Volume 161, number 1 FEBS 0771 September 1983

Calcium-induced Ca^{2+} release from sarcoplasmic reticulum of pigs susceptible to malignant hyperthermia

The effects of halothane and dantrolene

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Received 1 June 1983

Calcium-induced calcium release and halothane-induced calcium release from pig sarcoplasmic reticulum (SR) were studied. The SR prepared from pigs susceptible to malignant hyperthermia (MH) was shown to release calcium at a much lower level of calcium content than in normal pig SR. The concentration above which halothane can release calcium is 40 μM for both MH-SR and normal SR, although the latter required a high calcium content to demonstrate the calcium release. Dantrolene was shown to inhibit the halothane-induced calcium release. Results suggest that SR plays an important role in pathogenesis of MH.

Calcium-induced calcium release *Malignant hyperthermia* *Calcium release, from sarcoplasmic reticulum* *Halothane* *Dantrolene*

1. INTRODUCTION

It is theorized that malignant hyperthermia (MH) is triggered by abnormally high levels of ionized calcium in muscle [1]. Since the sarcoplasmic reticulum (SR) plays a key role in regulating the level of Ca^{2+} in the cytoplasm of muscle, many studies have been done to determine the effects of halothane and other drugs on SR. However, previous reports of the effects of halothane on calcium fluxes across isolated SR vesicular membranes are inconsistent (for an extensive list of references, see [2]). Because the results are so divergent, no clear-cut conclusion has been reached as to whether or not SR is really playing a role in triggering MH. Confusion in this area of study appears to result from several reasons; each investigator uses SR prepared by different methods and measures calcium fluxes with different techniques under different conditions. In addition, the most physiological method to release calcium from isolated SR has not been established. Furthermore, there is no clear evidence to prove that halothane (which triggers MH) and dantrolene (which inhibits the symptom of MH) regulate the calcium fluxes of isolated SR in vitro [2]. In fact, it is reported that dantrolene does not inhibit either calcium-induced or membrane potential-induced calcium release from vesicles of isolated SR of rabbits [3].

Since the contraction of skeletal muscle is caused by Ca^{2+} release from the SR, it seemed important to compare the effects of halothane and dantrolene on the Ca^{2+} release of SR preparations isolated from both normal and MH porcine muscles.

Although calcium-induced Ca^{2+} release is probably not the primary mechanism by which normal excitation–contraction coupling is initiated in vertebrate skeletal muscle, the presence of caffeine or other agents that enhance contractions might release additional calcium by this mechanism [7,13]. Therefore, we have used the calcium-

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00145793/83/$3.00

induced calcium release phenomenon, which can be observed in isolated SR at low Mg^{2+} concentrations [4–6] as a model to study the effects of halothane and dantrolene on Ca^{2+} release from vesicles of porcine SR. We compared the Ca^{2+}-induced Ca^{2+} release and halothane-induced Ca^{2+} release from isolated SR of genetically susceptible MH-pigs and their littermates. The results suggest that SR from normal and MH-pigs are different, and that SR may play an important role in the pathophysiology of MH.

2. MATERIALS AND METHODS

Three genetically susceptible MH-pigs and 3 littermate normal pigs (Pietrain: 20 to 50 kg) were used. Under thiopental anesthesia, we ventilated the animals and maintained arterial CO_2 tension at 40 mm Hg with a Harvard pump connected to an endotracheal tube. A mixture of 50% NO_2–50% O_2 was used for ventilation while the semitendinosus muscle was removed surgically. The muscle was subsequently used for preparing isolated SR which began immediately after excision of the muscle. Heavy fraction of SR was prepared by a differential centrifugation method [8] with the following modifications: muscle weighing 100 g was homogenized in a Waring blender with 400 ml solution containing 20 mM MES buffer (2-(*N*-morpholino)ethanesulfonate; pH 6.8); and 2.5 mM NaOH for 180 s (30 s × 6). The pH of the homogenate was adjusted to 6.8 (using BTB paper) by the addition of a small amount of 1 M NaOH. The fraction sedimented between 10000 × *g* for 3 min and 17000 × *g* for 25 min was collected and wash-centrifuged once with a buffer solution containing 150 mM KCl and 20 mM MES (pH 6.8). The pellets were resuspended in the same buffer and used for the experiment. The protein concentration was determined by the Biuret method.

