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THE EFFECT OF HALOTHANE ON THE STABILITY OF Ca^{2+} TRANSPORT ACTIVITY OF ISOLATED FRAGMENTED SARCOPLASMIC RETICULUM*

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Abstract—The effects of the inhalation anaesthetic agent, halothane ($CF_3CHBrCl$), on the stability of the calcium transport system of isolated rabbit white skeletal muscle sarcoplasmic reticulum have been studied. Calcium transport activity was unaffected when suspensions of sarcoplasmic reticulum vesicles were preincubated at 37° and pH 6.8 at concentrations of halothane below 5 mM, but was progressively inactivated at higher concentrations. (Ca^{2+},Mg^{2+})-ATPase activity was enhanced during inactivation of calcium transport. At pH 6.3 and 5.8, halothane increased the first order rate constants of inactivation and effects were noted in the anaesthetic range of concentration (1–2 mM). The inulin inaccessible space of membrane vesicles did not change appreciably during the period of treatment with halothane, excluding increased permeability as an explanation of the inhibition of calcium accumulation. Inactivation was irreversible and highly temperature dependent, with an activation energy of 52.7 kcal/mol. Calcium ions had a protective effect against inactivation ($K_{0.5\ (Ca2+)} = 1.5 \times 10^{-6}$M), as did ATP ($K_{0.5\ (ATP)} \simeq 10^{-6}$M). It is concluded that mild acid conditions and halothane act synergistically during inactivation of the calcium transport system of sarcoplasmic reticulum membranes. These studies suggest that halothane interacts with the (Ca^{2+}, Mg^{2+})-ATPase protein at the ATP-specific binding site or that it disrupts protein–lipid associations in the membrane. In either case the destabilizing effect of halothane may be modified by the conformational state of the protein.

The interaction of both local and general anaesthetics with membranes is fundamental to the mechanism of anaesthesia. Interactions with both lipid [1–3] and protein [4, 5] moeities of the membrane have been suggested. Consequently, anaesthetic agents may be used as probes of membrane function. Halothane ($CF_3CHBrCl$), a widely used general anaesthetic agent, initiates the condition of malignant hyperthermia in susceptible humans and pigs [6, 7]. In addition to hyperthermia, the syndrome is characterized by rigor and severe lactacidosis. *In vitro*, halothane has been shown to potentiate contracture of muscle fibres from susceptible individuals [8]. It has been postulated that the triggering agent releases calcium from defective calcium-storing sarcoplasmic reticulum (SR)‡ membranes [9]. The resultant increase in cytoplasmic calcium concentration accelerates glycogenolysis and glycolysis by activation of phosphorylase *b* kinase and myosin ATPase and inhibits troponin, permitting contractures to occur. Calcium would then accumulate in mitochondria, causing uncoupling of oxidative phosphorylation. These events could liberate sufficient heat to account for the rise in body temperature. However, in spite of this attractive hypothesis, definitive studies [10, 11] have failed to demonstrate a defect in ATP-dependent active calcium transport by preparations of SR membranes isolated from malignant hyperthermia-susceptible individuals.

Halothane is known to inhibit mitochondrial function by blocking NADH-dependent electron transfer reactions [12, 13]. Mitchelson and Hird [14] have demonstrated that skeletal muscle mitochondria are especially sensitive to the effects of halothane under acid conditions. Mild acid conditions also irreversibly inactivate the calcium transport system of rabbit SR membranes [15]. In this study we have investigated the effects of halothane and acid conditions on the stability of the calcium transport system of rabbit white skeletal muscle SR *in vitro*. The findings suggest that halothane interacts with the (Ca^{2+},Mg^{2+})-ATPase (EC 3.6.1.3, ATP phosphohydrolase) protein which results in destabilization rather than direct inhibition of the transport system.

