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The Effect of Halothane on the Free Intracellular Calcium Concentration of Isolated Rat Heart Cells

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The free intracellular calcium concentration of suspensions of isolated rat heart cells was monitored during sequential exposures to halothane and caffeine to evaluate cellular mechanisms of the negative inotropic effect of halothane. The calcium-sensitive, fluorescent dye quin2 was used as the indicator of free intracellular calcium. The acute addition of halothane in concentrations ≥0.062 mM (0.19 vol%) to suspensions of quiescent rat heart cells at 37° C caused a transient (approximately 1.5 min) increase in free intracellular calcium concentration. The intracellular calcium concentration after the decay of this transient was not detectably different from that prior to the addition of halothane. Neither the reduction of extracellular calcium from 1 mM to 100 nM, nor the prior addition of verapamil (5 μM) decreased this halothane-induced calcium transient. The transient was completely blocked by the prior addition of 10 mM caffeine, which depletes the sarcoplasmic reticulum of calcium. Also, the prior addition of halothane caused a reduction in the calcium transient due to caffeine. The depression of the caffeine-induced calcium transient by halothane was independent of the time interval (up to 4 min) between the additions of halothane and caffeine. These results indicate that halothane causes a net loss of calcium from the sarcoplasmic reticulum of quiescent rat heart cells. Thus, halothane has a direct effect at the sarcoplasmic reticulum, probably an enhancement of calcium release, which may explain its depression of myocardial contractility. (Key words: Anesthetics, volatile: halothane. Heart: contractility. Ions, calcium: indicators; quin2. Muscle, cardiac: sarcoplasmic reticulum.)

HALOTHANE is widely recognized as a potent myocardial depressant,[1] yet the mechanisms responsible for halothane's depression of contractility have not been fully elucidated.[2] Recently, halothane has been shown to attenuate the transient increase in intracellular calcium concentration that occurs with each beat, and this reduction of the Ca transient qualitatively correlates with the observed decrease in contractility.[3] A decrease in the Ca transient with each beat could be due to inhibition of Ca influx across the sarcolemma or to reduction of the amount of Ca released from internal stores. Halothane has been shown to inhibit the slow inward current[4] (responsible for a major part of the transsarcolemmal Ca influx in cardiac muscle),[5] but decreases in contractility have been found at halothane concentrations that did not produce detectable decreases in this current.[6] Thus, a depression of Ca from internal sources may also be important. A decrease in the Ca made available to the myofilaments from the sarcoplasmic reticulum (SR; the major intracellular store of Ca in mammalian cardiac muscle)[7] could be due to a direct inhibition of Ca release from the SR or to a reduction in the quantity of Ca stored there. No evidence presently exists to support the former mechanism; however, data consistent with a reduction of SR Ca stores have been reported. Such a conclusion has been reached through evaluation of potentiated-state contractions in intact papillary muscles.[8] Further evidence for a halothane-induced reduction of SR Ca is based on alterations of caffeine-induced tension transients in functionally skinned myocardial fibers[9] or on Ca uptake measurements in fragmented SR.[10]

We have utilized intact cells to address the mechanism by which halothane might decrease the calcium transient with each beat. Our focus has been on the response of the SR to halothane in quiescent rat heart cells. The rat cardiac SR is extensive and accounts for the large part of the intracellular Ca transient with each beat.[11] Because the Ca content of the SR cannot yet be measured directly in intact and functional cells, changes in the cytoplasmic Ca concentration have been used to assess alterations in SR Ca. These cells possess a large capacity for Ca transport between SR and cytoplasm in order to repetitively contract. Thus, they can be reasonably pictured as a series, two-compartment system for calcium. That is, decreases in SR calcium will appear as increases in cytoplasmic Ca, and increases in SR calcium occur at the expense of cytoplasmic Ca. All other cellular mechanisms responsible for maintaining the cytoplasmic Ca concentration will tend to oppose any changes in this quantity. As long as the mechanisms that attempt to maintain a constant cytoplasmic concentration have less capacity than the Ca transport between SR and cytoplasm, changes in SR Ca will be at least transiently reflected in the cytoplasm in reciprocal fashion.

