Inhibition of Sodium/Calcium Exchange and Calcium Channels of Heart Cells by Volatile Anesthetics

Robert A. Haworth, Ph.D.,† Atilla B. Gokturk, Ph.D.‡

Background: Volatile anesthetics exert profound effects on the heart, probably through their effect on Ca\(^{2+}\) movements during the cardiac cycle. Ca\(^{2+}\) movements across the sarcolemma are thought to involve mainly Ca\(^{2+}\) channels and the Na\(^+/Ca\(^{2+}\) exchanger. We have therefore investigated the action of halothane, isoflurane, and enflurane on Na\(^+/Ca\(^{2+}\) exchange and Ca\(^{2+}\) channel activity to assess the contribution of these pathways to the observed effect of the anesthetics on the myocardium.

Methods: Sarcolemmal ion fluxes were investigated using radioisotope uptake by isolated adult rat heart cells in suspension. Na\(^+/Ca\(^{2+}\) exchange activity was measured from \(^{45}\)Ca\(^{2+}\) uptake by Na\(^+\)-loaded cells. Ca\(^{2+}\) channel activity was measured from verapamil-sensitive trace \(^{45}\)Mn\(^{2+}\) uptake during electric stimulation.

Results: Halothane, isoflurane, and enflurane inhibited Na\(^+/Ca\(^{2+}\) exchange completely, with similar potency when concentrations were expressed in millimolar units in aqueous medium but not when expressed as minimum alveolar concentration (MAC). The inhibition by enflurane was particularly strong, >50%, at 2 MAC. In contrast, the three anesthetics inhibited Ca\(^{2+}\) channels with similar potency when concentrations were expressed as MAC but not when expressed in millimolar units in aqueous medium. Hill plots of pooled data with all three anesthetics showed a slope of \(-3.87 \pm 0.50\) for inhibition of Na\(^+/Ca\(^{2+}\) exchange and \(-1.75 \pm 0.19\) for inhibition of Ca\(^{2+}\) channels.

Conclusions: Halothane, isoflurane, and enflurane inhibit both Na\(^+/Ca\(^{2+}\) exchange and Ca\(^{2+}\) channels at concentrations relevant to anesthesia, although they exhibit differences in potency and number of sites of action. At 1.5 MAC, halothane inhibits Ca\(^{2+}\) channels more than Na\(^+/Ca\(^{2+}\) exchange, whereas enflurane inhibits Na\(^+/Ca\(^{2+}\) exchange more than Ca\(^{2+}\) channels. Isoflurane inhibited both systems equally. The inhibition of Ca\(^{2+}\) influx by these agents is likely to contribute to their negative inotropic effect in the heart. The inhibition of Na\(^+/Ca\(^{2+}\) exchange by enflurane may account for its observed action of delaying relaxation in species lacking sarcoplasmic reticulum. (Key words: Anesthetics, volatile: enflurane, halothane, isoflurane. Ions: calcium; sodium. Ions, calcium: channels.)

VOLATILE anesthetics exert profound effects on the heart that are thought to be caused largely by their effect on Ca\(^{2+}\) movements during the cardiac cycle. Of interest, the various anesthetics affect cardiac function in different ways and to different degrees, suggesting a spectrum of actions at the subcellular level. Because Ca\(^{2+}\) flux into and out of the heart cell is mediated primarily by Ca\(^{2+}\) channels and by the Na\(^+/Ca\(^{2+}\) exchanger, we sought to measure and compare the effect of halothane, isoflurane, and enflurane on these two systems.

We were particularly interested in measuring the effects of the anesthetics on the Na\(^+/Ca\(^{2+}\) exchanger, because in previous work we had found that the inhibition of the exchanger by octanol was strongly potentiated by extracellular Na\(^+\). In these studies Na\(^+/Ca\(^{2+}\) exchange activity was measured from the rate of \(^{45}\)Ca\(^{2+}\) uptake by Na\(^+\)-loaded adult rat heart cells, in the presence of ouabain to inhibit the Na\(^+\) pump. This assay was judged to be specific for Na\(^+/Ca\(^{2+}\) exchange on the basis of its Na\(^+\) dependence and the insensitivity of the Ca\(^{2+}\) uptake to the Ca\(^{2+}\) channel blocker verapamil under these conditions of Na\(^+\) loading.

To measure the inhibition of Ca\(^{2+}\) channel activity by anesthetics, we have used a unique assay developed in this laboratory: Ca\(^{2+}\) channel activity is measured from the rate of uptake of trace concentrations of \(^{55}\)Mn\(^{2+}\) induced by electric stimulation of the cells in suspension. The principal of this assay is that trace concentrations of Mn\(^{2+}\) can pass through Ca\(^{2+}\) channels without significantly inhibiting them, and Mn\(^{2+}\) is not
carried by the Na\(^+\)/Ca\(^{2+}\) exchanger. The rate of Mn\(^{4+}\) efflux from cells is negligible. This results in a linear rate of Mn\(^{4+}\) uptake induced by electric stimulation. Because the uptake was found to be stimulated by isoproterenol and inhibited by verapamil we were able to conclude that this uptake is a specific measure of Ca\(^{2+}\) channel activity. An advantage of this assay over electrophysiologic methods is that the measurement is done under more physiologic conditions, without disturbing the intracellular composition or clamping the membrane potential.

