Anesthesiology
79:73–82, 1993

Effects of Isoflurane and Enflurane on Intracellular Ca^{2+} Mobilization in Isolated Cardiac Myocytes

Dixon W. Wilde, Ph.D., Bruce A. Davidson, B.S.,† Mitchell D. Smith, B.S.,‡ Paul R Knight, M.D., Ph.D.§*

Background: **Enflurane and isoflurane may reduce cardiac contractility by altering mobilization and clearance of intracellular Ca^{2+} (Ca^{2+}_i). It was hypothesized that the negative inotropic actions of these agents involve limiting both membrane Ca^{2+} entry and altering intracellular Ca^{2+} release.**

Methods: **The Ca^{2+}_i transients in rat ventricular myocytes loaded with fura-2 were recorded from a fluorescence microscope. Transients stimulated by membrane depolarization (suction electrode or elevated $[K^+]_o$) or 15 mM caffeine to release Ca^{2+} from the sarcoplasmic reticulum (SR) were analyzed for net amplitude, maximal rate of rise (V_R), average rate of decline (V_R) in $[Ca^{2+}]_i$, and duration.**

Results: **Enflurane and isoflurane reduced electrically stimulated Ca^{2+}_i transients in a dose-dependent manner. Enflurane depressed the Ca^{2+}_i transient amplitude more than isoflurane. Enflurane was more effective than isoflurane in reducing V_R and V_F in a concentration-dependent manner. At similar concentrations, both enflurane and isoflurane reduced the steady state elevation of $[Ca^{2+}]_i$ by 50 mM K^+_o. Similarly, enflurane and isoflurane depressed caffeine-sensitive release of Ca^{2+} from the SR. The reduction in the Ca^{2+}_i transient because of SR Ca^{2+} release was greater in enflurane than in equal concentrations of isoflurane. Rates of elevation and decline in $[Ca^{2+}]_i$ were also reduced in enflurane and isoflurane.**

Conclusions: **The negative inotropic actions of enflurane and isoflurane involve a depression of Ca^{2+} influx during membrane excitation, as well as a reduction in SR Ca^{2+} release. Slowed rates of elevation in $[Ca^{2+}]_i$ indicate that the latter mechanism may, in part, be caused by alterations in the kinetics of SR Ca^{2+} release. (Key words: Anesthetics, volatile: enflurane; isoflurane. Heart: myocardium. Ions: calcium. Measurement techniques: fura-2.)**

THE negative inotropic actions of isoflurane and enflurane on cardiac tissues have been examined, using a variety of techniques, in a range of animal species. Enflurane has been shown to exert a greater depressant effect than isoflurane on myocardium,[1,2] and both agents are thought to act by influencing sites along the excitation-contraction pathway. The purpose of this study was to analyze the actions of enflurane and isoflurane at different points in the excitation-Ca^{2+} mobilization pathway using single ventricular myocytes. We hypothesized that these anesthetics not only limit Ca^{2+}_i mobilization, but may also alter Ca^{2+}_i clearance, thereby altering the magnitude and time course of cardiac contraction.

Intracellular Ca^{2+} mobilization in cardiac myocytes is initiated by opening of sarcolemmal Ca^{2+} channels during the action potential. Anesthetics reduce both L- and T-type Ca^{2+} channel current.[3,4] Enflurane and, to a lesser extent, isoflurane depress atrioventricular conduction and contraction in a manner similar to that of diltiazem, indicating an interaction of these agents at the level of the L-type Ca^{2+} channel.[5] Isoflurane and enflurane also reduce dihydropyridine binding to L-type Ca^{2+} channels.[6,7] These results indicate that the sarcolemmal Ca^{2+} channel is a principal site of action of these anesthetic agents.

Coupling of the sarcolemmal signal to intracellular events is an essential component of the excitation-contraction coupling process. Sarcolemmal influx of Ca^{2+} during membrane depolarization leads to Ca^{2+}-induced release of Ca^{2+} from the cardiac sarcoplasmic reticulum (SR). In the rat heart, which depends primarily on SR release of Ca^{2+} for contraction,[8] halothane[9–11] and, to some extent, enflurane and isoflurane[12] may deplete SR stores of Ca^{2+}. The same effect has been observed in the rabbit heart.[13] Termination of the contractile event depends partly on clearance of free Ca^{2+} from the cy-

* Assistant Professor, Department of Anesthesiology, University of Michigan Medical Center.

† Research Instructor, Department of Anesthesiology, State University of New York at Buffalo.

D Research Associate, Department of Anesthesiology, University of Michigan Medical Center.

§ Professor and Chairman, Department of Anesthesiology, State University of New York at Buffalo.

Received from the Department of Anesthesiology, University of Michigan Medical Center, Ann Arbor, Michigan; and the Department of Anesthesiology, State University of New York at Buffalo, Buffalo, New York. Accepted for publication February 28, 1993. Supported by NIH/NIGMS GM 39227.

Address reprint requests to Dr. Wilde: Department of Anesthesiology, R4032 Kresge II, University of Michigan Medical Center, Ann Arbor, Michigan 48109-0572.

tosol. In cardiac myocytes, this clearance is accomplished, primarily, by reuptake of Ca^{2+} by the SR and, secondarily, by sarcolemmal Ca^{2+} extrusion mechanisms. The volatile anesthetics may alter SR reuptake of liberated Ca^{2+} under appropriate conditions of intracellular pH, $[Ca^{2+}]_i$, and intracellular ATP concentration by changing the activity of the SR Ca^{2+}-ATPase[14–16] or by altering clearance of Ca^{2+} from the cytosol by means of the sarcolemmal Na^+/Ca^{2+} exchanger.[17] There is some evidence to support (as well as debate regarding) alteration of Ca^{2+} sensitivity of the contractile filaments in the presence of enflurane and isoflurane—a putative effect that could reduce development of contractile force.[18–20]

These findings indicate multiple sites of action for enflurane and isoflurane to limit Ca^{2+} cycling in the cell, as well as a direct effect on the contractile proteins. Any or all of these mechanisms would contribute to a negative inotropic action. However, many of these studies have used detergent-skinned myocytes or isolated SR membranes, preparations that may lose or lack essential regulators of cardiac cell function. To eliminate these potentially confounding conditions, we performed the experiments described in this manuscript on intact single cardiac myocytes, in an effort to maintain the physiologic integrity of the cells. Instead of manipulating the cellular components involved in excitation-contraction coupling, we used pharmacologic and ionic manipulations to examine the actions of clinically relevant concentrations of isoflurane and enflurane on the entry of Ca^{2+} through sarcolemmal Ca^{2+} channels and on the direct release of Ca^{2+} from the SR.

