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Detection of Proton Release from Cultured Human Myotubes to Identify Malignant Hyperthermia Susceptibility

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Background: **Malignant hyperthermia (MH) is a pharmacogenetic disorder of skeletal muscle. During general anesthesia, a life-threatening hypermetabolic state may occur resulting from increased release of Ca^{2+} from the sarcoplasmic reticulum in skeletal muscle. Diagnosis of MH susceptibility requires surgical muscle biopsies to measure force in response to chemical stimulation (*in vitro* contracture test, IVCT). Here, the authors investigated an alternative way of discriminating MH-susceptible (MHS) from normal (MHN) subjects by using cultured human myotubes and measuring proton release as an indicator of cellular metabolism.**

Methods: **Myotubes were stimulated with the Ca^{2+} release channel agonist 4-chloro-m-cresol (4-CmC), leading to metabolic activation and proton secretion. The rate of extracellular acidification was recorded with a silicon sensor chip.**

Results: **A stepwise increase in 4-CmC concentration led to a phasic–tonic increase in the acidification rate. The response, measured at different concentrations of 4-CmC, was considerably larger in cultures from MHS compared with MHN subjects and correlated well with the force response in the IVCT.**

Conclusions: **The enhanced metabolism of cultured skeletal myotubes, likely originating from an increased myoplasmic Ca^{2+} concentration, can be monitored by studying the proton secretion rate. Because the method seems to be able to distinguish normal from pathologic phenotypes, it is a promising technique for possible future use in less invasive MH testing.**

MALIGNANT hyperthermia (MH) is a life-threatening hypermetabolic condition that can occur in genetically predisposed persons during general anesthesia.[1–3] MH is characterized by hypercapnia, hypoxemia, metabolic acidosis, muscle contractures, and hyperthermia. In many of the affected families, the susceptibility to MH shows genetic linkage to the skeletal muscle ryanodine receptor (RyR1), the Ca^{2+} release channel of the sarcoplasmic reticulum (SR). More than 30 point mutations in RyR1 causing MH susceptibility have been reported to date. In some families, linkage to RyR1 could be excluded, and alternative genetic loci are being considered.[2] During an MH crisis, the commonly used volatile anesthetic agents and depolarizing muscle relaxants trigger excessive release of Ca^{2+} from the SR. This, in turn, activates major ATP-consuming processes like myosin cross-bridge cycling and Ca^{2+} reabsorption by the SR. The metabolic reactions leading to replenishment of ATP, primarily glycolysis and respiration, contribute significantly to the MH symptoms.[4]

It is important to accurately evaluate and label the members of affected families regarding their MH susceptibility. MH-susceptible (MHS) patients can then be anesthetized with nontriggering agents in the event of surgery. On the other hand, all unevaluated members of a family with MH incidents must provisionally be labeled MHS. They may, therefore, be subject to inconveniences, resulting from the use of alternative anesthetic agents, or disadvantages pertaining to employment or insurance questions.[5] Further, only if the phenotype is well defined is it possible to carry out reliable genetic linkage analysis. In North America and Europe, MH diagnosis is based on a standardized *in vitro* contracture test (IVCT). In the IVCT,[6,7] the MHS phenotype is characterized by significantly reduced threshold concentrations of halothane and caffeine for force generation in excised muscle bundles, resulting from a stronger activation of ryanodine receptor-mediated Ca^{2+} release.[8–10] 4-chloro-m-cresol (4-CmC), which activates Ca^{2+} release and force at considerably lower concentrations than caffeine, can likewise discriminate between normal and MHS muscle.[11,12] In a recent multicenter study,[13] this substance has been claimed to improve phenotypic classification and recommended to be included in the contracture test as an additional diagnostic tool.

The IVCT is expensive, confined to specialized testing centers, and inconvenient because of the necessary surgery. In particular, it should not be done on those at greatest risk, *i.e.*, young children. Replacement by a less invasive procedure would be a significant advance. In the present investigation, we explored the possibility of monitoring the metabolic activity of cultured human myotubes. Adult muscle stem cells (satellite cells), which form the basis of a primary myotube culture, can be derived in sufficient quantities by enzymatic dissoci-

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ation from needle biopsies.[14] We made use of a silicon biosensor device (Cytosensor® microphysiometer; Molecular Devices, Ismaning, Germany)[15–17] to measure the rate of proton release during activation by different concentrations of the Ca^{2+}-releasing agent 4-CmC. Normal myotubes (MHN) and cells of persons who had been labeled MHS by the IVCT were compared.

Materials and Methods

Patients

The patients were selected randomly among the persons tested for MHS by IVCT during a 2-yr period. The ethics committee of the University of Ulm, Ulm, Germany, approved the procedures. Informed consent was obtained from the patients. Indications for MH testing were an adverse anesthetic event of the patient (three persons) or of one of his or her close relatives (three persons), membership of an established MHS family (eight persons), or isolated familial creatine kinase elevations (two persons). Eight of the patients were MHS and eight MHN according to the IVCT results. Mutation screening of the RyR1 gene was performed by restriction analysis or single strand conformation analysis (SSCA),[18] checking for most of the known MH mutations including the 15 mutations recognized as causative in heterologous expression experiments.[8] In two of the MHS patients, the G2434R mutation was identified; two further patients carried the R614C mutation. In the other subjects, no mutation could be detected.

Solutions and Culture Media

The following solutions were used in dissection, culture, and experimental measurements (temperature, 37°C).

Krebs-Ringer's solution: NaCl, 118 mM; KCl, 3.4 mM; $MgSO_4$, 0.8 mM; KH_2PO_4, 1.2 mM; glucose, 11.1 mM; $NaHCO_3$, 25.0 mM; $CaCl_2$, 2.5 mM; pH, 7.40.