METHODS

Preparation of SR vesicles and treatment with halothane. Rabbits were stunned by sharp blows to the back of their necks. No anaesthetic agents were employed. SR vesicles were prepared from longissimus dorsi muscle by differential centrifugation and purified by 0.6 M KCl extraction and centrifugation through a continuous sucrose density gradient [16]. Stock suspensions of SR, approximately 3–4 mg protein/ml, were stored at 0° in 0.3 M sucrose, 10 mM imidazole, pH 7.4. The SR suspension, 0.1 ml, was diluted 30 fold into buffered ice-cold halothane solution containing either 40 mM ammonium acetate or 20 mM histidine, and 50 mM KCl at the stated pH values. The diluted suspension was immediately

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‡ Abbreviations: Sarcoplasmic reticulum (SR); phosphoenol pyruvate (PEP); pyruvate kinase (PK); ethylene glycol-bis-(2-aminoethyl)-tetra-acetic acid (EGTA).

aspirated into a thick walled glass coil (2 mm internal diameter) with thermostat. This procedure minimized losses of halothane during the inactivation process and ensured rapid temperature equilibration. Inactivation was studied at 37° unless otherwise indicated. Samples (50 μl) were delivered from the coil at timed intervals directly into either calcium uptake buffer (425 μl) for measurement of calcium transport or into the ATPase assay buffer (2.65 ml), both kept at 0°. Under these experimental conditions the residual transport or ATPase activity remained constant until assay (within 60 min).

Assay of ATP-dependent calcium transport. Calcium transport in the presence of oxalate was determined by the millipore filtration method of Martonosi and Feretos [17]. The reaction at 25° was initiated by adding 25 μl of 100 mM ATP to the test tube containing SR suspension in the uptake medium. The uptake medium consisted of 20 mM histidine, pH 7.4, 50 mM KCl, 5 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM $^{45}CaCl_2$, and 5 mM potassium oxalate. Transport was measured over a 2 min period. The ^{45}Ca radioactivity remaining on the millipore filter was determined in a Beckman LS 233 scintillation spectrometer after dissolving the filters in 4 ml of Instagel (Packard Instruments).

Assay of ATPase activity. Both Mg^{2+}-ATPase and total ATPase activity were measured spectrophotometrically by monitoring the decrease in absorbance of NADH at 340 nm in the presence of an ATP regenerating system [18]. The (Ca^{2+},Mg^{2+})-ATPase activity was calculated by deducting the Mg^{2+}-ATPase from the total activity.

Determination of membrane permeability. Membrane permeability was studied by measuring the inulin inaccessible space of packed vesicles according to the method of Duggan and Martonosi [19] using inulin ^{14}C-carboxylic acid. This determination required relatively large amounts of purified SR. The method of Eletr and Inesi [20] was used in this case since it resulted in higher yields of SR vesicles than that of Boland *et al.* [16] employed for other experiments. Vesicles were used at a concentration of 15 mg protein/ml.

Preparation of solutions of known free Ca^{2+} concentrations. Calcium–EGTA buffers were made up by mixing varying amounts of 10 mM $CaCl_2$ with a fixed volume of 10 mM EGTA. Free calcium ion concentrations were calculated using a value of 10^{-11}M for the true dissociation constant K, of the Ca–EGTA complex [21] and an apparent dissociation constant, K', at pH 7.4 of 4.13×10^{-8}M.

Halothane solutions. Halothane in aqueous solution was assayed by gas–liquid chromatography by the method of Gadsen *et al.* [22]. Buffer solutions were saturated with halothane at 30°. These were found to contain similar concentrations to halothane-saturated distilled water, previously determined to be 21 mM [23]. The saturated solutions were diluted with buffer to the appropriate halothane concentration immediately before use.

Calcium-free ATP. Calcium-free ATP was prepared by passing 100 mM disodium ATP through a 10×1 cm Chelex 100 (K^+ form) column, equilibrated with ammonium acetate (10 mM) at pH 7.0.

Chemicals. EGTA was obtained from Fluka AG, Switzerland; Halothane from Halocarbon Laboratories Inc., Hackensack, NJ; ATP, PEP and pyruvate kinase from Sigma Chemical Co., St. Louis, MO and Chelex 100 cation exchange resin from Bio Rad Laboratories, Richmond, CA. $^{45}CaCl_2$ and inulin [^{14}C]-carboxylic acid were purchased from The Radiochemical Centre, Amersham, England. The ionophore X 537A was a gift from Roche Products (Pty) Ltd., Isando, Republic of South Africa. All other chemicals were of Analytical Reagent grade.

