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Effects of Calcium-free Solution, Calcium Antagonists, and the Calcium Agonist BAY K 8644 on Mechanical Responses of Skeletal Muscle from Patients Susceptible to Malignant Hyperthermia

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The purpose of this investigation was to determine if alteration in the function of the dihydropyridine receptor may in turn modify halothane-induced contractures in muscle bundles from patients susceptible to malignant hyperthermia (MH). The effects of Ca^{2+}-free Krebs Ringer (KR) solution, 5 μM verapamil, 5 μM nifedipine, and 10 μM of the Ca^{2+} agonist BAY K 8644 on halothane-induced contracture were therefore investigated. The halothane-induced contracture was prevented in the absence of extracellular Ca^{2+} and significantly reduced in the presence of verapamil or nifedipine. BAY K 8644 significantly enhanced the 0.5-, 1.0-, and 1.5-vol % halothane-induced contracture in MH-susceptible muscle bundles. When BAY K 8644 was dissolved in Ca^{2+}-free KR solution, no contracture was observed in MH-susceptible muscle bundles. These results on cut MH-susceptible human muscle bundles support the hypothesis that halothane-induced contracture in MH can be modified by the binding of Ca^{2+} agonists or antagonists to the dihydropyridine receptor. The role of Ca^{2+} entry phenomena remains unclear, but the results suggest that extracellular Ca^{2+} is required to reprime or to bind to some sites of the dihydropyridine receptor. (Key words: Anesthetics, volatile: halothane. Calcium agonist: BAY K 8644. Hyperthermia: malignant. Ions: Ca^{2+}. Pharmacology, calcium antagonists: verapamil; nifedipine.)

SEGMENTS OF MUSCLE BUNDLES are used for *in vitro* contracture tests to diagnose malignant hyperthermia (MH) susceptibility. The tests determine the sensitivity of cut muscle fibers to halothane or caffeine added to the bathing solution. Muscle bundles from patients susceptible to MH have a lower contracture threshold for these drugs than do those from normal patients.[1,2] There is general agreement that contractures elicited by caffeine reflect facilitated opening of the Ca^{2+}-release channels of the terminal cisternae of the sarcoplasmic reticulum (SR).[3] This channel opening leads to release of stored Ca^{2+}, similar to the pathophysiologic defect believed to be present in MH.[4,5] Mechanisms whereby halothane induces contractures in MH muscle *in vitro* remain unclear. It is assumed that the effects of halothane on MH muscle are due also to an abnormal regulation of the Ca^{2+} release channels of the SR, which in MH muscle have a lower threshold of response to release Ca^{2+}.[6] However, it is not known if halothane causes abnormal muscle contracture *in vitro* by acting directly on the Ca^{2+}-release channel.

Recently, it has been proposed that, in normal skeletal muscle, the dihydropyridine receptor (DHPR) (which is functionally and structurally different from cardiac DHPR[7]) may initiate charge movements and act as the voltage-sensor controlling the Ca^{2+} release from SR.[8–11] This DHPR also mediates the entrance of a very small current of extracellular Ca^{2+} in the muscle fiber. Although extracellular Ca^{2+} entry may not be the mechanism of excitation–contraction (E–C) coupling, it may have some secondary role.[12]

Therefore, the purpose of this investigation was to determine whether modulation of the DHPR can influence the Ca^{2+}-release channel response of MH muscle bundles to halothane. To this end, the effects of Ca^{2+} antagonists, Ca^{2+} agonist, and Ca^{2+}-free Krebs' Ringer's (KR) solution were examined on the *in vitro* halothane contracture test.

Materials and Methods

Patients presenting for a diagnostic muscle biopsy as part of investigation for MH participated in the study. The study was approved by the Lille University Studies Ethics Committee, and informed consent was obtained from the patients for removing extra muscle.

