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Anesthetic alteration of ryanodine binding by cardiac calcium release channels

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Abstract

Differential cardiac contractile depression by volatile anesthetics is well documented, and evidence points to differing actions on the myocardial sarcoplasmic reticulum (SR). Since the Ca^{2+}-release channel (CaRC) of the SR binds ryanodine with high-affinity when opened by micromolar Ca^{2+} concentrations, ryanodine binding to cardiac SR membrane vesicles was employed as an assay of anesthetic modulation of CaRC activity. Canine ventricle was homogenized, centrifuged preparatively and then differentially on a sucrose gradient. A fraction enriched with CaRCs was defined by: the presence of a ~ 450 kDa protein consistent with CaRC; ~ 3-fold enhancement of vesicular $^{45}Ca^{2+}$ uptake by ruthenium red; Ca^{2+}-activated [^{3}H]ryanodine binding. Specific binding of 10 nM ryanodine was activated by > 0.5 μM Ca^{2+} and was maximal at ~ 6 pmol/mg protein in ≥ 20 μM Ca^{2+}. Halothane (1.5%), but not isoflurane, shifted the Ca^{2+}-dependence of ryanodine binding to lower [Ca^{2+}]. With submaximal activation by 5 μM Ca^{2+}, 1.5% and 0.75% halothane enhanced binding of 10–80 μM ryanodine, while 2.5% isoflurane and 3.5% enflurane did not. A plot of bound/free vs. bound ryanodine suggests that halothane causes a dose-dependent increase in ryanodine binding to a high-affinity site, while isoflurane has no such action. In intact myocardium, this effect will decrease Ca^{2+} retention in the SR so that less Ca^{2+} will be available to activate contractions, consistent with halothane's depressant action.

Key words: Anesthetic, volatile; Halothane; Isoflurane; Enflurane; Contractility; Calcium; Ryanodine receptor; (Heart)

1. Introduction

Inhalational anesthetic depression of myocardial contractility results from alterations in sarcolemmal Ca^{2+} entry, sarcoplasmic reticulum (SR) uptake and release of Ca^{2+}, as well as direct effects upon contractile proteins [1]. The latter effect appears to be relatively minor at clinically relevant concentrations [2–4], while substantial evidence suggests that multiple effects are present with regard to Ca^{2+} uptake and release by the SR [1,5–11]. Su et al. [5] demonstrated that in the presence of halothane there is decreased Ca^{2+} available for subsequent caffeine stimulated release in mechanically skinned rabbit myocardial fibers, an effect which is less prominent with isoflurane [12]. A number of mechanical studies on intact myocardium have suggested that halothane causes decreased accumulation of Ca^{2+} within the SR [5,8,9,13], while studies have also suggested other specific effects of isoflurane on Ca^{2+} release from the SR [6,14,15]. In studies on isolated SR vesicles, anesthetics caused decreased retention of Ca^{2+} in both actively or passively loaded vesicles [7,11]. The presence of a possible non-specific leak [11] obscured potential anesthetic effects on the Ca^{2+} release channels (CaRC), which are located at the SR membrane junction with the T-tubules and which regulate release of Ca^{2+} [16–18]. With Ca^{2+}-dependent CaRC activation and opening, CaRCs develop high-affinity sites for the plant alkaloid ryanodine [19–22]. Connelly et al. [23] have reported that halothane, isoflurane, and enflurane differentially enhance ryanodine binding by the skeletal and cardiac CaRC. To more fully elucidate how anesthetics may alter cardiac CaRC behavior under various intracellular conditions, we determined anesthetic effects on the affinity, number and Ca^{2+}-dependence of ryanodine binding sites in

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isolated junctional SR from canine ventricle. A preliminary report has been presented [24].

2. Methods

2.1. SR isolation

Dogs were euthanized and hearts removed according to the regulations approved by the Animal Research Committee of the University of Virginia. SR vesicles were prepared from canine ventricular tissue using a modified version of Alderson and Feher [19]. The tissue was homogenized in 10 mM imidazole at pH 7.0. All solutions were maintained at 4°C and contained the following proteinase inhibitors: 200 μM phenylmethylsulfonylflouride (PMSF), 77 nM aprotinin, 0.83 mM benzamidine, 1 mM iodoacetamide, 1.1 μM leupeptin, 0.70 μM pepstatin A. The supernatant solutions from two separate 20 minute $6000 \times g$ centrifugations were combined and centrifuged for 2 h at $100\,000 \times g$, which resulted in a crude microsomal pellet. This pellet was subsequently homogenized in 1 M KCl and 10 mM imidazole, layered on a discontinuous sucrose gradient consisting of 0.6, 0.8, 1.0, 1.1, 1.6 mM sucrose, and centrifuged for 10 h at $150\,000 \times g$. The proteins from each resulting interfacial fraction and from the pellet were solubilized, separated electrophoretically on a SDS-PAGE gel constructed with a linear 6–12% polyacrylamide gradient, and stained with Coomassie blue. Each fraction was also characterized by Ca^{2+} uptake capabilities at 37°C employing the method of Feher et al. [25]. SR vesicles (50 μg protein/ml of buffer) were added to assay buffer consisting of (mM) 100 KCl, 10 sodium azide, 5 mM potassium oxalate, 5 $MgCl_2$, 0.2 $^{45}CaCl_2$ (0.8 μCi/ml) and 20 imidazole (pH 7.0). Uptake was initiated with the addition of 5 mM ATP and duplicate 100 μl samples were removed and filtered at appropriate time intervals. Protein concentrations were determined using the method of Bradford [26].