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TABLE 1. Composition of Solutions

Solution	Purpose	Na (mM)	K (mM)	Cl (mM)	HCO_3 (mM)	H_2PO_4 (mM)	Glucose (mM)	Ca (mM)	Albumin (g/l)	Collagenase (g/l)	HEPES (mM)
Ca-free Earle's	Initial washout	142	5.4	116	26.2	1.0	5.6	—	—	—	—
Collagenase	Disaggregation	142	5.4	116	26.2	1.0	5.6	0.050	—	0.4	—
Earle's with low Ca	Collagenase washout, initial suspension	142	5.4	116	26.2	1.0	5.6	0.200	—	—	—
Earle's with albumin	Resuspensions	142	5.4	116	26.2	1.0	5.6	1.0	10	—	—
Modified Earle's	Final cell suspension	142	5.4	116	26.2	1.0	20	1.0	2.5	—	10

Methods

Each experiment was performed using one male Wistar rat, 2–3 mo of age. The rat was decapitated by guillotine and its heart rapidly excised. The aortic root was cannulated, and the heart was perfused by gravity, initially with Ca-free Earle's salt solution. Once the effluent became free of blood, the perfusion solution was changed to Earle's salts (GIBCO, Grand Island, NY) containing collagenase 0.4 g/l (Type I, C-0130, Sigma Chemical Co., St. Louis, MO) and 50 μM Ca. After approximately 20 min of perfusion with collagenase the perfusate was changed to Earle's salts with 200 μM Ca. After 5 min of exposure to this latter solution perfusion was discontinued, the atria and great vessels were trimmed and discarded, and the ventricles were minced with fine scissors in the 200 μM Ca solution. The resulting slurry was then gently agitated by pipetting and filtered through Nitex gauze. The suspension was allowed to settle, then resuspended twice in Earle's salts with 1 mM Ca and 10 g/l bovine serum albumin (Fraction V, Miles Scientific, Naperville, Illinois) and lastly, resuspended in a modified Earle's salt solution with 1 mM Ca (table 1). All settling procedures were carried out at 37° C, and solutions were bubbled with 95% O_2/5% CO_2 and warmed to 37° C. The total volume of cell suspension obtained ranged from 8 to 16 ml at cell densities of 250–450 cells · mm^{-3}. The average yield of rod-shaped cells (morphologically and physiologically similar to cells in intact tissue) was 64%; the remainder consisted of rounded cells, presumably in contracture related to membrane damage. Suspensions with <60% rod-shaped cells were not used for quantitative comparisons between suspensions.

The cell suspension was divided into 2 ml aliquots and placed in sealed vials in a shaker bath at 37° C. Periodically, the head space of each vial was purged with a 95% O_2/5% CO_2 gas mixture. Each aliquot was loaded with the Ca-sensitive dye quin2 by a 30 min exposure to a 50 μM concentration of the acetoxymethyl ester of quin2 (Calbiochem®, La Jolla, California). At the end of dye loading the cells were centrifuged (40 g) for 1.5 min and resuspended in 2 ml of the modified Earle's solution. The aliquot of cell suspension was placed in a stirred cuvette in the chamber of a fluorometer (Ratio fluorometer-2, Farrand Optical Co., New York, New York). This chamber was maintained at 37° C and continually gassed with a 95% O_2/5% CO_2 mixture. Additions to the cuvette were made by microliter syringe through a diaphragm in the top of the chamber.