Dissociation solution: A Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline solution (L1825; Biochrom, Berlin, Germany) supplemented with 0.1% bovine serum albumin (BSA) (A4503; Sigma, Steinheim, Germany), 2.5 mg/ml Trypsin (0153–61; Difco, Sparks, MD), and 220 U/ml collagenase type A (C6885, Sigma).

Ham F12 solution bicarbonate-buffered basic medium (F0815, Biochrom).

Growth medium: Skeletal muscle cell growth medium kit (C-23060; PromoCell, Heidelberg, Germany) containing 5% fetal calf serum (FCS) and various growth factors.

Differentiation medium: Skeletal muscle cell differentiation medium kit (C-23161, PromoCell) containing no FCS and fewer growth factors.

Cytosensor® test solution: A weakly pH-buffered basic nutrient F12 medium (N6760, Sigma) with equimolar substitution of Na-bicarbonate by NaCl (total osmolarity, 290 mOsm; pH, 7.35).

Stock solutions of the trigger reagents 4-CmC (10 mM) and caffeine (100 mM) were prepared in Cytosensor® test solution or bi-distilled water (for IVCT). Halothane was applied using a vaporizer (Vapor 19.1; Draeger, Lübeck, Germany). Caffeine was purchased from Merck (Darmstadt, Germany), halothane from Zeneca (Plankstadt, Germany), and 4-CmC from Fluka (Neu-Ulm, Germany).

In Vitro *Contracture Test*

Testing for MHS was carried out according to the protocol of the European Malignant Hyperthermia Group.[6] Briefly, biopsies were excised near the distal insertion of the m. vastus lateralis during regional anesthesia (3-in-1 block). Small muscle bundles were prepared, and mechanical tension was recorded while increasing concentrations of the test agents (halothane, caffeine) were applied. In most of the investigated biopsies, a dose–response curve for 4-CmC was recorded in addition. Halothane concentrations were monitored during the test by infrared spectroscopy (Iris; Draeger, Lübeck, Germany). In separate measurements, the concentrations of the test agents in the bath solutions were checked by gas chromatography (halothane) and by ultraviolet spectrophotometry (caffeine, 4-CmC).

MH-susceptible muscle showed abnormal contractures of 2 mN or more in at least one muscle specimen exposed to 0.44 mM halothane or less *and* one exposed to 2 mM caffeine or less, whereas MHN muscle strips showed no abnormal response.

Cell Culture

Two or 3 h after surgery, excised muscle tissue sections (50–100 mg) were cut in small pieces in Krebs-Ringer's solution and transferred to dissociation solution. The enzymatic dissociation (30 min at 37°C) was stopped by F12 solution containing 10% FCS. After filtration (50 and 20 μm pore size) and centrifugation (100 *g*, 5 min), the pellet containing the satellite cells was resuspended in growth medium. Satellite cells were cryopreserved using 10% DMSO. They were rethawed for culture and seeded onto micronets (Capsule cups; Molecular Devices, Sunnyvale, CA) for proliferation. Staining with crystal violet (according to Molecular Devices instructions) showed a cell number of about 50 myotubes per micronet. The medium was exchanged every 2 days. After 4–6 days in culture, the growth medium was exchanged for differentiation medium to induce fusion and differentiation. Experiments were carried out on days 4–6 after induction of differentiation. Microscopic inspection showed that growth and density of cells on the micronets was similar to standard cultures in Petri dishes. Petri dish cultures were always run in addition to the micronet cultures to check for reasonable growth (cell viability).

Proton Release Measurements

The Cytosensor® microphysiometer was used to detect proton secretion from the cultured myotubes. Each measurement unit of the instrument contains a silicon chip with a pH-sensitive surface (fig. 1A) on the bottom of a perfusable microchamber. Light pulses from an infrared light-emitting diode (LED) induce transient currents driven by a variable voltage. Because a change in the proton concentration of the bath alters the surface potential at the solution interface, a pH change shifts the current–voltage relation. The device was calibrated automatically at the start of each experiment. Test measurements with solutions of different pH showed that the voltage shift was proportional to pH (approximately 60 mV/pH unit) in the pH range 6–8 and that changes were highly reproducible.

The measuring compartment of the microchamber containing the cells had a volume of 1.4 μl and was perfused discontinuously with a weakly buffered culture medium (see above) at a rate of 50 μl/min and a temperature of 37°C. Figure 1B shows four consecutive perfusion cycles. The perfusion time of each cycle was 36 s. During this time (pump on), the chamber pH increased because of equilibration with the bath. On stopping the perfusion (pump off, 24-s interval), the biosensor voltage decreased (*i.e.*, pH decreased) because protons that were extruded by the cells accumulated in the chamber. One digitally stored raw data point (pH measurement) was acquired per second. The mean time derivative of a 20-s interval within each 24-s, pump-off interval was calculated by linear regression to determine the rate (Rate 1, Rate 2, and so on) of proton extrusion.[16,19] The instrument allowed accurate measurements of the acidification rate in the milli-pH units/min range. The recorded signal amplitudes were of the order of 0.01–0.1 pH units/min. The timing of the perfusion cycle was a compromise between temporal resolution and the time required to determine the acidification rate in the pump-off interval. Pump cycle control, data acquisition, and rate calculation were carried out with the system's microcomputer (Macintosh Power PC 7600/132) and the Cytosoft® program supplied with it. Further analysis was carried out off-line using Microsoft Excel spreadsheets.

