Ethanol effects on cardiomyocyte contractility

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ABSTRACT

Little is known about the direct cardiac effects of socially common sub-intoxication levels of ethanol. Previous studies evaluating the responses of normal cardiomyocytes to short-term ethanol exposure have utilized ethanol concentrations equivalent to extreme intoxication or lethal levels in vivo. The purpose of the present study was to investigate the contractile responses of isolated rat ventricular cardiomyocytes during exposure to relatively low concentrations of ethanol in the range 0.05–0.5% (v/v) (8.6–86 mM) under physiological conditions (3 Hz stimulation; 36 °C; BSA vehicle). High-speed imaging techniques were used to study the kinetics of myocyte contraction, and shortening parameters were calculated for mechanistic evaluation. The concentration–response relationship was not linear and exhibited two plateau phases, suggesting at least two mechanisms of action of ethanol on cardiomyocyte contraction. At 0.05% (8.6 mM), ethanol treatment produced a 14.4% decrease in maximum myocyte shortening. The maximum rates of cell shortening and lengthening were similarly impaired, but there was no effect on contraction cycle timing at this low concentration. At 0.30% (51 mM), ethanol reduced maximum shortening by 40.2%, prolonged excitation–contraction coupling latency and abbreviated the contraction cycle time by 38%. The inotropic modulatory effect of ethanol was exaggerated in the absence of protein in the superfusion buffer. This is the first report which identifies ethanol at 0.05% (v/v) as a modulator of cardiac contractility. Kinetic analyses indicate that the mechanism of action involves disturbance of sarcoplasmic reticulum function, and this may contribute to arrhythmogenic vulnerability – especially in an in vivo context of heightened compensatory sympathetic drive.

INTRODUCTION

The acute in vivo cardiovascular response to moderate levels of ethanol intake involves sympathetic activation, probably due to peripheral vasodilation, and usually results in an increase in heart rate and maintained or elevated cardiac output [1]. In experimental situations where sympathetic or autonomic blockade is applied, it has been demonstrated both in vivo and in vitro that ethanol reduces myocardial contractility independent of neural influence [2–5]. Studies on isolated cardiomyocytes from a number of species have confirmed that acute ethanol treatment has a direct negative inotropic effect which cannot be attributed to the release of secondary mediators by other cell types [6–9]. The cellular mechanisms underlying the effects of ethanol on excitation–contraction coupling are only partially understood.

It is well established that chronic exposure of the myocardium to ethanol produces basal contractile...
dysfunction and electrophysiological abnormalities [10,11]. Alterations in myocardial contractile function associated with long-term alcohol exposure primarily reflect changes in the myocyte sarcolemmal phospholipid structure, rather than a specific effect of ethanol on the excitation–contraction coupling processes. Alcoholism is associated with increased arrhythmogenic vulnerability [12], and alcoholic cardiomyopathy results in blunted responsiveness of the myocardium to acute ethanol exposure [13].

Little is known about the direct cardiac effects of socially common sub-intoxication levels of ethanol. Studies evaluating the responses of normal cardiomyocytes to short-term ethanol exposure have used ethanol at concentrations equivalent to extreme intoxication or lethal levels in vivo [14]. Systematic in vitro evaluation of the acute effects of ethanol on the inotropic responses of cardiomyocytes under relatively physiological conditions has not been undertaken previously. Further study is needed to determine how levels of ethanol frequently encountered socially impact on basic cardiac myocyte cellular processes which may be implicated in arrhythmogenesis in non-alcoholics [15].

The purpose of the present study was to carry out a detailed investigation of the contractile responses of isolated rat ventricular cardiomyocytes during exposure to relatively low concentrations of ethanol in the range 0.05–0.5 % (v/v) (8.6–86 mM). High-speed imaging techniques were used to study the kinetics of myocyte contraction cycles, and contractile parameters were calculated for mechanistic evaluation. The time course of the response to ethanol and the sensitivity of myocytes to repeated exposure was assessed. The high temporal resolution of the present study allows a detailed kinetic analysis of the effects of ethanol on cardiomyocyte contractility that has not been possible previously, and provides new mechanistic insight.

