Anesthesiology
77:316–323, 1992

Halothane and Isoflurane Effects on Ca^{2+} Fluxes of Isolated Myocardial Sarcoplasmic Reticulum

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To elucidate better the differential myocardial depressant actions of halogenated volatile anesthetics, anesthetic-induced changes in Ca^{2+} accumulation, release, and Ca-ATPase (Ca^{2+} pump) activity of isolated canine cardiac sarcoplasmic reticulum (SR) vesicles were examined. An initial crude microsomal fraction of homogenized canine ventricle was subfractionated on a discontinuous sucrose gradient after Ca^{2+} loading in the presence of phosphate. Junctional SR (JSR) enriched with terminal cisternae was identified by its content of an electrophoretically verified ~450-kDa protein, the Ca^{2+}-release channel (CaRC). When the CaRC of JSR was blocked by 1 μM ruthenium red (RR), the rate of Ca^{2+} uptake increased 47% as measured spectrophotometrically using the Ca-sensitive dye antipyrylazo III. A second fraction was identified as primarily longitudinal SR (LSR) based on its trace content of 450-kDa protein and 11% increase of Ca^{2+} uptake with RR. Halothane (0.75–2.5%) or isoflurane (2.5–4%) decreased net Ca^{2+} accumulation rate by either LSR or JSR, and the decrease in uptake rate of JSR was only partially reversed by addition of 1 μM RR (27% increase for isoflurane, 7% increase for halothane). Both halothane and isoflurane increased JSR ATP consumption as measured by a coupled-enzyme assay. $^{45}Ca^{2+}$ efflux from passively loaded SR vesicles was then determined to verify that the decreased net uptake rate was due to enhanced Ca^{2+} efflux from vesicles. Both anesthetics increased passive Ca^{2+} efflux from SR vesicles in which the CaRC was blocked by 10 μM RR as well as those in which Ca^{2+} release *via* the CaRC was activated by 10 μM Ca^{2+}. By enhancing nonspecific Ca^{2+} efflux, halothane and isoflurane decrease Ca^{2+} retention of isolated cardiac SR vesicles, an effect that may mask specific actions on Ca^{2+} flux through the CaRC. (Key words: Anesthetics, volatile: halothane; isoflurane. Heart: contractility; sarcoplasmic reticulum. Ions, calcium; release; uptake.)

THE MECHANISMS by which volatile anesthetics depress myocardial contractility are manifold.[1] Alteration in myofibrillar responsiveness to Ca^{2+}, mediated *via* binding of Ca^{2+} to troponin, which controls actin–myosin interaction, may contribute to the depression.[2–5] However, the primary mechanisms underlying myocardial depression involve alterations in the cellular control of $[Ca^{2+}]$ by the surface membrane (sarcolemma)[6–10] and the sarcoplasmic reticulum (SR).[4,7,11–16] The SR is the cellular organelle primarily responsible for release of Ca^{2+} to initiate tension development as well as uptake of Ca^{2+} from the myoplasm to relax the myocardium. Release of Ca^{2+} results from the opening of specific Ca^{2+}-release channels located in the junctional SR (JSR) or terminal cisternae, where SR membrane is juxtaposed to the t-tubules.[17] Accumulated Ca^{2+} passes down its approximately 10^4 concentration gradient (0.1 μM in the myoplasm *vs.* 1 mM in the SR lumen) into the myoplasm, where it binds to troponin on the actin myofibrils to activate tension development. The Ca^{2+}-release channel opening is stimulated by entry of Ca^{2+} through the depolarized sarcolemma. Relaxation results from uptake of Ca^{2+} as it diffuses off of the troponin and is accumulated by the Ca-ATPase (Ca^{2+} pump) into the SR, primarily the longitudinal SR (LSR), which envelops each myofibril along its entire length (fig. 1). Once relaxation has occurred, the Ca^{2+} is retained in the SR until release is again activated. Thus the function of the SR membrane system can be separated into processes of Ca^{2+} release, Ca^{2+} uptake, and Ca^{2+} retention.

When myocardium is homogenized, the disrupted membrane systems will reassemble into intact vesicles. These vesicles of various membranes (sarcolemma, JSR, LSR) as well as the mitochondria can be centrifuged and separated from each other based on their density. As shown schematically in figure 1, JSR and LSR can be prepared, and such vesicles will retain the functional characteristics of Ca^{2+} uptake and retention.[18] While vesicles from both LSR and JSR contain a high density of Ca-ATPase proteins, which are responsible for Ca^{2+} accumulation by the SR, only junctional vesicles contain the Ca^{2+}-release channel. This separation permitted the examination of vesicles in which Ca^{2+} efflux *via* the Ca^{2+}-release channel was present or absent. Alternately, the polycationic molecule ruthenium red (RR) binds tightly to and blocks the Ca^{2+}-release channel, permitting determination of effects in the presence and absence of Ca^{2+} release. The purpose of the present study was to determine the effects of halothane and isoflurane on the specific myocardial SR processes of Ca^{2+} uptake and release.

Materials and Methods

ISOLATION OF SARCOPLASMIC RETICULUM

The procedure for cardiac membrane subfractions was a modification of that of Alderson and Feher.[19] Dogs were killed with sodium pentobarbital according to a protocol approved by the University of Virginia Animal Research Committee. The heart was immediately excised and put

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Received from the Department of Anesthesiology, University of Virginia Health Sciences Center, Charlottesville, Virginia. Accepted for publication March 30, 1992. Supported in part by National Institutes of Health grant R01-31144.

