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Activation of the Ca^{2+} Release Channel of Cardiac Sarcoplasmic Reticulum by Volatile Anesthetics

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Background: Depression of myocardial contractility associated with the volatile anesthetics is well established clinically and experimentally. The molecular mechanisms underlying this effect, however, have not been completely characterized. Whereas the Ca^{2+} release channel of cardiac sarcoplasmic reticulum (SR) has been implicated as a potential target contributing to anesthetic-induced myocardial depression, the effect of the volatile anesthetics on this protein have not been characterized at the isolated, single-channel level. The authors sought to identify changes in channel gating and conductance resulting from exposure to halothane, enflurane, and isoflurane that would contribute to the associated negative inotropy, as well as to explain the observation that isoflurane causes less contractile depression than either halothane or enflurane.

Methods: Vesicles enriched in SR were prepared from porcine left ventricular tissue. Fusion of these vesicles with artificial lipid bilayers under the experimental conditions provided single-channel recordings of the SR Ca^{2+} release channel. The gating properties and the conductance of these channels were determined in the presence and absence of clinical concentrations of halothane, enflurane, and isoflurane.

Results: Halothane (1.2 vol%) and enflurane (1.6 vol%) activated the Ca^{2+} release channel by increasing the open probability (fraction of time that the channel is open) without altering the channel conductance. These agents altered channel gating by increasing the duration of open events, rather than the number of open events. Isoflurane (1.4 vol%) had no effect on channel gating or conductance. Halothane caused dose-dependent channel activation (0.2–1.5 vol%), and channel activation was found to be reversible upon washout of halothane from the solutions bathing the lipid bilayer.

Conclusions: Halothane and enflurane gate the Ca^{2+} release channel into the open state without altering the channel con-

ductance. An increase in the duration of open events results from halothane and enflurane, but does not occur in the presence of isoflurane. Activation of the SR Ca^{2+} release channel would lead to loss of SR stores of Ca^{2+} into the cytoplasm, which is rapidly mobilized to the extracellular space. A net depletion of Ca^{2+} available for excitation-contraction coupling would result. The observation that isoflurane does not alter gating of this channel contributes to the understanding of the molecular mechanisms by which isoflurane depresses myocardial contractility less than halothane and enflurane. (Key words: Anesthetics, volatile: enflurane; halothane; isoflurane. Heart: contractility; myocardial depression.)

CLINICAL use of the volatile anesthetics halothane, enflurane, and isoflurane is limited in certain patient populations by the associated depression of myocardial contractility.^{1–3} In experimental preparations ranging from isolated papillary muscle through the intact animal model, halothane and enflurane have been shown to cause more negative inotropy than isoflurane at equivalent anesthetic concentrations. In an isolated, perfused rat heart preparation, halothane and enflurane cause a dose-dependent decrease in pressure generated by the left ventricle.⁴ Similarly, peak force developed in isolated papillary muscle is depressed almost twice as much by equivalent anesthetic concentrations of halothane and enflurane (40–50% of control) when compared to that produced by isoflurane.^{5–7} The negative inotropy resulting from exposure to these agents has been examined in a variety of preparations and species, and an alteration in intracellular Ca^{2+} homeostasis rather than an interaction with contractile proteins is felt to be causative (reviewed by Rusy and Komai, 1987).⁸ However, the mechanisms by which volatile anesthetics alter Ca^{2+} regulation to cause contractile depression remain incompletely characterized and the specific target sites explaining their relative depressant potencies have not been identified.

Several Ca^{2+} permeability sites normally associated with excitation-contraction coupling have been identified as potential targets for the volatile anesthetics. At the sarcolemma, anesthetics reduce inward Ca^{2+}

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currents of both L-type and T-type by as much as 20 to 60% at clinical concentrations.⁹⁻¹¹ However, since halothane, enflurane, and isoflurane depress sarcolemmal Ca^{2+} currents equally,⁹ an interaction at this site alone cannot explain the different potencies of the volatile anesthetics as negative inotropic agents. Moreover, because the sarcolemmal Ca^{2+} current is amplified inside the cell 20 to 50 fold by Ca^{2+} release from the sarcoplasmic reticulum (SR), even a 60% reduction in the Ca^{2+} current would appear to be insufficient to eliminate completely the intracellular Ca^{2+} transient that triggers myocardial contraction.¹² These observations and those of Lynch *et al.*,¹³ showing that contractile force is more sensitive to halothane than is the slow action potential, suggest that interactions other than that with the sarcolemmal Ca^{2+} channel contribute to the documented myocardial depression.

The effect of volatile anesthetics on SR function has been investigated in multiple preparations. Measurements of caffeine-induced tension developed in skinned myocardial fiber bundles,¹⁴⁻¹⁶ first indicated that halothane and enflurane decrease the Ca^{2+} loading capacity of the SR more so than isoflurane. More recently, Herland *et al.*¹⁷ demonstrated that the depression of caffeine-induced tension generated by skinned myocardium resulting from exposure to halothane was blocked by the SR Ca^{2+} release channel blocker ruthenium red, suggesting that halothane stimulated the Ca^{2+} release mechanism of SR. Studies of anesthetic effects on intracellular Ca^{2+} studied in myocytes with Ca^{2+} -sensitive fluorescence dyes have shown that halothane and enflurane cause greater depression of the caffeine-induced intracellular Ca^{2+} transient than isoflurane, and suggest that this results from stimulation of the SR Ca^{2+} release mechanism that depletes the SR of Ca^{2+} stores.¹⁸⁻²⁰ The cardiac ryanodine receptor-calcium release channel complex of SR, which is distinct from that in skeletal muscle and brain,²¹ may thus be critical in explaining the degree of myocardial depression associated with the different anesthetics.

We previously demonstrated that halothane and enflurane, but not isoflurane, increase binding of radio-labeled ryanodine to porcine SR vesicles without altering the Ca^{2+} sensitivity.²² Other investigators have described similar results in canine tissue.²³ Because ryanodine binds to the open state of the channel,²⁴ this pharmacologic interaction should reflect anesthetic-related channel activation. The purpose of the present study was to test the hypothesis that alterations in conductance and gating of the SR Ca^{2+} release channel re-

sult from exposure to halothane and enflurane, but not isoflurane, and thereby definitively identify the role of this channel in the established negative inotropy resulting from volatile anesthetics.