**Materials and Methods**

**Cell Isolation**

This protocol was approved by our institutional Animal Care Committee. Female retired breeder rats were anesthetized by intraperitoneal injection with 30 mg sodium thiamylal. The chest was opened and the heart removed. Heart cells were isolated from the excised hearts according to our original method, as modified. According to the modification (condition 5 in table 2 of that report), the perfusion buffers contained 25 mM N-2-hydroxyethylpiperezine-N'-ethanesulfonic acid (HEPES), adjusted to pH 7.4 with NaOH, in place of bicarbonate, plus basic Eagle's medium amino acids. Ca\(^{2+}\) (1 mM) was restored to the recirculating perfusate 15 min after enzyme addition. This method gave a high yield of cells with a high percentage (74.3 ± 6.0%) of rod-shaped cells, when the cells were resuspended in our standard HEPES buffer medium, which contains 1 mM Ca\(^{2+}\). The remaining cells, 25.7%, were round. Only 2.5% of the round cells in this preparation exclude trypan blue. Thus the contribution of round cells to Na\(^+\)-sensitive Ca\(^{2+}\) uptake and verapamil-sensitive Mn\(^{4+}\) uptake, both of which require an intact sarcolemma, is expected to be small.

**HEPES Buffer Medium**

The buffer was composed of (in millimolar units) NaCl 118, KCl 4.8, HEPES 25, KH\(_2\)PO\(_4\) 1.2, MgSO\(_4\) 1.2, CaCl\(_2\) 1.0, pyruvic acid 5, and glucose 11 and (in micromolar units) insulin 1, adjusted to pH 7.4 with NaOH.

**Anesthetic Solutions**

For each experiment a stock solution of anesthetic-equilibrated HEPES buffer was prepared by adding liquid anesthetic to a glass bottle containing glass beads and filled with a known volume of buffer. The stopped bottle was shaken vigorously until the anesthetic was visibly dissolved. The values used for minimum alveolar concentration (MAC) and other relevant conversion factors are given in table 1.

**Verification of Anesthetic Concentration**

Anesthetic concentrations were also monitored by gas chromatography of head-space extracts of anesthetic-buffer mixtures incubated in the chamber under the conditions described below. Such measurements showed that loss of anesthetic from the chamber over the duration of Ca\(^{2+}\) uptake or Mn\(^{4+}\) uptake was negligible.

**Measurement of Na\(^+\)/Ca\(^{2+}\) Exchange Activity by \(^{45}\)Ca\(^{2+}\) Uptake**

**Na\(^+\) Loading.** Cells were resuspended (4–5 mg protein/ml) in HEPES buffer medium without Mg\(^{2+}\) or Ca\(^{2+}\), and containing 0.1 mM EDTA. Ouabain (1 mM) was added, and cells were incubated 10 min. Suspensions were maintained aerobic by equilibration with air in a shaking incubator at 37°C. We have previously found these conditions to cause complete equilibration of monovalent ions across the sarcolemma via Ca\(^{2+}\) channels, resulting in complete Na\(^+\) loading. After 10 min Mg\(^{2+}\) (1.3 mM) was restored, extra ouabain (2.5 mM final) and ruthenium red (12.5 μM) were added, and cells were stored on ice until used. The extra ouabain and the ruthenium red concentrations were such as to give final concentrations of 1 mM and 5 μM, respectively, under conditions of Ca\(^{2+}\) uptake (see below). Ruthenium red was included to prevent any contribution to measured uptake of Ca\(^{2+}\) by mitochondria of damaged cells. Length of storage on ice (<1 h) did not affect measured rates of \(^{45}\)Ca\(^{2+}\) uptake. An aliquot of cells was additionally exposed to rotenone (4 μM).

| Table 1. Rat MAC Values Used in the Present Study |
|---------------------------------|-------|-------|-------|
|                                | Halothane | Isoflurane | Enflurane |
| 1 MAC (% atm)*               | 1.03    | 1.52   | 2.17   |
| Buffer/gas partition coefficient | 0.75    | 0.55   | 0.74   |
| 1 MAC† (μM)                   | 0.303   | 0.328  | 0.631  |
| Standard solution (μl)        | 35.2    | 45.9   | 88.9   |

