Antagonistic Actions of Halothane and Sevoflurane on Spontaneous Ca^{2+} Release in Rat Ventricular Myocytes

Mark D. Graham, B.Sc.,* Philip M. Hopkins, M.D.,† Simon M. Harrison, Ph.D.‡

Background: Halothane has been reported to sensitize Ca^{2+} release from the sarcoplasmic reticulum (SR), which is thought to contribute to its initial positive inotropic effect. However, little is known about whether isoflurane or sevoflurane affect the SR Ca^{2+} release process, which may contribute to the inotropic profile of these anesthetics.

Methods: Mild Ca^{2+} overload was induced in isolated rat ventricular myocytes by increase of extracellular Ca^{2+} to 2 mM. The resultant Ca^{2+} transients due to spontaneous Ca^{2+} release from the SR were detected optically (fura-2). Cells were exposed to 0.6 mM anesthetic for a period of 4 min, and the frequency and amplitude of spontaneous Ca^{2+} transients were measured.

Results: Halothane caused a temporary threefold increase in frequency and decreased the amplitude (to 54% of control) of spontaneous Ca^{2+} transients. Removal of halothane inhibited spontaneous Ca^{2+} release before it returned to control. In contrast, sevoflurane initially inhibited frequency of Ca^{2+} release (to 10% of control), whereas its removal induced a burst of spontaneous Ca^{2+} release. Isoflurane had no significant effect on either frequency or amplitude of spontaneous Ca^{2+} release on application or removal. Sevoflurane was able to ameliorate the effects of halothane on the frequency and amplitude of spontaneous Ca^{2+} release both on application and wash-off.

Conclusions: Application of halothane and removal of sevoflurane sensitize the SR Ca^{2+} release process (and *vice versa* on removal). Sevoflurane reversed the effects of halothane, suggesting they may act at the same subcellular target on the SR.

HALOTHANE and sevoflurane have been reported to induce both positive and negative inotropic effects in isolated ventricular myocytes when these agents are either applied or removed, the mechanisms of which are not fully elucidated. For example, application of halothane induces an initial positive inotropic effect[1-6] thought to result from sensitization of the sarcoplasmic reticulum (SR) Ca^{2+} release process,[3,4,7,8] followed by a sustained negative inotropic effect due primarily to reductions in SR Ca^{2+} content,[3,4,9,10] the L-type Ca^{2+} current (I_{Ca}),[1,11-15] and myofilament Ca^{2+} sensitivity.[4,6,16,17] In contrast, sevoflurane initially depresses contractility, which then recovers toward control during the exposure,[5,6,18] and on removal, a short-lived positive inotro-

pic effect is induced[5,6,18] before contractions return to control values. In cells equilibrated with ryanodine to inhibit SR function, the initial positive inotropic effect of halothane is blocked, the extent of initial inhibition of contraction on exposure to sevoflurane is reduced, and the magnitude of the positive inotropic effect on removal is greatly diminished, suggesting a pivotal role of SR Ca^{2+} regulation in these inotropic effects.[5] The aim of this study was to investigate whether halothane, isoflurane, and sevoflurane affected the sensitivity of the SR Ca^{2+} release process. To assess this function, we used unstimulated single ventricular myocytes in which mild Ca^{2+} overload was induced by increase of extracellular Ca^{2+}.[19,20] This leads to the induction of spontaneous Ca^{2+} transients, which are reproducible in their frequency and amplitude. It has been shown previously that these spontaneous Ca^{2+} transients reflect Ca^{2+} release from the SR as they are abolished by ryanodine.[4] Using this model, changes in the frequency and/or amplitude of spontaneous Ca^{2+} transients in the presence of the anesthetics will provide evidence for the contribution of the sensitivity of the SR Ca^{2+} release process to the inotropic profiles of these anesthetics.

