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Calcium dynamics in cardiac myocytes as a target of dichloromethane cardiotoxicity

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Abstract The purpose of the present study was to determine if cardiac actions of dichloromethane (DCM) in vivo correlate with in vitro alterations of Ca^{2+} dynamics in cardiac myocytes. Neonatal rat ventricular myocytes were obtained from 2- to 4-day-old rats, and electrically induced fluctuations of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in single cardiomyocytes were investigated using spectrofluorometric analysis of fura-2-$[Ca^{2+}]_i$ binding. In cultured myocytes, cumulative exposure to 0.64–40.96 mM DCM resulted in a concentration-dependent and reversible decrease in the magnitude of $[Ca^{2+}]_i$ transients with IC_{10} and IC_{50} values of 7.98 and 18.82 mM, respectively. Total inhibition of $[Ca^{2+}]_i$ transients and cessation of beating were observed at 40.96 mM DCM. Suffusion with DCM for 40 min did not cause morphological alterations of the myocytes. In a urethane-anesthetized rat model, left ventricular pressure was measured by introducing a tip catheter via the carotid artery into the left ventricle, the ECG was recorded by two needle electrodes applied subcutaneously to the chest wall, and arterial pressure was measured via the femoral artery. Oral administration of 3.1–12.4 mmol DCM/kg resulted in DCM blood concentrations between 1.0 and 1.6 mM, accompanied by a dose-dependent decrease in contractile force and heart rate without influencing blood pressure and ECG tracings. Moreover, DCM treatment provided significant protection against arrhythmia development due to $CaCl_2$-infusion. In spite of the slight discrepancy between DCM blood concentrations and in vitro concentrations of DCM for $[Ca^{2+}]_i$ transient inhibition, present data are consistent with the view that cardiac effects after DCM exposure are mediated by alterations of Ca^{2+} dynamics during excitation-contraction coupling.

Key words Dichloromethane · Cardiotoxicity · $[Ca^{2+}]_i$ transients · Myocardial contraction · Cardiac arrhythmia

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Introduction

Dichloromethane (DCM) is widely used as an industrial solvent and as vehicle in household products. It possesses low hepatic, renal, neural and genetic toxicity potential. Despite being relatively nontoxic, DCM shares cardiac adverse effects, such as myocardial depression and cardiac arrhythmia after high-level exposure, with other halogenated hydrocarbons (HC) (WHO 1984; Zakhari 1992). In vitro studies revealed that depression of Ca^{2+} dynamics in cardiac myocytes is a common action of HC including DCM (Bosnjak and Kampine 1986; Hoffmann et al. 1994). Moreover, it has been shown, in vitro, that depression of Ca^{2+} dynamics by 1,1,1-trichloroethane, halothane, and carbon tetrachloride correlates with depression of contractility. These findings support the hypothesis that depression of intracellular Ca^{2+} dynamics is responsible for the negative inotropic and arrhythmogenic actions of HC in vivo (Bosnjak and Kampine 1986; Toraason and Breitenstein 1991; Hoffmann et al. 1992).

The purpose of the present study was to substantiate this hypothesis by examining cardiac actions of DCM in vitro and in vivo. Therefore, effects of DCM on fluctuations of cytosolic free Ca^{2+} concentration

($[Ca^{2+}]_i$) in electrically stimulated cardiac myocytes and effects of DCM on cardiac function in anesthetized rats were compared.

Preliminary results from this study have been reported at the 8th International Workshop on in Vitro Toxicology (Hoffmann et al. 1995).

Materials and methods

Chemicals

DCM was of certified grade purchased from Fisher Scientific, Fair Lawn, N.J., USA. Hanks' balanced salt solution was purchased from Gibco, Grand Island, N.Y., USA. New-born calf serum was purchased from Hyclone, Logan, Utah, USA. Urethane was obtained from Philopharm Quedlinburg GmbH, Germany, and $CaCl_2$ was purchased from Aldrich, Germany. All other chemicals and reagents were purchased from Sigma Chemical Company, St Louis, Mo., USA.

