Halothane Alters Control of Intracellular Ca\textsuperscript{2+} Mobilization in Single Rat Ventricular Myocytes

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In an attempt to understand the cellular mechanisms underlying volatile anesthetic-induced myocardial depression, halothane-induced negative inotropy was investigated in an animal model through continuous monitoring of intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]) in rat ventricular myocytes loaded with fura-2. Single cells were stimulated with 15 mM caffeine or 50 mM extracellular K\textsuperscript{+} (K\textsubscript{o}) or were paced by extracellular glass suction pipette electrode. With each stimulus modality, halothane (0.6–1.5%) caused a significant (P < 0.05) and dose-dependent depression of the Ca\textsuperscript{2+} transient. Caffeine and electrically stimulated Ca\textsuperscript{2+} transients were reduced, in 1.5% halothane, to 35 ± 14 and 42 ± 8% of control, respectively. Resting or basal [Ca\textsuperscript{2+}] was unaffected by halothane. Halothane did not elicit spontaneous Ca\textsuperscript{2+} transients in these cells. Single cells stimulated by trains of electrical stimuli at 1.0, 1.5, and 2.0 Hz showed a change in [Ca\textsuperscript{2+}] from prestimulus levels to a stimulated baseline steady state that appeared to increase with stimulus frequency. Halothane at 0.7% increased the change in resting to stimulated baseline [Ca\textsuperscript{2+}], and depressed net transients (P < 0.05) at 1.0 and 1.5 Hz. In contrast, 0.1 µM ryanodine depressed the Ca\textsuperscript{2+} transients in myocytes stimulated by trains of stimuli, but did not potentiate the change in stimulated baseline [Ca\textsuperscript{2+}], at any pacing rate. The results are consistent with the hypothesis that halothane reduces Ca\textsuperscript{2+} availability by causing a net loss of Ca\textsuperscript{2+} from the sarcoplasmic reticulum. The results from experiments using onset of pacing to induce a sudden increase in Ca\textsuperscript{2+} load in previously quiescent myocytes suggest that halothane may act to limit sarcoplasmic reticulum and/or sarcosomal uptake/extrusion mechanisms, as compared to ryanodine, which depletes sarcoplasmic reticulum Ca\textsuperscript{2+} stores without affecting reuptake and extrusion. (Key words: Anesthetics, volatile: halothane. Heart: Ca\textsuperscript{2+}, homeostasis; negative inotropy; sarcoplasmic reticulum. Ions: calcium.)

HALOTHANE, isoflurane, and enfurane are cardiovascular depressants\textsuperscript{1,2} that have different efficacies as negative inotropic agents,\textsuperscript{3,4} and may exert their effects through different mechanisms. Halothane is a commonly studied anesthetic that depresses cardiac force by acting at both the sarcolemma and the sarcoplasmic reticulum (SR).\textsuperscript{5–7} Modulation of cardiac force results from a depression of the mobilization of activator Ca\textsuperscript{2+} during the excitation–contraction cycle that may occur through a number of mechanisms acting singly or jointly. Halothane depresses sinoatrial node firing rate,\textsuperscript{8} reduces papillary muscle tension,\textsuperscript{9} decreases beating in cultured cardiac myocytes,\textsuperscript{10} reduces slow inward Ca\textsuperscript{2+} current,\textsuperscript{11} and in SR from malignant hyperthermic skeletal muscle, induces the SR Ca\textsuperscript{2+} release channel to a low-conductance open state.\textsuperscript{12} These effects all implicate halothane’s alteration of intracellular Ca\textsuperscript{2+} (Ca\textsuperscript{2+}) availability from both sarcolemmal and cytosolic pools. Although suppression of the slow inward current alone would contribute to the negative inotropic effect of halothane, it remains unclear whether this mechanism is a primary or secondary target for halothane action.

Differences between species in the primary source of Ca\textsuperscript{2+} for cardiac cell contraction may be the determinant for the principal site of halothane’s action. Rat ventricular myocytes, for example, are primarily dependent upon release of Ca\textsuperscript{2+} from the SR to provide Ca\textsuperscript{2+} for contraction.\textsuperscript{13,14} Studies of halothane’s negative inotropic effects have exploited this characteristic behavior of rat cardiac myocytes and have focused on the mechanism by which halothane affects release of Ca\textsuperscript{2+} from the SR.\textsuperscript{4,15,16} The development of intracellular, Ca\textsuperscript{2+}-sensitive, fluorescent indicators has afforded a number of noninvasive techniques for monitoring [Ca\textsuperscript{2+}] in living, isolated cells without significantly altering the function of those cells. However, the results obtained from experiments using indicators such as quin-2 and fura-2 may be significantly affected by the choice of preparation used, i.e., cell populations versus single, isolated cells. Recent investigations using quin-2 have used stirred suspensions of ventricular myocytes.\textsuperscript{15} Although this technique yields a larger fluorescence signal from the cells when they are stimulated by, for example, caffeine, these cells have the disadvantage of representing a nonhomogeneous population. Unfortunately, all cell isolation procedures result in some cell death, and these dead or dying cells may produce responses to various stimuli different from those of cells in healthy condition. The examination of halothane effects on isolated cardiac myocytes in suspension by introduction of halothane-containing solutions to a closed, nonrecirculating environment raises the possibility of variable cellular responses to locally high concentrations of anesthetic. Therefore,
the manner of anesthetic treatment of the cells is also of critical importance.

The experiments described in this report were performed to examine the effects of clinically relevant concentrations of halothane on [Ca²⁺] in single, quiescent, rat ventricular myocytes loaded with fura-2. The objective was to characterize some of the putative mechanisms by which halothane depresses availability of activator Ca²⁺ during the stimulus–excitation–contraction cycle. To this end, a comparison was made of halothane’s effects on Ca²⁺ transients elicited via caffeine-stimulated Ca²⁺ release from the SR and Ca²⁺ influx through L-type Ca²⁺ channels during membrane depolarization by elevated extracellular potassium (K⁺) and during direct electrical membrane excitation via suction pipette intended to elicit a normal excitation–contraction cycle. The effects of halothane on reuptake of Ca²⁺ by the SR were also examined indirectly by using the onset of electrical pacing in previously quiescent cells to abruptly elevate [Ca²⁺]. The results support the hypothesis that halothane limits the availability of Ca²⁺ from the SR during the excitation–contraction cycle and that this mechanism may principally underlie the negative inotropic action of halothane on rat ventricular myocytes.

