Biochimica et Biophysica Acta, 1064 (1991) 175–183

ADONIS 000527369100184X

BBAMEM 75232

Abnormal rapid Ca^{2+} release from sarcoplasmic reticulum of malignant hyperthermia susceptible pigs

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(Received 13 December 1990)

Key words: Calcium ion release; Caffeine; Halothane; Malignant hyperthermia; Sarcoplasmic reticulum

Using the rapid filtration technique to investigate Ca^{2+} movements across the sarcoplasmic reticulum (SR) membrane, we compare the initial phases of Ca^{2+} release and Ca^{2+} uptake in malignant hyperthermia susceptible (MHS) and normal (N) pig SR vesicles. Ca^{2+} release is measured from passively loaded SR vesicles. MHS SR vesicles present a 2-fold increase in the initial rate of calcium release induced by 0.3 μM Ca^{2+} (20.1 ± 2.1 vs. 6.3 ± 2.6 nmol mg^{-1} s^{-1}). Maximal Ca^{2+} release is obtained with 3 μM Ca^{2+}. At this optimal concentration, rate of Ca^{2+} efflux in absence of ATP is 55 and 25 nmol mg^{-1} s^{-1} for MHS and N SR, respectively. Ca^{2+}-induced Ca^{2+} release is inhibited by Mg^{2+} in a dose-dependent manner for both MHS and N pig SR vesicles ($K_{1/2}$ = 0.2 mM). Caffeine (5 mM) and halothane (0.01% v/v) increase the Ca^{2+} sensitivity of Ca^{2+}-induced Ca^{2+} release. ATP (5 mM) strongly enhances the rate of Ca^{2+} efflux (to about 20–40-fold in both MHS and N pig SR vesicles). Furthermore, both types of vesicles do not differ in their high-affinity site for ryanodine (K_d = 12 nM and B_{max} = 6 pmol/mg), lipid content, ATPase activity and initial rate of Ca^{2+} uptake (0.948 ± 0.034 vs. 0.835 ± 0.130 μmol mg^{-1} min^{-1} for MHS and N SR, respectively). Our results show that MH syndrome is associated to a higher rate of Ca^{2+} release in the earliest phase of the calcium efflux.

Introduction

Malignant hyperthermia (MH) is a muscular disease of genetically predisposed human and pig. This disease leads to a catastrophic crisis under anesthesia with certain agents such as halothane (for review, see Ref. 1). MH involves abnormal intracellular Ca^{2+} movements in skeletal muscles. An increase in the myoplasmic Ca^{2+} concentration has been measured with a specific electrode [2] though disputed by the measurements using the calcium indicator fura-2 [3]. It has been suspected that the primary defect is located in the sarcoplasmic reticulum (SR) which releases and accumulates Ca^{2+} during the contraction-relaxation cycle [4–11].

Studies comparing Ca^{2+} accumulation by the SR Ca^{2+}-ATPase from malignant hyperthermia susceptible (MHS) and normal (N) skeletal muscles are controversial (for review, see Ref. 12): although some groups found a diminution of the Ca^{2+} uptake by MHS human [13] and MHS pig SR vesicles [14], others have reported no difference in this aspect between MHS and N human [15] and pig SR vesicles [6,8,9].

Several studies have demonstrated a defect in Ca^{2+} release from the MHS SR. Using skinned fibers from a MHS patient, Endo et al. have shown an increase of Ca^{2+}-induced Ca^{2+} release [4], and similar results have been obtained using porcine skinned fibers [16]. Spectrophotometric studies using arsenazo III [5–7] or calcein [11] as Ca^{2+} indicators have shown a greater sensitivity to Ca^{2+}, caffeine, or halothane for Ca^{2+} release from MHS pig SR. Using filtration technique, Mickelson et al. have reported an increase of the rate and the amplitude of Ca^{2+} release induced by Ca^{2+} in MHS pig SR vesicles [9,10]. One report using stopped-flow method indicated higher initial rates of Ca^{2+} release induced by Ca^{2+}, halothane, or by membrane depolarization in MHS pig SR vesicles [8]. However, they studied Ca^{2+} release from actively loaded SR

Abbreviations: EGTA, ethyleneglycol bis(β-aminoethyl ether)tetraacetic acid; MH, malignant hyperthermia; MHS, malignant hyperthermia susceptible; Mops, 4-morpholinepropanesulfonic acid; N, normal; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene; Pipes, 1,4-piperazinediethanesulfonic acid; PPO, 2,5-diphenyloxazole; SDS, sodium dodecyl sulfate; SR, sarcoplasmic reticulum.

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vesicles and they were thus unable to separate the release from the uptake mechanism.

The aim of this study is to investigate whether the defect in MHS pig SR results from an abnormality in the Ca^{2+} release, in the Ca^{2+} uptake or both. We used the rapid filtration technique to study the initial rates of Ca^{2+} release and Ca^{2+} uptake [17,18]. This technique permits to measure these processes separately, and also permits to change instantaneously a medium by an other. Ca^{2+} release was determined from passively loaded SR vesicles without interferences from the process of Ca^{2+} uptake [17]. There was no interaction between release and uptake because of the absence of ATP in the loading solution. We investigated the effects of Mg^{2+}, caffeine, halothane and ATP on Ca^{2+}-induced Ca^{2+} release from MHS and N pig SR vesicles.