Calcium-induced Ca^{2+} release experiments were performed at 25°C as in [4,5] using arsenazo III as an indicator [9]. The ordinate of figures in this paper represents total [Ca^{2+}] in the SR suspension as measured by the indicator. Thymol-free halothane was prepared by distilling commercial halothane solution. This was dissolved in ice-cold water to make a final concentration of 10 mM. The solution was made daily. This solution was added directly to the suspension of SR in the cuvette. To the SR suspension (containing 0.5 mg/ml of protein, 10 mM $MgCl_2$ and 100 μM Ca^{2+}), 100 μM ATP was added. From the recorder trace of Ca^{2+} uptake and release [10], the maximum amount of Ca^{2+} sequestered by the SR was measured and used as an index of Ca^{2+}-uptake capacity of SR. The amount of endogenous Ca^{2+} content of the SR was determined from a separate experiment whereby 10 μM A23187 was added to the SR suspension and the amount of Ca^{2+} release was measured.

3. RESULTS

Fig.1 demonstrates ATP-induced Ca^{2+} uptake and subsequent Ca^{2+} release induced by halothane

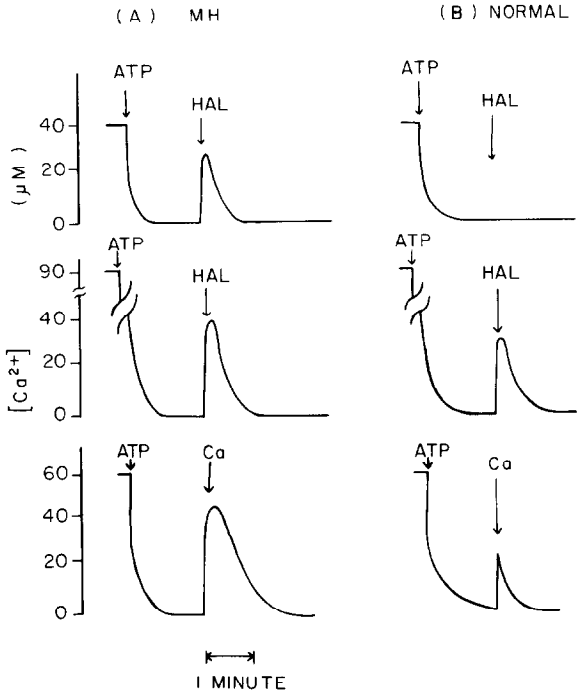

Fig.1. Halothane-induced Ca^{2+} release and Ca^{2+}-induced Ca^{2+} release from SR prepared from (A) MH muscle and (B) normal muscle. Top and middle traces demonstrate the halothane effects and bottom traces Ca^{2+}-induced Ca^{2+} release. Ca^{2+} concentrations were 40 μM (top), 90 μM (middle) and 60 μM (bottom). Additions of 0.5 mM ATP, 200 μM halothane and 20 μM Ca^{2+} were made at positions indicated by arrows. Experimental conditions: 150 mM KCl, 20 mM MES buffer (pH 6.8), 0.5 mM $MgCl_2$, 1.5 mg SR protein/ml, 9 μM arsenazo III, at 25°C.

