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Depression of Calcium Dynamics in Cardiac Myocytes–a Common Mechanism of Halogenated Hydrocarbon Anesthetics and Solvents

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Cellular Toxicology Section, National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention, 4676 Columbia Parkway, Cincinnati, OH 45226, and [1]National Research Council Research Associate, National Institute for Occupational Safety and Health, Cincinnati, OH, USA

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P. Hoffmann, K. Heinroth, D. Richards, P. Plews and M. Toraason. Depression of Calcium Dynamics in Cardiac Myocytes–a Common Mechanism of Halogenated Hydrocarbon Anesthetics and Solvents. *Journal of Molecular and Cellular Cardiology* (1994) **26**, 579–589. Individual halogenated hydrocarbons (HC) have recently been demonstrated to depress Ca^{2+} dynamics in cardiomyocytes during excitation–contraction coupling. In the present study, eight widely used HC were systematically compared for their effects on Ca^{2+} dynamics in neonatal rat cardiomyocytes by means of spectrofluorometric analysis of fura-2-Ca^{2+}-binding. Cells were exposed to dichloromethane (DCM), dichloroethane (DCE), 1,1,2-trichloroethane (112-TCE), trichloroethylene (TRI), halothane (HAL), 1,1,1-trichloroethane (111-TCE), perchloroethylene (PER), or pentachloroethane (PCE) in an environmentally controlled chamber. All HC tested decreased the height of electrically induced cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) transients in a concentration-dependent and reversible manner (IC_{50} 0.15–18.06 mM) without significant effects on diastolic $[Ca^{2+}]_i$. The increase in $[Ca^{2+}]_i$ induced by depolarization with 90 mM KCl was inhibited to a lesser degree. Investigations with thapsigargin (100 nM) and ryanodine (1 μM)–inhibitors of Ca^{2+} release from the sarcoplasmic reticulum–provided evidence that the tonic Ca^{2+} response after KCl depolarization depends mainly on sarcolemmal Ca^{2+} influx. The potency of the eight HC to inhibit Ca^{2+} dynamics in cardiomyocytes correlated with their octanol/water partition coefficients. Results support the hypothesis that alteration of Ca^{2+} dynamics in cardiomyocytes is a common mechanism of cardiotoxic HC actions.

Key Words: Cardiomyocytes; Fura-2; Ca^{2+} transients; KCl depolarization; Halogenated hydrocarbons; Sarcolemmal membrane; Sarcoplasmic reticulum.

Introduction

Aliphatic halogenated hydrocarbons (HC) have widespread use as inhalation anesthetics, vehicles in household products, and industrial solvents. The myocardial depressant and arrhythmic actions of HC have been known for over 80 years (Levy and Lewis, 1911–12). Recently, studies with HAL (Bosnjak and Kampine, 1986; Wilde *et al.*, 1991), carbon tetrachloride (Toraason and Breitenstein, 1991) and 111-TCE (Hoffmann *et al.*, 1992) suggested that the basis of myocardial depression and arrhythmia could be attributed to an impairment of Ca^{2+} flux across sarcolemmal and sarcoplasmic reticulum membranes during excitation–contraction coupling. Disrupted Ca^{2+} fluxes by these substances are manifested as depressed systolic $[Ca^{2+}]_i$ and reduced myocardial contraction (Bosnjak and Kampine, 1986; Toraason *et al.*, 1990; Hoffmann *et al.*, 1992).

HC are also potent inhibitors of gap junctional intercellular communication (GJIC) (Burt and Spray, 1989; Spray and Burt, 1990; Toraason *et al.*, 1992). Inhibition of GJIC uncouples cells from one another resulting in action potentials becoming increasingly dictated by the intrinsic membrane

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properties of individual cells (Spray and Burt, 1990), thereby sensitizing the heart to reentrant type arrhythmias (Reynolds, 1984).

The varying structures of the lipophilic HC which inhibit GJIC suggest a mechanism involving "nonspecific" lipid effects in their actions. According to the calculations of Burt and Spray (1989), there is apparently no correlation between effective aqueous inhibitory concentrations and lipophilicity for alkanols and HC. They proposed that lipid soluble substances have a direct interaction with susceptible membrane proteins. A systematic comparison of 11 HC, however, revealed a high correlation between the calculated HC concentrations inhibiting GJIC in 50% of cells examined and their lipid/aqueous partition coefficients (Toraason *et al.*, 1992). This indicates that regardless of whether there is a direct or indirect effect on membrane proteins, lipid/aqueous partitioning is a critical physicochemical property of HC associated with inhibition of GJIC.

The purpose of the present study was to determine if a comparable association occurred for HC inhibition of $[Ca^{2+}]_i$ transients during excitation–contraction coupling. We examined the effects of eight widely used HC on $[Ca^{2+}]_i$ transients in electrically stimulated cardiomyocytes from neonatal rats. We also examined the effects of HC on $[Ca^{2+}]_i$ during KCl-induced depolarization to assess the role of the sarcolemmal membrane in HC-induced alterations of Ca^{2+} dynamics. Present findings support the hypothesis that depression of Ca^{2+} dynamics in cardiomyocytes is a common mechanism of cardiotoxic HC actions (Hoffmann *et al.*, 1992). The results demonstrate a high correlation between the potency (effective aqueous concentration) of the eight HC for Ca^{2+} dynamics depression and their lipophilic properties (octanol/water partitioning). Depression of Ca^{2+} dynamics appears to be due to alterations of sarcolemmal and sarcoplasmic reticulum Ca^{2+} fluxes.

Results from this study were presented in part at the 32nd Annual Meeting of the Society of Toxicology, March 1993, New Orleans, LA, USA (Hoffmann *et al.*, 1993b).

Materials and Methods

Preparation of myocytes

Cardiac ventricular cells were isolated from 2- to 4-day-old Sprague-Dawley rats by the method previously described (Toraason *et al.*, 1990; Hoffmann *et al.*, 1992). Rat pups were obtained from a breeding colony maintained in the animal quarters of the National Institute for Occupational Safety and Health, which is accredited by the American Association for Accreditation of Laboratory Animal Care.

