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Effect of halothane on Ca^{2+}-induced Ca^{2+} release from sarcoplasmic reticulum vesicles isolated from rat skeletal muscle

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Halothane induces the release of Ca^{2+} from a subpopulation of sarcoplasmic reticulum vesicles that are derived from the terminal cisternae of rat skeletal muscle. Halothane-induced Ca^{2+} release appears to be an enhancement of Ca^{2+}-induced Ca^{2+} release. The low-density sarcoplasmic reticulum vesicles which are believed to be derived from nonjunctional sarcoplasmic reticulum lack the capability of both Ca^{2+}-induced and halothane-induced Ca^{2+} release. Ca^{2+} release from terminal cisternae vesicles induced by halothane is inhibited by Ruthenium red and Mg^{2+}, and require ATP (or an ATP analogue), KCl (or similar salt) and extravesicular Ca^{2+}. Ca^{2+}-induced Ca^{2+} release has similar characteristics.

Introduction

Muscle contraction is triggered by the release of Ca^{2+} from the sarcoplasmic reticulum following depolarization of the transverse tubule system by the action potential [1–3]. Relaxation follows the removal of cytoplasmic Ca^{2+} by the sarcoplasmic reticulum. Ca^{2+} transport into the sarcoplasmic reticulum is mediated by the $(Ca^{2+} + Mg^{2+})$-ATPase [4–6]. The mechanism of Ca^{2+} release from the sarcoplasmic reticulum is not known.

The interaction of the general anesthetic, halothane (CF_3-CHBrCl) with sarcoplasmic reticulum vesicles has been extensively studied [7–26]. Sarcoplasmic reticulum is one of the best characterized biomembranes [3–6] and therefore has been used as a model system to investigate the effects of general anesthetics on membrane function and structure. In addition, understanding the effect of halothane on sarcoplasmic reticulum function may help elucidate the apparent role that sarcoplasmic reticulum plays in malignant hyperthermia. Halothane triggers the malignant hyperthermia syndrome in susceptible individuals. It is generally believed that the abnormality in malignant hyperthermia involves the release of Ca^{2+} from the sarcoplasmic reticulum leading to an increased metabolic rate and muscle contractions [17,19,20,27,28].

Halothane at high, nonclinical concentrations (2–20 mM) alters many of the functional and physical properties of the sarcoplasmic reticulum membrane. High concentrations of halothane have been reported to increase membrane fluidity [25], increase membrane permeability [9,12], decrease ATP-dependent Ca^{2+} accumulation [8,11–15,23] and inhibit the activity of the $(Ca^{2+} + Mg^{2+})$-ATPase [7,9–11,14,15]. Studies on the effect of halothane at lower, more clinically relevant concentrations have led to conflicting conclusions. Halothane ($\equiv$ 1 mM) has been reported to slightly

Abbreviations: ATP, adenosine 5′-triphosphate; Ado*PP*[NH]*P*, 5′-adenylyl imidodiphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether) *N*,*N*′-tetraacetic acid; Mops, 4-morpholinepropanesulfonic acid.

stimulate [12,13,17,19–21], to have no significant effect [7,10,14,18], or to decrease Ca^{2+} accumulation [11,9,16,22,26] by sarcoplasmic reticulum vesicles. These discrepancies may be a result of different procedures for preparing sarcoplasmic reticulum vesicles or different experimental conditions for assaying their functional properties.

It has been proposed that muscle contractions induced by halothane are related to Ca^{2+}-induced Ca^{2+} release from sarcoplasmic reticulum [22,29, 30]. Ca^{2+}-induced Ca^{2+} release is a process in which Ca^{2+} on the outside of the sarcoplasmic reticulum initiates the release of internal Ca^{2+} [31,32]. Therefore, to investigate the effect of halothane on sarcoplasmic reticulum function, one should pay particular attention to those factors that alter Ca^{2+}-induced Ca^{2+} release. In the study reported here, we find that halothane at clinical concentrations alters Ca^{2+} accumulation by a subpopulation of sarcoplasmic reticulum vesicles by enhancing Ca^{2+}-induced Ca^{2+} release.

Materials and Methods

Materials. Adenosine 5′-triphosphate (ATP), 5′-adenylyl imidodiphosphate (Ado*PP*[NH]*P*), lactate dehydrogenase and pyruvate kinase were obtained from Sigma (St. Louis, MO). The ionophore A23187 was purchased from Calbiochem-Behring (La Jolla, CA). Arsenazo III and Ruthenium red were supplied by Aldrich (Milwaukee, WI). Halothane came from Halocarbon Laboratories (Hackensack, NJ).

Preparation of sarcoplasmic reticulum. Sarcoplasmic reticulum membranes released from skeletal muscle fibers during homogenization consist of a heterogenous population [33–38]. In this paper, three main subcellular fractions derived from sarcoplasmic reticulum membrane are prepared. Most of the studies were performed with sarcoplasmic reticulum vesicles prepared from the crude mitochondrial fraction. This fraction will be called 'heavy sarcoplasmic reticulum vesicles'. This fraction is enriched with terminal cisternae vesicles as indicated the its large content of calsequestrin [33,39]. This fraction also contains intact triad junctions as indicated by the enrichment of transverse tubule markers [40–44] (nitrendipine receptor (18 pmol/mg protein), $(Na^+ + K^+)$-ATPase (0.3 μmol ATP hydrolyzed/mg per min at 37°C) and cholesterol (0.2 mol ratio cholesterol-to-phospholipid)). Triad-like structures (as described by Mitchell et al. [45,46] and Caswell et al. [36,47]) are commonly seen in electron micrographs of this fraction. In addition, French-press treatment followed by sucrose gradient centrifugation as described by Caswell and co-workers [36,47] separates the T-tubule vesicles from the sarcoplasmic reticulum vesicles. A more complete description of the properties of this fraction will be published elsewhere. The other sarcoplasmic reticulum fractions are prepared from the microsomal fraction and are called 'low-density sarcoplasmic reticulum vesicles' and 'high-density sarcoplasmic reticulum vesicles'.