Materials and Methods

These studies conformed to the *Guide for the Care and Use of Laboratory Animals*,[21] and all animal procedures conformed to schedule 1 regulations described in the Animals (Scientific Procedures) Act, 1986, of the United Kingdom government Home Office. Rat ventricular myocytes were prepared as described previously.[6]

Recording Cytosolic Ca^{2+}

Freshly dissociated cells were loaded with fura-2 by gentle agitation of a 2-ml aliquot of cell suspension with 6.25 μl fura-2 AM, 1 mM, in dimethyl sulfoxide for 12 min followed by centrifugation and resuspension in the physiologic salt solution (see Solutions, first sentence). Cells were transferred to a tissue chamber (volume, 0.1 ml) attached to the stage of an inverted microscope (Nikon Diaphot; Nikon, Surrey, United Kingdom) and allowed to settle for several minutes onto the glass bottom of the chamber before being superfused at a rate of approximately 3 ml/min with the physiologic salt solution. Solutions were delivered to the experimental chamber by magnetic drive gear metering pumps (Micropump, Concord, CA), and solution level and temperature were maintained by feedback circuits.[22] All experiments were

conducted at 30°C to maximize the retention of cytosolic fura-2. To record Ca^{2+} transients, the fura-2–loaded cells were excited alternately with light at 340 and 380 nm using a monochromator based system (Cairn; Faversham, Kent, United Kingdom) and fluorescence detected at 510 nm. The ratio of fluorescence at 510 nm in response to excitation at 340 and 380 nm was used as a measure of the intracellular Ca^{2+} concentration. Ca^{2+} transients were digitized at 1 kHz and analyzed using Ionoptix software (Milton, MA).

Solutions

During experimentation, cells were perfused with the following physiologic salt solution: 140 mM NaCl, 5.4 mM KCl, 1.2 mM MgCl$_2$, 0.4 mM NaH$_2$PO$_4$, 5 mM HEPES, 10 mM glucose, and 1 or 2 mM CaCl$_2$, pH 7.4 (NaOH) at 30°C. Halothane, isoflurane, and sevoflurane were delivered from stock solutions made up in dimethyl sulfoxide; the final concentration of dimethyl sulfoxide never exceeded 0.2%, which had no significant effect on Ca^{2+} transients (not shown). The maximum final bath concentration of anesthetic used in the experiments, 0.6 mM, approximates to twice the minimum alveolar concentration of halothane, isoflurane, and sevoflurane, and therefore, these concentrations are both clinically relevant and broadly equianesthetic in rat ventricle.[23] Concentrations of anesthetics in the superfusate were verified with gas–liquid chromatography[4] and found to be stable over the course of an experiment.

Experimental Protocol

When cells were perfused with solution containing increased (2 mM) Ca^{2+}, spontaneous Ca^{2+} transients were evoked, which propagated down the cells as Ca^{2+} waves. The frequency and amplitude of these transients were recorded in the absence of anesthetic to establish baseline conditions. If frequency or amplitude was not regular over a period of at least 2 min, the cell was discarded. When steady baseline conditions were achieved, the cells were exposed to 0.6 mM of halothane, isoflurane, or sevoflurane (or a mixture of anesthetics with a total anesthetic concentration of 0.6 mM) for a period of 4 min before control perfusate was restored. The frequency and amplitude of spontaneous Ca^{2+} transients were recorded during periods of 30 s. For purposes of analysis of Ca^{2+} transient amplitude, if a cell did not display any spontaneous transients during a 30-s period (e.g., see fig. 1A), that cell was not included in the calculation of mean amplitude for that time period because this would artificially reduce amplitude of transients in cells where they did occur. However, to give a more complete picture of total SR Ca^{2+} release, data are also presented as the product of frequency and amplitude. In these experiments, each cell acted as its own control and was only exposed to one anesthetic (or one anesthetic mixture) except for the protocols in electri-
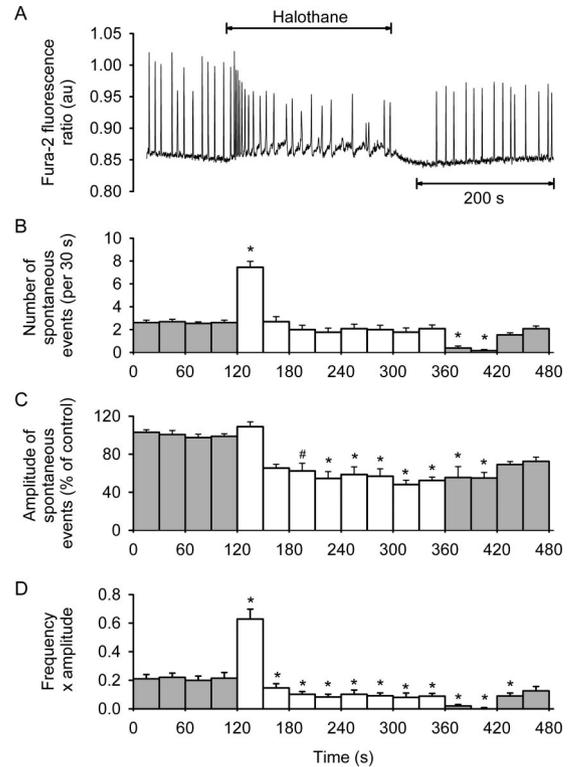