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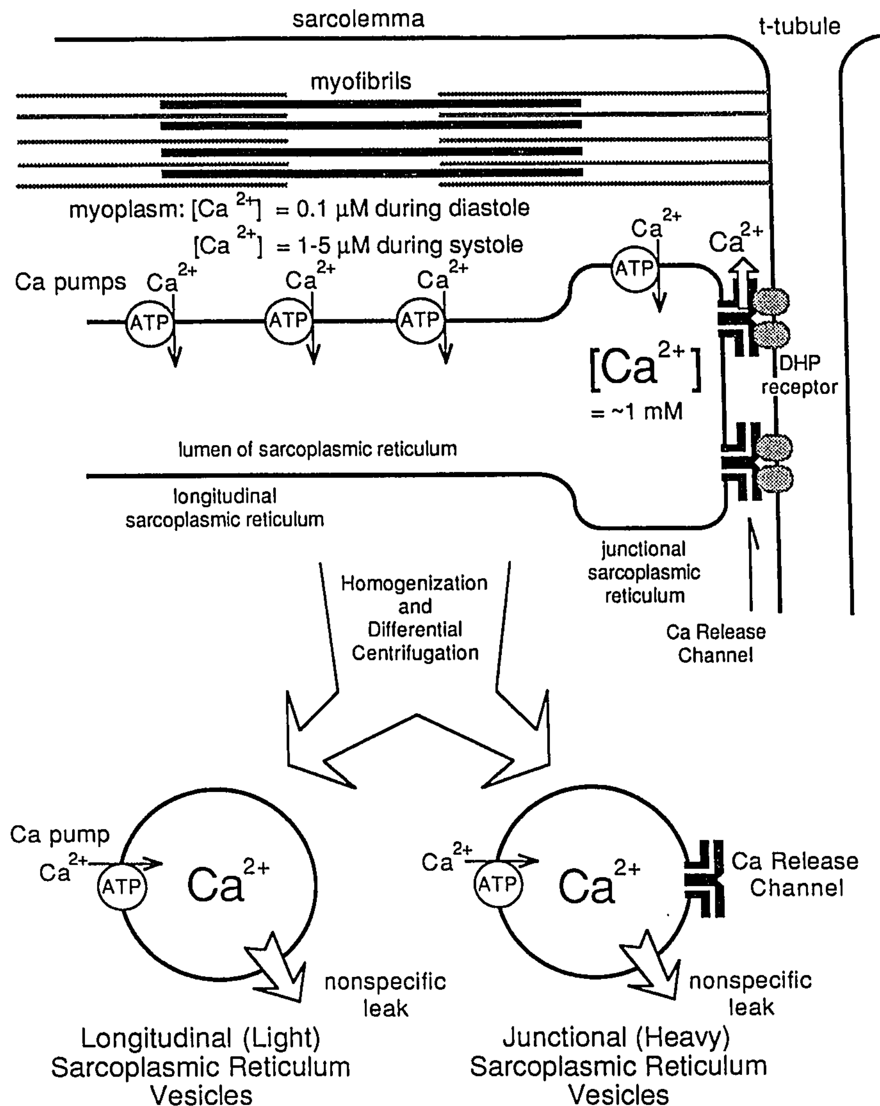

FIG. 1. Schematic diagram of a partial myocardial sarcomere showing myofibrils, a t-tubule, and the sarcoplasmic reticulum (SR), the membrane system that provides the major control of the Ca^{2+} fluxes that activate tension, by orchestration of sequential Ca^{2+} release (from the junctional SR or terminal cisternae), uptake, and retention (both longitudinal and junctional SR). When homogenized and centrifuged on a sucrose gradient, not only can the mitochondria and sarcolemma be separated from the SR membranes, but also the SR membrane itself can be subfractionated into longitudinal SR vesicles and junctional SR vesicles, the latter retaining the Ca^{2+}-release channels through which Ca^{2+} can rapidly efflux. Both vesicles also possess some component of nonspecific leak. Dihydropyridine (DHP) receptor couples depolarization to Ca release channel.

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in 0.9% sodium chloride solution (4° C). All solutions and centrifuge tubes were kept on ice, and the entire preparation was conducted in a cold room (4° C). The ventricles were minced into large chunks, weighed, and combined with three volumes of 10 mM imidazole at *p*H 7.0, and homogenized in a Waring blender for 1 min at top speed.

The homogenate was centrifuged in a Beckman JS-7.5 rotor at 6,000 × g for 20 min. The supernatant was poured through four layers of gauze and stored on ice, and the pellets resuspended in three volumes of 10 mM imidazole and homogenized for 1 min as above. This second homogenate was centrifuged as above, and the supernatant was poured through four layers of gauze and combined with the first supernatant. This mixture was centrifuged in a Beckman 50.2Ti rotor at 15,400 × g for 15 min. Following Inui *et al.*,[18] this supernatant was divided into 26-ml tubes and centrifuged for 2 h at 32,000 rpm in the 50.2Ti rotor. The pellets were homogenized and resuspended (30 g initial tissue weight/22 ml of 0.29 M sucrose, 0.65 M KCl, 0.5 mM dithiothreitol, 3 mM NaN_3, 10 mM imidazole) and spun at 41,500 rpm. The pellets were homogenized and then incubated (2 mg protein/ml 150 mM KCl, 10 mM ATP, 10 mM $MgCl_2$, 10 mM $CaCl_2$, 10 mM EGTA, 125 mM K_2HPO_4) at *p*H 7.4 and 37° C for 10 min to permit a period of Ca^{2+} uptake and then centrifuged for 30 min at 41,500 rpm at 4° C.

The pellet was resuspended (0.15 M KCl, 5 mM ATP, 5 mM $MgCl_2$, 2 mM $CaCl_2$, 2 mM EGTA, 50 mM K_2HPO_4), and ~50 mg protein was placed on discontinuous gradients of 0.6, 0.8, 1.1, and 1.6 M sucrose and spun at 23,000 rpm overnight to yield the following bands at the respective interfaces: fraction A, 0:0.6 M; fraction B, 0.6:0.8 M; fraction C, 0.8:1.1 M; fraction D, 1.1:1.6 M; and fraction E, a dense buff band on top of the 1.6 M

layer. A small aliquot of each fraction was fixed in 2% glutaraldehyde and 100 mM cacodylate followed by 1% OsO_4 in 60 mM NaCl and 100 mM veronal acetate and was embedded for electron microscopic examination. The rest of each fraction was placed in 0.3 M sucrose, 0.3 M KCl, and 20 mM HEPES, of which 10-μl aliquots were placed on sodium dodecyl sulfate gels for electrophoresis. Figure 2 shows the electron micrographs of fractions D and E and the electrophoretograms of the fractions. Samples were stored at −80° C until use.