The excitation wavelength of the fluorometer was 333 nm (mercury lamp with narrow bandpass interference filter) and emissions >480 nm were collected through the use of a sharp-cut yellow filter (Farrand 3-71). The fluorescence output was continuously recorded on chart paper. Halothane (Ayerst, New York, NY) was dissolved in the modified Earle's salt solution at a calculated aqueous phase concentration of 9.8 mM (16 vol% in gas phase) or 4.9 mM (8.2 vol% in gas phase) in a sealed septum vial prior to addition to the cell suspensions. Halothane concentrations in the stock solution and in the fluorometer cuvette were verified by gas chromatography. The measured halothane concentration of the more concentrated stock solution was 8.9 ± 2.3 mM (mean ± SD; n = 8; gas phase concentration: 15 ± 4 vol%). This result is in reasonable agreement with the calculated value, and calculated concentrations are used in reporting the results. A slow decrease in halothane concentration in the fluorometer cuvette occurred over time and amounted to a 15% loss over 4 min. (No experimental intervals involving halothane exceeded 4 min.) The halothane used contained thymol as a preservative. The maximum thymol concentration in any cell suspension was 72 nM. Addition of thymol in amount sufficient to achieve this concentration had no effect on the fluorescence of seven aliquots taken from two cell suspensions. Thus, any change in fluorescence found with halothane was not due to the preservative. Caffeine was added to the suspensions by injection of a 250 mM solution. Heating was required to dissolve the caffeine, which would then remain in solution for several hours at 37° C.

The experimental protocol for each aliquot of cells proceeded as follows: a fluorescence baseline was established, test solutions were added to the suspension in sequence, and a calibration was performed. The calibration sequence consisted of the addition of 100 μM $MnCl_2$ to quench the fluorescence of extracellular quin2 and thereby obtain (by difference) F_{ec}, the fluorescence of ex-

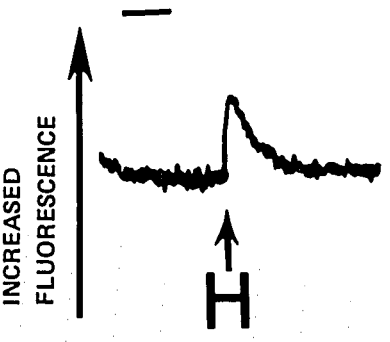

FIG. 1. Quin2 fluorescence tracing of a suspension of rat heart cells. Increased fluorescence is upward. Horizontal calibration bar represents 1 min. At the point marked H, 100 μl of 9.8 mM halothane is added to the 2 ml suspension to produce a final halothane concentration of 0.47 mM (1.5% at 37° C).

tracellular quin2, then 5 μM digitonin was added to permeabilize the sarcolemma and allow the Mn to become intracellular, thereby quenching intracellular quin2 fluorescence and resulting in a minimum fluorescence (F_{min}), and finally 1 mM diethylenetriaminepentaacetate (Ca salt) was added to chelate the Mn and thus expose the quin2 to saturating levels of Ca, giving a fluorescence maximum (F_{max}). In the present study this calibration sequence was used to provide a scaling factor for the fluorescence from each suspension and not to calculate a free intracellular calcium concentration. Sufficient uncertainty exists as to the accuracy of any calibration of quin2 fluorescence in heart cells that we have avoided the actual calculation of concentration.[12] The scaling factor used here was the total intracellular fluorescence. That is, to compare different aliquots of cells, the fluorescence obtained was divided by ($F_{max} - F_{ec}$).¶

Where possible, comparisons were made between different aliquots of the same cell suspension. Paired *t* tests were used to analyze such comparisons with the pairs being formed by adjacent cell aliquots in the experimental sequence. One-way analysis of variance and modified *t* tests[13] were used to detect differences in results at different doses of halothane, with the critical *t* values based on the Bonferroni method to account for multiple comparisons.

Animal use was in accordance with the Guiding Principles published by the American Physiological Society. The protocol was reviewed and approved by the institutional Animal Care and Use Committee. Because all anesthetic agents have the potential to alter myocardial function and Ca metabolism, and given the speed and reliability of the method of killing, it was judged proper to kill the animals without prior anesthesia.