Fig. 1. (A) Spontaneous Ca^{2+} transients before, during, and after a 4-min exposure to 0.6 mM halothane. Mean data (± SEM, n = 13) describing the number (B), amplitude (C), and product of amplitude and frequency (D) of spontaneous events per 30-s periods. Control perfusate is shown as *gray bars*, and anesthetic exposure is shown as *white bars*. * $P < 0.05$ to three or more of the four 30-s precontrol periods. # $P < 0.05$ to two of the four precontrol periods and at least one postcontrol period.

cally stimulated cells (see Discussion). In all groups of cells, there were no significant differences in either the frequency or amplitude of spontaneous Ca^{2+} release under baseline conditions (analysis of variance).

Statistical Analysis

Data are presented as mean ± SEM, and statistical comparisons were conducted with SigmaStat, (SPSS, Chicago, IL) using one-way repeated-measures analysis of variance followed by Tukey tests for multiple comparisons or Friedman repeated-measures analysis of variance on ranks followed by Tukey tests for multiple comparison if data failed normality or equal variance tests. Independent sample t tests were used for comparison of the effects of halothane alone *versus* halothane–sevoflurane mixtures on the frequency of spontaneous Ca^{2+} release. All figures were prepared using SigmaPlot (SPSS).

Results

Effects of Halothane, Sevoflurane, and Isoflurane on Spontaneous Ca^{2+} Release

Figure 1A illustrates spontaneous Ca^{2+} transients before, during, and after a 4-min exposure to 0.6 mM halo-
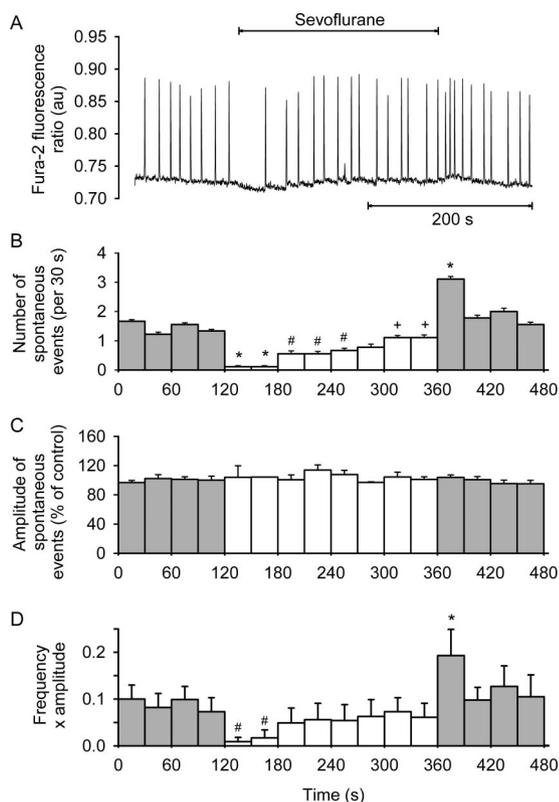
Fig. 2. (*A*) Spontaneous Ca^{2+} transients before, during, and after a 4-min exposure to 0.6 mM sevoflurane. Mean data (± SEM, n = 10) describing the number (*B*), amplitude (*C*), and product of amplitude and frequency (*D*) of spontaneous events per 30-s periods. Control perfusate is shown as *gray bars*, and anesthetic exposure is shown as *white bars*. * *P* < 0.05 to three or more of the four 30-s precontrol periods. # *P* < 0.05 to two of the four precontrol periods and at least one postcontrol period. + *P* < 0.05 *versus* initial exposure (*columns 5* and *6*).