Materials and Methods

The protocol for this procedure was approved by the University Committee onUse and Care of Animals at the University of Michigan. Female Sprague-Dawley rats (200–250 g) were given anticoagulant (200 units/kg sodium heparin intraperitoneally) and were anesthetized with halothane. During anesthesia, the heart was removed and cannulated by the aorta on a modified Langendorff perfusion apparatus. Single, quiescent ventricular myocytes were dispersed from the heart using a modification of the procedure described by Mitra and Morad. The enzyme perfusate contained, in HEPES-buffered Tyrodes, 50 μM Ca²⁺, 150 U/ml collagenase (type I, Sigma, St. Louis, MO), 15 mM taurine (Sigma) and 0.1% bovine serum albumin (BSA, Sigma). The standard Tyrodes solution contained, in millimolar concentrations: NaCl 150.0, KCl 5.4, CaCl₂ 0, MgCl₂ 1.2, glucose 5.0, and HEPES 5.0. The solution pH was adjusted to 7.29 with NaOH. Ca²⁺ was added from a 1 M CaCl₂ stock. All water used was Milli-Q—filtered, 16–18 MΩ cm⁻¹. Incubation temperature was maintained at 37°C. After Langendorff perfusion, the ventricles were removed to a secondary enzyme incubation medium containing all of the above components plus 1% BSA. The secondary digestion medium was periodically examined for intact, quiescent cells, which were then harvested and washed with Tyrodes solution containing 1% BSA. Ca²⁺ tolerance of isolated myocytes was ensured by slowly raising the [Ca²⁺]₀ over 1 h. At this point, cells were allowed to cool to room temperature (22°C) until final preparation for use.

Myocytes in suspension were loaded with the Ca²⁺-sensitive fluorescent dye fura-2 by incubation with the membrane permeant acetoxy methylester form, fura-2 AM (Molecular Probes, Eugene, OR) at a concentration of 4 μM for 10 min at 25°C. Excess fura-2 AM was removed by washing the cells in 1.8 mM Ca²⁺–1% BSA Tyrodes solution. Culture tubes containing fura-2–loaded cells were lightproof to minimize degradation of the dye.

Once loaded, cells were removed to the stage of a Leitz Diavert inverted fluorescence microscope equipped with quartz optics and a 75-W Xe lamp. Cells were placed in an environmental chamber (3-mI volume) and warmed to 30°C. For experimentation, cells were continuously superfused with oxygenated 1.8 mM Ca²⁺–HEPES Tyrodes solution at 3 ml/min.

Cytosolic [Ca²⁺] (i.e., [Ca²⁺]) was measured by alternately exciting fura-2 fluorescence at wavelengths of 340 and 380 nm. An emission cutoff filter of 530 nm was used. Excitation filters were housed in a filter wheel rotated in steps at a maximum rate of 100 ms/alternation. Fluorescence emissions were captured by a Leitz MPV scanning photometer and digitized for storage by computer. Ca²⁺ transients were captured with either of two computer-based acquisition systems. For long-term recording (> 30 min), control of data acquisition was accomplished with software obtained from Leitz and subsequently modified. This system recorded photometer output at a rate of 200 ms/point. Short-duration (~ 1 s) records of fast acquisition of Ca²⁺ transients was accomplished by digitization and storage on a Macintosh II FX computer. The software for this recording system allows sampling at either excitation wavelength at up to 40 μs/point. If we arbitrarily assume a high intracellular fura-2 concentration of 1 mM and a maximum [Ca²⁺] of 10 μM, then the time constant (τ) for Ca²⁺ binding to fura-2 at 35°C becomes ~ 160 μs, or four times slower than the fast acquisition rate. High-speed acquisition of fast Ca²⁺ transients was facilitated by maintaining the preparation temperature at 30°C, which slowed Ca²⁺ release/reuptake events in these cells and increased cell longevity.

At the start of an experiment each cell was observed for normal morphology and quiescence for approximately 10 min while being superfused with 1.8 mM Ca²⁺–HEPES Tyrodes. Only cells meeting strict criteria for normality were used. Trypan blue exclusion was not considered an adequate indicator of cell viability. Cells with optimal morphology were free of vacuoles and possessed clear striations and sharp borders (fig. 1). Cells in suboptimal condition had rounded ends and were often slightly contracted. An initial observation period of 8–10 min was used to certify quiescence: this step was essential, since many cells exhibited slow spontaneous rhythms. Sponta-
neously active cells were considered to be damaged and possibly leaky to extracellular Ca\(^{2+}\), characteristics that could bias the observed effects of halothane on [Ca\(^{2+}\)]. The basal, nonstimulated ratio of fluorescence at 340 and 380 nm was the final criterion for cell usability. Only cells exhibiting 340/380-nm ratios, indicating resting [Ca\(^{2+}\)]_i of ~50–150 nM, were used. Cells with high [Ca\(^{2+}\)]_i usually exhibited spontaneous contractions and poor survival. The initial, resting [Ca\(^{2+}\)]_i for the cells used in these investigations was 75 ± 4 nM (n = 153). Therefore, the value for resting [Ca\(^{2+}\)]_i is lower than that reported by other investigators\(^{20,21}\) and may reflect a complication in [Ca\(^{2+}\)]_i measurement induced by mitochondrial uptake of fura-2 AM.\(^{22}\) Although loading concentrations of Fura-2 were kept to a minimum, there may have been significant artifact introduced in some cells by mitochondrial uptake of the dye providing a source of fura-2 in an environment with relatively unchanging [Ca\(^{2+}\)].

During the control period, the 340/380-nm ratio was allowed to stabilize before application of control stimuli. Stimulation protocols were designed to activate either release of Ca\(^{2+}\) from SR alone (15 mM caffeine), or a combination of membrane Ca\(^{2+}\) entry leading to SR release of Ca\(^{2+}\); depolarization by 50 mM K\(^+\) and direct extracellular electrical stimulation of the sarcolemma via glass suction pipette. All stimulus protocols were tested to ensure that consistent responses were elicited over time.