Materials and Methods

The methodology for anesthesia and handling of experimental animals was approved by the University of Michigan Committee on Use and Care of Animals. The procedure for enzymatic isolation of single quiescent ventricular myocytes has been described previously[21] and is based on the method of Mitra and Morad.[22] Briefly, adult female Sprague-Dawley rats (250 g) were anesthetized with halothane, and each rat's heart was removed and mounted by the aorta on a modified Langendorff apparatus. The heart was perfused at 35° C with bicarbonate buffered Tyrodes solution containing 150 U/ml collagenase (Type I; Sigma, St. Louis, MO), 50 μM Ca^{2+} (nominal), 0.1% bovine serum albumin (Sigma), and 15 mM taurine (Sigma). After collagenase perfusion was performed, the ventricles were removed, minced, and shaken in a water bath at 35° C to release single cells. Cells were washed in Dulbecco's Modified Essential Medium (DMEM, bicarbonate buffer, no phenol red indicator; Gibco, Grand Island, New York) containing 1.8 mM Ca^{2+} and kept at 22° C until use.

For measurement of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), cells in suspension were loaded with fura-2 AM (Molecular Probes, Eugene, OR) at a concentration of 4 μM for 10 min (22° C). Excess fura-2 AM was removed by washing the cells in DMEM, and the cells were allowed a minimum of 20 min to deesterify the membrane permeant acetyoxy methylester form of the fluophore to its membrane impermeant potassium salt. Aliquots of the loaded cell suspension were placed in a 1-ml recording chamber on the stage of an inverted fluorescence microscope. The temperature of this chamber was maintained at 35° C and the atmosphere was controlled. A superfusion system delivered solution to the cells in the chamber at a rate of ~3 ml/min. The superfusion solutions were continuously gassed with 95% O_2/5% CO_2 alone or with isoflurane or enflurane supplied by vaporizer (Ohio Medical Products, Madison, WI). The airspace above the bath was also supplied with carrier gas or anesthetic plus carrier gas. Anesthetic concentration in the bathing medium was determined by gas chromatography and monitored continuously in the vapor phase with a Rascal II gas analyzer (Ohmeda, Salt Lake City, UT).

For each experiment, a single, quiescent myocyte with normal morphology was selected.[20] Under fluorescence epiillumination, the emission from this cell was isolated in the window aperture of a Leitz MPV photomultiplier (PMT). Cell autofluorescence was corrected following manganese quenching after cell calibration. Quenched cells had background fluorescence equivalent to unloaded cells, which was approximately one-tenth the fluorescence intensity of loaded cells. Excitation of intracellular fura-2 was provided by 340- and 380-nm quartz filters mounted in a computer-controlled filter wheel. A low-pass emission filter of 530 nm was used. Filter-wheel control and data acquisition were provided by software written in Labview (National Instruments, Houston, TX) and run on a Macintosh IIfx computer (Apple Computer, Cupertino, CA). The time required for wheel switching was 100 ms. Isosbestic recording was also used to take advantage of faster sampling rates for measuring rates of change in $[Ca^{2+}]_i$-dependent fura-2 fluorescence. The isosbestic point of a ratiometric fluorescent dye is that

wavelength at which the fluorescent output of both forms (bound and unbound) of the dye are equal. Setting recorder sensitivity at the isosbestic excitation wavelength before recording the dye signal at either the bound or unbound wavelength compensates for changes in the amount of functional dye available to the ligand. For these experiments, the PMT voltage level was set to yield a PMT output of 5 V using a 360-nm excitation filter. Recording was then performed at an excitation wavelength of 340 nm. These data are expressed in units of PMT output voltage at 340 nm. Data acquisition at either excitation wavelength could be performed as fast as 10 μs/point. Analyses of fluorescence emission ratios were performed off line. Acquired data were processed through a nonlinear median filter, the ratio of 340 to 380 nm emission was determined (when appropriate), and the $[Ca^{2+}]_i$ was calculated according to the formula of Grynkiewicz *et al*:[23]

$$[Ca^{2+}] = \left(K_d \frac{F - F_{min}}{F_{max} - F}\right)\left(\frac{Sf_2}{Sb_2}\right)$$

where F = fluorescence ratio; F_{max} = the maximum ratio in ionomycin; F_{min} = the minimum in EGTA-Ca^{2+} free solution; and the relationship Sf2/Sb2 = the ratio of fura-2 fluorescence intensities at 380 nm excitation in the free (Sf2) and fully bound (Sb2) states. Before calibration, cells were poisoned with the protonophore carbonyl cyanide 4-(trifluoromethoxy)-phenylhydrazone (FCCP, Sigma) to deplete ATP stores and prevent Ca^{2+}-ATPase activity from antagonizing the action of the ionomycin. Maximal and minimal fura-2 signals were determined after permeabilization of the cell membrane with ionomycin (Calbiochem, San Diego, CA), a Ca^{2+} ionophore, and subsequent treatment with 15 mM EGTA (Sigma). The K_d of fura-2 was determined to be 391 nM for the optical system used in these experiments. This K_d is higher than the value of 224 nM reported by Grynkiewicz *et al.*, and represents an *in vitro* approximation of the intracellular K_d for fura-2. Determination of the intracellular K_d for fura-2 in a living cell is difficult because of dye compartmentalization, cytosolic viscosity, and inactivation of dye.[24,25]

Cells were stimulated in one of three ways in either the presence or absence of volatile anesthetics in the superfusing solution. Intracellular Ca^{2+} transients were elicited by steady state sarcolemmal depolarization *via* 50 mM extracellular K^+ (K^+_o), or by extracellular glass suction electrode producing transient membrane excitation (3 ms, 1 Hz, constant current pulses at 1.2 × threshold for cell). In electrical pacing experiments, fluorescence signals proportional to Ca^{2+}_i transients were recorded in sequential pairs with excitation at 340 nm/380 nm; a ratio trace was produced off line for analysis, assuming that the Ca^{2+}_i transients were identical for each stimulus. Release of SR stores of Ca^{2+} was accomplished by superfusing the cell with 15 mM caffeine. To take advantage of maximal computer sample rates during caffeine experiments, responses were recorded isosbestically. Data were recorded in an anesthetic concentration-response format. After a control stimulation, each cell was exposed to either isoflurane or enflurane for 10 min. The superfusate in the reservoirs and the recording chamber atmosphere equilibrated to the anesthetic in less than one-half of this time. After stimulation of the myocyte during exposure to anesthetic, the anesthetic was removed by washing and a recovery stimulation series was performed. Time-control experiments revealed that healthy cells meeting our criteria for use yielded consistent responses over the period for the experiments.