RESULTS

Effect of halothane on the in vitro *stability of Ca^{2+} transport and (Ca^{2+},Mg^{2+})-ATPase activity of SR membranes.* Figure 1 shows that over a 5 min period at 37° in the presence of 10 mM halothane, calcium transport by SR was abolished. Over the same time interval the (Ca^{2+},Mg^{2+})-ATPase activity increased from 1.05 to approximately 3 μmoles/min/mg protein, while the basal activity (Ca absent) remained constant at 0.3 μmoles/min/mg protein. The ATPase activities in the presence of the ionophore X537A were unaltered. The inclusion of a calcium specific ionophore in the assay ensured that the ATPase activity as measured represented the maximal calcium stimulated activity (cf. [24]). In the absence of an ionophore, accumulation of intravesicular calcium leads to inhibition of ATPase activity.

In a separate experiment, the effect of halothane on the inulin inaccessible space of SR vesicles was determined. Incubation with 10 mM halothane for 15 min decreased the inulin inaccessible water space slightly from 4.0 to 3.7 μl/mg protein, indicating that the observed inactivation of nett calcium transport could not be accounted for by increased membrane permeability (cf. [19]).

The inactivation of calcium transport activity of SR by halothane was shown to be irreversible. Incubation with 7 mM halothane for 5 min reduced the calcium transport activity from 1200 nmoles/min/mg protein to 50 nmoles/min/mg protein. Removal of the halothane by bubbling nitrogen through the suspension for 10 min did not restore the transport activity nor was recovery evident after storage of vesicles at 0° for up to 20 hr. In control experiments, bubbling of nitrogen through the vesicle suspension did not affect calcium transport activity.

Halothane concentration dependence and the effect of pH on the stability of SR calcium transport. The effect of prior incubation of suspensions of SR vesicles at various halothane concentrations was investigated at three different pH values, viz. 6.8, 6.3 and 5.8. In these and subsequent experiments, the decline in calcium transport was first order with respect to residual activity, as was previously shown for acid inactivation [15]. In Fig. 2, the first order rate constants of inactivation are shown as a function of halothane concentration. Preincubation of vesicles at pH 5.8 in the absence of halothane caused a significantly enhanced rate of inactivation as previously shown [15]. At pH 6.8, calcium transport was unaffected by concentrations of halothane below approx. 5 mM. Between 5 and 10 mM, the transport of calcium was more rapidly inactivated. At the more