Although the initial rate of Ca^{2+} uptake by MHS and N SR vesicles appeared similar, the rate of rapid Ca^{2+} efflux induced by Ca^{2+} was larger in the MHS SR vesicles. Ca^{2+}-induced Ca^{2+} release was inhibited by Mg^{2+} and potentiated by caffeine, halothane and ATP. The effects of these agents were similar for MHS and N SR vesicles. Our results suggest that caffeine and halothane increase the affinity of Ca^{2+} for its binding site. In contrast, ATP did not increase the Ca^{2+} affinity of the Ca^{2+} binding site but the effects of Ca^{2+} and ATP were added to produce a strong release of Ca^{2+} from the SR vesicles.

Our results suggest that the disorder of the Ca^{2+} release mechanism in MHS SR vesicles is due to an abnormal regulation of the SR calcium channel affecting primarily the earliest phase of calcium efflux.

Materials and Methods

Materials. Calcium-45 was obtained from Amersham. [^{3}H]Ryanodine was obtained from New England Nuclear and unlabeled ryanodine from Calbiochem. Caffeine, Mops and Pipes were obtained from Sigma Chemical Co., and ATP-Na_2 from Boehringer (Mannheim, F.R.G.). EGTA, KCl, KOH, NaCl, $MgCl_2$, and $CaCl_2$ were from Prolabo (Paris, France). Halothane was obtained from Aldrich (Strasbourgh, France). Halothane concentration was expressed at the percent volume of the reaction mixture. Halothane 0.01% (v/v in solution) used in this study would be 1 mM if all molecules would remain in solution. According to the solubility coefficient (water-air) of 1.35 at 20 °C [19], 1 mM halothane in water is in equilibrium with 1.91% (v/v) in air at 20 °C [20]. Halothane was introduced at the very last moment with an Hamilton syringe directly into the final solution, and immediately enclosed in the cylinder of the rapid filtration system without contact with air.

Animals. Five Pietrain MHS pigs, and four Large White N pigs were obtained from the Institut National de la Recherche Agronomique (Theix, Clermont-Ferrand, France). Their MH susceptibility was assessed by halothane challenge according to the procedure described by Ref. 21.

Preparation of sarcoplasmic reticulum vesicles. Within 5 min after the death of the animal, longissimus dorsi muscles were removed. SR vesicles were prepared from these skeletal muscles by the technique of Ref. 22 as modified by Ref. 23. The vesicles (about 20 mg protein/ml) were suspended in 20 mM Mops (3-(*N*-morpholino)propane sulfonic acid), 100 mM KCl, and 300 mM sucrose (10% w/w), at pH 6.8. Samples (0.2–0.5 ml) were rapidly frozen and stored in liquid nitrogen until used. Protein concentration was determined spectrophotometrically by the absorbance at 280 nm in the presence of 1% SDS [24]. Cholesterol and phospholipids contents were determined as described previously [25,26].

ATPase activity measurements. ATPase activity was followed by monitoring with a pH-stat the release of H^+ due to the clivage of ATP [23]. The measurements were made under a continuous flow of argon at room temperature in 3 ml of a solution containing 50 μM $CaCl_2$, 5 mM $MgCl_2$, 5 mM $ATPNa_2$, 2 mM Mops, 60 mM KCl (pH 7.2). About 150 μg of SR vesicles preincubated with 1% A23187 (w/w of the SR) were added to the medium. Titration was made with 10 mM KOH.

Ca^{2+}-uptake measurements. Ca^{2+} uptake was measured using the rapid filtration system (Bio-Logic, Echirolles, France) [27]. This system permits to measure ionic flux down to very short times, from 10 to 20 ms in the best cases.

The SR vesicles (0.2 mg/ml) were first incubated in a solution containing 50 μM $^{45}CaCl_2$ (0.4 μCi/ml), 5 mM $MgCl_2$, 20 mM sucrose, 20 mM Mops, 100 mM KCl (pH 7.0). 190 μg of SR vesicles were then deposited on a Millipore filter (0.65 μm), and the loading solution was passed through the filter for various time. Loading solution contained 50 μM $CaCl_2$, 10 mM $MgCl_2$, 5 mM $ATPNa_2$ (5 mM free Mg^{2+}, 5 mM MgATP), 20 mM Mops, 100 mM KCl (pH 7.0). Filtration time varied from 0.1 and 3 s. The radioactivity of the Ca^{2+} remaining on the filter was determined with a SL 3000 scintillation counter, after dissolution of the filter in 10 ml of a mixture composed of dioxan/naphthalene/POPOP/PPO/water. Background was obtained by the same way but in the absence of ATP.