METHODS

Myocyte preparation and measurement of cell contraction
Isolated ventricular myocytes were prepared from the hearts of adult Wistar Kyoto rats (body weight 325 ± 4 g (mean ± S.E.M.), n = 29). Experiments were conducted in compliance with the principles outlined in the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and with the approval of the University of Melbourne Animal Experimentation Ethics Sub-Committee. Hearts were excised under ether anaesthesia, and enzymic dissociation of cells was commenced by retrograde aortic perfusion with Krebs-buffered solution containing collagenase (Type 2; Worthington, Freehold, NJ, U.S.A.). After dissociation, myocytes were filtered, washed and resuspended in Hepes-buffered medium containing trypsin inhibitor.

Cells were allowed to settle and adhere lightly to the glass base of the experimental chamber, and were then bathed with pre-oxygenated, buffered solution containing (mM): 118 NaCl, 4.8 KCl, 1.2 MgSO_4, 1.2 KH_2PO_4, 25.0 Na-Hepes, 11.0 glucose and 1.0 CaCl_2. Using a temperature- and flow-controlled superfusion system described previously [16], the chamber temperature was maintained at 36.0 ± 0.5 °C and the flow at 2.0 ± 0.01 ml/min. Cells were stimulated to contract at 3 Hz by platinum field electrodes delivering 1 ms pulses at 30% suprathreshold. Switching (exchange time 1.5 s) between control and test superfusates was achieved using a hydraulically operated valve located at the chamber inflow port. Except where indicated, all cell recordings (including time controls) were carried out in the presence of 0.125 % (w/v) BSA vehicle.

Measurements of cell length during contraction were carried out using a line-scan camera and digital imaging technique similar to that described previously [17], but upgraded to operate on a Unix platform (Sun OS V4.4.4) with an Ultrad AD1205SS 5bus data acquisition board. Briefly, a 1 x 512 element photodiode array was positioned along the longitudinal axis of the cell by means of a rectangular mask located in the optical path of the microscope phototube. Cell length was scanned at intervals of 1.088 ms throughout the contraction cycle, with scanning synchronized to commence with stimulus delivery. The camera output of successive scans was digitized and displayed as an image. Calibration at 1 pixel = 0.36 μm was determined by scanning a stage graticule. Cell boundary positions were determined for each scan. For each contraction recorded this information was analysed to derive values for a series of parameters as detailed below, and as described previously [17].

A number of contraction parameters were defined. Maximum shortening (%S) was expressed as a percentage of initial resting cell length (L_o), and the time of first occurrence of %S was designated t_w. The detection of departure from and return to L_o (times designated t_d and t_p respectively) was set at a length change of 0.005 x L_o. Maximum rates of shortening (MRS) and lengthening (MRL) were determined by step differentiation (4.35 ms differential interval) and were normalized with respect to cell length. All time parameters were referred to the commencement of scanning, which was synchronized with the delivery of the stimulus to the cell.

Recording protocols and data analysis
Cell performance was allowed to achieve steady state after the start of stimulation (3 min allowed), and was then monitored during the test periods of ethanol exposure or vehicle-only application. Contraction parameters were averaged over 30 s periods immediately…
before and during the test periods at 1.0 min intervals. The average parameter values for each interval of the test period were normalized with respect to the initial average values determined before the test period for each cell. Mean normalized (average) parameter values were determined for each experimental group and are expressed as percentage change from the initial level (designated 100%). This procedure allowed each cell to serve as its own control by eliminating differences in initial contractility, and also minimized variation in the data arising from short-term fluctuations in contractility.

The effects of five concentrations of ethanol were investigated. The molar concentrations (mM) equivalent to the clinically utilized ‘% (v/v)’ values tested were 0.05% (8.6), 0.10% (17.1), 0.20% (34.3), 0.30% (51.4) and 0.50% (85.6). Myocytes were exposed to a single ethanol concentration, and where recordings from multiple cells from the same preparation were made, these cells were assigned to different treatment groups. In an additional series of experiments the effects of washout of ethanol and repeated treatment with selected concentrations was evaluated.