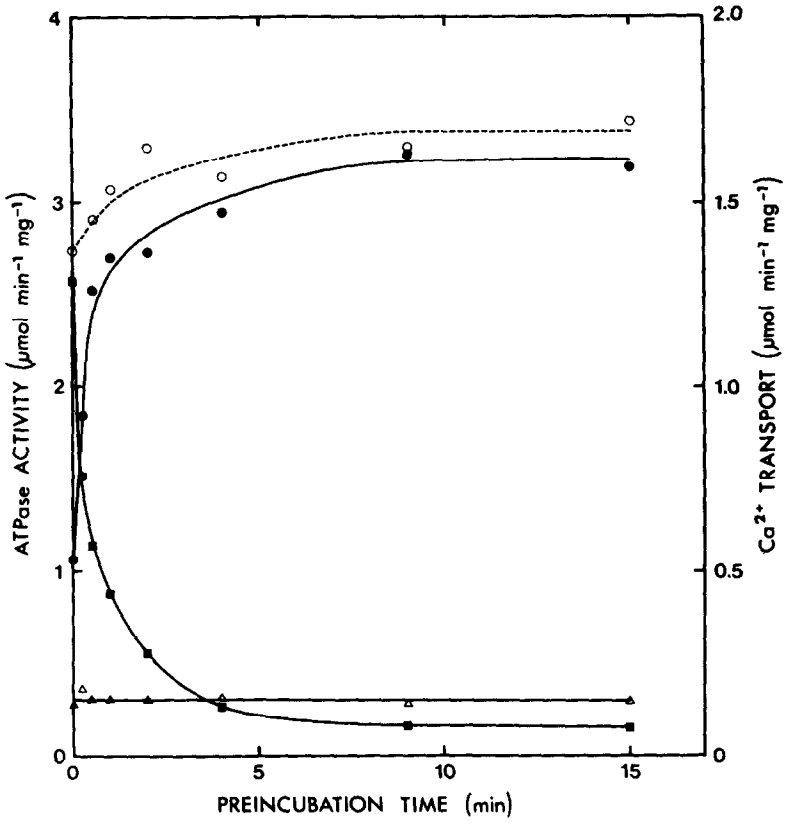

Fig. 1. Effect of preincubation of SR vesicles with halothane on calcium transport, (Ca^{2+}, Mg^{2+})-ATPase and Mg^{2+}-ATPase activities. Suspensions of SR vesicles (0.1 mg/ml) were preincubated at 37° in the presence of 10 mM halothane (see Methods) in 40 mM ammonium acetate, pH 6.8. At timed intervals samples were taken for the measurement of calcium transport at 26° (—■—■—), (Ca^{2+}, Mg^{2+})-ATPase (—●—●—), Mg^{2+}-ATPase (—△—△—) and (Ca^{2+}, Mg^{2+})-ATPase in the presence of X537A (—○—○—).

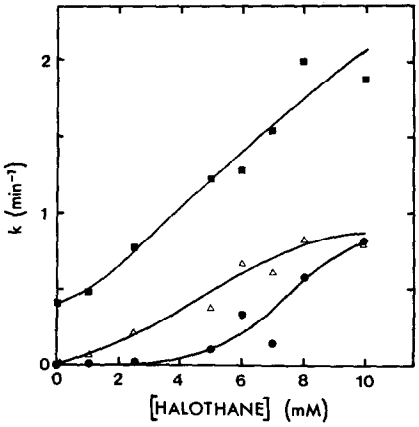

Fig. 2. Halothane concentration dependence and effect of pH on inactivation of Ca^{2+} transport by SR vesicles. Suspensions of SR vesicles (0.1 mg/ml) were incubated at 37° in 40 mM ammonium acetate buffers at pH 5.8, 6.3 and 6.8 at halothane concentrations between 0 and 10 mM. Calcium uptake was assayed as described before at 26° and the rate constant for inactivation, k, at the various pH values and halothane concentrations was calculated (—●—●—, pH 6.8; —△—△—, pH 6.3; —■—■—, pH 5.8).

acidic pH values, 6.3 and 5.8, two effects could be noted: (a) the rate constants of inactivation were increased above the values obtained at pH 6.8 and (b) there was increased inactivation in the lower range (1–2 mM) of halothane concentrations. This is well within the concentration range reached in muscle during clinical anaesthesia [25]. Thus halothane and H^+ ions appear to have a synergistic effect in inactivating the calcium transport mechanism of SR vesicles.

The temperature dependence of halothane inactivation of calcium transport activity of SR. Inactivation of calcium transport activity by halothane was measured at various temperatures between 25° and 40°. The temperature dependence of the first order rate constants of inactivation, k, are shown in Fig. 3 in the form of an Arrhenius plot. As can be seen, the plot was linear, indicating no gross change in thermodynamic parameters of the inactivation process within the temperature limits investigated. Furthermore, the process was highly temperature dependent with an activation energy *Ea* of 52.7 kcal mol^{-1}.

Effect of free Ca^{2+} *and* Mg*ATP on halothane inactivation of* Ca^{2+} *transport activity of SR.* The SR

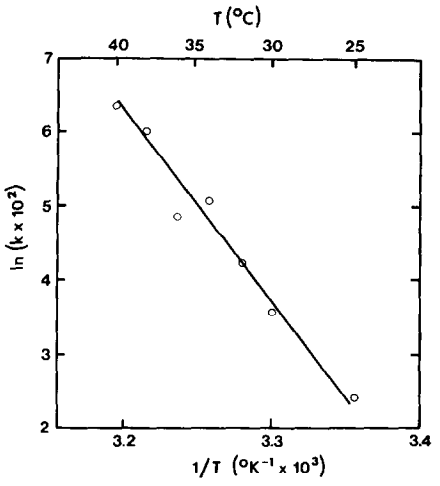

Fig. 3. Temperature dependence of halothane inactivation of calcium transport by SR vesicles. Suspensions of SR vesicles (0.1 mg/ml) were incubated in 40 mM ammonium acetate, pH 6.8, with 7 mM halothane at various temperatures. Aliquots were removed at intervals during a 2 min incubation period. Calcium uptake was assayed as described before at 26° and the temperature dependence of the first order rate constant of inactivation, k, in the form of an Arrhenius plot is shown where k is expressed as min^{-1}. (Ea = 52.7 kcal mol^{-1}.)

vesicles were incubated at 37° at pH 7.4 in the presence of 10 mM halothane with concentrations of free Ca^{2+} varying between 2×10^{-8}M and 3×10^{-4}M. The rate constant of inactivation, plotted against the free Ca^{2+} concentrations, is shown in Fig. 4. Calcium ions protected against halothane inactivation, with a half maximal effect $K_{0.5\,(Ca^{2+})}$ of 1.5×10^{-6}M.