Materials and Methods

Preparation of Cardiac SR

With approval of the University of Wisconsin Research Animal Resources Committee, adult swine were anesthetized with an intramuscular injection of tiletamine and zolazepam. After skin incision, sternotomy and dissection of the intrathoracic structures, the great vessels were ligated, the aorta cross-clamped and the aortic root injected with a standard cardioplegic solution (154 mM NaCl, 20 mM KCl, 16 mM MgCl_2 , 2 mM CaCl_2 , 2 mM EGTA, pH 7.2). After cardiac arrest, the heart was excised and placed in iced cardioplegia solution. The left ventricular free wall was dissected free of connective tissue, and used exclusively in the remainder of the preparation. Total SR rather than heavy SR (*i.e.*, terminal cisternal) was employed to minimize the period during which proteolytic degradation might occur prior to membrane storage. Cardiac microsomes were prepared by a modification of the methods described by Harigaya and Schwartz.²⁵ Ventricular tissue was minced in a precooled food processor, then homogenized in 5 volumes of buffer (5 mM TRIS, 0.1 M NaCl, pH titrated to 6.8 with maleate) in a commercial blender. This material was centrifuged at $2600 \times g$ (Sorvall, DuPont Instruments, Hoffman Estates, IL), for 30 min at 4°C to remove connective tissue, cellular debris and nuclei. The supernatant was next centrifuged at $100,000 \times g$ for 60 min at 4°C in a Beckman L7 centrifuge (Beckman Instruments, Palo Alto, CA) and the resulting pellet was resuspended as a crude microsomal fraction at approximately 10 mg/ml in 0.3 M sucrose, 0.4 M KCl, 20 μM CaCl_2 , 5 mM TRIS titrated to pH 6.8 with MES, snap frozen in liquid nitrogen and stored at -80°C . Protein concentrations were determined by the Bradford method using a Bio-Rad Kit (Richmond, CA). Six animals were used in the course of these experiments and anesthetic effects of each drug were determined in microsomal preparations from at least three animals.

Planar Bilayer Recording of Ca^{2+} Release Channels.

Planar bilayer formation and recording was performed as previously described at $23-25^\circ\text{C}$ ²⁶ with modifica-

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tions. Demonstration of high cationic conductance, calcium sensitivity and channel gating into a subconductance state in the presence of ryanodine verified SR Ca^{2+} release channel activity.²⁶ Bilayers composed of equal concentrations of phosphatidylethanolamine and phosphatidylserine (Avanti Polar Lipids, Birmingham, AL) were formed across a 150 μm diameter aperture in a Delran^R chamber using a 20 mg/ml mixture of the phospholipids in decane (Gold label, Aldrich Chemical, Milwaukee, WI). After bilayer formation, 100–200 μg of SR microsomal fraction was added to the cis solution composed of 500 mM CsCH_3SO_3 , 10 mM CsCl , 10 mM HEPES titrated with TRIS to pH 7.2. The trans solution was identical except for the presence of 50 mM CsCH_3SO_3 . Free Ca^{2+} was nominal (contaminant free, 1–3 μM) in all experiments, except where otherwise stated. Ca^{2+} channels transfer from vesicles to the bilayer through a process of membrane adhesion and fusion such that the extravascular face of the vesicular proteins face the side of the bilayer to which the vesicles were added.²⁷ In this case, the cytosolic face of the Ca^{2+} release channel projects into the cis chamber, while the SR luminal side of the channel is oriented toward the trans chamber. After identification of SR Ca^{2+} release channel activity in the bilayer, recordings were made on VCR tape using an analog to digital processor (PCM-2, Medical Systems, Greenvale, NY). For analysis, replayed data was filtered through a low-pass 8-pole Bessel (Frequency Devices, Haverhill, MA) at 1 kHz and digitized at 4 kHz using a TL-125 interface with pClamp and Axotape software (Axon Instruments, Foster City, CA). Digitized data was analyzed using Transit (Baylor College of Medicine, Houston, TX) and NFIT (University of Texas, Galveston, TX) software. Statistical analysis employed a Student's *t*-test for paired observations and statistical significance was established for $P < 0.05$.

Delivery and Measurement of Anesthetic Concentrations

Stock solutions containing anesthetic were prepared prior to each experimental session. Samples of cis recording solution were equilibrated with liquid anesthetic at room temperature in Teflon-capped glass vials for three hours. During this interval, cis solution became saturated with vapor-phase anesthetic, which was confirmed by gas chromatography. Anesthetic-saturated cis solution, free of liquid-phase anesthetic, was used to determine anesthetic effects, since liquid phase anesthetic obtained commercially is poorly miscible with

the cis solution and because the addition of liquid anesthetic to the cis chamber could expose the bilayer to foci with high concentrations of anesthetic (>5 vol %) that have been shown to irreversibly destroy SR function.¹⁷ Since baseline channel activity varies between channels (even from the same preparation), anesthetic effects were determined by recording the activity of the same channel before and after the addition of that anesthetic. In each experiment, a period of recording from 1 to 3 min was used as control after the incorporation of a channel into the bilayer. Subsequently, 300 μl of stock, anesthetic-saturated cis solution was added to the 3.5 ml cis chamber with a glass syringe. The cis chamber was sealed from the external environment and after 30 s of stirring with a magnetic bar previously placed in the cis chamber, channel activity was recorded for a period similar to the control period. At the completion of this period, a 625 μl aliquot of cis solution was aspirated with a glass syringe and placed in a 650 μl Teflon^R-capped vial.

Experiments were designed to determine anesthetic effects at equianesthetic concentrations in the range of 1–2 MAC (0.5–1 mM), since myocardial depression associated with the volatile agents has been characterized in this manner.^{5–8} After each experiment, vials containing cis solution were incubated and agitated in a water bath at 37°C for 30 min, a period that was adequate for equilibration between the gas and liquid phases. Since MAC values are gas phase measurements at 37°C, we measured and report gas phase concentrations in equilibrium with the experimental solutions at 37°C. However, the anesthetic partial pressure at 23°C would be 55–70% of that at 37°C. Although our experiments were performed at 23–25°C, this correction would alter aqueous phase concentrations minimally because the volume of gas in the measurement vials was small (<5%) relative to the total volume. Anesthetic concentrations in the gas phase were measured using gas chromatography with a flame ionization detector (Varian Model 3700, Varian Instruments, Sugar Land, TX) that had been calibrated with gas-phase standards (Scott Medical Products, Plumsteadville, PA). Anesthetic aqueous-gas partition coefficients determined at 37°C by gas chromatography (halothane and enflurane, 0.75; isoflurane, 0.70) were consistent with reported values²⁸ and the aqueous-phase concentration at 25°C was obtained by correcting for the diffusion of anesthetic into the gas volume in the sample vials (a small correction).

Gas phase calibration standards obtained commercially (0.5, 1.5 and 3 vol%, Scott Medical Products, Plumsteadville, PA) demonstrated that the gas chromatographic measurements were linear over the range studied. To assure that channels were exposed to a uniform concentration of the anesthetic during the period of observation, pilot experiments were performed to assess the loss of anesthetic from the experimental chamber during the recording period. In these experiments, interval sampling from the cis chamber demonstrated that the anesthetic concentration declined less than 10% over the duration of the typical experiment. Anesthetic in the trans chamber was not detectable over a similar time course. The concentration of anesthetic at the end of each experiment was recorded in each case.

Chemicals

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified. Preservative-free halothane was the generous gift of Halocarbon Laboratories (North Augusta, SC) and enflurane and isoflurane were purchased commercially from Anaquest (Madison, WI).

