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DEPRESSION OF CONTRACTILITY IN CULTURED CARDIAC MYOCYTES FROM NEONATAL RAT BY CARBON TETRACHLORIDE AND 1,1,1-TRICHLOROETHANE*

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Abstract—The cardiac depressant effects of carbon tetrachloride (CCl_4) and 1,1,1-trichloroethane (CH_3CCl_3) were evaluated in cultured heart cells from neonatal rats. Heart cells were grown on glass coverslips and formed a confluent monolayer that beat spontaneously, rhythmically and in synchrony. Contractility was assessed by video-motion analysis. Stock solutions of CCl_4 or CH_3CCl_3 were prepared in dimethylsulphoxide (DMSO) and aliquoted (final DMSO concentration 0.2%) into medium (M199 supplemented with 5% serum) immediately prior to perfusion across myocytes in an environmentally controlled chamber. CCl_4 and CH_3CCl_3 had a negative chronotropic effect on myocytes by prolonging the relaxation phase of beating. Duration of the contraction phase of beating, and peak velocity of cell wall movement were not affected by these halocarbons. Beating was stopped by 2.5 mM-CCl_4 or 5 mM-CH_3CCl_3, and washout of these compounds resulted in a resumption of beating activity. Increasing (3.6 mM) or decreasing (0.6 mM) the calcium concentration of the medium (normal = 1.8 mM) significantly affected the duration of contraction and relaxation phases of beating, but did not alter the concentration-dependent action of CCl_4. A positive chronotropic effect of isoproterenol was evident from 10^{-9} to 10^{-6} M, but contractility was depressed by isoproterenol concentrations greater than 10^{-8} M in the presence of 750 μM-CCl_4. This study demonstrates the usefulness of cultured heart cells for assessing the cardiac depressant and sensitizing actions of halogenated hydrocarbons.

Introduction

It has been more than 75 years since Levy and Lewis (Levy, 1913; Levy and Lewis, 1911) first reported that chloroform sensitized the heart to epinephrine-induced arrhythmias. Since that time the myocardial depressant and sensitizing effects of various chlorinated, fluorinated and brominated hydrocarbons have been thoroughly described (Katz and Epstein, 1968; Merin, 1977; Reynolds, 1984; Rinehardt *et al.*, 1971 and 1973; Steffey, 1982; Zakhari and Aviado, 1982). In addition to acting directly on the heart, halogenated hydrocarbons affect the vascular, respiratory, and central nervous systems, which can have a feedback effect on the heart and modulate or even produce myocardial effects. For the most part, information on halogenated hydrocarbons has been derived from experimental investigations with anaesthetized animals (Kobayaashi *et al.*, 1986 and 1988) and humans (Dornette and Jones, 1960), from case reports of accidental occupational exposures (McCarthy and Jones, 1983; Wright and Strobl, 1984) or from self-inflicted recreational exposures (Bass, 1970; MacDougall *et al.*, 1987). As a result, the direct effects on the myocardium have not been separated from the various reflexes arising from these other systems. Therefore, the underlying mechanisms responsible for attenuated contractility and sensitization to catecholamines have not been well elucidated.

In recent years, cultured cardiac myocytes have been used extensively to demonstrate cardiac events at the cellular level (Sperelakis, 1982). The present report, to our knowledge, is the first to demonstrate the direct effects of carbon tetrachloride (CCl_4) and 1,1,1-trichloroethane (CH_3CCl_3) on contractility of cultured cardiac myocytes. The purpose of this study was to determine the value of using isolated cells for investigating the cardiac effects of halogenated hydrocarbons.

Materials and Methods

Hearts were harvested aseptically from two- to four-day-old Sprague–Dawley rats obtained from our own breeding colony as previously described (Toraason, 1989). Briefly, excised hearts were minced into 1-mm^3 pieces, and refrigerated (4°C) overnight in Hanks' balanced salt solution (Hazelton, Logan, UT, USA) containing 0.1% crude trypsin (Sigma Chemicals Co., Ltd, St Louis, MO, USA). The following morning minced hearts were washed and then incubated at 35°C for 25 min in M199 (Hazelton, KS, USA) containing 10% foetal bovine serum (Hyclone, Lenexa, KS, USA) and 100 U penicillin-streptomycin/ml (Hazelton). Following incubation, cardiac tissue was dispersed by rapid re-pipetting to produce a single cell suspension. The cell suspension was

*Mention of company names does not constitute endorsement by the National Institute for Occupational Safety and Health.

