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Effect of Calcium on Halothane-Depressed Beating in Heart Cells in Culture

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SUMMARY

Heart cells in culture need no external stimulation to contract; they beat rhythmically at a rate and intensity dependent on culture conditions. These cells respond to the general anesthetic 2-bromo-2-chloro-1,1,1-trifluorethane (halothane), with a loss of beating intensity and a lessening of beating rate. Increased calcium concentrations in growth medium reversed the halothane-depressed beating intensity of heart cells in culture; however, increased calcium concentrations had no effect on the halothane-depressed beating rate. Calcium uptake and release took place in two phases, fast and slow. Only the fast calcium uptake was affected by halothane. Like halothane-depressed beating intensity, the halothane-depressed fast calcium uptake also can be reversed by increased calcium in the growth medium of beating heart cells in culture. Data in this manuscript support the theory that general anesthetics dissolve in membranes and thus disrupt membrane function. The anesthetic halothane appears to affect myocardial beating intensity through its ability to disrupt fast calcium uptake. Halothane also depresses the cardiac beating rate, but the data collected do not relate beating rate with calcium metabolism.

INTRODUCTION

Anesthetics are known to depress myocardial beating rate and beating intensity. Among the older anesthetics, nitrous oxide, cyclopropane, diethyl ether, and thiopental have been shown to depress myocardial function (1). The newer halogenated anesthetic agents, which include halothane, are even stronger depressants of cardiac contractile force (2). General anesthetics are now known to alter lipid bilayer membranes (3), cardiac sarcoplasmic reticulum function (4), oxygen consumption (5), and tissue concentrations of neurohumoral substances (6).

Clinically, halothane depresses myocardial function at the concentrations used to maintain anesthesia [below 3.0% (v/v) halothane]. Price (7) demonstrated a relationship between anesthesia and calcium when he showed that increased calcium concentrations in the external medium bathing a kitten papillary muscle preparation antagonized halothane-induced depression of contractility. During cardiac muscle contraction these calcium ions flow across the plasma membrane, triggering the release of internal calcium ions (8). After activating the contractile proteins this calcium is taken up by the sarcoplasmic reticulum, where either it is made available for release to the contractile proteins on subsequent beats or the calcium is transported out of the cell by a Na^+-Ca^{2+} transport mechanism (8). Price (7) in 1974 suggested that general anesthetics affect calcium metabolism during contraction, and previous experiments have demonstrated that alterations in calcium metabolism result in changes in myocardial beat response (9, 10). Thus anesthetics could affect beat response in cardiac tissue by affecting calcium metabolism. Pentobarbital is known to decrease the amount of superficially located calcium in cardiac tissue (11), and there is some evidence that general anesthetics also decrease the amount of calcium bound to the cardiac plasma membrane (7, 12). Langer *et al.* (13) have shown, using calcium washout studies, that membrane-bound or membrane-associated calcium plays an important role in cardiac muscle contraction.

Myocardial inotropic and chronotropic responses (responses in beating intensity and rate) can be observed in beating heart cells in culture (14). Other properties and the morphology of these cultures have been described elsewhere (15). Heart cell cultures offer the relative simplicity of a tissue culture preparation while retaining physiological function (beating) characteristically present only in whole-body or whole-organ preparations (16).

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An optical-electronic monitoring system permits measurements of the iontropic and chronotropic responses of cells to various chemicals, including anesthetics (17). Other investigators also have used monitoring systems to measure beating rate and the relative contractile force of heart cells in culture (18–21). Our monitor provides an indirect measurement of contractile force but a direct measurement of beating rate. Ethrane, nitrous oxide, methoxyflurane and halothane were shown to depress both beating rate and intensity in these cells in a manner similar to anesthetic-induced myocardial depression (17).

Heart cell cultures also have been used by Langer and Frank (22) to measure calcium flux across the cardiac plasma membrane. Langer and Frank found that approximately 75% of cellular calcium is exchangeable in two kinetically defined flux phases, a fast calcium flux and a slow calcium flux.

The relationships between halothane-induced depression of cardiac function, external calcium requirements for cardiac contraction, and calcium exchange across the cardiac plasma membrane are examined in this paper. Heart cell cultures provide a unique system with which to study these relationships. Data from these studies possibly could reveal a mechanism by which anesthetic agents affect cardiac function.