Measurement of $[Ca^{2+}]_i$

Measurement of $[Ca^{2+}]_i$ was performed as described recently (Hoffmann *et al.*, 1992, 1993a). In brief, myocytes were loaded with 3 μM fura-2 by a 10-min incubation at 37°C with the acetoxymethyl ester dissolved in DMSO. The low fura-2 acetoxymethyl ester concentration and short loading time were used to prevent significant intracellular compartmentalization of fura dye. A rapid loss of intracellular fura-2 (>90%) within 5 min of digitonin (10 μM) treatment indicates that there is not significant compartmentalization of the dye. However, we cannot exclude some compartmentalization in intracellular organelles. Cover slips containing myocytes loaded with fura-2 were transferred to a temperature-controlled (32°C) suffusion chamber on the stage of an inverted microscope (Nikon) that was coupled with a dual excitation spectrofluorometer (Deltascan, Photon Technology International Inc., South Brunswick, NJ, USA). Myocytes were suffused with Hanks' balanced salt solution at 2 ml/min. Cells were paced at 1 Hz by field stimulation with pulses from two platinum electrodes (S88 stimulator, Grass Instruments, Quincy, MA, USA) at 60 V for 10 ms. The Deltascan system provided UV excitation at 340 nm and 380 nm. Emission from a single cell at the two excitation wavelengths was filtered at 510 nm and collected by a photomultiplier tube. Excitation ratios were collected at a rate of 20 Hz for all experiments. $[Ca^{2+}]_i$ was calculated from the 340/380 fluorescence emission ratios of the Ca^{2+}-bound and Ca^{2+}-free forms of fura-2 according to Grynkiewicz *et al.* (1985) as previously described (Hoffmann *et al.*, 1992). Calibrations in single cells were carried out at the conclusion of each experiment. R_{min} and R_{max} values were obtained from data derived by adding a metabolic inhibitor (2 μM carbonyl cyanide *m*-chlorophenylhydrazone) and an ionophore (10 μM ionomycin) first to Ca^{2+}-free (5 mM EGTA) suffusate, then to suffusate containing 1.2 mM Ca^{2+}. R_{min} was highly reproducible between cells (0.54 ± 0.04), but R_{max} was more variable (5.93 ± 1.32, $n = 24$). These values are in agreement with those obtained by other authors for rat and guinea pig ventricular myocytes (Li *et al.*,

1987; Siri *et al.*, 1991). Potential sources of error using intracellular fluorescence probes have been described by Groden *et al.* (1991) and Martinez-Zaguilán *et al.* (1991). Control experiments with the fura-2 pentapotassium salt in a cell-free medium indicated that the HC in the highest concentrations used did not quench or enhance fura-2-fluorescence. Autofluorescence of the myocytes was negligible relative to background counts (approximately 20 000 CPS eliminated by background correction). Therefore, changes in fura-2-fluorescence transients of ventricular myocytes upon addition of the HC represent changes in $[Ca^{2+}]_i$.

Chemicals

The HC were of certified grade purchased from Fluka Chemical Corp., Ronkonkoma, NY [1,1,2-trichloroethane (112-TCE), trichloroethylene (TRI)], Fisher Scientific, Fair Lawn, NJ [dichloromethane (DCM)], Aldrich Chemical Company, Inc., Milwaukee, WI [dichloroethane (DCE), halothane (HAL), perchloroethylene (PER), 1,1,1-trichloroethane (111-TCE)], or Chem Service, Inc., West Chester, PA, USA [pentachloroethane (PCE)]. Ryanodine was provided by Agrisystems International, Wind Gap, PA. Hanks' balanced salt solution was purchased from GIBCO BRL, Grand Island, NY. New born calf serum was purchased from Hyclone Laboratories, Inc., Logan, UT, USA. Thapsigargin and all other chemicals and reagents were purchased from Sigma Chemical Company, St. Louis, MO, USA.

Exposure to test solutions

Prior to exposure to the HC, cells loaded with fura-2 were allowed to equilibrate for 30 min in the suffusion chamber. HC were dissolved in DMSO, added to Hanks' balanced salt solution and vortexed in an air-tight vial immediately prior to the start of the suffusion. The final concentration of DMSO in the suffusion medium was 0.1%. All control experiments were performed with Hanks' balanced salt solution containing 0.1% DMSO, which was without effect on $[Ca^{2+}]_i$ transients. Cumulative concentration–response data were obtained by doubling the exposure concentrations of HC at 5-min intervals starting with a non-effective concentration. The lowest concentration used was 0.00025% (v/v) for PCE.

Data analysis

Fluorescence data were acquired continuously with a PC computer and analysed using Deltascan software. For calculating basal and peak height of the electrically induced $[Ca^{2+}]_i$ transients, ten transients obtained under steady state were averaged. Least squares linear regression, Student's *t*-test or ANOVA with subsequent Scheffe test were performed on commercial statistical analysis software (Statistical Analysis Services, Statgraphics or SigmaPlot). $P<0.05$ was considered to indicate a significant difference.

Results

Effects of HC on electrically induced $[Ca^{2+}]_i$ transients

The concentration–response studies with the eight HC were conducted in a cumulative manner with an exposure period of 5 min for every concentration. Five minutes were found to be more than adequate to ensure that maximum effects on $[Ca^{2+}]_i$ transients were established. Figure 1 illustrates typical $[Ca^{2+}]_i$ transients measured in a fura-2 loaded ventricular myocyte. A sequential series of three transients elicited by electrical stimulation is shown at steady state. Under control conditions the basal and peak $[Ca^{2+}]_i$ drift of the transients over a period of 1 h was less than 10%. Addition of HAL decreased the height of electrically induced $[Ca^{2+}]_i$ transients in a concentration-dependent manner. This effect was reversible upon washout. Comparable observations on $[Ca^{2+}]_i$ transients were made after the exposure to the other seven HC (data not shown). Figure 2 illustrates summary concentration-response data for inhibition of $[Ca^{2+}]_i$ transient height of single ventricular myocytes during the exposure to the eight HC. The inhibitory effect of the eight HC on electrically induced $[Ca^{2+}]_i$ transients was reversible after a 15-min washout of the chemicals (data not shown).

IC_{50} values for reducing the height of the $[Ca^{2+}]_i$ transients were calculated from the least squares linear regression lines of the concentration-response data obtained from individual cells. The means ± S.E. of the IC_{50} values are summarized in Table 1 together with the octanol/water partition coefficients of the eight HC. Log transformed partition coefficients of the eight HC were compared with the log of the IC_{50} values using least squares regression analysis according to Martin (1983). Figure 3 reveals a high correlation between partition coefficients and IC_{50} values.