At the beginning of the third pump cycle in figure 1B, the flow system was switched from a drug-free solution to a solution containing 600 μM 4-CmC. It can be seen that in the following stop interval, the pH decreased more rapidly, indicating a higher rate of proton secretion (Rate 3, Rate 4) and, hence, metabolic activity. In initial experiments, we applied caffeine as in the IVCT to activate myotubes. Caffeine produced variable responses and mostly a decrease rather than an increase in the proton release rate. Also, other studies indicate that caffeine is not an ideal tool to study Ca^{2+} turnover in cultured cells because of unspecific effects.[9,20] Because the membrane-permeant cAMP derivative dibutyryl cyclic AMP (D0627, Sigma) showed similar inhibition of proton extrusion (two experiments at 500 μM and 1 mM), we tentatively attributed the observed behavior to the known inhibitory effect of caffeine on phosphodiesterases and discontinued experiments with this drug.

The acidification rates were normalized to the rate at the beginning of the experiment, *i.e.*, before adding 4-CmC (predrug condition) to account for differences in rate resulting from varying numbers of myotubes in the samples. Measurements were included in the analysis when an increase in rate by 50% of the predrug baseline value was reached at any of the applied concentrations. Myotube cultures from 20 patients were measured using the described protocol; 4 patients were excluded because responses remained below threshold.

Statistical Analysis

Results are presented as means and SD. Differences in location between groups were evaluated in an exploratory data analysis using exact two-sided Wilcoxon rank sum tests. *P* values less than 0.05 were interpreted as indicating relevant differences. Because of the exploratory nature of the comparison, no adjustment for multiple testing was carried out. *P* values were calculated using StatXact®, Version 5 (Cytel Software, Cambridge, MA).

Results

4-CmC–induced Acidification Responses in Normal and MHS Myotubes

Figure 1C shows the time-resolved change in the acidification rate of primary cultured human myotubes caused by 600 μm 4-CmC. Each measurement point corresponds to a single pump cycle of the perfusion system. It can be seen that the proton secretion rate increases rapidly from baseline to a peak and then decreases to a lower steady level. After removing the stimulus, the rate recovered close to the baseline value.

The 4-CmC effect on Ca^{2+} release is attributed to a direct action on the Ca^{2+} release channel (RyR1) of the SR,[11] and the threshold for activation of RyR1 by 4-CmC has been shown to be decreased in preparations with MH mutations.[11,12] Because the increase in the proton secretion rate, shown in figure 1C, likely results from the activated cell metabolism in response to the 4-CmC–triggered release of Ca^{2+}, differences in the dose dependence of the acidification response between normal and MH myotubes should also be expected.

Figure 2 illustrates the acidification rate responses of an MHS (fig. 2A) and an MHN (fig. 2B) culture at different 4-CmC concentrations. The drug concentration was gradually increased from a minimum value of 10 μM to 600 μM. At the end of the experiment, a considerably higher concentration (1,200 μM) was applied, which

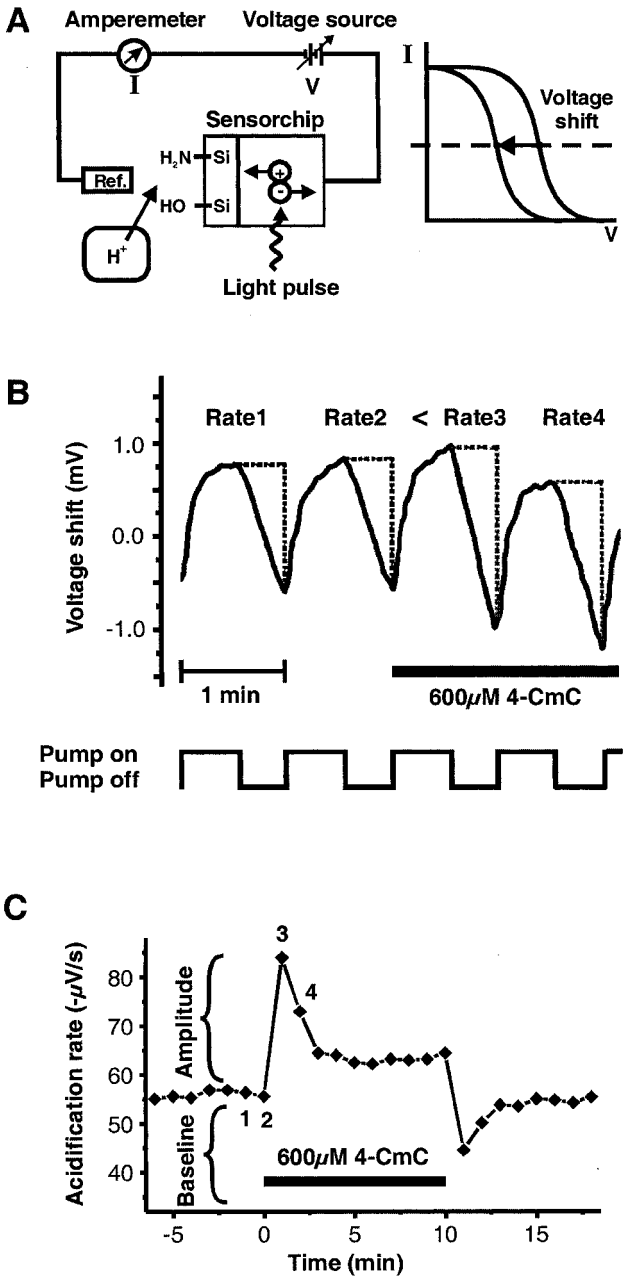

Fig. 1. Time-resolved determination of the proton secretion rate from human myotubes. (*A*) Principle of pH recording. A charge movement, induced by pulsed illumination of the sensor chip is modulated by the voltage V (*left*). The current–voltage relation is displaced along the voltage axis (*right*) when protons that are secreted into the bath solution compensate negative surface charges of the chip. The charged groups originate from a thin insulating layer of silicon oxide and nitride. Ref = reference electrode. (*B*) Four consecutive pump cycles of the perfusion system and corresponding pH-dependent alterations in the inflection points of the current–voltage relation resulting from the combined action of proton extrusion from the myotubes in the microchamber and of the flow rate of the perfusion system. During the intervals labeled "pump-on," the chamber was perfused with the experimental solution. The acidification rate was determined from the pH decrease in the "pump-off" intervals. (*C*) Acidification rates as determined in *B* plotted *versus* time. The numbers 1–4 indicate the four perfusion cycles shown in *B*. 4-CmC = 4-chloro-m-cresol.