PREPARATION OF HUMAN MUSCLE BIOPSIES

The biopsies were taken from the vastus lateralis muscle under combined block with lidocaine (6 mg/kg) of the

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femoral nerve and lateral cutaneous nerve of the thigh. Muscle bundles (approximately 15–20 mm in length and 2–3 mm in diameter) were carefully dissected from the biopsies. One end was pinned to the silicone bottom of the tissue bath (4 ml) perfused continuously (4–5 ml/min) with KR solution at 37° C. The *p*H was adjusted to 7.35 ± 0.05, and the KR solution was bubbled with carbogen (95% oxygen and 5% carbon dioxide). The other end of the strip was attached by a thin silk thread to a force transducer (Bioscience Dynamometer UFI and Biological Amplifier 120). The preparations were stimulated directly using silver electrodes with rectangular current pulses of 2-ms duration delivered at a frequency of 0.2 Hz by a stimulator CEA-DAM model GPI-GE2198. The optimal length was determined by increasing the voltage to supramaximal twitch tension and then increasing the length to achieve absolute twitch tension. Baseline and twitch tension were recorded continuously at low speed on a Siemens C1013 polygraph. The smallest acceptable twitch for both the diagnostic procedure and the experiment was 1 g (0.0098 N).

Diagnosis of MH Susceptibility

All patients were investigated according to the protocol of the European MH group.[1] The criteria of MH susceptibility (MHS) were an increase in resting tension of at least 0.2 g (0.0019 N) both with a halothane threshold concentration ≤ 2% and a caffeine threshold concentration ≤ 2 mM. A normal response (MH-negative [MHN]) was defined as a halothane threshold > 2% and a caffeine threshold > 2 mM. Other results (*i.e.*, one abnormal response either with halothane or with caffeine) were classified MH-equivocal (MHE), but these patients were not included in this study since the significance of these MHE results is not currently known.

Materials

The normal KR solution was of the following composition (millimolar): NaCl 118.1, KCl 3.4, $CaCl_2$ 2.5, $MgSO_4$ 0.8, KH_2PO_4 1.2, $NaHCO_3$ 25, and glucose 11.1. Distilled water was further filtered through two deionizing glass distillations to provide Ca^{2+}-free solution. Halothane was mixed with carbon dioxide in oxygen by means of a calibrated vaporizer (Fluotec Mark III) in concentrations of 0.5, 1, 1.5, 2, and 3 vol % as measured by gas chromatography, corresponding to 0.099 ± 0.010, 0.202 ± 0.018, 0.366 ± 0.024, 0.536 ± 0.021, and 0.680 ± 0.069 mM (mean ± standard error of the mean [SEM] for ten determinations), respectively. Each concentration was maintained for 3 min when the desired concentration of halothane was reached. Verapamil and nifedipine were added to the normal KR solution at a concentration of 5 μM. BAY K 8644 was used at a concentration of 10 μM and added either to normal or to Ca^{2+}-free KR solution. Verapamil hydrochloride was used in ready-mixed solution and supplied in 2-ml ampules (Biosedra) containing 5 mg in 2 ml distilled water. Pure nifedipine and BAY K 8644 powder (Bayer Pharma) were dissolved in dimethyl sulfoxide (DMSO) as stock solutions (10 mM) and were kept refrigerated. The desired bath concentrations were achieved by diluting the stock solution with KR solution. The nifedipine and BAY K 8644 solutions were protected from exposure to light during the experiments. As these drugs were dissolved in 0.1% DMSO, a preliminary test in this solution was necessary. DMSO 0.1% in KR solution did not show any effect on twitch and baseline tension in both MHN and MHS preparations (n = 4 for each group).

Preliminary Experiments

Muscle bundles from patients susceptible to MH were used to determine the concentration of verapamil and nifedipine that would induce an observable effect on halothane-induced contracture. The 2% halothane contracture response was reduced by 1 μM verapamil, whereas 5 μM verapamil abolished it completely in a time-dependent manner. Although 10 μM verapamil attenuated contracture more rapidly, high concentrations have been shown to affect sites other than DHPR.[13] A concentration of 1 μM nifedipine significantly reduced the 2% halothane contracture, and the attenuation was greater with 5 μM. Therefore, we used 5 μM verapamil and nifedipine in our experiments to minimize the nonspecific effects of both drugs. The effects of 1, 5, and 10 μM BAY K 8644 were studied on MHS muscles (n = 4). It was found that increases in twitch tension and potentiation of halothane contracture were dose-dependent. Therefore, we used 10 μM BAY K 8644 in our experiment, since this concentration is still compatible with a specific action on DHPR.[14]

