To evaluate the reliability of the in vitro contracture test for susceptibility to malignant hyperthermia, we studied muscles from normal pigs and those susceptible to malignant hyperthermia. We performed the contracture test with various muscles from the same animal. Trapezius and intercostal muscles gave similar results, whereas the extensor digiti II muscle had lower sensitivities to both caffeine and halothane. Thus, the muscle chosen to determine susceptibility to malignant hyperthermia is important. In several animals, a false negative diagnosis would have resulted if only the distal muscle had been studied, and this was true even if weak contractures (<200 mg) were considered significant. In addition, we compared the response to caffeine or halothane of cut and intact muscle fibers. Although the cut fibers were depolarized, the sensitivity to these drugs was unchanged. Hence, results of the in vitro contracture test are independent of the resting membrane potential.
Key words: malignant hyperthermia • caffeine • halothane • contracture
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THE IN VITRO DETERMINATION OF SUSCEPTIBILITY TO MALIGNANT HYPERTHERMIA

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The most widely accepted test for susceptibility to malignant hyperthermia in man is the so-called in vitro contracture test. The test determines the sensitivity of a muscle specimen to caffeine or halothane applied to the bathing solution. Muscles from persons susceptible to malignant hyperthermia have lower contracture thresholds for these agents than normal muscle. Much work has been done to determine which parameters influence the reliability of this test.[4,8,10,12,14,15,18] Unfortunately, each of these studies employed a slightly different protocol. However, several European laboratories are currently investigating which parameters affect the diagnosis of susceptibility to malignant hyperthermia using a defined test protocol; the one supported by the European Malignant Hyperpyrexia Group (EMHG).[5] For example, the dynamic version of the in vitro contracture test which includes cyclic stretching and releases of the muscle was not found superior to the simple static one.[17]

To gain further insight into the applicability of the static version of the in vitro contracture test, we studied whether different muscles of the same animal have different sensitivities to halothane and caffeine. In addition, we examined the influence of the resting membrane potential on the test results. Finally, we evaluated contracture amplitude using several different criteria to determine which best describe a threshold response to these drugs.

MATERIALS AND METHODS

Biopsies of the external intercostal muscles were taken from 14 pigs susceptible to malignant hyperthermia and from 11 normal German Landrace swine. In addition, from some of these animals biopsies of the trapezius and/or the extensor digiti II were also taken. The muscle specimens were removed while the animals were anesthetized with thiopental (Hormon-Chemie, Munchen, FRG).

The excised muscles were transported to the laboratory in Krebs-Ringer solution[5] at room temperature under continuous gassing (carbogen: 95% O_2, 5% CO_2). Bundles of cut fibers from the trapezius and extensor digiti II muscles were studied in less than 4 hours after biopsy (2 hours after dissection). Such bundles were 2–3 cm long and

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had diameters of approximately 2 mm. Bundles of intact fibers of intercostal muscle were prepared up to 5 hours after surgery. These bundles were 1–1.5 cm long and also had diameters of approximately 2 mm. Preparations of cut intercostal muscle were obtained by cutting one or two ends of the intact fibers 10–15 minutes prior to use. Thus, these bundles had similar diameters, but were 1–3 mm shorter. Resting membrane potentials were recorded from the center of the fibers as previously described.[13]

Halothane was bubbled through the bath via a fluothane vaporizer (Vapor, Drager, Lubeck, FRG). The concentration of the vaporized halothane was monitored on line with a digital sensor (Iris, Drager, Lubeck, FRG). The rate of bubbling was controlled by teflon flow meters (ROTA Apparate, FRG). The vaporizer was adjusted to levels which resulted in bath concentrations of halothane suggested by the EMHG.[5] The concentration of halothane in the bath was determined as described by Van Dyke and Wood.[19] Caffeine (dehydrated; Carl Roth Karlsruhe, FRG) was added to the bathing solution in single doses to produce final concentrations of 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 8.0, and 32 m*M*. In a few experiments, intermediate caffeine concentrations between 4.0 and 10.0 m*M* were used. All solutions were maintained at 37°C.