Mean values for data were compared by unpaired Student's *t* test, with a *P* value of less than 0.05 considered significant. Analyses of recorded Ca^{2+}_i transients included: amplitude, maximal rate of rise, maximal rate of fall, and transient duration. Transient duration was measured from 10% on the rising phase to 90% recovery on the falling phase (or 10% above baseline). The rate of rise (V_R) for the Ca^{2+}_i transient was determined as the maximum slope of a curve fitted to points from 10 to 90% of maximum amplitude on the rising phase of the transient. The rate of decline of the Ca^{2+}_i transient was determined as an average value from peak transient amplitude to 50% amplitude. The rate of decline in $[Ca^{2+}]_i$ is caused by Ca^{2+} clearance from the cytosol by way of multiple mechanisms, including SR and sarcolemmal Ca^{2+} ATPase and sarcolemmal Na^+/Ca^{2+} exchange.[15,26] In addition, the use of caffeine to release SR Ca^{2+} *en masse* limits SR reuptake of Ca^{2+},[27] and shunts Ca^{2+}_i clearance to sarcolemmal Ca^{2+} extrusion processes. All data are presented as mean ± SEM.

Results

Isoflurane and Enflurane Alter Amplitude and Time Course of Electrically Stimulated Ca^{2+} Transients

The average resting $[Ca^{2+}]_i$ in quiescent myocytes was 75 ± 5 nM (n = 161). Throughout these experiments,

no evidence was obtained that showed that enflurane or isoflurane altered resting $[Ca^{2+}]_i$. Electrical stimulations delivered *via* extracellular glass suction pipettes produced large, rapidly rising Ca^{2+}_i transients. Electrically stimulated Ca^{2+}_i transients, recorded in the absence of anesthetic, had a mean amplitude, for all control runs, of 233 ± 18 nM. The maximum rate of change for the upstroke of the Ca^{2+}_i transient (V_R) was 24 ± 4 μM/s (n = 107), and the average rate of fall of the transient (V_R) was 0.79 ± 0.07 μM/s. The duration of the Ca^{2+}_i transient averaged 510 ± 36 ms in the absence of anesthesia.

Isoflurane and enflurane caused a concentration-dependent reduction in the amplitude of the electrically stimulated Ca^{2+}_i transients, with enflurane being more depressant. Figure 1 shows representative recordings of single Ca^{2+}_i transients from myocytes untreated or exposed to isoflurane or enflurane (both 0.5% in fig. 1). Figure 2a compares the concentration-dependent effects of isoflurane and enflurane (0.5–2.5%) on the peak Ca^{2+}_i transient amplitude (*i.e.*, peak $[Ca^{2+}_i$ minus baseline $[Ca^{2+}]_i$). Isoflurane, at a concentration of 2.0%, depressed peak transient amplitude from 260 ± 21 nM to 108 ± 24 nM ($P < 0.05$, n = 26), and 1.0% enflurane depressed amplitude to 142 ± 6 nM ($P < 0.05$, n = 7). At the equivalent concentrations of 1.5% enflurane and 1% isoflurane (0.9 MAC for each agent), enflurane was significantly more effective at reducing the amplitude of the net Ca^{2+} transient ($P < 0.001$). Both isoflurane and enflurane reduced the rates of increase and decline in the Ca^{2+}_i transient in a concentration-dependent manner (fig. 2b and c). Enflurane at 2% reduced V_R from a control value of 24 ± 4 μM/s to 7 ± 1 μM/s ($P < 0.05$, n = 5). Isoflurane, at a concentration of 2%, reduced V_R to only 18 ± 3 μM/s ($P < 0.05$ compared with control and with 2% Enflurane, n = 22). Both enflurane and isoflurane reduced the mean duration of the electrically stimulated Ca^{2+}_i transient in a concentration-dependent manner (fig. 2d). Enflurane was, again, more effective than isoflurane. The transient duration in 1.5% enflurane was reduced from 454 ± 72 ms to 264 ± 45 ms ($P < 0.05$, n = 8), and 1.5% isoflurane reduced the transient duration from 530 ± 40 ms to 463 ± 57 ms ($P < 0.05$, n = 17).

Effect of Isoflurane and Enflurane on K^+-Stimulated Ca^{2+} Transients

The response of myocyte $[Ca^{2+}]_i$ to elevation of extracellular K^+_o is a rapid rise in $[Ca^{2+}]_i$ to a plateau or steady state level that is maintained until the extracellular $[K^+]$ is returned to physiologic levels (fig. 3). Cells pretreated with 1 μM nitrendipine exhibited blunted or abolished steady state increases in $[Ca^{2+}]_i$ in response to elevated K^+_o, indicating that the steady state elevation in $[Ca^{2+}]_i$ is caused by L-channel activation. The steady state increase in $[Ca^{2+}]_i$ was preceded by a "spike" transient (fig. 3) that could be prevented by 1-μM ryanodine pretreatment, indicating that the "spike" resulted from Ca^{2+} influx-induced release of SR Ca^{2+} stores. Elevated extracellular K^+ at 50 mM depolarizes a healthy myocyte sarcolemma to a steady level of approximately −25 mV (according to the Nernst equation and assuming a normal resting potential of ~ −70 mV). This is the point along the current-voltage relationship at which inward, Ca^{2+} current is approximately 20–30% of maximum.[28]

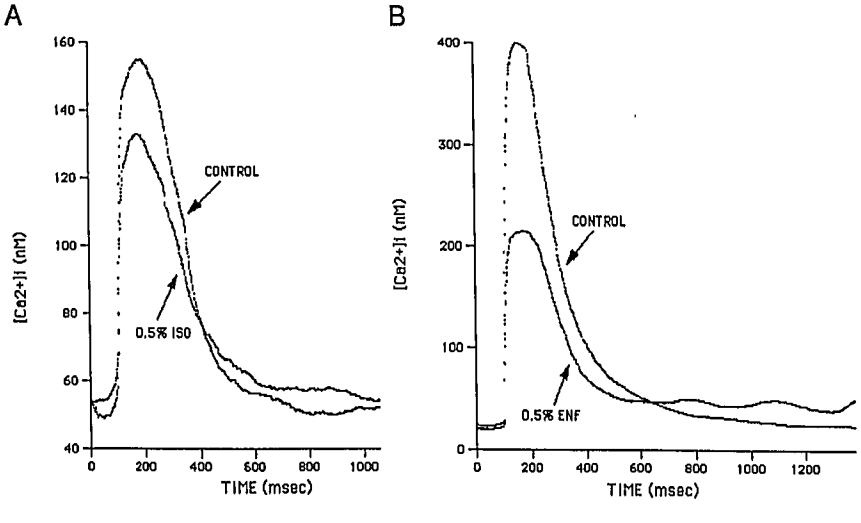

Fig. 1. Intracellular Ca^{2+} transients recorded from electrically stimulated rat ventricular myocytes in the absence (control) or presence of 0.5% isoflurane (*A*) or 0.5% enflurane (*B*). Transients were recorded as pairs of responses at 340 nm and 380 nm excitation wavelengths. Ratio data were processed through a nonlinear median filter at a rank order of 20. Transients were recorded at 100 μs/point. Anesthetic was supplied by vaporizer for 10 min between control and anesthetic recordings and monitored by gas chromatography and a Rascal II gas monitor.