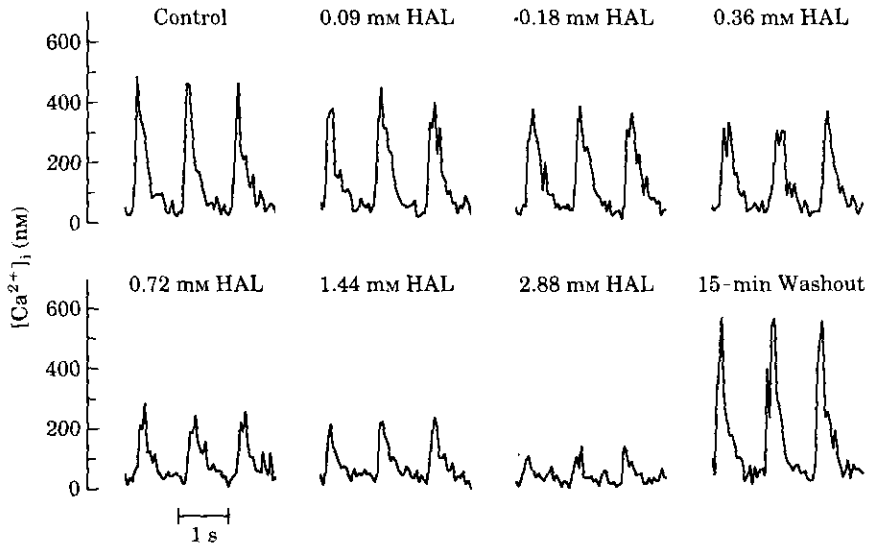

Figure 1 $[Ca^{2+}]_i$ transients in a single electrically stimulated (1 Hz) ventricular myocyte exposed to HAL. Cumulative concentration–response data were obtained by doubling the exposure concentration at 5-min intervals. HAL was washed out with Hanks' balanced salt solution. Fura-2-fluorescence measurements were used to calculate $[Ca^{2+}]_i$ as described in Materials and Methods.

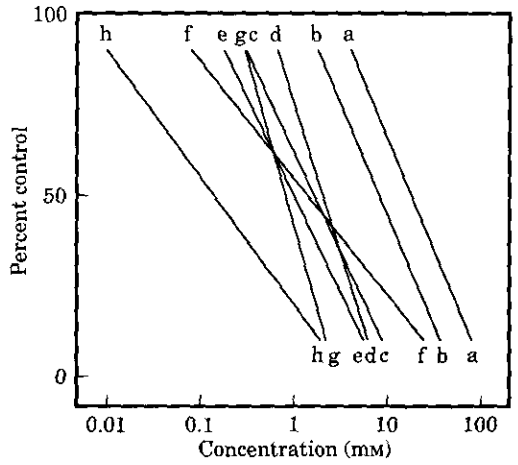

Figure 2 Concentration–response graphs for inhibition of electrically induced $[Ca^{2+}]_i$ transient peak height in single ventricular myocytes by eight HC. Least squares regression lines were calculated using the mean ($n = 4$) percent control values at the corresponding concentrations of HC. Lines shown are for a, DCM; b, DCE; c, 112-TCE; d, TRI; e, HAL; f, 111-TRI; g, PER; h, PCE.

Basal (diastolic) $[Ca^{2+}]_i$ tended to be lower after HC exposure, but varied considerably within the experimental groups. As a result, reduction of basal $[Ca^{2+}]_i$ after HC exposure did not attain statistical significance ($P > 0.05$).

Arrhythmic events occurred after exposure to the HC at concentrations > 1 mM. Regular $[Ca^{2+}]_i$ transients were interrupted by premature beats, periods of rapid beating (tachyarrhythmia), or elevated $[Ca^{2+}]_i$ with irregular oscillations (fibrillations) as judged by $[Ca^{2+}]_i$ registration and the phase contrast image observed under the microscope.

The HC did not cause overt damage to the ventricular myocytes during the 30- to 40-min exposure period. This conclusion is supported by the morphological integrity of the cells as indicated by an absence of fura-2 loss through leaky membranes and the reversibility of the effects on $[Ca^{2+}]_i$ transients after washout.

Role of the sarcoplasmic reticulum in the KCl-induced $[Ca^{2+}]_i$ response

Depolarizing quiescent ventricular myocytes with high extracellular KCl (90 mM) induced a cluster of $[Ca^{2+}]_i$ oscillations and the damped oscillations formed a plateau (Figs 4 and 5). The plateau level of cytosolic $[Ca^{2+}]_i$ after KCl depolarization can be returned to basal levels by the addition of verapamil or nitrendipine (Stewart *et al.*, 1991; Hoffmann *et al.*, 1992) indicating the involvement of voltage-sensitive Ca^{2+} channels in the manifestation of the $[Ca^{2+}]_i$ plateau. To define the significance of the sarcoplasmic reticulum in the increase in $[Ca^{2+}]_i$ after KCl depolarization more clearly, we conducted experiments with two inhibitors of Ca^{2+} release from the sarcoplasmic reticulum.

The plant-derived sesquiterpene lactone thapsigargin prevents sarcoplasmic reticulum Ca^{2+} release by inhibiting sarcoplasmic reticulum Ca^{2+}

Table 1 IC_{50} values for inhibition of the amplitude of electrically induced $[Ca^{2+}]_i$ transients and octanol/water partition coefficients

Chemical	CAS No.	Log P (oct)[1]	IC_{50} (mM)[2]
Dichloromethane CH_2Cl_2	75-09-2	1.25	18.06 ± 4.63
Dichloroethane $CH_2Cl—CH_2Cl$	107-06-2	1.48	8.21 ± 0.49
1.1.2-Trichloroethane $CHCl_2—CH_2Cl$	79-00-5	2.17	1.70 ± 0.07
Trichloroethylene $CCl_2=CHCl$	79-01-6	2.29	2.05 ± 0.23
Halothane $CHBrCl—CF_3$	151-67-7	2.30	1.01 ± 0.15
1,1,1-Trichloroethane $CCl_3—CH_3$	71-55-6	2.49	1.39 ± 0.10
Perchloroethylene $CCl_2=CCl_2$	127-18-4	2.60	0.99 ± 0.05
Pentachloroethane $CHCl_2—CCl_3$	76-01-7	3.58	0.15 ± 0.01

[1] Log of the octanol/water partition coefficients obtained from Hansch and Leo (1979) except 1,1,2-trichloroethane (Tute, 1971) and pentachloroethane (De Wolf *et al.*, 1988).

[2] IC_{50} values ± the standard error of the IC_{50} estimate were calculated from the least squares regression lines of the concentration responses illustrated in Figure 2.