(200 μM) or 20 μM Ca^{2+}. The concentrations of Ca^{2+} added to the SR suspension prior to the addition of ATP were 40 μM (top traces), 90 μM (middle traces) and 60 μM (bottom traces). When ATP is added to the SR suspension, Ca^{2+} in the suspension is taken up by the SR (Ca^{2+}-loading). When [Ca^{2+}] reaches a near-zero level, 200 μM halothane (top and middle traces) or 20 μM Ca^{2+} (bottom traces) are added to examine whether Ca^{2+} is released from inside the SR [4,5]. As shown, the SR prepared from normal pig (right-side traces) did not release Ca^{2+} by halothane at the Ca^{2+}-loading of 40 μM (for 1.5 mg/ml SR protein). It requires a Ca^{2+}-loading of 90 μM to respond to the halothane addition. On the contrary, with SR prepared from susceptible pig (left-side traces), halothane released Ca^{2+} even at the Ca^{2+}-loading of 40 μM. The same phenomenon was observed with the Ca^{2+}-induced Ca^{2+} release. At the Ca^{2+}-loading level of 60 μM, MH–SR released 24 μM Ca^{2+} from inside on addition of 20 μM Ca^{2+} to the SR suspension (bottom left). At the same condition, normal SR did not release any Ca^{2+} from inside; added Ca^{2+} (20 μM) was simply sequestered by the SR.

Fig.2 shows the relationship between the level of Ca^{2+}-loading and the amount of Ca^{2+} release upon the addition of 200 μM halothane. There is a threshold value, below which halothane was unable to release Ca^{2+} from the SR. Above the threshold value, the amount of Ca^{2+} release increased with the increased level of Ca^{2+}-loading, but the amount quickly reaches a plateau. By extrapolating the initial increasing portion of the curve, the threshold value could be estimated. The major difference between MH–SR and normal SR is that the threshold value for halothane-induced Ca^{2+} release of MH–SR is much less than that of normal SR. For the addition of 200 μM halothane, the threshold values of Ca^{2+}-loading for Ca^{2+} release are 13.3 ± 3.0 nmol/mg SR protein for MH–SR and 47.0 ± 6.5 for normal SR, respectively ($p < 0.001$). The Ca^{2+} uptake capacities are 155.0 ± 15.5 nmol/mg SR protein for MH–SR and 160.5 ± 12.3 for normal SR ($p > 0.2$); the endogenous Ca^{2+} contents are 52.0 ± 4.0 nmol/mg SR protein for MH–SR and 52.7 ± 6.4 for normal SR, respectively ($p > 0.2$).

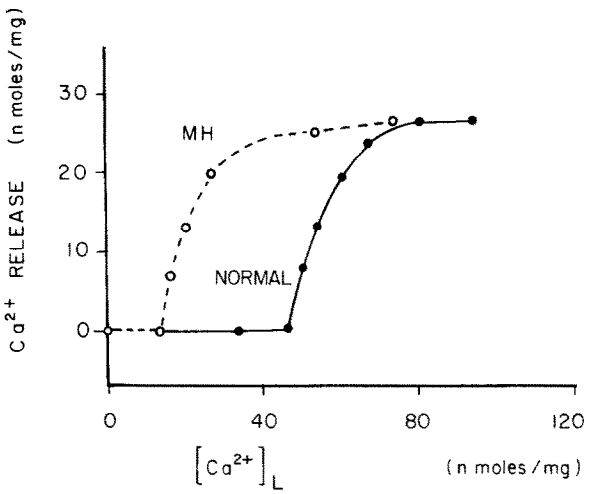

Fig.2. Relationship between the level of Ca^{2+}-loading and the amount of halothane-induced Ca_2^{+} release. Experimental conditions are the same as those in fig.1 (top or bottom traces), except for the Ca^{2+} concentration which was varied. The halothane concentration used to induce the Ca^{2+} release was 200 μM.

