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Effects of halothane, caffeine, dantrolene and tetracaine on the calcium permeability of skeletal sarcoplasmic reticulum of malignant hyperthermic pigs

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Preparing skeletal sarcoplasmic reticulum from both normal and malignant hyperthermia susceptible pigs, the effects of various drugs on the passive calcium permeability of these sarcoplasmic reticulum preparations were studied. It was found that, in the absence of halothane, the permeability of heavy sarcoplasmic reticulum prepared from malignant hyperthermia susceptible pigs was much higher than that of normal pigs. It was observed that halothane, at concentrations above 10 μM (well below anesthetic concentrations, which are on the order of 1 mM), increased the permeability of sarcoplasmic reticulum. The Hill coefficient of the effect of halothane ranged from 1.96 to 2.25, suggesting that some kind of cooperativity was involved in this reaction. The effects of caffeine were similar to those of halothane. Inhibitors, such as tetracaine and ruthenium red inhibited both the calcium permeability and the halothane-induced increment. The Hill coefficient of the effect of tetracaine was 1.75. The mode of inhibition suggests that tetracaine directly binds with the calcium channel to inhibit the calcium efflux. On the contrary, dantrolene did not affect the calcium permeability of the sarcoplasmic reticulum. However, it inhibited the halothane-induced and caffeine-induced increments of the permeability. The Hill coefficient of inhibition by dantrolene ranged from 2.3 to 3.9, suggesting that several molecules of dantrolene may interact cooperatively with one calcium release channel to inhibit the effect of halothane. These results suggest that dantrolene has a unique inhibitory action, which may be related to its efficacy in ameliorating the syndrome of malignant hyperthermia.

Introduction

The effect of certain general anesthetics to abnormally increase the cytoplasmic Ca^{2+} concentration in muscle of malignant hyperthermia susceptible man and pig has been well documented [1–6]. Since the sarcoplasmic reticulum plays a major role in regulating the cytoplasmic Ca^{2+} concentration, it might be assumed that the sarcoplasmic reticulum of malignant hyperthermic muscle is abnormal. The fact that dantrolene has been found to be an effective prophylactic and therapeutic agent for malignant hyperthermia in both man and pig [7–12] stimulated us to undertake studies of the effects of various agents on the Ca^{2+} release phenomena of the sarcoplasmic reticulum.

Using genetically susceptible malignant hyperthermic pigs, we have previously demonstrated that halothane could release Ca^{2+} from the sarcoplasmic reticulum (under conditions in which it did not cause release from the sarcoplasmic reticulum of normal pigs [13]). It was also shown that dantrolene inhibited the halothane-induced

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Ca^{2+} release [13]. We have found that the putative Ca^{2+} release channel of the sarcoplasmic reticulum in malignant hyperthermic pigs has a much higher permeability than that of normal sarcoplasmic reticulum [14]. Other investigators also reached the same conclusion [15,16].

In our previous work [13,14] and that of others [15,16], the sarcoplasmic reticulum was loaded with Ca^{2+} in the presence of ATP. However, since Ca^{2+} release was taking place simultaneously with the ATP-induced Ca^{2+} uptake, the interpretation of the data was complicated. Therefore, we developed a method to measure Ca^{2+} release in the absence of ATP [22]. In this paper, we used this method to study the effects of release stimulating agents, such as halothane and caffeine [17–21] as well as those of inhibitors, such as dantrolene, ruthenium red [18,19] and tetracaine [17,18].

Materials and Methods

Chemicals. Halothane was purchased from Ayerst Laboratories (New York, NY). Dantrolene was obtained from Norwich-Eaton Pharmaceuticals (New York, NY). All other chemicals were purchased from Sigma (St. Louis, MO).