¶ The F_{min} was not used in the scaling because it was less consistent than either F_{max} or F_{ec}. A possible explanation for the variability of F_{min} is that digitonin and similar agents significantly and variably alter the autofluorescence of the cardiac myocytes in the absence of quin2 (data not shown). Because autofluorescence makes up the major part of F_{min} in our fluorometric system, if digitonin also changes autofluorescence in the presence of quin2, a significant error would be introduced into F_{min}.

Results

When halothane was acutely introduced to heart cell suspensions loaded with quin2, the fluorescence immediately increased, then gradually decayed toward its prehalothane baseline over approximately 90 s (fig. 1). The fluorescence of the quin2–Ca complex in the absence of cells was not altered by the addition of 0.47 mM halothane (1.5 vol% at 37° C**). Nor was the fluorescence of rat cardiac myocytes in the absence of quin2 changed by the addition of halothane. Therefore, this quin2 fluorescence transient in cell suspensions upon addition of halothane likely represents a transient increase in cytoplasmic calcium.

The peak height of the fluorescence transient was used as a semiquantitative measure of the change in cytoplasmic Ca. The heights of the transients reveal a clear dependence on the halothane concentration achieved in the suspension (fig. 2). Fluorescence transients could be detected at halothane concentrations as low as 0.024 mM (0.075 vol% at 37° C), and clearly significant results were found at a concentration of 0.062 mM (0.19 vol%).

After decay of the halothane-induced fluorescence transient the fluorescence level was near to that prior to the addition of halothane. There was, though, a small decrease in the fluorescence baseline in many cases. This decrease in baseline was not significantly different from that observed when the same volume of solution without halothane was added to the suspension. Thus, this small decrease in baseline fluorescence is due to dilution of the suspension, and the intracellular calcium concentration is not detectably different from normal after decay of the halothane-induced transient.

The configuration of the transient increase in cytoplasmic Ca upon addition of halothane was similar to that obtained when caffeine was added to these cell suspensions (fig. 3). Although caffeine has many effects on cardiac cells, this transient increase in free intracellular Ca after caffeine is likely due to a sudden release of Ca from the SR.[15,16] The magnitude of the response reaches a plateau at a caffeine concentration of approximately 5 mM, and no additional Ca transients can be elicited by more caffeine once this concentration is present. It was therefore concluded that caffeine concentrations of 5 mM or above effectively deplete the SR of releasable Ca.

If halothane was added to cell suspensions containing 10 mM caffeine, no change in fluorescence was found. In the presence of lower concentrations of caffeine (1 mM or less) a fluorescence transient was obtained upon addition of halothane. If caffeine was added after halothane,

** Based on a normal saline/gas partition coefficient of 0.83 at 37° C.[14]

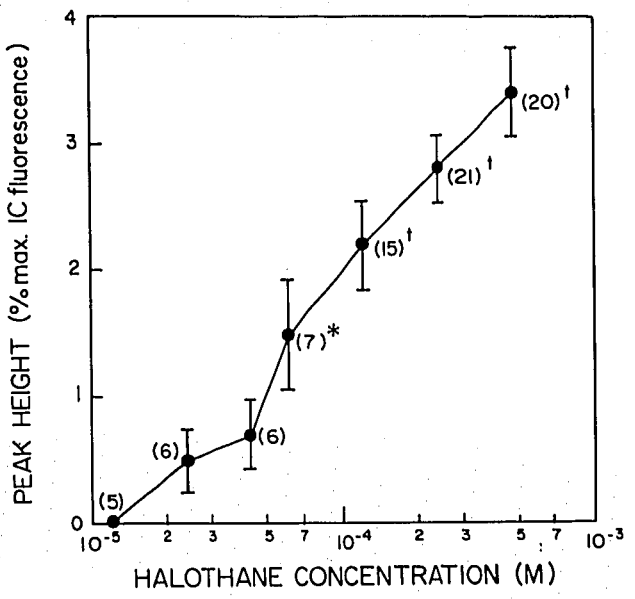

FIG. 2. Dose–response curve for halothane-induced fluorescence transients. The peak height of the transient is expressed as a percentage of the maximum intracellular fluorescence; that is, height equals $[\Delta F/(F_{max} - F_{ec})] \times 100$. Data points represent means ± SE. The numbers in parenthesis are the n's for each point. *$P < 0.05$; †$P < 0.001$. The statistical comparisons were made to null hypothesis (no effect of halothane on fluorescence).