* Values were taken from reference 45.
† Calculated from: 1 MAC in mm = k × (part. coefficient) × (1 MAC in % atm), where k = 0.393 at 37°C and 760 mmHg.
plus carbonylcyanide p-trifluoromethoxyphenylylhydrazone (2 μM) for 8 min before storage on ice, to deplete them of adenosine triphosphate (ATP). ATP depletion blocks \(^{45}\text{Ca}^{2+}\) uptake by Na\(^+\)/Ca\(^{2+}\) exchange and hence allowed us to use this condition to define zero uptake.\(^9\)

\(^{45}\text{Ca}^{2+}\) Uptake. Time-course Experiments. \(^{45}\text{Ca}^{2+}\) uptake was measured on cell suspensions in a 5-ml glass syringe placed on a rocking mixer and kept at 37°C in an incubator. This arrangement prevented loss of the volatile anesthetic from the cell suspension during \(^{45}\text{Ca}^{2+}\) uptake. Na\(^+\)-loaded cells (2 ml) were drawn into the syringe with an air space and incubated at 37°C for 2 min. The air space was then excluded, and buffer (3 ml) with or without halothane was drawn into the syringe. After 2 min incubation with mixing, \(^{45}\text{Ca}^{2+}\) uptake was initiated by adding Ca\(^{2+}\), \(^{45}\text{Ca}^{2+}\), and \(^3\text{H}2\text{O}\) to give a free Ca\(^{2+}\) concentration of 1 mM, and final isotope concentrations of 0.5 and 1 μCi/ml respectively. At intervals 0.5-ml aliquots of cell suspension were dispensed from the syringe into plastic tubes where they were immediately centrifuged through a bromoethylene layer into perchloric acid, as previously described.\(^10\) The \(^3\text{H}2\text{O}\) was included to allow automatic compensation for dispensing errors.\(^10\) Because no air space was created when the aliquots were dispensed, anesthetic concentration remained constant during the time course of \(^{45}\text{Ca}^{2+}\) uptake.

Dose-Response Curves. Dose response curves were generated by measuring \(^{45}\text{Ca}^{2+}\) uptake for a fixed time (2 min) by cell suspensions incubated with various concentrations of anesthetic in a water-jacketed chamber at 37°C. The chamber, volume 1.5 ml, was stirred continuously and was free of significant air space when closed with a glass stopper. This chamber\(^1\) was the same one we use for electric field stimulation of cells in suspension. Na\(^+\)-loaded cells (0.6 ml) were put into the chamber, followed by (0.9 – x) ml buffer and x ml anesthetic-equilibrated buffer, and the chamber was closed with the stopper. The volume x was varied in steps of 0.1 ml to give different final concentrations of anesthetic. \(^{45}\text{Ca}^{2+}\) uptake was initiated by introducing 4 μl \(^{45}\text{Ca}^{2+}\) through the bleed hole in the stopper with a Hamilton syringe. The addition contained Ca\(^{2+}\), \(^{45}\text{Ca}^{2+}\), and \(^3\text{H}2\text{O}\) to give a free Ca\(^{2+}\) concentration of 1 mM, and final isotope concentrations of 0.5 and 1 μCi/ml, respectively. After 2 min the stopper was removed, and 0.5-ml aliquot was taken for immediate centrifugation. This chamber allowed better control of suspension temperature than the syringe system used in time-course experiments, but only a single sample could be taken for a well-defined concentration of anesthetic, because taking a sample created an air space. The rate of ATP-depletion-sensitive \(^{45}\text{Ca}^{2+}\) uptake in the presence of anesthetic was expressed as a percentage of the rate of ATP-depletion-sensitive \(^{45}\text{Ca}^{2+}\) uptake in the absence of anesthetic. Anesthetics had no effect on baseline \(^{45}\text{Ca}^{2+}\) uptake in ATP-depleted cells (fig. 1).

Measurement of Ca Channel Activity by \(^{51}\text{Mn}^{2+}\) Uptake

Cells after isolation were suspended (4–5 mg protein/ml) in our standard HEPES buffer medium and maintained aerobically at 37°C in a shaking incubator. Aliquots were exposed to isoproterenol (1 μM) for 3 min and transferred to the stimulation chamber.\(^1\) This was the same chamber as was used for the measurement of \(^{45}\text{Ca}^{2+}\) uptake by Na\(^+\)/Ca\(^{2+}\) exchange (see above). Ruthenium red (5 μM) was added, and verapamil if needed (10 μM), followed by (0.9 – x) ml buffer. Ruthenium red was included to prevent any contribution to measured uptake of \(^{51}\text{Mn}^{2+}\) by mitochondria of damaged cells. The chamber was then closed with the stopper and x ml anesthetic-equilibrated buffer was added.