Animals. Three genetically susceptible malignant hyperthermic pigs and three littermates (Pietrain; 20–50 kg) were obtained from the Animal Science Department, University of Minnesota. Under thiopental anesthesia, semitendinosus muscle was removed surgically while the animal was ventilated with 50% NO_2/50% O_2. The heavy sarcoplasmic reticulum fraction was immediately prepared from the muscle according to the method described previously [13].

Ca^{2+} permeability measurement. A calcium indicator (calcein) was encapsulated inside sarcoplasmic reticulum by an osmotic shock method [22]. The heavy sarcoplasmic reticulum was incubated in a medium containing 0.8 M sucrose and 2 mM 4-morpholineethanesulfonate (Mes) buffer (neutralized with tris(hydroxymethyl)aminomethane (Tris) to a pH of 6.8) for several hours. Then the suspension was diluted 10 times with a hypotonic solution containing 140 μM calcein, 1 mM Ca^{2+}, and 2 mM Mes-Tris buffer (pH 6.8) to induce osmotic swelling and inclusion of both calcein and Ca^{2+}. After 2 hours, the diluted suspension was centrifuge-washed with a buffer containing 120 mM KCl, 1 mM $CaCl_2$, and 40 mM Mes buffer (neutralized with KOH; pH 6.8) and incubated overnight. (The sarcoplasmic reticulum seemed to have equilibrated with 1 mM Ca^{2+}, because the incubation longer than overnight did not significantly change the result.) All procedures were carried out below 4°C. The calcein-Ca^{2+}-encapsulating sarcoplasmic reticulum suspension (300 μl) was pipetted into a cylindrical cuvette (6 mm i.d.) inside a dual-wavelength spectrophotometer (Aminco DW-2) equipped with a magnetic stirring device. The wavelength combination was 478–502 nm and the temperature was 25 ± 0.1°C. After temperature equilibration, a 10 μl solution of EGTA was added to decrease the free Ca^{2+} concentration from 1 mM to desired values (from 0.01 to 100 μM). The binding of Ca^{2+} to EGTA released H^+ from EGTA moleucles, thus causing a pH drop in the suspension. We corrected this effect by pre-mixing a suitable amount of KOH with the EGTA solution which would keep the final pH constant. As shown in Fig. 1, after an instant change of absorption (a dilution effect), a gradual decrease of the intravesicular

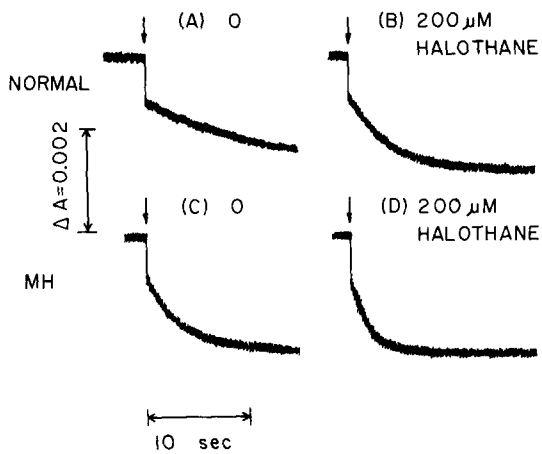

Fig. 1. Measurement of the decrase of intravesicular Ca^{2+}. At time zero indicated by arrows, 10 μl of an EGTA-calcium buffer was added to a 300-μl suspension of calcium-calcein-encapsulating sarcoplasmic reticulum (SR) to decrease the extravesicular Ca^{2+} concentration to 1 μM. (A) and (B) Normal pig SR; (C) and (D) malignant hyperthermic (MH) pig SR. Halothane concentration: (A) and (C) 0; (B) and (D) 200 μM. Other conditions were 2 mg protein/ml, 120 mM KCl, 40 mM Mes buffer (pH 6.8) at 25°C. The wavelength combination was 478–502 nm (see text for details).