Measurement of cytosolic free Ca^{2+} concentration in isolated cardiac myocytes

Isolation of cardiac ventricular cells from 2- to 4-day-old rats and measurement of $[Ca^{2+}]_i$ were performed as described recently (Toraason et al. 1990; Hoffmann et al. 1992, 1993). In brief, cultured cardiac myocytes were loaded with fura-2 by a 10-min incubation at 37°C with 3 µM of the acetoxymethyl ester. 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) which was coupled with a dual-excitation spectrofluorometer (Deltascan, Photon Technology International, South Brunswick, N.J.). Myocytes were suffused with Hanks' balanced salt solution at 2 ml/min. Cells were paced at 1 Hz by field stimulation with 10-ms pulses at 60 V from two platinum electrodes connected to a Grass S88 stimulator (Grass Instruments, Quincy, Mass.). 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. $[Ca^{2+}]_i$ was calculated from the 340/380 fluorescence emission ratios of the Ca^{2+}-bound and Ca^{2+}-free forms of fura-2 according to the method of Grynkiewicz et al. (1985), as described earlier (Hoffmann et al. 1992). Calibrations in single cells were carried out at the conclusion of each experiment by adding a metabolic inhibitor (2 µM carbonyl cyanide *m*-chlorophenylhydrazone) in conjunction with an ionophore (10 µM ionomycin) first to the perfusate containing very low $[Ca^{2+}]$ (5 mM EGTA) and then to the perfusate containing 1.2 mM Ca^{2+}. It must be noted, however, that $[Ca^{2+}]_i$ values obtained using this method are only estimates. Potential sources of error using intracellular fluorescence probes have been described by Groden et al. (1991) and Martínez-Zaguilán et al. (1991).

Control experiments with the fura-2 pentapotassium salt in a cell-free medium indicated that DCM in the highest concentration 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 DCM represent changes in $[Ca^{2+}]_i$.

Prior to exposure to DCM, cells loaded with fura-2 were allowed to equilibrate for 30 min in the suffusion chamber. DCM was 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. This concentration was without effect on $[Ca^{2+}]_i$ transients. Cumulative concentration-response data were obtained by doubling the exposure concentrations of DCM at 5-min intervals. A 5-min exposure period was more than adequate to ensure that maximum effects of DCM on $[Ca^{2+}]_i$ transients were established.

Measurement of cardiovascular parameters in urethane-anesthetized rats

Cardiovascular parameters were recorded 15 min after DCM administration in urethane-anesthetized (0.9 g/kg i.p.) male Wistar rats according to a protocol originally proposed by Zbinden (1981) and described recently in detail (Hoffmann and Müller 1990). Blood pressure was measured via a catheter introduced into the femoral artery. The ECG (lead II) was recorded by two needle electrodes applied subcutaneously at both sites of the chest wall. Left ventricular pressure of the heart was measured by introducing a Millar tip catheter (SPR 249/3F, Hugo Sachs Elektronik, Germany) via the carotid artery into the left ventricle. Pressure tracings and ECG were recorded on a polygraph (Watanabe Linear Recorder WR 3310, Hugo Sachs Elektronik, Germany). The following contractility parameters were calculated using commercial software (PO-NE-MAH software HD5, Hugo Sachs Elektronik, Germany): Maximum velocity of left ventricular pressure rise (dp/dt_{max}, mm Hg/s), time from the onset of pressure rise to the point of dp/dt_{max} (t-dp/dt_{max}, ms) and Crayenbühl index (dp/dt_{max} divided by instantaneous pressure, 1/s).

$CaCl_2$ arrhythmia

Fifteen minutes after DCM administration, $CaCl_2$ (50 mg/kg per min at a concentration of 205.9 mg/ml $CaCl_2$ in 0.9% NaCl) was infused into the jugular vein of urethane-anesthetized rats at a rate of 85 µl/min, until death (Marmo 1971). The ECG (lead II) was recorded by two needle electrodes applied subcutaneously at both sides of the chest wall. Rhythm disturbances were analyzed according to the Lambeth Conventions (Walker et al. 1988).