In all experimental regimes, the responses to exogenous stimuli were compared before, during, and after exposure to halothane. Halothane was equilibrated to both the closed atmosphere above the cell and to the superfusion reservoirs by a Dräger vaporizer using 100% O\(_2\) as carrier. A superfusion rate of 3 ml/min in a 3-ml bath provided one bath exchange per minute; therefore, a 10-min exposure to halothane more than allowed for bath equilibration of the anesthetic. Halothane concentration in the bath was determined by gas chromatography of N-heptane–extracted anesthetic. Since residual halothane remains in the superfusion reservoirs for a time after washout with 100% O\(_2\), the solutions in the reservoirs were
replaced at the time of halothane washout to negate any
effects of residual anesthetic. Direct effects of halothane
against fura-2 fluorescence were determined in vitro
by exposing Ca\(^{2+}\)-ethyleneglycol bis-(β-aminoethyl ether)
-N,N',N'-tetraacetic acid (EGTA) buffers containing 5
µM fura-2 pentapotassium salt to 3% halothane. We ob-
served no effect of halothane on the fura-2 ratio.

Caffeine and high K\(^+\)o were applied to cells by super-
fusion. The 50 mM K\(^+\) solution had reduced Na\(^+\) to main-
tain isotonicity, and was intended to depolarize the sar-
colemma to ∼-25 mV according to the Nernst equation.
Glass suction pipettes for extracellular membrane stim-
ulation were pulled on a Kopf model 720 vertical puller
from 2.0-mm OD borosilicate glass tubing (World Pre-
cision Instruments, New Haven, CT). Pipettes with a tip
resistance of 0.5–1 MΩ were filled with Ca\(^{2+}\)-free HEPES
Tyrode. Constant current extracellular stimuli were ap-
plied to cells from a Grass S9 stimulator with stimulus
isolation. Cells were paced with 3-ms square-wave depo-
larizing pulses. Stimulus strength was adjusted to ∼1.2
times threshold for initiation of fura-2 Ca\(^{2+}\) transients.
For constant pacing, cells were stimulated at 1 Hz. In
other experiments to examine cellular responses to the
sudden onset of pacing, trains of stimuli at 1.0, 1.5, and
2.0 Hz were generated from rest. [Ca\(^{2+}\)]i was allowed to
return to the prestimulus level between trains. Although
suction micropipettes may be used to dialyze cells with
fura-2 pentapotassium salt, it was our intention to mini-
mize perturbation of the sarcolema and transmembrane
ionic gradients. Therefore, only extracellular stimuli were
administered, and fura-2 AM was used to deliver the
fluophore to the cytosol.

Maximum and minimum [Ca\(^{2+}\)]i were determined for
each cell after ATP depletion using glucose-free HEPES
Tyrodes with 2 µM carbonyl cyanide 4-(trifluoromethoxy)-
phenylhydrazone (FCCP, Sigma). Maximum and mini-
 mum [Ca\(^{2+}\)]i were then recorded by treating the cell with
10 µM ionomycin (Calbiochem, La Jolla, CA) (1.8 mM
Ca\(^{2+}\)) and 15 mM EGTA (Sigma) (0 mM Ca\(^{2+}\)) solution,
respectively. The ratios of 340/380 nm were then used
to calculate [Ca\(^{2+}\)] according to the equation of Grynk-
iewicz et al.:

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

where \(F_{\text{max}}\) = the fluorescence ratio in ionomycin; \(F_{\text{min}}\)
= the ratio in EGTA; and \(Sf_2/Sb_2\) = the ratio of 380-nm
signals in ionomycin and EGTA solutions. We have cal-
culated the dissociation constant for fura-2 in our system
to be 391 nM. A comparison of different calibration
methods was made. Pretreatment of myocytes with FCCP
followed by ionomycin yielded slightly larger maxima than
with cell permeabilization by ionomycin alone, but sub-
sequent EGTA treatment produced no lower minima than
in the absence of FCCP treatment. The ionophore Br-
A23187 was also tested and produced smaller maxima
than ionomycin. The ratio \(Sf_2/Sb_2\) can significantly affect
calculated [Ca\(^{2+}\)]. In our experiments, optimal maxima
and minima were obtained with ionomycin and EGTA;
however, the \(Sf_2/Sb_2\) ratio never approached that of Li
et al., and must have significantly affected our final values
of [Ca\(^{2+}\)].

Caffeine (Sigma) was prepared fresh daily in 1.8 mM
Ca\(^{2+}\) Tyrodes. Rynadone (Progressive Agri Systems,
Wind Gap, PA) was prepared as a 10 mM stock in 100%
ethanol and diluted for use. The final ethanol concentra-
tion was 0.001%. All ryanodine was kept in lightproof
containers to minimize photolysis.

Because each cell acted as its own control, simple sta-
tistical comparisons via the paired Student’s t test were
possible. Differences were considered significant at \(P
< 0.05\). Results are presented as means ± standard error
of the mean.

**Results**

**HALOTHANE DOES NOT ALTER BASAL [Ca\(^{2+}\)]
IN QUIESCENT MYOCYTES**

The effects of halothane on resting or basal [Ca\(^{2+}\)]i were
examined in cells (n = 25) in the absence of any stimulus.
Prior to halothane exposure, the [Ca\(^{2+}\)]i in these cells was
70 ± 9 nM. Exposure of cells to 0.7% halothane-equili-
brated superfusate caused no significant change in resting
[Ca\(^{2+}\)]i ([Ca\(^{2+}\)]i after halothane was 56 ± 22 nM) nor was
any effect observed in halothane at 1% concentration
([Ca\(^{2+}\)]i after halothane was 67 ± 12 nM). Higher con-
centrations of halothane (as great as 1.5%) also were with-
out effect on resting [Ca\(^{2+}\)]. Halothane-elicited Ca\(^{2+}\)
transients were not observed in any of the cells used for
these studies.