Fractions from the 1.0/1.1 and 1.1/1.6 interfaces and from the pellet showed prominent ATP-supported uptake of $^{45}Ca^{2+}$. On electrophoresis of these three fractions, the most prominently stained proteins were at 105 and 45 kDa, characteristic for the Ca-ATPase and the calcium binding protein (calsequestrin) of cardiac SR, respectively. The densest fraction (No. 3) obtained from the pellet showed an additional band at 500–450 kDa corresponding to the Ca release channel (CaRC). In three heavy SR vesicle preparations, the control $^{45}Ca^{2+}$ uptake (0.97 ± 0.34 μmol/mg per min) was enhanced by almost 3-fold (2.9 ± 0.7 times) when 10 μM ruthenium red (RR) was added to block Ca^{2+} efflux via the CaRC. The lighter SR fractions showed enhancement with 10 μM RR of only 10–30%.

Binding experiments were performed at 37°C in 100, 250 mM, or 1 M KCl with 200 μM PMSF, 25 mM Pipes (pH 7.4), and addition of 200 μg protein/ml. Concentrations of [^{3}H]ryanodine (60 Ci/mmol) were made by dilution of labeled ryanodine with unlabeled drug. Ca^{2+} concentrations were buffered with the appropriate amount of EGTA to produce the free Ca^{2+} stated at the pH employed [27]. The quantity of bound ryanodine was determined from the SR retained after filtration (Whatman GF/B glass fiber) of the 0.5 ml aliquots. Filters were then washed three times with 5 ml of ice cold assay buffer, placed in 7 ml scintillation cocktail, and counted in a Beckman LS 230 scintillation counter. Results were expressed as pmol/mg SR protein. In control experiments, the incubation period was varied from five to 120 min to verify ryanodine binding had reached a steady state. Subsequent studies employed a 90 min incubation period. Non-specific binding was measured at each ryanodine concentration with the inclusion of 10 μM untritiated ryanodine and/or 10 mM EGTA and no added Ca^{2+}.

2.2. Reagents and anesthetic administration

Reagents and proteinase inhibitors were obtained from Sigma (St. Louis, MO). Radioisotopically labeled $^{45}Ca^{2+}$ and [^{3}H]ryanodine were obtained from New England Nuclear (Boston, MA), with a single batch of the latter used in all studies comparing anesthetic effects. Halothane was supplied by Halocarbon Industries (Newark, NJ); isoflurane and enflurane by Anaquest (Madison, WI). For a minimum 20 min equilibration period, buffer solution at the experimental temperature (usually 37°C) was bubbled via a fritted glass tube with filtered air (0.2 μm porosity filter) which was passed through an appropriate calibrated, temperature-compensated anesthetic vaporizer, providing approximately equipotent anesthetic concentrations [28]: 1.5% (of atmosphere) halothane; 2.5% isoflurane; 3.5% enflurane. The appropriate quantity of ryanodine was placed in a 1 ml glass vial, followed by an aliquot of SR on the opposite side, with final addition of a 0.5 ml aliquot of anesthetic-equilibrated buffer. The mixture was promptly sealed with Teflon-lined caps to prevent loss of anesthetic. The presence of anesthetic concentrations in solution appropriate for the known buffer/gas partition coefficient [29] were verified by gas chromatography. Halothane at 0.75% and 1.5% yielded approximately 0.25 and 0.5 mM in solution; whereas 2.5% isoflurane and 3.5% enflurane yielded 0.6 mM and 0.9 mM, respectively.

2.3. Statistical and binding analysis

Comparison among control and various anesthetics of ryanodine binding was made by ANOVA with Fish-

ers PLSD (protected least significant difference) test for planned comparisons (Statview, Abacus Concepts, Berkeley, CA). The dependence of specific bound ryanodine (bRy) on ryanodine concentration ([Ry]) was employed to estimate the changes in binding properties of the SR caused by anesthetics. Although complex multiple interacting ryanodine binding sites have been described [20], in this case the simplest model of ligand binding which approximated the results was assumed, in which ryanodine could bind to either a high- or low-affinity site (1 and 2, respectively) with maximum capacity B_{max} and dissociation constant K_d where:

$$K_d = \frac{[Ry](B_{max} - bRy)}{[bRy]} \quad (1)$$

so that

$$bRy_{total} = [Ry]\left\{ \frac{(B_{max1} - bRy_1)}{K_{d1}} + \frac{(B_{max2} - bRy_2)}{K_{d2}} \right\} \quad (2)$$

As an added constraint on the system, it was assumed that for the ryanodine binding sites:

$$\text{total } B_{max} = B_{max1} + B_{max2} \quad (3)$$

Control values for K_{d1}, K_{d2}, B_{max1}, and B_{max2} were obtained using a least-squares fitting program (Sigma Plot, Jandel Scientific) for a single ligand-two site model as described by Feldman (see Eq. 12) [30]. Binding of ryanodine reduced [Ry] by less than 1% (~ 3% with 20 μM Ca) and this small change was not included in the calculation of bRy/[Ry].