DCM administration and determination of blood concentration

DCM was administered by gavage at doses of 3.1 mmol/kg, 6.2 mmol/kg, or 12.4 mmol/kg (1 : 10 in (Oleum pedum tauri) to three groups of urethane-anesthetized rats (300–350 g). The control animals received 4.2 ml/kg of the vehicle. This volume was equal to that received by the high DCM dose treatment group. DCM blood concentrations were determined by gas chromatography, as described recently (Müller et al. 1991). Blood samples (retroorbital plexus) were taken 15 min and 45 min post-dosing and extracted with 1,3-dimethylbenzene containing trichloromethane as an internal standard.

Data analysis

Results are presented as mean ± SEM. Fluorescence data were analyzed using Deltascan software. Least squares linear regression was performed on commercial statistical analysis software (Statistical Analysis Services, Statgraphics and Abstat). IC_{10} and IC_{50} values were calculated from the regression analysis of the concentration-response curve using a non-linear/logarithmic model. Statistical analysis was performed using multiple *t*-test or the χ^2-method. In the statistical analyses, $p < 0.05$ was accepted to indicate a significant difference. The investigation conforms with the *Guide for the care and use of laboratory animals* published by the US National Institutes of Health (NIH publication No 85-23, revised 1985).

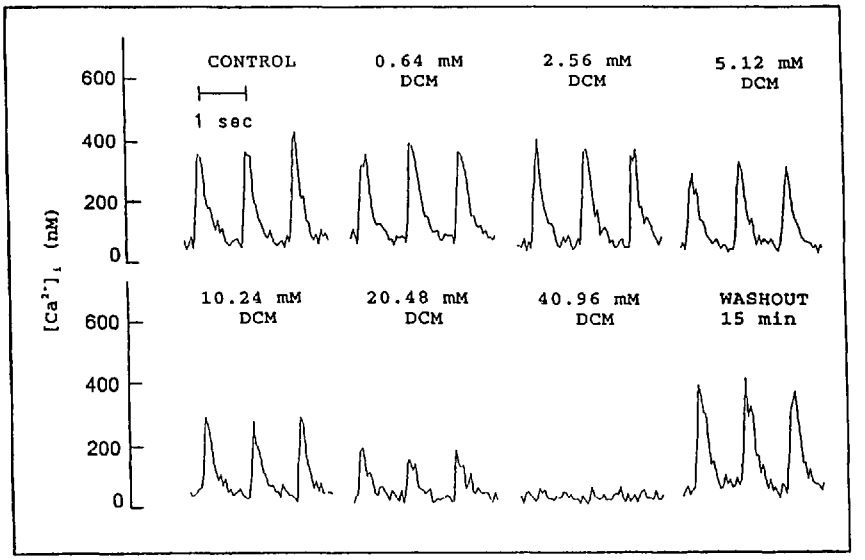

Fig. 1 $[Ca^{2+}]_i$ transients in a single electrically stimulated (1 Hz) ventricular myocyte exposed to DCM. Cumulative concentration-response data were obtained by doubling the exposure concentration at 5-min intervals. DCM was washed out with Hanks' balanced salt solution. Fura-2 fluorescence measurements were used to calculate $[Ca^{2+}]_i$ as described in Materials and methods. A representative original tracing from four experiments using separate ventricular myocyte preparations is shown

Results

Effects of DCM on Ca^{2+} dynamics in isolated cardiomyocytes

Figure 1 illustrates typical $[Ca^{2+}]_i$ transients in a single ventricular myocyte. A sequential series of three electrically induced transients under steady state is shown under control conditions and after cumulative exposure to 0.64–40.96 mM DCM. Addition of DCM to the suffusion medium decreased the height of $[Ca^{2+}]_i$ transients in a concentration-dependent manner. Total inhibition of $[Ca^{2+}]_i$ transients was observed after exposure to 40.96 mM DCM in two out of four experiments. Contractility studies were not performed in the present in vitro experiments. However, it was observed under the inverted microscope that total inhibition of $[Ca^{2+}]_i$ transients was accompanied by a cessation of beating. The inhibitory actions of DCM on $[Ca^{2+}]_i$ transients and mechanical properties were reversible after a 15-min washout period.