**EFFECTS OF HALOTHANE ON THE CAFFEINE-
stimulated \(\text{Ca}^{2+}\) TRANSIENTS**

Treatment of single ventricular myocytes with 0.6–
1.3% halothane reduced the amount of \(\text{Ca}^{2+}\) released
from the SR upon application of 15 mM caffeine. As shown
in figure 2A, superfusion of cells at 3 ml/min with 1.8
mM Ca\(^{2+}\) HEPES Tyrodes containing 15 mM caffeine
equilibrated with 100% \(\text{O}_2\) produced a transient elevation
in [Ca\(^{2+}\)]i, with a mean change of 100 ± 26 nM over base-
line (n = 32). This Ca\(^{2+}\) transient coincided with a small
phasic contraction of the myocyte, as determined by direct
observation. The contraction was small compared to those
observed in electrically paced myocytes. The Ca\(^{2+}\)
transient response decayed rapidly and was followed by an
“undershoot” in [Ca\(^{2+}\)], which probably reflects stimu-
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Patients increased with halothane concentration (fig. 2B). Halothane caused significant reductions \((P < 0.05)\) in caffeine-stimulated net Ca\textsuperscript{2+} transients at concentrations as low as 0.6% \((67 \pm 12\% \text{ of control transient amplitude, } n = 5)\), with the attenuation most pronounced at 1.3% halothane \((35 \pm 14\% \text{ of control transient amplitude, } n = 6)\).

**Effects of Halothane on the K+-stimulated Ca\textsuperscript{2+} Transients**

Superfusion of single myocytes with 1.8 mM Ca\textsuperscript{2+}-Tyroses containing 50 mM K\textsuperscript{+} induced a tonic increase in [Ca\textsuperscript{2+}] that was inhibited by 0.6–1.3% halothane. As shown in figure 3A, 1.3% halothane treatment dramatically and reversibly reduced the K\textsuperscript{+}-induced Ca\textsuperscript{2+} increase. The tonic increase in [Ca\textsuperscript{2+}] reflects both activation of L-channels and Ca\textsuperscript{2+}-induced release of Ca\textsuperscript{2+} from the SR. To confirm this, positive control experiments in which cells were treated with 1 \(\mu\)M ryanodine to deplete SR stores of Ca\textsuperscript{2+} (caffeine-releasable pool) showed that cells still produced tonic Ca\textsuperscript{2+} transients after ryanodine treatment.

Release of SR stores of Ca\textsuperscript{2+} occurs during the initial, rapid phase of Ca\textsuperscript{2+} entry, whereas the tonic component of the response reflects sustained L-channel activation.

**Fig. 2.** A: Effect of 0.6% halothane on Ca\textsuperscript{2+} transient elicited by superfusion with 15 mM caffeine in 1.8 mM Ca\textsuperscript{2+}-HEPES-Tyroses. This typical trace from a fura-2-loaded myocyte depicts the caffeine response; a rapid increase in [Ca\textsuperscript{2+}], followed by a sharp drop in concentration and an "undershoot," probably resulting from hyperstimulation of the Ca\textsuperscript{2+} reuptake/extrusion processes. The trace is presented as a ratio plot of the fluorescence emissions at 340 and 380 nm and gives [Ca\textsuperscript{2+}], in nanomolar units. B: Effect of increasing halothane concentration on the net caffeine-stimulated Ca\textsuperscript{2+} transient. Results are presented as percent of control. Significant difference from control at \(P < 0.05\), according to a paired Student's t test.

The effect of halothane on the caffeine-stimulated Ca\textsuperscript{2+} transient was reversed upon return to 0% halothane (100% O\textsubscript{2}) for 10 min, and treatment with caffeine to obtain a washout response. The caffeine-induced Ca\textsuperscript{2+} transient following the halothane washout period returned to 94% of the net transient in the control period. The morphology of the cell remained unchanged throughout, and the recovery of the Ca\textsuperscript{2+} transient to near control level indicates that cell viability was maintained. The reduction in caffeine-stimulated Ca\textsuperscript{2+} transient

**Fig. 3.** A: Ratio plot of the effect of 1.3% halothane on the elevation of [Ca\textsuperscript{2+}], induced by 50 mM extracellular K\textsuperscript{+}. In these experiments, superfusion with 50 mM K\textsuperscript{+}-Tyroses continued until the rise of [Ca\textsuperscript{2+}] reached a steady state. At that point, K\textsuperscript{+} washout with normal Tyroses (with or without anesthetic) commenced. [Ca\textsuperscript{2+}] is presented in nanomolar units. B: Effect of increasing halothane concentration on the tonic rise in [Ca\textsuperscript{2+}], induced by 50 mM K\textsuperscript{+}. Results are expressed as percent of control. *Significant difference from control, \(P < 0.05\).
At increasing halothane concentrations, the tonic Ca\(^{2+}\) transients were progressively decreased (fig. 5B). The net Ca\(^{2+}\) transient (peak, steady state [Ca\(^{2+}\)]\(_i\); minus baseline [Ca\(^{2+}\)]\(_i\)) was significantly reduced at all concentrations of halothane above 0.6% (table 1). For example, exposure of the cell to 0.7% halothane reduced the net transient from a control value of 242 ± 81 nM to 173 ± 58 nM (P < 0.05). Attenuation of the transient reached 83% in 1.3% halothane. This effect was reversible. As in the caffeine experiments, comparisons of mean resting [Ca\(^{2+}\)]\(_i\) in the presence of halothane at concentrations of 0.6–1.3% caused no significant change in resting [Ca\(^{2+}\)]\(_i\) and did not elicit spontaneous transients. The experimental record shown in figure 3A demonstrates a slight decrease in [Ca\(^{2+}\)] when halothane is removed. This artifact was produced, in some cells, when the superfusion solution in the reservoir was replenished with fresh Tyrodes solution. The small change in [Ca\(^{2+}\)] probably reflects a slight decrease in temperature and was not consistently observed. In contrast to the caffeine results, washout of 50 mM K\(^+\) did not produce an undershoot in [Ca\(^{2+}\)]\(_i\).