Once a muscle bundle was in the experimental chamber, it was stimulated with supramaximal pulses of 1 msec duration at a frequency of 0.1 Hz. Force was recorded simultaneously from up to eight muscle bundles using a battery of force transducers (Grass FT03, Quincy, MA), standard amplifiers, and a chart recorder (Linseis, Selb, FRG). Each bundle was stretched until the twitch amplitude was considered maximal. At this length, slight shortening or lengthing did not alter the force amplitude. At least four bundles from each muscle were studied: two were exposed to halothane and two to caffeine. The static version of the test protocol supported by the EMHG was strictly followed. In total, 243 muscle bundles were examined.

The amplitudes of the contractures were quantified in grams and also calculated as fractions of the maximal contracture amplitude elicited in response to 32 m*M* caffeine.[10] The baseline force level from which the amplitudes of all contractures were calculated was defined as the force level just prior to the addition of 0.5 m*M* caffeine.

RESULTS

Different Muscles from the Same Animal. When the data were evaluated according to the criteria supported by the EMHG, in which a contracture >0.2 g is considered significant, there was no significant difference between the results from the cut trapezius, cut intercostal, or intact intercostal muscle bundles prepared from the same animal ($P > 0.5$, paired t-tests). Thus, the diagnosis of susceptibility to malignant hyperthermia was the same when the test results from either muscle were considered (see Table 1 and Fig. 1). Only in one pig (F) did the intact intercostal bundles have slightly higher thresholds to caffeine (malignant hyperthermia equivocal) than the cut trapezius bundles, which were clearly positive.

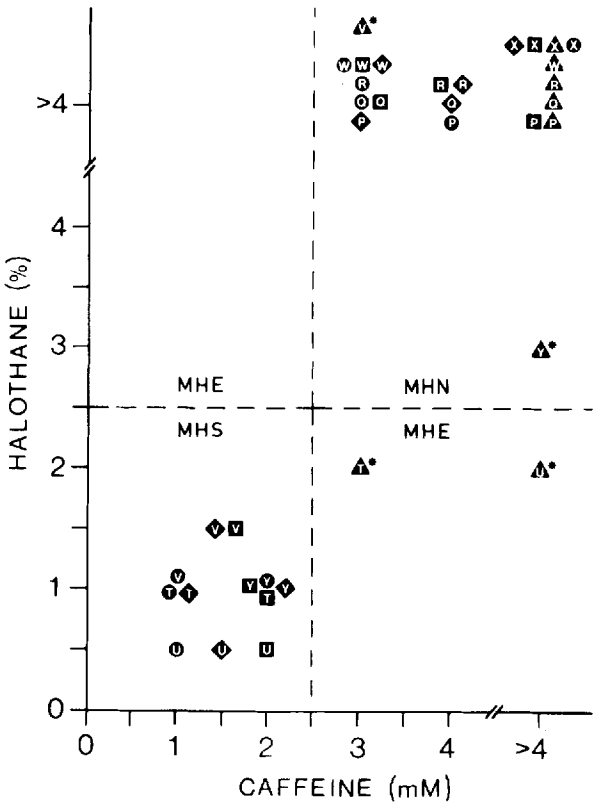

FIGURE 1. Results of in vitro tests for these animals in which bundles were prepared from cut extensor digiti II (▲), cut trapezius (●), cut intercostal (◆), and intact intercostal (■) muscles. The results for both intact and cut fibers from intercostal muscle were plotted. The dashed lines divide the plot into four different regions as described by the EMHG. The upper right region includes the test results that are considered normal (malignant hyperthermia negative: MHN); the lower left region includes the test results that are clearly positive (malignant hyperthermia susceptible: MHS); and the other two regions include the test results that are unclear or considered malignant hyperthermia equivocal (MHE). The * identifies a muscle which was classified different from that of the other muscles from the same animal.