Abbreviations: CCl_4 = carbon tetrachloride; CH_3CCl_3 = 1,1,1-trichloroethane; DMSO = dimethylsulphoxide.

decanted from the flask leaving undigested tissue fragments behind. Following a cell count, the suspension was diluted to 10^6 cells per ml. Viability was consistently about 90% as determined by trypan blue exclusion. Heart cells were plated at a density of 2×10^6 cells per 35-mm well in 6-well plastic trays (Falcon) containing 19-mm round coverglasses.

Video-motion analysis was used to measure contractility of isolated cardiac myocytes. The round coverglass to which cardiac myocytes were attached, was placed in a Dvorak–Stotler controlled-environment culture chamber (Nicholson Percision Instrument, Inc., Gaithersburg, MD, USA) that was then placed on an inverted microscope stage (Nikon). The microscope stage was encased in a plexiglass chamber and maintained at 37°C with a thermocoupled controlled heater (Nikon). A single spontaneously contracting cell was brought into focus, and the image captured with a video camera (Dage) and transferred to a video monitor. Movement of the cell wall was measured using an image processor (model 604) and motion analyser (model 633) with sync-stripper (model 302-2), manufactured by Colorado Video (Boulder, CO, USA). Pixel-to-pixel movement of the image along a single raster line on the monitor was measured, and the analogue voltage fluctuation was fed through a DC driver amplifier (model 7DA, Grass Instruments, Quincy, MA, USA) to a personal computer (IBM-AT) containing a DASA 4600 data acquisition/signal analysis system (Gould Electronics, Cleveland, OH, USA).

Test solutions were aerated with 5% CO_2 in air and pumped through the flow-through chamber at 0.5 ml/min. Perfusion media was M199 containing 5% foetal bovine serum. Cells were perfused for 30 min in culture medium prior to initiation of experiments to allow cells to equilibrate with the perfusion chamber. Calcium and halocarbon concentrations were adjusted as indicated in the Figures. Concentration–response data were obtained by increasing the concentration of the test compounds at 10-min intervals.

Statistical comparisons were made using analysis of variance and Duncan's Multiple-Range test to identify specific treatments that were significantly different ($P < 0.05$).

Results

Contractility of cardiac myocytes was evaluated by taking the first derivative of the motion of the cell wall during contraction. This was expressed in volts per sec, and was used as an index of cell wall velocity rather than distance per sec since motion of the cardiac myocyte was transferred and saved as voltage fluctuations. No attempt was made to convert the signal to distance, since all comparisons were relative. Comparisons of experimental variables were made on the basis of peak velocity attained during contraction, frequency of contraction (beats/min), and duration of contraction and relaxation (sec). Changes in contraction amplitude appeared to be independent of experimental variables, and dependent on several variables in the measuring technique. The motion analysis is performed in a single plane along a single raster line on the video display, yet the contraction is evident in two planes on the video monitor, and occurs in three dimensions on the culture dish. Therefore, the actual extent of movement of the myocyte cell wall is not always accurately reflected by the signal output used for analysis. However, the direction and velocity of motion from pixel to pixel can be accurately determined.

The spontaneous beating of cardiac myocytes in rhythmic and synchronous fashion was dependent on the presence of serum in the culture media. As little as 2% serum induced beating, but beating was slow and somewhat variable culture to culture. Supplementing the media with 5% serum resulted in a consistent beating. Increasing the serum concentration above 5% did not improve the performance of the cells and this concentration was used for contractility work.