3. Results

Fig. 1A shows the [^{3}H]ryanodine (2 nM) retained by SR vesicles after varying times of incubation, and is similar to that reported previously [19]. When the ryanodine receptor was activated by 25 μM free Ca^{2+}, the rate of association of ryanodine with SR in 1 M KCl buffer was described by a single exponential with a time constant of 16 min (solid line). The presence of anesthetics did not alter the rate of association, while ruthenium red (1 μM), which binds to the ryanodine receptor, markedly depressed binding. Since stable binding was present beyond 75 min to at least 120 min (not shown), the 90 min incubation period employed in subsequent studies was sufficient to ensure equilibration. Fig. 1B shows the Ca^{2+}-dependence of ryanodine binding in 1 M KCl and 250 mM KCl buffer, which in this SR preparation resulted in activation of binding at > 0.1 μM Ca^{2+}. The presence of 1.5% halothane during the incubation significantly enhanced the binding of ryanodine at the threshold Ca^{2+} concentrations for each study. In contrast and significantly different

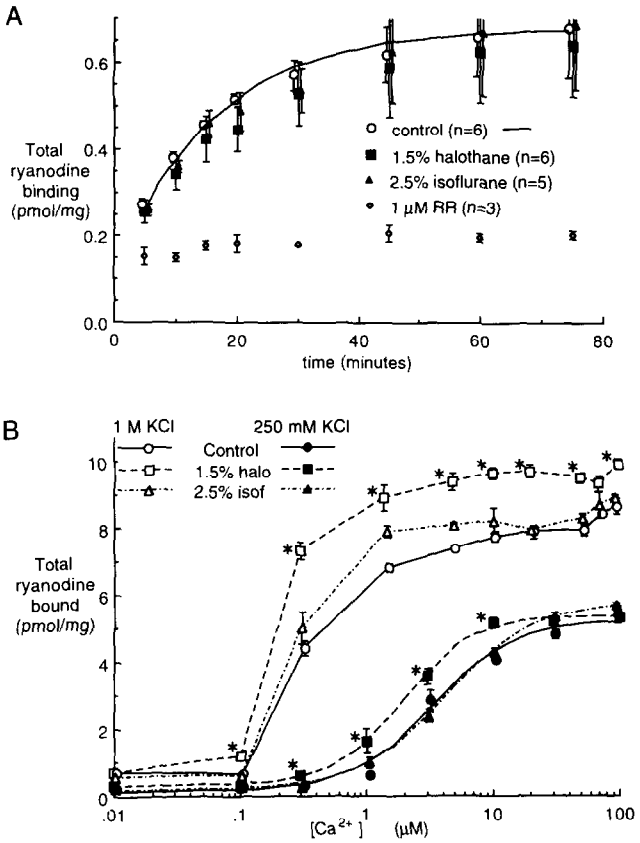

Fig. 1. (A) Time dependence of total ryanodine association with SR vesicles using 25 μM Ca^{2+} for activation and labeling with 2 nM ryanodine. The non-specific binding was estimated at 0.09 pmol/mg protein. The solid line describes binding to a single site with a time constant (τ) of 16 min. Amount and rate of ryanodine binding was unaltered by the anesthetics with such maximal Ca^{2+} activation. (B) Ca^{2+} dependence of ryanodine (10 nM) binding to isolated SR vesicles in either 1 M ($n = 4$) or 250 mM ($n = 5$) KCl at 37°C. The vesicles employed were derived from two different heavy SR preparations; the preparation in 1 M KCl demonstrated activation at unusually low Ca^{2+} concentrations. In both cases, halothane significantly enhanced binding versus control or isoflurane ($^*P < 0.05$). As indicated by the curves describing ryanodine binding in 250 mM KCl, the data was well described by a Hill equation in which: bound ryanodine = B_{max} $[Ca^{2+}]^n/(([Ca^{2+}]_{50})^n + [Ca^{2+}]^n)$). According to a least-squares analysis fit to the data, n (1.34 to 1.58) and B_{max} (5.0 to 5.6 pmol/mg) did not differ significantly. The $[Ca^{2+}]$ for half maximal binding ($[Ca^{2+}]_{50}$) decreased from a control value 3.4 ± 0.7 to 2.0 ± 0.1 μM in the presence of halothane; isoflurane (4.3 ± 0.2 μM) did not differ from control.

from halothane, the equivalent concentration of isoflurane (2.5%) had less effect. The $[Ca^{2+}]$ for activation of half-maximal ryanodine binding was clearly shifted to lower $[Ca^{2+}]$ by halothane, but not by isoflurane.