Sprague-Dawley rats (150–200 g) were killed by decapitation. The back and hind leg muscles were removed, and trimmed of connective, adipose and predominately red skeletal muscle tissue. Preparation of muscle subcellular fractions were performed at 4°C. The muscle tissue was chopped into small pieces (approx. 0.5 cm^3) and homogenized in 15 vol. of 0.15 M KCl/10 mM Mops (pH 6.8)/5 mM $MgSO_4$ (KCl solution) with a Polytron set at low speed for 2 min. Every 15 s, the connective tissue bound to the Polytron probe was removed and discarded. The muscle was further homogenized using a Potter-Elvehjem homogenizer (five strokes). The homogenate was centrifuged at $1500 \times g$ for 30 min. The pellet was discarded and the supernatant was centrifuged $10\,000 \times g$ for 20 min. The crude mitochondrial fraction ($10\,000 \times g$ pellet) was used to prepare the heavy sarcoplasmic reticulum fraction. The supernatant was centrifuged at $53\,000 \times g$ for 1 h to obtain the microsomal fraction. The low-density and high-density sarcoplasmic reticulum vesicles were prepared by fractionation of the microsomes by sucrose gradient centrifugation (30–45% sucrose in KCl solution, $130\,000 \times g$, 15 h).

Heavy sarcoplasmic reticulum vesicles were prepared from the crude mitochondrial fraction. The $10\,000 \times g$ pellet was resuspended in KCl solution and centrifuged at $1500 \times g$ for 30 min to remove most of the contractile filaments. The supernatant was centrifuged once again at $10\,000 \times g$ for 20 min. The pellet was resuspended in KCl solution containing 25% Percoll and centrifuged for 30 min

at 30000 × *g*. The sarcoplasmic reticulum vesicles which floated to the top of the Percoll gradient were removed and diluted with KCl solution. The Percoll was then removed by collecting the heavy sarcoplasmic reticulum vesicles once again by centrifugation (10000 × *g*, 20 min). The final pellet was resuspended in KCl solution.

Measurement of Ca^{2+} accumulation by sarcoplasmic reticulum. Ca^{2+} uptake was measured using arsenazo III [48,49] as an indicator of extravesicular Ca^{2+}. Measurements were performed using an Aminco DW-2 dual-beam spectrophotometer. The change in the absorbance of the arsenazo III-Ca^{2+} complex was monitored at 660 nm using 685 nm as a reference wavelength. The baseline (zero Ca^{2+} uptake) was established at the end of each measurement by adding 1 μM ionophore A23187 to release all the accumulated Ca^{2+}. The arsenazo III signal was then calibrated by successive additions of 1.25 μM Ca^{2+}

The fluorescent Ca^{2+} indicator, quin II [50] was used to monitor Ca^{2+} uptake in solutions containing Ca^{2+} concentrations below 1.0 μM. Measurements were performed with a Perkin-Elmer Model 640 fluorescence spectrophotometer. The excitation and emission wavelengths were set at 339 and 492 nm, respectively. The quin II signal was calibrated using EGTA/Ca^{2+} buffers to establish free Ca^{2+} concentration in the range of 1–0.1 μM. The free Ca^{2+} was calculated as described by Steinhardt et al. [51].

Measurement of Ca^{2+} efflux from sarcoplasmic reticulum using chlortetracycline as Ca^{2+} indicator [52–55]. Sarcoplasmic reticulum vesicles were passively loaded with Ca^{2+} by incubating them with 15 mM $CaCl_2$ overnight at 4°C. Ca^{2+} efflux was monitored using chlortetracycline as an indicator of the intravesicular Ca^{2+} concentration. Ca^{2+} efflux was initiated by diluting the vesicle 50-fold into solutions containing 10 μM chlortetracycline. The fluorescence of the chlortetracycline-Ca^{2+} complex was monitored using a Perkin-Elmer fluorescence spectrophotometer (excitation 390 nm, emission 530 nm).

Results

As previously reported [16,22,26], halothane (25–500 μM) reduces ATP-dependent Ca^{2+} accumulation by heavy sarcoplasmic reticulum vesicles (Fig. 1). Ca^{2+} accumulation was monitored spectrophotometrically using the Ca^{2+}-indicator arsenazo III to measure changes in the extravesicular Ca^{2+} concentration. The decrease in Ca^{2+} uptake was not transient. The same steady-state Ca^{2+} loading level in the presence of halothane was eventually reached regardless of the time of halothane addition (data not shown). It is possible that the transient Ca^{2+} release observed by others [22,26] is due to the re-uptake of Ca^{2+} by halothane-insensitive vesicles. For the same reason, the 'threshold' Ca^{2+} loading level (the Ca^{2+} loading level required before one observes halothane-induced Ca^{2+} release) may also depend on the amount of halothane-insensitive vesicles that are present. The half-optimal halothane concentration for Ca^{2+} release was 150 μM, which is