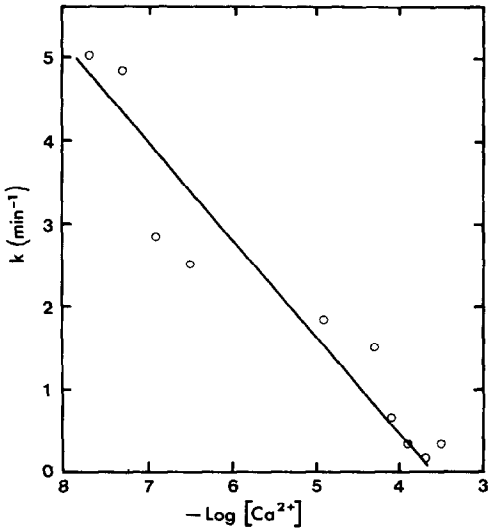

Fig. 4. The effect of free Ca^{2+} on the halothane inactivation of calcium transport by SR vesicles. Suspensions of SR vesicles (0.1 mg/ml) were incubated with 10 mM halothane at pH 7.4 in 20 mM histidine, 50 mM KCLl and 10 mM EGTA. Varying free calcium ion concentration was achieved by adding $CaCl_2$ (see Methods). Calcium transport was assayed at 26°, as described previously, and the first order rate constant of inactivation, k, plotted as a function of the free Ca^{2+} concentration.

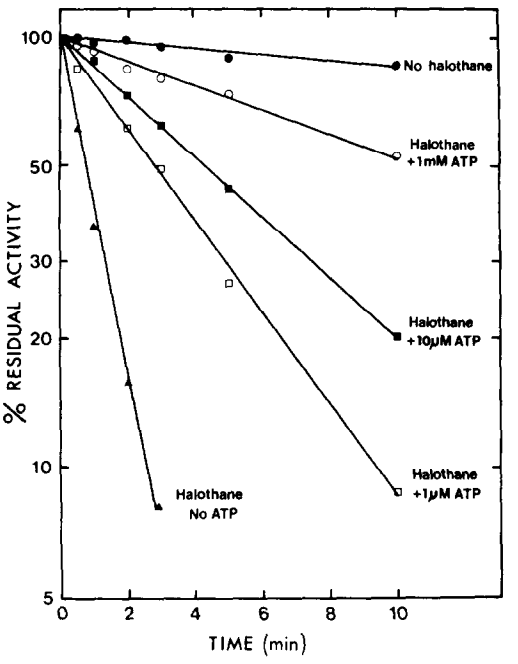

Fig. 5. The effect of ATP on the halothane inactivation of calcium transport by SR vesicles. Suspensions of SR vesicles (0.1 mg/ml) were incubated at 37° in 20 mM histidine, pH 7.4, 50 mM KCl, 5 mM $MgCl_2$, 4 mM PEP, 3 units/ml PK in the presence or absence of 10 mM halothane and concentrations of ATP indicated alongside the curves. Aliquots were taken at timed intervals and calcium transport immediately assayed by the millipore filtration method at 26°.

Figure 5 shows the effect of incubating vesicles in the presence of 10 mM halothane and varying concentrations of MgATP. In the presence of the PEP/PK ATP regenerating system, micromolar concentrations of ATP significantly decreased the first order rate constant of inactivation of calcium uptake by SR vesicles. ATP, 1 mM, almost completely blocked the effect of 10 mM halothane. ATP, 1×10^{-6}M, halved the rate constant of inactivation, indicating that the affinity of the ATP protective site has a dissociation constant K_D, of this order.

Effect of ageing of SR vesicles on their susceptibility in inactivation of Ca^{2+}*-transport by halothane.* Suspensions of SR which had been aged at 0° were exposed to 10 mM halothane and the effects measured (Fig. 6). Control preparations of SR aged for up to 102 hr, when incubated at pH 6.8 in the absence of halothane, showed no decline in transport activity. In the presence of 10 mM halothane, the degree of inactivation was related to the age of the preparation. An increasing degree of inactivation was noted after longer periods of ageing.