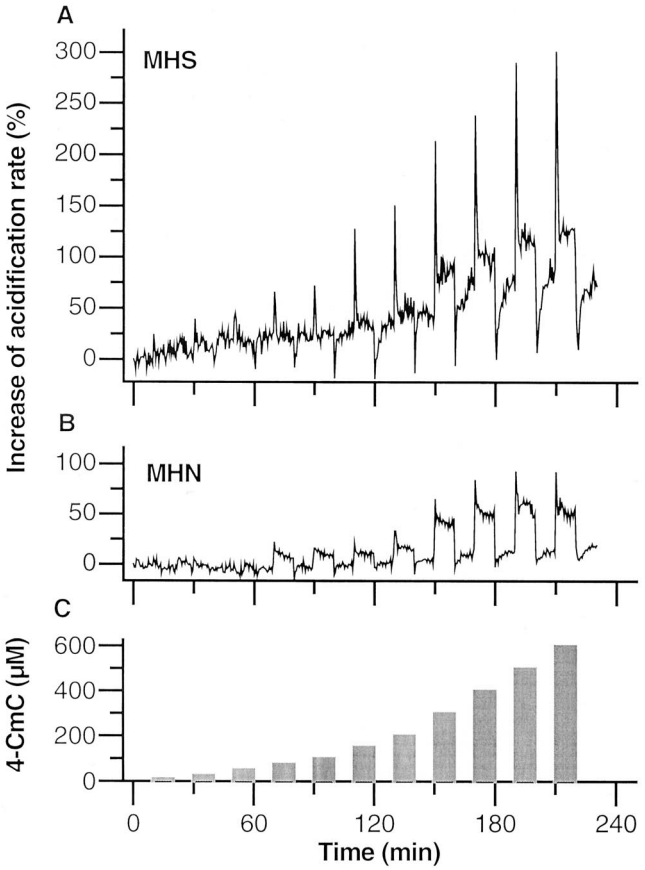

Fig. 2. Acidification responses at increasing 4-chloro-m-cresol (4-CmC) concentrations. (*A*) Normalized increase in proton secretion rate recorded from a malignant hyperthermia–susceptible (MHS) culture. (*B*) Rate recordings from a malignant hyperthermia–normal (MHN) culture. (*C*) Concentrations of 4-CmC applied in the test.

caused highly variable reactions with occasional signs of damage. Therefore, this concentration was not included in the analysis. Each drug application consisted of a 10-min exposure with the respective concentration and a 10-min washout with 4-CmC–free solution (fig. 2C). The ordinate shows the change in acidification rate as a percentage of the initial (predrug) basal rate. The responses were qualitatively similar. A step change in 4-CmC concentration was answered by a rapid increase in the acidification rate and a subsequent rapid decrease. The rate remained increased, although at a lower level, during the time of the stimulus and decreased to below that of baseline after return to the control solution.

Figure 3 shows the relative change in acidification rate as a function of 4-CmC concentration using mean values obtained from cultures of eight MHS patients (filled symbols). For comparison, the concentration dependence obtained from cultures of eight MHN subjects is shown (open symbols).

In figure 3A, the total change in acidification rate is shown, whereas figure 3B shows only the amplitude of the rapid response reached immediately on application