[^{3}H]Ryanodine binding assay. SR vesicles (50 μg/ml) were incubated at 37 °C, 2 h (i.e. long enough to reach equilibrium), with 5 nM [^{3}H]ryanodine (spec. act. 60 Ci/mmol) and various concentrations of unlabeled ryanodine to have the final ryanodine concentrations desired in a solution containing 50 μM $CaCl_2$, 0.5 M NaCl, 10 mM Pipes (pH 7.4) [28]. Samples (40 μg of SR) were filtered onto Whatman GF/C filters. Filters were then washed with 15 ml of a solution containing

50 μM $CaCl_2$, 0.5 M NaCl, and 10 mM Pipes (pH 7.4). The amount of the [^{3}H]ryanodine retained on the filters was determined using SL 3000 scintillation counter. Non specific binding of ryanodine was measured in the presence of 10 μM of unlabeled ryanodine.

Ca^{2+} release measurements. Ca^{2+} release was measured from passively loaded SR vesicles using the rapid filtration system as described in Ref. 17. Vesicles (1 mg protein/ml) were passively loaded by incubation for a minimum of 1 h at room temperature (22°C) in a solution containing 5 mM $^{45}CaCl_2$ (5 μCi/ml), 21 mM sucrose, 20 mM Mops, 100 mM KCl, at pH 6.8 (Solution A). The passively loaded vesicles were then 40-fold diluted in the solution A without $^{45}CaCl_2$. Then, 1.9 ml of the diluted vesicles (50 μg of protein) were deposited on a Millipore filter (0.65 μm). This filter was then washed with 5 ml of the rinsing medium (solution A without $^{45}CaCl_2$) to eliminate non specific Ca^{2+} binding. 25 s after vesicles dilution, Ca^{2+} release was induced by passing a solution through the filter for different time using the rapid filtration system. The free Ca^{2+} concentration of the various solutions between pCa 9 and pCa 3 was established with 2 mM EGTA (pH 6.8), and calculated using the dissociation constants given by Vianna [29], although pCa 2.3 corresponds actually to the rinsing medium. Filtration time varied from 20 ms and 10 s. The radioactivity of the Ca^{2+} remaining on the filter was then determined as described above.

SR vesicles from MHS and N pig muscles did not differ in their passive Ca^{2+}-loading ability: under our passive loading conditions, we obtained after 1 hour loading levels of 50 to 70 nmol Ca^{2+} per mg protein for both MHS and N pig SR vesicles. This Ca^{2+} loading level remained stable throughout the experiment which lasts 2 or 3 h.

Statistical analysis. All comparisons of mean values of MHS and N populations were analyzed by a Student's *t*-test ($P < 0.1$).

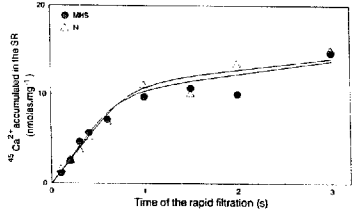

Fig. 1. Time course of active Ca^{2+} uptake. 0.2 mg/ml of SR vesicles were incubated in a solution containing 50 μM $^{45}CaCl_2$, 5 mM $MgCl_2$, 85 mM sucrose, 20 mM Mops, 100 mM KCl (pH 7.0) and then deposited (190 μg) on a 0.65 μm Millipore filter. The loading solution containing 50 μM $CaCl_2$, 10 mM $MgCl_2$, 5 mM $ATPNa_2$, 20 mM Mops, 100 mM KCl (pH 7.0) was then passed through the filter for various times (between 0.1 and 3 s) using the rapid filtration system. The $^{45}Ca^{2+}$ accumulated in the vesicles was then measured. Experiments were performed with MHS (●) and N (△) pig SR vesicles.

Results

Characterization of the SR vesicles

Three criteria were chosen for the characterization of SR vesicles: ATPase activity, Ca^{2+} uptake, and lipid contents. Table I shows the different values obtained in each case for MHS and N SR preparations. There was no significant difference between the two types of vesicles in all the parameters tested. Our values of cholesterol and phospholipids contents were consistent with those found by others on pig [8,9], and on rabbit SR vesicles [30]. Fig. 1 shows typical experiments of Ca^{2+} uptake obtained with the rapid filtration system in MHS and N pig SR vesicles. The initial rate of Ca^{2+} uptake was evaluated from the slope of the curve at origin. There was no difference between MHS and N

TABLE I

Characterization of pig MHS and N SR vesicles

The ATPase activity, Ca^{2+}-uptake and lipid content were measured as described under Materials and Methods. All values are reported as mean ± S.E. (for Student's *t*-test, $P < 0.1$). The number of pigs tested is given in parenthesis.