Cultured cardiac myocytes used in the present study responded in a positive chronotropic fashion to increases in calcium concentration. Figure 1 shows velocity fluctuations during several contractions and the effect of increasing and then reducing the calcium concentration. Beating ceases in calcium-free medium, but cells contract in a synchronous and rhythmic fashion when 0.6 mM-calcium is added. Frequency of contraction increases when calcium is elevated to 1.8 and 3.6 mM. Increasing calcium to 5.4 mM did not increase the beating rate above the rate obtained with 3.6 mM-calcium (data not shown).

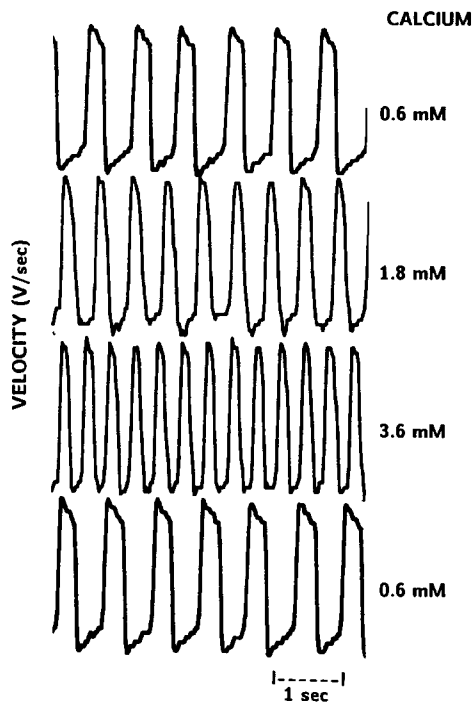

Fig. 1. Positive chronotropic response of a single cardiac myocyte to increased extracellular calcium concentration. The cell was monitored in an environmentally controlled flow-through chamber and calcium concentration of the medium was adjusted as indicated. The wave is the first derivative of the voltage fluctuation obtained as a result of movement of a spontaneously beating cardiac myocyte. Movement of the cell wall along a single video raster line was used as an index of cell wall velocity (V/sec).

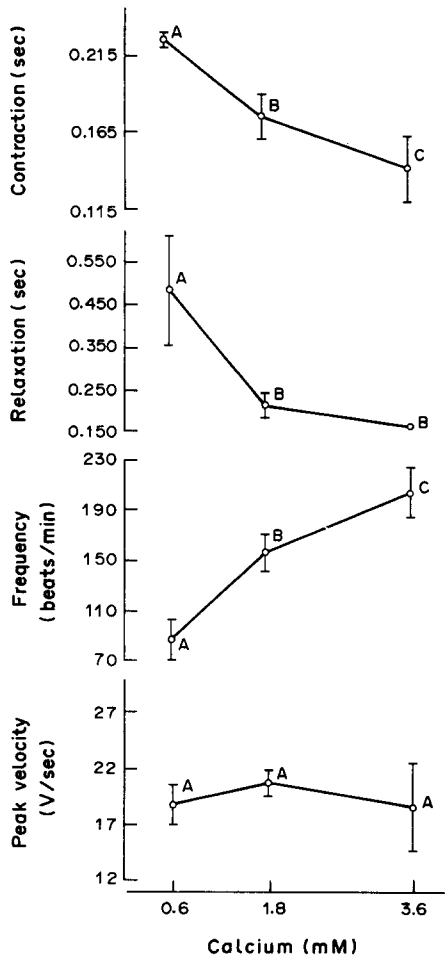

Fig. 2. Summary of the positive chronotropic effects of elevated calcium concentration on cardiac myocytes. Symbols represent means ± SD of four experiments at each calcium concentration. In some cases, error bars were smaller than the symbol designating the mean. Means noted with a common letter for each variable are not significantly different ($P < 0.05$).

Figure 2 summarizes contractility changes induced by calcium in 12 independent experiments such that each cell was exposed to only one concentration of calcium. A positive chronotropic effect indicated by increased frequency of beating stemming from shortened contraction and relaxation duration, was induced by increasing the calcium concentration from 0.6 mM to 3.6 mM. Peak velocity of contraction was not significantly changed by alterations in calcium concentration.