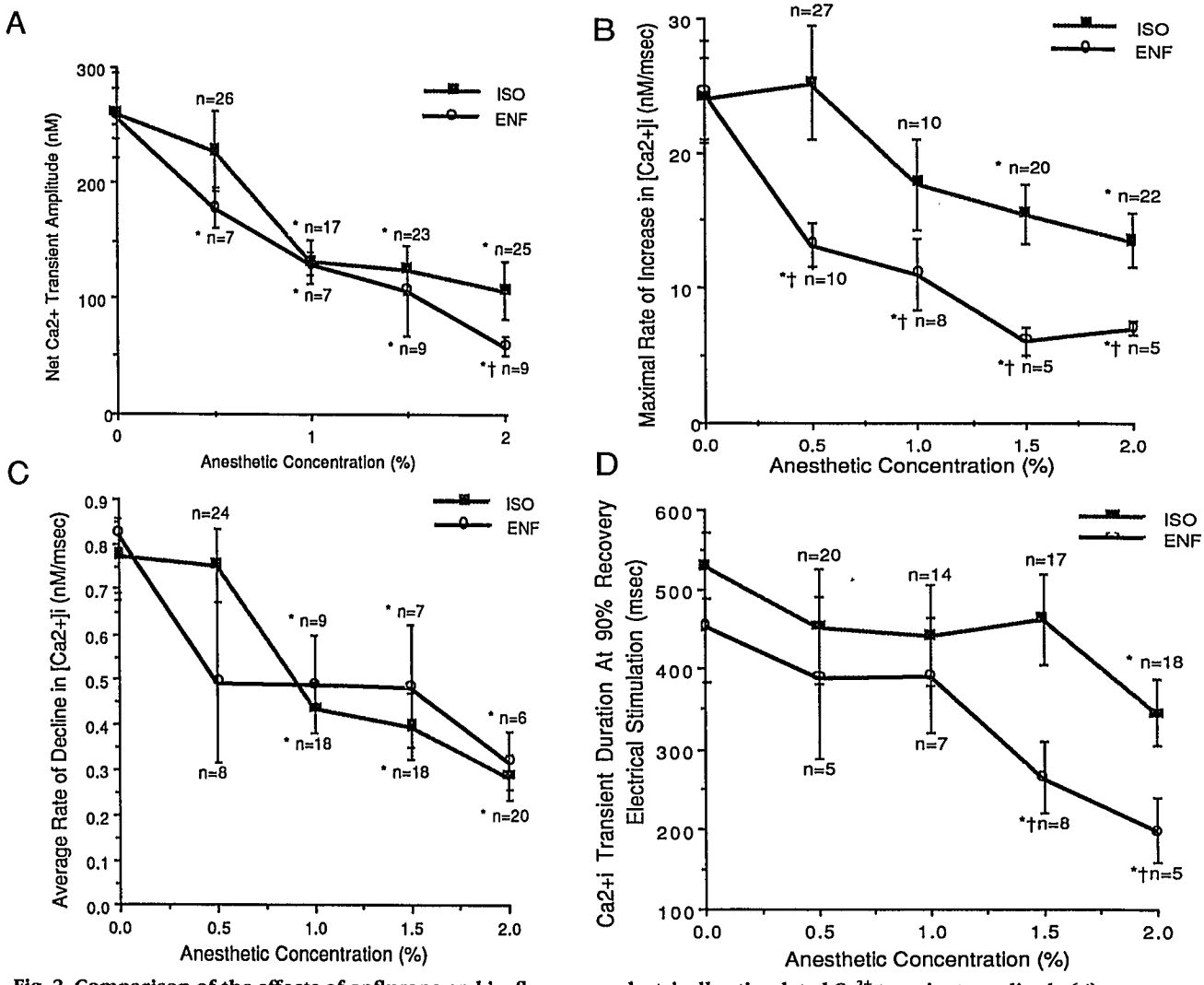

Fig. 2. Comparison of the effects of enflurane and isoflurane on electrically stimulated Ca^{2+} transient amplitude (*A*), on rates of $[Ca^{2+}]_i$ elevation (*B*) and decline (*C*), and transient duration (*D*). Data are presented as mean ± SEM. *Significant difference from control at $P < 0.05$; †significant difference from same concentration of isoflurane at $P < 0.05$.

Both isoflurane and enflurane reversibly reduced the K^+_o-stimulated steady state elevation of $[Ca^{2+}]_i$. Figure 3 shows both the concentration-dependent (0.25–2.5%) effects of these anesthetic agents on the Ca^{2+} transient amplitude and representative recordings. The steady state level of the K^+_o stimulated rise in $[Ca^{2+}]_i$ was 79.2 ± 6.5% of control in 0.25% enflurane and 83.4 ± 9.6% of control in 0.25% isoflurane. The dose effect for both anesthetics had reached a plateau at concentrations of 0.5–2.5%. At equivalent concentrations of 1.5% enflurane and 1% isoflurane, there was no significant difference in the amplitude of the $[Ca^{2+}]_i$ increase because of elevation of extracellular $[K^+]$. Rate parameters for K^+_o stimulated changes in $[Ca^{2+}]_i$ were not measured, because termination of the steady state $[Ca^{2+}]_i$ response was determined by the onset of washout of high $[K^+]_o$ solution (as compared with the spontaneous $[Ca^{2+}]_i$ clearance after peak electrical or caffeine-stimulated elevation in $[Ca^{2+}]_i$).