In contrast, the extensor digiti II muscles from both normal and susceptible animals had a lower sensitivity to caffeine than the proximal muscles ($P < 0.05$, paired t-tests). The extensor digiti II from animals susceptible to malignant hyperthermia also showed a lower sensitivity to halothane. In the case of one susceptible animal (V), even the maximal concentration of halothane (>4%) did not induce contractures in the extensor digiti II preparations. We do not know if the normal extensor digiti II has a higher threshold to halothane than the proximal muscles because we never observed a contracture of a normal muscle during exposure to halothane in the concentration range we used.

Thus it turned out to be critical which muscle was chosen to determine the susceptibility to malignant hyperthermia. In a given animal, if the diagnosis had been based solely on the results obtained from the extensor digiti II, it would have been wrong for all the susceptible animals tested. This is shown in Fig. 1, which indicates the results of the in vitro test for those animals in which preparations from all three muscles were studied.

Cut Versus Intact Fibers. Bundles of cut or intact fibers from the same muscle responded with contractures to similar concentrations of caffeine or halothane. There was no significant difference in the contracture test results using the EMHG protocol between the intact or cut fiber preparations ($P > 0.5$, paired t-test). This was true whether the animal was susceptible to malignant hyperthermia or not (see Table 1 and Fig. 1). The average resting membrane potential of the cut fibers was −60 mV or less negative during the test period, whereas in intact fibers it was between −75 and −90 mV for more than 10 hours after biopsy. For

Table 1. Caffeine and halothane thresholds determined by using the static version of the contracture test protocol supported by the EMHG.

Pig	Muscle	Halothane (%)	Caffeine (m*M*)	Diagnosis
A	Intercostal (I)	>4.0	8.0	MHN
B	Intercostal (I)	>4.0	4.0	MHN
C	Trapezius	>4.0	4.0	MHN
	Intercostal (I)	>4.0	8.0	MHN
D	Trapezius	2.0	4.0	MHE
E	Trapezius	2.0	1.0	MHS
	Intercostal (I)	2.0	1.0	MHS
F	Trapezius	1.0	1.0	MHS
	Intercostal (I)	1.0	3.0	MHE
G	Trapezius	1.0	1.0	MHS
	Intercostal (I)	1.0	1.0	MHS
H	Trapezius	1.0	1.0	MHS
	Intercostal (I)	1.0	1.0	MHS
I	Trapezius	1.0	1.0	MHS
	Intercostal (I)	1.0	2.0	MHS
J	Trapezius	0.5	0.5	MHS
	Intercostal (I)	2.0	1.0	MHS
K	Trapezius	1.0	0.5	MHS
	Intercostal (I)	0.5	0.5	MHS
L	Trapezius	0.5	1.0	MHS
	Intercostal (I)	0.5	1.0	MHS
M	Trapezius	2.0	1.5	MHS
	Intercostal (I)	1.0	1.5	MHS
N	Trapezius	>4.0	4.0	MHN
	Intercostal (I)	>4.0	>4.0	MHN
	Intercostal (C)	>4.0	4.0	MHN
O	Trapezius	>4.0	4.0	MHN
	Intercostal (I)	>4.0	4.0	MHN
	Intercostal (C)	>4.0	4.0	MHN
S	Trapezius	>4.0	>4.0	MHN
	Intercostal (I)	>4.0	>4.0	MHN
	Intercostal (C)	>4.0	>4.0	MHN

Note: I = intact fibers, C = cut fibers, MHN = malignant hyperthermia negative, MHS = malignant hyperthermia susceptible, MHE = malignant hyperthermia equivocal. Alphabetical order indicates sequence of study.