Figure 3 illustrates the negative chronotropic effect of CCl_4 on a single cardiac myocyte. It is apparent from Fig. 3 that reduced beating frequency is due to an increased duration of the relaxation or lengthening phase of cell beating. There appeared to be little or no change in the duration of contraction or in peak velocity. Figure 4 illustrates the reversibility of the effects of CCl_4 on beating activity of a single cardiac myocyte. Spontaneous beating was repeatedly stopped and started by alternating between control medium and medium containing 2.5 mM-CCl_4.

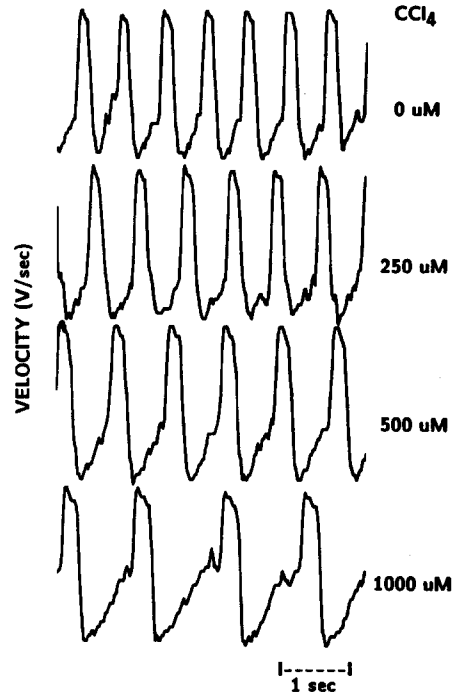

Fig. 3. Negative chronotropic effect of CCl_4 on a single cardiac myocyte. The signal was obtained as noted in Fig. 1.

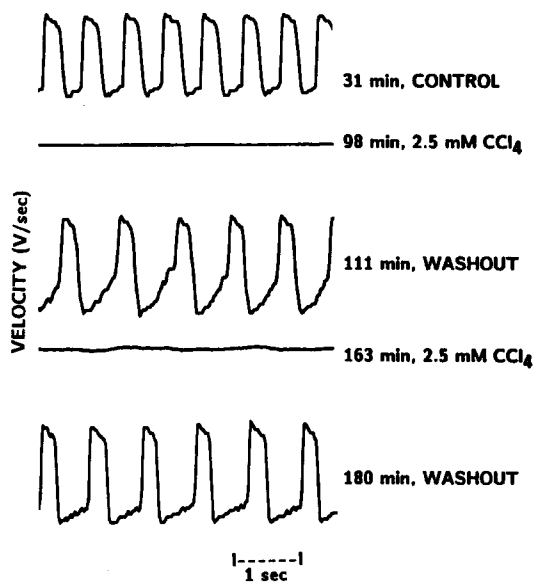

Fig. 4. Reversible effect of 2.5 mM-CCl_4 on beating activity of a spontaneously contracting myocyte. Signal shown was obtained as described for Fig. 1 and was taken from a single myocyte. A is the signal obtained after a 30-min perfusion with control medium. B was recorded at the end of a concentration–response experiment when beating was halted by perfusion with 2.5 mM-CCl_4. C shows the recovery of beating after a 13-min washout of 2.5 mM-CCl_4 with control medium. The exposure and washout were repeated two more times and beating was again arrested with 2.5 mM-CCl_4 (D). E shows the final recovery of beating in the same myocyte after 3 hr of repeated perfusions with CCl_4.

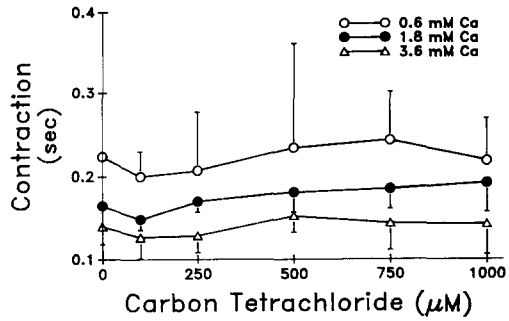

Fig. 5. Lack of a CCl_4 effect on duration of contraction of spontaneously beating cardiac myocytes. Symbols represent means ± SD of four experiments at each calcium concentration. In some cases, error bars were smaller than the symbol designating the mean, or were omitted for clarity.