Effects of Enflurane and Isoflurane on Caffeine-Stimulated Ca^{2+}_i Transients

Quiescent myocytes exposed to 15 mM caffeine in the superfusion solution responded with large Ca^{2+}_i transients, followed by a smooth exponential decay. Before application of anesthetic, the amplitude of the

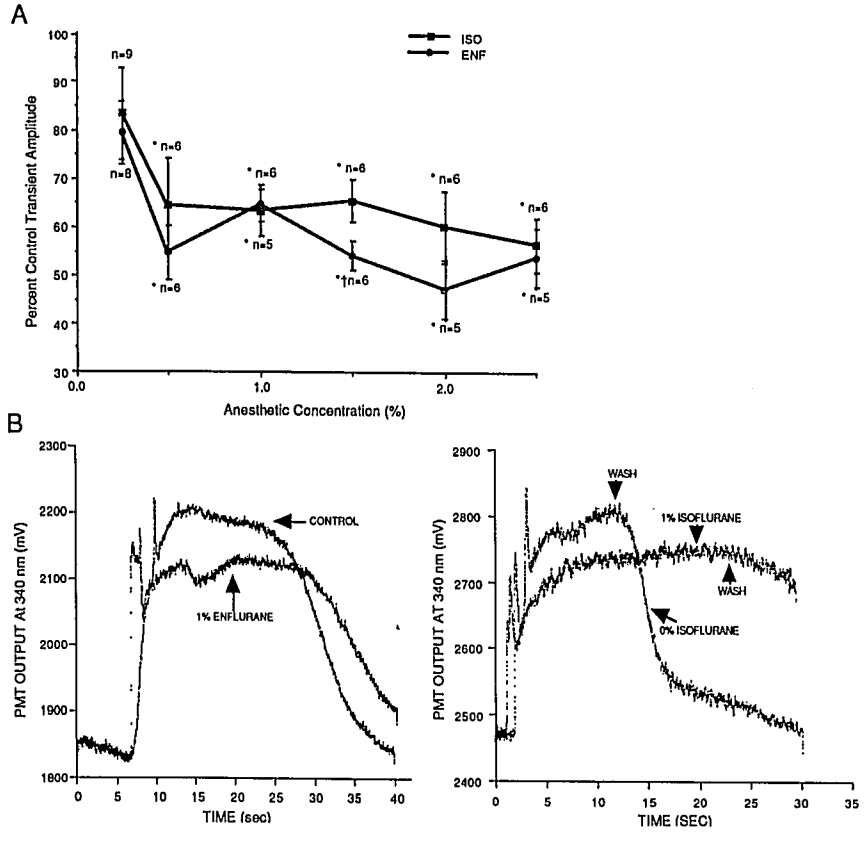

Fig. 3. Comparison of the effects of enflurane and isoflurane on the amplitude of the peak rise in $[Ca^{2+}]_i$ stimulated by superfusion of single cells with 1.8 mM Ca^{2+} Tyrodes containing 50 mM K^+ (*A*). *Significant difference from control at $P < 0.05$; †Significant difference from same concentration of isoflurane at $P < 0.05$. This treatment produces a steady state depolarization of the cell membrane allowing examination of sustained Ca^{2+} entry through voltage-gated sarcolemmal channels. Representative recordings depicting the effects of these agents on the cellular response of $[Ca^{2+}]_i$ to 50 mM K^+_o (*B*). The traces in *b* were recorded isosbestically at 100 μs/point. Wash indicates the point at which 50 mM K^+_o was removed. Measurements of peak response were made at steady state. Note the "spike" preceding the tonic Ca^{2+}_i transient which is caused by SR Ca^{2+} release.

caffeine-stimulated Ca^{2+}_i transients was 605 ± 38 mV (PMT output at 340 nm). The characteristic response to the application of caffeine is shown in figure 4. The transient elevation of $[Ca^{2+}]_i$ always reached a peak and began a decline before washout of caffeine commenced. Duration of the caffeine-stimulated transient in the absence of anesthetic averaged 5.86 ± 0.34 s (n = 66). Measurements of maximal upstroke and average

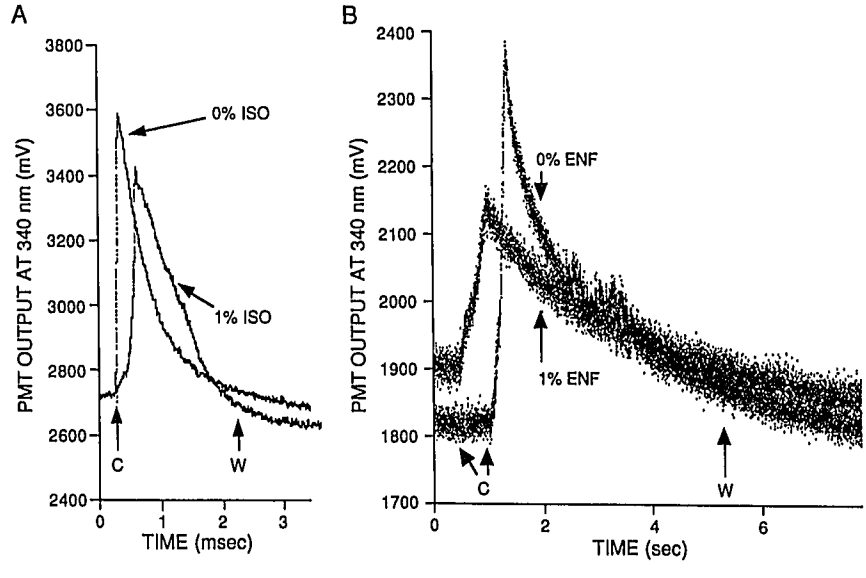

Fig. 4. Effects of isoflurane (*A*) and enflurane (*B*) on the caffeine-stimulated rise in $[Ca^{2+}]_i$. C = the point of caffeine application; and W = the point at which caffeine was removed by washing the cell with normal, 1.8 mM Ca^{2+} Tyrodes solution. The recordings were made isosbestically to allow maximal sample rates (100 μs/point).

downstroke rates for caffeine-stimulated Ca^{2+}_i transients recorded isosbestically had mean values of 26.6 ± 3.1 mV/ms (n = 68) and 0.345 ± 0.045 mV/ms (n = 68), respectively.

Enflurane and isoflurane produced noticeable concentration-dependent reduction in the caffeine-stimulated Ca^{2+}_i transient in the concentration range of 0.5–2.5% (fig. 5a). Enflurane, at a concentration of 1.5%, reduced the caffeine-stimulated Ca^{2+}_i transient amplitude from a control value of 605 ± 38 mV to 339 ± 45 mV (n = 10, $P < 0.05$ compared with control) (fig. 5a). In contrast, isoflurane at a concentration of 1.5% reduced caffeine-stimulated Ca^{2+}_i transient amplitude from a control mean of 594 ± 33 mV to 523 ± 38 mV ($P > 0.05$ compared with control, n = 12). Therefore, it appears that, in the clinical range of these anesthetics, there is a significant depression of the amount of Ca^{2+} released from the SR by caffeine.