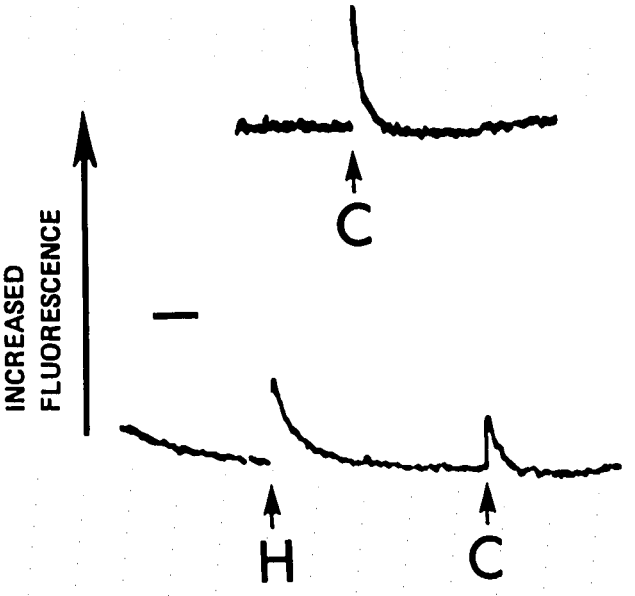

FIG. 3. Quin2 fluorescence tracings of two aliquots of the same suspension of rat heart cells. Increased fluorescence is upward. Horizontal calibration bar represents 1 min. At the point marked H, 100 μl of 9.8 mM halothane was added to the suspension (final concentration 0.47 mM or 1.5 vol%). At the points marked C, 80 μl of 250 mM caffeine was added for a final concentration of 10 mM. The maximum intracellular fluorescence is similar for both aliquots.

a fluorescence transient was always obtained, although the height of the caffeine-induced fluorescence peak was reduced compared to the peak obtained with no prior halothane (figs. 3 and 4). The maximal caffeine (10 mM) peak was more depressed by greater halothane concentrations.

To determine whether the depression of the caffeine-induced Ca transient by halothane remained constant over time, 10 mM caffeine was added at various times after the halothane-induced Ca transient had occurred. The height of the caffeine-induced fluorescence transient was independent of time after addition of halothane for at least 4 min (fig. 5).

The source of the Ca responsible for the halothane-induced transient is crucial to the interpretation of these experiments. EGTA, in a concentration sufficient to decrease extracellular calcium from 1 mM to approximately 100 nM, was added to some cell suspensions prior to exposure to halothane. Any sarcolemmal Ca influx caused by halothane should be clearly reduced by this 10,000-fold decrease in extracellular Ca. Similarly, experiments were conducted with verapamil to block sarcolemmal Ca channels and evaluate their role in the Ca transient. Neither the prior addition of EGTA (2 mM) nor verapamil (5 μM) had a significant effect on the halothane-induced fluorescence transient (fig. 6). The trend after EGTA was