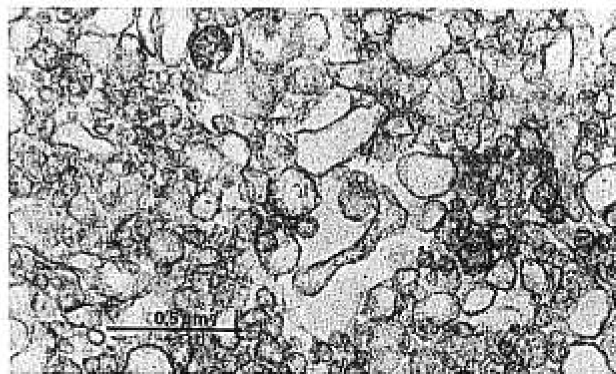
Determination of Ca^{2+} Uptake

The rate of Ca^{2+} uptake (nanomoles per microgram protein per minute) from the media surrounding isolated SR vesicles was spectrophotometrically measured using antipyrylazo III by determining the absorbance ratio at 710 nM and 790 nM in a dual-beam spectrophotometer.[18] The 1 ml assay mix consisted of 125 mM Na_2HPO_4, 1 mM $MgCl_2$, 5 mM NaN_3, 10 μl 100 mM ATP, 10 μg SR vesicles (5–12 μl), and 0.5 mM antipyrylazo III (Sigma, St. Louis, MO) at pH 7.0. After the initial uptake of contaminating Ca^{2+} in the assay mix, the rate of uptake at 22° C was observed after addition of increments of 5–40 nmol Ca^{2+} (5–40 μl of 1 mM $CaCl_2$). The vesicles take up Ca^{2+} to an extravesicular concentration of 0.2–0.5 μM and appear to reach an equilibrium between uptake and efflux. A calibration assay for $[Ca^{2+}]$ was performed using addition of known Ca^{2+} aliquots to assay mix in the absence of vesicles. RR was used to bind to the Ca^{2+}-release channel and block Ca^{2+} efflux from vesicles.[20,21] Since it absorbs light in the same wavelength region as the antipyrylazo III used to determine $[Ca^{2+}]$, a concentration of 1 μM RR was used to minimize interference in this assay. This concentration should be sufficient to block ~90% of Ca^{2+}-release channels, since half-maximal enhancement of Ca^{2+} uptake is achieved at 0.08 μM.[20]

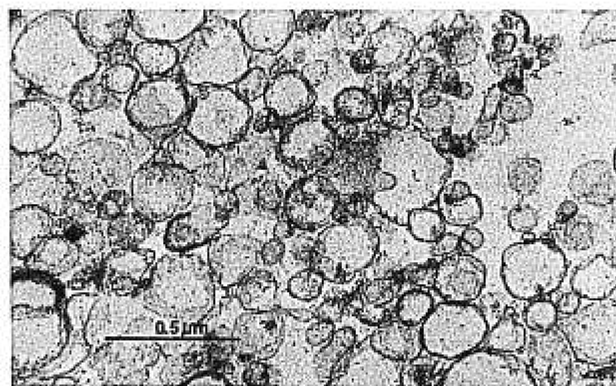
ATPase Determination

The ATPase activity of the preparations was determined by using an enzyme-coupled scheme[22] in which ATP regeneration from ADP resulted in formation of pyruvate, the conversion of which to lactate resulted in oxidation to NAD^+ of NADH. The latter reaction rate was quantified by monitoring fluorescence at 340 nm. Each 2 ml assay (pH 7.4, 22° C) contained 100 mM triethanolamine, 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM EGTA, 5 mM NaATP, 5 mM NaN_3, 1.5 mM phospho(enol)pyruvate, 0.04 units lactate dehydrogenase, 0.26 units pyruvate kinase, 0.27 mg NADH, and 20 μg SR vesicles. Background ATPase activity was determined on addition of SR vesicles. Addition of $CaCl_2$ to a concentration of 500 μM activated the Ca-ATPase, and values

A Fraction D



B Fraction E



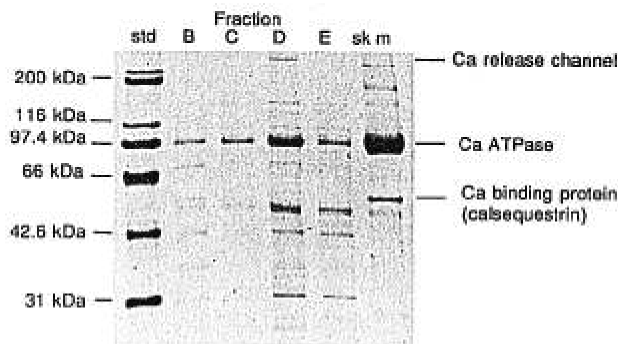


FIG. 2. Electron micrographs and electrophoresis of sarcoplasmic reticulum (SR) vesicles. *A:* Fraction D represents more predominantly junctional SR, based on the presence of the Ca^{2+}-release channel (see electrophoresis) and improvement in Ca^{2+} uptake caused by blockade of Ca^{2+}-release channel by addition of ruthenium red. *B:* Fraction E represents more longitudinal SR. *C:* Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of isolated cardiac membrane fractions B–E, as well as skeletal muscle SR vesicles (sk m). The molecular weight standards (left column) in order of increasing molecular weight are carbonic anhydrase, ovalbumin, bovine serum albumin, phosphorylase B, β-galactosidase, and myosin. std = standard.

reported are the observed increases above the unstimulated ATPase activity. Non–Ca^{2+}-stimulated ATPase activity of LSR averaged 0.04 nmol · mg $protein^{-1}$ · min^{-1}, 10–15% of the Ca^{2+}-stimulated activity. The Ca^{2+}-de-

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pendent activity was also determined in the presence of 10 μM A23187, a Ca^{2+} ionophore that prevents Ca^{2+} accumulation inside the vesicles and thereby resulted in maximal ongoing activity.