thane. The frequency (fig. 1B) and amplitude (fig. 1C) of spontaneous Ca^{2+} transients assessed over consecutive 30-s periods were stable over the initial control period (0–120 s). Addition of 0.6 mM halothane led to an immediate increase in frequency (from 2.6 ± 0.21 to 7.5 ± 0.5 transients per 30 s; *P* < 0.05 *vs.* control), which then declined to a level not significantly different from control (fig. 1B). At the end of the 4-min exposure, Ca^{2+} transient amplitude was significantly reduced to 54 ± 10% of control (*P* < 0.05). On removal of halothane, the frequency of spontaneous Ca^{2+} release declined (*P* < 0.05) for a period of 60 s before returning to control. The amplitudes of events (when they occurred, see Materials and Methods) remained below control but after 2 min of control perfusate were restored. The product of frequency and amplitude (fig. 1D), which gives a measure of total Ca^{2+} release, reflected changes in both variables.

In contrast, sevoflurane initially reduced the frequency of spontaneous Ca^{2+} release (*P* < 0.05) over a period of 60 s (figs. 2A and B), which returned toward control levels during the 4-min exposure, with no significant change in the amplitude of spontaneous Ca^{2+} transients (fig. 2C). On removal of sevoflurane, frequency was

enhanced transiently (*P* < 0.05) before returning to control, but again, this was not accompanied by any change in Ca^{2+} transient amplitude. Figure 2D illustrates that total Ca^{2+} release reflected the effect of sevoflurane on frequency, not amplitude, of release.

Isoflurane (0.6 mM) had no significant effect on the frequency or amplitude of spontaneous Ca^{2+} release when first applied, during the exposure, or on removal (not shown), suggesting that isoflurane had no net influence on the sensitivity of SR Ca^{2+} release.[7]

SR Ca^{2+} Content at the Point of Spontaneous Release

Because spontaneous release of Ca^{2+} from the SR is strongly dependent on SR Ca^{2+} content,[24] this was assessed by rapid application of 20 mM caffeine at the time point when the next spontaneous Ca^{2+} release was expected. Figure 3A illustrates this protocol; under control conditions, 20 mM caffeine was applied to the cell under study to discharge SR Ca^{2+} content. After removal of caffeine, spontaneous Ca^{2+} transients were restored. Anesthetic, 0.6 mM, was then applied, and the caffeine protocol was repeated at the beginning and at the end of the 4-min anesthetic exposure. Figures 3B and C illustrate that sevoflurane significantly increased the magnitude of caffeine-induced release of Ca^{2+} from the SR (a measure of SR Ca^{2+} content) during the exposure (to 110 ± 12%, n = 10; *P* < 0.05), as would be expected if SR Ca^{2+} release were inhibited, whereas halothane reduced content to 77 ± 24% (n = 9; *P* < 0.05), consistent with a halothane-induced enhancement of SR Ca^{2+} release (*i.e.*, spontaneous Ca^{2+} release evoked at a lower SR Ca^{2+} content).