**Effects of Halothane on Electrically Stimulated Ca\(^{2+}\) Transients**

To avoid perturbation of sarcolemmal K\(^+\) and Na\(^+\) gradients, single, quiescent cells were electrically stimulated with extracellular, glass suction pipette electrodes. Paced cells exhibited robust phasic contractions corresponding to the recorded Ca\(^{2+}\) transients. The contractions, with the cell shortening to approximately 70% of its resting length, were a good indicator that buffering of [Ca\(^{2+}\)]\(_i\) by excess fura-2 was not occurring. In cells paced constantly at 1 Hz, halothane at 0.4–1.5% concentration reduced the amplitude of the net Ca\(^{2+}\) transients (peak stimulated [Ca\(^{2+}\)]\(_i\); minus baseline [Ca\(^{2+}\)]\(_i\); averaged over 15 transients) in a dose-dependent manner but did not consistently or significantly affect baseline [Ca\(^{2+}\)]\(_i\) (fig. 4A). Depression of the net Ca\(^{2+}\) transient compared to control was significant at all halothane concentrations, with the greatest inhibition (to 22 ± 4% of control) at 1.5% halothane. The modulation of the Ca\(^{2+}\) transients by halothane (0.4–1.5%) was reversible, with the transients returning to 80–90% of their control values after 10–15 min of washout of the anesthetic. However, the time to recovery depended on both the concentration of halothane and the duration of halothane exposure. In some experiments at 1.5% halothane, the transients were totally inhibited.

Figure 4B depicts a typical experiment, in which a single myocyte, which met all criteria for use, was stimulated constantly at 1 Hz. The onset of stimulation changed the [Ca\(^{2+}\)]\(_i\) from its resting, basal level of 119 nM to a new, stimulated baseline level of 128 nM. Net amplitude of the Ca\(^{2+}\) transient averaged 179 nM during the control phase. After a period of control pacing, the superfusate and chamber atmosphere were equilibrated with 1.5% halothane in 100% O\(_2\). Net transient amplitude decreased over the course of the initial 3 min of halothane exposure to a steady level of 58 nM. Transient amplitudes recovered to 90% of control after removal of halothane by washout using 100% O\(_2\). The record shown in figure 4B shows a slight decrease in stimulated baseline or baseline [Ca\(^{2+}\)]\(_i\), from 128 nM in the control to 89 nM in halothane; however, as in the results from experiments with other protocols, there was no consistent, significant effect on baseline [Ca\(^{2+}\)]\(_i\). In 35 cells, halothane, at bath concentrations of 0.4–1.5%, did not significantly alter stimulated baseline [Ca\(^{2+}\)]\(_i\), nor did halothane affect the threshold for stimulation.

Figure 4C demonstrates the effect of halothane on single Ca\(^{2+}\) transients recorded at 40 μs/poin. Transient amplitude was reduced, but it is unclear whether the time course for the rising phase of the transient was also affected. In some cells, however, higher concentrations (> 1.5%) of halothane produced total inhibition of the stimulated transients. Elevation of stimulus strength during halothane exposure could increase net transient amplitude in the presence of the anesthetic, but doing this usually precipitated cell death.

**Effects of Halothane on Baseline [Ca\(^{2+}\)] Following Transition of Quiescent Myocytes to Phasic Electrically Stimulated Ca\(^{2+}\) Transients**

The findings described above as well as those reported from other laboratories are consistent with the hypothesis that halothane decreases the amount of Ca\(^{2+}\) available for release from the SR. The mechanism by which the anesthetic decreases available SR Ca\(^{2+}\) in the cardiac myocyte, however, is not apparent. In the experiments described in this report it was noted that baseline [Ca\(^{2+}\)]\(_i\) increased when quiescent cells were first presented with repetitive electrical stimulation. A likely explanation for this observation is that when quiescent myocytes, not
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**Fig. 4.** A: Effect of increasing halothane on the amplitude of electrically stimulated, net Ca\(^2+\) transients (systolic [Ca\(^2+\)], minus stimulated baseline [Ca\(^2+\)], averaged over 15 transients). Results are presented as mean ± SEM. *Significant difference from control, \(P < 0.05\). B: Effect of 1.5% halothane on Ca\(^2+\) transients in a single myocyte paced extracellularly at 1 Hz. A dual excitation wavelength trace is presented as an example of a typical fura-2 recording. C: Ca\(^2+\) transients recorded at high sample rate (10,000 samples at 40 μs/point) at 340-nm excitation, to maximize sample rate. The onset of 1% halothane inhibition is rapid as anesthetic containing superfuse reaches the cell. In this experiment, baseline [Ca\(^2+\)] did not shift during halothane exposure. Traces are offset for clarity. Control = 0% halothane.

exhibiting Ca\(^2+\) transients, are suddenly exposed to periodic increases in [Ca\(^2+\)], time is required by the cellular Ca\(^2+\) uptake and extrusion processes to adjust to the new physiologic state of phasic changes in [Ca\(^2+\)].

In order to examine the mechanism by which halothane may decrease available Ca\(^2+\) from the SR, we examined the effects of the anesthetic on baseline changes and transient increases in [Ca\(^2+\)], following the onset of phasic electrical stimulation in previously quiescent myocytes, and compared them to changes observed in cells treated with ryanodine, an agent known to directly deplete the SR of Ca\(^2+\) by increasing the leakage of Ca\(^2+\) through the SR release channel.\(^{26,27}\) The rationale for these experiments was based on the hypothesis that if halothane induced only direct leakage of Ca\(^2+\) from the SR, as has been described for ryanodine,\(^{26,27}\) then only the phasic Ca\(^2+\) transients should be decreased, in a manner similar to that observed in ryanodine-exposed myocytes and without any effect on baseline [Ca\(^2+\)]). However, if exposure of the myocytes to halothane decreased available Ca\(^2+\) by inhibiting SR reuptake and/or extrusion of Ca\(^2+\) by the sarcolemma in conjunction with direct leakage of Ca\(^2+\) from the SR, then the stimulus-induced increase in baseline [Ca\(^2+\)] should be exacerbated upon initiation of phasic electrical stimulation of previously quiescent cells. Any effects on baseline [Ca\(^2+\)]; that result from inhibition of SR reuptake and/or plasmalemmal extrusion of Ca\(^2+\) should not occur during treatment with ryanodine since this agent has no demonstrated effect on these processes.