Ryanodine binding as a function of [Ry] is shown in Fig. 2. For the SR preparation employed, the inset in Fig. 2A indicates a clear component of Ca^{2+}-dependent activation at $[Ca^{2+}] \geq 1$ μM. With 10 nM ryanodine, 5 μM Ca^{2+} resulted in 1.0 ± 0.1 pmol/mg total bound ryanodine, compared to 6.2 ± 0.3 with 20 μM Ca^{2+}. An obvious component of specific high-affinity

binding activated by 20 μM Ca^{2+} appeared saturated in 10–50 nM ryanodine (Fig. 2A), with further increase in specific binding observed only at 100 and 500 nM ryanodine suggesting the presence of an additional binding site. Activation of ryanodine binding with 100 μM Ca^{2+} was no greater than that seen with 20 μM Ca^{2+}(not shown for clarity). In contrast, 5 μM Ca^{2+} resulted in a gradual ryanodine-dependent increase in binding up to 100 nM and beyond. Non-specific binding was linearly related to ryanodine concentrations studied (10 nM–2000 nM). In the presence of excess unlabeled ryanodine with either 5, 20 or 100 μM free [Ca^{2+}], or in the absence of activating Ca^{2+}, there was similar nonspecific binding which averaged 0.0475 pmol/mg per nM ryanodine.

Since the action of halothane was absent or less profound with maximal Ca^{2+} activation (Fig. 1B), anesthetic actions on ryanodine binding were compared at submaximally stimulating 5 μM Ca^{2+}. The effect of 1.5% halothane is indicated in Fig. 2A and magnified in 2B, with enhancement of ryanodine binding evident at 10 nM–80 nM. Fig. 2B shows actions of the various anesthetics over the 10–100 nM ryanodine range. Both 0.75% and 1.5% halothane enhanced the amount of total ryanodine bound above control, which was also significantly higher than the binding with 2.5% isoflurane. At 10, 30, and 70 nM [^{3}H]ryanodine, 1.5% halothane also differed significantly from 3.5% enflurane which caused no change in ryanodine binding. Non-specific binding was unaltered by the presence of halothane or enflurane (one experiment each, data not shown). Under identical conditions, 5 mM caffeine clearly caused profound activation of ryanodine binding.

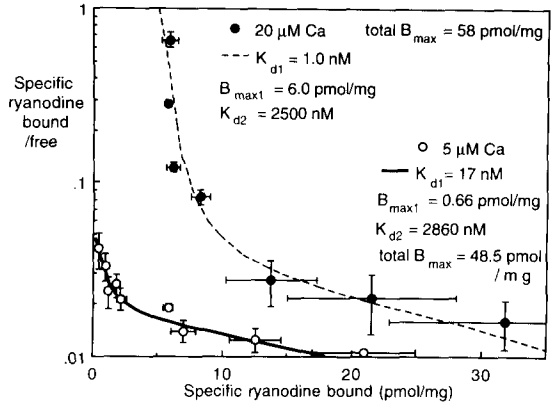

Fig. 3. Scatchard analysis of specific [^{3}H]ryanodine binding to junctional SR vesicles activated by 5 or 20 μM Ca^{2+} in 1 M KCl ($n = 5$). The lines represents a least-squares fit to the data points for a model in which [^{3}H]ryanodine binds to either a high- or low-affinity site with the values indicated (see text for equations).

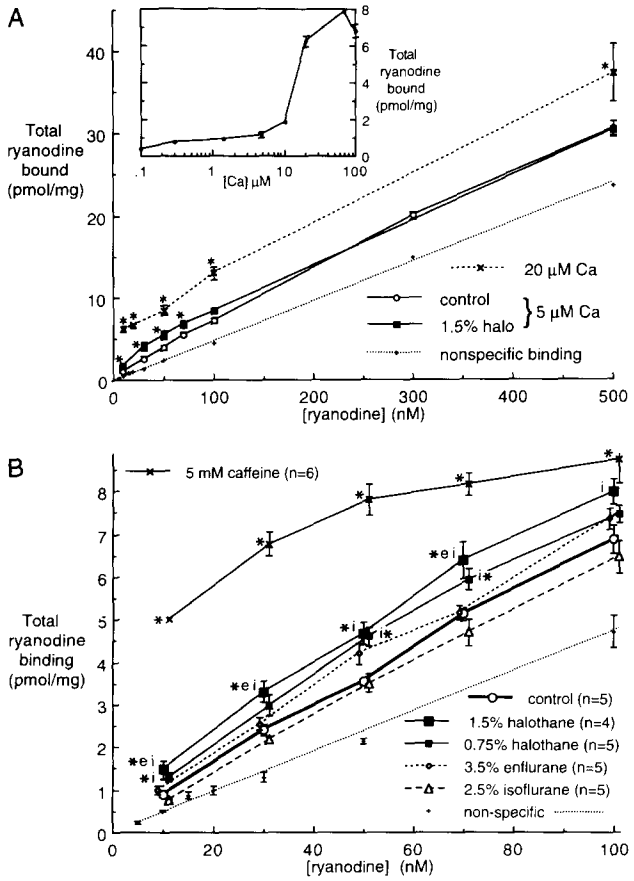

Fig. 2. (A) Total [^{3}H]ryanodine binding in the presence of 20 μM Ca^{2+} ($n = 5$) and 5 μM Ca^{2+} (control, $n = 5$, and with 1.5% halothane (~ 0.5 mM), $n = 4$) in 1 M KCl. The inset shows the Ca^{2+} dependence of ryanodine binding in this particular SR vesicle preparation ($n = 3$). Ryanodine binding in the presence of 10 μM excess unlabeled ryanodine is also shown as non-specific binding ($n = 5$), which was a linear function of [^{3}H]ryanodine, being 0.0475 pmol/mg per nM ($R^2 = 0.998$). (B) Anesthetic effects on total [^{3}H]ryanodine binding in 5 μM Ca^{2+}. Halothane significantly enhanced ryanodine binding: * $P < 0.05$ for difference from control; i, $P < 0.05$ for difference from isoflurane; e, $P < 0.05$ for difference from enflurane.