Experimental Procedure

Additional muscle strips obtained from the same biopsies were pretreated with 1) Ca^{2+}-free solution until twitch tension was reduced by 50%; 2) 5 μM verapamil, 5 μM nifedipine, or 10 μM BAY K 8644 for 10 min prior to the administration of halothane; or 3) 10 μM BAY K 8644 dissolved in Ca^{2+}-free KR solution until twitch tension was reduced by 50%. The halothane test was then performed according to the same procedure as described above. Each protocol was performed on a separate muscle bundle. The tests of halothane alone and of halothane plus antagonist or agonist always were performed on different muscle strips. However, all studies with antagonist or agonist were paired with controls from the same biopsy specimen. The sequence of the tests was not randomized because it first was necessary to secure enough viable tissue

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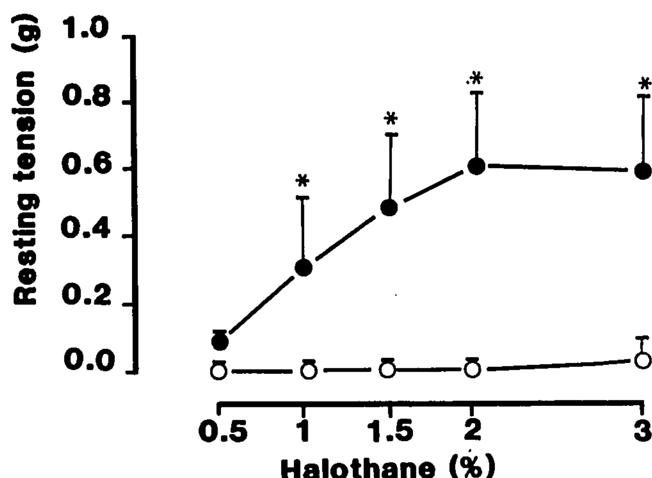

FIG. 1. Responses to cumulative concentrations of halothane in muscle bundles from six MHS patients in normal KR solution (filled circles) and in Ca^{2+}-free solution (open circles). The halothane-induced contracture was prevented by the exposure to the Ca^{2+}-free solution. Results with Ca^{2+}-free solution were paired with MHS muscle bundles from the same patients in normal KR solution. Data are presented as mean ± SEM. $*P < 0.05$ *versus* Ca^{2+}-free solution (paired *t* test).

for the diagnostic tests. The experimental procedures were performed on tissue remaining after the diagnostic tests.

Data were expressed as means ± SEM. Statistical analysis was performed using a Student's paired *t* test within groups of either MHS or MHN patients and using an unpaired Student's *t* test between groups of MHS and MHN patients. A P value < 0.05 was considered significant.

Results

In Vitro Discrimination of MH Susceptibility

Muscle bundles from 23 patients developed a caffeine contracture of 0.31 ± 0.05 g at 2 mM caffeine and a contracture of 0.40 ± 0.08 g at 2% halothane. According to the protocol supported by the European MH Group, these patients were classified as MHS. Twenty patients did not develop any significant contracture at the above concentrations and were classified as MHN.

Effects of Ca^{2+}-free Solution

The time course of twitch decay observed with Ca^{2+}-free solution was similar in MHS and MHN muscle bundles and was relatively slow. A 50% reduction of the twitch tension occurred within approximately 15 min (15 ± 5 min). As illustrated in figure 1, the halothane-induced contracture in MHS muscle bundles was prevented by the exposure to the Ca^{2+}-free solution; this effect was observed consistently in the six muscle bundles from the six MHS patients. At the end of the halothane test, normal KR solution (containing Ca^{2+}) was restored to the organ bath, and the preparation was allowed to stabilize for progressive recovery of the twitch tension. The halothane contracture test was repeated after 80% recovery of twitch tension, which was achieved within 30 min (30 ± 10 min).

