The importance of calcium ions for *in vitro* malignant hyperthermia testing

Jeffrey E. Fletcher PhD, Florence J. Huggins MS, Henry Rosenberg MD

Intracellular Ca^{++} levels in skeletal muscle are elevated during the in vitro *contracture response of muscle from subjects with malignant hyperthermia. The role of Ca^{++} in the bathing medium and the consequences of substitution of Sr^{++} for Ca^{++} in the response to agents associated with malignant hyperthermia were examined. When Ca^{++} was omitted from the bathing medium the contractures induced in human vastus lateralis by halothane (three per cent) or succinylcholine (50 mM) were reduced by 80 and 100 per cent, respectively, while contractures induced by caffeine (8 mM) were only reduced by 50 per cent. Substitution of Ca^{++} by another divalent cation, Sr^{++}, completely restored contractures induced by caffeine, but only partially restored contractures induced by halothane or succinylcholine (to 50 and 30 per cent of Ca^{++}-containing medium, respectively). Mepacrine (10 μM) was effective in antagonizing contractures by caffeine, whereas verapamil and nifedipine (10 μM) were not. These results support an essential role for extracellular Ca^{++} not fulfilled by Sr^{++} in contracture induction by halothane and succinylcholine, but not by caffeine.*

Les taux de Ca^{++} intracellulaire dans le muscle squelettique est élevé lors de la contracture in vitro *du muscle des sujets atteints d'hyperthermie maligne. Le rôle du Ca^{++} dans le bain où le muscle est étudié et les conséquences de la substitution du Sr^{++} au lieu du Ca^{++} en réponse aux agents associés avec l'hyperthérmie maligne a été examiné. Quand le calcium a été omis du bain, les contractures induites chez l'homme par l'halothane (trois pour cent) ou la succinylcholine (50 μM) étaient réduites de 80 à 100 pour cent respectivement alors que les contractures induites par la caféine (8 mM) ont été réduites de seulement 50 pour cent. La substitution du Ca^{++} par un autre cation divalent, Sr^{++}, a complètement restauré les contractures induites par la caféine mais a restauré seulement partiellement les contractures induites par l'halothane ou la succinylcholine (à 50 et 30 pour cent respectivement). La mépacrine (10 μM) était efficace pour antagoniser les contractures dues à la caféine alors que le vérapamil et la nifédipine (10 μM) ne l'étaient pas. Ces résultats démontrent le rôle essentiel du Ca^{++} extracellulaire dans les contractures induites par l'halothane et la succinylcholine mais non la caféine.*

Key words
ANAESTHETICS, VOLATILE: halothane;
COMPLICATIONS: MH;
HYPERTHERMIA: malignant;
NEUROMUSCULAR RELAXANTS: succinylcholine.

From the Laboratory for the Study of Malignant Hyperthermia, Department of Anesthesiology, Hahnemann University, Philadelphia, PA 19102-1192 U.S.A.

This research was funded by the Hahnemann Anesthesia Research Foundation and NIH grant #GM34872.

Address correspondence to: Dr. Jeffrey E. Fletcher, Department of Anesthesiology MS-310, Hahnemann University, Broad and Vine, Philadelphia, PA 19102-1192 USA.

Malignant hyperthermia (MH) is currently diagnosed by the *in vitro* contracture response of biopsied skeletal muscle to halothane and caffeine.[1,2] Halothane and succinylcholine act in synergy *in vivo* to induce MH,[3] and *in vitro* to induce contractures in skeletal muscle.[4,5]

The contracture response correlates with elevated myoplasmic Ca^{++} concentrations.[6] The source of Ca^{++} has been suggested to be either release from the sarcoplasmic reticulum by a decreased threshold of the Ca^{++} release process,[7,8] or leakage across the sarcolemma.[9] Since the threshold of the contracture response to halothane and caffeine is reduced in muscle from MH susceptibles, an understanding of the source of Ca^{++} in this response is of importance in interpreting the results obtained in isolated organelles.