	N	MHS
ATPase activity		
ATP hydrolysis (μmol mg^{-1} min^{-1})	0.356 ± 0.182 ($n = 3$)	0.312 ± 0.089 ($n = 4$)
Ca^{2+}-uptake		
Ca^{2+} accumulated (μmol mg^{-1} min^{-1})	0.835 ± 0.130 ($n = 3$)	0.948 ± 0.034 ($n = 4$)
Lipids measurements		
Cholesterol (nmol mg^{-1})	60 ± 17 ($n = 4$)	88 ± 31 ($n = 4$)
Phospholipids (nmol mg^{-1})	505 ± 46 ($n = 4$)	525 ± 37 ($n = 4$)
Cholesterol/phospholipids	0.12 ± 0.03 ($n = 4$)	0.16 ± 0.06 ($n = 4$)

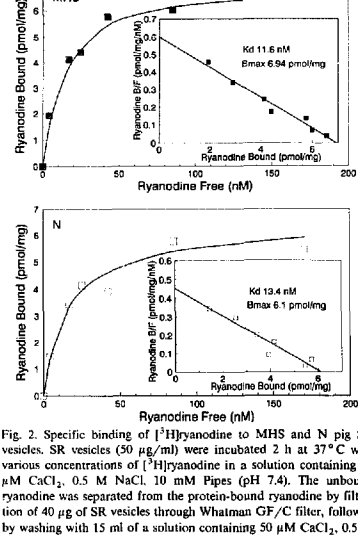

Fig. 2. Specific binding of [^{3}H]ryanodine to MHS and N pig SR vesicles. SR vesicles (50 μg/ml) were incubated 2 h at 37°C with various concentrations of [^{3}H]ryanodine in a solution containing 50 μM $CaCl_2$, 0.5 M NaCl, 10 mM Pipes (pH 7.4). The unbound ryanodine was separated from the protein-bound ryanodine by filtration of 40 μg of SR vesicles through Whatman GF/C filter, followed by washing with 15 ml of a solution containing 50 μM $CaCl_2$, 0.5 M NaCl, and 10 mM Pipes (pH 7.4). The count retained on the filters were determined using SL 3000 scintillation counter. Non specific binding of ryanodine was measured in the presence of 10 μM of unlabeled ryanodine. The inserts show Scatchard plots for specific [^{3}H]ryanodine binding.

pig SR vesicles in the initial rate of Ca^{2+} uptake, and Table I confirms this for many animals.

[^{3}H]Ryanodine binding

An example of [^{3}H]ryanodine binding to pig SR vesicles is shown in Fig. 2. Scatchard analysis of specific ryanodine binding revealed a single class of high-affinity binding sites with a K_d of 11.6 and 13.4 nM for MHS and N SR vesicles, respectively. The B_{max} was 6.94 and 6.1 pmol mg^{-1} for MHS and N SR vesicles, respectively. Table II shows the values from three different pigs reported as mean ± S.E. The difference of the ryanodine affinity for its receptor between MHS and N pig SR vesicles was not significant even if it was slightly higher for MHS vesicles (10.13 ± 3.09 vs. 15.13 ± 3.67 nM). Furthermore, there was no significant difference of the density of receptor between the two types of vesicles (6.87 ± 2.48 vs. 4.87 ± 1.82 pmol mg^{-1}). The binding of ryanodine was dependent of Ca^{2+} concentrations. No binding of [^{3}H]ryanodine was observed

TABLE II

Ryanodine binding and calcium release rate measurements

Specific [^{3}H]ryanodine binding and rapid calcium release were measured as described under Materials and Methods. All values are reported as mean ± S.E. (for Student's *t*-test. $P < 0.1$). The number of pigs tested is given in parenthesis.

	N	MHS
[^{3}H]Ryanodine binding		
K_d (nM)	15.13 ± 3.67 ($n = 3$)	10.17 ± 3.09 ($n = 3$)
B_{max} (pmol mg^{-1})	4.87 ± 1.82 ($n = 3$)	6.87 ± 2.48 ($n = 3$)
Initial rate of calcium release induced by 0.3 μM Ca^{2+} (nmol mg^{-1} s^{-1})	6.30 ± 2.59 ($n = 4$)	20.10 ± 2.09 ($n = 5$)

in the absence of Ca^{2+} even at high concentrations of ryanodine (not shown).

Ca^{2+} efflux induced by extravesicular Ca^{2+}

Fig. 3 shows the time course of a calcium release experiment as measured with the rapid filtration system, for MHS and N pig SR vesicles. The initial rate of Ca^{2+} release was calculated from the slope of the curve at the origin. 0.1 μM free Ca^{2+} (pCa 7) induced a slow Ca^{2+}