The specific ryanodine binding under various conditions could be most simply described by binding to a high- and low-affinity site. In 20 μM Ca^{2+}, the specific binding with 2000 nM ryanodine was 24 $\pm$ 6 pmol/mg of which ~ 6 pmol/mg represents binding to the high-affinity site saturated $\leq$ 20 nM ryanodine. If the low-affinity ryanodine binding is assumed to have a K_d of 2000–6000 nM [16,17,31], in 2000 nM ryanodine the remaining 18 pmol/mg bound would represent 25–50% of B_{max2}, giving an estimated range of 36–72 pmol/mg. Fig. 3 shows the plot of bound/free vs. bound ryanodine in the presence of 5 or 20 μM Ca^{2+} with the low and high binding evident (a log scale on the ordinate is employed for clarity). The affinity and number binding sites was clearly modulated by Ca^{2+}: for the high-affinity ryanodine binding in 20 μM Ca^{2+}, $K_{d1} = 1.0$ nM and $B_{max1} = 6$ pmol/mg, whereas in 5 μM Ca^{2+}, the binding was best described by $K_{d1} = 17$ nM and B_{max1} 0.66 pmol/mg. While the total number of binding sites in 5 μM appeared slightly lower (58 vs. 48.5 pmol/mg) the difference was well within the error of the approxi-

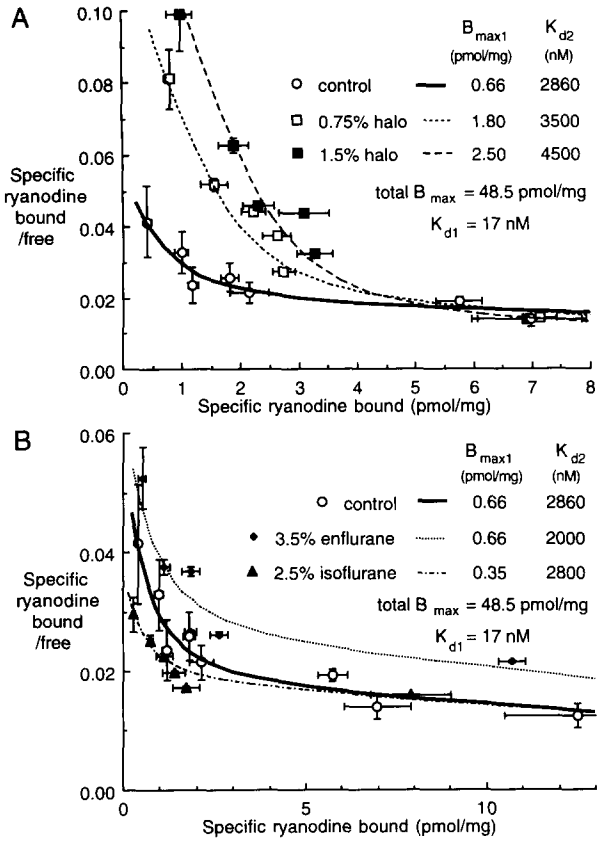

Fig. 4. Scatchard analysis of 0.75 and 1.5% halothane (A), or 2.5% isoflurane and 3.5% enflurane (B) effects on specific [^{3}H]ryanodine binding to junctional SR vesicles activated by 5 μM Ca^{2+} in 1 M KCl. The lines in both plots are results generated assuming [^{3}H]ryanodine binds to either a high- or low-affinity site according to text equations using the values indicated in the panels. The heavy solid line (control) is the same as that indicated in Fig. 3. Anesthetic effects on ryanodine binding beyond 500 nM were not explored in this particular preparation, so the changes in low-affinity binding by the anesthetics are less certain. Note different scales are employed in A and B.

mation; K_{d2} also showed little difference in 5 μM vs. 20 μM Ca^{2+}. Since [ryanodine] did not exceed K_{d2}, the value of K_{d2} may be underestimated.

The Scatchard analysis of anesthetic effects on specific ryanodine bound in 5 μM Ca^{2+} is shown in Fig. 4. The dose-dependent enhancement of total ryanodine binding caused by halothane (10 nM-100 nM [^{3}H]ryanodine) is clearly evident in the Scatchard analysis (Fig. 4A). The major effect of halothane could be adequately described simply by an increase in the number of high-affinity (K_{d1} = 17 nM) sites, with no apparent change in binding affinity. For 0.75 and 1.5% halothane, B_{max1} increased by 2.7 and 3.8 times from an estimate of 0.66 pmol/mg, to 1.8 and 2.5 pmol/mg, respectively. The lack of a major effect of halothane on the affinity of the high-affinity site is evident from the similar slopes representing high-affinity binding in Fig. 4A. However, since the ryanodine binding was not increased at 500 nM by 1.5% halothane, a decreased binding to the low-affinity sites also would appear to have occurred to compensate for the increased high-affinity binding. In Fig. 4A an increase in K_{d2} was employed to approximate this behavior, although it could also be described by an decrease in B_{max2}.