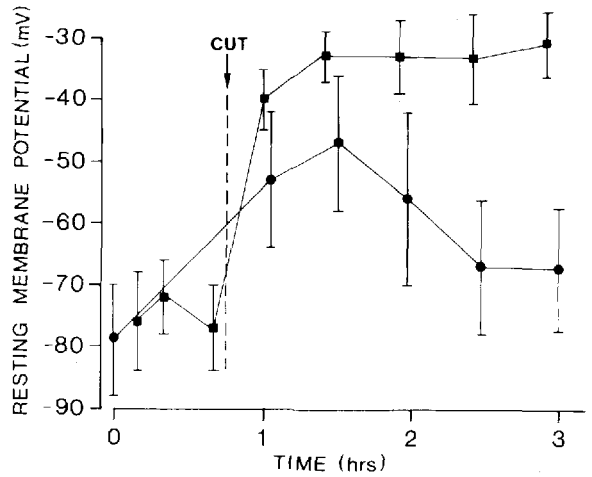

FIGURE 2. The effects of fiber transsection on resting membrane potential. The resting membrane potentials were recorded from several intact fibers from the intercostal muscle. Then the fibers were cut, either one or both ends. Those fibers which had both ends cut were depolarized beyond the threshold level of an action potential. Potentials were recorded from the center of the fibers. Time zero corresponds to 8 hours after biopsy. (•) One end cut: 1.4 → 1.3 cm; (■) both ends cut: 1.4 → 1.1 cm.

example, the mean resting membrane potential of intact fibers in two intercostal preparations was −80.0 ± 6.6 mV ($\bar{X}$ ± SD; n = 51). When one of the ends of these bundles were cut, the fibers depolarized to −50.1 ± 10.7 (n = 108; values were recorded between 5 and 120 minutes following transsection). When only one end of the fibers was transsected, a repolarization of the fibers occurred after several hours (Fig. 2). Nevertheless, the contracture test was completed on all cut intercostal bundles within 2 hours after transsection. Hence, most cut intercostal fibers would have had resting membrane potentials more positive than −60 mV (as in Fig. 2). It was noted that if both ends of the intercostal fibers were cut, the magnitude of the depolarization was even greater. However, such preparations were not routinely studied using the contracture test, because they were very difficult to mount into the experimental chamber and did not elicit twitch contractions. Such fibers were depolarized beyond the threshold level of an action potential. It was not possible to assess the viability of such a preparation.

Contracture Amplitude. When we considered smaller contracture amplitudes (<200 mg) as sig-