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Fig. 1. Inhibition by halothane (open triangles and solid triangles) of \(^{45}\text{Ca}^{2+}\) uptake by Na\(^+\)-loaded cells, measured with \(^{45}\text{Ca}^{2+}\). Values shown are the means and standard deviations of three experiments. ATP = adenosine triphosphate; MAC = minimum alveolar concentration.
through the bleed hole. The volume x was varied in steps of 0.1 ml to give different final concentrations of anesthetic. $^{14}$Mn$^{2+}$ (5 $\mu$mol final concentration, 0.5 $\mu$Ci/ml) was introduced through the bleed hole in the stopper with a Hamilton syringe, and after 30-s electric field stimulation of the suspension at 5 Hz was initiated. After 2 min the stopper was removed, and a 1 ml aliquot was taken and added to 80 $\mu$l 40 mm ethyleneglycol-bis(\(\beta\)-aminoethyl ether)N,N',N'-tetraacetic acid. The mixture was incubated for 2 min on ice, and two 0.5 ml aliquots of the mixture were centrifuged as for the measurement of $^{40}$Ca$^{2+}$ uptake. The rate of verapamil-sensitive $^{51}$Mn$^{2+}$ uptake in the presence of anesthetic was expressed as a percentage of the rate of verapamil-sensitive $^{51}$Mn$^{2+}$ uptake in the absence of anesthetic. The magnitude of verapamil-sensitive $^{51}$Mn$^{2+}$ uptake was 18.4 $\pm$ 2.2 pmol $^{51}$Mn$^{2+}$/mg protein, and the magnitude of verapamil-insensitive $^{51}$Mn$^{2+}$ uptake was 20.0 $\pm$ 1.2 pmol $^{51}$Mn$^{2+}$/mg protein. Anesthetics had no effect on the latter, the baseline $^{51}$Mn$^{2+}$ uptake in the presence of verapamil (data not shown).

Data Analysis

Values shown are the mean and standard deviation of experimental points about that mean, using experimental points from at least three experiments. Data from each experiment were converted to percentage inhibition before data from different experiments were pooled.

Data were fit to the Hill equation:

$$\log(v/(100 - v)) = -n \times \log[\text{anesthetic}] + n \times \log[IC_{50}]$$

where v = the initial rate of $^{40}$Ca$^{2+}$ uptake, expressed as a percentage of the rate measured without anesthetic; n = the slope; and IC$_{50}$ = the concentration of anesthetic producing 50% inhibition. Because the Hill plot distorts errors, the fit was weighted by weight (w), where:

$$w = 1/sdh^2$$

where sdh = the standard deviation of $v/(100 - v)$, which is given by:

$$sdh = \text{sd}v \times (1 + (100 - v)^2)^{1/2}/(100 - v)^2$$

where sdv = the standard deviation of the measurement v.

Test for Linearity of Data. The test statistic for lack of fit of data to a straight line is given by the ratio of the mean square error for lack of fit to the mean square error for pure error.$^{14}$

The test for different slopes of linear fits in figure 1 was by analysis of covariance.$^{14}$

Results

When 1 mm free Ca$^{2+}$ containing $^{40}$Ca$^{2+}$ was added to Na$^+$-loaded cells, the rate of $^{40}$Ca$^{2+}$ uptake was rapid for 2 min, and was near zero in cells after ATP depletion (fig. 1), as was observed previously.$^9$ We concluded in that study that the intercept of $^{40}$Ca$^{2+}$ uptake measured on ATP-depleted cells corresponds to $^{40}$Ca$^{2+}$ that is bound to the extracellular surface, and thus can be considered as the baseline for intracellular uptake of Ca$^{2+}$. When halothane (0.88 mm, 3 MAC) was present, $^{40}$Ca$^{2+}$ uptake by cells with ATP was reduced by half, whereas there was no effect on the cells without ATP (fig. 1). Because we were interested in measuring the initial rate of $^{40}$Ca$^{2+}$ uptake and the effect of anesthetics on the initial rate, we tested the data in figure 1 for linearity (see Methods). First we fit the data for ATP-depleted cells to a straight line, to give a y-axis intercept that we could use as a point of origin for the other data. We then tested whether the other data deviated significantly from a fit to a straight line that went through this point of origin. We found that the control curve was not significantly different from linear for as much as 2 min (F = 0.65; F = 7.71 for P < 0.05), and the curve for uptake with 3 MAC halothane was not significantly different from linear for as much as 5 min (F = 0.56; F = 4.07 for P < 0.05). The slopes of the best fit lines were, however, significantly different (P < 0.05) between conditions, except for the two ATP depletion conditions.

A difficulty with the experimental design of figure 1 was that temperature control was uncertain, because the syringe had to be removed from the incubator for samples to be taken. Also, we wished to study the effect of anesthetics at several different concentrations. Therefore, we used water-jacketed closed chambers we had previously developed for subjecting cells in suspension to electric field stimulation,$^{11}$ to measure the effect of anesthetics on $^{40}$Ca$^{2+}$ uptake by Na$^+$-loaded cells. Preliminary experiments using a similar chamber containing an O$_2$ electrode showed that the cell suspension remained aerobic for the duration of the $^{40}$Ca$^{2+}$ uptake measurement, which we chose as 2 min on the basis of the result in figure 1. Also, we determined by gas chromatography that anesthetic concentrations in the chamber remained constant for the duration of the $^{40}$Ca$^{2+}$ uptake measurement.
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Fig. 2. Concentration dependence of inhibition by anesthetics of \textsuperscript{45}Ca\textsuperscript{2+} uptake by Na\textsuperscript{+} loaded cells. Concentrations are expressed (A) as rat minimum alveolar concentration (MAC) and (B) in millimolar units. Conversion factors are given in Table 1.