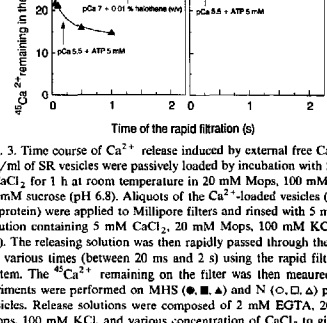

Fig. 3. Time course of Ca^{2+} release induced by external free Ca^{2+}. 1 mg/ml of SR vesicles were passively loaded by incubation with 5 mM $^{45}CaCl_2$ for 1 h at room temperature in 20 mM Mops, 100 mM KCl, 15 mM sucrose (pH 6.8). Aliquots of the Ca^{2+}-loaded vesicles (50 μg of protein) were applied to Millipore filters and rinsed with 5 ml of a solution containing 5 mM $CaCl_2$, 20 mM Mops, 100 mM KCl (pH 6.8). The releasing solution was then rapidly passed through the filter for various times (between 20 ms and 2 s) using the rapid filtration system. The $^{45}Ca^{2+}$ remaining on the filter was then measured. Experiments were performed on MHS (●, ■, ▲) and N (○, □, △) pig SR vesicles. Release solutions were composed of 2 mM EGTA, 20 mM Mops, 100 mM KCl, and various concentration of $CaCl_2$ to give the free Ca^{2+} concentration desired, at pH 6.8: (○, ●) pCa 7 (0.34 mM $CaCl_2$), (□, ■) pCa 7 + 0.01% halothane (v/v), (△, ▲) pCa 5.5 + ATP 5 mM (1.86 mM $CaCl_2$).

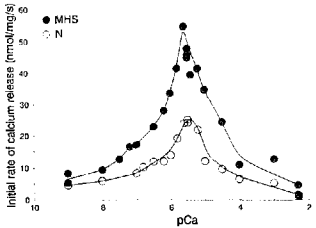

Fig. 4. Dependence of Ca^{2+} release on external free Ca^{2+}. Passive loading and Ca^{2+} release measurements were performed as described in the legend to Fig. 3, on MHS (●) and N (○) pig SR vesicles. Release solutions contained 2 mM EGTA, 20 mM Mops, 100 mM KCl, and various concentrations of $CaCl_2$ to give the indicated free Ca^{2+} concentrations, at pH 6.8.

release from both types of SR vesicles (17.2 and 8.4 nmol Ca^{2+} mg^{-1} s^{-1}, for MHS and N SR vesicles, respectively). The addition of 0.01% halothane (v/v) increased the initial rate of Ca^{2+} release (92 and 18 nmol Ca^{2+} mg^{-1} s^{-1}, for MHS and N SR vesicles, respectively). In the presence of 3 μM Ca^{2+} (pCa 5.5) and 5 mM ATP, we observed a rapid Ca^{2+} release from the SR vesicles (1000 and 700 nmol Ca^{2+} mg^{-1} s^{-1}, for MHS and N SR vesicles, respectively). In the presence of ATP, the rates of calcium release reached the physiological rates estimated by Meissner and McKinley [31]. The initial rates of Ca^{2+} release induced by the different Ca^{2+} releasing media were higher for MHS pig SR vesicles than for N SR vesicles.

The Ca^{2+} release from the SR vesicles was influenced by the extravesicular Ca^{2+} concentration as shown in Fig. 4, for one MHS and one normal (N) pig. The initial rate of Ca^{2+} efflux increased with the Ca^{2+} concentration up to 3 μM Ca^{2+} (pCa 5.5) and then decreased with higher concentrations (pCa < 5.5) for both MHS and normal (N) pig SR. MHS pig SR vesicles had about a 2-fold increase of the initial rate of Ca^{2+} efflux for each concentration of Ca^{2+} compared to N SR vesicles. Maximal rate of Ca^{2+} release were 55 and 25 nmol mg^{-1} s^{-1}, for MHS and N SR vesicles, respectively. The half-maximal activation of calcium release was obtained with about pCa 6.0 and the half-maximal inhibition was obtained with about pCa 4.6 for both MHS and N SR vesicles.

To become confident in the difference of curves revealed in Fig. 4, we have made the choice to look at 0.3 μM free Ca^{2+} (pCa 6.5) the initial rate for all the available pigs (5 MHS and 4 N). These data are shown in Fig. 4 and in Table II which show that MHS SR vesicles had a 2–3-fold increase of the initial rate of Ca^{2+} release (20.1 ± 2.1 vs. 6.3 ± 2.6 nmol mg^{-1} s^{-1} for MHS vs. N pig SR vesicles).

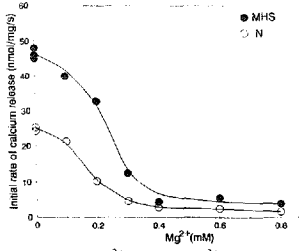

Fig. 5. Dependence of Ca^{2+} release on Mg^{2+} concentrations. Passive loading and Ca^{2+} release measurements were performed as described in the legend to Fig. 3, on MHS (●) and N (○) pig SR vesicles. Releasing solution contained 2 mM EGTA, 20 mM Mops, 100 mM KCl (pH 6.8), 3 μM free Ca^{2+} (1.735 mM $CaCl_2$), and various concentrations of $MgCl_2$.