Ca^{2+} concentration was recorded through absorption changes of encapsulated calcein. Using a calibration curve (which was made in a separate experiment in the presence of A23187 [22]) the initial Ca^{2+} efflux was estimated. In order to estimate the efflux rate in terms of nmol/mg sarcoplasmic protein, it was necessary to know the internal volume of the sarcoplasmic reticulum. We used 4.4 μl/mg sarcoplasmic reticulum protein, which was the value determined by Miyamoto and Kasai [23]. We did not use the more usual method of measuring Ca^{2+} efflux with a stopped-flow apparatus combined with a metallochromic indicator to detect a minute change of Ca^{2+} concentration in the extravesicular space (which is at micromolar levels, requiring 10 millisecond resolution [24,25]). Our method allows us to measure the rate of depletion of intravesicular Ca^{2+} without using a stopped-flow apparatus (because the intravesicular concentration is very high – millimolar levels – and therefore, the timebase is stretched to 10 seconds).

Addition of halothane and dantrolene. Thymol-free halothane was prepared by distilling commercial halothane. This was dissolved in dimethyl sulfoxide and the solution was added directly to the sarcoplasmic reticulum suspension. Dantrolene was also dissolved in dimethyl sulfoxide, and the solution was added to the suspension. The concentration of total dimethyl sulfoxide in the suspension was less than 1%. It was confirmed that this concentration of dimethyl sulfoxide did nto interfere with the permeability measurement (data not shown). For the study of the effect of drugs, they were added to the sarcoplasmic reticulum suspension one minute prior to the addition of the EGTA-Ca buffer.

Results

Effects of halothane on calcium permeability

Fig. 2 shows how halothane affects the calcium permeability of malignant hyperthermia sarcoplasmic reticulum and normal sarcoplasmic reticulum. In normal sarcoplasmic reticulum (Fig. 2B), the permeability peaks maximum at 10 μM of Ca^{2+} concentration in the absence of halothane. As pointed out previously [19], the permeability increased upon addition of halothane, and at the same time, the peak concentration shifted toward the left (to 3 μM in the presence of 200 μM halothane). In case of malignant hyperthermia sarcoplasmic reticulum, the permeability was much higher than that of normal sarcoplasmic reticulum and the peak concentration was already reduced (3 μM) in the absence of halothane (Fig. 2A). The addition of halothane also increased the permeability and shifted the curve further toward the left.

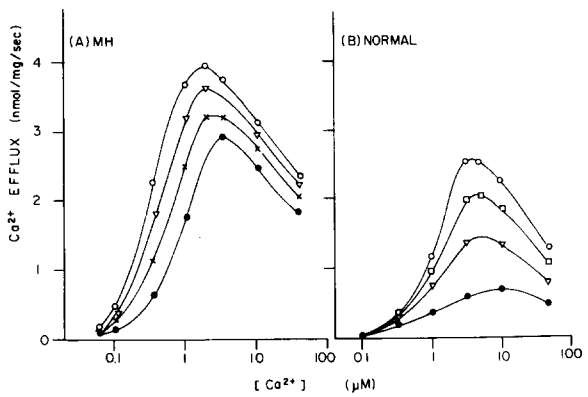

Fig. 2. Effect of extravesicular calcium concentration on the calcium permeability of sarcoplasmic reticulum prepared from (A) malignant hyperthermic pig and (B) normal pig. Experimental conditions: 120 mM KCl, 40 mM Mes buffer (pH 6.8), 25°C. Halothane concentrations: ●, ○; ×, 25 μM; ▽ 50 μM; □, 100 μM and ○, 200 μM.