DISCUSSION

The mechanism whereby anaethetics act on membranes has been much debated in recent years. Basically, the question is whether they react with the protein [4, 5] or lipid [1–3] components of the membrane. Seeman [26] has proposed that local and

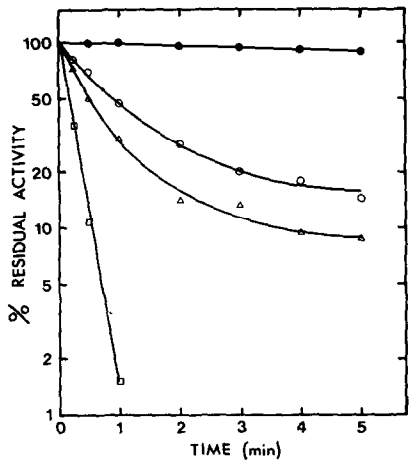

Fig. 6. Effect of ageing on the halothane inactivation of calcium transport by SR vesicles. Suspensions of SR vesicles were stored at 0° in 300 mM sucrose, 10 mM imidazole, pH 7.4 at a concentration of 4.2 mg of protein/ml. Aliquots were diluted 30 fold with 40 mM ammonium acetate buffer pH 6.8 at 30 hr (—○—○—) (fresh preparation), 54 hr (—△—△—) and 102 hr (—□—□—) after initial homogenization of the muscle and calcium transport measured at 26° after preincubation of these aliquots at 37° in the presence of 10 mM halothane. Control suspensions of SR after 102 hr were incubated in the absence of halothane (—●—●—).

general anaesthetics have a common mechanism of action and that both proteins and lipids are affected by anaesthetic molecules which attach by hydrophobic bonding causing an expansion of the membrane and that the magnitude of the lipid expansion is less than that of the hydrophobic site within the membrane proteins. Franks and Lieb, however, using X-ray and neutron diffraction analysis, found no effect of general anaesthetics on lipid bilayers and have suggested that the primary site of action has both polar and nonpolar characteristics and probably involves protein [27].

Halothane, at anaesthetic concentrations, interacts with tissues causing a variety of biochemical changes. It has been shown that halothane affects hepatic microsomal electron transfer by interaction with the cyanide-sensitive factor of the stearate desaturase pathway [28]. Hepatic carbohydrate metabolism is affected by halothane [29] which inhibits mitochondrial electron transport at the stage of NADH dehydrogenase [12]. The depressant effect of halothane on myocardial contractility has been explained on the basis that halothane reversibly inhibits oxidation of NADH-linked substrates by cardiac mitochondria, and decreases mitochondrial electron transfer and consequently ATP synthesis [13]. Volatile anaesthetic agents, including halothane, facilitate a structural transition of membrane bound acetylcholine receptor proteins of the electric organ of *Torpedo californica* and it has been suggested that the primary effect is a disruption of protein–lipid interactions [30].

Calcium ions have been implicated in the mechanism of anaesthetic action. Papahadjopoulos [31] has shown that the site of action of local anaesthetics on biological membranes involves acidic phospholipids and results in inhibition of their calcium binding properties. Blaustein and Goldman [32] have demonstrated that calcium and procaine compete with one another with respect to their action on the conductance mechanism of lobster axons. Calcium competes *in vitro* with procaine and its analogues for binding to phospholipids [33]. Grist and Baum have suggested [34] that the molecular basis of the halothane dependent efflux of calcium from rat liver mitochondria involves the partition of the anaesthetic in the apolar phase of the membrane causing its expansion and impairment of its binding capacity for calcium via the phospholipid membrane components. The data of Vanderkooi *et al.* [35] support Seeman's concept [26] that the hydrophobic interior of proteins has similar physical characteristics to lipid bilayers and that anaesthetics can be expected to partition equally well in both proteins and lipids.