Effect of Mg^{2+} on Ca^{2+}-induced Ca^{2+} release

Fig. 5 shows the effect of Mg^{2+} on the Ca^{2+} release induced by 3 μM Ca^{2+} from the SR vesicles isolated from one MHS and one normal pig. Mg^{2+} caused a dose-dependent decrease of the initial rate of Ca^{2+}-induced Ca^{2+} release in both MHS and N pig SR vesicles. In absence of ATP in the medium, the concentrations of free Mg^{2+} equaled the concentrations of added $MgCl_2$. The half-maximal inhibition was obtained with about 0.2 mM Mg^{2+}, for MHS and N SR vesicles, respectively. With both preparation, the maximal and total inhibition was obtained with concentrations of Mg^{2+} above 0.4 mM. This Mg^{2+}-dependence of Ca^{2+} release is consistent with previous results obtained using rabbit SR vesicles [17].

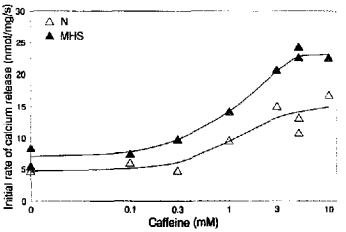

Fig. 6. Dependence of Ca^{2+} release on caffeine concentrations. Passive loading and Ca^{2+} release measurements were performed as described in the legend to Fig. 3, on MHS (▲) and N (△) pig SR vesicles. Releasing solution contained 2 mM EGTA (no added $CaCl_2$, i.e. free Ca^{2+} estimated to be less than 1 nM), 20 mM Mops, 100 mM KCl, and various concentrations of caffeine, at pH 6.8.

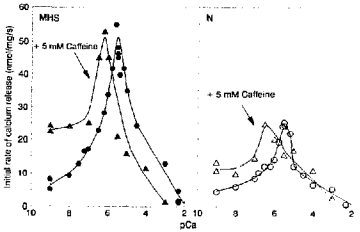

Fig. 7. Effect of 5 mM caffeine on the Ca^{2+} dependence of Ca^{2+} release. Passive loading and Ca^{2+} release measurements were performed as described in the legend to Fig. 3 on MHS (closed symbols) and N (open symbols) pig SR vesicles. Releasing solution contained 2 mM EGTA, 20 mM Mops, 100 mM KCl, various concentrations of $CaCl_2$ to give the indicated free Ca^{2+} concentrations, and 0 (○, ●) or 5 mM (△, ▲) caffeine, at pH 6.8.

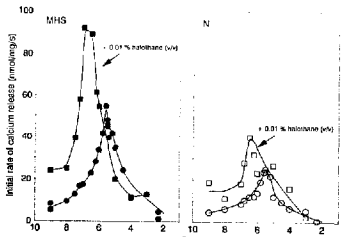

Fig. 8. Effect of 0.01% halothane (v/v) on the Ca^{2+} dependence of Ca^{2+} release. Passive loading and Ca^{2+} release measurements were performed on MHS (closed symbols) and N (open symbols) pig SR vesicles as described in the legend to Fig. 3. Releasing solution contained 2 mM EGTA, 20 mM Mops, 100 mM KCl, various concentrations of $CaCl_2$ to give the indicated free Ca^{2+} concentrations, and 0% (○, ●) or 0.01% (□, ■) halothane (v/v), at pH 6.8.

Effect of caffeine on Ca^{2+}-induced Ca^{2+} release

Caffeine concentration dependance of the initial rate of Ca^{2+} release is represented in Fig. 6. Caffeine induced a release of Ca^{2+} from both types of vesicles, and the Ca^{2+} efflux rate increased with the caffeine concentrations. The half-maximal activation was obtained with 1 mM caffeine, and V_{max} with 5 to 10 mM, for both MHS and N SR vesicles. No significant difference of caffeine sensitivity was observed between MHS and N SR vesicles. The effect of 5 mM caffeine on the Ca^{2+} dependence of Ca^{2+} release from SR vesicles is shown in Fig. 7 for one MHS and one normal pig. Caffeine increased the sensitivity to Ca^{2+} by shifting the Ca^{2+} dependence of Ca^{2+} release to lower extravesicular Ca^{2+} concentrations, so that maximal effect was obtained with about 0.3 μM Ca^{2+} (pCa 6.5) for both MHS and N SR vesicles. Furthermore, caffeine increased the initial rate of Ca^{2+} efflux in the low Ca^{2+} concentrations range (pCa 9 to 7) but did not change the maximal rate of Ca^{2+} release for both MHS and N SR vesicles. Caffeine also shifted the curve to the left so that the inhibition of the Ca^{2+} release by high Ca^{2+} was obtained with lower Ca^{2+} concentrations (pCa > 6.5 instead of pCa > 5.5).