The difference between malignant hyperther-

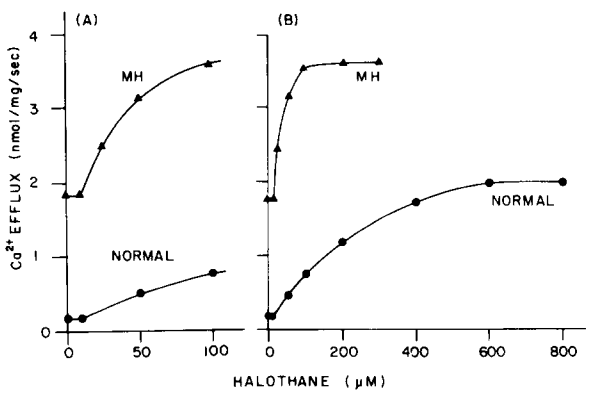

Fig. 3. Effect of halothane concentration on the calcium permeability of malignant hyperthermia and normal sarcoplasmic reticulum. (A) at low halothane concentrations and (B) at higher halothane concentrations. The free Ca^{2+} concentration was 1 μM. Other conditions were the same as those in Fig. 1.

mia sarcoplasmic reticulum and normal sarcoplasmic reticulum is also seen in the dose-response relationship of the halothane effect. As shown in Fig. 3, above a critical concentration (10 μM), halothane increased permeability of both types of sarcoplasmic reticulum (Fig. 3A magnifies a low concentration portion of Fig. 3B). The maximal stimulation of Ca^{2+} permeability of normal sarcoplasmic reticulum is reached at 600 μM, whereas in malignant hyperthermia sarcoplasmic reticulum, it was reached only at about 100 μM.

Effects of caffeine

Effects of caffeine were basically the same as those of halothane. Caffeine shifted the calcium permeability curve upward, and at the same time toward the left (Fig. 4). The dose-response relationship between the caffeine concentration and the permeability was similar to that of halothane. The critical concentration for caffeine was 210 μM in both sarcoplasmic reticulum preparations. The stimulating effect on normal sarcoplasmic reticulum leveled off at 6 mM, while that on malignant hyperthermia sarcoplasmic reticulum saturated at 1 mM (Fig. 4B).

Hill plot of the effects of halothane and caffeine

Hill plots were made from Fig. 3 and Fig. 4B. As shown by the plot (Fig. 5), the range of the Hill coefficients for normal sarcoplasmic reticulum was 1.71–1.96 and that for malignant hyperthermia sarcoplasmic reticulum was 2.25–2.48.

Effects of dantrolene and other inhibitors

As shown in Fig. 6A, dantrolene was shown to inhibit the halothane-increased permeability. However, dantrolene did not change the permeability of sarcoplasmic reticulum in the absence of halothane. The dose-response curve of dantrolene effects on malignant hyperthermic sarcoplasmic reticulum is shown in Fig. 6B. The effect was not remarkable at lower concentrations (below 5 μM), while it started showing an increased inhibitory effect at higher concentrations (above 20 μM). After the halothane-induced permeability-increment was abolished at higher concentrations of dantrolene, the permeability did not decrease any further. The effect of dantrolene on the caffeine-induced permeability was similar to that with halothane and is shown in the same figure by the dotted line. The effects of dantrolene on normal