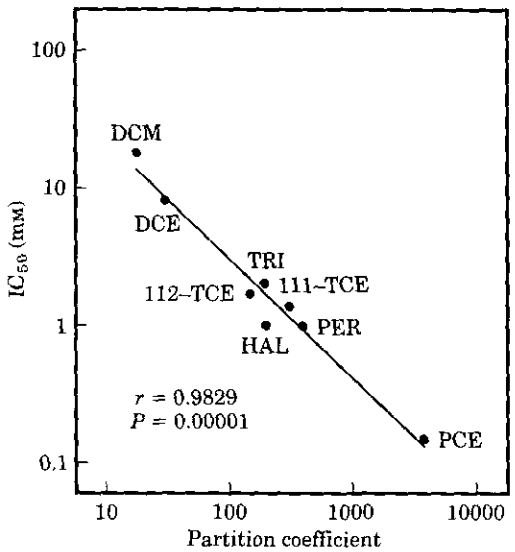

Figure 3 Correlation of the IC_{50} values, obtained from regression lines shown in Figure 2, with octanol/water partition coefficients. Source of partition coefficients are indicated in Table 1.

ATPase (Takemura *et al.*, 1989; Kirby *et al.*, 1992). Pretreatment of the cells with 100 nM thapsigargin for 10 min increased basal (diastolic) $[Ca^{2+}]_i$ of the electrically induced $[Ca^{2+}]_i$ transients and produced a marked widening [Fig. 4(a), upper part]. However, thapsigargin pretreatment did not significantly affect the level of the KCl-induced $[Ca^{2+}]_i$ plateau [Fig. 4(a), lower part]. The KCL-induced increase in $[Ca^{2+}]_i$ from resting to plateau level was 123 ± 15 nM Ca^{2+} in non-pretreated control cells compared with 150 ± 44 nM Ca^{2+} in cells pretreated for 10 min with 100 nM thapsigargin ($P > 0.05$, $n = 4$).

The plant alkaloid ryanodine decreases Ca^{2+} release from the caffeine-releasable pool of the sarcoplasmic reticulum by locking the Ca^{2+} release channel in an open state (Hilgemann *et al.*, 1989). Pretreatment of the cells with 1 μM ryanodine for 10 min resulted in an increase in basal (diastolic) $[Ca^{2+}]_i$ as well as a widening and irregular configuration of electrically induced $[Ca^{2+}]_i$ transients, but the plateau level after KCl depolarization was not influenced by ryanodine pretreatment [Fig. 4(b)]. After KCl addition, $[Ca^{2+}]_i$ was increased by 156 ± 44 nM Ca^{2+} in control cells compared with 158 ± 32 nM Ca^{2+} in cells pretreated for 10 min with 1 μM ryanodine ($P > 0.05$, $n = 5$). Taken together, these results indicate that there was little or no contribution of sarcoplasmic reticulum Ca^{2+} pools in the tonic $[Ca^{2+}]_i$ response after KCl depolarization.

Effects of HC on KCl-induced increase in $[Ca^{2+}]_i$

The initial cluster of oscillations as well as the plateau level after KCl depolarization were markedly reduced in HAL exposed cells (Fig. 5). An inhibition of the KCl response, i.e., decline of initial cluster of oscillations and reduction of plateau $[Ca^{2+}]_i$ was also observed after exposure of cells to the other HC. The potencies of the eight HC at 1 mM for reducing the KCl-induced $[Ca^{2+}]_i$ plateau are illustrated in Figure 6. DCM and DCE were not effective at this concentration.

Under control conditions, the plateau level of $[Ca^{2+}]_i$ after KCl addition remained constant over an investigation period of 30 min. Addition of solvents during this period reduced the $[Ca^{2+}]_i$ plateau. The reduction by HC of the plateau $[Ca^{2+}]_i$ level after KCl depolarization was often preceded by a small brief increase in $[Ca^{2+}]_i$ as shown for HAL in Figure 7. This effect was observed in 61% of the myocytes treated with 1 mM 112-TCE, TRI, HAL,

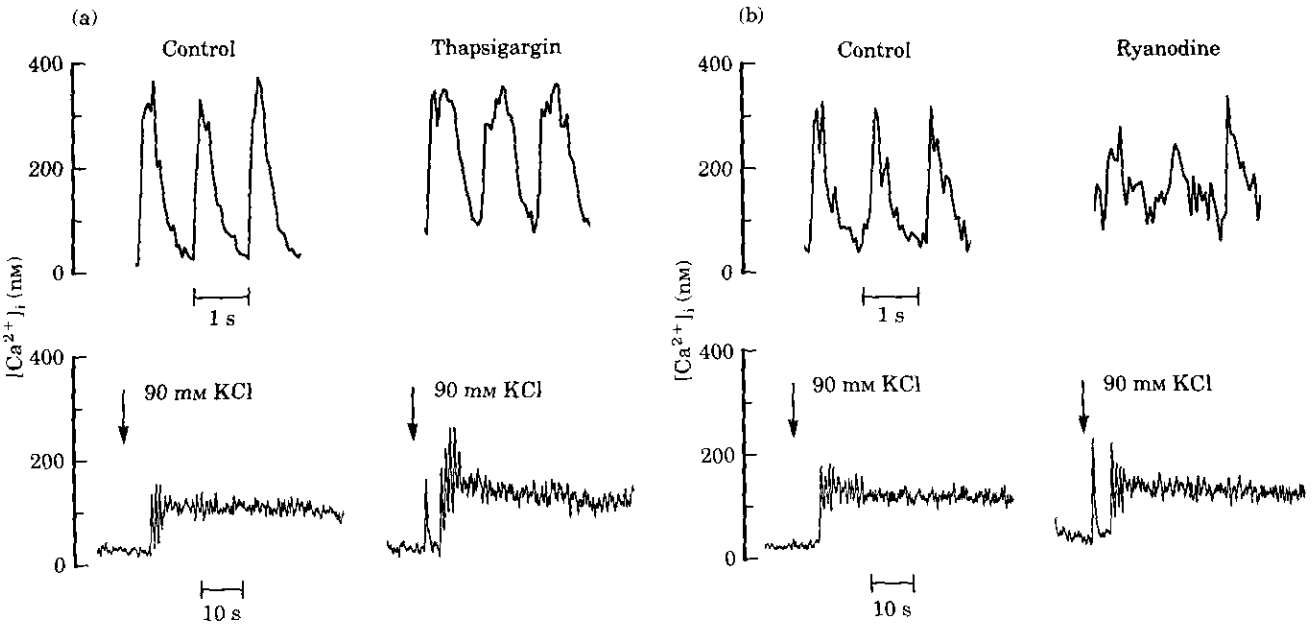

Figure 4 Effects of thapsigargin (a) and ryanodine (b) on electrically induced $[Ca^{2+}]_i$ transients (upper part) and KCl (90 mM) induced $[Ca^{2+}]_i$ increases (lower part) in ventricular myocytes. Electrically induced $[Ca^{2+}]_i$ transients and the effects of KCl addition were registered in a non-pretreated cell (left, control) and after a 10-min suffusion of the same cell with 100 nM thapsigargin or 1 μM ryanodine (right). Each tracing is representative of a series of four separate experiments.