Figure 5 shows the response of a single myocyte to a train of electrical stimuli at 1.5 Hz. The data acquisition rate was 2 KHz. There is a slight, progressive elevation in the baseline [Ca\(^2+\)]; toward the end of the seven-pulse train. The same cell, after treatment with 0.7% halothane for 10 min, exhibited a greater shift in baseline [Ca\(^2+\)]; during the train than in the control portion of the experiment. In addition, this cell displayed "aftertransients" after cessation of the stimulus train. The effect of 1-, 1.5-, and 2-Hz pulse trains on the elevation of baseline [Ca\(^2+\)]; was examined further using the slower acquisition system, which produced a more time-averaged Ca\(^2+\) signal but allowed continuous recording of the responses to pulse trains in the presence and absence of 0.7% halothane without saturating the available memory of the computer.

Figure 6A depicts a typical experiment using this protocol. In the control phase, the cell, stimulated by suction pipette at 1.0, 1.5, and 2.0 Hz, demonstrated a progressive elevation of stimulated baseline [Ca\(^2+\)]; and a concomitant...
REVERSAL OF HYPERTROPHY BY RYANOCLINE

Fig. 5. Response of a single myocyte to a train of electrical stimuli at 1.5 Hz in the absence and presence of 0.7% halothane recorded at 540-nm excitation to allow maximal sample rate. The slight elevation of baseline [Ca$^{2+}$] by the end of the control train is exacerbated in the presence of halothane. In the lower panel, the net Ca$^{2+}$ transients were reduced in the presence of 0.7% halothane and the cell continued to give two to three “aftertransients” at the termination of the stimulus train. These records were taken at a sample rate of 2 kHz. Stimulus voltage was 0.5 V, constant current with stimulus isolation (1.2 X threshold for the cell).

reduction in net transient amplitude. The stimulus and recording conditions were the same for both the control and halothane-treated periods. Observation of the cell during stimulus trains revealed that cell shortening also decreased from an initially large value to lower values as steady state was achieved. Exposure of the cell to superfusate equilibrated with 0.7% halothane (fig. 6A) significantly potentiated the change in baseline [Ca$^{2+}$] at 1.0 and 1.5 Hz (P < 0.05) and reduced net transient amplitude in a manner similar to that observed in the constant-pulse experiments. A summary of the effects of halothane is presented in Table 2. Halothane, at 0.4%, produced significant reductions in net transient amplitude but did not affect the change in stimulated baseline [Ca$^{2+}$] at the onset of pacing. The differences in results at these two halothane concentrations suggest a possible dose effect, but this was not explored because use of higher halothane concentrations limited the ability to consistently activate and measure stimulated Ca$^{2+}$ transients.

In a set of comparative experiments, myocytes were exposed to ryanodine, in place of halothane, at a concentration that depletes SR Ca$^{2+}$ stores. Myocytes were subjected to the same stimulation protocol described in the preceding section with the exception that a 10-min treatment with 0.1 μM ryanodine replaced halothane treatment. Comparison of the results of the ryanodine experiments to those with halothane are important since ryanodine exerts no limiting effect on SR uptake of Ca$^{2+}$ via Ca$^{2+}$-adenosine triphosphatase (Ca$^{2+}$-ATPase). In the rat heart, the ryanodine depletion of SR Ca$^{2+}$ stores has a critical effect on cardiac function and results in a negative inotropic response. An initial series of experiments confirmed the negative inotropic action of ryanodine. In these experiments myocytes were paced continuously at 1 Hz and, after an initial control period, were exposed to 0.1 μM ryanodine for 10 min. Ryanodine reduced net Ca$^{2+}$ transient amplitude from a mean value of 338 ± 128 nM to 189 ± 68 nM (P < 0.05, n = 5) representing a 56 ± 11% reduction. This effect is roughly equivalent to that produced by 0.7% halothane, which reduced net transient amplitude to 61 ± 29% of control.

The responses of cells exposed to 0.1 μM ryanodine stimulated to a sudden increase in baseline [Ca$^{2+}$] by the onset of electrical pacing contrasted sharply with those obtained in the experiments with 0.7% halothane. Ryanodine did not potentiate the shift in baseline [Ca$^{2+}$] at the onset of electrical pacing (fig. 6B). The results from cells treated in this manner are presented in Table 3. Ryanodine caused significant reduction in net transient amplitudes at 1.0, 1.5, and 2.0 Hz (P < 0.05) and there was actually a significant reduction in stimulated baseline amplitude.

Fig. 6. A: Effect of 0.7% halothane (10-min exposure) on the response to initiation of trains of electrical stimuli at 1.0, 1.5, and 2.0 Hz. In each stimulus train, the initial, large Ca$^{2+}$ transient decreased with successive stimuli until a new steady state was reached. The shift in baseline [Ca$^{2+}$] from resting to a new stimulated baseline level during the stimulus train appeared to increase with pacing frequency. Halothane reduced the net transient amplitude and, relative to control, increased the shift in [Ca$^{2+}$] from resting state to stimulated baseline level. Levels of [Ca$^{2+}$] are represented in nanomolar units on the ordinate. B: Same protocol as in A, except that 0.1 μM ryanodine replaced halothane. Ryanodine reduced the net Ca$^{2+}$ transient but did not increase the shift in [Ca$^{2+}$] from resting state to stimulated baseline levels.
Table 2. Effect of halothane on changes in [Ca^{2+}] induced by the onset of repetitive electrical stimulation

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<thead>
<tr>
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<th>0.7% Halothane</th>
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<tr>
<td></td>
<td>Δ Baseline</td>
<td>Net Transient</td>
<td>Δ Baseline</td>
<td>Net Transient</td>
</tr>
<tr>
<td>1.0 Hz</td>
<td>30 ± 4</td>
<td>221 ± 23</td>
<td>74 ± 26*</td>
<td>124 ± 21*</td>
</tr>
<tr>
<td>1.5 Hz</td>
<td>95 ± 32</td>
<td>222 ± 49</td>
<td>185 ± 40*</td>
<td>147 ± 40*</td>
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<td>2.0 Hz</td>
<td>84 ± 19</td>
<td>249 ± 46</td>
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<tr>
<th></th>
<th>0.0% Halothane</th>
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<th>0.4% Halothane</th>
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<td></td>
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<tr>
<td>1.0 Hz</td>
<td>67 ± 30</td>
<td>448 ± 98</td>
<td>95 ± 28</td>
<td>158 ± 49*</td>
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<td>1.5 Hz</td>
<td>89 ± 28</td>
<td>277 ± 88</td>
<td>95 ± 28</td>
<td>119 ± 32*</td>
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<tr>
<td>2.0 Hz</td>
<td>72 ± 26</td>
<td>317 ± 72</td>
<td>103 ± 30</td>
<td>124 ± 12</td>
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The changes in baseline represent the differences in stimulated baseline [Ca^{2+}], and resting [Ca^{2+}], in nanomolar units (mean ± SEM). Net transient amplitude represents [Ca^{2+}] at the peak of the transient minus the stimulated baseline [Ca^{2+}] (mean ± SEM). All measurements were taken at steady state.