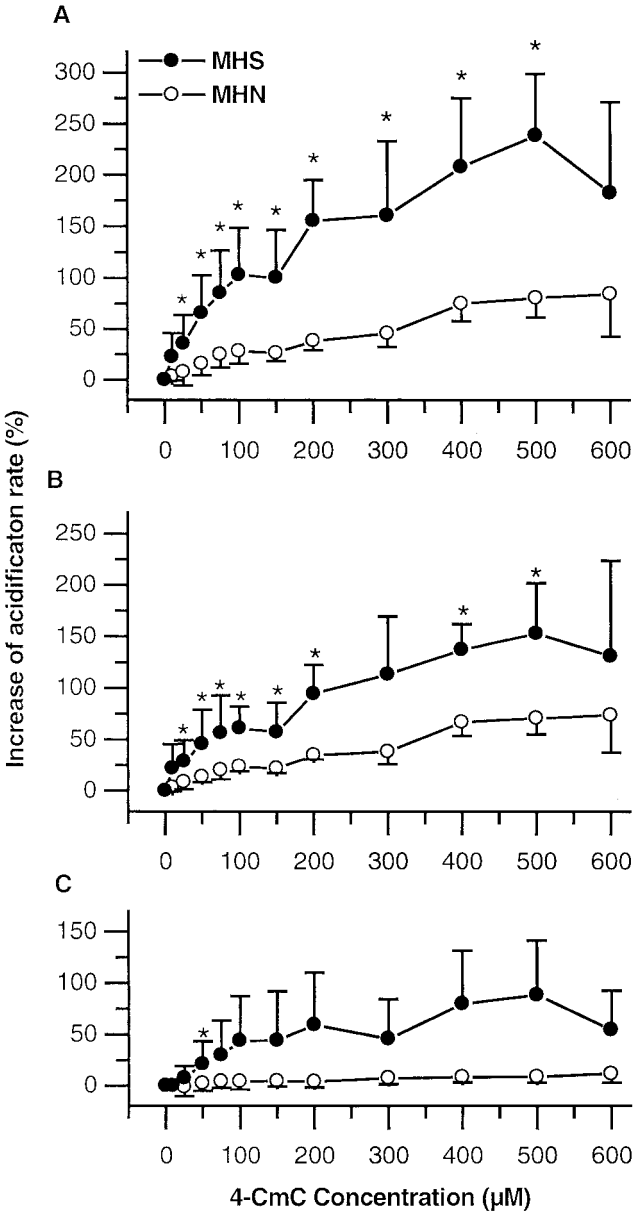

Fig. 3. Dose dependence of the acidification rate in malignant hyperthermia–normal (MHN) and malignant hyperthermia–susceptible (MHS) myotubes. Increase in acidification rates (in percent of predrug value) plotted *versus* 4-chloro-m-cresol (4-CmC) concentration. (*A*) Total normalized increase in acidification rate (sum of B and C). (*B*) Normalized amplitude, *i.e.*, 100 × amplitude/predrug baseline rate. (*C*) Normalized increase in baseline acidification rate, *i.e.*, 100 × (baseline rate − predrug baseline rate)/predrug baseline rate. Mean data from eight MHS patients (filled-in circles) and eight MHN (open circles). Error bars show SD; asterisks indicate $P < 0.05$ in the exact Wilcoxon rank sum test.

of 4-CmC. Figure 3C shows the change in baseline rate, measured immediately before the stimulation. In the MHN cultures, the baseline rate remained essentially constant, whereas in the MHS cultures, it gradually increased. In all three plots, larger mean values of the acidification rates could be observed for all concentrations in the MHS group. The best discrimination between MHS and MHN cells is evident from the total increase in rate (fig. 3A), *i.e.*, taking into account amplitude and baseline. The increase in the total rate for MHS cells was about fourfold, at a concentration of 200 μM 4-CmC. Exploratory statistical comparison of the data was carried out using the nonparametric exact Wilcoxon rank sum test. At 9 of 11 concentrations shown, *P* values for comparison of the total change in acidification rate were below 0.05, showing a clear difference between the two groups.

Comparison of Physiometer Test and Contracture Test Results

All biopsies used for this investigation had shown unambiguous results in the European IVCT test, *i.e.*, the concentration thresholds for contractures of muscle fiber bundles triggered by halothane and caffeine were either normal (MHN) or clearly shifted to lower concentrations (MHS). In addition, 4-CmC–induced force responses were measured in 13 of 16 biopsies. The results agreed with the results obtained using classic IVCT agents. Figure 4A shows the mean dose–response curves for these biopsies. On average, a contracture force comparable with the one reached at 150 μM 4-CmC in the MHN biopsies was already obtained at only 25 μM in the MHS fiber bundles.

Comparing the results of individual measurements, there was a clear correlation between the strength of the responses in the IVCT and in the acidification assay. As threshold for the acidification response, we chose an increase by 50% of the initial proton secretion rate. Figure 4B shows on the ordinate for each tested subject the concentration at which acidification rate first exceeded threshold, *i.e.*, reached 150% of the initial basal rate. This concentration value is plotted against the concentration at which contracture force in the IVCT first exceeded 2 mN.

The histogram of figure 5 shows the number of subjects who reached the given acidification threshold plotted *versus* the 4-CmC concentration (logarithmic scale). This again underlines the complete separation of the two groups. According to figure 5, the optimal concentration range of 4-CmC to distinguish the MHS phenotype from a normal response with the Cytosensor® test is between 150 and 200 μM. At 150 μM, all MHS patients and none of the MHN subjects had reached threshold.

Discussion

Extracellular pH as an Indicator of Altered Intracellular Calcium Turnover

In this first study to apply microphysiometry to cultured skeletal muscle, we recorded extracellular acidification of human myotubes with a sensitive pH-metric biosensor. Extracellular acidification is an integral consequence of cellular activity. Regardless of the pathway,