Several other agents are known to substitute for, or interact with, Ca^{++} and may be of use as probes in contracture studies. Strontium (Sr^{++}), a divalent cation, completely substitutes for Ca^{++} at most sites involved in the excitation-contraction coupling mechanism, although Sr^{++} is less efficient than Ca^{++} in activating the contractile proteins.[10] Despite a lesser effect on the contractile proteins, Sr^{++} can fully support caffeine contractures in frog skeletal muscle.[11] Organic Ca^{++} antagonists reduce halothane contractures in human skeletal muscle[9,12] and caffeine contractures in the cat,[13] but have not been examined as regards caffeine or succinylcholine contrac-

tures in human skeletal muscle. Mepacrine antagonizes contractures induced by halothane and succinylcholine.[14] The effects of mepacrine on caffeine contractures have not been examined.

The present study examined the role of extracellular Ca^{++} in contractures induced in human vastus lateralis by agents triggering or used in the diagnosis of MH (halothane, succinylcholine, caffeine). The methods employed include altering the ionic conditions of the bathing medium and adding agents affecting myoplasmic Ca^{++} levels.

Methods

Materials

Succinylcholine chloride, mepacrine, verapamil and nifedipine were purchased from Sigma Chemical Co. (St. Louis, Mo). Halothane was obtained from Ayerst (New York, NY). Caffeine was obtained from Eastman Kodak Co. (Rochester, NY). Strontium chloride was purchased from Johnson Matthey Chemicals Limited (England).

Human muscle biopsies

Approval by the Hahnemann University Human Studies Committee was obtained for this study. Patients were selected for biopsy based on suspicion of MH. Pharmacological studies were conducted on tissue remaining after diagnostic testing with halothane. The magnitude of the response of muscle strips to halothane, caffeine and succinylcholine was essentially unaltered if the response to halothane alone during the diagnostic test was <1 g. Control strips (also initially used for diagnostic testing) bathed in a Ca^{++} buffer with no antagonists added were always paired with experimental strips for each biopsy specimen to verify the reproducibility of responses under these conditions, as these strips would again be exposed to halothane. Preparations were washed three times and equilibrated for 30 min before conducting the pharmacological studies on muscle strips exhibiting <1 g contractures to halothane on initial exposure. The preparation and stimulation of the muscle strips (1.5–2.5 cm (length) by 2–4 mm (width) by 2–4 mm (thickness)) was the same as previously described.[4] Muscle strips were mounted at an initial resting tension of 2 g and stimulated with supramaximal pulses at 0.2 Hz in a 5 ml tissue bath containing Krebs solution at 37° C bubbled with O_2:CO_2 (95:5). Preparations were equilibrated for a minimum of 30 min following diagnostic testing and before beginning drug additions. When modified Krebs solution containing Sr^{++} (2.5 mM) in place of Ca^{++} (2.5 mM) was used, preparations were washed at least three times and equilibrated for 30 min in this medium before testing. Following the equilibration period verapamil, nifedipine, or mepacrine was added and remained in the bath throughout the remainder of the test. These agents were all dissolved immediately before use. Control preparations were exposed to the same volume of solvent as the experimental group, either 5 μl ethanol (nifedipine and verapamil studies), or 100 μl distilled water (mepacrine studies). The studies with nifedipine were done with the overhead lights turned off and the organ baths and stock solutions wrapped in aluminum foil, as this agent is very light-sensitive in solution. Five minutes after verapamil, nifedipine, or mepacrine addition to the bath the preparations were challenged with halothane three per cent, succinylcholine (50 mM), or caffeine (8 mM). Halothane at three per cent in O_2:CO_2 (95:5), when used, was bubbled through the chamber. The halothane concentration in the gas phase was confirmed by gas chromatography. Succinylcholine was dissolved in the appropriate bathing medium (Ca^{++} free, or containing Ca^{++} or Sr^{++}) and injected into the bath to a final concentration of 50 mM. For the succinylcholine and halothane interaction studies the first agent (either halothane or succinylcholine) was added to the bath. After five minutes, the other agent (succinylcholine or halothane) was added to the bath and the maximum increase in baseline tension (contracture) recorded during a second five-minute period. This response to the second agent added (halothane or succinylcholine) was used for the values in Table II. Due to the presence of both agents in the bath, the contractures during the pharmacological studies may be larger than those in diagnostic testing.[4] The results from MH-susceptible and -nonsusceptible patients (about half were from each population) were pooled, as there were no qualitative differences in the effects of agents or altered bathing media that could be attributed to diagnostic outcome, in agreement with previous studies with ketamine.[15]