111-TCE, PER, or PCE. DCM or DCE exposure of KCl-depolarized cells at 1 mM did not produce this brief increase in $[Ca^{2+}]_i$.

KCl depolarization experiments were also performed at the end of the concentration response studies on electrically induced $[Ca^{2+}]_i$ transients. After registration of the effects of the highest concentrations of the eight HC on electrically induced $[Ca^{2+}]_i$ transients, electrical stimulation was ceased, and cells were chemically depolarized by addition of 90 mM KCl. In Figure 8 the inhibitory effects of the HC on both amplitude of electrically induced $[Ca^{2+}]_i$ transients and plateau $[Ca^{2+}]_i$ level after KCl depolarization are compared. In the highest concentrations of the cumulative concentration-response studies, HC caused a 68–92% decrease of the amplitude of electrically induced transients, whereas the tonic $[Ca^{2+}]_i$ response to KCl depolarization was reduced only by 33–59% after exposure to the eight HC ($P < 0.05$).

Discussion

The present study describes the effects of eight widely used HC on electrically induced $[Ca^{2+}]_i$ transients in single neonatal rat ventricular myocytes. All HC rapidly reduced the amplitude of electrically induced $[Ca^{2+}]_i$ transients with IC_{50} values of 0.15–18.06 mM. Recovery of $[Ca^{2+}]_i$ occurred within 15 min of washout of the chemicals. Exposures did not produce overt signs of damage to the myocytes as judged by the reversibility of the effects and the phase contrast image observed under an inverted microscope. A loss of fura-2, which would indicate a defective sarcolemmal membrane, was also not registered. Of the eight HC tested, DCM, DCE, 112-TCE, TRI, PER, and PCE have not previously been reported to depress $[Ca^{2+}]_i$ transients in cardiac myocytes. The IC_{50} values of HAL and 111-TCE are comparable to previously described inhibitory *in vitro* concentrations of carbon tetrachloride, HAL and 111-TCE on electrically induced $[Ca^{2+}]_i$ transients in ventricular myocytes, and are also comparable to effective *in vivo* concentrations (Bosnjak and Kampine, 1986; Toraason and Breitenstein, 1991; Hoffmann *et al.*, 1992). The IC_{50} values of the eight HC for depression of Ca^{2+} dynamics also correspond to reported concentrations for GJIC inhibition in cardiomyocytes (Burt and Spray, 1989; Toraason *et al.*, 1992). There are a number of possible sites at which the HC might act to alter Ca^{2+} dynamics during excitation-contraction coupling. It has been reported that HAL and 111-TCE depress $[Ca^{2+}]_i$ transients by an inhibitory action on both the influx of extracellular

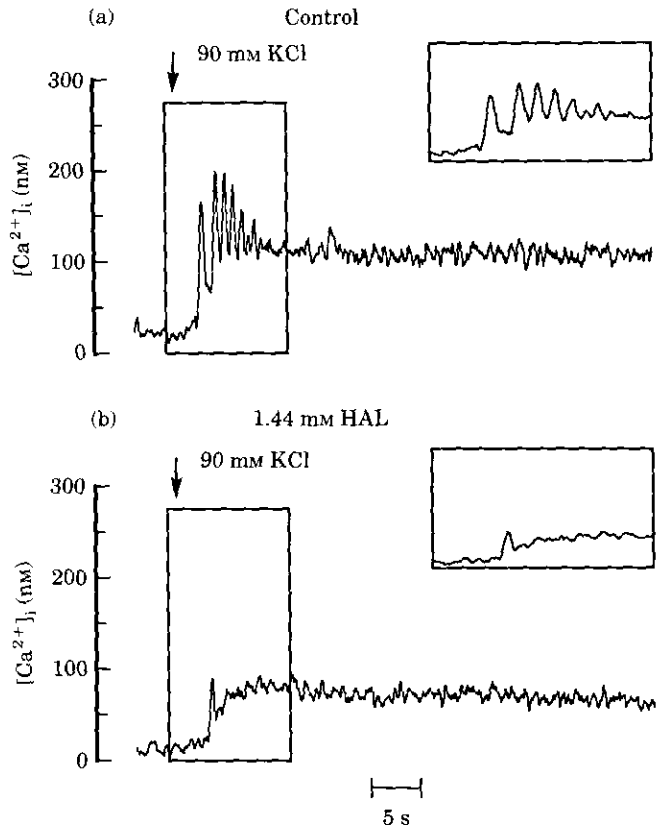

Figure 5 Effect of HAL pretreatment on KCl (90 mM) induced increases in $[Ca^{2+}]_i$ in a quiescent ventricular myocyte. Suffusion with KCl was started at the points indicated to a non-pretreated cell (a) or 5 min after treatment of the same cell with 1.44 mM halothane (b). The insets show the initial clusters of $[Ca^{2+}]_i$ oscillations at an expanded time scale. Representative tracings from four control KCl depolarizations and four KCl depolarizations after HAL pretreatment are shown.

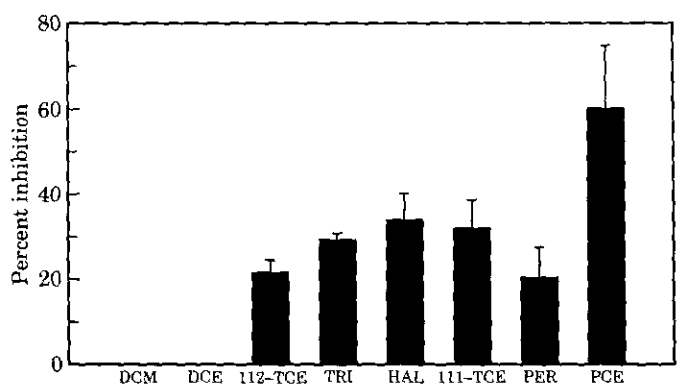

Figure 6 Inhibitory effects of the eight HC on KCl (90 mM) induced $[Ca^{2+}]_i$ plateau level. KCl-induced $[Ca^{2+}]_i$ increases observed at 1 mM of the HC are expressed as percent inhibition of the KCl induced $[Ca^{2+}]_i$ increases measured in the absence of HC for that cell preparation. Each column represents mean ± S.D. of values obtained from four separate myocyte preparations.