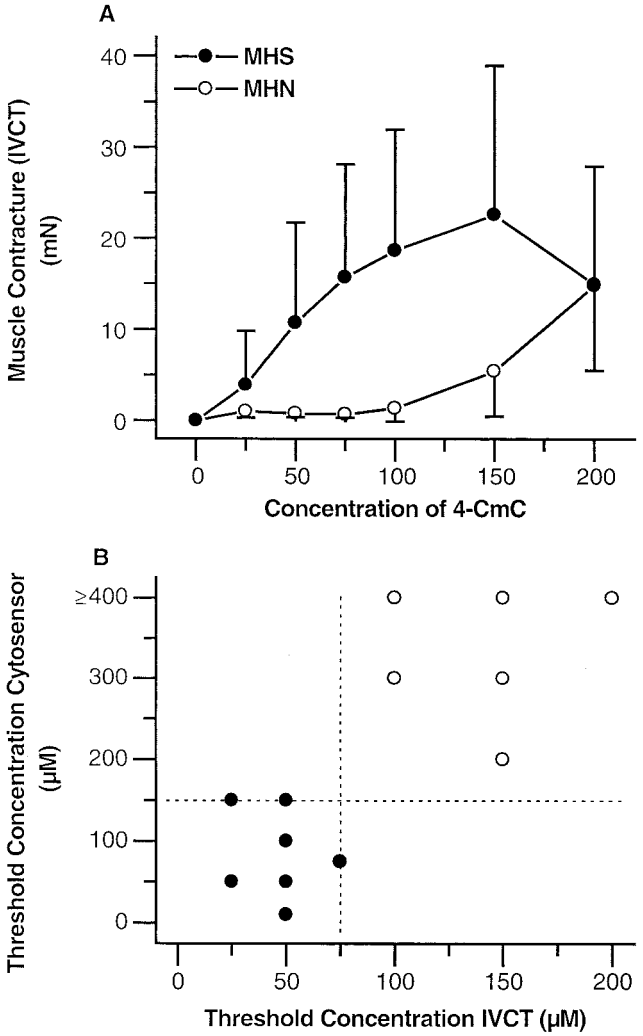

Fig. 4. Comparison of contracture test and acidification assay. (*A*) Contracture force in the *in vitro* contracture test (IVCT) plotted *versus* 4-chloro-m-cresol (4-CmC) concentration. Results from 13 of 16 biopsies (7 malignant hyperthermia–susceptible [MHS], 6 malignant hyperthermia–normal [MHN]) that were used for the myotube cultures. Error bars indicate SD. (*B*) Correlation between IVCT data and Cytosensor® (Molecular Devices, Ismaning, Germany) data given as 4-CmC concentrations that produce a suprathreshold drug response (threshold definitions, 50% increase above baseline value of acidification rate in the Cytosensor® test [ordinate] and 2 mN contracture force in the IVCT [abscissa]). The dashed lines indicate the concentrations that permitted full separation of the MHS and MHN groups in either test (150 µM and 75 µM, respectively).

cellular activation of mammalian cells goes along with an increased energy demand associated with ATP regeneration by anaerobic glycolysis or oxidative respiration.[16] The main metabolites of these events, lactic acid and carbon dioxide, lead to intracellular acidification, which in turn causes efflux of protons to the extracellular environment.[21] Therefore, increase of the energy metabolism of the activated cell usually leads to a higher rate of extracellular acidification.

Here, acidification responses of the myotubes were triggered by superfusion with 4-CmC, a substance that leads to highly specific activation of the ryanodine receptor[11] and releases Ca^{2+} from isolated SR vesicles.[12] In skeletal myotubes, 4-CmC has been shown to trigger large intracellular Ca^{2+} transients.[22] The ATP requiring processes that are likely to be primarily influenced by a 4-CmC–induced Ca^{2+} release are the active Ca^{2+} transport into the SR and out of the cell and the actin-modulated myosin ATPase. Therefore, the acidification signal can easily be traced back to the disturbance in Ca^{2+} turnover caused by 4-CmC. This is in agreement with our finding that MHS, which is known to modify the Ca^{2+} release and force response to 4-CmC, also modified the acidification response in a similar way.

Time Course of the Signal

On application of 4-CmC, a phasic concentration-dependent increase in the proton secretion rate was observed. The effect could be divided into an initial peak that was followed by a plateau for the duration of the 4-CmC stimulus and an undershoot below the baseline when the chemical stimulus was removed. The limited time resolution of about 1 min of the Cytosensor® microphysiometer may even have underestimated the phasic nature of the acidification response. A phasic–tonic response of the acidification rate as observed in the experiments is not uncommon in microphysiometric measurements of plasmalemmal receptor activation. In myotubes it may be a direct reflection of the intracellular Ca^{2+} transients. The decrease after the initial peak may be the result of a corresponding decrease of Ca^{2+} release caused by Ca^{2+}-dependent inactivation of the ryanodine receptor[23–25] or by depletion of the SR. On the other

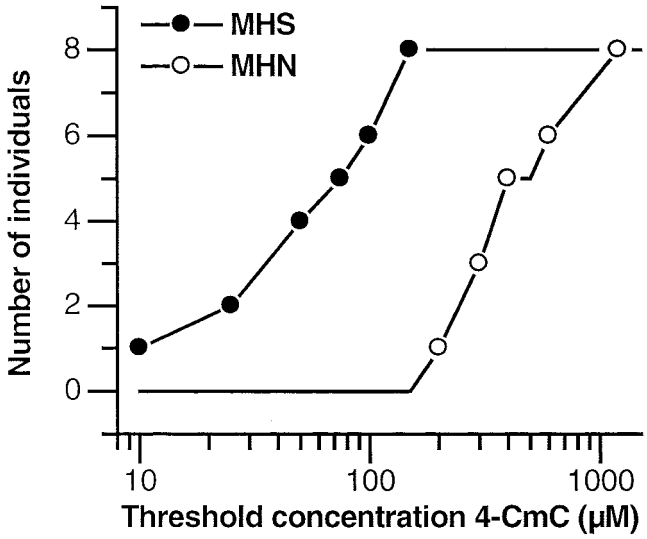

Fig. 5. Discrimination between malignant hyperthermia–normal (MHN) and malignant hyperthermia–susceptible (MHS) using a concentration threshold criterion. The number of subjects who reached threshold (*i.e.*, 50% total increase in acidification rate) plotted *versus* the logarithm of the 4-chloro-m-cresol (4-CmC) concentration tested. Note that MHS subjects required considerably lower concentrations.

hand, the undershoot at the end of the signal is difficult to explain in this way and would be more compatible with two antagonistic events affecting the release of protons. An inhibitory process may persist for some time after the end of the stimulus and lead to a transient decrease in the acidification rate. This may also explain a net decrease in the acidification rate that was observed rarely in cultures exhibiting subthreshold responses as seen with caffeine and dibutyryl cAMP (see Materials and Methods). The latter may indicate a possible involvement of cAMP in inhibiting proton extrusion. Net proton consumption rather than production may also occur if creatine phosphate instead of carbon sources is used to replenish ATP.[21] Finally, the phasic behavior of the 4-CmC response may also result from sarcolemmal changes that affect the mechanisms of outward flux of intracellularly generated protons in muscle.[26,27] Owicki and Parce[21] demonstrated that a simple transport model can generate an overshoot of extracellular acidification rate even though the intracellular pH decreases monotonically.