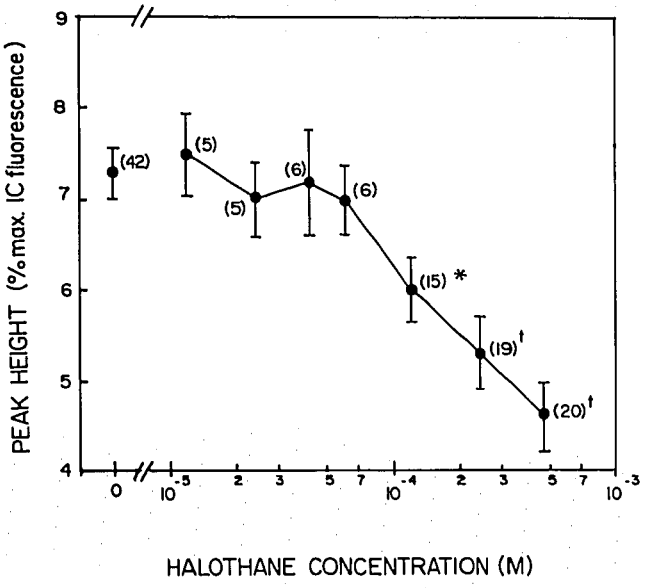

FIG. 4. Height of caffeine-induced fluorescence transients in the presence of various concentrations of halothane. The caffeine was added as 80 μl of a 250 mM solution for a final concentration of 10 mM. The caffeine-induced transient without any prior halothane had a height of 7.3 ± 0.3 (mean ± SE; n = 42). The points represent means ± SE, and the numbers in parenthesis represent n for each point. Points significantly different from caffeine transients without prior halothane are marked with *($P < 0.01$) and †($P < 0.001$).

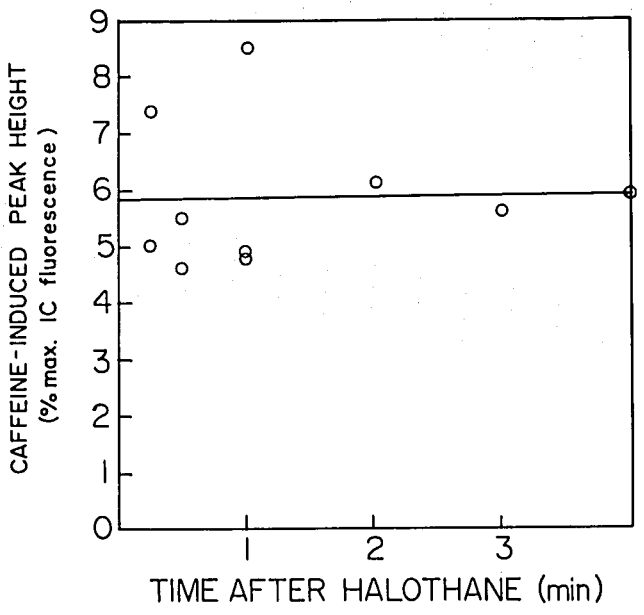

FIG. 5. Height of caffeine-induced fluorescence transients as a function of time after the addition of halothane to suspensions of rat heart cells. Each point represents the result of one aliquot of cells. A total of three rats were used to generate this data. In each case 50 μl of 9.8 mM halothane was added to the 2 ml suspension for a final concentration of 0.24 mM (0.75 vol%). The caffeine added was 80 μl of a 250 mM solution (final concentration 10 mM). The solid line was derived by linear regression and has a slope not significantly different from zero.

toward a larger halothane transient, although statistical significance was not achieved.

Discussion

The fluorescence transient found upon addition of halothane to quin2-loaded rat heart cells reflects a transient increase in the free intracellular Ca concentration. Halothane-induced autofluorescence or chemical interference between halothane and quin2 were ruled out as possible explanations. Because the Ca transients were not depressed by a 10,000-fold reduction of the extracellular Ca concentration nor by inhibition of the sarcolemmal Ca channel by verapamil, extracellular Ca is not the source of the halothane-induced transient. Caffeine induces a similar transient increase in intracellular Ca, which has been shown to be related to a release of Ca from the SR.[15,16] A dose of caffeine that produces maximal Ca release from the SR completely inhibits the halothane-induced transient. A submaximal caffeine dose does not produce complete inhibition. Prior addition of halothane reduces the response to caffeine, and this reduction remains constant for at least 4 min. The most straightforward interpretation of these results is that halothane and caffeine both cause a net Ca depletion from the same pool, specifically the SR. This sudden loss of Ca from the SR could result from an opening of Ca release channels in the SR membrane or from an inhibition of Ca uptake into the SR in the face of ongoing release (or outward leak). The present results do not discriminate between these two possibilities. However, other work involving isolated cardiac SR suggests that at the intracellular ATP concentration of resting muscle (5 mM), halothane has an inhibitory effect on the SR Ca uptake.[17] This effect was only apparent, however, at halothane concentrations above 1.13% (at 36° C). Our study reveals halothane-induced Ca transients at much lower halothane concentrations. Thus, an opening of Ca release mechanisms is the more likely mode of the halothane-induced Ca transient.