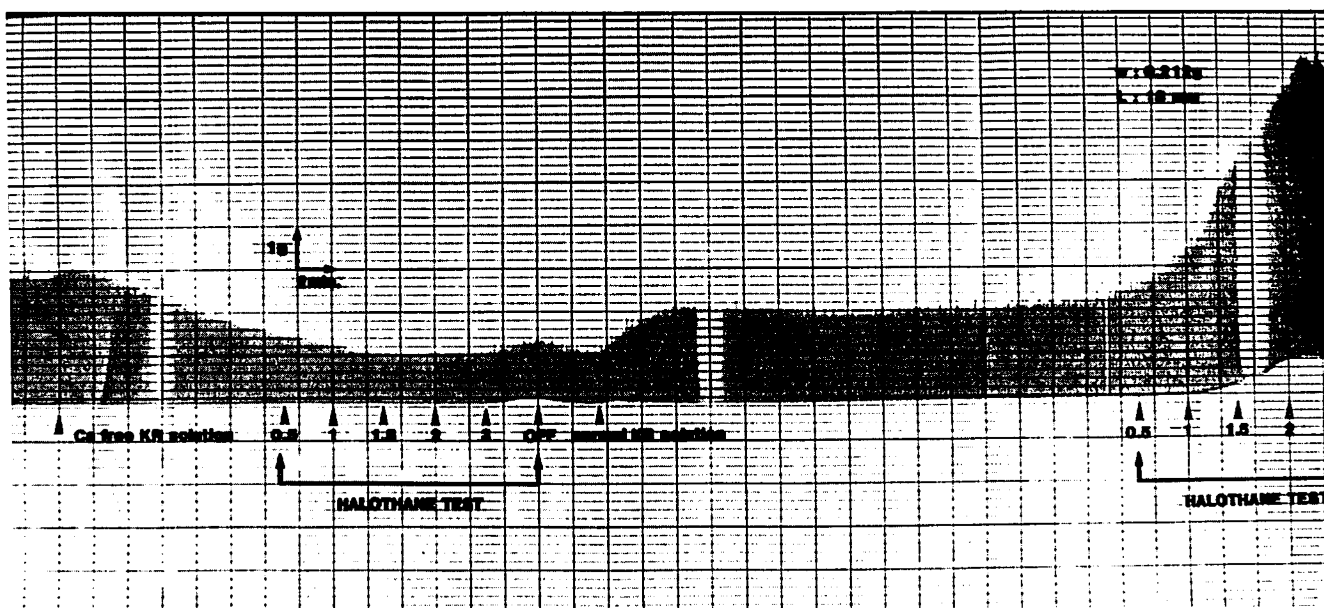

FIG. 2. Effect of Ca^{2+}-free solution on halothane-induced contracture in MHS muscle. The halothane test, performed after a 50% reduction of twitch tension, did not induce muscle contracture. When the same experiment was repeated after 80% recovery of twitch tension in normal KR solution, the MHS muscle bundle developed a significant contracture (≥ 0.2 g) at 2% halothane.

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After this recovery, the six MHS muscle bundles now developed a significant contracture at 2% halothane (fig. 2). In MHN muscle bundles, 80% recovery of twitch tension was achieved within the same delay but no contracture to halothane was observed.

Effect of Verapamil and Nifedipine

In the absence of halothane, verapamil and nifedipine did not exert significant effect on twitch tension in either MHS or MHN muscles. When halothane alone was applied to the muscle bundles, twitch tension usually was increased both in MHN and in MHS muscle preparations. In the presence of verapamil and of nifedipine, the halothane-induced potentiation of the twitch tension was diminished (data not shown). As shown in figure 3, 5 μM verapamil reduced the halothane contracture ($P < 0.05$) in 12 of 12 MHS patients. Furthermore, in 4 of the 12 MHS patients, the halothane-induced contracture was completely blocked. In the nifedipine experiments, the halothane-induced contracture in MHS muscle bundles was similarly reduced (fig. 3); this was observed in 10 of 10 MHS patients. In four muscle bundles, the halothane contracture was completely blocked with nifedipine.