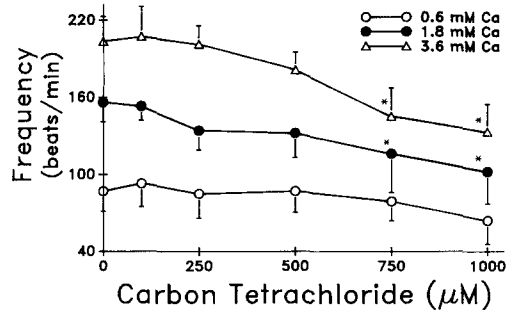

Fig. 7. CCl_4 concentration-dependent decrease in frequency of spontaneously beating cardiac myocytes. Symbols represent means ± SD of four experiments at each calcium concentration. In some cases, error bars were omitted for clarity. Asterisks denote means that are significantly less than the control mean ($P < 0.05$).

Figure 5 shows that there was no significant effect of CCl_4 on duration of contraction. Duration of relaxation was significantly increased by 750 μM and 1 mM-CCl_4 at 3.6 mM-calcium, and by 1 mM-CCl_4 at 0.6 and 1.8 mM-calcium (Fig. 6). Beating frequency was significantly reduced by 750 μM and 1 mM-CCl_4 at 1.8 mM and 3.6 mM-calcium, but not at 0.6 mM-calcium (Fig. 7). Peak velocity of contraction was not affected by exposure to CCl_4 (Fig. 8). Figure 9 illustrates the negative chronotropic effect of CH_3CCl_3 and CCl_4 on beating myocytes in culture in 1.8 mM-calcium. The concentration–response curves of these two halocarbons are comparable except that 2.5 mM-CCl_4 arrested beating, whereas 5 mM-CH_3CCl_3 was required to stop myocytes from beating. The effects of CH_3CCl_3 on duration of contraction and relaxation, and on peak velocity were comparable with those of CCl_4 (data not shown). As with CCl_4, washout of CH_3CCl_3 resulted in a resumption of spontaneous, rhythmic and synchronous beating of cardiac myocytes.

Isoproterenol had a positive chronotropic effect on cardiac myocytes between the concentrations of 10^{-8} and 10^{-6} M (Fig. 10). Increasing the isoproterenol concentration to 10^{-5} M caused a reduction in beating relative to that attained at lower isoproterenol concentrations. In the presence of 750 μM-CCl_4, only 10^{-8} M-isoproterenol stimulated beating, and increasing the isoproterenol concentration further depressed beating.

Discussion

Depression of contractility, enhanced sensitization to catecholamine-induced arrhythmias and rapid recovery have been consistently reported following exposure to halogenated hydrocarbons (Zakhari and Aviado, 1982). To some extent, all three were demonstrated here in cultured cardiac myocytes exposed to CCl_4 or CH_3CCl_3. Each of these halocarbons had a concentration-dependent inhibitory effect on heart cell contractility. Concentrations of CCl_4 or CH_3CCl_3 that could completely arrest beating were attained, and washout of the compounds resulted in resumption of normal beating activity even after repeated and prolonged exposure. Since only the contractility of a single cell could be monitored, it was not possible to adequately evaluate the effect of the halocarbons on synchronous beating. Arrhythmic beating was occasionally noticed in the cell being monitored, but no attempt was made to record the frequency of occurrence because it was sporadic and infrequent. Nonetheless, increased sensitivity to catecholamine-stimulated contractility was demonstrated. Heart cells responded in a positive chronotropic fashion to

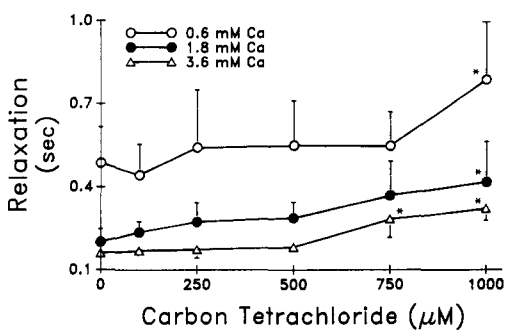

Fig. 6. CCl_4 concentration-dependent increase in the duration of relaxation of spontaneously beating myocytes. Symbols represent means ± SD of four experiments at each calcium concentration.