Amelioration of Halothane-induced Effects by Sevoflurane

These results illustrate that halothane and sevoflurane have opposing effects on the SR Ca^{2+} release process, in which case the effects of halothane might be ameliorated or reversed by coapplication of sevoflurane. The experiments in figure 4 illustrate the results of experiments in which mixtures of halothane and sevoflurane were applied to spontaneously active cells; application of a mixture of 0.3 mM halothane and 0.3 mM sevoflurane (fig. 4A) significantly increased frequency of release (*P* < 0.05) from an average value of 1.8 (over the four 30-s control periods) to 3.1 over the first 30-s period of exposure, before returning to control (fig. 4B). This compares to 0.6 mM halothane alone (fig. 1B), where frequency increased from an average value of 2.6 (over four 30-s periods) to 7.5 (over the first 30-s period). This could result from the lower actual concentration of halothane, but this was investigated in a separate series of experiments (see next paragraph, second sentence). The mixture of 0.1 mM halothane and 0.5 mM sevoflurane (fig. 4C) effectively abolished the initial transient increase in
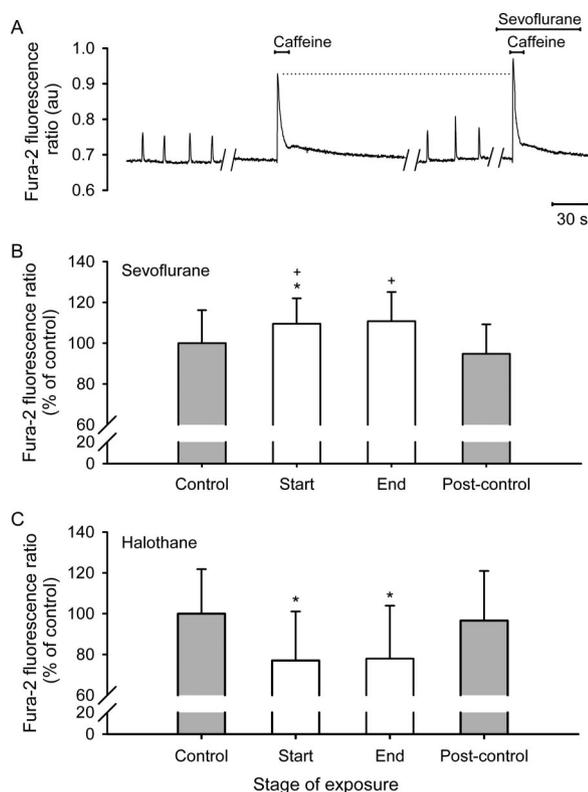
Fig. 3. (*A*) Original trace of protocol for measurement of sarcoplasmic reticulum Ca²⁺ content. Caffeine, 20 mM, was rapidly applied under control conditions at the time when the next spontaneous Ca²⁺ release was expected. Caffeine was then removed, and control conditions were restored. Anesthetic was added, and the caffeine pulse was applied again at the start and end (not shown) of the anesthetic exposure and after removal of anesthetic (postcontrol, not shown). (*B* and *C*) Mean data (± SEM) describing the peak caffeine-induced fura-2 fluorescence under control conditions and at the Start and End of a 4-min exposure to sevoflurane (*B*) or halothane (*C*). For Start data with sevoflurane (n = 10), caffeine was applied at the time when the first spontaneous transient was expected after the addition of the anesthetic, and for halothane (n = 9), caffeine was applied after the initial burst of Ca²⁺ release. For End data, caffeine was applied at the end of the anesthetic exposure. * *P* < 0.05 to precontrol. + *P* < 0.05 to postcontrol.

Fig. 4. Spontaneous Ca²⁺ transients before, during, and after a 4-min exposure to 0.3 mM halothane plus 0.3 sevoflurane (*A*) and 0.1 mM halothane plus 0.5 mM sevoflurane (*C*). Mean data (± SEM) describing the number of spontaneous events per 30-s periods for 0.3 mM halothane plus 0.3 sevoflurane (*B*, n = 15) and 0.1 mM halothane plus 0.5 mM sevoflurane (*D*, n = 16). Control perfusate is shown as *gray bars*, and anesthetic exposure is shown as *white bars*. * *P* < 0.05 to three or more of the four 30-s precontrol periods. # *P* < 0.05 to two of the four precontrol periods and at least one postcontrol period.

frequency that we observed with 0.1 mM halothane alone and figure 4D shows that during the first 30-s period, frequency of release was actually reduced. After removal of this anesthetic mixture, there was no effect on the frequency of release.

Figure 5A illustrates that as the proportion of sevoflurane was increased in the mixture, the frequency of spontaneous events on application declined with the line crossing baseline conditions (dotted line) at approximately 0.1 mM halothane–0.5 mM sevoflurane. The effect of 0.1 and 0.3 mM halothane alone (filled symbols) on the initial increase in frequency was significantly (*P* < 0.05) greater than that induced by the anesthetic mixtures containing the same level of halothane. Data for frequency of events on removal (fig. 5B) also illustrates that sevoflurane ameliorates the effect of halothane on suppression of SR Ca²⁺ release, and the line again inter-

cepts baseline conditions at approximately 0.1 mM halothane–0.5 mM sevoflurane.