METHODS

The procedure for preparing heart cell cultures is described elsewhere (23, 24). All experiments were performed with cultures 7 to 10 days after cells were placed in culture. Six hours prior to the start of an experiment the growth medium was replaced with fresh growth medium. Where halothane is used in an experiment it is introduced onto a plate of cells by means of a modified Petri dish lid with a carrier gas of 95% air and 5% CO_2 and a Dräger halothane vaporizer (North American Dräger, Teiford, Pa.). The vaporized halothane, at the appropriate concentration, was passed over the culture medium. Halothane concentrations in the culture medium can be determined by the simple equation formulated by Price and Ohnishi (5).

In experiments that require monitoring the beating rate and beating intensity of cells, the response curves were drawn from data obtained using the optical electronic monitor that was previously described.

Halothane (Ayerst Laboratories, New York, N.Y.) was purchased from Mountainview Hospital (State College, Pa.). All calcium solutions were made from anhydrous $CaCl_2$ (Fisher Scientific Company, Pittsburgh, Pa.). Choline chloride was obtained from the Sigma Chemical Company (St. Louis, Mo.).

The relationship between calcium concentration in the growth medium and beating was examined by varying the calcium concentration from 1.0 to 3.2 mM by addition of either EGTA[4] or $CaCl_2$ to growth medium and measuring the beating response. Normal growth medium contains 2 mM $CaCl_2$. Stepwise additions of 30 μl of a 30.0 mM EGTA solution (in calcium-free growth medium) were made to normal growth medium (3 ml) to decrease the concentration of calcium from 2.0 mM through 1.0 mM. An apparent dissociation constant of 2.13×10^{-8} M was used to calculate calcium bound with each addition of EGTA. To ensure that EGTA was not directly affecting the cultures, beating was monitored by additions of calcium to calcium-free growth medium which was devoid of EGTA; i.e., beating was monitored from 1.0 mM to 2.0 mM $CaCl_2$. $CaCl_2$ was added to the growth medium at levels similar to those obtained during the use of EGTA. In all experiments, beating rate and intensity were measured 10 min after addition of calcium or EGTA.

Studies on calcium's effect on halothane-depressed beating were performed on heart cell cultures exposed to 0.8% or 1.5% (v/v) halothane. Beating rate and beating intensity were determined after the cell cultures had been allowed to equilibrate on the monitor for 10 min. Values obtained under 5% CO_2, 95% air, and 0% halothane were used as the basis for further comparison and were assigned a value of 100%. Halothane was introduced onto the plate of cells for 10 min prior to beating and intensity measurements. Stepwise additions of 20 μl of 15 mM $CaCl_2$ were made to the growth medium (0.15 mM increase per addition, to 2.0 ml of growth medium) to study the effect of increased external calcium on halothane-depressed beating. Beating measurements were made 10 min after each incremental addition of $CaCl_2$. Cultures not treated with halothane were exposed also to increasing concentrations of growth medium calcium.

Calcium titrations were performed using $CaCl_2$. This also introduced chloride ions into the growth medium. Cells were titrated with choline chloride as a control for chloride ions. Choline chloride was added from a 30 mM solution in 20-μl increments (to 2.0 ml of growth medium). Final concentrations of chloride ions were the same as those achieved during the addition of $CaCl_2$ to halothane-treated and untreated cultures. Cellular beating response was recorded using the procedure described for calcium titrations.

Calcium exchange experiments were performed using a special incubator constructed to hold up to 12 culture plates, each in individual compartments. Each compartment was connected to a common gas source supplying 5% CO_2/95% air. Anesthetic was introduced into each compartment by means of this carrier gas and a Dräger halothane vaporizer. Culture medium was swirled every 30 sec to ensure complete mixing of the growth medium. Cells are tightly attached to the tissue culture dishes and few, if any, detach during the experiments (15). Radioactive calcium chloride was obtained from Amersham Corporation (Arlington Heights, Ill.).