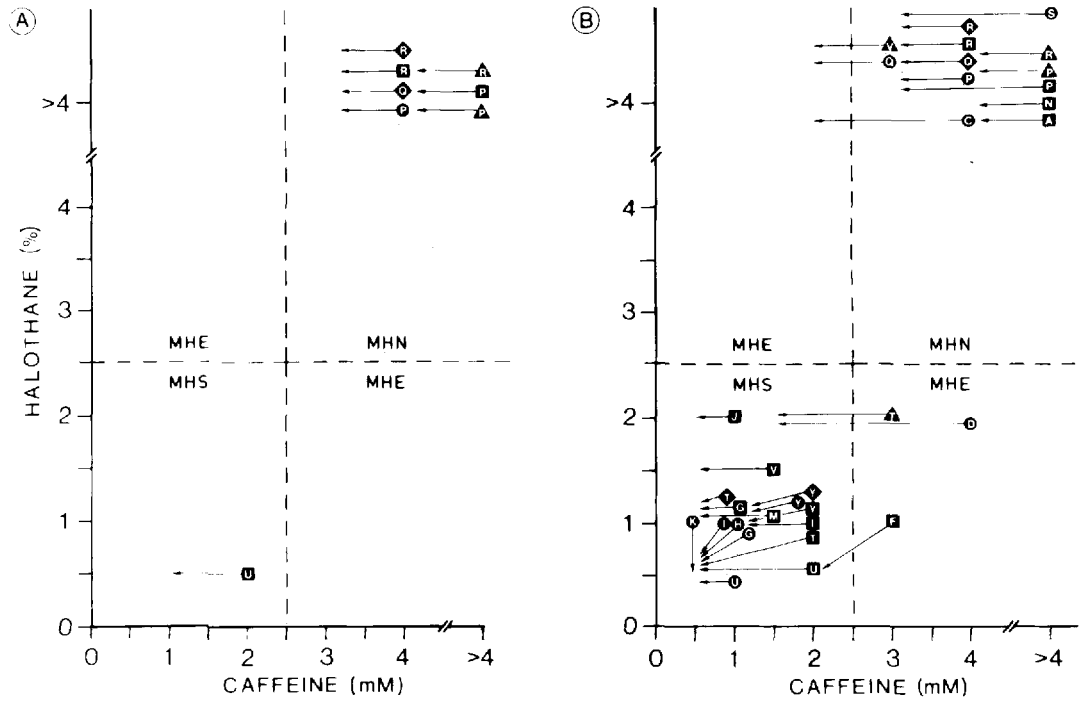

FIGURE 3. Shifts in caffeine and halothane thresholds by considering different criteria. The dashed lines divide each plot into four regions as in Fig. 1. **(A)** The shifts in threshold were plotted for those muscles in which the fractional contracture amplitude (produced by 32 m*M* caffeine) was ≥0.04. **(B)** The shifts in thresholds were plotted for those muscles in which a contracture as small as 10 mg was considered significant. Only test results in which a shift occurred were plotted. (•) Cut trapezius, (♦) cut intercostal, (■) intact intercostal, (▲) cut extensor digiti II.

nificant, changes in contracture thresholds occurred. Figure 3A shows such changes when contractures that had amplitudes less than 200 mg but 4% or greater of the 32 m*M* caffeine response were considered significant. However, using this criterion, the diagnosis of susceptibility was not altered for any test. In contrast, if the threshold value was lowered to 3% or greater of the 32 m*M* caffeine response, the test result from the trapezius muscle from pig Q resulted in a false positive diagnosis (the fractional ratio was 0.033). If the smallest detectable contracture was considered significant, then the test result of an additional normal pig (C) would have been incorrectly considered equivocal (Fig. 3B). Nevertheless, the consideration of the smallest detectable contracture did not alter the statistical significance of any of the comparisons in test results between individual muscles (i.e., proximal versus distal) or the comparison between the cut and intact intercostal fibers.

From pig D an intercostal muscle biopsy was taken, but it appeared very deteriorated and was not studied. Only bundles of cut fibers from the trapezius were used, and the test result was equivocal (EMHG protocol). However, this animal was considered to be susceptible to malignant hyperthermia for several reasons: (1) a contracture greater than 200 mg occurred upon exposure to 2% halothane, and we did not observe contractures induced by halothane in normal muscles; (2) smaller amplitude contractures were induced by 2.0 and 3.0 m*M* caffeine, and (3) several littermates of this animal were diagnosed as susceptible to malignant hyperthermia.

DISCUSSION

Although we observed differences in the results of the contracture test from different muscles, our findings are not in agreement with others who reported that the trapezius and forelimb extensor muscles showed greater dysfunction associated with malignant hyperthermia than intercostal muscle.[8,16] Stiffness of the limbs has been described as one of the hallmarks of a porcine MH episode. However, we observed that the extensor digiti II muscle had the lowest sensitivity to caffeine or halothane. This was true even when a force production of 10 mg was considered significant.

In one animal susceptible to malignant hyperthermia, cut fibers from the extensor digiti II muscle did not go into contracture when they were exposed to halothane. It may be that the more proximal limb muscles are most responsible for the "extensor rigidity." This indicates that not all muscles in animals susceptible to malignant hyperthermia are affected to the same degree. The muscles that are spared in malignant hyperthermia may be those that are typically spared in other myopathies or muscle disorders (i.e., progressive muscular dystrophy). However, more work is needed to verify this suggestion. If this were true it may also suggest that the pattern of distribution in a myopathy may be related to differences in the way these muscles regulate myoplasmic calcium concentration.