In contrast to halothane, 2.5% isoflurane and 3.5% enflurane did not increase high-affinity binding of ryanodine (Fig. 4B). Clearly, neither the affinity nor the number of the high-affinity sites appear to be enhanced by the presence of these two ether anesthetics. Increased specific binding in 500 nM ryanodine caused by enflurane could be approximated by a decrease in K_{d2} (increased affinity), resulting in increased binding to the low-affinity site. For isoflurane, there is the suggestion that the number or affinity of high-affinity sites is decreased, while K_{d2} may also have decreased in order to account for unchanged binding in 500 nM ryanodine. However, the description of actual 10–50 nM ryanodine binding was relatively insensitive to similar changes in K_{d2} and B_{max2}, as expected since low-affinity binding accounts for 30% of bound ryanodine at most in this range. Over the range 10–100 nM ryanodine, K_{d2} = 2100 nM and total B_{max} = 32 pmol/mg protein, or K_{d2} = 8000 nM and total B_{max} = 80 pmol/mg protein would also describe the low-affinity binding.

In order to verify that the action of halothane was not limited to effects in 1 M KCl, the effect of 1.5% halothane on ryanodine binding was examined in 100 mM KCl. In this setting total binding was enhanced (Fig. 5A), as was specific binding at in 5, 10, 20 and 100 nM ryanodine. Binding at 300, 500 and 1000 nM ryanodine was actually depressed by the anesthetic (not shown). The plot of bound/free vs. bound (Fig. 5B) is similar to that observed in 1 M KCl study, although control B_{max1} was considerably higher (4.8 vs. 0.66 pmol/mg). As in the higher ionic strength, halothane caused an apparent increase in the number of high-affinity binding sites of 36% with no apparent change in affinity. In addition, there appeared to be a decrease in binding to low-affinity sites, which could be explained by a decrease in affinity (increase in K_{d2} as shown in Fig. 5B) or a decrease in the number of binding sites (not shown).

To further verify the differential enhancement of high and low-affinity binding by anesthetics, the effects of anesthetics were explored with 10 and 1000 nM ryanodine with varied activation by Ca^{2+}. In this SR vesicles preparation in 300 mM KCl buffer, binding of 5 nM ryanodine activated by 0.5 or 10 μM Ca^{2+} was 19% and 98% of the maximal binding (5.2 pmol/mg at 50 μM Ca^{2+}). As indicated in Table 1, 1.5% halothane alone of the three anesthetics was able to increase specific ryanodine binding when activating Ca^{2+} was 0.5 μM. In the presence of 10 μM Ca^{2+}, both halothane and enflurane enhanced binding. When low-affinity

Table 1
Anesthetic effects on specific ryanodine binding to cardiac SR vesicles (pmol/mg)

	n	10 nM ryanodine		1000 nM ryanodine	
		0.5 μM Ca^{2+}	10 μM Ca^{2+}	0.5 μM Ca^{2+}	10 μM Ca^{2+}
Control	8	1.21 ± 0.06	5.42 ± 0.14	2.0 ± 1.6	8.6 ± 0.5
1.5% halothane	4	2.44 ± 0.11 *	7.49 ± 0.35 *	2.8 ± 2.4	8.2 ± 1.8
2.5% isoflurane	4	1.37 ± 0.10	5.91 ± 0.26	5.0 ± 1.9	14.5 ± 1.3 *
3.5% enflurane	4	1.08 ± 0.09	7.15 ± 0.22 *	12.0 ± 2.6 *	22.9 ± 2.9 *

Experiments performed in 300 mM KCl buffer at pH 7.0. Non-specific binding was 0.037 pmol/mg per nM ryanodine. * $P < 0.05$ for difference from control by ANOVA.

binding was assessed with 1000 nM ryanodine, 3.5% enflurane demonstrated a clear ability to activate low-affinity ryanodine binding in the presence of 0.5 or 10 μM Ca^{2+}, consistent with the results in Fig. 4B. This activation of low-affinity binding was also seen to a lesser extent with 2.5% isoflurane in the presence of 10 μM Ca^{2+} activation. Although halothane increased high-affinity binding, the absence of enhanced binding with 1000 nM ryanodine suggests that low-affinity binding was inhibited by halothane to result in no increase, confirming the effect suggested in Figs. 4A and 5B.