Fig.3 indicates dose–response relationship of halothane-induced Ca^{2+} release. At the Ca^{2+}-loading level of 27 nmol/mg, MH–SR started to release calcium at the halothane concentration of 40 μM, whereas no release is observed with normal SR. When the level of Ca^{2+}-loading of normal SR is increased (e.g., 60 nmol/mg) halothane-induced

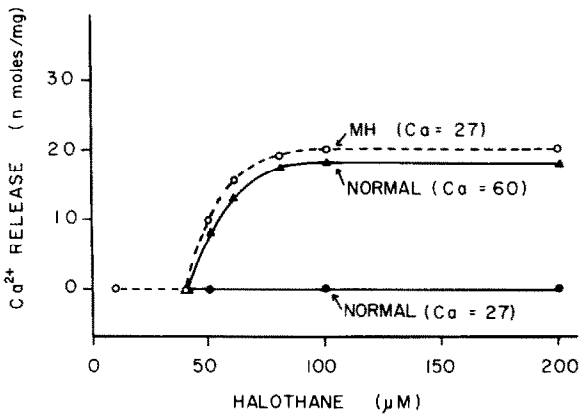

Fig.3. The dose–response relationship of halothane-induced Ca^{2+} release. The levels of Ca^{2+}-loading (nmol/mg) are indicated in the figure. The experimental conditions are the same as those in fig.1, except for the halothane concentration which was varied.

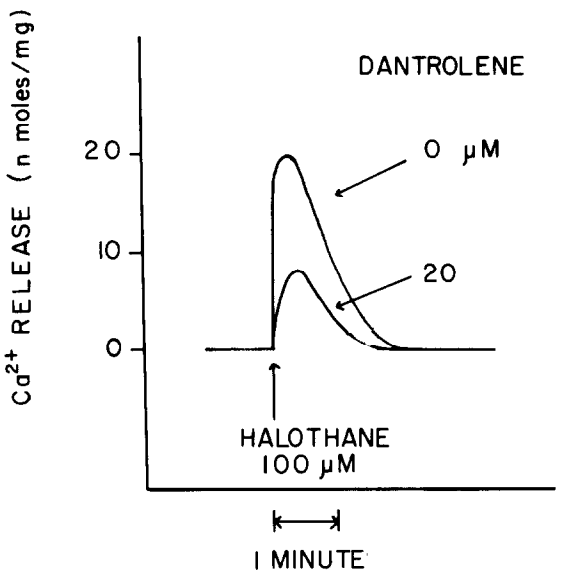

Fig.4. Inhibition of halothane-induced Ca^{2+} release by dantrolene. Concentration of halothane: 100 μM. Concentrations of dantrolene: 0–40 μM. Other experimental conditions: 150 mM KCl, 20 mM MES (pH 6.8), 2 mM $MgCl_2$, 1.5 mg SR protein/ml, 9 μM arsenazo III, 40 μM $CaCl_2$ and 1 mM ATP.

Ca^{2+} release was initiated again at 40 μM halothane.

Fig.4 demonstrates the effect of dantrolene on the halothane-induced Ca^{2+} release. Both the velocity and the amount of Ca^{2+} release were inhibited by dantrolene.

4. DISCUSSION

At low Mg^{2+} concentrations (e.g., 0.5 mM), striated muscle demonstrates the phenomenon called calcium-induced Ca^{2+} release [11,12]. This phenomenon has been extensively studied in skinned muscle fibers of skeletal muscle [13] and cardiac muscle [14]. This concentration of Mg^{2+} is not a physiological one.

However, since a reproducible calcium-induced Ca^{2+} release and halothane-induced Ca^{2+} release from isolated SR is observed with this condition, we have been using this as an in vitro model for Ca^{2+} release [4,5].

It has been proposed that the mechanisms of halothane-induced and calcium-induced Ca^{2+} release involve the same channel [5]. These release phenomena appear to be transient. The fact that released Ca^{2+} is sequestered again by the SR suggests that the channel closes shortly after it opens. However, we cannot rule out the possibility that the halothane-induced Ca^{2+} release occurs in a subpopulation of SR and the released Ca^{2+} is then reaccumulated by another (halothane-insensitive) subpopulation.