Several studies have indicated that the response of a muscle to caffeine and halothane depends on the fiber-type composition.[2,3,16,18] Muscles with a higher percentage of type 1 fibers responded more strongly to caffeine. Similarly, in our study the composition of a given muscle appeared to be well correlated with their sensitivities to halothane and caffeine. We determined that the extensor digiti II muscles were composed of 80% type 2 fibers, whereas the trapezius and intercostal muscles were about 50% type 1 and 50% type 2 (unpublished observations). We observed that the trapezius and intercostal muscles which had similar fiber type compositions had similar sensitivities to halothane and caffeine and that the extensor muscle which varied in composition also varied in its response to these agents. Our comparisons were made on muscles from the same animal, and we used the same experimental setup and protocol. However, we feel that additional study is required to verify the role of fiber type on the results of the in vitro contracture test. For example, it would be of interest to compare the sensitivities to halothane and caffeine of two distal muscles with very different fiber-type compositions (i.e., the tibialis anterior and the extensor digiti II).

In the past it was proposed that the sequence of events which initiates episodes of malignant hyperthermia included the progressive depolarization of the sarcolemma.[7,9] However, more recent evidence has indicated that this is not likely to be the case; resting membrane potentials in muscle fibers susceptible to malignant hyperthermia were unchanged during halothane-induced contractures.[11] It was of interest to consider the alternative order of events, whether or not an initial depolarization influenced the concentration of caffeine or halothane required to induce a contracture. It has been reported that cut fibers from both normal and susceptible pigs are more sensi-

tive to halothane than intact fibers and that this difference may affect the reliability of the in vitro contracture test.[6] It was also reported that cut and intact fibers differed in their contracture response to the exposure to halothane.[6,8] However, we observed no effect of fiber transsection on response to caffeine or halothane application. This was true for intercostal fibers from either normal animals or those susceptible to malignant hyperthermia. Intact and cut fibers prepared from the same muscle from an animal susceptible to malignant hyperthermia went into contracture of relatively similar amplitudes at nearly the same bath concentration of halothane. The difference between these findings is not clear but may be related to our improved monitoring of the administration of halothane. Nevertheless, the caffeine and halothane contractures observed here were not depolarization-induced contractures, which is consistent with the previous observations for caffeine[1] and halothane.[11]

Finally, we observed contractures with amplitudes less than 200 mg in 20% of the preparations studied. When these weak contractures (10–200 mg) were considered as significant, the diagnosis of susceptibility to malignant hyperthermia was altered in 9% of the muscles studied. The diagnosis of two muscles from normal pigs were equivocal. This was not a desirable shift, because a human with an equivocal test result is usually considered susceptible. Thus, our data support the idea that not all weak contractures are relevant, and a defined contracture amplitude is required.[5,10] The EMHG proposes the use of a somewhat arbitrary level of significance: 0.2 g of force.[5] Unfortunately, such a value does not take into account that the preparations used in various laboratories may be of different dimensions and hence not capable of producing the same amount of force. Therefore, it might be more useful to determine a threshold contracture from the fractional contracture amplitude (the amplitude of a contracture relative to the response observed in 32 m*M* caffeine) to determine a threshold contracture.[10] Yet, this brings up the question of what is an applicable fractional value. We noted an undesirable shift in diagnosis if a value of 0.03 was used; hence, this is too low. In contrast, the diagnosis of susceptibility to malignant hyperthermia was not altered in any case when a fractional contracture ≥ 0.04 was considered relevant. Thus, if the dimensions of preparations vary considerably or if the maximal contracture amplitude is relatively small, we suggest that the determination of the fractional contracture amplitude might be a better discriminator to determine the significance of an observed contracture. We suggest that a contracture with a fractional value ≥ 0.04 may be significant.

We conclude that the sensitivities to caffeine and/or halothane within a given animal vary from muscle to muscle, and that this variability is independent of the membrane potential.

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