Examination of rates of rise and decline of the caffeine-stimulated Ca^{2+}_i transients in the absence and presence of enflurane and isoflurane revealed that enflurane caused a concentration-dependent reduction in the maximal rate of rise in $[Ca^{2+}_i]$ (fig. 5b). The effects of isoflurane on the rate of increase in the $[Ca^{2+}]_i$ response to 15 mM caffeine were less defined. The average rate of decline in $[Ca^{2+}]_i$ was not significantly affected by either agent. Similarly, neither agent appeared to produce any significant change in transient duration after caffeine stimulation ($P > 0.05$ at all concentrations).

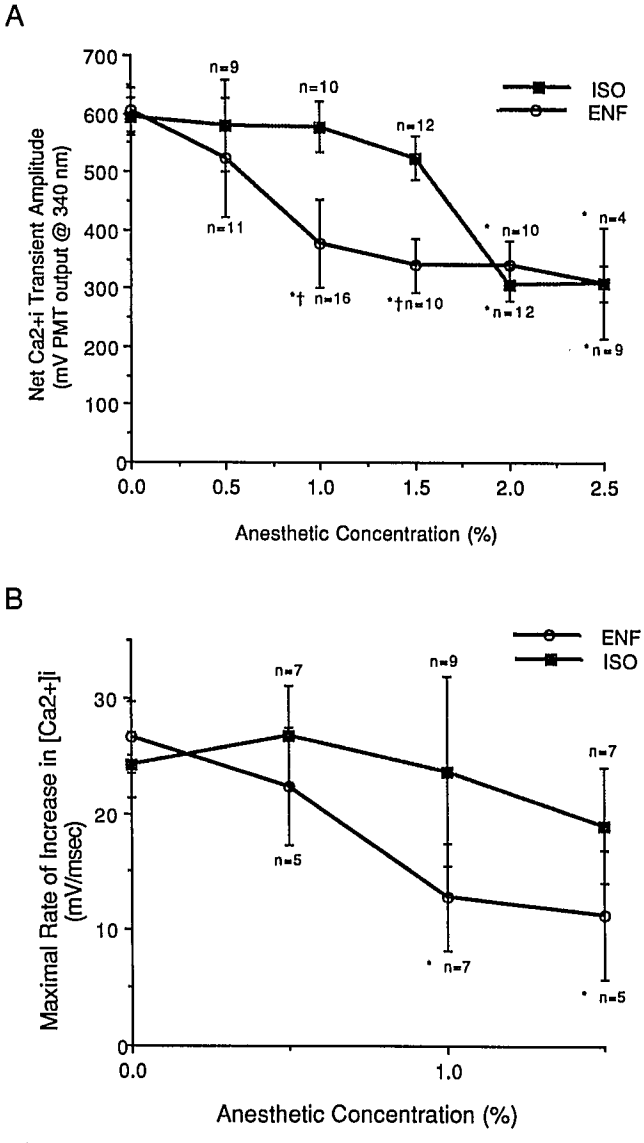

Fig. 5. Comparison of enflurane and isoflurane at increasing concentrations on the amplitude (*A*) and maximal rate of elevation (*B*) in caffeine-stimulated Ca^{2+} transients. Data are presented as mean ± SEM. *Significant difference from control at $P < 0.05$; †Significant difference from same concentration of isoflurane at $P < 0.05$.

Discussion

Isoflurane and enflurane depress stimulated elevations in $[Ca^{2+}]_i$ in intact, single ventricular myocytes loaded with fura-2. This depression of Ca^{2+}_i transients is most evident during stimulation of a "normal" excitation-$[Ca^{2+}]_i$ elevation cycle in the rat ventricular myocytes. The results of our experiments are generally consistent with reports from other laboratories describing the negative inotropic effects of enflurane and isoflurane.[1,2,18,29] However, the negative inotropic efficacy of isoflurane and enflurane differs, and this may reflect different primary mechanisms of action. Our results confirm that, over a range of clinically used concentrations, enflurane inhibits depolarization-initiated Ca^{2+}_i transients to a greater degree than isoflurane. However, because enflurane and isoflurane depress steady state depolarization-stimulated elevations in $[Ca^{2+}]_i$ equally, the greater effect of enflurane on electrically stimulated Ca^{2+}_i transients may be caused by its actions against other components of the excitation-contraction coupling cycle, such as the release of Ca^{2+} from the SR. The Ca^{2+} transients produced in response to membrane depolarization *via* extracellular suction electrode are a result of both membrane Ca^{2+} entry *via* voltage-gated channels and intracellular release of Ca^{2+}

stored in the sarcoplasmic reticulum. It has been hypothesized that enflurane and isoflurane interfere with the cardiac excitation-contraction coupling process at each step: the sarcolemma, the sarcoplasmic reticulum, and the contractile proteins.[18]

A number of studies have demonstrated depletion of SR Ca^{2+} stores by the halogenated volatile anesthetics, with halothane being the most potent, followed by enflurane and isoflurane.[9,12] The experiments of Katsuoka and Ohnishi[12] and Katsuoka *et al.*,[30] for example, demonstrated that the supraclinical concentrations of 6.5% enflurane and 14.8% isoflurane depressed sarcoplasmic reticulum stores of $[Ca^{2+}]_i$ in rat ventricular myocytes. We have also observed depression of the caffeine-releasable Ca^{2+} pool, but in the clinical range for both anesthetics (0.5–2.5%). However, within the clinically relevant-concentration range, these anesthetics appear, as well, to have significant effects on the rates of release of Ca^{2+} from the SR (as determined by measurements of V_R in caffeine-stimulated cells). The maximal rate of elevation of $[Ca^{2+}]_i$ after myocyte exposure to caffeine was reduced in a dose-dependent manner by enflurane. Isoflurane also reduced the rate of rise in the caffeine-induced transient, but the effect was minimal and not, apparently, dose dependent. These findings indicate that enflurane and, perhaps, isoflurane alter the conductance or gating properties for the Ca^{2+} release channel in the SR. The actions of enflurane and isoflurane on the kinetics of the Ca^{2+} transients were also observed in electrically paced cells; this effect may involve not only SR Ca^{2+} release, but, also, reduction of inward Ca^{2+} current.