Calcium release (exchange of internal calcium with growth medium calcium) was measured from cells that had been loaded with radioactive calcium by exposure to 10 μCi $^{45}Ca^{2+}$ (each plate contained 3 ml of growth medium, 3.33 μCi/ml) for 48 hr. The radioactivity had no visible effect on cell morphology or beating characteristics. At the start of each experiment the growth medium containing radioactive calcium was removed and the cells were washed twice with 3 ml of growth medium devoid of radioactive calcium. Three milliliters of growth medium were then placed on the cells, the cultures were placed in the incubator, and 50-μl samples of growth

[4] The abbreviation used is: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

medium were removed at various times between 0 and 60 min. Radioactive calcium in each sample was then determined. At the end of 60 min each plate was washed eight times with 2 ml of ice-cold 0.9% NaCl, the cells were scraped into 3 ml of 0.9% NaCl and sonicated, and a 50-μl aliquot was counted to determine the amount of $^{45}Ca^{2+}$ left inside the cells. The amount of internal calcium that had exchanged with external calcium was then calculated. Total calcium concentrations in the heart cells were determined by atomic absorption. The experiment as described was then repeated with the cells exposed to various halothane concentrations between 1.0% and 3.0% (v/v). Exposure to halothane began 10 min before the radioactive medium was removed, and exposure was continued until the 60-min sample was removed.

To study the effect of halothane on calcium uptake (exchange of external calcium with internal calcium), 10 μCi of $^{45}Ca^{2+}$ were added to the growth medium (3.0 ml). At various times between 0 and 120 min the growth medium containing $^{45}Ca^{2+}$ was removed from the plate and the cells were washed eight times with 2 ml of an ice-cold solution containing 0.9% NaCl and 2.0 mM $CaCl_2$ (wash time was approximately 45 sec). Cells from each plate were scraped into 3 ml of ice-cold saline solution. After sonication, a 50-μl sample was counted to determine the amount of $^{45}Ca^{2+}$ taken up by the cells. Cell protein was measured by the method of Lowry *et al.* (25). The effect of halothane (0.6%–2.0%, v/v) on calcium exchange was then measured. Halothane exposure began 10 min prior to the addition of $^{45}Ca^{2+}$ and continued until the end of the experiment.

RESULTS

Total calcium in the normal growth medium is 2.0 mM, and the free calcium ion concentration, as measured using an Orion 90-02 calcium ion-specific electrode, is 1.25 mM. All results are reported using the total growth medium calcium concentration (2.0 mM) and do not reflect the 38% reduction in measured calcium activity caused by the presence of other substances in the growth medium. Cultured heart cells beat at their maximal rate (2–3 beats/sec) and maximal intensity when the calcium concentration is 2.0 mM. A decrease in the growth medium calcium concentration caused by the addition of EGTA results in a decrease in the beating rate and a lowering of beating intensity, whereas an increase in calcium concentration over 2.0 mM has no effect on either intensity or beating rate. The addition of calcium to calcium-free growth medium indicated that cells respond to different levels of calcium (1 mM through 3.2 mM) in the same manner and magnitude as when the medium was depleted of calcium by additions of EGTA. These results agree with what has been previously found with other cardiac tissue preparations (26) in that a certain calcium concentration gives maximal contraction, and increases above this level have no additional effect.

Both beating intensity and beating rate were depressed in the presence of halothane in a manner similar to the loss of beating intensity and lessening of beating rate seen when the heart cells are deprived of external calcium (Fig. 1).

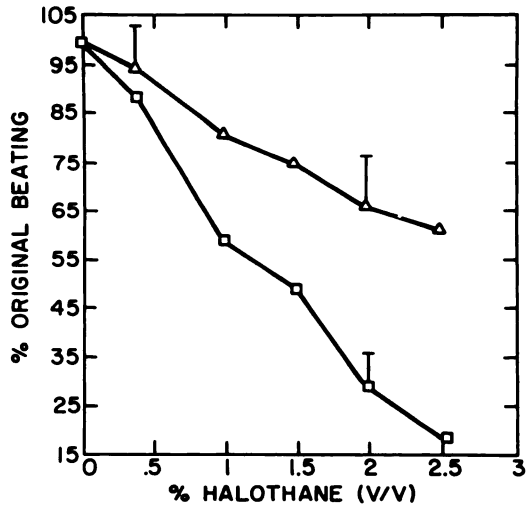

FIG. 1. *Dose-response curves for halothane-treated cells in culture*
Heart cells were exposed to 0, 0.5, 1.0, 1.5, and 2.0% (v/v) halothane. Inotropic and chromotropic response was measured as described in text. □, inotropic response; △, chromotropic response. Each point represents at least eight experiments ± standard deviation.

Cultures exposed to 1.5% halothane responded to increasing levels of external calcium with a restoration of the halothane-depressed beating intensity to its original value (Fig. 2). The beating intensity of cells depressed by exposure to 1.5% halothane responded to addition of calcium ions in a dose-related manner. An increase from 2.0 mM to 2.5 mM calcium was required to obtain a 50% restoration of beating intensity depressed by exposure to 1.5% halothane. Heart cell cultures treated with 0.8% halothane responded to an increase in external calcium in a similar manner.