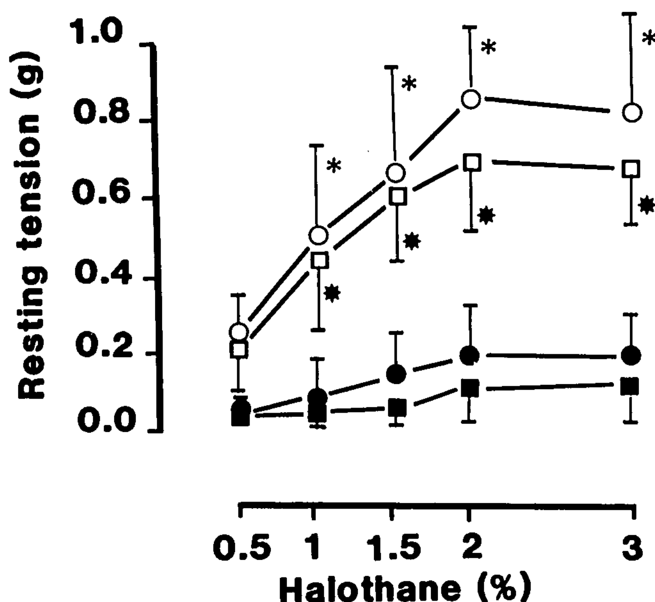

FIG. 3. Effects of 5 μM verapamil or 5 μM nifedipine on the response to halothane in muscle bundles from MHS patients. Open squares = muscle bundles without verapamil (group 1) (n = 12); filled squares = muscles bundles with verapamil from the same biopsies of group 1 (n = 12); open circles = muscle bundles without nifedipine (group 2) (n = 10); filled circles = muscle bundles with nifedipine from the same biopsies of group 2 (n = 10). Data are presented as mean ± SEM. Asterisk = $P < 0.05$ *versus* nifedipine muscle bundles from group-2 biopsies; starburst = $P < 0.05$ *versus* verapamil muscle bundles from group-1 biopsies (paired *t* test).

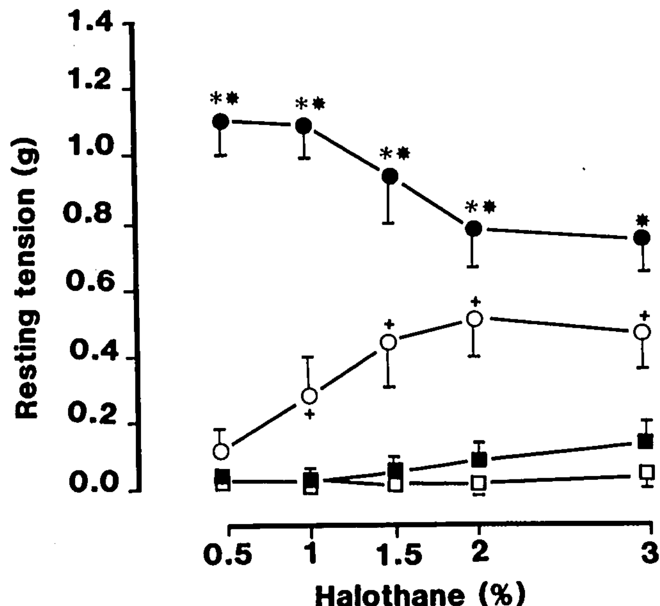

FIG. 4. Effects of BAY K 8644 on the response to cumulative concentrations of halothane in muscle bundles from MHS and MHN patients. Open symbols (circles: MHS, n = 15; squares: MHN, n = 15) = halothane dose-response curves in the absence of BAY K 8644; closed symbols (circles: MHS, n = 15; squares: MHN, n = 15) = halothane dose-response curves in the presence of BAY K 8644. Data are presented as mean ± SEM. Asterisk = $P < 0.05$, statistically significant differences between MHS muscle bundles with and without BAY K 8644 (paired *t* test); starburst = $P < 0.05$, statistically significant differences between groups of MHS and MHN muscle bundles for halothane contracture in the presence of BAY K 8644 (unpaired *t* test); dagger = $P < 0.05$, statistically significant differences between groups of MHS and MHN muscle bundles for halothane contracture in the absence of BAY K 8644 (unpaired *t* test).

Effects of BAY K 8644 in Normal KR Solution

BAY K 8644 alone consistently enhanced the amplitude of the twitch. However, no difference in the percentage of the maximal twitch increase was observed between normal and abnormal muscle bundles (150 ± 30% for MHS muscle and 162 ± 40% for MHN muscle). BAY K 8644 did not induce muscle contractures on its own but considerably enhanced the halothane contracture of MHS muscle bundles. This effect occurred as soon as halothane was added to the carbogen flow and reached its maximal level (1.1 ± 0.15 g) at the lowest concentration (0.5%) of halothane (fig. 4). This response was significantly higher than the 0.1 ± 0.05 g obtained in the absence of BAY K 8644. It was observed in 15 muscle preparations from 15 MHS patients. In contrast, in 15 MHN patients, no contracture was observed in the presence of combined 10 μM BAY K 8644 and 0.5% halothane, whereas a nonsignificant contracture (0.15 g ± 0.1) developed after increasing the concentration of halothane beyond 1.5% in the presence of BAY K 8644.