With these chambers we examined the concentration dependence of the inhibition of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange-mediated \textsuperscript{45}Ca\textsuperscript{2+} uptake by the anesthetics halothane, isoflurane, and enflurane. We found (fig. 2) that although all these anesthetics inhibited \textsuperscript{45}Ca\textsuperscript{2+} uptake completely, they varied considerably in their potency, when concentrations were expressed in units of MAC (fig. 2A). In contrast, when expressed in millimolar units, a similar concentration dependence of \textsuperscript{45}Ca\textsuperscript{2+} uptake inhibition was seen for all three anesthetics (fig. 2B). A further feature of the dose response curves was their bell shape, suggesting a multisite action of the anesthetics. This was more quantitatively evident when the data were plotted as a Hill plot (fig. 3). The slope

in the region >50% inhibited had a best fit value of $-3.46 \pm 0.04$, indicating at least four sites of action of the anesthetic.\textsuperscript{12} For comparison, the data we obtained previously for inhibition of the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger by octanol under these conditions are also shown. The slope for octanol in the region >50% inhibition was $-1.78 \pm 0.06$, suggesting at least two sites of inhibition. Studies on the reversibility of this inhibition were not undertaken.

When the effect of the anesthetics on Ca\textsuperscript{2+} channel activity was assessed with the \textsuperscript{55}Mn\textsuperscript{2+} uptake assay, an inhibitory action was observed but with characteristics different from those of the inhibition of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange (fig. 4). When anesthetic concentrations were expressed as MAC, halothane, isoflurane, and enflurane were approximately equipotent (fig. 4A). Expressed in millimolar units, enflurane was considerably weaker than the other two (fig. 4B). Also, the shape of the curves by inspection was less bell-shaped. We have previously found that the uptake of \textsuperscript{55}Mn\textsuperscript{2+} induced by electric stimulation was linear to 5 min.\textsuperscript{3} The uptake time of 2 min used here is therefore within the linear range, as for \textsuperscript{45}Ca\textsuperscript{2+} uptake by Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange, and the concentration dependence of anes-

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thetic effects on these two systems may be compared with validity.

To compare the action of the anesthetics at the exchanger and at Ca\(^{2+}\) channels, we first derived best fit IC\(_{50}\) values for each curve from a linear fit of the Hill plot data for >50% inhibition, and then plotted the combined data for all anesthetics on a Hill plot, with concentrations for each anesthetic normalized by expression as a factor of its own IC\(_{50}\). Figure 5 shows these plots, for inhibition at the exchanger (fig. 5A) and at Ca\(^{2+}\) channels (fig. 5B). The visual impression of a steeper concentration dependence for inhibition of the exchanger (fig. 2) than for inhibition of Ca\(^{2+}\) channels (fig. 4) is born out quantitatively in this plot.

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Fig. 4. Concentration dependence of inhibition by anesthetics of stimulation-induced Mn\(^{2+}\) uptake by normal cells, measured with \(^{55}\)Mn\(^{2+}\). Mn\(^{2+}\) uptake was measured after 2 min of electric stimulation. Concentrations are expressed (A) as rat minimum alveolar concentration (MAC) and (B) in millimolar units. Conversion factors are given in table 1.

Fig. 5. Hill plot of inhibition by anesthetics of (A) \(^{45}\)Ca\(^{2+}\) uptake by Na\(^{+}\)-loaded cells and (B) stimulation-induced \(^{55}\)Mn\(^{2+}\) uptake by normal cells. Concentrations of anesthetics are expressed relative to their concentrations producing 50% inhibition (IC\(_{50}\)) (table 3). Lines are best linear fits to data for >50% inhibition; the slopes of these lines are given in table 2. \(v\) = the initial rate of uptake, expressed as a percentage of the rate measured without anesthetic.
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(fig. 5) as a steeper slope at >50% inhibition, being \(-3.87 \pm 0.50\) for Na\(^+\)/Ca\(^{2+}\) exchange and \(-1.73 \pm 0.19\) for Ca\(^{2+}\) channels, for data pooled for halothane, isoflurane, and enflurane (table 2). The IC\(_{50}\) values found are given in table 3.

Finally, because the data for inhibition of the exchanger were obtained on separate days and with different heart cell preparations from that obtained for inhibition of the Ca\(^{2+}\) channel, we sought to confirm our essential findings by measuring both kinds of inhibition in the same preparation, for all anesthetics, on the same day. To do this we chose a single level of anesthetic, 1.5 MAC. We found (fig. 6) a similar level of inhibition of Ca\(^{2+}\) channels with each anesthetic, and a greater degree of inhibition of Na\(^+\)/Ca\(^{2+}\) exchange by enflurane than by isoflurane, consistent with the results of figures 2 and 4.