The halothane-depressed beating rate did not respond to increased concentrations of calcium in the growth medium. Upon removal of halothane from cultures which had been treated with concentrations of calcium above

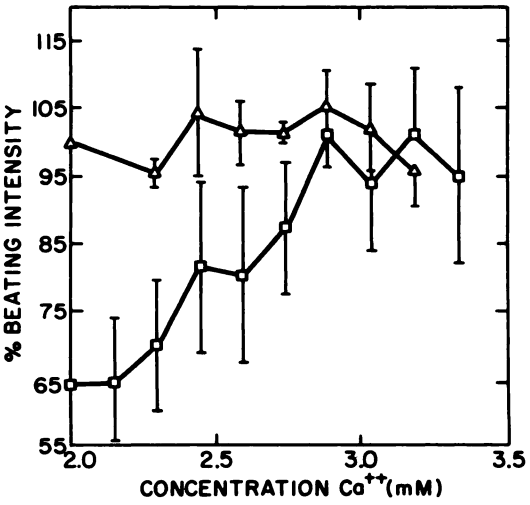

FIG. 2. *Dose-response curve for increased growth medium calcium concentration on the inotropic response of 1.5% halothane-treated cultures*
□, 1.5% halothane-treated cultures; △, no halothane present. Each point represents at least eight experiments ± standard deviation.

2.0 mM, the beating rate returned to at least 95% of its original rate.

When cultures exposed to 1.5% halothane were titrated with choline chloride there was no change in the depressed beating rate or beating intensity caused by exposure to halothane.

Calcium release from beating heart cell cultures exposed to 0% and 1.5% halothane is shown in Fig. 3. These results are expressed as the percentage of total internal calcium that has exchanged with external calcium. Calcium release takes place in two phases, fast (occurring between 0 and 10 min) and slow (occurring between 20 and 60 min). Table 1 represents further calcium release data (nanomoles of calcium exchanged per milligram of protein) collected with cultures exposed to various halothane concentrations. Neither the fast nor the slow release of calcium was altered by the halothane concentrations examined.

Calcium uptake occurs also in two phases, fast and slow (Fig. 4). Halothane at 2% concentration has no effect on the slow phase of calcium uptake, but the fast phase is depressed a little more than 80% by the anesthetic. Table 2 contains data on calcium uptake rates at various halothane concentrations, and the effect of halothane on the observed fast and slow calcium uptake rates are summarized in Fig. 5. Various halothane concentrations between 0% and 2.0% were tested. All concentrations of halothane caused a depression of the fast calcium uptake rate. The magnitude of the depression is dependent on the concentration of halothane to which the heart cells were exposed. At no halothane concentration tested was the slow calcium uptake depressed.

Halothane affects the fast rate of calcium uptake, the rate of beating, and the beating intensity. The beating intensity is related to the amount of calcium available at the myofibril site. Halothane at 1.5% decreased the fast calcium uptake rate from 1.2 nmoles/min/mg of protein to 0.43 nmole/min/mg of protein. It also depressed the beating rate by 25%, from 2 beats/sec to 1.5 beats/sec. Furthermore, the calcium uptake per beat was depressed 52%, from 0.0100 nmole/beat/mg of protein to 0.0048 nmole/beat/mg of protein. It is clear that halothane has depressed both the uptake of calcium per minute per milligram of protein and calcium per beat per milligram of protein. Thus, the halothane-depressed beating intensity may be a consequence of a decreased concentration of calcium at the site of contraction.

Calcium uptake rates varied from culture to culture. Fast uptake ranged from 0.69 nmole/min/mg of protein to 1.65 nmoles/min/mg of protein, and the slow uptake ranged from 0.03 nomole/min/mg of protein to 0.10 nmole/min/mg of protein. While uptake rates between cultures varied, the uptake rates for the 60–100 plates of heart cells from any one culture were constant. Each plate of cells contained between 1.8 mg and 2.2 mg of protein with total calcium at 27.0 ± 0.9 nmoles of calcium

TABLE 1

Nanomoles of internal calcium exchanged with growth medium calcium

Time	Control (0% halothane)[a]	Halothane-treated cells[a, b]
min	*nmoles calcium exchanged/mg protein*	
0	0	0
1	7.2 ± 0.8	8.9 ± 1.9
2	11.1 ± 2.7	9.9 ± 1.9
5	13.3 ± 2.4	11.4 ± 1.7
10	15.1 ± 2.7	13.1 ± 1.9
20	15.9 ± 1.6	14.7 ± 1.4
40	16.4 ± 2.2	16.1 ± 1.6
60	18.4 ± 1.6	17.5 ± 1.6

[a] Results are averages ± standard deviation. $p > 0.25$ compared with control.