Changes in Diastolic Ca²⁺

Halothane (0.6 mM) significantly (*P* < 0.05) increased diastolic Ca²⁺ throughout the exposure (figs. 1A and 6A), which returned to control levels on removal of halothane. In contrast, 0.6 mM sevoflurane significantly reduced diastolic Ca²⁺ for a period of 120 s, after which diastolic Ca²⁺ returned to control levels (figs. 2A and 6B). In cells pretreated with 1 μM ryanodine to inhibit SR function, no significant change in diastolic Ca²⁺ occurred during exposure to halothane (n = 14) or sevoflurane (n = 12). Figure 6C shows that 0.1 mM halothane also led to a sustained increase in diastolic Ca²⁺ similar to that observed with 0.6 mM halothane. Application of a mixture of 0.1 mM halothane and 0.5 mM sevoflurane also produced a sustained increase in diastolic Ca²⁺; however, there was a 90-s delay before the increase in diastolic Ca²⁺ reached significance.
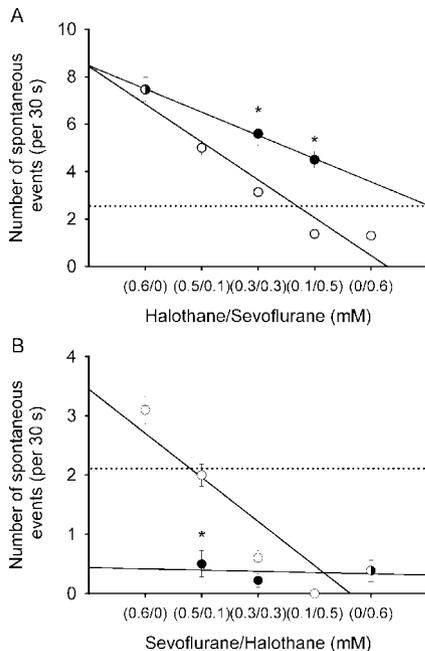
Fig. 5. Composite curves for mixtures of halothane and sevoflurane (total anesthetic concentration of 0.6 mM) on the number of spontaneous Ca^{2+} transients induced on initial exposure (*A*) and upon removal of anesthetic(s) (*B*). *Open symbols* identify data describing the effects of halothane–sevoflurane mixtures, and *filled symbols* identify concentrations of halothane alone. Data are mean ± SEM (n = 15 or 16). * P < 0.05 between halothane alone and halothane–sevoflurane mixtures. *Solid lines* are the result of linear regression of these data. *Dotted lines* represent the mean frequency of spontaneous release under baseline conditions.

Discussion

Halothane and Sevoflurane on Spontaneous Ca^{2+} Release

These data describe the effects of halothane, isoflurane, and sevoflurane on the properties of spontaneous Ca^{2+} release in isolated ventricular myocytes and the associated changes in diastolic Ca^{2+} that accompany the application and removal of anesthetic, which help to further our understanding of the inotropic actions of these agents in ventricular muscle. One intriguing aspect of these data is that the effects of halothane on SR Ca^{2+} regulation can be reversed by coapplication of sevoflurane.

During normal excitation–contraction coupling, Ca^{2+} entry *via* I_{Ca} induces the release of Ca^{2+} from the SR *via* the process of calcium-induced calcium release.[25,26] However, in certain pathologic conditions (*e.g.*, glycoside toxicity), cellular Ca^{2+} overload occurs, which can result in arrhythmogenic activity. This results from spontaneous release of Ca^{2+} from the SR, which induces delayed after depolarizations (*via* inward Na^+–Ca^{2+} exchange current[27] as Ca^{2+} is extruded from the cell), potentially leading to the generation of spontaneous action potentials. The extent of spontaneous release of Ca^{2+} from the SR is thought to be heavily influenced by the level of Ca^{2+} within the lumen of the SR,[28] and it has been proposed that luminal Ca^{2+} modulates the sensi-
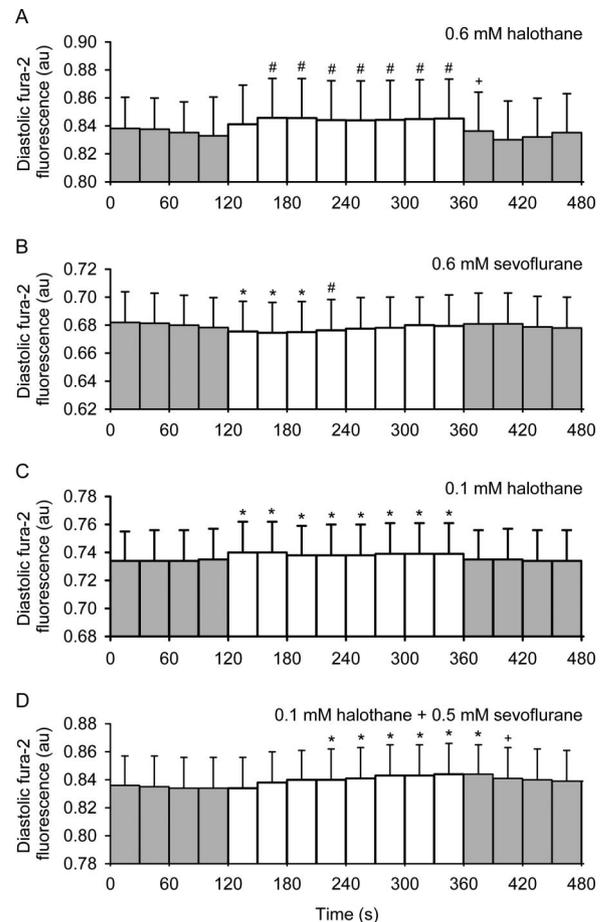
Fig. 6. Mean data (± SEM) describing diastolic fura-2 fluorescence ratio before, during, and after a 4-min exposure to 0.6 mM halothane (*A*, n = 13), 0.6 mM sevoflurane (*B*, n = 9), 0.1 mM halothane (*C*, n = 6), and 0.1 mM halothane plus 0.5 mM sevoflurane (*D*, n = 16). Control perfusate is shown as *gray bars*, and anesthetic exposure is shown as *white bars*. * P < 0.05 to three or more of the four 30-s precontrol periods. # P < 0.05 to two of the four precontrol periods and at least one postcontrol period. + P < 0.05 *versus* steady state (*column 12*).