DETERMINATION OF Ca^{2+} EFFLUX

JSR vesicles (5 μg/μl) were incubated for 2 h at 22° C in 20 mM piperazine-N,N'-bis[2-ethane-sulfonic acid] (*p*H 7.0), 100 mM KCl, 0.1 mM EGTA, and 5 mM $^{45}CaCl_2$ (20 mCi/mmol), which had a free Ca^{2+} concentration of approximately 4.9 mM. During this period, Ca^{2+} passively diffused into the vesicles, since no ATP was present to support active (ATPase-mediated) uptake. Ca^{2+} release was initiated by adding 30 μl of SR mix to 9 ml of release medium (22° C). Release was observed in medium in which Ca^{2+} release was inhibited (20 mM PIPES, 100 mM KCl, 10 mM $MgCl_2$, 10 μM RR, *p*H 7.0), or in which Ca^{2+} release was activated (20 mM PIPES, 100 mM KCl, 1 mM EGTA, 0.96 mM $CaCl_2$ (10 μM free Ca^{2+}), *p*H 7.0). Aliquots (0.5 ml) of efflux mix were filtered through 0.45 μm HAWP Millipore® filters and washed twice with 5 ml of ice-cold efflux mix. The amount of $^{45}Ca^{2+}$ retained on the filter was determined by liquid scintillation counting. Control experiments were performed in parallel with anesthetic exposure experiments.

ANESTHETIC ADMINISTRATION

Nitrogen gas was conducted through the appropriate calibrated vaporizer, from which the outflow gas was passed through the buffer used for the respective assay (Ca^{2+} uptake, Ca^{2+} efflux, or ATPase activity). Previous chromatographic studies showed that anesthetic concentrations in solution were equilibrated in ≤20 min. In Ca^{2+} uptake experiments, 1.3%, 2.5%, or 4% isoflurane, or 0.75%, 1.5%, or 2.5% halothane was used, these concentrations being approximately equianesthetic in a variety of species and representing 0.65–1, 1.3–2, or 2–3 times the minimal alveolar concentration (MAC).[23] To prevent loss of the volatile anesthetic from the solution during uptake studies, which lasted 5–10 min, anesthetic-containing gas was passed over the previously equilibrated solution in the cuvette *via* a port in the Teflon stopper. Gas exited from a second port, while additions to the solution were made through a third port. In ATPase and Ca^{2+} efflux studies, release media was equilibrated with either 1.5% halothane or 2.5% isoflurane. ATPase and efflux studies were concluded within 2 min of initiation, and anesthetic loss to the atmosphere was minimal during that interval. At the room temperature (20–22° C) at which studies were performed, aqueous concentrations were approximately 1.1 mM for 2.5% isoflurane and 0.75 mM for 1.5% halothane.

Differences among control and anesthetics were compared by ANOVA using Fisher's protected least significant difference test to verify differences at the <0.05 probability level (Statview, Abacus Concepts, Inc.). Differences from control when expressed as percent control employed a one-sample *t* test *versus* 100%.

Results

Electrophoresis of the different membrane fractions (B–E) demonstrated the highest concentration of SR Ca-ATPase in fractions D and E (fig. 2C), which also showed the greatest Ca^{2+} uptake activity. Fraction D showed a small but distinct band at ~450 kDa, corresponding to the Ca^{2+}-release channel protein; thus, this fraction was considered enriched with JSR. Fraction E contained little discernible Ca^{2+}-release channel protein and was considered to be predominantly LSR. Electron microscopy showed similar vesicular structures in the two fractions (figs. 2A and 2B), although vesicles enriched with JSR (fraction D) were typically smaller in diameter (0.08–0.3 μm) than the predominantly LSR (0.2–0.4 μm), as previously described.[18] The brief period of Ca^{2+} loading at 37° C prior to the sucrose gradient centrifugation apparently resulted in the LSR having a higher density (fraction E) than the JSR (fraction D), since in the absence of Ca^{2+}-release channels, the LSR retained more accumulated Ca^{2+} (as $CaHPO_4$).

Ca^{2+} UPTAKE STUDIES

The control tracings in figure 3 show the change in the antipyrylazo III absorbance ratio observed for LSR or JSR after addition of 25 nmol of Ca^{2+}. Typically observed was a stable rate of Ca^{2+} uptake into the vesicles, as indicated by the constant slope of the tracing. JSR-enriched vesicular Ca^{2+} uptake was increased by 1 μM RR (top tracing), confirming the functional presence of Ca^{2+}-release channels. In contrast, subsequent addition of 1 μM RR did not appear to enhance Ca^{2+} uptake by LSR (bottom tracing). The average RR-induced increase in uptake by JSR was 49 ± 3%, in contrast to an increase of only 12 ± 3% in LSR (table 1).

Figure 3 also illustrates the alteration in Ca^{2+} uptake induced by 2.5% isoflurane or 1.5% halothane. Either anesthetic decreased both the uptake rate as well as the response to addition of RR. Table 1 summarizes the effects halothane and isoflurane on Ca^{2+} uptake by JSR and LSR before and after addition of 1 μM RR. There was not statistical difference or dose-dependence among anesthetic doses. Only 2.5% and 4% isoflurane specifically caused significant depression of JSR Ca^{2+} uptake rate, reducing it to 63 and 68% of control. Since there was no dose-dependence, uptake rates of all concentrations of either anesthetic were combined for analysis: both isoflurane and halothane significantly reduced Ca^{2+} uptake rates, with no difference between agents. Addition of RR

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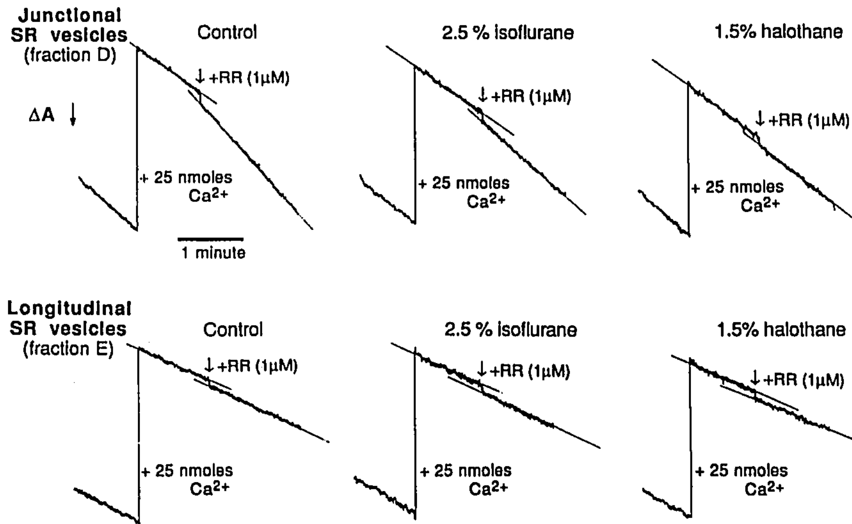