Top, n = 10; bottom, n = 4.
* Compared to control, P < 0.05.

[Ca^{2+}] at 1.5 Hz. These data contrast markedly to those obtained in halothane-treated cells. Ryanodine elicited no change in resting [Ca^{2+}] in unstimulated cells.

Halothane Effects on Spontaneously Contracting Cells

The effects of 1.5% halothane on cells (n = 6) exhibiting spontaneous Ca^{2+} transients and contractions were examined in order to compare the results with those from quiescent cells. The effect of halothane on spontaneous cells was unpredictable and variable. In some spontaneous cells, halothane reduced the amplitude of the Ca^{2+} transients corresponding to cell contraction (fig. 7A). This response is consistent with halothane’s effect on electrically paced cells. However, some spontaneous cells examined showed little change in Ca^{2+} transient amplitude or even an increase in basal [Ca^{2+}], subsequent to elimination of the Ca^{2+} transients (i.e. steady state [Ca^{2+}] was not achieved; figs. 7B and 7C).

Discussion

The results of these experiments support the hypothesis that halothane, in clinically relevant concentrations, produces its negative inotropic effect by limiting the availability of activator Ca^{2+} in cardiac myocytes in response to direct sarcolemmal stimulation or from stimulated release from intracellular stores. Halothane reduced the increase in [Ca^{2+}]; in response to membrane depolarization (50 mM K+ and electrical stimulation) and to caffeine-induced release of SR Ca^{2+} stores. Reduction in cell shortening, observed microscopically, was concomitant to depression of the Ca^{2+} transient. The phasic transient increase of [Ca^{2+}]; in response to caffeine, as compared to the tonic increase in [Ca^{2+}]; induced by 50 mM K+, depends on the principal site of action of the stimulating agent. Caffeine acts on the SR Ca^{2+}-release channel while 50 mM K+ depolarizes the sarcolemma to ~25 mV (assuming a ~90-mV resting potential) and activates voltage-gated Ca^{2+} channels. The Ca^{2+} influx initiates sarcoplasmic signal transduction cascades to release Ca^{2+} from internal stores.

The effect of halothane to limit, reversibly, the Ca^{2+} transient in response to 15 mM caffeine is consistent with previously published reports and suggests that halo-
thane depletion of SR Ca\(^{2+}\) stores is a major contributor to the negative inotropic effects of the anesthetic. Direct effects of halothane on SR release of Ca\(^{2+}\) have been demonstrated in purified SR Ca\(^{2+}\) channel preparations from frog skeletal muscle.\(^{12}\) In these reconstituted lipid bilayer preparations under voltage clamp conditions, halothane increased channel open probability via a large decrease in channel closed time. The net effect, in this case, was to promote Ca\(^{2+}\) loss from the intrareticular compartment. The results of Wheeler et al.\(^{15}\) clearly indicate that halothane reduces the caffeine-induced Ca\(^{2+}\) transient in rat ventricular myocytes, which is directly proportional to a cell's ability to generate contractile force. Wheeler et al.\(^{15}\) showed that solutions preequilibrated with halothane could elicit caffeine-like Ca\(^{2+}\) transients in populations of cardiac myocytes in suspension. Subsequent caffeine treatment produced a greatly diminished Ca\(^{2+}\) transient.

Recently, Wheeler et al. showed a transient, positive inotropic response and increased Ca\(^{2+}\) transient amplitude in rat myocytes superfused by solutions preequilibrated with halothane vapor.\(^{29}\) The positive inotropic effect was short-lived and was followed by a reduction in cell shortening and Ca\(^{2+}\) transient amplitude. This effect is consistent with the hypothesis that halothane promotes loss of SR stores of Ca\(^{2+}\). These results showed that rapid elevation of the halothane concentration local to the cells has a stimulatory effect on intracellular Ca\(^{2+}\) release. Application of halothane by slow equilibration of superfusion solutions and recording chamber atmosphere does not result in spontaneous Ca\(^{2+}\) release, reinforcing the hypothesis that the manner of halothane application is of critical importance in experiments of this nature.

The effects of halothane are determined not only by the manner of halothane application but also by the condition of the cells exposed to the anesthetic. It is our contention that spontaneous cells are injured and possibly leaky to extracellular entry of Ca\(^{2+}\). Studies of cell populations, therefore, may unnecessarily bias the observed effects of anesthetics, as it is unlikely that a population is entirely homogeneous, and the response of injured cells to anesthetic application could have significant consequences. Regardless of the conditions under which heart cells are studied, it appears that halothane does limit availability of activator Ca\(^{2+}\) by a number of mechanisms, including reduction of slow inward Ca\(^{2+}\) current\(^{11}\) and depletion of SR stores of Ca\(^{2+}\).\(^{5,12,15,29,30}\) The mechanism of SR depletion by halothane is as yet undetermined.

In paced ventricular myocytes, halothane depletion of SR stores of Ca\(^{2+}\) may be exacerbated if SR Ca\(^{2+}\) reuptake mechanisms are inhibited by the anesthetic. The results of experiments in which [Ca\(^{2+}\)]\(_{i}\) was suddenly increased by a stimulus train presented to quiescent cells suggest indirectly that halothane may limit SR reuptake of Ca\(^{2+}\). In direct contrast, the results of experiments with ryanodine, which induces leakage of Ca\(^{2+}\) from the SR but has no effect to limit SR reuptake, demonstrated no potentiation of the shift in [Ca\(^{2+}\)]\(_{i}\) upon onset of pacing. Relative comparison of the [Ca\(^{2+}\)]\(_{i}\) response to trains of stimuli indicated no difference in the stimulated shifts in baseline [Ca\(^{2+}\)]\(_{i}\) between control and ryanodine. It appears, therefore, that halothane may have an action on myocytes in addition to inducing leakage of SR Ca\(^{2+}\) stores.