Discussion

The assays for Ca\(^{2+}\) channel activity and Na\(^+\)/Ca\(^{2+}\) exchange activity used here do not appear to overlap in what they measure, because Mn\(^{2+}\) cannot enter the cell through the Na\(^+\)/Ca\(^{2+}\) exchanger, and Ca\(^{2+}\) influx by Na\(^+\)-loaded cells under the conditions used here was not inhibited by verapamil. We should, however, consider the possibility that although the \(^{55}\)Mn\(^{2+}\) uptake is a measure of Ca\(^{2+}\) channel activity, the inhibition of \(^{55}\)Mn\(^{2+}\) uptake seen with anesthetics may be indirect. This could occur if the anesthetics were inhibiting Na\(^+\) channels, and Na\(^+\) channel activity was required to trigger Ca\(^{2+}\) channel activity under our conditions of electric field stimulation. We have indeed obtained some evidence in previous experiments that inhibition of Na\(^+\) channels with tetrodotoxin causes a reduction in Ca\(^{2+}\) channel activation in this system.\(^{15}\) We also found that this Na\(^+\) channel dependence could be overcome by \(\beta\)-adrenergic stimulation of the cells with isoproterenol.\(^{15}\) For the current experiments we therefore used cells treated with isoproterenol, and in control experiments (not shown) found that \(^{55}\)Mn\(^{2+}\) uptake under these conditions was insensitive to tetrodotoxin. We therefore conclude that the inhibitory action of the anesthetics on \(^{55}\)Mn\(^{2+}\) uptake observed here truly reflects their inhibition of Ca\(^{2+}\) channel activity.

A 66% inhibition of Na\(^+\)/Ca\(^{2+}\) exchange activity by 3% halothane has recently been reported in neonatal

Table 2. Comparison of Hill Plot Slopes for Inhibition by Anesthetics of the Na/Ca Exchanger and Ca Channels

<table>
<thead>
<tr>
<th>Inhibition of Na/Ca exchange</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Halothane (fig. 3)</td>
<td>(-3.46 \pm 0.04)</td>
</tr>
<tr>
<td>Octanol (fig. 3)</td>
<td>(-1.78 \pm 0.06)</td>
</tr>
<tr>
<td>Halothane, isoflurane, and enflurane combined (fig. 5A)</td>
<td>(-3.87 \pm 0.50)</td>
</tr>
<tr>
<td>Inhibition of Ca channels</td>
<td>(-1.73 \pm 0.19)</td>
</tr>
<tr>
<td>Halothane, isoflurane, and enflurane combined (fig. 5B)</td>
<td>(-1.73 \pm 0.19)</td>
</tr>
</tbody>
</table>

Slopes are best fit values for data showing >50% inhibition.

Table 3. Concentration of Anesthetic Required for 50% Inhibition (IC\(_{50}\))

<table>
<thead>
<tr>
<th>Anesthetic</th>
<th>Na/Ca Exchange</th>
<th>Ca Channel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Halothane</td>
<td>0.91</td>
<td>0.82</td>
</tr>
<tr>
<td>% in gas</td>
<td>3.09</td>
<td>2.78</td>
</tr>
<tr>
<td>Rat MAC</td>
<td>3.00</td>
<td>2.70</td>
</tr>
<tr>
<td>Isoflurane</td>
<td>0.69</td>
<td>0.98</td>
</tr>
<tr>
<td>% in gas</td>
<td>3.19</td>
<td>4.53</td>
</tr>
<tr>
<td>Rat MAC</td>
<td>2.09</td>
<td>2.98</td>
</tr>
<tr>
<td>Enflurane</td>
<td>0.87</td>
<td>1.43</td>
</tr>
<tr>
<td>% in gas</td>
<td>2.99</td>
<td>4.92</td>
</tr>
<tr>
<td>Rat MAC</td>
<td>1.38</td>
<td>2.27</td>
</tr>
</tbody>
</table>

Values were calculated from the best linear fit of data for each anesthetic on Hill plots like figure 3, for degrees of inhibition >50%.

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Fig. 6. Comparison of the inhibition by 1.5 minimum alveolar concentrations of anesthetics of \(^{45}\)Ca\(^{2+}\) uptake by Na\(^+\)-loaded cells and \(^{45}\)Mn\(^{2+}\) uptake by stimulated normal cells, measured in the same preparations. *P = 0.016, a significantly greater degree of inhibition of \(^{45}\)Ca\(^{2+}\) uptake by enflurane than by isoflurane, by a pooled t test.
rabbit ventricular myocytes, measured electrophysiologically at 23°C under conditions of physiologic concentrations of extracellular Na⁺. This level of inhibition is comparable, and actually somewhat greater, than the inhibition we report here (table 5).