Results

Figure 1A shows the activity of a typical Ca^{2+} release channel from porcine cardiac SR at a cis (myoplasmic) contaminant free Ca^{2+} concentration of 1 to 3 μM and 0 mV. The channel displayed a single conductance level with a mean slope of 340 pS in recording solutions containing cis 500 mM and trans (luminal) 50 mM CsCH_3SO_3 . The reversal potential was more negative than -40 mV, indicating that the cation Cs^+ was the main current carrier.²⁶ In contaminant free Ca^{2+} , the open probability, P_o , measured as the fraction of open time averaged during the recording period, was always low and in this case was 0.05. Adjustment of the free Ca^{2+} of the cis solution to 100 μM resulted in an increase in P_o from 0.05 to 0.55 (fig. 1B). This increase in activity consisted of bursts of high P_o interspersed with periods of quiescence similar to that described in canine cardiac channels.²⁹ Burstlike activity was also present in cis solutions containing contaminant free Ca^{2+} but were much more prevalent at higher Ca^{2+} concentrations. The addition of 10 μM ryanodine to the cis chamber (fig. 1C) resulted in a loss of the unmodified unit conductance and the appearance, instead, of

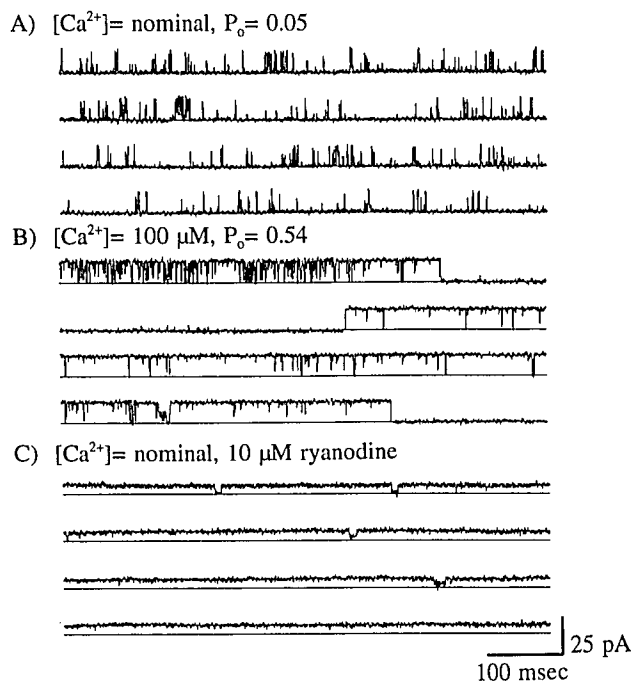


Fig. 1. Modulation of Ca^{2+} release channels by calcium and ryanodine. (A) Channel recordings at nominal free $[\text{Ca}^{2+}]$ of 1–3 μM demonstrate low channel activity. Upward deflections from the baseline represent channel openings. (B) Addition of Ca^{2+} to the same channel in A to raise free $[\text{Ca}^{2+}]$ to 100 μM results in a ten-fold increase in open probability and causes the channel to gate in bursts of high activity interspersed with period of quiescence. (C) Exposure of the Ca^{2+} release channel to 10 μM ryanodine activates a subconductance state with high P_o and long open dwell times.

a subconductance state with long-lived openings that results in a high open probability ($P_o > 0.95$). The high cationic conductance and the alterations in gating and conductance induced by ryanodine, as well as the modulation by Ca^{2+} demonstrated here for the porcine cardiac Ca^{2+} release channel are characteristic of cardiac Ca^{2+} release channels demonstrated in other species.^{30,31} However, the Cs^+ conductance was only 75% of that observed in canine,²⁹ bovine³² and ovine myocardium.³³

Activation of Channels by Volatile Anesthetics

Because halothane has been the most extensively studied volatile anesthetic, we first focused on the interaction of this agent with the cardiac SR Ca^{2+} release channel. P_o was determined in each experiment for the same channel prior to and after the addition of anesthetic-saturated cis solution and the gas-phase halo-

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thane concentration measured at 37°C at the conclusion of each experiment is reported. Continuous recordings of representative channel activity before and after the addition of halothane are shown in figure 2A, demonstrating an increase in P_o and the duration of each opening. Figure 2B demonstrates the activity of this channel over the entire experiment in the form of cumulative P_o , plotted as a function of recording time. In this plot, P_o was determined for each 500 ms interval, summed to the prior value and plotted against elapsed time. Time was rezeroed after the addition of halothane so that the plots in the presence and absence of halothane could be superimposed. The slope is proportional to the number of open events per unit time for each condition. From this plot it is apparent that halothane exposure results in channel activation that does not decay with time. This plot also demonstrates that long closed periods (segments parallel to x-axis) persist even in the presence of halothane and are interspersed with periods of high activity. Six separate experiments were performed in which a total of 25 min of channel activity was recorded in the presence and absence of halothane (1.2 ± 0.5 vol%, 0.64 mM in the aqueous phase). Halothane activated the channel in every experiment, and increased the mean P_o threefold from 0.026 ± 0.020 (mean \pm SD, $n = 6$) in the absence of halothane to 0.073 ± 0.034 ($P = 0.01$) in the presence of halothane. Halothane had no effect on either the unitary or slope conductance of the channels studied (data not shown).

We also investigated the effect of enflurane, since previous studies indicated that this anesthetic agent should exert an effect similar to that of halothane.^{15,20} Enflurane activated Ca^{2+} release channels in five experiments comprised of 20 min of recording time in the presence and absence of enflurane. At a mean concentration of 1.6 ± 0.4 vol% (0.85 mM aqueous), enflurane increased P_o from a control value of 0.024 ± 0.024 to 0.048 ± 0.047 ($P = 0.04$). Representative continuous channel recordings in the absence and presence of enflurane are shown in figure 3A and the cumulative P_o versus time relationship for the same