[b] Cells were treated with 1.0, 1.5, 2.0, or 3.0% (v/v) halothane.

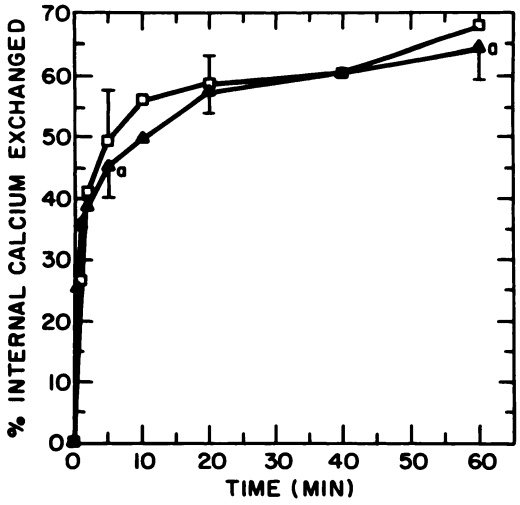

FIG. 3. *Effect of halothane on calcium release in beating heart cell cultures*

□, 0% halothane; ▲, 1.5% halothane. Results are expressed as the percentage of internal calcium exchanged with external calcium. Each time point represents the average of at least six plates ± standard deviation. *a*, $p > 0.25$ compared with 0% halothane value at the same time point.

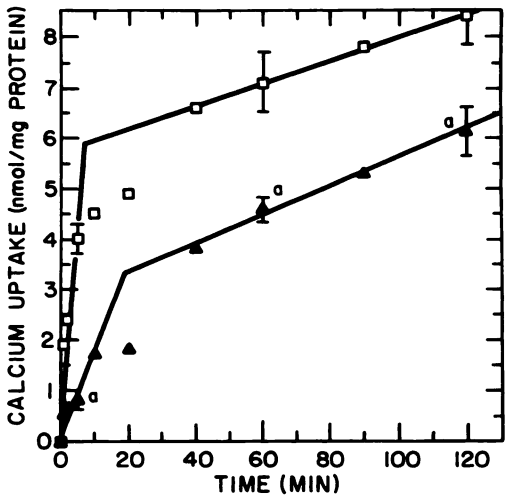

FIG. 4. *Effect of halothane on calcium uptake in beating heart cell cultures*

□, 0% halothane; ▲, 2.0% halothane. Results are the average of four plates ± standard deviation. *a*, $p < 0.005$ compared with 0% halothane value at the same time point.

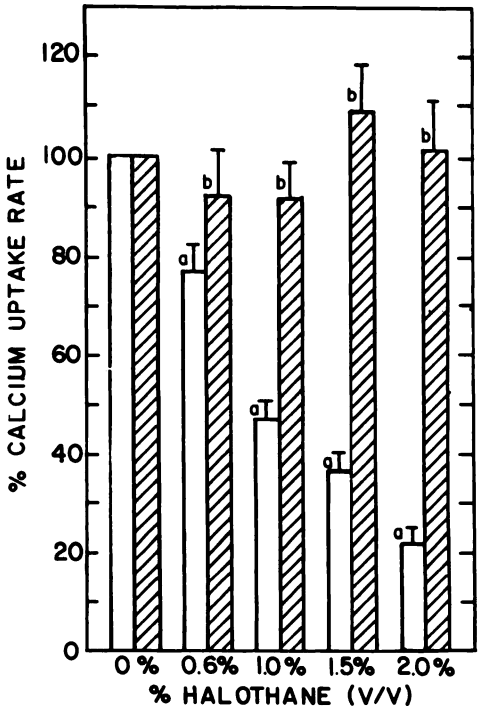

FIG. 5. *Effect of halothane on the fast (unshaded) and slow (shaded) calcium uptake in beating heart cells in culture*

Data are represented as percentage of control (0% halothane). Each point represents the average of at least four experiments ± standard deviation. a, $p < 0.005$ compared with 0% halothane; *b*, $p > 0.25$ compared with 0% halothane.

per milligram of protein (atomic absorption flame photometry). Beating intensity, beating rate, and the fast calcium uptake rate were all independent of calcium concentration at calcium concentrations above 2.0 mM.

