preparations.

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