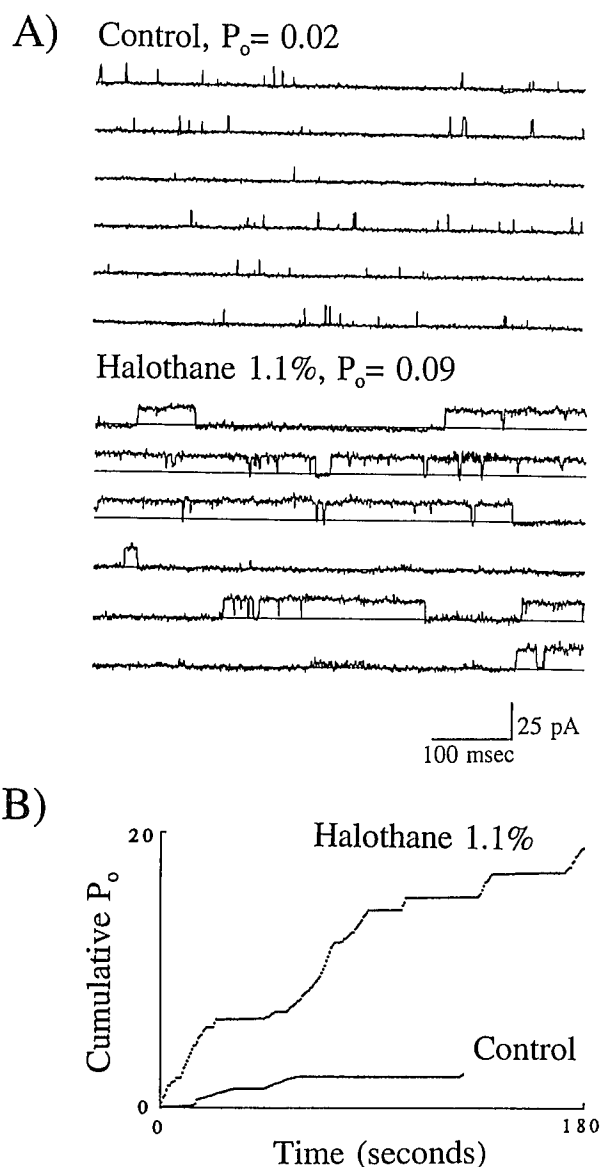


Fig. 2. Activation of Ca^{2+} release channels by halothane. (A) Representative tracings where upward deflections from the baseline represent channel openings recorded from single Ca^{2+} release channels demonstrate low activity and short duration of openings in the absence of halothane at nominal free Ca^{2+} and 0 mV. Activity of the same channel is demonstrated in the lower tracings after the addition of 1.1% halothane (gas phase at 37°C). Both P_o and the duration of open events increase after halothane exposure. P_o values noted are the mean values for that phase of the experiment. (B) Channel activity of the experiment shown in A are presented in their entirety in the form of cumulative P_o as a function of time. In this plot, the average P_o for each 500 ms interval is summed to the previous value, and plotted against the recording time in the absence of halothane. The same bilayer is exposed to halothane as described in Methods, and a cumulative P_o versus time plot is generated and superimposed on that for the control period. Anesthetic concentration measured at the end of the experiment is reported. Channel activation does not decay with time although the anesthetic does not prevent gating into a prolonged closed state (segments of plot that are parallel to the time axis).

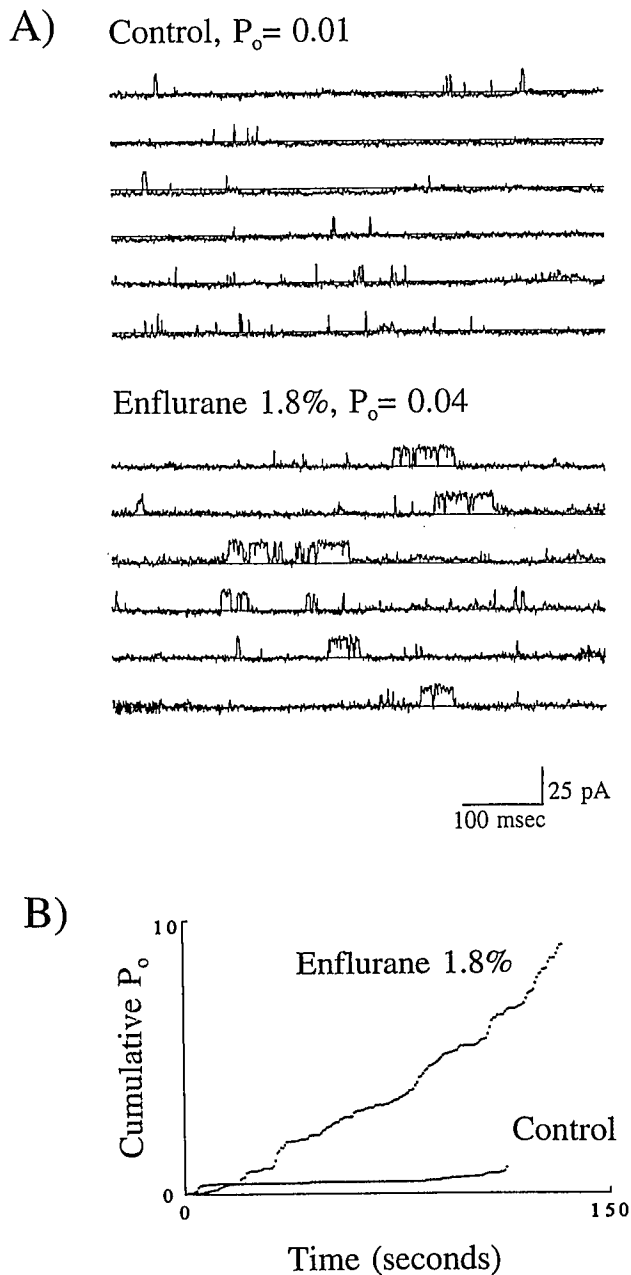


Fig. 3. Activation of Ca^{2+} release channels by enflurane. (A) Representative single-channel recordings from single Ca^{2+} release channels. Upper tracings demonstrate the channel activity in the absence of enflurane at nominal free Ca^{2+} and 0 mV. Exposure of the same channel to 1.8% enflurane (gas phase at 37°C) increases the open probability and increases the duration of open events, as illustrated in the lower tracings. (B) Cumulative P_o as a function of recording time for the same experiment as in panel A. The P_o for each 500 ms interval is summed to the previous value during prior to and after the addition of enflurane, and plotted as a function of recording time.

channel is shown figure 3B. Channel activation was similar to that produced by halothane in that there was no obvious decay with time, in that long closed periods persist and in that there was no effect on channel conductance.

If differences in myocardial depression caused by volatile anesthetics results from a selective interaction with the SR release channel, then isoflurane should have negligible effects on channel gating and conductance. This was observed in five experiments comprised of 16 min of channel activity recorded before and after exposure to isoflurane. Neither the gating or the unitary conductance of the Ca^{2+} release channel was altered by 1.4 ± 0.7 vol% (0.67 mM aqueous) isoflurane. The mean P_o decreased slightly from 0.041 ± 0.041 in the absence of isoflurane to 0.031 ± 0.030 after its addition. This change was not statistically significant ($P = 0.52$) and P_o increased after two exposures and decreased after three exposures to isoflurane. Channel recordings in the absence and presence of isoflurane are shown in figure 4A, while cumulative P_o as a function of time is shown for the same experiment in figure 4B.