An inhibitory action of halothane on Ca²⁺ currents has been observed in rat, guinea pig, and dog heart cells. These studies have shown degrees of inhibition of Ca²⁺ currents ranging from about 57% reduction with 0.88 mm halothane in dog cells stimulated at 0.1-0.2 Hz at room temperature to only 20% reduction in guinea pig cells stimulated at 0.3 Hz at 37°C, for the same liquid phase concentration. This range may be caused by differences in species and conditions of measurement. Also, anesthetics were found to inhibit Ca²⁺ channels much more strongly at a stimulation frequency of 3 Hz than at 0.3 Hz. The degree of inhibition of Ca²⁺ channels found in rat heart cells using a switched voltage clamp at room temperature was 35%, with 1% gas phase halothane (0.63 mm at 20°C). That is similar to the degree of inhibition observed here, where 0.46 mm halothane (1.46 MAC at 37°C) caused about 30% inhibition of verapamil-sensitive Ca²⁺ uptake. It is possible that the similarity of our result masks a cancellation of a tendency for the anesthetic to be more potent at the higher rate of stimulation and to be less potent at the higher temperature. The degree of inhibition found here with the Mn⁴⁺ method is more than that found with guinea pig cells at 37°C and less than that found with dog cells at room temperature.

Bosnjak et al. also found that equianesthetic concentrations of halothane, isoflurane, and enflurane each produced a similar degree of inhibition of Ca²⁺ current in dog heart cells. Our results with rat heart cells are in agreement with this observation (fig. 4). The significance of this may be that cardiac Ca²⁺ channels possess similar structural properties to the channels responsible for anesthetics. This similarity of anesthetic action is in striking contrast to the very different potency of the anesthetics as inhibitors of Na⁺/Ca²⁺ exchange (fig. 2). A further difference in action is indicated by the steeper Hill plots for inhibition of the exchanger than for inhibition of Ca²⁺ channels (fig. 5). The steeper Hill plot for inhibition of the exchanger (fig. 5A) suggests more sites of action for the volatile anesthetic on the exchanger than at the Ca²⁺ channel.

A potentially important consequence of this difference is that their relative degree of inhibition will vary with anesthetic concentration. At higher concentrations of anesthetic, inhibition of the exchanger will become increasingly important.

What light do our observations shed on the mechanisms of the observed effect of these anesthetics on the heart?

First, we consider how the action of anesthetics on Ca²⁺ channels and on Na⁺/Ca²⁺ exchange may contribute to their inotropic effect. Inotropy in the heart is controlled to a large extent by the regulation of the access of Ca²⁺ to the myofilaments. The inotropic effect of anesthetics is thought to be mediated primarily through their effect on Ca²⁺ homeostasis, because their effect on the myofilaments in skinned muscle preparations and intact preparations is relatively small. Ca²⁺ for activation of the myofilaments comes from two sources: Ca²⁺ influx from outside the cell and Ca²⁺ released from the sarcoplasmic reticulum (SR), an intracellular store. Recent experiments with thapsigargin, a specific inhibitor of SR Ca²⁺ uptake, show that the contribution of the two sources of Ca²⁺ to myofilament activation can be equally significant, and also that the contribution of each source varies between species. Ca²⁺ influx affects contractile strength not only by direct activation of the myofilaments but also by triggering SR Ca²⁺ release and further by replenishing SR Ca²⁺ stores. Ca²⁺ channels and the Na⁺/Ca²⁺ exchanger are the only two known pathways for Ca²⁺ influx in the heart. Because the exchanger is electrogenic, exchanging three Na⁺ ions for one Ca²⁺ ion, Ca²⁺ influx is favored by membrane depolarization. However, although the ability of Ca²⁺ channels to mediate Ca²⁺-induced Ca²⁺ release during excitation has long been recognized, the role of the exchanger in this process is only just emerging. With regard to SR filling, evidence has been gained for a role of Ca²⁺ influx both by Ca²⁺ channels and by the Na⁺/Ca²⁺ exchanger in replenishing SR Ca²⁺ stores. Thus Ca²⁺ channels and the Na⁺/Ca²⁺ exchanger both can potentially play a role in all three inotropic actions of Ca²⁺ influx: direct activation of the myofilaments, triggering SR Ca²⁺ release, and SR filling.