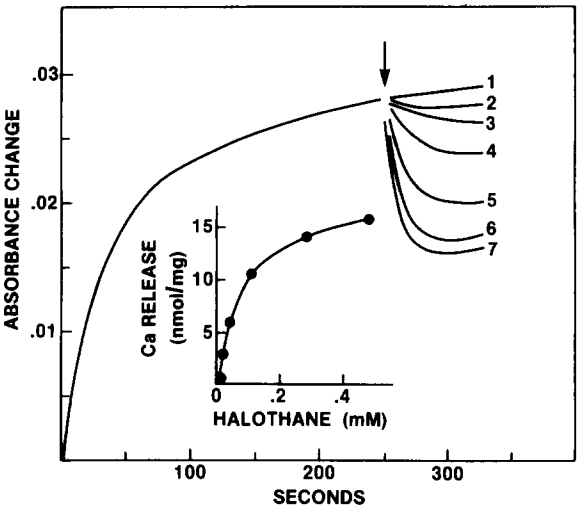

Fig. 1. Effect of halothane on Ca^{2+} accumulation by the heavy sarcoplasmic reticulum vesicles. The Ca^{2+} uptake medium contained 0.15 M KCl, 10 mM histidine (pH 6.8), 2 mM $MgSO_4$, 1 mM ATP, 25 μM $CaCl_2$ and 0.1 mM arsenazo III. Ca^{2+} transport was initiated by the addition of heavy sarcoplasmic reticulum vesicles (42 μg protein/ml) at 20°C. After 250 s, halothane was added to give a final concentration of 12.5 μM (2), 25 μM (3), 50 μM (4), 125 μM (5), 250 μM (6) or 500 μM (7). The halothane stock solution was made in ethanol and diluted 2000-fold into the Ca^{2+} uptake medium. Addition of ethanol (1) without halothane served as a control. The difference absorbance spectrum ($A_{685nm} - A_{660nm}$) of the Ca^{2+}-arsenazo complex was monitored. An absorbance change of 0.01 corresponds to an accumulation of 12 nmol Ca^{2+}/mg protein.

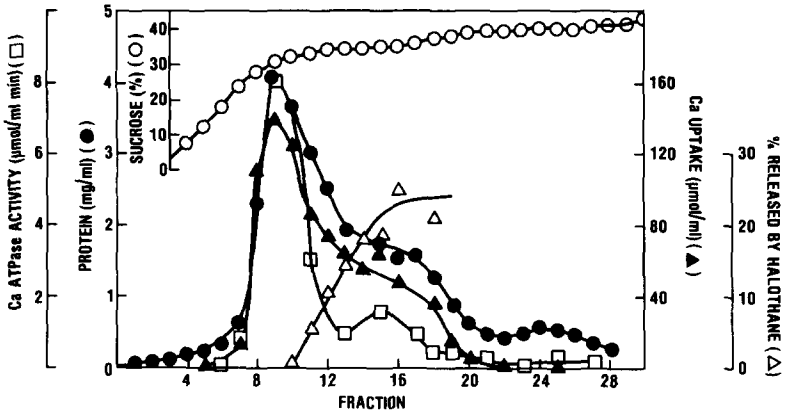

Fig. 2. Effect of halothane on Ca^{2+} accumulation by sarcoplasmic reticulum vesicles following fractionation by sucrose gradient centrifugation. The microsomal fraction was placed on a discontinuous sucrose gradient (5 ml 45%, 10 ml 40%, 10 ml 35%, 5 ml 30%) and centrifuged for 12 h at 53000 × *g*. The gradient was fractionated into 1.3-ml aliquots and the protein concentration (●), the Ca^{2+}-dependent ATPase activity (□), the Ca^{2+} loading level (3 min after initiating Ca^{2+} uptake at 20°C) (▲) and the percent Ca^{2+} released by 0.5 mM halothane (△) (as described in Fig. 1) was measured.

well within the clinically significant range [54,55]. The Ca^{2+}-dependent ATPase activity of the heavy sarcoplasmic reticulum vesicles was not altered by 10–500 μM halothane. Increasing the halothane concentration from 0.5 to 2 mM, did not significantly increase the amount of Ca^{2+} released. Concentrations of halothane about 2 mM made the vesicles leaky and inhibited the $(Ca^{2+} + Mg^{2+})$-ATPase.

Ca^{2+} accumulation by the heavy sarcoplasmic reticulum vesicles isolated from the 1500–10000 × *g* pellet was reduced 45% by 0.5 mM halothane. Ca^{2+} uptake by the sarcoplasmic reticulum vesicles in the microsomal fraction was reduced by less than 7% under the same conditions. The sarcoplasmic reticulum vesicles in the microsomal fraction constitute a heterogeneous population of vesicles which can be separated into low-density and high-density sarcoplasmic reticulum vesicles by sucrose gradient centrifugation [31] (Fig. 2). Halothane (0.5 mM) had essentially no effect on Ca^{2+} accumulation by the low-density sarcoplasmic reticulum vesicles, but did reduce Ca^{2+} uptake by the high-density sarcoplasmic reticulum (Fig. 2). Due to the high content of calsequestrin in the high-density sarcoplasmic reticulum vesicles and in the heavy sarcoplasmic reticulum vesicles, these membrane fractions are believed to contain vesicles derived from the terminal cisternae of the sarcoplasmic reticulum. The ability of extravesicular Ca^{2+} to induce Ca^{2+} release has also been shown to be a property of the high-density sarcoplasmic reticulum vesicle but not of the low-density vesicles [58–61]. These data show that only a subpopulation of sarcoplasmic reticulum vesicles is sensitive to 0.5 mM halothane. Halothane would have little effect on Ca^{2+} accumulation by preparations with a high content of nonjunctional, light sarcoplasmic reticulum vesicles. This is one reason why some investigators have failed to observe Ca^{2+} release with halothane.

The effect of activators and inhibitors of Ca^{2+}-induced Ca^{2+} release on the inhibition of Ca^{2+} accumulation by halothane was measured to determine if the two processes are related. Ruthenium

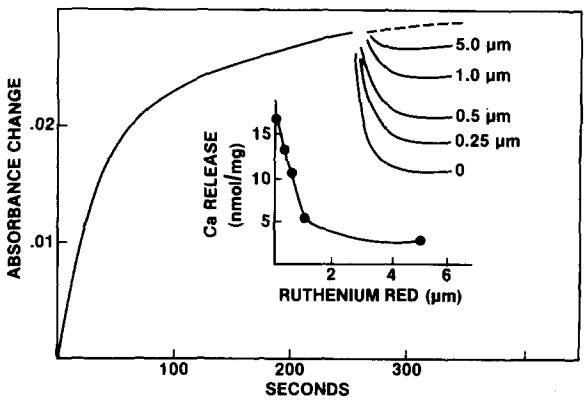

Fig. 3. Inhibition of halothane-induced Ca^{2+} release by Ruthenium red. The Ca^{2+} uptake medium contained 0.15 M KCl, 4 mM $MgSO_4$, 1 mM histidine (pH 6.8), 5 μM $CaCl_2$, 0.1 mM arsenazo III, 1 mM ATP, 40 μg protein/ml heavy sarcoplasmic reticulum vesicles, and Ruthenium red at the indicated concentration. Ca^{2+} transport was initiated by the additionof the vesicles. After 250 s, halothane (1.0 M in ethanol) was diluted 2000-fold into the Ca^{2+} uptake medium. Ethanol was added to the control (— — —) containing no Ruthenium red. Ca^{2+} accumulation at 20°C was monitored by measuring the absorbance difference ($A_{660\,nm} - A_{685\,nm}$) of the Ca^{2+}-arsenazo III complex as described in Fig. 1.