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EFFECT OF BAY K 8644 IN CA^{2+}-FREE KR SOLUTION

The Ca^{2+} agonist slightly lengthened the time course of twitch decay in both MHN and MHS muscle. A 50% reduction of the twitch tension occurred within approximately 20 min (20 ± 5 min). As shown in figure 5, BAY K 8644 did not modify the preventive effect of Ca^{2+}-free solution on halothane contracture in five MHS muscle bundles from five patients. Once normal KR solution was returned to the organ bath, the time for 80% recovery of twitch tension was not significantly shortened (25 ± 5 min). Then the halothane test was repeated, and BAY K 8644-potentiation of halothane-induced contracture was observed in all preparations.

Discussion

It is generally agreed that depolarization of the transverse-tubular system (T-system) triggers the release of Ca^{2+} from the SR.[15] This Ca^{2+} release is controlled by "voltage sensors" located in the T-system that detect any potential change of the muscle membrane.[16] One proposed mechanism suggests that an electrical alteration induces a movement of voltage sensors of the T-system and that the movement of these sensors is the first step in the Ca^{2+}-release control mechanism.[17,18] Recently, it has been suggested that the DHPRs, which also mediate the entrance of a very small current of extracellular Ca^{2+} into the muscle fiber,[12] act as (or in fact are) the voltage sensors controlling Ca^{2+} release from the SR.[8-11]

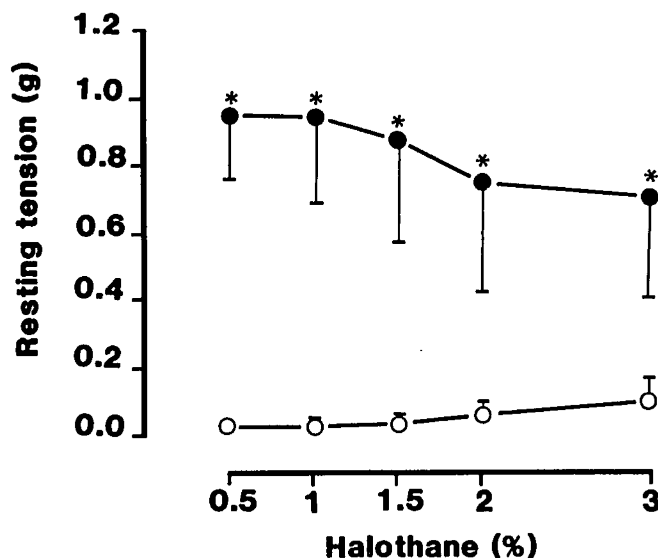

FIG. 5. Responses to cumulative concentrations of halothane in muscle bundles from six MHS patients in the presence of BAY K 8644 dissolved in normal Krebs Ringer solution (filled circles) and BAY K 8644 dissolved in Ca^{2+}-free solution (open circles). The BAY K 8644 plus halothane-induced contracture was prevented by the exposure to the Ca^{2+}-free solution. Results with Ca^{2+}-free solution were paired with those in normal KR solution from the same biopsies. Data are presented as mean ± SEM. Asterisk = $P < 0.05$ *versus* (paired *t* test).

Our findings that the twitch tensions of skeletal muscle bundles from both MHN and MHS patients decreased when they were exposed to Ca^{2+}-free solution may be less related to the E–C coupling process *per se* than to the instability of these cut-fiber preparations, since cut-fiber specimens are prone to depolarization at least during the 1st h after excision.[19] However, it has been demonstrated recently that such muscle cells may reseal with time and that these preparations may be far more physiologic than appreciated in the past.[19] Consequently, our results in MHS and MHN muscle may be consistent with previous results suggesting that extracellular Ca^{2+} is somehow required to reprime or bind to some site on the DHPR.[20,21] Nevertheless, the results of the current study may also be a consequence of a loss of intracellular Ca^{2+} stores, explaining the reduction of twitch tension in Ca^{2+}-free solution, as suggested by Nelson and Chausmer.[22]