Here, the halothane-induced and Ca^{2+}-induced Ca^{2+} release phenomena of SR preparations isolated from normal pigs and MH pigs are studied. All pigs used here were examined via biopsy and halothane exposure to determine that muscle was susceptible to halothane. The control pigs were littermates with a normal response to anesthesia. We observed that both the Ca^{2+} uptake capacity and the endogenous Ca^{2+} content of MH–SR and normal SR preparations were the same. The difference between MH and normal SR seemed to be the level of Ca^{2+}-loading at which halothane started inducing Ca^{2+} release. Halothane released Ca^{2+} from MH–SR at a low level of loading (13.3 nmol/mg compared to 47 nmol/mg for normal SR). If the level of Ca^{2+}-loading was less than 47 nmol/mg SR protein, Ca^{2+} release was not observed with normal SR by halothane triggering. According to [13], the level of Ca^{2+} in SR in vivo is less than 0.25 of its full capacity. It is, therefore, tempting to speculate that the level of Ca^{2+} in SR of normal pigs is such that halothane does not trigger Ca^{2+} release, whereas at the same level of Ca^{2+}, halothane induces the Ca^{2+} release in MH–SR.

At an elevated level of Ca^{2+}-loading, normal SR also released Ca^{2+} by halothane; the maximum amount of the release was the same as that from MH–SR. It is interesting to note that the threshold concentration of halothane required to trigger the release was also the same: 40 μM for both MH and normal SR. (The threshold concentration of halothane for Ca^{2+} release from rabbit skeletal SR is also 40 μM [5]. At room temperature, 40 μM halothane in water is in equilibrium with about 0.08% halothane vapor (v/v) in the air [15], which is well under the anesthetic concentration (0.3–1%).) This suggests that the affinity of halothane receptors on the putative Ca^{2+} channel [5] of both MH and normal SR is the same. A possible difference between MH and normal SR may be that the permeability of the Ca^{2+} channel

of MH–SR is greater than that of normal SR. This would allow the Ca^{2+}-efflux in MH–SR to start at a lower level of Ca^{2+}-loading than in normal SR. A phenomenon of regulation of Ca^{2+} by the internal Ca^{2+} concentration has been studied in rabbit SR [16,17]. An alternative explanation for this difference is that MH–SR is more labile than control SR so that MH–SR is damaged during the preparation procedure. Further study is needed to clarify this point.

Dantrolene has been used as a drug to effectively manage the MH symptom in both man and pig [1]. The drug was shown to slow down the spontaneous release of Ca^{2+} from the isolated SR [18]. We have obtained more direct evidence to show that dantrolene inhibits the halothane-induced Ca^{2+} release from the isolated SR (fig.4). Further support for the idea that dantrolene suppresses Ca^{2+} release was shown in isolated intact skeletal muscle fibers injected with Aequorin and inactivated via membrane depolarization [19].

Since SR is not the only organelle in muscle that influences the level of ionized calcium in the myoplasm, we cannot rule out the possibility that abnormalities of other organelles, or membrane systems in intact muscle may also be involved in the pathogenesis of MH [20]. However, the data presented here suggest that SR itself plays an important role in MH.

ACKNOWLEDGEMENTS

Authors would like to extend their sincere appreciation for the assistance provided by Mr Jeffrey L. Flick, Mr Jack Pyenson, Mr Joseph J. Sabadish, Dr Kazumi Horiuchi and Dr Shyh-rong G. Fang of Hahnemann University School of Medicine, and Mr James H. Milde of Mayo Medical School. Thanks are also due to Dr Thomas E. Nelson, University of Texas Medical Branch, Galveston, for his participation in the preliminary stage of this work. This work was supported in part by NIH grants GM30703, HL23788, AA04320, GM21729 and NS14268.

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