tivity of the ryanodine receptor (RyR) to cytoplasmic Ca^{2+}. In these studies, we have induced mild Ca^{2+} overload to assess the impact of anesthetics on the sensitivity of the SR Ca^{2+} release process.

Our data show that halothane induced an immediate release of SR Ca^{2+}, which was ryanodine sensitive and consistent with sensitization of the Ca^{2+} release process.[3,4,7] In contrast, application of sevoflurane (although less potent than halothane in molar terms) initially inhibited SR Ca^{2+} release. This result is similar to the decreased efficacy of SR Ca^{2+} release in response to rapid cooling induced by sevoflurane.[29] Coapplication of halothane and sevoflurane significantly ameliorated the sensitizing effect of halothane on the frequency of spontaneous SR Ca^{2+} release, with the mixture of 0.1 mM halothane and 0.5 mM sevoflurane having little impact on the frequency or amplitude of SR Ca^{2+} release, and effectively mimicked the results produced by 0.6 mM isoflurane.

On wash-off of halothane, spontaneous SR Ca^{2+} release was inhibited, whereas removal of sevoflurane induced a burst of Ca^{2+} release. As above, these effects of halothane were ameliorated by sevoflurane (figs. 4 and 5). Although these data do not permit a specific subcellular site to be identified, a plausible explanation of these results is that halothane and sevoflurane bind to the RyR, inducing either an increase[7] or a decrease in its open probability, respectively. Their binding seems antagonistic because 0.5 mM sevoflurane blocked the effects of 0.1 mM halothane almost completely, but again, this may not reflect antagonism at the same site of action as halothane. These data also suggest that isoflurane seems to have no net effect on the RyR under these conditions.

The transient effects of halothane on the frequency of spontaneous Ca^{2+} release are similar to those induced by a low dose of caffeine,[24,30] which are thought to involve luminal feedback of RyR sensitivity. To explain data with halothane (and also caffeine),[24,30] the initial increase in RyR opening would decrease SR Ca^{2+} content (fig. 3). The reduction in SR luminal Ca^{2+} would reduce RyR activation and therefore Ca^{2+} efflux from the SR and a new steady state would be reached when Ca^{2+} efflux was balanced by SR Ca^{2+} uptake. On wash-off of halothane, the decrease in Ca^{2+} release would result from a combination of lower SR Ca^{2+} content and reduced RyR activity.[24]

The effects of sevoflurane on the frequency of spontaneous Ca^{2+} release are similar to those of tetracaine,[24,31] which has been reported to decrease RyR open probability.[31-33] To explain data with sevoflurane (and tetracaine),[23,31] inhibition of SR Ca^{2+} release would increase SR Ca^{2+} content (fig. 3). The consequent increase of luminal Ca^{2+} would overcome the sevoflurane-induced decrease in open probability of the RyR, and Ca^{2+} efflux would increase. This would reach a new steady state when the enhanced efflux at the higher SR Ca^{2+} was balanced by SR Ca^{2+} uptake. On removal of sevoflurane, the burst of spontaneous Ca^{2+} release would result from the restoration of RyR sensitivity coupled with the increased SR Ca^{2+} content.