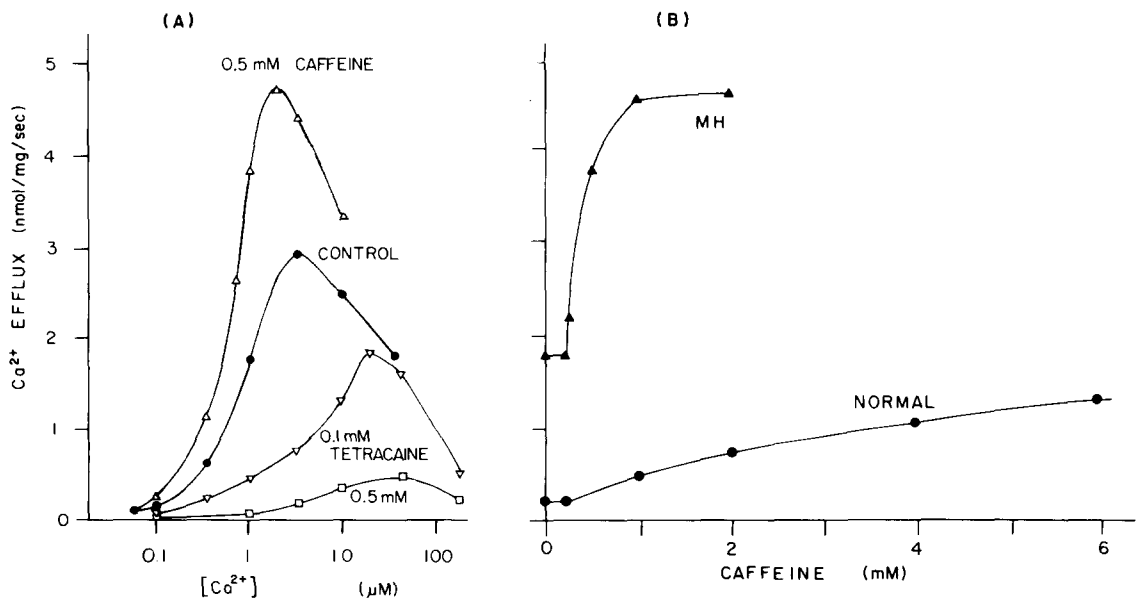

Fig. 4. (A) Effects of caffeine (500 μM) and tetracaine (0.1 mM and 0.5 mM) on the calcium permeability of malignant hyperthermia sarcoplasmic reticulum. (B) Effect of caffeine on the calcium permeability of malignant hyperthermia and normal sarcoplasmic reticulum. Conditions were the same as shown in Fig. 1.

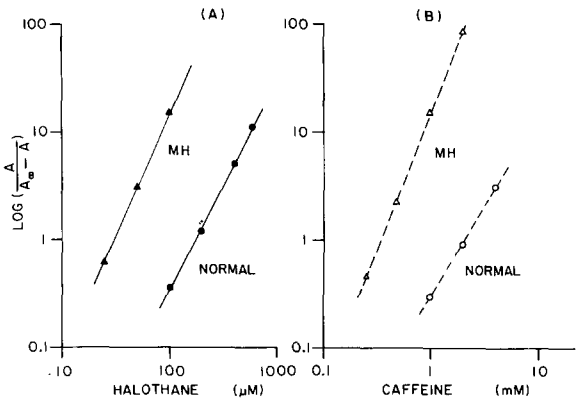

Fig. 5. Hill plots of the effects of (A) halothane and (B) caffeine on the calcium efflux from normal and malignant hyperthermia sarcoplasmic reticulum. The value A indicates the increment of calcium efflux in the presence of drugs, and A_∞ indicates its saturated value. Data were derived from Figs. 3 and 4.

sarcoplasmic reticulum permeability were similar to those in malignant hyperthermia sarcoplasmic reticulum (data not shown).

Tetracaine inhibited both the calcium permeability (in the absence of halothane; see Fig. 4A) and the halothane-induced increase of the calcium permeability. The mode of inhibition by tetracaine (as shown in Fig. 6C) was different from that by dantrolene (Fig. 6B). Tetracaine had a stronger inhibitory effect at lower concentrations below 100 μM) but the effects leveled off at higher concentrations (above 300 μM). The effect of tetracaine on the caffeine-induced permeability is shown by a dotted line in Fig. 6C. (The effect of ruthenium red was similar to that of tetracaine, data not shown).

Hill plot of the effects of dantrolene:

As shown in Fig. 7, the range of the Hill coefficient for tetracaine inhibition was 1.69–1.75, while that for dantrolene inhibition was 2.3–3.9.