The most likely explanation of these results favors a mechanism that would facilitate an increase basal [Ca\(^{2+}\)]\(_{i}\) during stimulation, such as a reduction in the cell's ability to remove free Ca\(^{2+}\) from the cytosol. One putative mechanism which would deplete SR Ca\(^{2+}\) and allow for increases in basal [Ca\(^{2+}\)]\(_{i}\) at the onset of repetitive stimulation is the inhibition of reuptake of Ca\(^{2+}\) by the SR Ca\(^{2+}\)-ATPase. Reduction in SR reuptake would favor prolonged or exaggerated elevation of [Ca\(^{2+}\)]\(_{i}\) after stimulation of SR Ca\(^{2+}\) release and would favor the net loss of Ca\(^{2+}\) from the cell by shifting cytosolic Ca\(^{2+}\) removal to the sarcotubular Ca\(^{2+}\)-ATPases and Na\(^+\)/Ca\(^{2+}\) exchange mechanisms. A putative effect of halothane on SR Ca\(^{2+}\) reuptake was postulated in an early report by Su and Kerrick,\(^{5}\) in which halothane depressed the caffeine-induced tension transient during the Ca\(^{2+}\) uptake phase or during the combined uptake--release phase. More recent evidence from Kargacin et al.\(^{31}\) has shown that halothane directly limits SR uptake of Ca\(^{2+}\) in SR membrane vesicles from both normal and malignant hyperthermic pig skeletal muscle preparations. The reduction in SR uptake of Ca\(^{2+}\) was dependent on halothane concentration although only at halothane levels greater than those used for clinical anesthesia. The inability of halothane-exposed myocytes to accommodate a sudden elevation in [Ca\(^{2+}\)]\(_{i}\) imposed by the onset of electrical stimulation suggests that the reduction of SR/sarcotubular Ca\(^{2+}\) pumping may be a mechanism contributing to the negative inotropism caused by halothane. Halothane and ryanodine both reduced the contraction in paced cells. Ryanodine depletes SR Ca\(^{2+}\) stores probably by direct action on the SR release channel and by increasing the opening probability of the SR release channels to low-conductance state.\(^{26,27}\) The net effect of ryanodine at submicromolar concentrations is to deplete SR Ca\(^{2+}\), and since no net changes were observed in resting [Ca\(^{2+}\)]\(_{i}\) during either halothane or ryanodine exposure, it seems likely that sarcotubular Ca\(^{2+}\) extrusion mechanisms remain relatively intact and can accommodate the relatively slow leak of Ca\(^{2+}\) from the SR induced by these compounds. In the event, however, that the high-affinity, low-capacity Ca\(^{2+}\)-ATPase of the sarcotubular were inhibited, the low-affinity, high-capacity Na\(^+\)/Ca\(^{2+}\) exchanger might accommodate any slow changes in SR Ca\(^{2+}\) leakage induced by halothane or ryanodine. Challenged by sudden, dramatic changes in
HALOTHANE EFFECTS ON \([\text{Ca}^{2+}]\) IN MYOCYTES

[\text{Ca}^{2+}]\) load, however, extrusion and reuptake mechanisms could become saturated. In constantly paced rat myocytes, increasing stimulus frequency results in a negative staircase.\textsuperscript{33} In our experiments, however, we initiated \text{Ca}^{2+} transients in quiescent cells, which required that the cells establish a new homeostatic set point for \([\text{Ca}^{2+}]\). Halothane modulation of one or more of the homeostatic processes controlling \([\text{Ca}^{2+}]\) could account for the observed potentiation of stimulated baseline \([\text{Ca}^{2+}]\) during bursts of electrical stimuli. Ryaneodine, which does not reduce SR \text{Ca}^{2+} reuptake, but may actually enhance it,\textsuperscript{34} did not potentiate stimulated baseline \([\text{Ca}^{2+}]\), during sudden onset of repetitive stimulation, suggesting that halothane may have the effect of exacerbating net loss of SR \text{Ca}^{2+} stores by reducing SR \text{Ca}^{2+} reuptake.

An effect of halothane on the rate of \text{Ca}^{2+} transport by the SR has been demonstrated.\textsuperscript{35} Halothane decreases the maximum rate of the SR \text{Ca}^{2+}-\text{ATPase} at any substrate concentration. Under conditions where the capacity of the \text{Ca}^{2+}-\text{ATPase} is saturated, a reduced maximum rate will decrease the maximal rate of uptake. Recent evidence suggests that general anesthetics, \textit{i.e.}, long-chain alcohols, depress \text{Na}/\text{Ca}^{2+} exchange.\textsuperscript{36} It is less clear whether the volatile anesthetics also inhibit \text{Na}/\text{Ca}^{2+} exchange. Using the fura-2 [\text{Ca}^{2+}]\textsuperscript{2-} measurement technique, it is difficult to assess halothane effects on sarcoplasmic \text{Ca}^{2+} transport. Ideally, each mechanism for clearing cytosolic \text{Ca}^{2+} should be selectively eliminated to examine.putative mechanisms of the action of halothane.

In summary, these experiments demonstrate possible mechanisms for the negative inotropic action of halothane. This negative inotropism may arise by the action of halothane at a number of loci in the excitation–contraction coupling process, including depletion of SR \text{Ca}^{2+} stores, inhibition of membrane entry of \text{Ca}^{2+} through voltage-gated channels, and reduction of SR \text{Ca}^{2+} reuptake. It is unclear which of these processes represents the primary site of halothane action. In rat myocytes, which depend primarily on SR \text{Ca}^{2+} release for contraction, the SR may be the principal site of action. It is also unclear whether halothane’s reported effect on the slow inward \text{Ca}^{2+} current results from a direct action on the channels or from feedback from some intracellular signal transduction cascade.

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