red, an inhibitor of Ca^{2+}-induced Ca^{2+} release [26,58,59] also inhibited the effect of halothane on Ca^{2+} uptake [26] (Fig. 3). Ca^{2+} uptake was measured in the presence of various ruthenium red concentrations. The assay conditions (relatively low [Ca^{2+}], high [Mg^{2+}]), were selected to minimize Ca^{2+}-induced Ca^{2+} release so that in the absence of halothane, Ruthenium red had little effect on Ca^{2+} uptake. The drop in Ca^{2+} accumulation upon addition of 0.5 mM halothane was reduced by Ruthenium red. Half-maximal inhibition was obtained at 0.6 μM Ruthenium red. Addition of Ruthenium red to vesicles after halothane-induced Ca^{2+} release restores the vesicles to the same Ca^{2+} loading level obtained in the absence of halothane, indicating that the halothane effect is reversible.

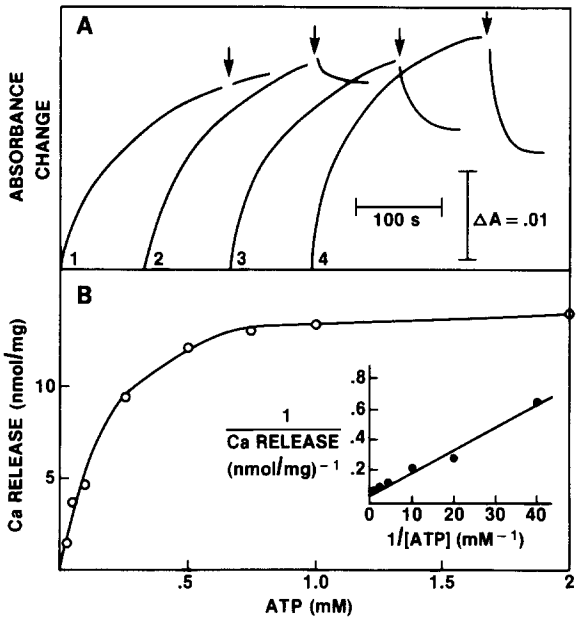

Fig. 4. The relationship between the ATP concentration and halothane-induced Ca^{2+} release. (A) The Ca^{2+} uptake medium contained 0.15 M KCl, 2.0 mM $MgSO_4$, 10 mM histidine (pH 6.8), 0.1 mM arsenazo III, 0.5 mM phospho*enol*pyruvate, 21 U/ml pyruvate kinase and either 12.5 μM MgATP (1), 100 μM MgATP (2), 250 μM MgATP (3) or 500 μM MgATP. Ca^{2+} transport was initiated by the addition of the heavy sarcoplasmic reticulum vesicles (80 μg protein/ml). Halothane (0.5 mM) was added 200 s after the initiation of Ca^{2+} uptake. Ca^{2+} accumulation at 20°C was monitored by measuring the absorbance difference ($A_{660nm} - A_{685nm}$) of the Ca^{2+}-arsenazo III complex. (B) The amount of Ca^{2+} released by 0.5 mM halothane as measured in (A) as a function of the ATP concentration.

Adenosine triphosphate (ATP) which is an activator of Ca^{2+}-induced Ca^{2+} release [59,62–64] is required for halothane-induced Ca^{2+} release (Fig. 4). Ca^{2+} accumulation was measured at various ATP concentrations. Phospho*enol*pyruvate and pyruvate kinase were included to maintain a constant ATP concentration. At 12.5 μM ATP, halothane had no effect on Ca^{2+} uptake by the heavy sarcoplasmic reticulum vesicles. The reduction of Ca^{2+} accumulation caused by halothane became greater as the ATP concentration was increased to 1.0 mM. The apparent K_d of ATP determined from a double-recipical plot of Ca^{2+} release as a function of the ATP concentration (Fig. 4B, inset) was 0.3 mM. The K_m of the (Ca^{2+} + Mg^{2+})-ATPase for ATP is about 10 μM so that at low ATP concentrations, maximum Ca^{2+} uptake levels can be reached with very little effect of halothane. The ATP analogue Ado*PP*[NH]*P* can substitute for ATP in stimulating halothane-induced Ca^{2+} release (data not shown).

The amount of Ca^{2+} released by halothane was dependent on both the KCl and Mg^{2+} concentrations (Fig. 5). Ca^{2+} uptake was measured at various KCl and Mg^{2+} concentrations. The osmolarity was kept constant with glycine. The decrease in Ca^{2+} accumulation was measured following the addition of 0.5 mM halothane. In the absence of KCl, halothane did not effect Ca^{2+} uptake. The reduction in Ca^{2+} accumulation caused by halothane increased as the KCl concentration was raised from 2 to 150 mM. Similar results were obtained when a constant osmolarity was maintained with sucrose or glucose, indicating that these results were not caused by the inhibition of the halothane effect by glycine. Other salts (NaCl, LiCl, RbCl, CsCl, choline chloride, NH_4Cl, lysine chloride, Tris-HCl, potassium aspartate, potassium acetate, potassium glutamate, Tris-aspartate, sodium glutamate) could substitute for KCl, indicating that there is not a specific requirement for K^+ or Cl^-, but a high concentration of a salt must be present for halothane to inhibit Ca^{2+} accumulation. The release of Ca^{2+} by low concentrations of Ag^+ also showed a similar dependency on the KCl concentration [65].