Discussion

It is the current concensus that the syndrome of malignant hyperthermia is linked to an abnormal increase of myoplasmic calcium cocnentration [1–6]. Therefore, many investigators studied the effect of halothane on the calcium metabolism of the sarcoplasmic reticulum. However, as reviewed by Gronert [6], the results were not consistent. Some discovered that halothane increased calcium metabolism, some reported a decrease and others reported no change. Based on the fact that the increase of myoplasmic clacium concentration is caused by a calcium release from the sarcoplasmic reticulum, we have focused on the effect of halothane on the calcium release from the sarcop-

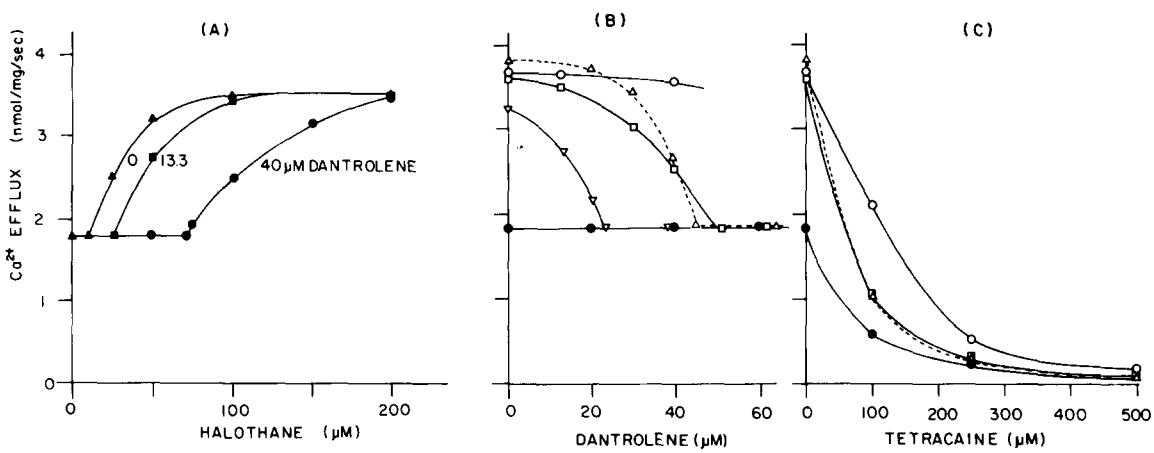

Fig. 6. (A) Effects of dantrolene on the dose-response relationship of halothane-induced increment of calcium permeability of malignant hyperthermia sarcoplasmic reticulum. Dantrolene concentrations: ▲, 0; ■, 13.3 μM and ●, 40 μM. (B) Dose-response relationship of the dantrolene effect on the calcium permeability. ●, no halothane; ▽, 50 μM halothane; □, 100 μM halothane; ○, 200 μM halothane and △, 500 μM caffeine. (C) Dose-response relationship of the tetracaine effect on the calcium permeability. ●, control; □, 100 μM halothane; ○, 200 μM halothane, and △, 500 μM caffeine. The conditions were the same as those in Fig. 1.

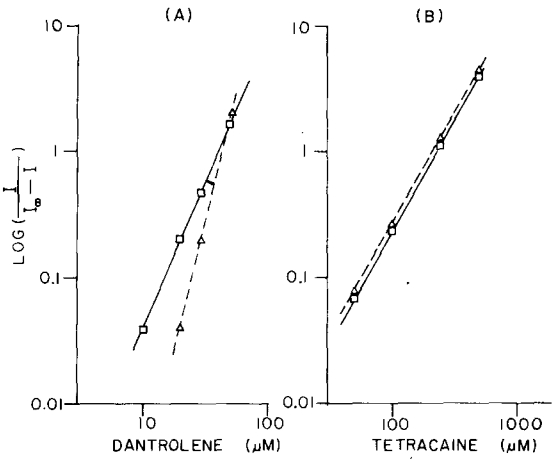

Fig. 7. Hill plots of the inhibitory effects of (A) dantrolene on the halothane-induced increase of the calcium permeability of malignant hyperthermia sarcoplasmic reticulum and (B) tetracaine on the calcium permeability of malignant hyperthermia sarcoplasmic reticulum. The value I represents the inhibitory effect and I_∞ its saturated level. Square symbols are for halothane (100 μM) and triangular symbols are for caffeine (500 μM). Data were derived from those in Figs. 6B and 6C.

lasmic reticulum. Since we had already developed a method to study the calcium-induced calcium release as well as halothane and caffeine-induced calcium release [13,18,19], we undertook the study of the effect of halothane and dantrolene on malignant hyperthermia sarcoplasmic reticulum.