We have found that the cells treated with 1.5% (v/v) halothane require an increase from 2.0 mM to 3.0 mM calcium to restore beating intensity lost due to halothane exposure. An increase in the concentration of calcium in the growth medium reversed halothane-depressed fast calcium uptake rate. Ten minutes after treatment with 1.5% halothane, the calcium concentration in the growth medium was raised from 2.0 mM to 3.0 mM. This treatment prevented the halothane depression of fast calcium uptake (Fig. 6) but did not change the fast calcium uptake rate of heart cells that had not been exposed to halothane (Fig. 6).

DISCUSSION

Previous literature reports (7) have related halothane's ability to depress myocardial function with external calcium bathing cells. Using beating heart cells in culture we have shown that halothane depresses both beating rate and beating intensity at anesthetic concentrations used clinically. Our heart cells in culture also show the two kinetically defined calcium flux phases observed by other investigators (22). Our results clearly demonstrate that halothane depresses the fast calcium uptake role in heart cells in culture. The slow calcium uptake rate and both phases of calcium release are unaffected by exposure

TABLE 2

Rates of calcium uptake by beating heart cells in culture

Calcium uptake	% Halothane (v/v)											
	Control[a]	0.6%		Control[a]	1.0%		Control[a]	1.5%		Control[a]	2.0%	
Fast rate (nmoles Ca^{2+}/min/mg protein ± SD)	1.55 ± 0.14	1.18 ± 0.07[b] (76%)[c]		0.84 ± 0.07 (44%)[c]	0.37 ± 0.04[b]		1.2 ± 0.1 (35%)[c]	0.43 ± 0.05[b]		0.71 ± 0.07 (21%)[c]	0.15 ± 0.02[b]	
Uptake rate (nmoles Ca^{2+}/beat/mg protein)	0.013	0.01 (77%)[c]		0.007 (54%)[c]	0.0038		0.01 (48%)[c]	0.0048		0.0059 (31%)[c]	0.0018	

[a] Controls were exposed to 0% halothane.
[b] $p < 0.005$ compared to control.
[c] Percentage of control.

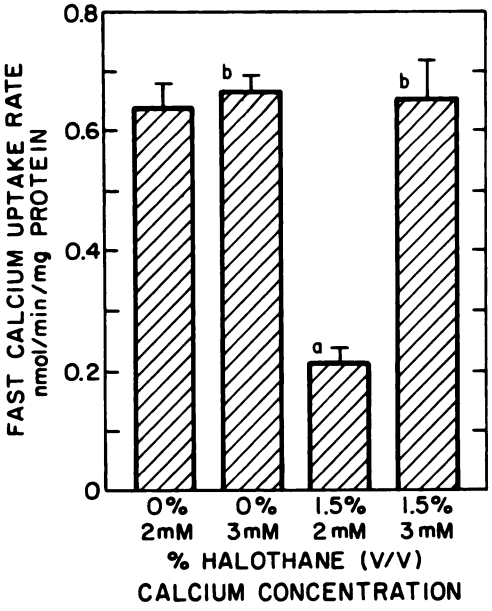

FIG. 6. *Dependence of fast calcium uptake on external calcium concentration*

The fast calcium uptake rates (± standard deviation) were measured at concentrations of 2.0 and 3.0 mM calcium and under 0 or 1.5% halothane. Uptake rates were measured using the methods described in the text for studying calcium exchange. Normal calcium concentration in growth medium is 2.0 mM. A 1.0 mM increase (to 3.0 mM) also reverses 1.5% halothane depression of beating intensity of the heart cell cultures. *a*, $p < 0.005$ compared with 0% halothane, 2 mM calcium; *b*, $p > 0.25$ compared with 0% halothane, 2 mM calcium.

to halothane. Increased calcium concentration in the growth medium of heart cell cultures restores halothane-depressed beating intensity and halothane-depressed fast calcium uptake rate to their original value but does not restore the halothane-depressed beating rate. In the absence of halothane, increased calcium concentrations in growth medium (above 2.0 mM) have no effect on beating rate, beating intensity, or fast calcium uptake rate.