Halothane-induced Ca^{2+} release was inhibited by Mg^{2+} (Fig. 5). Optimal Ca^{2+} release was obtained with the free Mg^{2+} concentration below 0.5

mM. In the presence of 75 mM KCl, 5 mM Mg^{2+} caused an 86% inhibition of halothane-induced Ca^{2+} release as compared to 0.5 mM Mg^{2+}.

The effect of the KCl concentration on Ca^{2+}-induced Ca^{2+} release is shown in Fig. 6. Miyamoto and Racker [58] demonstrated that the capacity of high-density sarcoplasmic reticulum vesicles to accumulate Ca^{2+} was dependent on the external Ca^{2+} concentration due to the action of Ca^{2+}-induced Ca^{2+} release. The higher the external Ca^{2+} concentration, the lower the Ca^{2+} loading level. Ruthenium red inhibited Ca^{2+}-induced Ca^{2+} release to permit maximum loading.

In the absence of KCl, the Ca^{2+} loading level was not dependent on the external Ca^{2+} concentration in the range of 3 to 50 μM (Fig. 6B) and was not significantly altered by Ruthenium red (Fig. 6A), indicating that Ca^{2+}-induced Ca^{2+} release also required KCl (or other salt). In the presence of 150 mM KCl but in the absence of Ruthenium red, the Ca^{2+} loading levels were reduced by Ca^{2+}-induced Ca^{2+} release as the Ca^{2+} concentration of the medium was increased from 3 to 50 μM. In the presence of 5 μM Ruthenium red, Ca^{2+}-induced Ca^{2+} release is inhibited and Ca^{2+} accumulation actually increases as the external Ca^{2+} concentration is raised from 3 to 50 μM (Fig. 6A), presumably due to competition between Ruthenium red and Ca^{2+} binding to the Ca^{2+} pump protein. These data indicate that Ca^{2+}-induced Ca^{2+} release requires the presence of a salt such as KCl.

Mg^{2+} inhibits Ca^{2+}-induced Ca^{2+} release [1,26,52,57]. The effect of Mg^{2+} on Ca^{2+} accumulation by the heavy sarcoplasmic reticulum vesicles is shown in Fig. 7. When Ca^{2+}-induced Ca^{2+} release is inhibited with Ruthenium red (Fig. 7B) or by the absence of KCl (Fig. 7A), varying the

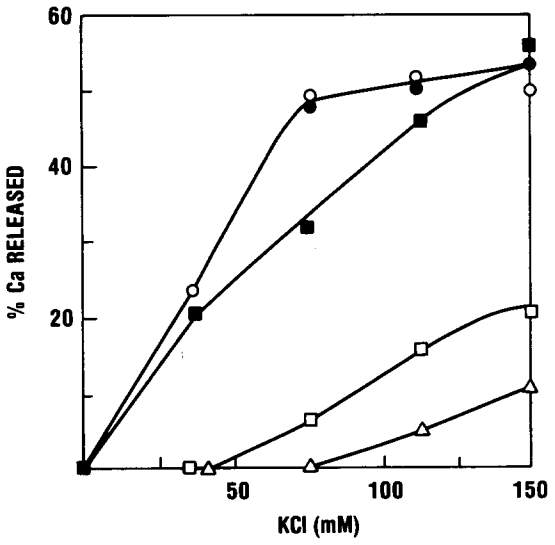

Fig. 5. The effect of KCl and $MgSO_4$ on halothane-induced Ca^{2+} release. The Ca^{2+} uptake medium contained 10 mM histidine (pH 6.8), 25 μM $CaCl_2$, 1.0 mM ATP (sodium salt), 40 μg protein/ml heavy sarcoplasmic reticulum vesicles, 100 μM arsenazo III, varying ratios of 0.15 M KCl and 0.3 M glycine, and either 10 mM (△), 5 mM (□), 2 mM (■), 1 mM (○) or 0.5 mM (●) $MgSO_4$. Halothane (0.5 mM) was added 180 s after Ca^{2+} uptake was initiated. Ca^{2+} accumulation was measured by monitoring the absorbance difference ($A_{660nm} - A_{685nm}$) of the Ca^{2+}-arsenazo III complex. The percent Ca^{2+} released 30 s after halothane addition is reported.

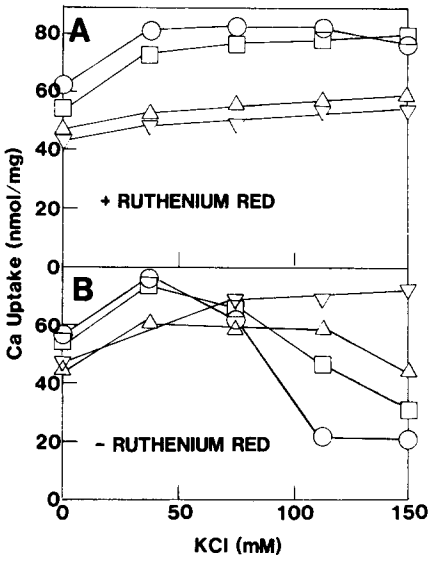

Fig. 6. Effect of Ruthenium red, extrnal Ca^{2+} and KCl on Ca^{2+} accumulation by heavy sarcoplasmic reticulum vesicles. The Ca^{2+} uptake medium contained 10 mM histidine (pH 6.8), 1 mM $MgSO_4$, 50 μg protein/ml heavy sarcoplasmic reticulum vesicles and different ratios of 0.15 M KCl and 0.3 M glycine. The external Ca^{2+} concentration was also varied (50 μM Ca^{2+}, ○; 25 μM Ca^{2+}, □; 25 μM Ca^{2+} + 20 μM EGTA, △; 25 μM Ca^{2+} + 30 μM EGTA, △). Ca^{2+} transport was initiated by the addition of 1 mM ATP (sodium salt). Ca^{2+} uptake was measured in the presence (panel A) and in the absence (panel B) of 5 μM Ruthenium red. Ca^{2+} accumulation at 20°C was measured by monitoring the absorbance change of the arsenazo III-Ca^{2+} complex at 660 nm using 685 nm as a reference wavelength. The Ca^{2+} loading level after 250 s is reported.