Once a 50% reduction of twitch tension in Ca^{2+}-free solution was achieved, the halothane-induced contracture in MHS muscle bundles was almost completely blocked. After restoration of normal KR solution, twitches increased to about 80% of their original height, whereas much greater increases were observed in the magnitude of the halothane contracture in MHS muscle. If such experiments were performed after 100% reduction of twitch tension, the restoration of twitch tension and the development of halothane contracture were never obtained in normal KR solution, suggesting a profound alteration of the muscle preparation. These findings suggest that the requirement for extracellular Ca^{2+} in halothane-induced contractures is crucial and is very different from that for twitches. However, since the muscle bundles continue to twitch when halothane contractures do not appear (in Ca^{2+}-free solution at 50% twitch depression), it could be argued that intracellular Ca^{2+} stores are more important to halothane contractures than is E–C coupling. Hence, it is possible that the small and slowly activated current of extracellular Ca^{2+} (not essential for twitch) somehow may influence an abnormal prolonged activation of MHS muscle induced by halothane. This latter possible explanation is strengthened by other studies demonstrating that extracellular Ca^{2+} and its influx may have some influence on prolonged skeletal muscle contraction.[23,24]

It is widely assumed that contraction of skeletal muscle depends essentially on intracellular storage of Ca^{2+}.[25,26] Although substantial data have suggested that binding of dihydropyridine or phenyl alkylamines to the DHPR in the T-system tubules alters E–C coupling,[8,11,27] these drugs do not affect muscle contractions under normal physiologic conditions. However, in abnormal conditions such as in our study (*i.e.*, application of halothane on MHS cut muscle bundles), verapamil and nifedipine did not

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significantly alter the amplitude of twitch tension but consistently reduced the halothane-induced contracture. This observation may indicate that these two different forms of E–C coupling probably are not mediated by a single process but more likely are mediated by different mechanisms. Indeed, twitch tension represents a relatively fast muscle contraction, which is not compatible with the low Ca^{2+} conductance of the T-system tubular DHPR.[28] Thus, while the DHPR mediates twitch tension without a large influx of extracellular Ca^{2+}, it still is possible that some Ca^{2+} entry may contribute to SR Ca^{2+} release as a result of halothane in MHS muscle. Hence, the effects of nifedipine and verapamil on halothane-induced contracture may be mediated by blockade of Ca^{2+} entry, and this mechanism also may explain the absence of significant effect of these Ca^{2+} antagonist on twitch tension.

The effects of Ca^{2+}-free solution on halothane-induced contracture have been investigated in skeletal muscle obtained from human subjects,[29,30] pigs,[22,31] and cats,[32] and the results are consistent with our findings. In contrast, the effects of Ca^{2+} antagonists on halothane-induced contractures suggest that there is considerable species variability in the action of these agents.[32–36]

Previous studies on human MHS muscle are consistent with our results. One, 8, and 10 μM verapamil,[29,30,37] 1–7 μM diltiazem,[38,39] and 1 μM nifedipine[38] have been reported to reduce or to prevent the halothane-induced contractures. Fletcher *et al.*[30] did not observe an effect of nifedipine on halothane contractures in human skeletal muscle. However, their study differed from the current study in many ways, including the manner in which halothane was administered, the type of solvent used, source of nifedipine, and the presence of succinylcholine.

BAY K 8644 strongly potentiated the halothane-induced contractures in skeletal muscle from MHS patients and had no effect on MHN muscle; BAY K 8644 is a dihydropyridine agonist that acts at the site of dihydropyridine antagonists.[40] The drug is structurally similar to dihydropyridine antagonist but exerts opposite effects.[14] It is interesting that the activation of the fast Na^{+} current by BAY K 8644 also has been reported.[41] This fast Na^{+} current has been implicated in the mechanism of MH[42] and may also be involved in the abnormal response of MH muscle to BAY K 8644 plus halothane. The blocking of the effects of combined BAY K 8644 and halothane in Ca^{2+}-free solution suggests that extracellular Ca^{2+} may be needed in the action or binding of BAY K 8644 to the DHPR and/or Na^{+} channel.

In the current study, the experimental procedures altered the capacity of DHPR to regulate the Ca^{2+} release channel or the SR. Consequently, the use of either verapamil and nifedipine or BAY K 8644 to bind to the DHPR led more likely to the depression or to the enhancement, respectively, of the release of Ca^{2+} from SR. The role of Ca^{2+} entry phenomena remains unclear, but the observed effects suggest that extracellular Ca^{2+} is required to reprime or to bind to some sites of the DHPR. Thus, our results do suggest that modulation of the DHPR can influence the Ca^{2+} release response of MH muscle to halothane.

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