In addition to the role of the exchanger in Ca²⁺ influx, its role in Ca²⁺ efflux must also be considered, because the effect of inhibition of the exchanger on inotropy will be a consequence of the net change in flux as a result of inhibition of both influx and efflux. A major role for the exchanger in Ca²⁺ efflux is generally accepted. The only other known efflux mechanism is the sarcolemmal Ca²⁺ pump, and its contribution appears to be small. Because at steady state the Ca²⁺ entering
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through Ca\(^{2+}\) channels and the Na\(^+\)/Ca\(^{2+}\) exchanger must equal the Ca\(^{2+}\) leaving through the Ca\(^{2+}\) pump and the Na\(^+\)/Ca\(^{2+}\) exchanger, the amount of Ca\(^{2+}\) leaving through the exchanger would appear to be greater than the amount entering through the exchanger if, as would commonly be assumed, the amount entering through Ca\(^{2+}\) channels was greater than the amount leaving through the Ca\(^{2+}\) pump. An inhibition of the exchanger would thus be expected to have a positive inotropic effect, although it is possible that total Ca\(^{2+}\) efflux by Na\(^+\)/Ca\(^{2+}\) exchange may be affected less by a partial inhibition of the exchanger than total Ca\(^{2+}\) influx by Na\(^+\)/Ca\(^{2+}\) exchange.

In light of the above considerations, we can evaluate how the inhibition of the Ca\(^{2+}\) channels and Na\(^+\)/Ca\(^{2+}\) exchange by anesthetics would be expected to affect inotropy. Because Ca\(^{2+}\) channels have no role in Ca\(^{2+}\) efflux, the inhibition of Ca\(^{2+}\) channels by anesthetics would be expected to have a negative inotropic effect. The effect of inhibition of the exchanger, on the other hand, is uncertain, depending on the relative magnitude of the role of the exchanger in Ca\(^{2+}\) influx and efflux. A role in both directions may mean that the inotropic effect of an inhibition would tend to cancel out.

The inotropic effect of halothane, isoflurane, and enflurane on rat papillary muscle has been investigated by Lynch and Frazer.\(^{40}\) Halothane and enflurane decreased the force of contraction by 28% and 24% respectively at 2 MAC and 3 Hz, whereas the negative inotropic effect of isoflurane was only 7%. A similarly small negative inotropic effect of isoflurane relative to that of halothane at high (but physiologic) frequencies of stimulation has also been noted with guinea pig \(^{41}\) and rabbit.\(^{42}\) In contrast, the reduction of Ca\(^{2+}\) channel activity observed here (at 4 Hz) was similar for all three anesthetics, being 30, 39 and 36%, for halothane, enflurane, and isoflurane respectively. This suggests to us that at these high frequencies of stimulation a partial inhibition of the Ca\(^{2+}\) channel may have little effect on inotropy, and thus the negative inotropic effect of halothane and enflurane under these conditions would have little to do with inhibition of the Ca\(^{2+}\) channel. Moreover, because we saw a similar degree of inhibition of the Na\(^+\)/Ca\(^{2+}\) exchanger by isoflurane as for Ca\(^{2+}\) channels, the lack of inotropic effect of isoflurane would suggest that Ca\(^{2+}\) influx by this route also is not limiting contractility at high stimulation frequencies. The negative inotropic effect of halothane and enflurane under these conditions may rather be related to their effect on the SR. Consistent with this, studies of potentiated state contractions, the strength of which is thought to reflect SR function, show a strong depressant effect of halothane, some effect of enflurane, and no effect of isoflurane.\(^{43,44}\) At low frequencies of stimulation, the anesthetics inhibited contraction more strongly. Halothane, isoflurane, and enflurane at 2 MAC were reported to inhibit rested state contractions by 53%, 26%, and 41%, respectively.\(^{45}\) The degree of inhibition of force by isoflurane is particularly stronger under these conditions than at high frequency, and is much more like the degree of inhibition of the Ca\(^{2+}\) channel. The inhibition by halothane and enflurane is still stronger than that by isoflurane, reflecting their inhibition at the SR as well as the Ca\(^{2+}\) channel. Terrar and Victory\(^{18}\) also concluded from their studies on guinea pig myocytes that the negative inotropic action of halothane was in part caused by inhibition of Ca\(^{2+}\) channels and in part caused by a reduction in SR Ca\(^{2+}\) stores.

Thus, in summary, the inotropic action of halothane, isoflurane, and enflurane on the rat heart can be understood in terms of their inhibition of Ca\(^{2+}\) channels and SR at low frequencies of stimulation and their inhibition of SR at high frequencies of stimulation. It is not clear if any of the observed inotropic effects can be attributed to an action on the Na\(^+\)/Ca\(^{2+}\) exchanger.

The action of anesthetics on Na\(^+\)/Ca\(^{2+}\) exchange may, however, contribute to their effect on relaxation. Relaxation in the heart is accomplished by Ca\(^{2+}\) removal from the myofilaments, primarily by the SR, in species that have a well-developed SR but also by Ca\(^{2+}\) extrusion from the cell by Na\(^+\)/Ca\(^{2+}\) exchange, as described above. Therefore, anesthetics may slow relaxation, especially in species with less well-developed SR. This possibility is consistent with the observations of Lynch and Frazer,\(^{40}\) who found that 2 MAC enflurane consistently delayed relaxation in the frog, whereas isoflurane and halothane did not, and that enflurane had no effect on relaxation in the rat.

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