Effect of halothane on Ca^{2+}-induced Ca^{2+} release

We have previously shown with skinned fiber experiments that 0.01% halothane (v/v in solution) produced a near maximal effect on Ca^{2+} release [32]. Fig. 8 shows the effect of 0.01% halothane (v/v) on the Ca^{2+} dependence of Ca^{2+} release from SR vesicles for one MHS and one normal pig. Like caffeine, halothane shifted the Ca^{2+} dependence of Ca^{2+} release to lower Ca^{2+} concentrations, so maximum efficiency was approximately obtained with 0.3 μM (pCa 6.5) for both types of vesicles. However, in contrast with caffeine, halothane produced an important increase in the initial rate of Ca^{2+} efflux and MHS SR vesicles were more sensitive to Ca^{2+} in the presence of halothane than N SR vesicles. The maximal rate of Ca^{2+} release was 90 and 40 nmol mg^{-1} s^{-1}, for MHS and N SR vesicles, respectively. Furthermore, halothane, like caffeine, shifted the inhibition of Ca^{2+} release by high free Ca^{2+} to lower free Ca^{2+} concentrations. Halothane increased the difference of efficiency of the Ca^{2+}-induced Ca^{2+} release existing between MHS and N SR vesicles.

Effect of ATP on Ca^{2+}-induced Ca^{2+} release

Fig. 9 shows the ATP concentration dependance of the initial rate of Ca^{2+} release induced by 3 μM Ca^{2+}. ATP accelerated the Ca^{2+}-induced Ca^{2+} release for

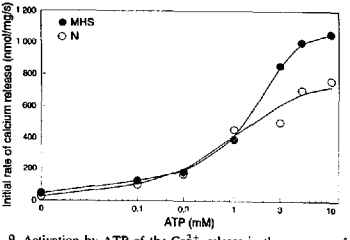

Fig. 9. Activation by ATP of the Ca^{2+} release in the presence of 3 μM free Ca^{2+}. Passive loading and Ca^{2+} release measurements were performed as described in the legend to Fig. 3. on MHS (●) and N (○) pig SR vesicles. Releasing solution contained 2 mM EGTA, 20 mM Mops, 100 mM KCl, various concentrations of $CaCl_2$ and various concentrations of $ATPNa_2$ to give 1 μM free Ca^{2+}, at pH 6.8.

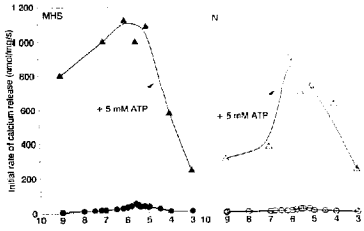

Fig. 10. Effect of 5 mM ATP on the Ca^{2+} dependence of Ca^{2+}-release. Experiments were performed on MHS (closed symbols) and N (opened symbols) pig SR vesicles. Experiments were performed as described in the legend to Fig. 3. Releasing solution was composed of 2 mM EGTA, 20 mM Mops, 100 mM KCl, various concentrations of $CaCl_2$ to give the indicated free Ca^{2+} concentrations, and 0 (○, ●) or 5 mM (△, ▲) $ATPNa_2$, at pH 6.8.

both types of vesicles. Half-maximal activation was obtained with about 1.5 mM ATP, the maximal effect being produced by ATP concentration above 5 mM for both types of vesicles. Fig. 10 shows the effect of 5 mM ATP on the Ca^{2+} dependence of Ca^{2+} release from SR vesicles isolated from one MHS and one normal pig. ATP stimulated the Ca^{2+}-induced Ca^{2+} release mechanism even at high Ca^{2+} concentrations (low pCa) for both MHS and N SR vesicles. Maximal initial rate of Ca^{2+} release were 1100 and 800 nmol mg^{-1} s^{-1}, for MHS and N SR vesicles, respectively. However, ATP slightly shifted – but not as strongly as caffeine and halothane – the Ca^{2+} dependence of Ca^{2+} release to lower Ca^{2+} concentrations. In the presence of ATP, the difference between MHS and N pig SR vesicles was accentuated in the low Ca^{2+} concentrations range. We also measured the effect of 5 mM caffeine or 0.01% halothane (v/v) in presence of 1 mM ATP on the Ca^{2+}-induced Ca^{2+} release. Our results showed that the effect of ATP was predominant over those of caffeine or halothane, i.e., in presence of ATP, there was no shift of the Ca^{2+} dependence of Ca^{2+} release to lower Ca^{2+} concentrations by caffeine or halothane (data not shown).

Discussion

The overall aim of this study was to investigate a possible abnormality of the Ca^{2+} movements across the SR membrane of MHS pig skeletal muscle by measuring the initial rates of Ca^{2+} uptake and Ca^{2+} release mechanisms. For this study, we have used a rapid filtration technique to measure the initial phases of these processes. This method, which was never used before in the context of MH susceptibility, allows to study the two mechanisms (uptake and release) independently from each other.

Contradictory results have been obtained by different laboratories concerning the Ca^{2+} uptake (rate and capacity) by MHS and N SR (for review see Ref. 12). However no data on the initial rate of active transport of Ca^{2+} in MHS pig SR have been obtained previously. Our results show no significant difference in the initial rate of Ca^{2+} uptake between MHS and N pig SR vesicles. Furthermore, no difference in ATP hydrolysis during this active transport was detected between MHS and N pig SR vesicles as previously described [9]. We obtained lower values of ATPase activity than those of Ref. 9, and the rate of Ca^{2+} uptake measured in our study were higher. But, there is a good correlation between our measurements of ATP hydrolysis with the pHstat and our measurements of Ca^{2+} uptake using the rapid filtration method. A stoichiometry of Ca^{2+} transported in the SR to ATP hydrolysed is in the range of 2 : 1. This suggests a good coupling between the ATPase activity and the Ca^{2+} transport by the Ca^{2+}-ATPase and suggests that the Ca^{2+}-ATPase functions of the SR are not altered in MHS pig SR. In contrast, MHS SR vesicles present a significant increase in the initial rate of Ca^{2+} release induced by Ca^{2+}.