TABLE I Effects of various agents and conditions on caffeine 8 mM-induced contractures in human skeletal muscle

Experimental conditions	*n*	*Control (In Ca^{2+} medium)*[a]	*Experimental condition*[a]
		Contracture in g (mean ± SEM)	
Ca^{2+}-free medium	4	2.1 ± 0.3	0.9 ± 0.3†
Strontium (2.5 mM)	11	2.7 ± 0.5	2.4 ± 0.5
Verapamil (10 μM)	3	1.6 ± 0.1	1.3 ± 0.4
Nifedipine (10 μM)	4	2.1 ± 0.2	2.9 ± 1.0
Mepacrine (10 μM)	5	2.7 ± 0.6	1.8 ± 0.6*

*Less than control ($P < 0.05$).

†Less than control ($P < 0.01$).

[a]For each medium or drug tested one muscle strip (control) was exposed to caffeine in a Ca^{2+}-containing medium. A second muscle strip from the same biopsy specimen was bathed in: (1) a Ca^{2+}-free; (2) a Sr^{2+}-containing medium, or (3) a Ca^{2+}-containing medium containing verapamil, nifedipine, or mepacrine and was then exposed to caffeine.

TABLE II Effect of a Ca^{2+}-free or a Sr^{2+}-containing bathing medium, or a Ca^{2+}-containing medium with organic Ca^{2+} antagonists on halothane- and succinylcholine-induced contractures in human muscle fibre bundles

Experimental conditions	*n*	*Control*[a]	*Experimental condition*[a]
		Contracture in g (mean ± SEM)[b]	
Ca^{2+}-free Medium			
Halothane 3%	6	0.5 ± 0.1	0.1 ± 0.0‡
Succinylcholine 50 mM	4	0.7 ± 0.1	0.0 ± 0.0*
Strontium (2.5 mM)			
Halothane 3%	11	1.0 ± 0.2	0.5 ± 0.1†
Succinylcholine 50 mM	13	0.9 ± 0.1	0.3 ± 0.1†
Verapamil (10 μM)			
Halothane 3%	4	2.0 ± 0.5	1.0 ± 0.4†
Succinylcholine 50 mM	4	0.6 ± 0.2	1.0 ± 0.7
Nifedipine (10 μM)			
Halothane 3%	4	1.1 ± 0.3	1.1 ± 0.6
Succinylcholine 50 mM	4	1.0 ± 0.2	0.9 ± 0.2

Less than control (*$P < 0.05$; †$P < 0.01$; ‡$P < 0.001$).
[a]For each medium or drug tested one muscle strip (control) was exposed to halothane and succinylcholine in a Ca^{2+}-containing medium. A second muscle strip from the same biopsy specimen was bathed in: (1) a Ca^{2+}-free; (2) a Sr^{2+}-containing medium, or; (3) a Ca^{2+}-containing medium containing verapamil or nifedipine and was then exposed to halothane and succinylcholine.
[b]The values indicate the maximum contracture within five minutes of adding the second agent.

Data analysis

A two-tailed paired t test was used for statistical comparisons of the experimental and control strips from each biopsy specimen.

Results

The caffeine-induced contractures were antagonized by about 50 per cent in a Ca^{++}-free medium (Table I). In contrast to caffeine contractures, halothane and succinylcholine contractures were almost completely blocked when Ca^{++} was excluded from the bathing medium (Table II).