Kinetics of Channel Activation

The open and closed state kinetics were studied to gain insight into the mechanism by which halothane and enflurane activate the Ca^{2+} release channel. Distributions of open and closed events before and after exposure to halothane were fit by exponential functions and are shown in figure 5. In these plots, the number of events of a specified duration were binned into 0.33 ms intervals and plotted against the duration of the events. Only events $> 500 \mu\text{s}$ were included in the fitting process because 50% of the events of shorter duration were missed due to the characteristics of the Bessel filter which was set at 1 kHz.³⁴ Open time distributions in the absence and presence of anesthetic were fit by the sum of two exponential functions, as demonstrated for one experiment with halothane in figure 5A. Employing a third exponential component did not improve the representation of the data, as determined by the chi-square value. The biexponential distribution of open events was consistent with previous reports indicating the presence of two open states in the cardiac Ca^{2+} release channel.^{29,31} Halothane (1.2 \pm 0.5 vol%, $n = 6$) increased the time constant representing the longer open events from a control value of 3.5 ± 1.3 ms to 7.4 ± 4.2 ms ($P < 0.05$) without altering the time constant representing the openings

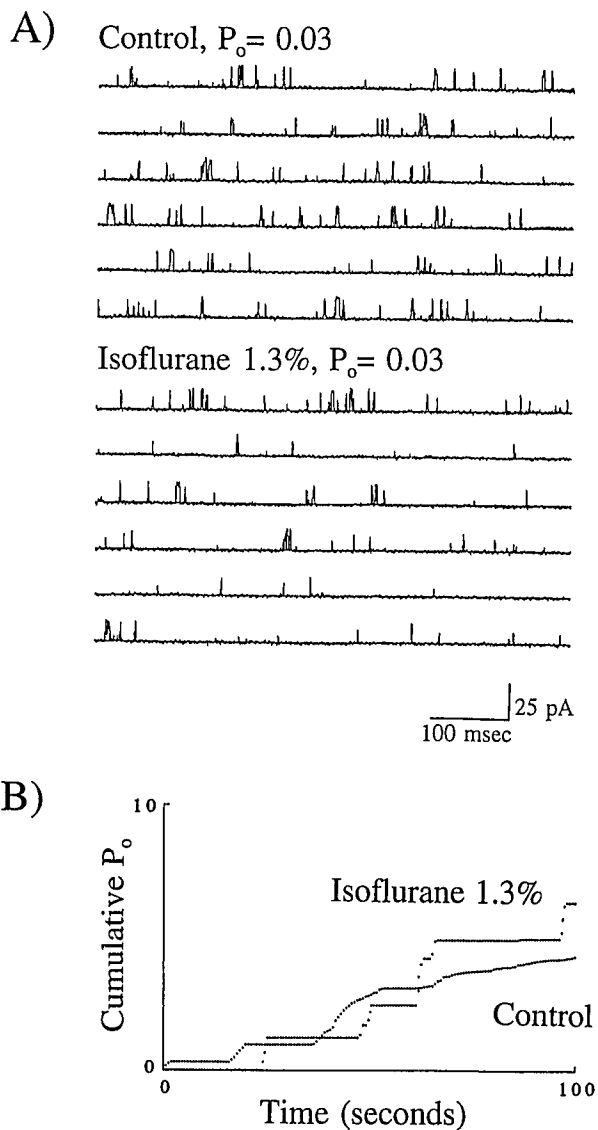
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Fig. 4. Effect of isoflurane on Ca^{2+} channel activity. (A) Representative tracings from a single Ca^{2+} release channel at nominal free Ca^{2+} and 0 mV in the absence (upper tracings) and presence of 1.3% isoflurane (gas phase at 37°C) demonstrate no change in channel activity resulting from anesthetic exposure. (B) Cumulative P_o as a function of recording time for the same experiment as shown in A. Superposition of the plots in the absence and presence of isoflurane demonstrates that isoflurane does not activate the channel. The channel gates in and out of long closed intervals both before and after the addition of isoflurane.

of short duration. Enflurane ($1.6 \pm 0.9\%$, $n = 5$) also shifted the duration of open events to longer times, as reflected by an increase in the long open time constant from a control value of 4.5 ± 2.8 ms to 7.3 ± 3.7 ms

($P < 0.05$) without altering the short open time constant. Isoflurane ($1.4 \pm 0.3\%$), on the other hand, did not effect the short or long time constants representing the open time distributions in five experiments.

Distributions of closed events before and after exposure to each anesthetic were represented by biexponential functions. Although a more prolonged closed state was evident in most recordings (best represented by horizontal segments in the cumulative P_o vs. time plots), the small number of events of this very long duration prevented reliable kinetic characterization. As shown for a representative channel in figure 5B, exposure to halothane resulted in no change in either the short or long time constants describing the closed time distributions (table 1).

The frequency of open events (number of events per millisecond of recording time) did not change from control values in the presence of halothane, enflurane or isoflurane. Therefore, based on the increase in open time constants observed after exposure to halothane and enflurane, we conclude that the increase in open probability resulting from channel exposure to halothane and enflurane can be attributed solely to an increase in the duration of open events.

Concentration Dependence and Reversibility of Channel Activation by Halothane

Separate experiments were performed to determine if activation of the Ca^{2+} release channel by halothane is dose dependent and whether the observed activation dissipates upon washout of the anesthetic from the experimental setup. In the experiments described above, bilayers were exposed to a single concentration of anesthetic targeted at 1–2 MAC for that anesthetic. Although some variability was found in the measured anesthetic concentrations of each anesthetic, the small number of observations and the variability in P_o between channels precluded the demonstration of dose dependency over this narrow concentration range. Experiments were therefore performed wherein a single bilayer was exposed to successively higher concentrations of halothane, each of which was confirmed by gas chromatography. Since microsomes with more than one channel fused with the bilayer as frequently as those with a single channel, and because only the analysis of channel kinetics precludes the use of experiments with multiple channels, we studied dose-dependency of halothane activation regardless of the number of channels in the bilayer. The results of these experiments are shown in figure 6, where separate experi-