The increase in cytoplasmic Ca seen after acute addition of halothane or caffeine is rapidly dissipated. Repletion of the SR Ca stores during halothane exposure is unlikely as a possible mechanism for recovery of the baseline cytoplasmic Ca concentration because the depression of caffeine-induced Ca release is independent of time after addition of halothane. Most probably, the excess Ca is extruded from the cell, in large part by sarcolemmal Na–Ca exchange, although the sarcolemmal Ca ATPase and uptake of Ca into mitochondria may play a role.[18] The net result is that the cytoplasmic Ca concentration in halothane is not detectably different from baseline once the transient has dissipated.

The present results can be compared with those of Su and Kerrick in a mechanically disrupted myocardial muscle preparation in which maximal caffeine-induced tension transients were used to assay the amount of Ca stored in the SR at 20° C.[9] When halothane was present during the portion of the experiment in which the SR was loading with Ca, a decreased tension transient resulted, indicating

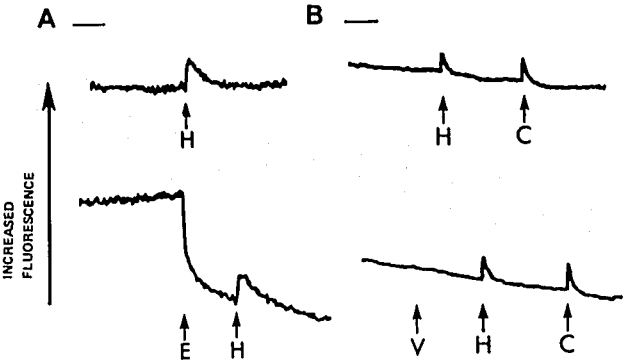

FIG. 6. *A.* Quin2 fluorescence tracings from two aliquots of the same rat heart cell suspension showing halothane(H)-induced transients. In the lower trace, EGTA (final concentration 2 mM) was added prior to halothane. The final halothane concentration in both traces was 0.24 mM (0.75 vol%). *B.* Quin2 fluorescence tracings from two aliquots of the same rat heart cell suspension showing halothane (H) and caffeine (C) transients. In the lower trace verapamil was added at the point marked V. The achieved concentration of halothane, caffeine, and verapamil are 0.24 mM (0.75 vol%), 10 mM, and 5 μM, respectively.

less Ca available in the SR. Our results demonstrate the same effect but in a preparation at 37° C with intact sarcolemma and with a more direct measure of Ca release. Su and Kerrick have also shown that halothane enhances the tension transients induced by a submaximal caffeine dose.[9] Therefore, halothane is not only capable of independently releasing Ca from the SR, as in the present results, but also potentiates caffeine-induced Ca release. Also related to the present results is the finding by Ohnishi in fragmented skeletal muscle SR that halothane enhances Ca-induced Ca release and also induces spontaneous Ca release from the SR.[19] Thus, halothane may cause or potentiate several different forms of Ca release from the SR.

We have shown that halothane causes a loss of Ca from the SR in rat cardiac cells. The releasable Ca stores of the SR remain depressed for at least several minutes while exposed to halothane. This reduction in SR Ca cannot be attributed to inhibition of the slow inward current (which could be the explanation of such a change in beating cells). A reduction of the SR Ca stores can potentially lead to a decrease in the amount of Ca released with each contraction. Such a reduction in the functional intracellular Ca transient has been related to halothane's reduction of contractility.[3] Thus, halothane has a primary effect at the SR, which may contribute to its reduction of contractility.

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