When Sr^{++} was added to the Ca^{++}-free medium caffeine-induced contractures were completely supported (Table I). Adding Sr^{++} to a Ca^{++}-free medium resulted in about 50 per cent of the contracture response to halothane and 30 per cent of the response to succinylcholine (Table II) as observed in normal Krebs solution.

At a concentration of 10 μM, the organic calcium channel antagonists verapamil and nifedipine had no effect on caffeine contractures (Table I). Likewise, succinylcholine contractures induced in preparations from humans were also not affected by verapamil or nifedipine (Table II). However, halothane contractures in the presence of succinylcholine were antagonized by verapamil, but not by nifedipine in human preparations (Table II).

Mepacrine has previously been shown to antagonize contractures to halothane and succinylcholine in preparations from humans.[14] In the present study mepacrine antagonized caffeine-induced contractures in human skeletal muscle (Table I).

Discussion

The present study investigated the role of extracellular Ca^{++} in contracture induction in skeletal muscle by agents associated with MH, including halothane, succinylcholine and caffeine. When the divalent cation Sr^{++}, which has properties similar to Ca^{++}, was added to the bathing medium, contractures were larger than those in a Ca^{++}-free medium. Contractures in a Sr^{++}-containing medium were about 100, 50 and 30 per cent of the maximum contractures to caffeine, halothane and succinylcholine, respectively. Therefore, Ca^{++} was essential for maximal contractures to halothane and succinylcholine, with the latter agent the most dependent (70 per cent) specifically on Ca^{++}. Contractures induced by halothane were unique, as they had a total of three components: (1) a Ca^{++}- and Sr^{++}-independent component (about 20 per cent of the total contracture); (2) a general divalent cation-dependent component that was supported by either Ca^{++} or Sr^{++} (about 30 per cent of the total contracture); and, (3) a component with an absolute dependence on Ca^{++} (about 50 per cent of the total contracture).

Previously, other investigators had demonstrated that contractures to halothane[9,16] and caffeine[16] in muscle from MH-susceptible patients were dependent on Ca^{++} in the bathing medium. None of these studies examined the effects of divalent cation replacement using Sr^{++}. Sr^{++} was used in the present study to distinguish if specific Ca^{++}-requiring processes were involved by contracture induction in human muscle. The present study suggests that Sr^{++} fully substitutes for Ca^{++} in contracture induction by caffeine, and only partially substitutes for Ca^{++} in contracture induction when contractures are induced by halothane or succinylcholine.

Mepacrine (quinacrine) exhibits a species-dependent effect, as halothane contractures are antagonized by this agent in human,[14] but not rat[5] muscle. Recently we have observed that mepacrine does not antagonize halothane contractures in MH-susceptible porcine muscle (unpublished observations). Caffeine contractures are decreased ($P < 0.05$) to about 65 per cent of control by mepacrine in rat preparations (unpublished observations; $n = 10$), in agreement with the effects of mepacrine on caffeine contractures in human preparations. These results suggest

that species differences must be accounted for with each drug tested when interpreting contracture studies.

Organic calcium channel antagonists have been used by other investigators to probe the mechanisms underlying MH. Our results are in agreement with those of others[9,12] who demonstrated that verapamil and diltiazam, respectively, antagonized contractures to halothane. In contrast, the two organic calcium channel antagonists tested had no effect on caffeine contractures in human skeletal muscle. Using the rat diaphragm model previously described,[5,15] we also did not observe a significant effect of either nifedipine (10 μM; $n = 7$) or verapamil (10 μM; $n = 5$) on caffeine contractures (unpublished observations). Since in the present study verapamil was only effective in antagonizing halothane-evoked contractures, it is possible that the antagonism of halothane contractures by verapamil may be independent of actual Ca^{++} channel blockade.