The effects of enflurane and isoflurane on depolarization-induced rises in $[Ca^{2+}]_i$ are, perhaps, of greatest interest, because they represent a "normal" excitation-Ca^{2+} release cycle for the myocyte. Although the maximum concentration of $[Ca^{2+}]_i$ mobilized after membrane depolarization is reduced in the presence of clinical concentrations of enflurane and isoflurane, examination of rates of elevation and decline in $[Ca^{2+}]_i$ provides another means for comparing the cardiotropic actions of these two anesthetics. The rate of change in $[Ca^{2+}]_i$ may be critical in the control of cellular events. For example, although the maximum $[Ca^{2+}]_i$ attained during a stimulation cycle is lowered in the presence of volatile anesthetics, a slowed rate of elevation or decline in $[Ca^{2+}]_i$ may result in the release of the same total amount of Ca^{2+} in the anesthetic-treated myocyte as in the untreated myocyte. Therefore, the SR content need not be depleted if the SR release channel kinetics were slowed; the end result could still be a depression of tension development.

During electrical pacing of single myocytes, the maximal rate of elevation in $[Ca^{2+}]_i$ was reduced to a greater degree by enflurane than by isoflurane, in similar concentrations. A slowing of the rate of depolarization-initiated rise of $[Ca^{2+}]_i$ is consistent with the findings that isoflurane and enflurane may interact with ^{3}H-nitrendipine binding sites at cardiac L-channels,[7] and may reduce both L- and T-type Ca^{2+} channel current in Purkinje fibers.[3] This interaction may reduce both the amount and the rate of inward Ca^{2+} current, which is a source of activator Ca^{2+} for Ca^{2+}-dependent release of SR Ca^{2+} stores. However, the work of Eskinder *et al.*[3] indicates that enflurane and isoflurane are equipotent at reducing the amplitude of inward Ca^{2+} channel current in ventricular myocytes. Therefore, it is likely that the differential alteration in rate of $[Ca^{2+}]_i$ elevation by enflurane and isoflurane results from changes in SR Ca^{2+} release.

The development of ventricular myocyte tension depends not only on the amount of Ca^{2+} released into the cytosol, but also on the rate of Ca^{2+} clearance from the cytosol at the end of an excitation-Ca^{2+} release-contraction cycle. Slowed Ca^{2+} clearance could result in a prolonged tension, but increased Ca^{2+} clearance would shorten the myocyte tension transient. The rate of decline in the $Ca^{2+}{}_i$ transient represents cytosolic Ca^{2+} clearance from all mechanisms: SR reuptake (which accounts for up to 90% of the cytosolic Ca^{2+}), sarcolemmal Ca^{2+} extrusion (Na^+/Ca^{2+} exchange Ca^{2+}-ATPase), and, to a lesser extent, Ca^{2+} binding by cytosolic proteins and mitochondrial Ca^{2+} storage. Enflurane and isoflurane were equally effective in reducing the maximal rate of $Ca^{2+}{}_i$ clearance in electrically paced myocytes and caffeine-stimulated myocytes, indicating that the difference in negative inotropic potency may not reside in anesthetic effects on cytosolic Ca^{2+} clearing mechanisms. In addition, the lack of prolongation of electrically stimulated $Ca^{2+}{}_i$ transient duration indicates that alteration of $Ca^{2+}{}_i$ clearance may not be critical to the cardiotropic actions of these anesthetics.

Our results indicate similar sites of action for the negative inotropic actions of enflurane and isoflurane. Both agents affect Ca^{2+} influx *via* sarcolemmal L-type Ca^{2+} channels (elevated $[K^+]_o$ and electrical pacing experiments) and SR Ca^{2+} release. Enflurane is more ef-

fective than isoflurane at reducing the amount and rate of the release of SR Ca^{2+} stores. Because enflurane and isoflurane depress sarcolemmal Ca^{2+} entry to an equivalent degree, the greater negative inotropic effect of enflurane, seen *in vivo,* must be caused by its effects on SR Ca^{2+} release. This effect, which may involve a reduction in SR Ca^{2+} content or the kinetics of SR Ca^{2+} release, or both, may occur *via* direct anesthetic-channel interaction or indirect action *via* other cytosolic messengers. Regarding the latter point, recent evidence indicates, for example, that inositol trisphosphate can increase Ca^{2+} release from cardiac SR.[31-33] Volatile anesthetics are known to interfere with this signal-transduction pathway in other cell systems, such as rat aortic smooth muscle stimulated by arginine vasopressin or platelet-derived growth factor.[34] The actions of volatile anesthetics against these intracellular signaling mechanisms remain to be investigated.

The measure of the effect of volatile anesthetics on stimulated cardiac myocytes or tissue preparations is dependent not only on the manner of stimulation, but also on the condition of the preparation and the manner of anesthetic application. This point is illustrated by the case of electrical stimulation of cardiac cells or tissues that initiates a more "normal" excitation-contraction cycle than caffeine stimulation. Caffeine stimulation can elevate cAMP *via* inhibition of phosphodiesterase. Elevation of cAMP may increase phospholamban-mediated SR Ca^{2+} uptake. However, in the presence of caffeine, SR Ca^{2+} uptake should also be "short-circuited" to allow examination of Ca^{2+} clearance, primarily by sarcolemmal transport mechanisms. In all phases of our experiment, the conditions for the caffeine stimulation of a $Ca^{2+}{}_i$ transient are the same, except for the presence or absence of anesthetic, and sufficient washout time is allowed for a recharging of SR Ca^{2+} stores before each subsequent phase of the experiment. Therefore, a change in rate of Ca^{2+} clearance (decline in $[Ca^{2+}]_i$) in the presence of anesthetic indicates that the anesthetic, directly or indirectly, alters some component of the cellular Ca^{2+} extrusion mechanism.

In conclusion, the full range of the molecular mechanisms producing the negative inotropic (and chronotropic) actions of isoflurane and enflurane is still not completely described. Although these volatile anesthetic agents are safely used in clinical situations, it is important to determine the mechanisms of their effects at the cell and molecular levels. In this way, the interactions of these compounds with pharmacologic adjuncts to patient therapy may become more predictable.