All data are presented as means (±S.E.M.). Analysis of variance was used to detect significant effects of treatment. Fischer’s LSD Test was applied post hoc to identify significant differences (P < 0.05) between treatment groups.

BSA (Fraction V, essentially fatty acid free) was obtained from Sigma-Aldrich Pty Ltd., Australia. Absolute ethanol (Analytic Reagent; triple distillation) was obtained from BDH Laboratories (cat no. 10107).

**RESULTS**

**Time course and concentration dependency of responses to ethanol**

The initial levels of contractile performance (%)S for cardiomyocytes in each treatment group were not different, and the mean value was similar (7.46 ± 0.17%; n = 72 cells) to that observed in previous studies under the same conditions [18]. The negative inotropic effects of all concentrations of ethanol tested were evident within the first 1 min of treatment and were observed to stabilize by 6 min (confirmed by a reduced number of extended period recordings; not shown). The mean time course of the development of the negative inotropic effect was dependent on the ethanol concentration, as illustrated in Figure 1. At the highest concentration tested (0.5%; 86 mM), ethanol reduced myocyte contraction by about 50% after 6 min, during which period myocytes treated with vehicle only demonstrated stable performance. At the lowest concentration tested, 0.05% (8.6 mM), ethanol induced about a time-averaged 10% decrease in contraction, which stabilized after 4 min.

![Figure 1](image1.png) **Figure 1** Mean time course of the effect of ethanol (0.05–0.50%, v/v) on %S during a 6 min test period The vehicle control group contained 0.125% (w/v) BSA. The value of %S for each cell is expressed as a proportion of the initial level of performance (100%) at the start of the test period (0 min).

![Figure 2](image2.png) **Figure 2** Concentration-dependence of the ethanol-induced decrease in %S %S was recorded after a 6 min test period in the presence of a 0.125% BSA vehicle (Veh; n = 16 myocytes), 0.05% ethanol (n = 15), 0.10% ethanol (n = 8), 0.20% ethanol (n = 9), 0.30% ethanol (n = 10) and 0.50% ethanol (n = 13).

The relationship between ethanol concentration and the normalized shortening responses of cardiomyocytes measured at the end of the 6 min test period is summarized in Figure 2. The concentration–response relationship is not linear and appears to exhibit two plateau phases. Exposure to low concentrations of ethanol (0.05–0.10%; 8.6–17.1 mM) elicited a modest negative inotropic response of the order of a 10% decline in contraction. A transition in the concentration range between 0.20 and 0.30% (34–51 mM) produced a stable 35–38% reduction in %S; beyond this, treatment with 0.5% ethanol (86 mM) resulted in a further significant decrement in performance. Higher concentrations exceeding the lethal range [19] were not tested, being of no physiological relevance.

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Alterations in contraction parameters

To describe more fully the effects of ethanol on the kinetics of cell contraction, an examination of the changes in contraction parameters was carried out at two concentrations (0.05 and 0.3%; 8.6 and 51 mM) that were selected to bracket the two plateau regions of the concentration–response relationship. Figure 3 shows the mean normalized maximum responses of myocytes at each of these ethanol concentrations for the parameters measured (relative to initial levels). Data recorded from untreated myocytes at 6 min are included as a time control. The maximum decreases in %S slightly exceeded the mean time-course responses presented in Figure 1, as the time-related variability in individual myocyte responses is eliminated with this analytical approach. Thus the mean maximum effects on %S were to reduce initial %S values by 14.4% and 40.2% respectively (Figures 3a). A similarly significant and graded dose effect of ethanol was observed for the contraction and relaxation rate parameters, although the magnitudes of the effects were slightly attenuated (Figures 3b and 3c). MRS and MRL were impaired by approx. 12–14% relative to control values at the lower ethanol concentration, and by approx 27–34% at the higher concentration.