Figure 2 illustrates summary concentration-response data for inhibition of $[Ca^{2+}]_i$ transient peak height of single ventricular myocytes during the cumulative exposure to DCM. The IC_{10} and IC_{50} values for reducing peak systolic $[Ca^{2+}]_i$ transient height were 7.98 ± 3.34 and 18.82 ± 5.02 mM, respectively.

Basal (diastolic) $[Ca^{2+}]_i$ tended to be slightly decreased by DCM exposure. However, effects varied considerably between the individual cardiac myocyte preparations. As a result, decrease of basal $[Ca^{2+}]_i$ by DCM was not statistically significant ($p > 0.05$).

Arrhythmic events were observed occasionally at DCM concentrations ≥ 1.28 mM. Regular electrically induced $[Ca^{2+}]_i$ transients were interrupted by premature transients, periods of rapid $[Ca^{2+}]_i$ transient rate (tachyarrhythmia) or elevated $[Ca^{2+}]_i$ with irregular oscillations (fibrillation). However, incidence and severity of arrhythmias were not correlated with the DCM concentrations in the suffusion medium.

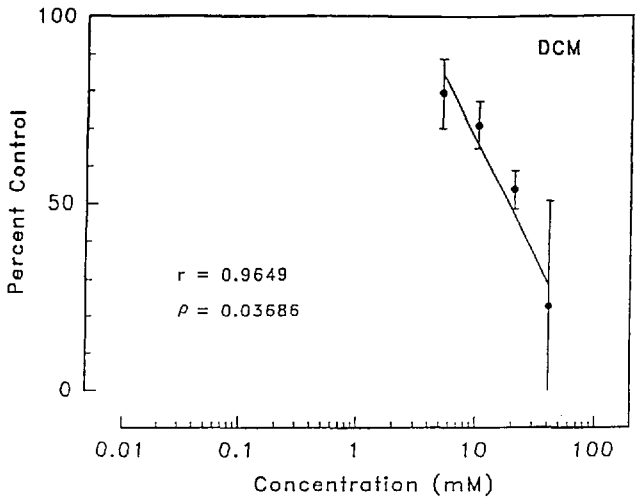

Fig. 2 Concentration-response curve for inhibition of electrically induced $[Ca^{2+}]_i$ transient peak height in single ventricular myocytes by DCM. Least squares regression line was calculated using the mean ($n = 4$) percent of the control values at DCM concentrations ≥ 5.12 mM

Addition of 0.64–40.96 mM DCM for 30–40 min did not cause morphological alterations of the cardiac myocytes as judged by the observation under the inverted microscope. Furthermore, normal transients resumed within 15 min of washout of DCM, and a loss of fura-2, which would indicate a defective sarcolemmal membrane, was also not registered.

DCM blood concentrations after oral administration

Fifteen minutes after treatment, DCM concentrations in blood obtained from the retroorbital plexus were 0.98 ± 0.11 mM, 1.54 ± 0.08 mM, and 1.6 ± 0.1 mM in animals treated with 3.1 mmol/kg, 6.2 mmol/kg and 12.4 mmol/kg, respectively ($n = 6$). Thirty minutes later, DCM blood concentrations in the three respective dose groups were significantly reduced to 0.51 ± 0.09 mM, 0.83 ± 0.05 mM, and 1.3 ± 0.15 mM ($p < 0.05$, $n = 6$). DCM was not detected in blood obtained from animals of the control group.