FIG. 3. Absorbance ratio curves (790–710 nm), using antipyrylazo III to monitor extravesicular Ca^{2+} concentration. The step increase in the absorbance change results from the 25 μM increase in [Ca^{2+}]; the downward slope is proportional to the rate of uptake of Ca^{2+} from the medium into the sarcoplasmic reticulum (SR) vesicles. In junctional SR, addition of 1 μM ruthenium red (RR), which blocks the Ca^{2+} release channels, increased the rate of Ca^{2+} uptake. *Left:* The control Ca^{2+} uptake. *Center and right:* Uptake in the presence of 2.5% isoflurane and 1.5% halothane, respectively.

to JSR increased the vesicular Ca^{2+} uptake rate in the presence of isoflurane (4% or combined) by 26–27%, whereas a significantly smaller effect was observed for RR addition with halothane (7% increase). For LSR, a reduction in uptake rate was statistically confirmed only for the combined isoflurane studies, and no significant change in behavior was induced by RR in the absence or presence of anesthetics. In two experiments, inclusion of 10 μM A23187 resulted in no measurable Ca^{2+} uptake by either LSR or JSR.

ATPase STUDIES

Table 2 presents the anesthetic effects on Ca^{2+}-stimulated ATP consumption. These assays showed substantial inherent variation from day to day, as suggested by the relatively large SEMs, which were typically 15–20% of the mean value. In terms of the mean absolute ATPase activity values, the anesthetics caused no significant variation from control; however, the activity of LSR with isoflurane was significantly less than in the presence of halothane. Vesicular leak of accumulated Ca^{2+} was increased by addition of the Ca^{2+} ionophore A23187 (10 μM), which in turn caused a 60–90% increase in Ca-ATPase activity. The initial control activity was 57 ± 3% of the maximum A23187-stimulated activity. However, in the presence of isoflurane and halothane, Ca-ATPase activity of JSR was increased to 69 ± 7 and 68 ± 8% of the maximum (A23187-stimulated), respectively. Such an increase was not apparent in LSR. In six experiments the effects of 1 μM RR were assayed in JSR and resulted in a nonsignificant 6 ± 3% decrease in ATPase activity.

TABLE 1. Effects of Isoflurane and Halothane on Rates of Ca^{2+} Uptake Before and After the Addition of 1 μM Ruthenium Red

	Fraction D (JSR)			Fraction E (LSR)		
		Before RR	1 μM RR added		Before RR	1 μM RR added
	n	($nmol \cdot mg^{-1} \cdot min^{-1}$, mean ± SEM)	(% increase, mean ± SEM)	n	($nmol \cdot mg^{-1} \cdot min^{-1}$, mean ± SEM)	(% increase, mean ± SEM)
Control	14	162 ± 16	49 ± 3*	11	123 ± 20	12 ± 3†
Isoflurane						
1.3%	4	117 ± 6	30 ± 13‡	4	87 ± 15	9 ± 6
2.5%	4	102 ± 9§	28 ± 11‡	4	68 ± 16	4 ± 4
4.0%	8	109 ± 11§	26 ± 7*‡	4	84 ± 21	11 ± 6
Combined	16	109 ± 6§	27 ± 5*‡	12	80 ± 10§	8 ± 3
Halothane						
0.75%	4	130 ± 9	14 ± 3†‡	4	84 ± 13	0 ± 0
1.5%	4	123 ± 11	8 ± 2†‡	4	75 ± 14	0 ± 0
2.5%	8	140 ± 12	4 ± 2‡¶	3	123 ± 31	0 ± 0
Combined	16	133 ± 7§	7 ± 2†‡¶	11	91 ± 12	0 ± 0

JSR = junctional sarcoplasmic reticulum; LSR = longitudinal sarcoplasmic reticulum; RR = ruthenium red.

* $P < 0.01$, †$P < 0.05$ increase above initial value (by single-value *t* test).

‡ $P < 0.05$ less than RR-induced increase observed in control (analysis of variance [ANOVA] and Fisher's protected least significant difference [PLSD] test).

§ $P < 0.05$ different from the control uptake rate (ANOVA and Fisher's PLSD test).

¶ $P < 0.05$ less than RR-induced change in the presence of isoflurane (ANOVA and Fisher's PLSD test).

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TABLE 2. Effects of Isoflurane and Halothane on Ca-ATPase Activity

	Condition	n	Ca-ATPase Activity (nmol · mg⁻¹ · min⁻¹)	Maximum Ca-ATPase Activity (in 10 μM A23187) (nmol · mg⁻¹ · min⁻¹)	Ca-ATPase Activity as % of Maximum
Junctional SR (fraction D)	Control	10	304 ± 37	541 ± 66	57 ± 3
	2.5% Isoflurane	5	300 ± 65	483 ± 140	69 ± 7*
	1.5% Halothane	6	282 ± 73	457 ± 148	68 ± 8*
Longitudinal SR (fraction E)	Control	10	255 ± 21	405 ± 33	65 ± 5
	2.5% Isoflurane	6	212 ± 32†	433 ± 47†	49 ± 5
	1.5% Halothane	6	314 ± 14	555 ± 15	57 ± 3

SR = sarcoplasmic reticulum.
* $P < 0.05$ different from control (by ANOVA and Fisher's PSLD test).
† $P < 0.05$ different from halothane (by ANOVA and Fisher's PSLD test).