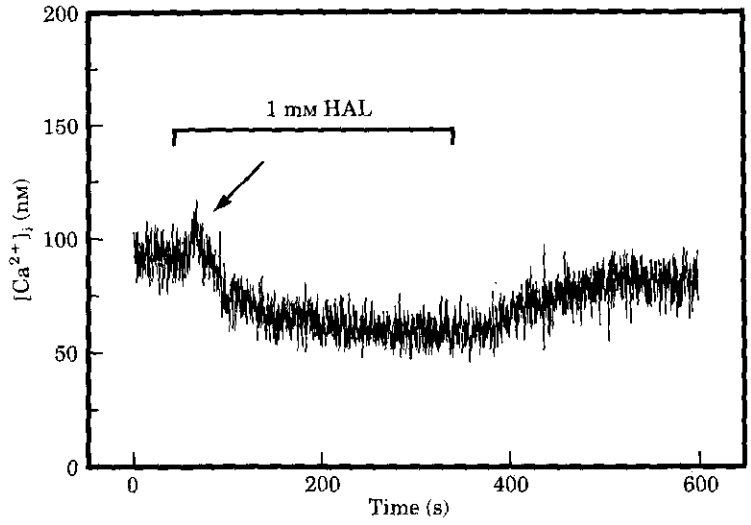

Figure 7 $[Ca^{2+}]_i$ response of a KCl (90 mM) depolarized ventricular myocyte after addition of 1 mM HAL. Suffusion with KCl (90 mM) increased $[Ca^{2+}]_i$ (not shown). In the KCl-depolarized cell, HAL at 1 mM produced a small brief increase in $[Ca^{2+}]_i$ (indicated by the arrow) followed by a reversible reduction of $[Ca^{2+}]_i$. A comparable small brief increase in $[Ca^{2+}]_i$ was observed in 61% of the KCl depolarized cells treated with the other HC except DCM and DCE.

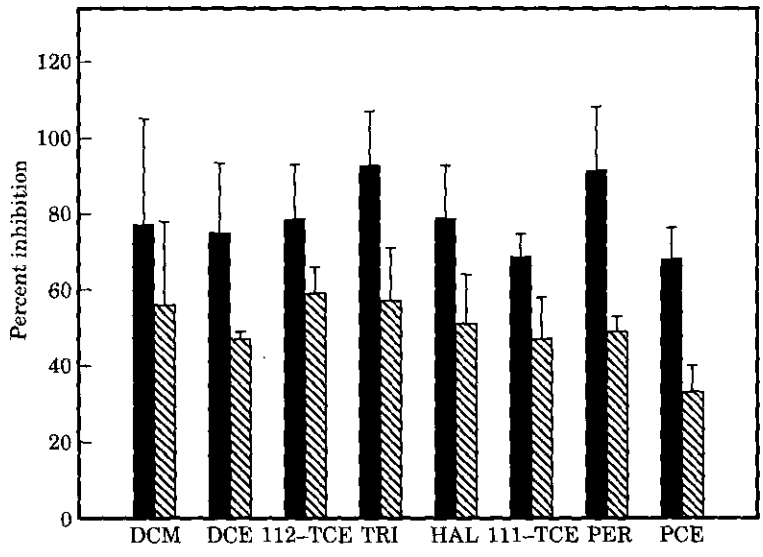

Figure 8 Comparison of the inhibitory effects of the eight HC on the height of (■) electrically induced $[Ca^{2+}]_i$ transients and (▨) KCl (90 mM) induced $[Ca^{2+}]_i$ plateau level. Average $[Ca^{2+}]_i$ transient peak height after addition of the highest concentration of the cumulative concentration-response experiments (see Fig. 2) is expressed as percent inhibition of the pre-exposure peak height. For each cell preparation, electrical stimulation was stopped 5 min after exposure to the highest HC concentration, and the magnitude of the $[Ca^{2+}]_i$ increase induced by addition of 90 mM KCl was determined. KCl-induced $[Ca^{2+}]_i$ increases observed at these HC concentrations are expressed as percent inhibition of the KCl-induced $[Ca^{2+}]_i$ increases measured in the absence of HC for that cell preparation. Each column represents mean ± S.D. of values obtained from four separate myocyte preparations. Except for DCM, the differences between corresponding electrically and KCl-induced responses are significant ($P<0.05$).

Ca^{2+} into the myocyte and the release and sequestration of Ca^{2+} by the sarcoplasmic reticulum (Bosnjak and Kampine, 1986; Wheeler *et al.*, 1988; Hoffmann *et al.*, 1992). In the experiments reported here, $[Ca^{2+}]_i$ increase after KCl depolarization was employed as a method for examining the role of the sarcolemmal membrane in HC-induced alterations of Ca^{2+} dynamics in cardiac myocytes. Previous experiments provided evidence that the plateau level of $[Ca^{2+}]_i$ after KCl depolarization can be inhibited by voltage sensitive Ca^{2+} channel blockers indicating the involvement of L type Ca^{2+} channels in this effect (Stewart *et al.*, 1991; Hoffmann *et al.*, 1992). Notwithstanding, sarcolemmal Ca^{2+} influx after depolarization with KCl might also trigger Ca^{2+} release from the sarcoplasmic reticulum. Contrary to this assumption, however, two recent reports suggest that there is little or no contribution of the sarcoplasmic reticulum Ca^{2+} pool to the tonic Ca^{2+} response after KCl depolarization (Stewart *et al.*, 1991; Wilde *et al.*, 1991). Present experiments with thapsigargin and ryanodine demonstrate that inhibition of the release of Ca^{2+} from the sarcoplasmic reticulum does not influence the tonic KCl depolarization response of the neonatal rat ventricular myocytes. Although it cannot be excluded that depolarization with KCl might provoke Ca^{2+} entry via Na^+-Ca^{2+} exchange, influence Ca^{2+} extrusion from the cell, or act on intracellular Ca^{2+} buffering systems, available evidence suggests that the tonic Ca^{2+} response after KCl depolarization depends mainly on sarcolemmal Ca^{2+} influx. All tested HC inhibited the tonic Ca^{2+} response after KCl depolarization. These findings support the conclusion that the eight HC inhibit sarcolemmal Ca^{2+} influx.