Contractures induced by halothane and succinylcholine differ from those induced by caffeine in that Sr^{++} does not completely substitute for Ca^{++} in the bathing medium (suggesting an essential role of sarcolemmal Ca^{++}) for halothane and succinylcholine. Contractures to succinylcholine differ from those induced by halothane in the Ca^{++}- and Sr^{++}-independent component (absent in succinylcholine contractures) and in the antagonism of halothane contractures by verapamil. The only contracture component common to halothane, succinylcholine and caffeine contractures is that supported by Sr^{++}. The results suggest that extracellular divalent cations play an essential, yet complex, role in contracture induction by these agents.

References

1 *Britt BA*. Muscle assessment of malignant hyperthermia susceptible patients. *In*: Britt BA (Ed.). Malignant Hyperthermia. Boston: Martinus Nijhoff Publishing, 1987: 193–267.

2 *Fletcher JE, Rosenberg H*. Laboratory methods for malignant hyperthermia diagnosis. *In*: Williams CH (Ed.). Experimental Malignant Hyperthermia, Berlin: Springer-Verlag, 1988: 121-40.

3 *Ørding H*. Incidence of malignant hyperthermia in Denmark. Anesth Analg 1985; 64: 700–4.

4 *Fletcher JE, Rosenberg H. In vitro* interaction between halothane and succinylcholine in human skeletal muscle: implications for malignant hyperthermia and masseter muscle rigidity. Anesthesiology 1985; 63: 190–4.

5 *Fletcher JE, Rosenberg H. In vitro* muscle contractures induced by halothane and suxamethonium: I. The rat diaphragm. Br J Anaesth 1986; 58: 1427–32.

6 *Iaizzo PA, Klein W, Lehmann-Horn F*. Fura-2 detected myoplasmic calcium and its correlation with contracture force in skeletal muscle from normal and malignant hyperthermia susceptible pigs. Pflugers Arch 1988; 411: 648–53.

7 *Nelson TE*. Abnormality in calcium release from skeletal sarcoplasmic reticulum of pigs susceptible to malignant hyperthermia. J Clin Invest 1983; 72: 862–70.

8 *Ohnishi ST, Waring AJ, Fang SRG et al*. Abnormal membrane properties of the sarcoplasmic reticulum of pigs susceptible to malignant hyperthermia: modes of action of halothane, caffeine, dantrolene, and two other drugs. Arch Biochem Biophys 1986; 247: 294–301.

9 *Gruener R, Blanck T*. Volatile anesthetics and skeletal muscle: evidence for sarcolemmal involvement in malignant hyperthermia. *In*: Fink BR (Ed.). Molecular Mechanisms of Anesthesia, New York: Raven Press, 1980: 423–7.

10 *Cognard C, Raymond G*. The strontium-induced calcium release process and its implication in contractility of skeletal muscle of *Rana ridibunda*. Proc R Soc Lond B 1985; 224: 489–509.

11 *Frank GB*. Utilization of bound calcium in the action of caffeine and certain multivalent cations on skeletal muscle. J Physiol 1962; 163: 254–68.

12 *Ilias WK, Williams CH, Fulfer RT, Dozier SE*. Diltiazam inhibits halothane-induced contractions in malignant hyperthermia-susceptible muscles *in vitro*. Br J Anaesth 1985; 57: 994–6.

13 *Deuster PA, Bockman EL, Biscardi H, Muldoon SM*. Verapamil and zero Ca^{2+} alter responses of cat muscle to halothane and caffeine. J Appl Physiol 1986; 60: 935–41.

14 *Fletcher JE, Rosenberg H. In vitro* muscle contractures induced by halothane and suxamethonium: II. Human skeletal muscle from normal and malignant hyperthermia susceptible patients. Br J Anaesth 1986; 58: 1433–9.

15 *Fletcher JE, Rosenberg H, Lizzo FH*. Effects of droperidol, haloperidol and ketamine on halothane, succinylcholine and caffeine contractures: implications for malignant hyperthermia. Acta Anaesthesiol Scand 1989; 33: 187–92.

16 *Nelson TE, Bedell DM, Jones EW*. Porcine malignant hyperthermia: effects of temperature and extracellular calcium concentration on halothane-induced contracture of susceptible skeletal muscle. Anesthesiology 1975; 42: 301–6.