Ca^{2+} EFFLUX STUDIES

Passively Ca^{2+}-loaded JSR vesicles were placed into release media, and samples were taken at the time intervals indicated in figure 4. When efflux through the Ca^{2+}-release channel was inhibited (10 μM RR and 10 mM Mg^{2+}, fig. 4A), Ca^{2+} retention in vesicles was markedly enhanced compared to media containing 10 μM Ca^{2+} (fig. 4B), although the rates of loss of Ca^{2+} appeared to be similar at 90–120 s. In the setting of Ca^{2+}-release channel blockade with RR and Mg^{2+}, A23187 reduced Ca^{2+} retention at 15 s, such that it resembled that seen with unblocked Ca^{2+}-release channels. Although not as profound in effect as the Ca^{2+} ionophore, halothane also reduced Ca^{2+} retention in the interval before the first sample was taken. In contrast, isoflurane did not alter Ca^{2+} retention from control levels until 90–120 s and differed significantly from halothane. Upon activation of the Ca^{2+}-release channel with 10 μM Ca, both 1.5% halothane and 2.5% isoflurane significantly increased Ca^{2+} efflux after 60–120 s. When plotted as a linear or as an exponential function of time (latter not shown), a rapid component and a slower component are evident both with and without Ca^{2+}-release channel blockade. However, no simple mono- or biexponential declines were present, and detailed quantitation or modeling of efflux rate was not pursued.

Discussion

In view of the considerable physiologic[4,7,11–14,16] and biochemical[24–26] evidence suggesting that volatile anesthetics alter cardiac contractility by effects on SR control of intracellular Ca^{2+}, we sought in these studies to determine more specifically whether anesthetic effects are mediated by alterations in Ca^{2+} uptake, retention, and/or release.

The spectrophotometric Ca^{2+} uptake assay that we used actually measured the decline in extravesicular [Ca^{2+}]. The observed rate represents the net rate of Ca^{2+} uptake: the rate of Ca^{2+} "pumped" into the vesicles by the ATPase minus the rate of Ca^{2+} efflux or leak from the vesicles back into the medium. If Ca^{2+} is taken up at a constant rate by ATPase activity, greater Ca^{2+} release or leak from the vesicles results in a less rapid decrease in extravesicular Ca^{2+}, which will be observed as a decreased uptake rate. Normally, the SR Ca-ATPase (Ca^{2+} pump) has a coupling

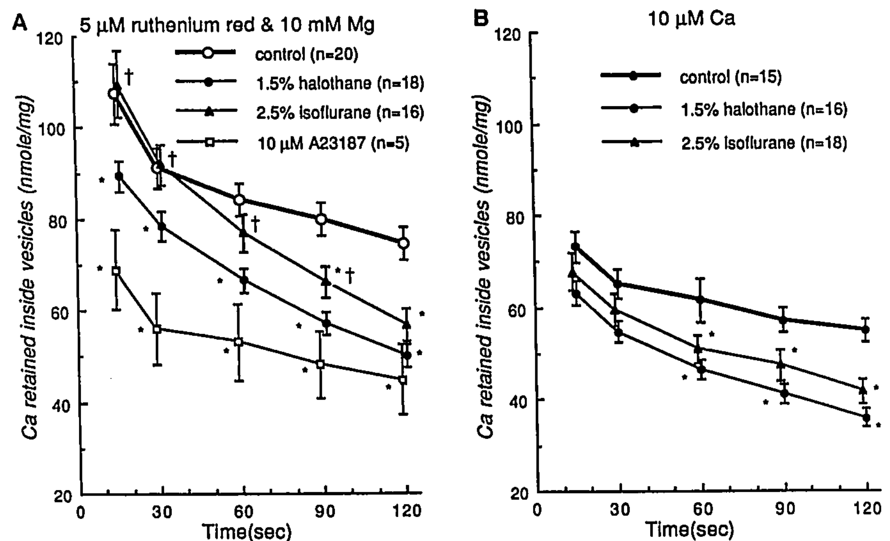

FIG. 4. Kinetics of $^{45}Ca^{2+}$ efflux from passively loaded sarcoplasmic reticulum (SR) vesicles after dilution in media in which the Ca^{2+}-release channel is blocked or activated. *A:* Retained $^{45}Ca^{2+}$ in junctional SR vesicles in the presence of Ca^{2+}-release channel blockade by 10 mM Mg^{2+} and 10 μM ruthenium red. Results under control conditions and in the presence of 1.5% halothane, 2.5% isoflurane, or 10 μM A23187. *B:* Retained $^{45}Ca^{2+}$ in junctional SR vesicles in the presence of Ca^{2+}-release channel activation in buffer with free [Ca^{2+}] = 5 μM. *$P < 0.05$ for difference from control. †$P < 0.05$ for difference from halothane, analysis of variance and Fisher's protected least significant difference test.

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ratio of 2; that is, two Ca^{2+} ions are translocated into the vesicle for each ATP molecule hydrolyzed.[27,28] Presuming no Ca^{2+} leaks from the vesicles, the Ca^{2+} uptake rate (nmol $Ca^{2+} \cdot mg^{-1} \cdot min^{-1}$) into the vesicles should ideally be twice the ATPase activity (nmol $P_i \cdot mg^{-1} \cdot min^{-1}$). Comparison of tables 1 and 2 instead gives coupling ratios ~0.5. Although the differing conditions between the assays may in part be responsible for the low ratio, ongoing efflux is probably a major contribution. Clearly the efflux rate was not so great that the Ca^{2+} pump could not overcome it, permitting effective accumulation into the vesicles. When the SR Ca^{2+}-release channel of JSR was blocked by RR, Ca^{2+} uptake increased, consistent with blockade of efflux, and resulting in an improved coupling ratio.