Changes in Diastolic Ca^{2+}

Although the above-proposed mechanism is capable of explaining the effects of halothane and sevoflurane on the frequency of SR Ca^{2+} release and SR Ca^{2+} content, it can not explain fully the effects on diastolic Ca^{2+}, because a mechanism centered around changes in the sensitivity of the RyR would be expected to induce only transient changes in diastolic Ca^{2+}, as were observed with sevoflurane (and caffeine[30]). Halothane induced a ryanodine-sensitive maintained increase in diastolic Ca^{2+} at doses between 0.1 and 0.6 mM, which suggests that additional mechanisms associated with the SR are affected. This could result from inhibition of SR Ca^{2+} uptake by halothane,[34] because other agents that inhibit
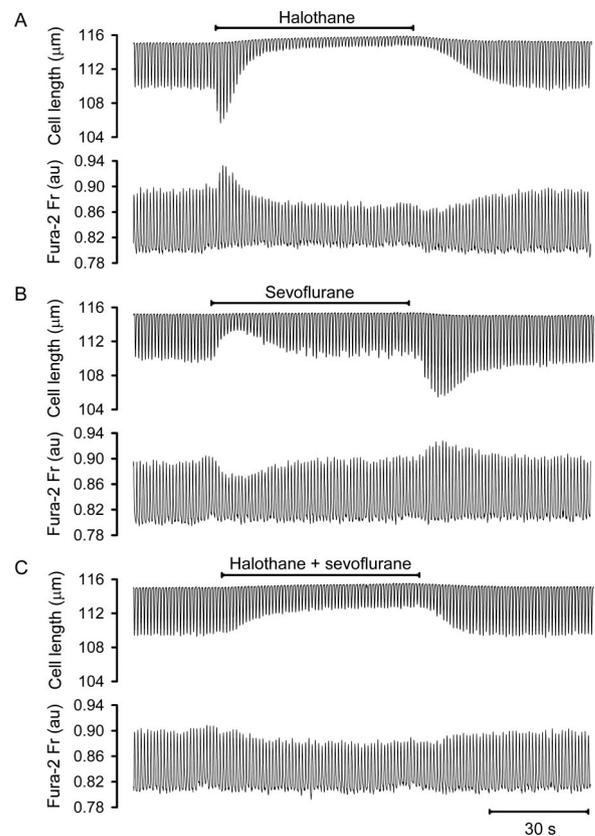
Fig. 7. Recordings of cell length and cytosolic fura-2 fluorescence ratio from a representative myocyte, stimulated electrically at 1 Hz (extracellular [Ca^{2+}] of 1 mM), during sequential 1-min exposures to 0.6 mM halothane (A), 0.6 mM sevoflurane (B), and a mixture of 0.3 mM halothane and 0.3 mM sevoflurane (C). Qualitatively similar results were seen in a total of 12 cells.

SR Ca^{2+} uptake[35,36] also increase diastolic Ca^{2+}. Figure 6D illustrates that sevoflurane was able to delay the increase in diastolic Ca^{2+}, suggesting that the effects of sevoflurane and halothane on diastolic Ca^{2+} were additive rather than antagonistic.

Limitations and Summary

One limitation is that these data might overstate the importance of SR-dependent mechanisms in the inotropic profiles of the anesthetics studied as excitation-contraction coupling is heavily SR dependent in the rat. Second, the experiments were conducted at 30°C rather than 37°C—a strategy used to maximize fura-2 retention by cells to allow accurate and consistent measurement of fluorescence signals during long protocols, and the balance of Ca^{2+} fluxes along the various pathways may differ slightly at 30°C compared with 37°C.

To summarize, these data suggest that halothane sensitizes, sevoflurane desensitizes, and isoflurane has no net effect on the SR Ca^{2+} release process. Figure 7 illustrates representative traces of cell shortening and the cytosolic fura-2 fluorescence ratio in an electrically stimulated cell exposed sequentially to halothane, then