Interestingly, evaluation of the parameters $t_0$, $t_M$, and $t_F$ revealed that an effect of ethanol on contraction cycle timing becomes evident only at higher ethanol concentrations (Figures 3d–3f). Ethanol-induced prolongation of excitation–contraction coupling latency, as measured by $t_0$, was significant in the 0.3% (51 mM) ethanol treatment group, but not in the low-concentration (0.05%, 8.6 mM) group. Associated with the delayed latency, ethanol induced an abbreviated contraction cycle time, with a reduced $t_M$ and $t_F$. Combining the significant effects of the extended $t_0$ and reduced $t_F$, the cycle time was reduced overall by about 38% in response to 0.3% (51 mM) ethanol, whereas 0.05% (8.6 mM) ethanol had no effect on cycle time.

Washout and repeat treatment effects

The reversibility of the effect of ethanol was examined in a separate series of ‘washout’ experiments. In Figure 4, a protocol involving several 5 min periods of repeated exposure to 0.05% (8.6 mM) ethanol with intervening 5 min washout periods is presented. Cell performance (%S) was tracked over the 30 min cumulative period of this protocol. After each successive treatment cycle there was a rapid recovery of contractile function to control levels. The negative inotropic effect of ethanol was certainly not diminished with repeated ethanol exposure, rather indicating a tendency towards increased sensitivity.

In a final series of experiments the role of extracellular protein in buffering the direct effects of ethanol on
Ethanol effects on cardiomyocyte contractility

**DISCUSSION**

Ethanol depresses myocyte contraction at low concentrations

In this study we demonstrate that acute cardiomyocyte exposure to ethanol at levels as low as 0.05% (v/v) (8.6 mM) under physiological conditions produces marked inotropic modulation of up to 20% of basal performance. Although the direct negative inotropic action of ethanol on the myocardium is well established [14], previous studies have investigated the effects of very high ethanol concentrations in the lethal and supra-lethal range [8,9]. Detailed characterization of the inotropic effects of ethanol at socially relevant non-intoxicating and clinically relevant moderately intoxicating levels has not been previously undertaken. Prior to the present study, the lowest ethanol concentration at which a statistically significant decrease in cardiomyocyte contractile function could be identified was 0.5% (v/v) [9]. An earlier report [6] offered an anecdotal suggestion that the threshold ethanol concentration for producing contractile depression in myocytes was between 0.1 and 0.2% (v/v).

Previous difficulties in detecting effects of ethanol on myocyte contraction are probably related to the use of unphysiological recording conditions. In the present study measurements were made at 36 °C using a 3 Hz stimulus frequency, whereas other investigations have been carried out at lower temperatures and/or utilizing lower frequencies [6,7,9]. The negative inotropic effect of ethanol is likely to be temperature- and frequency-dependent, although the present study does not directly analyse the effects of these variables. *In vivo*, rat body temperature and heart rate are slightly higher than the experimental levels utilized in this study (i.e. 38–39 °C compared with 36 °C, and 4–5 Hz compared with 3 Hz). Thus the inotropic responses to ethanol would be expected to be accentuated even further *in vivo*.

**Mechanisms of action of ethanol**

The extent of cardiomyocyte shortening during the contractile cycle is dependent on the level of free calcium (Ca²⁺) in the cytosol that is available to interact with the myofilaments to permit the formation of cross-bridges. The Ca²⁺ that enters the cytosol via voltage-gated Ca²⁺
channels during excitation directly activates the myofilaments, and also serves as the trigger for the release of a larger quantity of Ca$^{2+}$ from the sarcoplasmic reticulum (SR). Ethanol action at the sarcolemmal or SR sites of cytosolic Ca$^{2+}$ entry, on the SR Ca$^{2+}$ storage capacity and/or on the myofilament Ca$^{2+}$ interaction could potentially influence the contractile state.