When Ca^{2+} permeability was maximized with A23187, the capability for Ca^{2+} uptake was lost, and ATPase activity doubled as Ca^{2+} was translocated into the vesicle only to leak rapidly out. As Ca^{2+} is normally accumulated, establishment of a significant [Ca^{2+}] gradient feeds back to decrease Ca^{2+} pumping[28]; by preventing Ca^{2+} retention within the vesicle, A23187 enhances ATPase activity. Halothane and isoflurane had actions similar to A23187, decreasing Ca^{2+} uptake and increasing ATPase activity. Consistent with depressed Ca^{2+} uptake, the efflux experiments demonstrated enhanced passive efflux by the anesthetics in the presence of RR. However, anesthetic enhancement of Ca^{2+} efflux and of ATPase activity was not nearly to the extent observed with A23187, which permitted no Ca^{2+} accumulation. Blanck and Thompson[25] first reported an increase in Ca-ATPase activity in cardiac SR vesicles with clinical concentrations of isoflurane and halothane when ATP concentrations were less than 5 mM. While lipid actions of anesthetics in membrane bilayers that make the enzyme more accessible to Ca^{2+}[29] or change the Ca^{2+} binding constant[25] have been invoked to explain the increased ATPase activity, this effect may be explained largely by the enhancement of efflux, which prevents accumulating Ca^{2+} from causing feedback inhibition of the ATPase. The enhanced efflux is consistent with the previous results of Casella *et al.*,[26] who found that while anesthetics did not depress the initial $^{45}Ca^{2+}$ uptake by cardiac SR vesicles (not subfractionated), the ability of vesicles to retain Ca^{2+} in the presence of anesthetics was depressed when compared to control at 20 min.

The ability of RR to enhance JSR Ca^{2+} uptake was blunted by the presence of anesthetics. These observations are in contrast to studies by Herland *et al.*[15] in rat myocardial trabeculae in which the surface membranes were made permeable by saponin. In that study, halothane induced a dose-dependent release of Ca^{2+} accumulated in the still-functional SR membranes; this halothane effect was completely blocked by 10 μM RR so that it was attributed to an action on the Ca^{2+}-release channel. Although "nonspecific" SR permeability to Ca^{2+} was increased somewhat by permeabilization with saponin, halothane did not increase the RR-insensitive Ca^{2+} flux. Likewise, in skeletal muscle in which the Ca^{2+}-release channel is structurally and functionally similar to that in heart,[30,31] specific anesthetic actions on channel gating of Ca^{2+} flux have been reported.[32,33]

Regarding the lack of RR reversal of anesthetic effects in the present study, the following points must be considered. In the presence of Ca^{2+}-release channel block by 10 μM RR (and 10 mM Mg^{2+}), the halothane and isoflurane clearly increased passive RR-insensitive efflux. However, 1 μM RR used in the Ca^{2+} uptake study may permit a fraction of the Ca^{2+}-release channels to remain unblocked and to be activated by halothane, thereby contributing to the decreased Ca^{2+} uptake rate. It is possible that if Ca^{2+} efflux is enhanced both *via* the release channel and nonspecifically, incomplete blockade of the enhanced Ca^{2+}-release channel efflux by RR will not appear as significant increase in Ca^{2+} uptake against the background of an increased nonspecific leak.

The suggestion that anesthetics may cause a nonspecific increase in vesicle membrane permeability has been made previously based on observations in pig skeletal muscle[34] and in cardiac SR vesicles.[35] At clinically relevant concentrations, halothane caused changes interpreted as an increase in fluidity of lipid bilayer membranes[36] that might contribute to an increased Ca^{2+} permeability of membranes. The present results were obtained at room temperature in isolated and therefore structurally perturbed SR membrane systems. The presence of anesthetics in the membrane lipid may more readily increase ionic permeability and leak in such reaggregated vesicles. Such anesthetic-induced permeability changes may be less prominent or not occur in the highly structured SR of intact tissue, or in muscles with permeabilized surface membranes. Thus, the RR-resistant and apparently nonspecific anesthetic effects observed in these isolated vesicles may represent an inherent limitation in the application and interpretation of this technique. While the anesthetic-induced RR-resistant increase in Ca^{2+} efflux can be interpreted as increased nonspecific leakage, it is also possible that the anesthetics alter the Ca^{2+}-release channel so that it is less likely to be blocked by RR. Further studies focusing on anesthetic effects upon the cardiac Ca^{2+}-release channel will provide better definition of whether this is a distinct site of action.

The lack of dose-dependence in anesthetic effect on uptake is perhaps surprising and may represent some threshold phenomenon, such that only a modest concentration is necessary to initiate the process that increases efflux. Very modest anesthetic concentrations (<0.1 MAC) appear to activate release in skeletal muscle SR vesicles.[32] It is not unreasonable that SR actions predominate at low anesthetic concentrations and that other phenomenon such as inhibition of sarcolemmal Ca^{2+}

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entry[6,8,10] become prominent at higher concentrations, thereby contributing to depression.

The advice of Howard Kutchai, Lisa Geddes, and Chris Weis is gratefully acknowledged, as is the use of Dr. Kutchai's laboratory facilities. The authors also thank Dr. Kutchai and Dr. Joseph J. Pancrazio for review of the manuscript. The photomicrographs were kindly prepared by Dr. Michael Forbes.