Effects of DCM on cardiac function in anesthetized rats

Administration of DCM to anesthetized rats induced a negative inotropic and a negative chronotropic effect (Fig. 3). Compared with the control group, left ventricular dp/dt_{max} was significantly reduced in all DCM-treated animals. Crayenbühl index and heart rate were decreased and t-dp/dt_{max} was prolonged in the medium- and the high-DCM dose groups. Systolic blood pressure as well as amplitudes and intervals of the ECG were not significantly influenced by DCM administration (data not shown). No arrhythmic events were observed in either DCM-treated or control rats.

$CaCl_2$ arrhythmia

Table 1 describes the effects of DCM treatment on the development of arrhythmias during $CaCl_2$ infusion. Times till the beginning of arrhythmias (mostly second/third degree atrioventricular blocks) were prolonged in a dose-dependent manner. Appearances of ventricular fibrillation and death (defined as isoelectric recordings for 1 min) were significantly delayed in the high-dose DCM group.

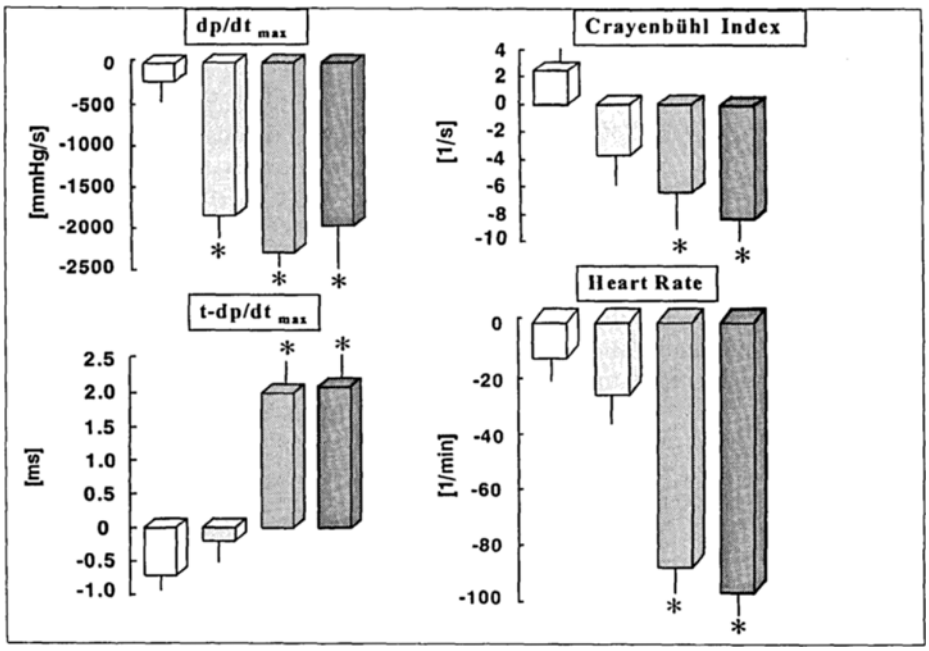

Fig. 3 Changes of left ventricular contraction and heart rate in urethane-anesthetized rats 15 min after oral DCM administration ($n = 9$–12). *Bars* shown represent change in absolute values from values obtained from individual animals prior to DCM administration. Initial values of contractility parameters and heart rate prior to DCM administration were not significantly different between the individual groups. Group means (± SEM) of initial values varied between 5719 ± 440 and 6120 ± 710 mmHg/s for dp/dt_{max}, 63.9 ± 3.4 and 70.0 ± 4.3 s^{-1} for Crayenbühl index, 18.6 ± 1.1 and 20.0 ± 0.8 ms for t-dp/dt_{max}, and between 392 ± 18 and 406 ± 21 min^{-1} for heart rate. □ Control, ▨ 3.1 mmol/kg, ▩ 6.2 mmol/kg, ■ 12.4 mmol/kg. dp/dt_{max} Maximum velocity of pressure rise, t − dp/dt_{max} Time from the onset of the pressure rise to the point of dp/dt_{max}, *$p < 0.05$ compared with the control group