Mg^{2+} concentration between 1 and 5 mM had little effect on the capacity of the heavy sarcoplasmic reticulum vesicles to accumulate Ca^{2+}. But in the absence of Ruthenium red and in the presence of 150 mM KCl, Ca^{2+} accumulation by the heavy sarcoplasmic reticulum vesicles increased as the Mg^{2+} concentration was raised from 0.5 to 5.0 mM. The experiments shown in Figs. 5 and 6 demonstrate that both Ca^{2+}-induced and halothane-induced Ca^{2+} release are blocked by high Mg^{2+} concentrations.

The rate of Ca^{2+} accumulation is dependent on both the rate of Ca^{2+} transport and the rate of Ca^{2+} efflux so that an apparent release of Ca^{2+} could be a result of a reduced rate of Ca^{2+} transport or a higher rate of Ca^{2+} efflux. Fig. 8 and 9 show that the rate of Ca^{2+} efflux is increased by external Ca^{2+} and halothane. Sarcoplasmic reticulumn vesicles were passively loaded with 15 mM Ca^{2+} and diluted into medium containing chlortetracycline to monitor the internal Ca^{2+} concentration. The fluorescence of chlortetracycline is proportional to the intravesicular Ca^{2+} concentration [55]. Fig. 8A shows the effect of MgATP and MgAdo*PP*[NH]*P* on the rate of Ca^{2+} release. Addition of ATP or Ado*PP*[NH]*P* to the dilution medium containing 18 μM free Ca^{2+} caused a rapid release of Ca^{2+} corresponding to a 25% drop in the chlortetracycline fluorescence. At low free Ca^{2+} concentrations ($\cong$ 0.1 μM), ATP had no effect on the rate of Ca^{2+} efflux (Fig. 9A). When Ruthenium red was included in the dilution medium, neither ATP nor Ado*PP*[NH]*P* influenced the initial rate of Ca^{2+} release (Fig. 8B). In the presence of ATP or Ado*PP*[NH]*P*, halothane (0.5 mM) increased the amount of

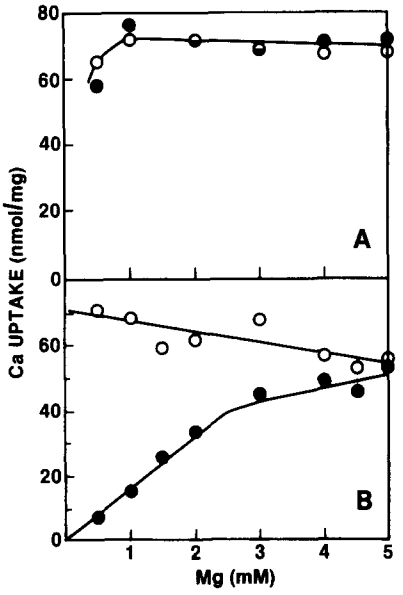

Fig. 7. Effect of Mg^{2+} on Ca^{2+} accumulation by heavy sarcoplasmic reticulum in glycine (A) or KCl (B) medium. The Ca^{2+} uptake medium contained 10 mM histidine (pH 6.8), 100 μM arsenazo III, 25 μM $CaCl_2$, 0.1 mg protein/ml heavy sarcoplasmic reticulum and 0.3 M glycine (panel A) or 0.15 M KCl (panel B) with (○) or without (●) 5 μm Ruthenium red at 20°C. The $MgSO_4$ concentration was varied as indicated. Ca^{2+} accumulation was measured by monitoring the absorbance difference of the arsenazo-Ca^{2+} complex ($A_{660nm} - A_{685nm}$). The Ca^{2+} loading level 250 s after Ca^{2+} transport was initiated is reported.

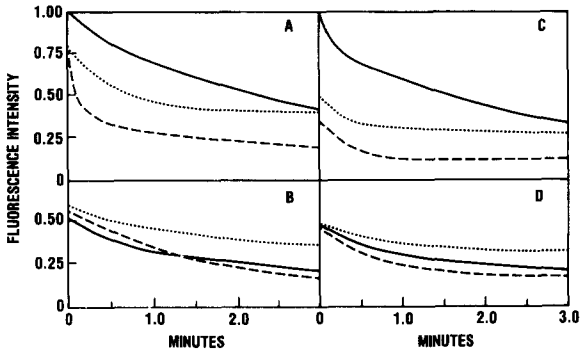

Fig. 8. Effect of ATP, Ado*PP*[NH]*P*, Ruthenium red and halothane on Ca^{2+} efflux from heavy sarcoplasmic reticulum vesicles. (A) Heavy sarcoplasmic reticulum vesicles were passively loaded with 15 mM $CaCl_2$ by incubating the vesicles for 15 h in 0.15 M potassium glutamate, 10 mM histidine (pH 6.8), 2 mM $MgSO_4$ and 15 mM $CaCl_2$. Ca^{2+} efflux was initiated by diluting the vesicle 50-fold into 0.15 M potassium glutamate, 1 mM $MgSO_4$, 10 mM histidine (pH 6.8), 0.3 mM EGTA and 10 μM chlortetracycline (———) at 20°C. In some experiments, 1.0 mM ATP (·····) or Ado*PP*[NH]*P* (— — —) was included in the dilution medium. Ca^{2+} efflux was monitored by measuring the decrease in the fluorescence intensity (excitation wavelength 390 nm, emission wavelength 530 nm) of the Ca^{2+}-chlortetracycline complex which depends of the concentration of internal Ca^{2+}. (B) Same as A, except 5 μM Ruthenium red was included in the dilution medium. The low fluorescence with Ruthenium red is due to the quenching of the chlortetracycline fluorescence by Ruthenium red. (C) Same as A, except 0.5 mM halothane was included in the dilution medium. (D) Same as B, except 0.5 mM halothane was included in the dilution medium

rapidly released Ca^{2+}, as indicated by the initial drop in the chlortetracycline signal to 50% of the control value (Fig. 8C). In the absence of ATP or Ado*PP*[NH]*P* (Fig. 8C) or in the presence of Ruthenium red (Fig. 8D), halothane had little effect on the rate of Ca^{2+} efflux. ATP or Ca^{2+} did not effect Ca^{2+} release from low-density sarcoplasmic reticulum vesicles [65,66].