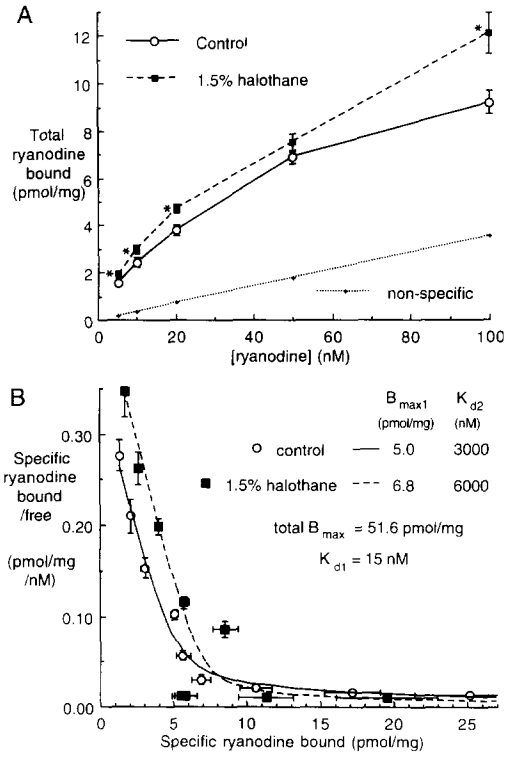

Fig. 5. (A) Effects of 1.5% halothane (~0.5 mM) on total [^{3}H]ryanodine binding activated by 5 μM Ca^{2+} in 100 mM KCl ($n = 7$). * $P < 0.05$ for difference from control. (B) Scatchard analysis of halothane effects. The control results (open circles) could be described by a model of [^{3}H]ryanodine binding to a high- and low-affinity site. The solid line represents a least-squares fit to the data using the values indicated (see text for equations). Halothane (filled squares) increased high-affinity binding and decreased low-affinity binding, an effect which could be described either by the decrease in affinity of site 2 (dashed line), or by a decrease in the number binding sites (not shown).

4. Discussion

The volatile anesthetics produce differential myocardial depression by exerting divergent effects at various cellular sites, particularly in altering Ca^{2+} fluxes through the sarcolemma and sarcoplasmic reticulum (SR) [6,8–12,14,32,33]. Studies in skinned myofibrils have suggested a greater effect of halothane in reducing the Ca^{2+} available for release from SR Ca stores when activated by caffeine [12]. However, anesthetic effects on isolated cardiac SR vesicle fluxes demonstrated that isoflurane and halothane decreased Ca^{2+} uptake and increased Ca^{2+} efflux, even in the presence of presumed blockade of the CaRC by the polycation ruthenium red [11]. While halothane appeared to have a greater action, it was not possible to determine if non-specific leak was more enhanced by halothane or whether RR binding to the CaRC might not have been altered. The aim of this study was to delineate any divergent effects on the SR Ca^{2+} release channel (CaRC) that controls release of Ca^{2+} which in turn activates tension development.

The plant alkaloid ryanodine has been a valuable tool to define the function and role of the CaRC, resulting in the moniker ryanodine receptor for these large homotetrameric proteins (~ 5000 amino acids/subunit), which appear to possess three isoforms [34]. Evidence from biochemical [20,35], electrophysiologic [36], and physiologic experiments [37] suggest that ryanodine binds to the CaRC with high-affinity when the channel opens, locking the channel in an open, yet less conductive state [36,38]. Binding of ryanodine (100 μM) to a low-affinity site correlates with channel closing [38] and even slower dissociation from the channel [39,40].

Agents (caffeine, ATP, anthraquinones) which lead to increased channel opening also enhance ryanodine binding [38,40–43], but of primary physiological relevance is Ca^{2+}, which in cardiac muscle serves to trigger opening of the channel [44], activating release of Ca^{2+}

stored in the SR. The Ca^{2+}-dependence of ryanodine binding observed in these canine cardiac SR preparations is similar to that reported previously with a 10–90% binding increase over a 6–8-fold increase in $[Ca^{2+}]$ [19]. In the present study, one SR preparation studied showed a very high Ca^{2+} sensitivity for activation in 1 M KCl, but this was not a constant finding. The increased ryanodine binding sensitivity to Ca^{2+} has been attributed to an action of ionic strength [40], but various reports suggest that this may not be a consistent effect in cardiac muscle [45,46]. In the presence of 50–100 μM activating Ca^{2+}, a variety of studies have documented a ryanodine binding site with an affinity of 1–5 nM [19,20,47]. Activation by ≥ 20 μM Ca^{2+} or 5 mM caffeine in this study also generated high-affinity binding which was saturated in < 10 nM ryanodine. Employing 25 μM Ca^{2+}, initial studies [16,48] in isolated 'heavy' cardiac SR vesicles documented high and low-affinity sites for ryanodine (K_d values of 17 and 1060 nM) similar to those estimated here with 5 μM Ca^{2+}.

A previous ryanodine binding study in isolated skeletal and cardiac SR vesicles suggested differing effects of halothane, isoflurane, and enflurane in modulating CaRC activation [23], effects which are verified and expanded in the present report. Halothane activated greater ryanodine binding at the threshold range of $[Ca^{2+}]$ which opens CaRCs and increases ryanodine binding, while isoflurane had no such effect. The action of halothane appears similar to that of caffeine and higher $[Ca^{2+}]$ (≥ 20 μM) which activate CaRC opening and enhance ryanodine binding. Unlike higher $[Ca^{2+}]$, caffeine, or anthraquinones which increase CaRC affinity for ryanodine to the higher level ($K_d < 4$ nM) [20,36,42,47,49], halothane did not appear to markedly increase ryanodine affinity. Due to the scatter in the data a modest increase in CaRC ryanodine affinity may have been missed, however, there is no appearance of any major component with a K_d of 1–2 nM as with 20 μM Ca^{2+}.