Acidification Assay as Diagnostic Tool

Adenosine triphosphate–regenerating reactions are thought to be the main causes for the characteristic detrimental events during a MH crisis, in particular the strong metabolic acidosis. Because cellular proton excretion is tightly coupled to energy consumption and replenishment by the cellular metabolism, we investigated the question as to whether the rate of extracellular acidification caused by the chemically stimulated cultured myocytes can be used to distinguish MHS from MHN preparations. The results show that the extent of the acidification response is in fact significantly different for the MHS myotubes.

The currently used test procedure for MH diagnosis (*i.e.*, IVCT) has been optimized to warrant high sensitivity (*i.e.*, very low number of false-negative results) at an acceptable specificity.[6] For phenotyping to support genetic linkage analysis, a more specific test would be desirable. In particular, the search for alternative MH loci that show no linkage to the currently known loci requires precise phenotyping of MHS.[5] Higher specificity (lower rate of false-positive results) can be gained by improving the diagnostic procedure. For instance, attempts have been made to include new test drugs (4-CmC, ryanodine) in the IVCT.[13,28] A noninvasive method of MH testing, using nuclear magnetic resonance (NMR) spectroscopy to assess parameters of muscle energy metabolism, has been considered[29,30] but failed to compete with the IVCT (for a recent summary, see Argov *et al.*[31]).

The microphysiometry procedure presented here is a promising new approach to MH testing based on cell metabolism. However, the effect measured (acidification) is also many steps removed from Ca^{2+} release. Therefore, in addition to diseases affecting Ca^{2+} turnover (*e.g.*, central core disease, Brody disease, Duchenne muscular dystrophy), all disorders that may affect energy metabolism (*e.g.*, mitochondrial myopathies) and proton handling can be expected to show changes in acidification rate. Thus, a test based on detection of proton release is likely to have similar problems of specificity as with the IVCT, which is known to show abnormal responses in patients with certain myopathies. On the other hand, it may be of additional help to study the cellular pathophysiology in such disorders. It may also be of value to detect physiologic changes in organisms expressing genetically altered muscle proteins.

Even though it may be applied to isolated muscle fibers or fiber bundles, the method seems particularly suited for work on cell cultures that are not easily accessible to contraction or Ca^{2+} measurements. A diagnostic test based on cultures derived from satellite cells would be less invasive than the standard IVCT because the necessary material can be acquired by needle biopsies.[14] In addition, repetitive tests are feasible because cultured myocytes can be stored in liquid nitrogen for further experiments unlike the mature muscle fiber preparations that can only be studied within 6 h after taking the biopsy. Also, cultured cells can more easily be sent to distant locations for testing. Recently, it has been reported that the skeletal muscle isoform of the ryanodine receptor (RyR1) is also present in B-lymphocytes,[32] opening the interesting possibility to perform a physiologic diagnostic test on blood cells to screen for forms of MHS that are linked to RyR1 mutations. We are currently exploring the use of microphysiometry for this purpose.[33]

In conclusion, this study describes a new approach to monitor Ca^{2+}-induced metabolic changes in muscle cells and its use for diagnosing MHS. It may also be of benefit to solve questions regarding other human myopathies that show defects in Ca^{2+} or H^{+} handling or in energy metabolism.

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References

1. MacLennan DH, Phillips MS: Malignant hyperthermia. Science 1992; 256: 789–94

2. Jurkat-Rott K, McCarthy T, Lehmann-Horn F: Genetics and pathogenesis of malignant hyperthermia. Muscle Nerve 2000; 23:4–17

3. Gronert GA, Antognini JF, Pessah IN: Malignant hyperthermia, Anesthesia, 5th edition. Edited by Miller RD. New York, Churchill Livingstone, 2000, pp 1033–52

4. Melzer W, Dietze B: Malignant hyperthermia and excitation-contraction coupling. Acta Physiol Scand 2001; 171:367–78

5. Larach MG, MacLennan DH: How carefully can we phenotype patients suspected of malignant hyperthermia susceptibility? ANESTHESIOLOGY 1999; 90: 645–8

6. Ørding H, Brancadoro V, Cozzolino S, Ellis FR, Glauber V, Gonano EF, Halsall PJ, Hartung E, Heffron JJ, Heytens L, Kozak-Ribbens G, Kress H, Krivosic-Horber R, Lehmann-Horn F, Mortier W, Nivoche Y, Ranklev-Twetman E, Sigurdsson S, Snoeck M, Stieglitz P, Tegazzin V, Urwyler A, Wappler F: *In vitro* contracture test for diagnosis of malignant hyperthermia following the protocol of the European MH Group: Results of testing patients surviving fulminant MH and unrelated low-risk subjects. The European Malignant Hyperthermia Group. Acta Anaesthesiol Scand 1997; 41:955–66

7. Allen GC, Larach MG, Kunselman AR: The sensitivity and specificity of the caffeine-halothane contracture test: a report from the North American Malignant Hyperthermia Registry. The North American Malignant Hyperthermia Registry of MHAUS. ANESTHESIOLOGY 1998; 88:579–88

8. Tong J, Oyamada H, Demaurex N, Grinstein S, McCarthy TV, MacLennan DH: Caffeine and halothane sensitivity of intracellular Ca^{2+} release is altered by 15 calcium release channel (ryanodine receptor) mutations associated with malignant hyperthermia and/or central core disease. J Biol Chem 1997; 272: 26332–9

9. Censier K, Urwyler A, Zorzato F, Treves S: Intracellular calcium homeostasis in human primary muscle cells from malignant hyperthermia-susceptible and normal individuals. Effect of overexpression of recombinant wild-type and Arg163Cys mutated ryanodine receptors. J Clin Invest 1998; 101:1233–42