Expernal Ca^{2+} is required for halothane-induced Ca^{2+} release (Fig. 9). At low Ca^{2+} concentrations ($\cong 0.1$ μM), 0.5 mM halothane had no effect on Ca^{2+} efflux. At 0.2 μM, Ca^{2+} only slightly increased the rate of Ca^{2+} efflux from the heavy sarcoplasmic reticulum vesicles (trace 2, Fig. 9A), but in the presence of 0.5 mM halothane, 0.2 μM Ca^{2+} caused a rapid release of Ca^{2+} corresponding to 20% drop in the chlortetracycline fluorescence (trace 2, Fig. 9C). In the presence of Ruthenium red, the rate of Ca^{2+} efflux was not dependent on the extravesicular Ca^{2+} concentration. The dependency of halothane-induced Ca^{2+} release on external Ca^{2+} may be another reason why some investigators fail to observe an effect of halothane on Ca^{2+} uptake. If the Ca^{2+} concentration is too high (50–100 μM), Ca^{2+}-induced Ca^{2+} release occurs even without halothane so that halothane addition has less of an effect on Ca^{2+} accumulation. If the Ca^{2+} concentration is too low, halothane-induced Ca^{2+} release is inhibited.

The effect of the external Ca^{2+} concentration on Ca^{2+} uptake by sarcoplasmic reticulum vesicles in the presence of halothane was further investigated by measuring the ability of sarcoplasmic reticulum vesicles to lower the free Ca^{2+} concentration. Ca^{2+} uptake was monitored using quin II as a fluorescent indicator. In the presence of 10 μM total Ca^{2+} and 10 μM quin II ($[Mg^{2+}] \cong 2$ mM), sarcoplasmic reticulum vesicles lowered the Ca^{2+} concentration to about 0.41 μM. The rate of Ca^{2+} uptake was about the same with or without halothane. Increasing the total Ca^{2+} concentration to 12.5 μM decreased the initial rate of Ca^{2+} uptake in the presence of halothane by 67%, but the final free Ca^{2+} concentration (0.49 μM) obtained was the same with or without halothane. In the presence of 15 μM total Ca^{2+}, halothane inhibited both the rate and capacity of heavy sarcoplasmic reticulum vesicles to accumulate Ca^{2+}. Ca^{2+} uptake was not altered by 0.5 mM

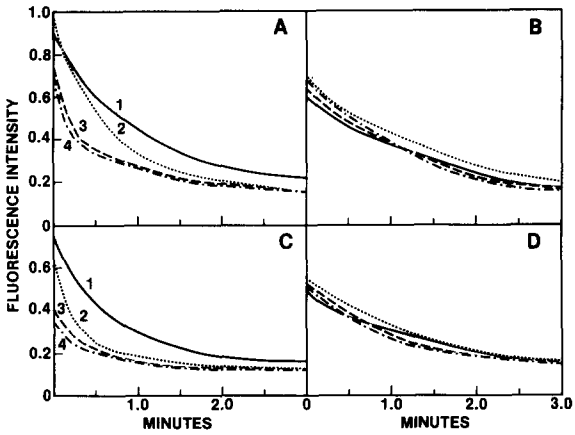

Fig. 9. Effect of the external Ca^{2+} concentration on the efflux of Ca^{2+} from heavy sarcoplasmic reticulum. (A) Heavy sarcoplasmic reticulum vesicles were loaded with 15 mM $CaCl_2$ as described in Fig. 8. Ca^{2+} efflux was initiated by diluting the vesicles 50-fold into medium containing 0.15 M potassium glutamate, 10 mM histidine (pH 6.8), 10 μM chlortetracycline, 1 mM MgAdo*PP*[NH]*P* and 1.5 mM (———), 1.0 mM (·····), 0.4 mM (— — —), or 0.3 mM (·–·–·) EGTA. The free Ca^{2+} concentrations of these solutions were calculated to be 0.11 μM (1), 0.18 μM (2), 0.61 μM (3) and 1.0 μM (4), respectively. Ca^{2+} efflux was monitored by measuring the fluorescence of the chlortetracycle-Ca^{2+} complex (excitation wavelength 390 nm, emission wavleength 530 nm). (B) Same as A, except 5 μM Ruthenium red was included in the dilution medium. (C) Same as A, except 0.5 mM halothane was included in dilution medium. (D) Same as C, except 5 μM Ruthenium red was included in the dilution medium.

halothane in the presence of 5 μM Ruthenium red. This experiment demonstrates that at lower Ca^{2+} loads, heavy sarcoplasmic reticulum can lower the free Ca^{2+} concentration to levels that inhibit halothane-induced Ca^{2+} release. But as the total Ca^{2+} increases, the sarcoplasmic reticulum vesicle is less able to lower the free Ca^{2+} concentration and halothane has a greater effect on Ca^{2+} accumulation.

Discussion

Halothane at clinical concentrations (0.1–0.5 mM) decreased Ca^{2+} accumulation by a subpopulation sarcoplasmic reticulum vesicles. This is due to an increased rate of Ca^{2+} efflux (Figs. 8 and 9). The (Ca^{2+} + Mg^{2+})-ATPase activity is not significantly effected by 0.5 mM halothane. Halothane appears to act at a specific site on the sarcoplasmic

reticulum membrane as opposed to a general perturbation of the membrane. Halothane does not affect Ca^{2+} uptake by light sarcoplasmic reticulum vesicles, indicating that neither the Ca^{2+} pump activity nor the permeability of the lipid bilayer is significantly altered by halothane. Ca^{2+} release induced by halothane was inhibited by Mg^{2+} (Fig. 5) and Ruthenium red (Figs. 3 and 10); and required ATP or an ATP analogue (Figs. 4 and 8), Ca^{2+} (Figs. 9 and 10), and KCl or similar salt (Fig. 5). The requirement for extravesicular Ca^{2+} and ATP is best explained by a Ca^{2+}-release protein which contains regulatory sites that bind Ca^{2+} and ATP. Activation of the Ca^{2+}-release protein by extravesicular Ca^{2+} may involve the dissociation of an internal salt-bridge located in a relatively hydrophobic environment. This would explain the requirement for KCl. Halothane penetration into the hydrophobic environment may make such a conformational change more favorable.