While two ryanodine site binding sites have been typically described for cardiac SR [16,48], the actual binding pattern for ryanodine to these receptors can be more complicated, with up to four sites demonstrable [20], and positive [39] or negative cooperativity [20] evident in dissociation studies. In the present study the two-site model adequately described the control ryanodine binding behavior, and was employed to estimate anesthetic-induced changes and to clarify whether enhanced binding is due to alteration in number or affinity of binding sites. The estimation of low-affinity binding might contain a substantial error, since binding was not explored above 2000 nM ryanodine which was less than the estimated low-affinity K_d. However, the value obtained for K_{d2} was similar to estimates by others [16,48]. The major action of halothane was an increase in the apparent number (B_{max1}) of ~ 20 nM K_d binding sites, with a greater effect at the higher concentration. If binding of Ca^{2+} to the channel (Ch) causes it to assume an open state (Ch*), to which ryanodine then binds with high-affinity (K_{d1}), it may be stated:

$$Ca^{2+} + Ch \leftrightarrow Ca^{2+} \cdot Ch \leftrightarrow Ca^{2+} \cdot Ch^*,$$

$$\text{and } Ca^{2+} \cdot Ch^* + Ry \leftrightarrow Ca^{2+} \cdot Ch^* \cdot Ry.$$

The results suggest that halothane shifts the first reaction scheme to the right, either by increasing the Ca^{2+} affinity of the CaRC or by increasing the subsequent rate at which it assumes the open (ryanodine binding) state. The greater number of open channels appear to bind ryanodine with the same affinity since K_{d1} showed little halothane-induced change, suggesting that halothane has little effect on the second reaction scheme. While an increased K_{d2} for the low-affinity site was employed to describe the halothane results (Figs. 4A anad 5B) and to permit better quantitation of changes in the high-affinity binding, assumptions about its value do not markedly alter the conclusions about high-affinity binding. We cannot rule out marked changes in K_{d2} and B_{max2} of the low-affinity site, which would require an decrease (or increase) in K_{d1} to describe the results. However, an increase in B_{max1} ($Ca^{2+} \cdot Ch^*$) combined with a modest increase in K_{d2} or decrease in B_{max} was the most parsimonious explanation for our observations. Higher halothane concentrations beyond the clinical levels employed might demonstrate the increases in affinity seen with caffeine. To explain the same total binding in 500–1000 nM ryanodine as seen with control, halothane apparently decreased low-affinity binding (increased K_{d2} or a decreased B_{max2}) coincident with the greater high-affinity binding. This reciprocal change in binding has been observed previously with activation of high-affinity binding by other agents [40]. Pessah et al. [20] have suggested that the ryanodine modulates its own binding when activated, with negative cooperativity being evident.

Due to the slow binding of ryanodine to activated CaRCs, the assay employed requires a prolonged incubation. Presumably, these effects of halothane occur rapidly in intact muscle to account for the observed physiological actions. When applied with submaximally stimulating caffeine to skinned cardiac fibers, halothane enhances SR release of Ca^{2+}[5]. When a high halothane concentration is applied abruptly to papillary muscles, halothane will briefly enhance the next few contractions [50,51]. The increased myocardial tension is all the more surprising since halothane depresses sarcolemmal Ca^{2+} currents [50,52,53] which trigger Ca^{2+}-induced Ca^{2+} release [54], suggesting that the Ca^{2+} sensitization of the CaRC by halothane overrides its

depression of Ca^{2+} current. Sustained opening of CaRCs during diastole will cause ongoing depletion of accumulated SR Ca^{2+} stores [5,9,55,56], reducing Ca^{2+} available for subsequent contraction. When combined with Ca^{2+} current depression, such CaRC-mediated effects provide a mechanism for myocardial contractile depression by sustained halothane administration.

The effects of isoflurane on the Ca^{2+}-dependence of ryanodine binding differed significantly from those of halothane, with no increased binding of ryanodine to higher affinity sites. Physiologically, application of isoflurane to regularly contracting myocardium causes no transient enhancement of tension as seen with halothane [51]. In contrast to halothane, enflurane markedly increased low-affinity ryanodine binding, as well as high-affinity binding with maximally stimulating Ca^{2+} (Table 1), implying an altered CaRC conformation that primarily augments low-affinity binding and that is distinct from the change induced by halothane. It is not clear if increased Ca^{2+} may contribute to this process, nor whether it represents a change in K_{d2} (which was used to describe the data, Figs. 4 and 5) or whether B_{max2} may have changed. Consistent with opening of CaRCs, release of SR Ca^{2+} is activated in isolated myocytes by halothane application [10,57]. However, similar effects were also found with enflurane and isoflurane in certain models [57]. The present results provide little support for such similar cellular effects among these agents being mediated via similar actions on CaRCs, unless some transient effect of isoflurane and enflurane on the CaRCs is not observed due to the sustained incubation. Since isoflurane and enflurane produce myocardial depression that has been linked in part to altered SR function [6,58], the effects of these agents also may be due to actions on other aspects of SR function such as Ca^{2+}-ATPase activity. In any case, the distinct effects of these different halogenated hydrocarbons on the CaRC suggests a direct protein interaction, rather than a similar generalized perturbation of membrane lipid which indirectly influences CaRC function.

Since ryanodine receptors have been located in neuronal tissue [34], these anesthetic actions on the CaRC may influence function in those tissues. Ca^{2+} mobilization in certain neuronal tissues has been demonstrated to be influenced by anesthetics [59]. As in cardiac tissue, such actions may be related to surface membrane Ca channel actions, as well as anesthetic-specific effects on internal Ca^{2+} stores modulated by CaRCs.

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