Table 1 Effect of oral DCM administration on arrhythmia development during infusion of $CaCl_2$ (50 mg/kg per min) in urethane-anesthetized rats ($n = 10$). Values are times (s) until appearance of the individual parameters after start of $CaCl_2$ infusion *ARRHYT* arrhythmia (any kind, mostly atrioventricular blocks), *AVB* 2nd and 3rd degree atrioventricular blocks, *ES* premature ventricular extrasystoles, *VT* ventricular tachycardia (≥ 4 consecutive ectopic beats), *FIB* ventricular fibrillation

DCM (mmol/kg)	ARRHYT	AVB	ES	VT	FIB	DEATH
0	66 ± 16	66 ± 16	288 ± 79	370 ± 10	585 ± 45	605 ± 24
3.1	110 ± 24	110 ± 24	351 ± 60	540 ± 73	641 ± 38	630 ± 39
6.2	130 ± 37*	138 ± 30*	345 ± 63	468 ± 61	597 ± 55	653 ± 32
12.4	143 ± 41*	148 ± 39*	363 ± 84	450 ± 30	757 ± 63*	747 ± 33*

*$p < 0.05$ compared with the control group

Discussion

Present in vivo findings confirm myocardial depression after oral DCM administration, as determined by different parameters of contractility, which evaluate time-dependent and quantitative properties of contraction (Adler et al. 1980). The negative inotropic effects were recorded at venous blood concentrations between 0.98 and 1.6 mM DCM. This concentration range is similar or even lower than DCM concentrations measured in venous blood of humans after high level inhalation exposure (Winek et al. 1981; Leikin et al. 1990; Manno et al. 1992). It must be taken into account that at any given DCM concentration in the alveolar space, the heart is probably exposed to substantially higher concentrations during the first passage of the blood through the heart than represented by measurements of DCM concentrations in mixed venous blood.

Present in vitro results confirm inhibitory actions of DCM on Ca^{2+} dynamics in isolated cardiac myocytes. The magnitude of electrically induced $[Ca^{2+}]_i$ transients was inhibited in a concentration-dependent and reversible manner. Compared with IC_{50} values for other HC, the value for DCM is relatively high, a condition which can be attributed to its lower lipophilicity (Hoffmann et al. 1994). Depression of $[Ca^{2+}]_i$ cycling in cardiac myocytes by DCM and other HC is caused mainly by an inhibitory action on Ca^{2+} influx into cardiac myocytes (Bosnjak and Kampine 1986; Wheeler et al. 1988; Wilde et al. 1991; Hoffmann et al. 1992). HC also seem to affect release and sequestration of Ca^{2+} by the sarcoplasmic reticulum.