We have attempted to find the effects of halothane (as well as caffeine) and dantrolene on the malignant hyperthermia sarcoplasmic reticulum, which are consistent with the malignant hyperthermia syndrome in man and pig. As reported in previous papers, we found that the heavy sarcoplsmic reticulum (which consists of terminal cisternae and longitudinal tubule, and is known to play a major role in calcium release in muscle) has an abnormally high calcium permeability [13,14]. We also found that halothane released calcium from the malignant hyperthermia sarcoplasmic reticulum, but that this release was inhibited by dantrolene [13]. This was the first evidence to show that malignant hyperthermia and normal sarcoplasmic reticulum were different and that the effect of halothane was inhibited by dantrolene at the level of the sarcoplasmic reticulum. However, at that time we could not further elucidate the mechanisms of action of halothane and dantrolene.

A difficulty of determining the mechanisms of action of these drugs came from the fact that the calcium flux of sarcoplasmic reticulum is determined by a balance of two phenomena, i.e. the ATP-induced calcium uptake and the calcium release. (As a matter of fact, one of the reasons why studies done by previous investigators on malignant hyperthermia sarcoplasmic reticulum were not consistent, was that these two phenomena were not properly separated). We have suggested a method of studying the calcium release from the sarcoplasmic reticulum by distinguishing the release channel from the calcium uptake [19,22,26]. For the study of the mechanism of drugs on the release phenomenon, we found it best to work on the system in which the pump does not play a role. We were able to do this by passively loading the sarcoplasmic reticulum with calcium and then initiating calcium release by a sudden decrease of the extra-vesicular calcium concentration with the addition of a Ca-EGTA buffer solution [19,22]. This method contributed to building model for gated calcium transport [19]. The effects of various agents which increase the permeability such as halothane, caffeine, alcohol [19,22] and inhibitors such as tetracaine and ruthenium red [18,19] were clearly demonstrated with this method.

The calcium permeability of rat skeletal muscle sarcoplasmic reticulum measured by this method was 0.3–0.8 nmol/mg per s at 1 μM of free calcium concentration [22], which was on the same order as that of rabbit skeletal muscle sarcoplasmic reticulum [27]. With normal pig sarcoplasmic reticulum (skeletal), it was observed that the permeability was about 3 nmol/mg per s under the same conditions (Fig. 2B). The calcium permeability seems to depend on the species. (We did not study the effect of Mg^{2+}. Preliminary data indicated that essentially similar results were obtained as long as the Mg^{2+} concentration was below 0.5 mM; data not shown).

Applying this method to malignant hyperthermia sarcoplasmic reticulum prepared from pigs, we found that malignant hyperthermia sarcoplasmic reticulum had a higher permeability even in the absence of halothane (Figs. 1–3). Upon the addition of halothane (above 10 μM, which was

the critical concentration for pig sarcoplasmic reticulum), the permeability of the sarcoplasmic reticulum remarkably increased. 10 μM of halothane in suspension is in equilibrium with 0.08% (v/v) halothane gas at 37°C [28]. Therefore, a concentration of halothane well below anesthetic levels (which are on the order of 1%) could trigger calcium release from the sarcoplasmic reticulum. The fact that the critical concentrations were the same for both normal and malignant hyperthermia sarcoplasmic reticulum may indicate that the minimum halothane sensitivity of putative receptors in both types of sarcoplasmic reticulum may be the same [13,19]. The situation was the same for caffeine (Fig. 4B).