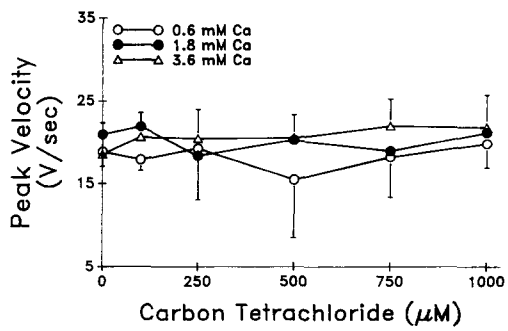

Fig. 8. Lack of a CCl_4 effect on peak velocity attained by the cell wall during contraction. Symbols represent means ± SD of four experiments at each calcium concentration. In some cases, error bars were smaller than the symbol designating the mean, or were omitted for clarity.

isoproterenol. However, increasing the concentration to 10^{-5} M decreased beating activity compared with that observed at 10^{-6} M. In the presence of CCl_4, cells responded positively to isoproterenol, but only at 10^{-8} M. Greater concentrations of isoproterenol depressed beating activity. In this respect, the concentration-dependent response curve of myocytes to isoproterenol was shifted to the left indicating increased sensitivity of myocytes to isoproterenol in the presence of CCl_4. To what extent this *in vitro* effect is analogous to increased sensitization to catecholamine-induced arrhythmias observed *in vivo* is not apparent.

Positive inotropic and chronotropic effects of increased extracellular calcium on cultured heart cells have been previously reported (Barry *et al.*, 1975; Marsh *et al.*, 1982). Marsh *et al.* (1982), in an evaluation of several studies, noted that considerable variation in contraction amplitude and frequency has been reported in cultured cardiac myocytes treated with known positive inotropic and chronotropic agents. In the present study, calcium and isoproterenol exhibited only a positive chronotropic effect.

Undoubtedly, the limitation of the measuring technique (see Results) was a major factor that prevented accurate evaluation of the effects of calcium and isoproterenol on amplitude of contraction. The chronotropic action of calcium was due to a significant decrease in both the duration of contraction and the duration of relaxation of cardiac myocytes, which suggests enhanced release and uptake of calcium by the sarcoplasmic reticulum (Shue and Blaustein, 1986). The decreased duration of contraction occurs as a result of an increased inward current of calcium arising from the elevated extracellular calcium. The shortened relaxation phase is apparently a consequence of the decreased contraction phase (Katz, 1977).

CCl_4 concentration-dependent response curves for contraction, relaxation and frequency of beating were essentially parallel at all three calcium concentrations. However, the responses were less marked at lower calcium concentrations as indicated by the lack of statistically significant effects for relaxation and beating frequency at the higher CCl_4 concentrations.

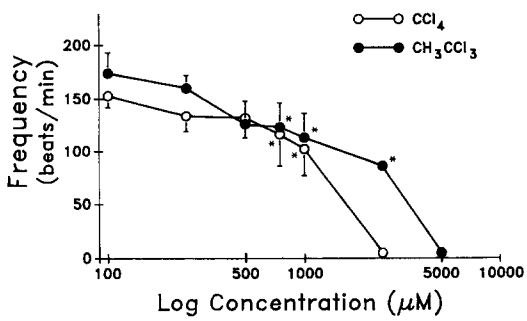

Fig. 9. Comparison of the negative chronotropic effect of CCl_4 and CH_3CCl_3 on spontaneously contracting myocytes. Symbols represent means ± SD of four experiments. In some cases, error bars were omitted for clarity. Asterisks denote means that are significantly less than the control mean ($P < 0.05$). Beating was arrested by 2.5 mM-CCl_4 or 5 mM-CH_3CCl_3.