In the present experiments, DCM blood concentrations were quite comparable with effective in vitro concentrations. The maximum concentrations of DCM measured in the blood were approximately one-fifth of the IC_{10} and 1/12 of the IC_{50} for $[Ca^{2+}]_i$ transient inhibition. This discrepancy between DCM blood concentrations for myocardial depression and in vitro concentrations for $[Ca^{2+}]_i$ transient inhibition may be explained by a number of differences between the in vitro and in vivo models utilized. The values obtained from in vitro experiments with cardiac myocytes are to a certain extent dependent upon both the techniques used to isolate and culture cells, and the specific conditions used during the examination of cell function. Myocytes were maintained in culture for 3–4 days, a condition which might have caused dedifferentiation to some embryonic state with lower sensitivity. To optimize the method of $[Ca^{2+}]_i$ measurement, in vitro experiments were performed at 32°C, and this slightly lower temperature relative to the in vivo situation may have contributed to the lower potency of DCM in vitro (Franks and Lieb 1993). Developmental changes in the regulation of $[Ca^{2+}]_i$ fluctuations during excitation-contraction coupling have been described (Chin et al. 1990) and these changes may have contributed to variation in the DCM response in adult rat versus neonatal rat cardiomyocytes. This hypothesis is supported by a recent report which described developmental changes of HC effects in rabbit hearts (Palmisano et al. 1994). We also cannot completely exclude that slight amounts of DCM might have evaporated from the suffusion medium or been absorbed by the polyethylene tubing system. On the other hand, it is not clear whether the DCM concentration in coronary artery blood was exactly the same as measured in the blood obtained from the retroorbital plexus. Moreover, blood/myocyte partitioning of DCM in vivo and buffer/myocyte partitioning in vitro are not identical (Gargas et al. 1989). However, the partition coefficients indicate DCM would move more readily from buffer to myocyte than from blood to muscle. Another potential factor of variation is related to DCM metabolism. Formation of active DCM metabolites in vitro is not likely because cardiac myocytes possess a very limited metabolic capacity. It has also been demonstrated that the potencies of individual HC for the depression of $[Ca^{2+}]_i$ dynamics in vitro were determined by the lipophilic properties of the HC, independent of their metabolic pattern after systemic administration (Hoffmann et al. 1994). In vivo, DCM is metabolized via an oxidative microsomal and a glutathione-dependent cytosolic pathway (Gargas et al. 1986) and might exert cardiotoxic actions also through its metabolites, as demonstrated for trichloroethylene (White and Carlson 1979). Although extensive metabolism in present experiments 15–25 min after DCM administration is unlikely, it cannot be excluded completely as a possible source of variation. A further aspect is a possible interaction between DCM and the general anesthetic. HC anesthetics have been demonstrated to depress $[Ca^{2+}]_i$ dynamics in cardiac myocytes (Bosnjak and Kampine 1986; Katsuoka et al. 1989). In present experiments, low-dose urethane anesthesia was employed. There is some indirect evidence that urethane may interfere with Ca^{2+} fluxes in myocytes (Maggi et al. 1984). Thus, it is not possible to exclude that urethane modifies DCM actions on Ca^{2+} dynamics in the myocardium. Finally, it has to be taken into account that cardiac function in vivo is modified by interrelated neural, paracrine, and humoral regulators, which are influenced by DCM exposure (WHO 1984). Therefore, in addition to direct actions of DCM on cardiac myocytes, cardiac function in vivo may also be influenced indirectly via primary effects of DCM on these regulatory parameters.

Previously reported in vitro and in vivo experiments revealed that increased extracellular Ca^{2+} concentrations counteracted HC-induced depression of $[Ca^{2+}]_i$ transients and contractility (Herd et al. 1974; Bosnjak and Kampine 1986; Hoffmann et al. 1992; Pagel et al. 1993). As an explanation, it has been postulated that elevation of extracellular Ca^{2+} concentration antagonizes HC-induced inhibition of Ca^{2+} influx into cardiac myocytes and the resulting increase in systolic $[Ca^{2+}]_i$

restores contractility. Present data which demonstrate that $CaCl_2$-induced arrhythmia development is delayed in DCM-treated rats are also in accordance with the assumption of an inhibitory action of DCM on Ca^{2+} influx into cardiac myocytes. Elevation of extracellular Ca^{2+} concentration stimulates Ca^{2+} influx into cardiac myocytes, resulting in an increase in $[Ca^{2+}]_i$ eventually followed by rhythm disturbances (Thandroyen et al. 1991). Inhibition of Ca^{2+} influx by DCM attenuates the increase in $[Ca^{2+}]_i$ after $CaCl_2$-infusion and thereby causes a delay of the $CaCl_2$-induced arrhythmia development. Taken together, present observations are consistent with the view that both the negative inotropic effects of DCM and the protection from $CaCl_2$-induced arrhythmias are mediated by DCM inhibition of $[Ca^{2+}]_i$ dynamics in the myocardium during excitation-contraction coupling.

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