An unexpected observation was the modest inhibition of the electrically induced $[Ca^{2+}]_i$ transients in thapsigargin- or ryanodine-treated myocytes. Both substances were used at concentrations previously demonstrated to inhibit Ca^{2+} release from the sarcoplasmic reticulum in rat cardiac myocytes and thereby reduce $[Ca^{2+}]_i$ transients (Hilgemann *et al.*, 1989; Stewart *et al.*, 1991; Kirby *et al.*, 1992). Most (80–90%) of the $[Ca^{2+}]_i$ transient is due to the sarcoplasmic reticulum Ca^{2+} in the adult rat heart (Bers *et al.*, 1990). Kirby *et al.* (1992) observed that addition of 100 nM thapsigargin to adult rat cardiomyocytes decreased the amplitude of the $[Ca^{2+}]_i$ transients by 63%. The absence of such a marked effect on $[Ca^{2+}]_i$ transients in the present study can be attributed to the less developed sarcoplasmic reticulum of the neonatal heart (Chin *et al.*, 1990). Moreover, there is evidence that depletion of intracellular Ca^{2+} pools promotes Ca^{2+} influx into the cell, possibly via activation of voltage-sensitive Ca^{2+} channels (Takemura *et al.*, 1989; Malcolm and Fitzpatrick, 1992; Low *et al.*, 1993). As a result, transients in thapsigargin- or ryanodine-treated myocytes cannot be considered to reflect sarcolemmal Ca^{2+} influx under physiological conditions.

It appears that the eight HC act also on Ca^{2+} release from the sarcoplasmic reticulum. This conclusion is supported by two findings of the experiments reported here. First, the tonic Ca^{2+} response after KCl depolarization was less inhibited by the HC than the amplitude of the electrically induced $[Ca^{2+}]_i$ transients. It seems reasonable to assume that the more marked effects of the HC on electrically induced $[Ca^{2+}]_i$ transients are due to an action on the sarcoplasmic reticulum in addition to their effects on the sarcolemmal membrane. Second, a small brief increase in $[Ca^{2+}]_i$ immediately following addition of 1 mM 112-TCE, TRI, HAL, 111-TCE, PER, or PCE was observed in 61% of the KCl-depolarized myocytes. DCM or DCE were not effective at this concentration due to their much higher IC_{50} values. Previous experiments in quiescent myocytes demonstrated that the small brief increase in $[Ca^{2+}]_i$ immediately after the addition of HAL or 111-TCE is due to a Ca^{2+}-releasing effect of the HC on the sarcoplasmic reticulum (Freeman and Li, 1991; Hoffmann *et al.*, 1992; Wheeler *et al.*, 1988). Present results with KCl-depolarized myocytes are in accordance with the assumption that acute addition of 112-TCE, TRI, PER, or PCE may also cause a sarcoplasmic reticulum Ca^{2+} release. A stimulation by the HC of sarcolemmal Ca^{2+} influx as the mechanism of the small brief increase in $[Ca^{2+}]_i$ is unlikely because 90 mM KCl caused a maximal depolarization of the cell membrane. Taken together, these results are consistent with the view that the HC tested in this study act on Ca^{2+} release from the sarcoplasmic reticulum. A depletion of sarcoplasmic reticulum Ca^{2+} stores would result in a decrease of the amount of Ca^{2+} released during excitation-contraction coupling. Such a release could contribute to a depression of Ca^{2+} dynamics in HC-exposed cardiac myocytes. However, it must be realized that the extent to which changes in sarcoplasmic reticulum Ca^{2+} handling are secondary to effects of HC on sarcolemmal Ca^{2+} flux cannot be determined from the present results. This is because effects on sarcolemmal Ca^{2+} flux ultimately affect sarcoplasmic reticulum Ca^{2+} load of the cardiomyocyte.

The mechanisms underlying the alteration of Ca^{2+} fluxes through sarcolemmal and sarcoplasmic reticulum membranes by HC are uncertain. The

ability of HC to inhibit GJIC among cardiac myocytes correlates with their octanol/water partition coefficient. It was postulated that incorporation of lipophilic substances into the sarcolemmal membrane may block intercellular communication through modulation of the immediate environment of the gap junctions (Spray and Burt, 1990; Massey *et al.*, 1992; Toraason *et al.*, 1992). Present experiments demonstrate that the potency of the eight HC to depress Ca^{2+} dynamics in neonatal rat ventricular myocytes also parallels their octanol/water partition coefficients. This correlation suggests a mechanism involving "nonspecific" lipid effects. Since the classic works of Meyer (1899) and Overton (1901) on narcosis, the traditional view has been that alcohols, solvents and anesthetic gases "nonspecifically" perturb lipid membranes. However, recent evidence (Franks and Lieb, 1991) suggests that lipophilic anesthetics and solvents might act also on particularly sensitive protein targets in the membranes. Our data do not allow distinction between these possibilities.

In conclusion, we observed a reversible inhibition of electrically induced $[Ca^{2+}]_i$ transients by eight widely used HC in single neonatal rat ventricular myocytes using spectrophotometric analysis of fura-2 fluorescence. Evidence has been provided that the effect is mediated by an inhibitory action on Ca^{2+} influx into the myocytes. The HC also seem to effect release and sequestration of Ca^{2+} by the sarcoplasmic reticulum. Because depressed $[Ca^{2+}]_i$ during excitation–contraction coupling decreases the force of myocardial contraction and modifies the configuration of the action potential (duBell *et al.*, 1991), the results support our hypothesis that an alteration of intracellular Ca^{2+} dynamics could be a common mechanism underlying the well known negative inotropic and arrhythmogenic actions of HC.

References

Bers DM, Lederer WJ, Berlin JR, 1990. Intracellular Ca transients in rat cardiac myocytes: role of Na-Ca exchange in excitation-contraction coupling. *Am J Physiol* **258**: C944–C954.

Bosnjak ZJ, Kampine JP, 1986. Effects of halothane on transmembrane potentials, Ca^{2+} transients, and papillary muscle tension in the cat. *Am J Physiol* **251**: H374–H381.

Burt JM, Spray DC, 1989. Volatile anesthetics block intercellular communication between neonatal rat myocardial cells. *Circ Res* **65**: 829–837.

Chin TK, Friedman WF, Klitzner TS, 1990. Developmental changes in cardiac myocyte calcium regulation. *Circ Res* **67**: 574–579.

De Wolf W, Canton JH, Deneer JW, Wegman RCC, Hermens JLM, 1988. Quantitative structure-activity relationships and mixture-toxicity studies of alcohols and chlorohydrocarbons: Reproducibility of effects on growth and reproduction of *Daphnia magna*. *Aquat Toxicol* **12**: 39–49.

duBell WH, Boyett MR, Spurgeon HA, Talo A, Stern MD, Lakatta EG, 1991. The cytosolic calcium transient modulates the action potential of rat ventricular myocytes. *J Physiol* **436**: 347–369.

Franks NP, Lieb WR, 1991. Stereospecific effects of inhalational general anesthetic optical isomers on nerve ion channels. *Science* **254**: 427–430.