We have investigated the effect of Mg^{2+}, caffeine, halothane, and ATP on the initial rate of Ca^{2+}-induced Ca^{2+} release from pig SR vesicles.

The Ca^{2+} dependence of the Ca^{2+} release follows a bell-shaped curve with a maximal effect at 3 μM external Ca^{2+} for MHS and N pig SR vesicles. MHS SR vesicles have a potentiated Ca^{2+}-induced Ca^{2+} release compared to N SR vesicles as shown before [8,9,11]. The higher rate of Ca^{2+} release in MHS SR can be explained by a larger open probability of the Ca^{2+} channel.

Mg^{2+} induces a dose-dependent decrease of the Ca^{2+}-induced Ca^{2+} release in both MHS and N pig SR vesicles.

Caffeine increases the Ca^{2+} sensitivity of the Ca^{2+}-induced Ca^{2+} release (by shifting the Ca^{2+} dependence to lower Ca^{2+}), but does not increase the maximal rate of Ca^{2+} release, in both MHS and N SR, as shown before in rabbit SR in comparable conditions [33]. This suggests that caffeine increases the affinity of the Ca^{2+} binding sites (of the high and the low sites) but does not change the permeability of the open channel.

Halothane, as caffeine, increases the Ca^{2+} sensitivity of the Ca^{2+}-induced Ca^{2+} release but also increases the rate of Ca^{2+} release in both MHS and N SR vesicles as observed before with other muscular preparations [4,11,32,33]. It has been reported using reincorporation of frog SR vesicles into planar lipid bilayer that halothane increased the open time probability of the Ca^{2+} channel without changing the conductance of the channel [34]. Our results suggest that halothane may

increase both the Ca^{2+} affinity of the high affinity Ca^{2+}-binding site and the permeability of the channel. Furthermore, in MHS SR vesicles, halothane also increases the affinity of the low Ca^{2+} binding site.

In contrast to caffeine and halothane, ATP does not shift the inhibition of the Ca^{2+} release by high Ca^{2+} concentrations as observed before with other tissues [17,33]. ATP strongly accelerates the mechanism by producing a 20–40-fold increase of the rate of Ca^{2+} efflux in both MHS and N SR vesicles. ATP, like halothane, may increase the permeability of the Ca^{2+} channel. The fact that the effect of ATP are still obtained when the concentration of Ca^{2+} is optimum suggests that ATP binds to a site of the Ca^{2+} channel different from that of Ca^{2+}.

Moreover, our results show that all the effectors investigated affect the Ca^{2+}-induced Ca^{2+} release in different ways; all types of Ca^{2+} release tested have higher rate in MHS when compared to N vesicles. Furthermore, the magnitude of the enhancement of Ca^{2+}-induced Ca^{2+} release by caffeine, halothane or ATP in MHS SR vesicles is almost the same as that in N SR.

We show that there is no significant modification in the binding of ryanodine to the MHS SR membranes in contrast to the results of Ref. 10, suggesting that the ryanodine receptor is not altered in our vesicles. The discrepancy can be explained in the frame of the results of Ref. 35 which have revealed a difference in binding for suboptimal conditions of binding, though in presence of higher Ca^{2+} or ATP this difference disappeared. Further experiments would be necessary to determine by which mechanism a regulation could potentiate Ca^{2+} release from the MHS SR vesicles. In this context, the activation of a G-protein would be possible: in the laboratory, we have shown that G-protein activate Ca^{2+} release induced by Ca^{2+} or caffeine [36]. A regulation by other mechanisms such as by glycolytic enzymes may also be possible because these enzymes are particularly abondant at the triades [37]. Ervasti et al. have reported an altered dihydropyridine receptor or decreased content of this protein in MHS T-tubules vesicles [38]. The T-tubules dihydropyridine receptor are implicated as the voltage sensor for the excitation-contraction coupling [39]. The coupling between the excitation of the muscle cell and the Ca^{2+} release from the SR is altered in MHS skeletal muscle fibers. A better knowledge of the defect in MHS skeletal muscle could therefore help to understand the actual coupling and the regulations involved between T-tubules receptor and ryanodine receptor in physiological conditions.

Acknowledgments

We thank the staff of the Institut National de la Recherche Agronomique of Theix (Clermont-Ferrand, France) for supply of animals, Mylène Robert for her help in the preparation of SR vesicles, and Michel Vivaudou and Michel Ronjat for fruitful discussions. This work was supported by grants from the Association Française contre les Myopathies, and the Muscular Dystrophy Association of the U.S.A.

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