Although the critical concentrations were the same for both types of sarcoplasmic reticulum, the stimulating effects of halothane (and of caffeine) on the calcium permeability were much different. In malignant hyperthermia sarcoplasmic reticulum, the increase of permeability leveled off at a halothane concentration of 100 μM with the half maximal stimulation at 30 μM. In the case of normal sarcoplasmic reticulum, the permeability increase reached a plateau at 600 μM with the half maximum stimulation at 230 μM (Fig. 3). The effects of caffeine on the permeability were similar to those of halothane. The critical cocnentrations were the same for both types of sarcoplasmic reticulum (0.21 mM). The half maximal stimulations were observed at 0.4 and 2.7 mM with malignant hyperthemia and normal sarcoplasmic reticulum, respectively (Fig. 4B).

Even though calcium-induced calcium release may not be the primary calcium release mechanism in skeletal sarcoplasmic reticulum [17], it could enhance contractility through increasing the myoplasmic calcium cocnentration. It has been proposed that the mechanism of calcium-induced calcium release and the mechanism of halothane-induced calcium release is the same [13,17,19,27]. Therefore, the onset of malignant hyperthermia etiology might be described as follows: Halothane increases the myoplasmic calcium concentration in malignant hyperthermia susceptible man (or pig) by stimulating calcium release from the sarcoplasmic reticulum. The increased calcium concentration then stimulates calcium-induced calcium release from the sarcoplasmic reticulum thus causing more calcium to be released. This positive feed-back nature of calcium release [19] will rapidly increase the calcium concentration in the myoplasm. This would cause hypercontraction and (because of the inefficiency of energy conversion in contraction) an abnormal rise of the temperature. This precipitates the malignant hyperthermia syndrome.

An important finding in our study is that dantrolene did not inhibit the calcium permeability, but inhibited only the halothane-induced increment of the calcium permeability (Fig. 6B). Unlike tetracaine and ruthenium red [18,19], dantrolene did not affect the calcium permeability itself. Another important difference between tetracaine and dantrolene was shown by the mode of inhibition (Fig. 6B and C). Namely, tetracaine inhibited the Ca^{2+} efflux by some kind of a 'binding-type' reaction as shown in Fig. 6C. A Hill coefficient in the range of 1.69–1.75 suggests that two molecules of tetracaine may bind with a calcium-release chanel, thereby blocking the calcium efflux. On the other hand, in the case of dantrolene, several molecules seem to be involved in the inhibition of a channel.

The inhibitory effect of dantrolene was seen at concentration levels of 20–40 μM. According to Norwich-Eaton Pharmaceuticals (Company's technical information), a total dose of 10 mg/kg dantrolene (i.v.) is recommended when a malignant hyperthermic reaction is recognized in the operating room. If we assume that 1/10 of the body weight is blood, this dosage is approximatley equivalent to 250 μM in the blood. Therefore, it is possible that dantrolene concentration in the lipid constituents of the myoplasm could reach 40 μM to inhibit halothane effect on the sarcoplasmic reticulum.

Tetracaine and ruthenium red are positively charged cations, and thus they may directly bind to negatively charged sites on the channel. Dantrolene, being a neutral molecule, would not interact with these charged sites. Since dantrolene is a lipophilic compound (dantrolene has a low solubility in water at a neutral pH), it may interact with a lipophilic region of the channel through hydrophobic interactions. The fact that the dantrolene interaction has a high Hill coefficient suggests that several molecules may be involved in

this interaction. An EPR spin-probe study offers additional supportive evidence. A neutral molecule like halothane (as well as caffeine) causes a conformational change in the sarcoplasmic reticulum membrane, and the change seems to be related to the calcium release [29]. Therefore, both halothane and dantrolene would act on the same site of the sarcoplasmic reticulum Ca^{2+} release channel to compete with each other based upon their hydrophobic interactions. This unique property of dantrolene may be related to its efficacy in subsiding the malignant hyperthermia syndrome.

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