References

1. Lynch C III: Differential depression of myocardial contractility by halothane and isoflurane *in vitro.* ANESTHESIOLOGY 64:620–631, 1986

2. Housmans PR, 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

3. Eskinder H, Rusch NJ, Supan FD, Kampine JP, Boznjak ZJ: The effects of volatile anesthetics on L- and T-type calcium channel currents in canine cardiac Purkinje cells. ANESTHESIOLOGY 74:919–926, 1991

4. Ikemoto Y, Yatani A, Arimura H, Yoshitake J: Reduction of the slow inward current of isolated rat ventricular cells by thiamylal and halothane. Acta Anaesthesiol Scand 29:583–586, 1985

5. Gallenberg LA, Stowe DF, Marijic J, Kampine JP, Boznjak ZJ: Depression of atrial rate, atrioventricular nodal conduction, and cardiac contraction by diltiazem and volatile anesthetics in isolated hearts. ANESTHESIOLOGY 74:519–530, 1991

6. Drenger B, Quigg M, Blanck TJ: Volatile anesthetics depress calcium channel blocker binding to bovine cardiac sarcolemma. ANESTHESIOLOGY 74:155–165, 1991

7. Nakao S, Hirata H, Kagawa Y: Effects of volatile anesthetics on cardiac calcium channels. Acta Anaesthesiol Scand 33:326–330, 1989

8. Fabiato A: Calcium induced release of calcium from the cardiac sarcoplasmic reticulum. Am J Physiol 245:C1–C14, 1983

9. Wilde DW, Gutta R, Haney MF, Knight PR: Effects of volatile anesthetics on the intracellular Ca^{2+} concentration in cardiac muscle cells, Mechanisms of Anesthetic Action in Skeletal, Cardiac, and Smooth Muscle. Edited by Blanck TJJ, Wheeler DM. New York, Plenum Press, 1991, pp 125–141

10. Wheeler DM, Rice RT, Hansford RG, Lakatta EG: The effect of halothane on the free intracellular calcium concentration of isolated rat heart cells. ANESTHESIOLOGY 69:578–583, 1988

11. Wheeler DM, Rice RT, Lakatta EG: The action of halothane on spontaneous contractile waves and stimulated contraction in isolated rat and dog heart cells. ANESTHESIOLOGY 72:911–920, 1990

12. Katsuoka M, Ohnishi ST: Inhalation anaesthetics decrease calcium content of cardiac sarcoplasmic reticulum. Br J Anaesth 62: 669–673, 1989

13. Su JY, Kerrick WGL: Effects of halothane on caffeine-induced tension transients in functionally skinned myocardial fibers. Pflugers Arch 380:29–34, 1979

14. Nelson TE, Sweo T: Ca^{2+} uptake and Ca^{2+} release by skeletal muscle sarcoplasmic reticulum: Differing sensitivity to inhalational anesthetics. ANESTHESIOLOGY 69:571–577, 1988

15. Blanck TJ, Thompson M: Enflurane and isoflurane stimulate calcium transport by cardiac sarcoplasmic reticulum. Anesth Analg 61:142–145, 1982

16. Blanck TJJ, Thompson M: Calcium transport by cardiac sarcoplasmic reticulum: Modulation of halothane action by substrate concentration and pH. Anesth Analg 60:390–394, 1981

17. Haworth RA, Goknur AB: Inhibition of Na/Ca exchange in heart cells by enflurane, isoflurane and halothane (abstract). ANESTHESIOLOGY 75:A576, 1991

18. Murat I, Ventura-Clapier R, Vassort G: Halothane, enflurane, and isoflurane decrease calcium sensitivity and maximal force in detergent-treated rat cardiac fibers. ANESTHESIOLOGY 69:892–899, 1988

19. Murat I, Lechenĕ P, Ventura-Clapier R: Effects of volatile anesthetics on mechanical properties of rat cardiac skinned fibers. ANESTHESIOLOGY 73:73–81, 1990

20. Blanck TJJ, Chiancone E, Salviati G, Heitmiller ES, Verzili D, Luciani G, Colotti G: Halothane does not alter Ca^{2+} affinity of troponin C. ANESTHESIOLOGY 76:100–105, 1992

21. 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

22. Mitra R, Morad M: A uniform enzymatic method for dissociation of myocytes from hearts and stomachs of vertebrates. Am J Physiol 249:H1056–H1160, 1985

23. Grynkiewicz G, Poenie M, Tsien RY: A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 260:3440–3448, 1985

24. Moore EDW, Becker PL, Fogarty KE, Williams DA, Fay FS: Ca^{2+} imaging in single living cells: Theoretical and practical issues. Cell Calcium 11:157–179, 1990

25. Virgilio FD, Steinberg TH, Silverstein SC: Inhibition of fura-2 sequestration and secretion with organic anion transport blockers. Cell Calcium 11:57–62, 1990

26. Meissner G, Henderson JS: Rapid calcium release from cardiac sarcoplasmic reticulum is dependent on Ca^{2+} and is modulated by Mg^{2+}, adenine nucleotide, and calmodulin. J Biol Chem 262:3065–3073, 1987

27. Bond M, Kitazawa T, Somlyo AP, Somlyo AV: Release and recycling of calcium by the sarcoplasmic reticulum in guinea-pig portal vein smooth muscle. J Physiol (Lond) 355:677–695, 1984

28. Isenberg G, Klockner U, Mascher D, Ravens U: Changes in membrane currents as studied with a single patch-electrode whole cell clamp technique, Electrophysiology of Single Cardiac Cells. Edited by Noble D, Powell T. London, Academic, 1987, pp 25–67

29. Lynch C III, Frazer MJ: Depressant effects of volatile anesthetics upon rat and amphibian ventricular myocardium: Insights into anesthetic mechanisms of action. ANESTHESIOLOGY 70:511–522, 1989

30. Katsuoka M, Kobayashi K, Ohnishi ST: Volatile anesthetics decrease calcium content of isolated myocytes. ANESTHESIOLOGY 70: 954–960, 1989

31. Nosek TM, Williams MF, Zeigler ST, Godt RE: Inositol trisphosphate enhances calcium release in skinned cardiac and skeletal muscle. Am J Physiol 250:C807–C811, 1986

32. Zhu Y, Nosek TM: Inositol trisphosphate enhances Ca^{2+} oscillations but not Ca^{2+}-induced Ca^{2+} release from cardiac sarcoplasmic reticulum. Pflugers Arch 418:1–6, 1991

33. Gilbert JC, Shirayama T, Pappano AJ: Inositol trisphosphate promotes Na-Ca exchange current by releasing calcium from sarcoplasmic reticulum in cardiac myocytes. Circ Res 69:1632–1639, 1991

34. Sill JC, Uhl C, Eskuri S, VanDyke R, Tarara J: Halothane inhibits agonist-induced inositol phosphate and Ca^{2+} signaling in A7r5 cultured vascular smooth muscle cells. Mol Pharmacol 40:1006–1013, 1991