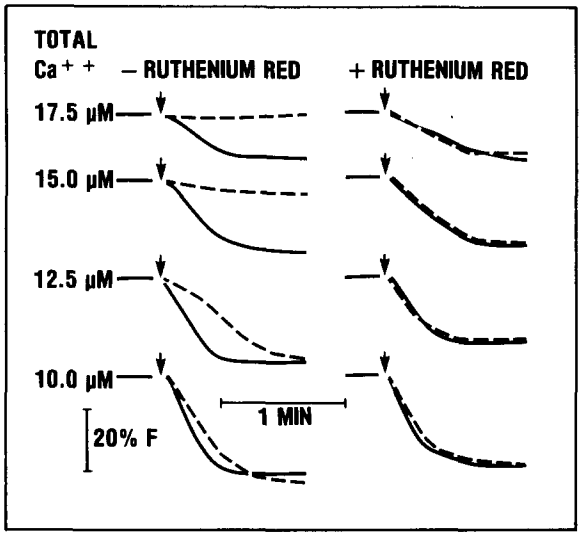

Fig. 10. Effect of halothane on Ca^{2+} uptake by heavy sarcoplasmic reticulum. (A) The Ca^{2+} uptake medium contained 0.15 M KCl, 10 mM histidine (pH 6.8), 2 mM $MgSO_4$, 10 μM quin II and 0.2 mg protein/ml heavy sarcoplasmic reticulum vesicles. Ca^{2+} uptake was initiated by the addition of 1 mM MgATP (↓). The traces on the left were obtained without Ruthenium red present, while the traces on the right were with 10 μM Ruthenium red included in the Ca^{2+} uptake medium. In some experiments (— — —), 0.5 mM halothane was included in the Ca^{2+} uptake medium, while the controls lacked halothane (———). The total Ca^{2+} in the Ca^{2+} uptake medium was varied as indicated. The free Ca^{2+} concentration was monitored by measuring the fluorescence of the quin II-Ca^{2+} complex (excitation wavelength 339 nm, emission wavelength 492 nm). The response of quin II fluorescence to [Ca^{2+}] was calibrated using EGTA-Ca^{2+} buffers. The initial fluorescence was the same for each sample, since the quin II was saturated with Ca^{2+} before Ca^{2+} transport was initiated.

Halothane appears to enhance Ca^{2+}-induced Ca^{2+} release, since both Ca^{2+}-induced and halothane-induced Ca^{2+} release was influenced by Mg^{2+}, Ruthenium red, ATP and KCl in a similar manner. In addition, halothane-induced Ca^{2+} release had a requirement for extravesicular Ca^{2+} (Fig. 9).

The relationship between the halothane-induced Ca^{2+} release from isolated sarcoplasmic reticulum vesicles and halothane-induced muscle activity of individuals with malignant hyperthermia is unclear, but it is possible that at least some of these individuals have a slightly increased resting level of cytoplasmic Ca^{2+}. Halothane may enhance Ca^{2+}-induced Ca^{2+} release in all individuals but only in those with increased cytoplasmic Ca^{2+} concentration is hyperthermia triggered by halothane. This is best illustrated by the experiment described in Fig. 10. At low Ca^{2+} loads, the ability of sarcoplasmic reticulum vesicles to lower the extravesicular Ca^{2+} is not effected by halothane, but at higher Ca^{2+} loads, the external Ca^{2+} concentration at steady state is higher and halothane inhibits Ca^{2+} accumulation. Therefore, anything that increases the resting cytoplasmic Ca^{2+} concentration could contribute to halothane-triggered malignant hyperthermia.

The physiological role of Ca^{2+}-induced Ca^{2+} release is also unclear. It has been proposed that Ca^{2+} entering from the T-tubule may activate Ca^{2+} release. There is evidence that the concentration of the voltage-gated Ca^{2+} channel in the T-tubule is high [40,41]. But removal of the extracellular Ca^{2+} does not prevent excitation-contraction coupling [68–71]. Therefore, the trigger Ca^{2+} would have to equilibrate with the extracellular medium very slowly. The existence of such a Ca^{2+} compartment has not been shown. Perhaps depolarization of the transverse tubule alters the affinity of the Ca^{2+}-binding regulatory site of the Ca^{2+} release protein analogous to the enhancement of Ca^{2+}-induced Ca^{2+} release by halothane. Under resting conditions, the affinity of the Ca^{2+} regulatory site would be too low to bind Ca^{2+}. Upon activation, the

affinity would increase, allowing Ca^{2+} to bind to the regulatory site initiating Ca^{2+} release. After repolarization of the transverse tubule, the Ca^{2+}-affinity of the regulatory site would return to the low-affinity state.

It is possible that Ca^{2+}-induced Ca^{2+} release acts to amplify Ca^{2+} release once the release process is initiated by another mechanism. If this is so, one would have to explain how Ca^{2+}-induced Ca^{2+} release is terminated. With isolated sarcoplasmic reticulum vesicles, Ca^{2+} release induced by Ca^{2+} or halothane was not transient unless there was an excess of halothane-insensitive vesicles capable of accumulating the released Ca^{2+}, or Ruthenium red or Mg^{2+} was added to block Ca^{2+} release. Perhaps Ca^{2+}-induced Ca^{2+} release is turned off by a mechanism which is lost during the preparation of the vesicles such as a Ca^{2+}-activated protein kinase.

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