References

1. Rusy BF, Komai H: Anesthetic depression of myocardial contractility: A review of possible mechanisms. ANESTHESIOLOGY 67: 745–766, 1987
2. Su JY, Kerrick WGL: Effects of halothane on Ca^{2+}-activated tension development in mechanically disrupted rabbit myocardial fibers. Pfluegers Arch 375:111–117, 1978
3. Pask HT, England PJ, Prys-Roberts C: Effects of volatile inhalational anesthetic agents on isolated bovine cardiac myofibrillar ATPase. J Mol Cell Cardiol 13:293–301, 1981
4. Su JY, Bell JG: Intracellular mechanism of action of isoflurane and halothane on striated muscle of the rabbit. Anesth Analg 65:457–462, 1986
5. Housmans PE, Murat I: Comparative effects of halothane, enflurane, and enflurane at equipotent anesthetic concentrations on isolated ventricular myocardium of the ferret: I. Contractility. ANESTHESIOLOGY 69:451–463, 1988
6. Lynch C III, Vogel S, Sperelakis N: Halothane depression of myocardial slow action potentials. ANESTHESIOLOGY 55:360–368, 1981
7. Lynch C III: Differential depression of myocardial contractility by halothane and isoflurane *in vitro*. ANESTHESIOLOGY 64:620–631, 1986
8. Terrar DA, Victory JGG: Influence of halothane on membrane currents associated with contraction in single myocytes isolated from guinea-pig ventricle. Br J Pharmacol 94:500–508, 1988
9. Terrar DA, Victory JGG: Isoflurane depresses membrane currents associated with contractions in myocytes isolated from guinea pig ventricle. ANESTHESIOLOGY 69:742–749, 1988
10. Bosnjak ZJ, Supan FD, Rusch NJ: The effects of halothane, enflurane and isoflurane on calcium currents in isolated canine ventricular cells. ANESTHESIOLOGY 74:340–345, 1991
11. Su JY, Kerrick WGL: Effects of halothane on caffeine-induced tension transients in functionally skinned myocardial fibers. Pfluegers Arch 380:29–34, 1979
12. Su JY, Kerrick WGL: Effects of enflurane on functionally skinned myocardial fibers from rabbits. ANESTHESIOLOGY 52:385–389, 1980
13. Luk HN, Lin CI, Chang CL, Lee AR: Differential inotropic effects of halothane and isoflurane in dog ventricular tissues. Eur J Pharmacol 136:409–413, 1987
14. DeTraglia MC, Komai H, Rusy BF: Differential effects of inhalational anesthetics on myocardial potentiated-state contractions *in vitro*. ANESTHESIOLOGY 68:534–540, 1988
15. Herland JS, Julian FJ, Stephenson DG: Halothane increases Ca^{2+} efflux via Ca^{2+} channels of sarcoplasmic reticulum in chemically skinned rat myocardium. J Physiol (Lond) 426:1–18, 1990
16. Lynch C III: Differential depression of myocardial contractility by volatile anesthetics *in vitro:* Comparison with uncouplers of excitation-contraction coupling. J Cardiovasc Pharmacol 15: 655–665, 1990
17. Moravec CS, Bond M: Calcium is released from the junctional sarcoplasmic reticulum during cardiac muscle contraction. Am J Physiol 260(Heart Circ Physiol 29):H989–H997, 1991
18. Inui M, Wang S, Saito A, Fleisher S: Characterization of junctional and longitudinal sarcoplasmic reticulum from heart muscle. J Biol Chem 263:10843–10850, 1988
19. Alderson BH, Feher JJ: The interaction of calcium and ryanodine with cardiac sarcoplasmic reticulum. Biochim Biophys Acta 900: 221–229, 1987
20. Chamberlain BK, Volpe P, Fleischer S: Inhibition of calcium-induced Ca^{++} release from purified cardiac sarcoplasmic reticulum vesicles. J Biol Chem 259:7547–7553, 1984
21. Pessah IN, Waterhouse AL, Casida JE: The calcium-ryanodine receptor complex of skeletal and cardiac muscle. Biochem Biophys Res Commun 128:449–456, 1985
22. Madden TD, Chapman D, Quinn PJ: Cholesterol modulates activity of calcium-dependent ATPase of the sarcoplasmic reticulum. Nature (Lond) 279:538–541, 1979
23. Firestone LL, Miller JC, Miller KW: Appendix: Tables of physical and pharmacological properties of anesthetics, Molecular and Cellular Mechanisms of Anesthetics. Edited by Roth SH, Miller KW. New York, Plenum Medical Book Co., 1986, pp 455–470
24. Blanck TJJ, Thompson M: Calcium transport by cardiac sarcoplasmic reticulum: Modulation of halothane action by substrate concentration and pH. Anesth Analg 60:390–394, 1981
25. Blanck TJJ, Thompson M: Enflurane and isoflurane stimulate calcium transport by cardiac sarcoplasmic reticulum. Anesth Analg 61:142–145, 1982
26. Casella ES, Suite DA, Fisher YI, Blanck TJJ: The effect of volatile anesthetics on the *p*H dependence of calcium uptake by cardiac sarcoplasmic reticulum. ANESTHESIOLOGY 67:386–390, 1987
27. Inesi G, Kurzmack M, Verjovski-Almeida S: ATPase phosphorylation and calcium ion translocation in the transient state of sarcoplasmic reticulum activity. Ann N Y Acad Sci 307:224–227, 1978
28. Hasselbach W, Oetlicker H: Energetics and electrogenicity of the sarcoplasmic reticulum calcium pump. Ann Rev Physiol 43:325–339, 1983
29. Petreski JG, Wolosker H, de Meis L: Activation of Ca uptake and inhibition of reversal of the sarcoplasmic reticulum Ca^{2+} pump by aromatic compounds. J Biol Chem 264:20339–20343, 1989
30. Otsu K, Willard HF, Khanna VK, Zorzato F, Green NM, MacLennan DH: Molecular cloning of cDNA encoding of the Ca^{2+} release channel (ryanodine receptor) of rabbit cardiac muscle sarcoplasmic reticulum. J Biol Chem 265:13472–13483, 1990
31. Zorzato F, Fujii J, Otsu K, Phillips M, Green NM, Lai FA, Meissner G, MacLennan DH: Molecular cloning of cDNA encoding human and rabbit forms of the Ca^{2+} release channel (ryanodine receptor) of skeletal muscle sarcoplasmic reticulum. J Biol Chem 265:2244–2256, 1990
32. Nelson TE, Sweo T: Ca^{2+} uptake and Ca^{2+} release by skeletal muscle sarcoplasmic reticulum: differing sensitivity to inhalational anesthetics. ANESTHESIOLOGY 69:571–577, 1988
33. Nelson TE: Halothane effect on calcium release channel from human malignant hyperthermia skeletal muscle. Biophys J 59: 85a, 1991
34. Rock E, Mammar MS, Thomas MA, Viret J, Vignon X: Halothane-induced functional and structural modifications in sarcoplasmic reticulum membranes from pig skeletal muscle. Biochimie 72: 245–250, 1990
35. Katsuoka M, Ohnishi ST: Inhalation anaesthetics decrease calcium content of cardiac sarcoplasmic reticulum. Br J Anaesth 62: 669–673, 1989
36. Mastrangelo CJ, Trudell JR, Edmeunds HN, Cohen EN: Effect of clinical concentrations of halothane on phospholipid cholesterol membrane fluidity. Mol Pharmacol 14:463–467, 1978

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