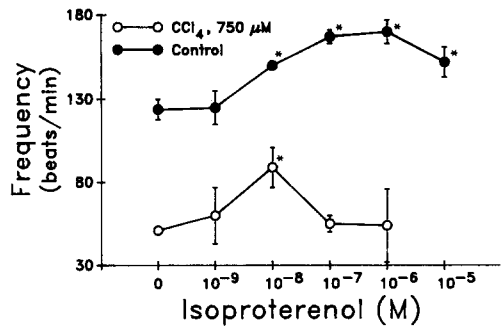

Fig. 10. Positive chronotropic effect of isoproterenol in the presence and absence of 750 μM-CCl_4. Symbols represent means ± SD of four experiments. Asterisks indicate means are significantly greater than control means for each curve ($P < 0.05$).

A critical observation was that CCl_4 only affected beating frequency by prolonging the interval between contractions, and did not alter duration of contraction even when it was markedly altered by changes in extracellular calcium. This suggests that CCl_4 does not effect the contractile response that is dependent on rapid release and uptake of calcium. However, CCl_4 and CH_3CCl_3 could inhibit the slow inward current of calcium that initiates excitation–contraction (Shue and Blaustein, 1986). The negative chronotropic effect of these halocarbons appears to be more analogous to a decrease in pacing (Katz, 1977), which has been observed in anaesthetized animals exposed to CH_3CCl_3 (Kobayashi *et al.*, 1988). However, CH_3CCl_3 has also been reported to reduce the peak rate of ventricular pressure increase (dp/dt) in anaesthetized dogs (Herd *et al.*, 1974; Kobayashi *et al.*, 1988). In the present study, peak velocity of cell wall movement is analogous to dp/dt, yet this variable was not affected by CCl_4 or CH_3CCl_3. However, maximum dp/dt is also dependent on vascular resistance (Katz, 1977), and aortic blood pressure declines during CH_3CCl_3 exposure (Kobayashi *et al.*, 1986). Therefore, the drop in dp/dt observed in anaesthetized dogs could be due to decreased aortic resistance. It has also been reported that calcium injection ameliorates the cardiac depressant effects of CH_3CCl_3 in anaesthetized dogs (Herd *et al.*, 1974). Examination of Fig. 7 reveals a comparable effect in cultured cardiac myocytes exposed to CCl_4. Although CCl_4 decreased frequency of contraction at all calcium concentrations, the concentration-dependent elevation in beating frequency due to extracellular calcium was maintained even at 1 mM-CCl_4.

An observation made repeatedly throughout this study and illustrated in Fig. 4 was that after CCl_4 and CH_3CCl_3 were removed myocytes completely recovered. Clark and Tinston (1973 and 1982) attributed this and other effects of halogenated hydrocarbons to their physicochemical properties. They found that the effective cardiac sensitizing effect of 14 halogenated compounds was highly correlated with each compound's vapour pressure. Aviado (1981) came to the same conclusion regarding the cardiac toxicity of 18 fluorocarbons. In both reports, it was noted that the lower the vapour pressure the lower the dose required to affect the heart. Therefore, the

halogenated hydrocarbons have a non-specific effect that is rapidly reversible and is related to the potential of the compound to saturate the lipid phase of cardiac tissue. The presence of the halogenated hydrocarbon in the membrane may affect membrane fluidity, which in turn could affect ion movement and receptor mediated responses. Such effects would then be manifested as depressed contractility or enhanced sensitization to catecholamine challenge.

The present study demonstrates the cardiac depressant and sensitizing effects of halogenated hydrocarbons on cultured cardiac myocytes. The observed effects are comparable with those previously reported in animals and humans and open the door to investigative research into the cellular and molecular action of these chemicals on contractility. Furthermore, the perfusion system employed provides a convenient method for obtaining concentration–response data, and could serve as a screening system for ranking halogenated hydrocarbons on the basis of their effects on myocardial cells.

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