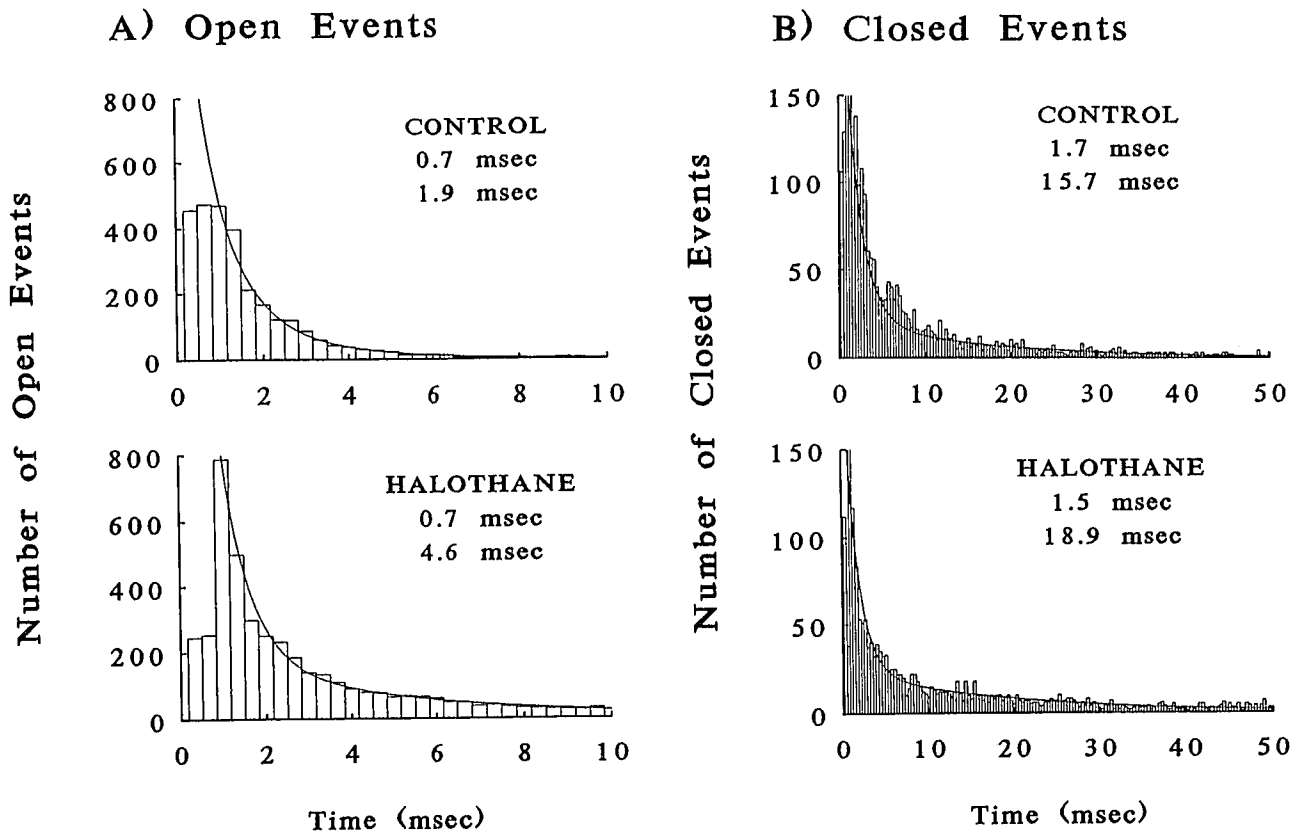


Fig. 5. Halothane effects on open and closed time distributions. (A) Open time distributions for the same channel in the absence (upper) and presence (lower) of halothane (0.9 vol%, gas phase at 37°C). Events longer than 500 μ s were fit by a biexponential function, and the short and long open decay constants are presented. Only the long open time decay constant is prolonged by halothane. (B) Closed time distributions for the same experiment. Biexponential fits of the distributions demonstrate no change in either decay constant resulting from exposure to halothane.

ments show channel activity as a function of halothane concentration when two or three channels are present in the bilayer. Channel activity for multiple channels was determined by

$$\sum n(P_o)_n = (P_o)_1 + 2*(P_o)_2 + 3*(P_o)_3 + \dots,$$

where $(P_o)_n$ is the fraction of time that n channels are open simultaneously. Figure 6 demonstrates that channel activation is minimal at subanesthetic concentrations and becomes pronounced at higher concentrations.

To demonstrate that volatile anesthetics do not cause irreversible changes in either the lipid or protein constituents of the bilayer that might result in the described alterations in gating, we performed experiments wherein channels were exposed to halothane and the anesthetic was subsequently removed. Washout of

halothane from the cis solution reversed the channel stimulation induced by the anesthetic in the two experiments where washout did not rupture the bilayer. In these experiments, after suitable periods of recording in the absence and presence of halothane, the cis chamber was perfused with 2–3 volumes of anesthetic-free cis solution, and channel activity was recorded after washout. The anesthetic concentrations were measured in the presence of halothane and after washout by gas chromatography. The results of one such experiment with three channels is shown in figure 7, where $\sum n(P_o)_n$ is plotted for each 500 ms interval during the periods before and after anesthetic exposure, and after washout. Channel activity (mean $\sum n(P_o)_n$) increased dramatically from control (0.08) values in the presence of 0.6% halothane (0.36) but decreased to control levels (0.09) after washout despite persistent

ANESTHETIC ACTIVATION OF CARDIAC SR Ca^{2+} CHANNELS**Table 1. Alterations in Channel Gating Resulting From Anesthetic Exposure**

	P_o	Fitted Parameters	
		Open Time τ_o (ms)	Closed Time τ_c (ms)
Control (n = 6)	0.026 ± 0.020	0.7 ± 0.4 3.5 ± 1.3	1.7 ± 0.4 20.0 ± 12.0
Halothane (1.2 ± 0.5%; n = 6)	0.073 ± 0.034*	0.6 ± 0.1 7.4 ± 4.2*	1.5 ± 0.1 19.1 ± 7.0
Control (n = 5)	0.024 ± 0.024	0.5 ± 0.1 4.5 ± 2.8	1.4 ± 0.6 25.0 ± 18.0
Enflurane (1.6 ± 0.9%; n = 5)	0.048 ± 0.047*	0.6 ± 0.1 7.3 ± 3.7*	1.1 ± 0.3 21.0 ± 18.0
Control (n = 5)	0.041 ± 0.041	0.6 ± 0.1 4.3 ± 1.3	1.4 ± 0.4 38.3 ± 31.5
Isoflurane (1.4 ± 0.3%; n = 5)	0.031 ± 0.030	0.7 ± 0.1 4.7 ± 2.8	1.7 ± 1.2 36.4 ± 34.3

Values are mean ± SD.

* Statistically significant changes ($P < 0.05$).

low levels of halothane (0.1%), suggesting that the lower concentration of halothane is below the threshold for channel activation.

Discussion

We previously demonstrated stimulation of [^3H]ryanodine binding to porcine cardiac SR resulting from exposure to halothane and enflurane but not isoflurane, at concentrations similar to those employed here.²² Single-channel recordings in planar bilayers were employed to establish the functional consequences of the interaction between the anesthetics and the receptor-channel complex. We sought to identify channel-anesthetic interactions at anesthetic concentrations within the range used in clinical practice. The mean concentrations measured in this study approximate 2 MAC for halothane, 1.3 MAC for isoflurane and 1 MAC for enflurane. Although this deviation from the targeted concentrations represents a small change in actual anesthetic concentration and reflects the difficulty in delivering anesthetics to a bilayer, the relatively lower concentrations of enflurane may help to explain why the alterations in channel gating resulting from enflurane were less impressive than those for halothane. Despite the fact that the alterations in channel kinetics were characterized for halothane at higher MAC concentrations than enflurane or isoflurane, the dose dependency experiments demonstrate that channel acti-

vation by halothane occurs at concentrations well below 1 MAC (<0.5 vol%).