Halothane is known to depress functions involving cell membranes. The best studied membrane-halothane interactions have been done on sarcoplasmic reticulum membrane. Two parameters have been studied: calcium uptake (27, 28) and the calcium-ATPase (4) activity. The effect of halothane was attributed to decreased calcium binding during membrane transport. The data from studies reported here support this theory on the halothane action.

Experiments (7, 29) have shown that modification of calcium uptake causes a subsequent change in myocardial beat intensity. Heart cells in culture exhibit a negative inotropic response to halothane treatment. A possible mechanism by which halothane depresses beat intensity is disruption of calcium exchange across the cardiac plasma membrane. In support of this mechanism we have shown that halothane does depress one phase of calcium exchange, viz. fast calcium uptake.

Fast calcium uptake is thought to involve the exchange of external calcium with the calcium associated with the plasma membrane (10, 11). On the basis of available data (10, 11, 13) the major fraction of calcium involved in contractile activation is localized on or near the plasma membrane, so a depression in the free calcium uptake rate may result in the depression of cardiac beat intensity. A comparison of the ability of halothane to depress fast calcium uptake and to depress beat intensity in beating heart cells in culture is shown in Fig. 7. A given concentration of halothane depresses both the beating intensity and the fast calcium uptake rate by the same magnitude.

Although halothane slows the fast calcium uptake rate, an increase in the external calcium concentration can restore the halothane-depressed fast calcium uptake rate. There are two possible mechanisms by which halothane can depress the calcium transport process: (a) by reducing the number of calcium transport sites or (b) lowering calcium transport efficiency across the plasma membrane. Restoration of the halothane-depressed fast calcium uptake rate by increased external calcium concentration is not explained by a halothane-induced reduction of the number of calcium binding sites. We have shown that, in the absence of halothane, at calcium concentrations above 2.0 mM, the fast calcium uptake is at its maximal rate; when halothane is present an increase in calcium concentrations should not increase the fast calcium uptake rate of the remaining calcium transport sites. The lowering of the efficiency of calcium transport does explain the present data. Halothane may affect the efficiency of calcium binding and thus depress the fast calcium uptake rate. If halothane affects the fast calcium uptake rate in a competitive manner, and data from Price (7) and Malinconico and McCarl (4) indicate that halothane does affect myocardial function in this manner, then an increase in the external calcium concentration should restore the fast calcium uptake to its maximal rate. This is what is seen in Fig. 6.

Halothane also depresses the beating rate of heart cells in culture, but the data presented here do not explain this aspect of myocardial depression. The beating rate is determined by changes in the membrane potential (8). The potential is determined by the permeability of the cell membrane not only to calcium but also to chloride, potassium, and sodium ions (30). Major changes in the

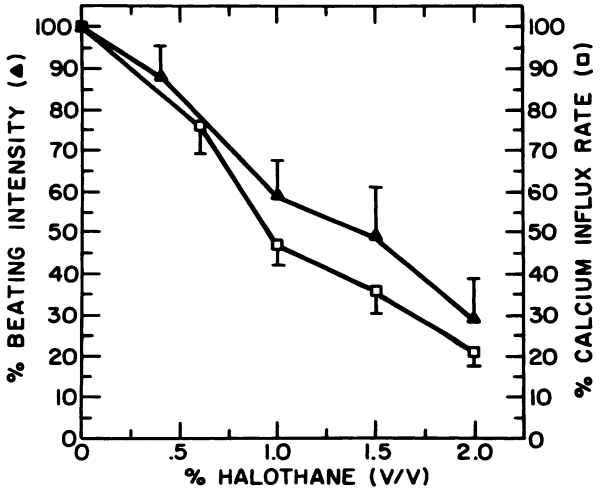

FIG. 7. *Comparison of the depressive effect of halothane on beating intensity (▲) and calcium uptake rate (□)*

Results are expressed as percentage of control (0% halothane) ± standard deviation.

calcium permeability do not take place during the initial phase of membrane depolarization. If halothane affects other permeabilities and/or transport as it does calcium uptake, this might explain the ability of halothane to affect myocardial beating rate.

In summary, the anesthetic halothane appears to affect myocardial beating intensity through its ability to disrupt the fast calcium uptake rate. Our data on how halothane affects cardiac calcium exchange support the theory that general anesthetics dissolve in membranes and disrupt their proper functioning. The effect of halothane on fast calcium uptake may in part explain the ability of halothane to depress cardiac function.

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