10. Herrmann-Frank A, Lüttgau HC, Stephenson DG: Caffeine and excitation-contraction coupling in skeletal muscle: A stimulating story. J Muscle Res Cell Motil 1999; 20:223–37

11. Herrmann-Frank A, Richter M, Sarközi S, Mohr U, Lehmann-Horn F: 4-Chloro-m-cresol, a potent and specific activator of the skeletal muscle ryanodine receptor. Biochim Biophys Acta 1996; 1289:31–40

12. Tegazzin V, Scutari E, Treves S, Zorzato F: Chlorocresol, an additive to commercial succinylcholine, induces contracture of human malignant hyperthermia-susceptible muscles via activation of the ryanodine receptor Ca^{2+} channel. ANESTHESIOLOGY 1996; 84:1380–5

13. Baur CP, Bellon L, Felleiter P, Fiege M, Fricker R, Glahn K, Heffron JJ, Herrmann-Frank A, Jurkat-Rott K, Klingler W, Lehane M, Ording H, Tegazzin V, Wappler F, Georgieff M, Lehmann-Horn F: A multicenter study of 4-chloro-m-cresol for diagnosing malignant hyperthermia susceptibility. Anesth Analg 2000; 90:200–5

14. Magistris MR, Kohler A, Pizzolato G, Morris MA, Baroffio A, Bernheim L, Bader CR: Needle muscle biopsy in the investigation of neuromuscular disorders. Muscle Nerve 1998; 21:194–200

15. Parce JW, Owicki JC, Kercso KM, Sigal GB, Wada HG, Muir VC, Bousse LJ, Ross KL, Sikic BI, McConnell HM: Detection of cell-affecting agents with a silicon biosensor. Science 1989; 246:243–7

16. McConnell HM, Owicki JC, Parce JW, Miller DL, Baxter GT, Wada HG, Pitchford S: The cytosensor microphysiometer: Biological applications of silicon technology. Science 1992; 257:1906–12

17. Hafner F: Cytosensor microphysiometer: Technology and recent applications. Biosens Bioelectron 2000; 15:149–58

18. Brandt A, Schleithoff L, Jurkat-Rott K, Klingler W, Baur C, Lehmann-Horn F: Screening of the ryanodine receptor gene in 105 malignant hyperthermia families: Novel mutations and concordance with the *in vitro* contracture test. Hum Mol Genet 1999; 8:2055–62

19. Hafeman DG, Parce JW, McConnell HM: Light-addressable potentiometric sensor for biochemical systems. Science 1988; 240:1182–5

20. Sei Y, Gallagher KL, Daly JW: Multiple effects of caffeine on Ca^{2+} release and influx in human B lymphocytes. Cell Calcium 2001; 29:149–60

21. Owicki JC, Parce JW: Biosensors based on the energy metabolism of living cells: The physical chemistry and cell biology of extracellular acidification. Biosens Bioelectron 1992; 7:255–72

22. Fessenden JD, Wang Y, Moore RA, Chen SR, Allen PD, Pessah IN: Divergent functional properties of ryanodine receptor types 1 and 3 expressed in a myogenic cell line. Biophys J 2000; 79:2509–25

23. Schneider MF, Simon BJ: Inactivation of calcium release from the sarcoplasmic reticulum in frog skeletal muscle. J Physiol 1988; 405:727–45

24. Meissner G, Rios E, Tripathy A, Pasek DA: Regulation of skeletal muscle Ca^{2+} release channel (ryanodine receptor) by Ca^{2+} and monovalent cations and anions. J Biol Chem 1997; 272:1628–38

25. Laver DR, Lamb GD: Inactivation of Ca^{2+} release channels (ryanodine receptors RyR1 and RyR2) with rapid steps in $[Ca^{2+}]$ and voltage. Biophys J 1998; 74:2352–64

26. Juel C: The effect of beta 2-adrenoceptor activation on ion-shifts and fatigue in mouse soleus muscles stimulated *in vitro*. Acta Physiol Scand 1988; 134:209–16

27. Juel C: Intracellular pH recovery and lactate efflux in mouse soleus muscles stimulated *in vitro*: the involvement of sodium/proton exchange and a lactate carrier. Acta Physiol Scand 1988; 132:363–71

28. Hopkins PM, Hartung E, Wappler F, and the European Malignant Hyperthermia Group: Multicentre evaluation of ryanodine contracture testing in malignant hyperthermia. The European Malignant Hyperthermia Group. Br J Anaesth 1998; 80:389–94

29. Galloway GJ, Denborough MA: Phosphorus-31 nuclear magnetic resonance studies of muscle metabolism in malignant hyperpyrexia. Br J Anaesth 1984; 56:663–4

30. Olgin J, Argov Z, Rosenberg H, Tuchler M, Chance B: Non-invasive evaluation of malignant hyperthermia susceptibility with phosphorus nuclear magnetic resonance spectroscopy. ANESTHESIOLOGY 1988; 68:507–13

31. Argov Z, Lofberg M, Arnold DL: Insights into muscle diseases gained by phosphorus magnetic resonance spectroscopy. Muscle Nerve 2000; 23:1316–34

32. Sei Y, Gallagher KL, Basile AS: Skeletal muscle type ryanodine receptor is involved in calcium signaling in human B lymphocytes. J Biol Chem 1999; 274:5995–6002

33. Hack G, Jurkat-Rott K, Baur C, Melzer F, Lehmann-Horn F, Klingler W: Measurements of the acidification rate of human B-lymphocytes for the identification of the Malignant Hyperthermia Susceptibility. Pflügers Archiv (European Journal of Physiology) 2001; 441(Suppl):R147