Freeman LC, Li Q, 1991. Effects of halothane on delayed afterdepolarization and calcium transients in dog ventricular myocytes exposed to isoproterenol. *Anesthesiology* **74**: 146–154.

Groden DL, Guan Z, Stokes BT, 1991. Determination of fura-2 dissociation constants following adjustment of the apparent Ca-EGTA association constant for temperature and ionic strength. *Cell Calcium* **12**: 279–287.

Grynkiewicz G, Poenie M, Tsien RY, 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* **260**: 3440–3450.

Hansch C, Leo A, 1979. *Substituent Constants for Correlation Analysis in Chemistry and Biology*. John Wiley & Sons, Inc., New York, 173–175.

Hilgemann DW, Roos KP, Brady AJ, 1989. Slowly relaxing caffeine responses in rat ventricle: relationships of ryanodine and caffeine actions. *Am J Physiol* **256**: H1100–H1109.

Hoffmann P, Breitenstein M, Toraason M, 1992. Calcium transients in isolated cardiac myocytes are altered by 1,1,1-trichloroethane. *J Mol Cell Cardiol* **24**: 619–629.

Hoffmann P, Heinroth-Hoffmann I, Toraason M, 1993a. Alterations by a thromboxane A_2 analog (U46619) of calcium dynamics in isolated rat cardiomyocytes. *J Pharmacol Exp Ther* **264**: 336–344.

Hoffmann P, Richards D, Plews P, Heinroth K, Toraason M, 1993b. Depression of calcium dynamics in cardiac myocytes by halogenated hydrocarbons (HC). (Abstract) *Toxicologist* **13**: 411.

Kirby MS, Sagara Y, Gaa S, Inesi G, Lederer WJ, Rogers TB, 1992. Thapsigargin inhibits contraction and Ca^{2+} transient in cardiac cells by specific inhibition of the sarcoplasmic reticulum Ca^{2+} pump. *J Biol Chem* **267**: 12545–12551.

Levy AG, Lewis T, 1911-12. Heart irregularities, resulting from the inhalation of low percentages of chloroform vapour, and their relationship to ventricular fibrillation. *Heart* **3**: 99–111.

Li Q, Altschuld RA, Stokes BT, 1987. Quantitation of intracellular free calcium in single adult cardiomyocytes by fura-2 fluorescence microscopy: calibration of fura-2 ratios. *Biochem Biophys Res Commun* **147**: 120–126.

Low AM, Darby PJ, Kwan C-Y, Daniel EE, 1993. Effects of thapsigargin and ryanodine on vascular contractility: cross-talk between sarcoplasmic reticulum and plasmalemma. *Eur J Pharmacol* **230**: 53–62.

Malcolm KC, Fitzpatrick, FA, 1992. Epoxyeicosatrienoic acids inhibit Ca^{2+} entry into platelets stimulated

by thapsigargin and thrombin. *J Biol Chem* **267**: 19854–19858.

MARTIN YC. 1983. Studies of relationships between structural properties and biological activity by Hansch analysis. In: Golberg L ed., *Structure–Activity Correlation as a Predictive Tool on Toxicology*. Hemisphere Publishing Corp., New York, 77–92.

MARTÍNEZ-ZAGUILÁN R, MARTÍNEZ GM, LATTANZIO F, GILLIES RJ. 1991. Simultaneous measurement of intracellular pH and Ca^{2+} using the fluorescence of SNARF-1 and fura-2. *Am J Physiol* **260**: C297–C307.

MASSEY K, MINNICH BN, BURT JM. 1992. Arachidonic acid and lipoxygenase metabolites uncouple neonatal rat cardiac myocyte pairs. *Am J Physiol* **263**: C494–C501.

MEYER H. 1899. Zur Theorie der Alkoholnarkose. Erste Mittheilung. Welche Eigenschaft der Anästhetica bedingt ihre narkotische Wirkung? *Arch Exp Pathol Pharmakol* **42**: 109–118.

OVERTON CE. 1901. Studien über die Narkose zugleich ein Beitrag zur allgemeinen Pharmakologie. G. Fischer, Jena, Germany.

REYNOLDS AK. 1984. On the mechanism of myocardial sensitization to catecholamines by hydrocarbon anesthetics. *Can J Physiol Pharmacol* **62**: 183–198.

SIRI FM, KRUEGER J, NORDIN C, MING Z, ARONSON RS. 1991. Depressed intracellular calcium transients and contraction in myocytes from hypertrophied and failing guinea pig hearts. *Am J Physiol* **261**: H514–H530.

SPRAY DC, BURT JM. 1990. Structure-activity relations of the cardiac gap junction channel. *Am J Physiol* **258**: C195–C205.

STEWART G, RUBIN E, THOMAS AP. 1991. Inhibition by cocaine of excitation-contraction coupling in isolated cardiomyocytes. *Am J Physiol* **260**: H50–H57.

TAKEMURA H, HUGHES AR, THASTRUP O, PUTNEY, JW, JR. 1989. Activation of calcium entry by the tumor promotor thapsigargin in parotid acinar cells. Evidence that an intracellular calcium pool, and not an inositol phosphate, regulates calcium fluxes at the plasma membrane. *J Biol Chem* **264**: 12266–12271.

TORAASON M, BREITENSTEIN M. 1991. Intracellular calcium transients in cardiac myocytes exposed to carbon tetrachloride (Abstract). *Toxicologist* **11**: 310.

TORAASON M, BREITENSTEIN MJ, WEY HE. 1992. Reversible inhibition of intercellular communication among cardiac myocytes by halogenated hydrocarbons. *Fundam Appl Toxicol* **18**: 59–65.

TORAASON M, KRUEGER JA, BREITENSTEIN MJ, SWEARENGIN TF, 1990. Depression of contractility in cultured cardiac myocytes from neonatal rat by carbon tetrachloride and 1,1,1-trichloroethane. *Toxicol In Vitro* **4**: 363–368.

TUTE MS, 1971. Principles and practice of Hansch analysis: a guide to structure-activity correlation for the medicinal chemist. *Adv Drug Res* **6**: 1–77.

WHEELER DM, RICE RT, HANSFORD RG, LAKATTA EG, 1988. The effect of halothane on the free intracellular calcium concentration of isolated rat heart cells. *Anesthesiology* **69**: 578–583.

WILDE DW, KNIGHT PR, SHETH N, WILLIAMS BA. 1991. Halothane alters control of intracellular Ca^{2+} mobilization in single rat ventricular myocytes. *Anesthesiology* **75**: 1075–1086.