Our findings are consistent with the results of studies in skinned myocardium¹⁷ implicating the SR Ca^{2+} release mechanism as a target for halothane that results in depression of myocardial contractility. Halothane increased the mean P_o three-fold while isoflurane caused no increase in mean P_o of the five experiments performed. At molar concentrations greater than those employed for halothane and isoflurane, but lower concentrations in terms of relative anesthetic potencies, enflurane caused a two-fold increase in mean channel P_o . We therefore suggest that activation of the SR Ca^{2+} release channel within the myocyte by halothane and enflurane but not by isoflurane would result in a depletion of Ca^{2+} that is to be released from the SR upon depolarization of the sarcolemma. Such a specific and reversible interaction of the anesthetics with the channel, much like the interaction of the channel with other ligands, would appear sufficient to explain the established relative potencies of volatile anesthetics as myocardial depressants.^{5,7} Since isoflurane had no effect on the SR Ca^{2+} release channel in this study, myocardial depression associated with its use must result from the

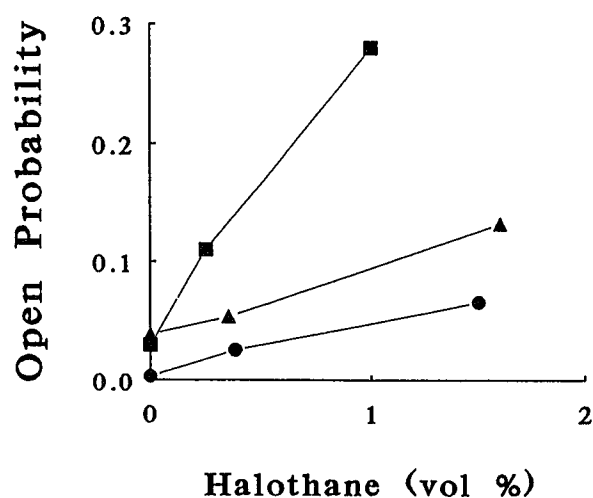


Fig. 6. Dose dependency of channel activation by halothane. Three experiments are presented wherein sequentially increasing concentrations of halothane (gas phase concentrations at 37°C) were delivered to the bilayer. Two or more channels incorporated into the bilayer with the primary fusion event in each of these experiments, and open probability is calculated as $\sum n(P_o)_n$, as defined in the text. Channel activation is evident at low halothane concentrations (0.2–0.5 vol%) and increases markedly in the range of 1–2 vol %. Closed circles and triangle represent experiments with two channels, while closed squares depict an experiment with three channels.

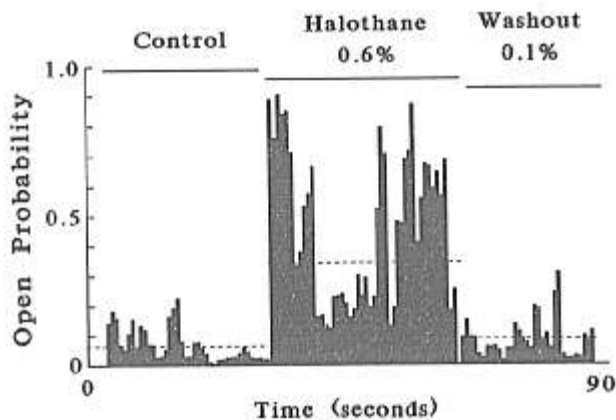


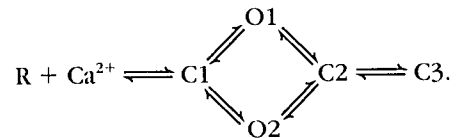
Fig. 7. Reversibility of halothane-induced channel activation. Open probability for the three channels incorporated into the bilayer ($\Sigma n(P_o)_{n,}$ as defined in the text) is plotted for each 500 ms interval in the absence and presence of halothane and after washout of the anesthetic from the cis solution. Periods of anesthetic washin and washout are omitted. The mean open probability for the control (0.08), anesthetic (0.36) and washout (0.09) phases of the experiment is plotted as a dashed line. Three conductance levels were evident in the recordings from each phase of this experiment, indicating that the washout process had not altered the number of channel in the bilayer. Detectable concentrations of halothane indicate that washout was incomplete, but at this low concentration, open probability is no different from control values.

inhibition of I_{Ca} that results from all these anesthetics or from a selective decrease in myofibril Ca^{2+} sensitivity that has been reported to occur only in the presence of isoflurane.⁷

Although stimulation occurred with every exposure of the Ca^{2+} release channel to halothane and enflurane, the extent of stimulation varied between channels. This is not unexpected, as channel activity in the absence of anesthetic is also variable.³⁵ While the origin of this wide range of responsiveness of the cardiac Ca^{2+} release channel to activating anesthetics is unknown, biochemical changes in the channel not under control in these experiments that are part of the normal cellular regulatory systems, such as the phosphorylation state of the channel,³⁶ may underlie the variation in sensitivity to anesthetics. These as of yet undefined conditions may explain the large standard deviations in P_o , and the kinetic parameters shown in table 1. This variability also provides further explanation, in addition to the fact that the number of observations was small, for why dose dependency was not demonstrated for the in the range of 1–2 MAC. While others have demonstrated dose dependency within this range,⁷ their observations represent the response of a cellular popu-

lation of Ca^{2+} release channels that cannot be approached using planar bilayer techniques.

Some insight into the mechanism through which halothane and enflurane activate the SR Ca^{2+} release channel can be obtained from the kinetics of channel activation. Both drugs caused a shift to longer open times without an obvious increase in the number of events per unit time. However, the activation observed occurred in bursts that were interspersed with long periods of quiescence. This complex behavior is best explained by the gating model for the cardiac SR Ca^{2+} release channel developed by Ashley and Williams³¹ and modified by Zahradnikova and Palade.²⁹



In this kinetic model, the channel exists predominantly in an inactive, resting state (R) in the absence of Ca^{2+} . The equilibrium is shifted away from the resting state in the presence of Ca^{2+} toward short-lived open (O1,O2) and closed (C1,C2) states, and to a long-lived closed (C3) state. We propose that halothane and enflurane preferentially reduce the rate of departure from one of the two open states, which results in longer openings. The analysis of closed time histograms did not reflect a change in the duration of short-lived closures upon exposure to anesthetics. Thus, neither C1 nor C2 seem to be affected by volatile anesthetics. In addition, the plots of cumulative open probability as a function of time demonstrated that the long-lived closed state, C3, persisted in the presence of halothane and enflurane. However, the relatively small number of long-lived events precluded the need for a third exponential component to fit the histograms of closed events. Qualitatively it would appear, though, that volatile anesthetics did not interact with the long-lived closed state of the channel.

Halothane, enflurane, and isoflurane are highly lipophilic compounds that readily partition into the lipid bilayer in the vicinity of channels. Considerable controversy exists over whether volatile anesthetics exert their action on ion channels by direct protein binding or secondarily by nonspecific perturbations of lipids. Although the results of this study demonstrate an interaction with the open state of the channel and would suggest a drug-protein interaction, a nonspecific interaction between the anesthetics, the hydrophobic core of membrane phospholipids and the channel protein cannot be excluded.