We have shown (Fig. 4) that calcium ions have a protective effect against inactivation of calcium transport activity of SR vesicles by halothane with $K_{0.5\,(Ca^{2+})} = 1.5 \times 10^{-6}$M. This value is similar to that of a calcium site which protects against thermal inactivation and of a high affinity transport site located on the (Ca^{2+},Mg^{2+})-ATPase component of the membrane [36]. If the protection were solely against a site on the lipid component of the membrane, one would have expected [37] a calcium concentration of at least two orders of magnitude greater than that found in this study for conferring stability on the transport system. Changes of pH in the range 5.8–6.8 have a marked effect on inactivation of calcium transport activity of SR [13]. The p*K*s of the ionizable groups of phospholipids are in the ranges 3,7–4.5 and 7.5–8 [38]. Consequently, halothane, whose action on SR is potentiated between pH 5.8 and 6.8, would appear to be interacting with the protein moiety. There is other evidence for the interaction of halothane with soluble proteins. It has been shown to bind to hydrophobic sites in haemoglobin [39] which are not on the surface of the molecule. In addition, Sachsenheimer *et al.* have shown that halothane can bind within a hydrophobic pocket of crystalline adenylate kinase which has been identified by crystallographic means as the adenine-specific niche [40].

The present study on the effects of halothane on the stability of the (Ca^{2+},Mg^{2+})-ATPase does not allow us to differentiate between the two possibilities that halothane may be interacting either directly with a hydrophobic domain of the ATPase protein or at the protein–lipid interface. The results, however, indicate that the conformational state of the ATPase has an effect on this interaction. In this respect it is significant that μM concentrations of ATP protected against inactivation of calcium transport, indicating that this protection is mediated via the substrate binding site.

The mechanism of inactivation of transport would appear to be similar to that proposed for the acid [13] and EGTA [36] mediated uncoupling of transport. Increased permeability does not appear to play a role in halothane mediated uncoupling since direct measurements using inulin ^{14}C-carboxylic acid did

not demonstrate any significant increase in permeability when transport, but not ATPase activity, was inhibited. These findings also indicate that the ATPase protein is primarily involved in the effect. Both ether [41, 42] and butanol [43] at relatively high concentrations abolished calcium transport, but their effects have been shown to be associated with increased membrane permeability.

Several factors could have contributed to the apparently conflicting reports [8, 10, 11] on SR function with respect to halothane and its role in the production of malignant hyperthermia. Firstly, as we show in Fig. 3, the inactivation of calcium transport by SR is highly temperature dependent with little inactivation below 25°. Consequently studies done at this temperature, which is usual in routine measurements of calcium transport, would create the impression that halothane does not in fact affect calcium transport by SR vesicles. Furthermore, in our studies we preincubated the vesicles prior to measuring calcium uptake. Other workers have equilibrated the uptake medium with halothane and performed the assays in the presence of halothane. The uptake medium contains free calcium ions ($\simeq$50 μM) and ATP (1–5 mM). We have shown that these concentrations protect significantly against inactivation by halothane.

The data in Fig. 6 suggest that ageing of SR vesicles did not decrease the absolute number of active molecular species but rather caused an increased susceptibility to halothane inactivation. This could imply progressive unfolding of the (Ca^{2+}, Mg^{2+})-ATPase with the hydrophobic region of the protein becoming more readily accessible to halothane. The possibility exists that the sarcoplasmic reticulum of individuals who are predisposed to malignant hyperthermia has a similar increased susceptibility to halothane. The normal concentrations of ATP in sarcoplasm of skeletal muscle ($\simeq$5 mM) would be expected to stabilize sarcoplasmic reticulum. The concentrations of calcium, however, fluctuate in the range 10^{-7}–10^{-6}M, which may confer instability. Mitchelson and Hird [14] have shown that muscle mitochondrial function is impaired in the presence of halothane if there is a predisposing intracellular acidosis. Increasing proton concentrations lower the affinity of the high-affinity calcium site which stabilizes the SR membrane [36]. Here we have demonstrated a similar synergistic effect of hydrogen ions and halothane on the inactivation of calcium transport. This synergism may be related to the development of the halothane sensitivity of malignant hyperthermia where intracellular acidosis could potentiate halothane inactivation of calcium transport. The resultant accumulation of sarcoplasmic calcium would promote glycogenolysis, lactate acidosis and myosin ATPase activity and thus constitute a vicious cycle.

Malignant hyperthermia has not been described in rabbits and the results obtained here may not be directly extrapolated to situations arising in other species. Preliminary experiments in our laboratory on SR isolated from skeletal muscle of normal and malignant hyperthermia-susceptible pigs indicate inactivation of calcium transport similar to that observed in rabbit SR.

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