ANESTHETIC ACTIVATION OF CARDIAC SR Ca^{2+} CHANNELS

Despite the fact that isoflurane and enflurane are chemical isomers, their physical properties are not identical. Therefore, the protein channel cannot be conclusively identified as the sole target for the activating anesthetics. Such a conclusion could only be drawn from differential effects resulting from exposure to stereoisomers, as described by Franks and Lieb.³⁷

References

1. Brown BR, Jr, Crout JR: A comparative study of five general anesthetics on myocardial contractility. *ANESTHESIOLOGY* 34:236–245, 1971
2. Eger EI II, Smith NT, Stoelting RK, Cullen DJ, Kadis LB, Whitcher CW: Cardiovascular effects of halothane in humans. *ANESTHESIOLOGY* 32:396–409, 1970
3. Calverley RK, Smith NT, Prys-Roberts C, Eger EI, Jones CW: Cardiovascular effects of enflurane anesthesia during controlled ventilation in man. *Anesth Analg* 57:619–628, 1978
4. Katsuoka M, Ohnishi ST: Inhalational anesthetics decrease calcium content of the cardiac sarcoplasmic reticulum. *Br J Anaesth* 62:669–673, 1989
5. Lynch C III: Differential depression of myocardial contractility by halothane and isoflurane *in vitro*. *ANESTHESIOLOGY* 64:620–631, 1986
6. Housmans PE, Murat I: Comparative effects of halothane, enflurane, and isoflurane at equipotent anesthetic concentrations on isolated ventricular myocardium of the ferret: I. Contractility. *ANESTHESIOLOGY* 69:451–463, 1988
7. Bosnjak ZJ, Aggarwal A, Turner LA, Kampine JM, Kampine JP: Differential effects of halothane, enflurane, and isoflurane on Ca^{2+} transients and papillary muscle tension in guinea pigs. *ANESTHESIOLOGY* 76:123–131, 1992
8. Rusy BF, Komai H: Anesthetic depression of myocardial contractility: A review of possible mechanisms. *ANESTHESIOLOGY* 67:745–766, 1987
9. Bosnjak ZJ, Supan FD, Rusch NJ: The effects of halothane, enflurane, and isoflurane on Ca^{2+} currents in isolated canine ventricular cells. *ANESTHESIOLOGY* 74:340–345, 1991
10. Terrar DA, Victory JGG: Isoflurane depresses membrane currents associated with contraction in myocytes isolated from guinea-pig ventricle. *ANESTHESIOLOGY* 69:742–749, 1988
11. Ikemoto Y, Yatani A, Arimura H, Yoshitake J: Reduction in the slow inward current of isolated rat ventricular cells by thiamylal and halothane. *Acta Anaesthesiol Scand* 29:583–586, 1986
12. Beuckelmann DJ, Weir WG: Mechanism of release of calcium from sarcoplasmic reticulum of guinea-pig cardiac cells. *J Physiol (Lond)* 405:233–255, 1988
13. Lynch C III, Vogel S, Sperelakis N: Halothane depression of myocardial slow action potentials. *ANESTHESIOLOGY* 55:360–368, 1981
14. Su JY, Kerrick WGL: Effects of halothane on Ca^{2+} -activated tension development in mechanically disrupted rabbit myocardial fibers. *Pflügers Arch* 375:111–117, 1978
15. Su JY, Kerrick WGL: Effects of enflurane on functionally skinned myocardial fibers from rabbits. *ANESTHESIOLOGY* 52:385–389, 1980
16. Su JY, Bell J: Intracellular mechanism of action of isoflurane and halothane on striated muscle of rabbit. *Anesth Analg* 65:457–462, 1986
17. Herland JS, Julian EJ, 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
18. Wheeler DM, Rice RT, Hansford RG, Lakatta EG: The effect of halothane on the free intracellular Ca^{2+} concentration of isolated rat heart cells. *ANESTHESIOLOGY* 69:578–583, 1988
19. Wilde DW, Knight PR, Sheth N, Williams BA: Halothane alters control of intracellular Ca^{2+} mobilization in single rat ventricular myocytes. *ANESTHESIOLOGY* 75:1075–1086, 1991
20. Wilde DW, Davidson BA, Smith MD, Knight PR: Effects of isoflurane and enflurane on intracellular Ca^{2+} mobilization in isolated cardiac myocytes. *ANESTHESIOLOGY* 79:73–82, 1993
21. McPherson PS, Campbell KP: The ryanodine receptor/ Ca^{2+} release channel. *J Biol Chem* 268:13765–13768, 1993
22. Connelly TJ, El-Hayek R, Rusy BF, Coronado R: Volatile anesthetics selectively alter [^3H]ryanodine binding to skeletal and cardiac ryanodine receptors. *Biochem Biophys Res Commun* 186:595–600, 1992
23. Frazer MJ, Lynch III C: Ca^{2+} release channels of cardiac sarcoplasmic reticulum are activated by halothane, but not by isoflurane (abstract). *ANESTHESIOLOGY* 77:A630, 1992
24. Valdivia HH, Kirby MS, Lederer J, Coronado R: Scorpion toxins specifically targeted against the Ca^{2+} release channel of skeletal and cardiac sarcoplasmic reticulum. *Proc Natl Acad Sci USA* 89:12185–12189, 1992
25. Harigaya S, Schwatz A: Rate of calcium binding and uptake in normal animal and failing human cardiac muscle. *Circ Res* 25:781–794, 1969
26. Coronado R, Kawano S, Lee CJ, Valdivia C, Valdivia, HH: Planar bilayer recordings of ryanodine receptors of sarcoplasmic reticulum. *Methods Enzymol* 207:699–707, 1992
27. Niles WD, Cohen FS: Video fluorescence microscopy studies of phospholipid fusion with a planar phospholipid membrane. *J Gen Physiol* 90:703–735, 1987
28. Smith RA, Porter EG, Miller KW: The solubility of anesthetic gases in lipid bilayers. *Biochim Biophys Acta* 645:327–338, 1981
29. Zahradnikova A, Palade P: Procaine effects on single sarcoplasmic reticulum Ca^{2+} release channels. *Biophys J* 64:991–1003, 1993
30. Rousseau E, Smith JS, Henderson JS, Meissner G: Single channel and $^{45}\text{Ca}^{2+}$ flux measurements of the cardiac sarcoplasmic reticulum Ca^{2+} channel. *Biophys J* 50:1009–1014, 1986
31. Ashley RH, Williams AJ: Divalent cation activation and inhibition of single Ca^{2+} release channels from sheep cardiac sarcoplasmic reticulum. *J Gen Physiol* 95:981–1005, 1991
32. Kawano S, Coronado R: Ca^{2+} dependence of Ca^{2+} release channel activity in the sarcoplasmic reticulum of cardiac and skeletal muscle (abstract). *Biophys J* 59:600a, 1991
33. Lindsay ARG, Manning SD, Williams AJ: Monovalent cation conductance in the ryanodine receptor-channel of sheep cardiac muscle sarcoplasmic reticulum. *J Physiol (Lond)* 439:463–480, 1991
34. Colquhoun D, Sigworth FJ: Fitting and statistical analysis of single channel records, *Single Channel Recording*. Edited by Sakmann B, Neher E. New York, Plenum Press, 1983, pp 191–263
35. El-Hayek R, Valdivia C, Valdivia HH, Hogan K, Coronado R: Activation of the Ca^{2+} release channel of skeletal muscle sarcoplasmic reticulum by palmitoyl carnitine. *Biophys J* 65:779–789, 1993
36. Witcher DR, Kovacs RJ, Schulman H, Cefali DC, Jones LR: Unique phosphorylation site on the cardiac ryanodine receptor regulates calcium channel activity. *J Biol Chem* 266:11114–11152, 1991
37. Franks NP, Lieb WR: Stereospecific effects of inhalational general anesthetic optical isomers on nerve ion channels. *Science* 254:427–430, 1991