The biphasic concentration relationship and the differential dose sensitivity of contraction parameters to ethanol indicates at least two mechanisms of action of ethanol on cardiomyocyte contraction. At the lowest ethanol concentration (i.e. 0.05%, v/v), the negative inotropic effect is observed in association with unchanged excitation–contraction coupling latency and stable timing of peak twitch. This suggests that the reduced response reflects decreased release of Ca$^{2+}$ from the SR, without a change in the timing or magnitude of the early trigger Ca$^{2+}$ influx. An impaired contribution of the SR to activator Ca$^{2+}$ would be expected to result in the reduction of rate parameter values, MRS and MRL, which were also observed at 0.05% ethanol. At higher ethanol concentrations the cycle time effects, i.e. delayed excitation–contraction coupling and reduced time to peak shortening, suggest a decrease in Ca$^{2+}$ channel influx together with diminished myofilament Ca$^{2+}$ sensitivity. This mechanistic interpretation is consistent with related findings reported by other investigators. An effect of ethanol on myofilament sensitivity has been described and may contribute to the negative inotropic effect observed at 0.3% ethanol [6,20,21]. Electrophysiological experiments investigating the effects of ethanol on the L-type Ca currents have shown a threshold of 0.15% ethanol for current inhibition at 37 °C [22]. Analyses of the effects of ethanol on the timing of peak shortening in multicellular preparations have produced discrepant findings, probably due, at least partly, to the use of low stimulus frequencies [23,24].

An effect of a low concentration of ethanol on SR function would be expected to result in a decrease in the amplitude of the Ca$^{2+}$ transient. An effect on the Ca$^{2+}$ transient has been observed previously only at higher ethanol concentrations [6]. However, these observations have been restricted to low-frequency stimulation conditions, and it is likely that the functional sensitivity of the SR to ethanol is frequency-dependent.

An influence of a low concentration of ethanol on SR Ca$^{2+}$ homoeostasis has not been reported previously in intact myocytes. Studies with SR vesicular fractions have demonstrated that ethanol can uncouple SR Ca$^{2+}$ transport [25] and unload SR Ca$^{2+}$ stores by potentiating spontaneous SR Ca$^{2+}$ release events [26]. The destabilizing effect of ethanol on intracellular Ca$^{2+}$ stores may be the cellular basis for the arrhythmogenic response to acute ethanol exposure — a response which is more evident in the normal than in the myopathic myocardium [13].

Role of extracellular BSA in modulating cardiomyocyte contraction

*In vitro* administration of ethanol in a crystalloid buffer is associated with a marked rebound positive inotropic response following ethanol washout (Figure 5). This rebound effect has been noted previously (albeit only at ethanol concentrations double the highest used in our study) [6]. The present study demonstrates that the rebound effect is not seen when a more physiological superfusate containing BSA is used to mimic the *in vivo* environment (Figure 4). The mechanistic basis for the rebound effect is unclear. In concert with plasma and interstitial Ca-buffering proteins, the cardiomyocyte sarcolemmal glyocalyx has an important role in regulating cation binding at the membrane. Disruption of this extracellular Ca buffering may alter the function of various channels and transporters by a surface charge effect. Interference by ethanol in glyocalyx Ca regulation may be more marked in the absence of superfusate BSA, and may be due to transiently increased extracellular Ca binding [27]. A surface charge effect on the operation of other membrane transporters (i.e. Na$^+–$K$^+$-ATPase [28]) may also contribute to the rebound response.

With repeated exposure to ethanol in BSA-containing buffer, the negative inotropic response to ethanol can be elicited reproducibly (Figure 4). The absence of tachyphylaxis in this response is consistent with the interpretation above, of a direct non-receptor-mediated effect on excitation–contraction coupling processes.

*In vivo* and *in vitro* implications

The findings reported in the present study are of both clinical and experimental importance. These data indicate that, at plasma levels of ethanol encountered commonly in social contexts, ethanol would be expected to be acting directly as a myocardial depressant. This is the first report which identifies ethanol at 0.05% (v/v) as a modulator of cardiac contractility, capable of depressing contraction by about 14% under physiological conditions. The kinetic analyses indicate that the mechanism of action involves disturbance of SR function, and this may contribute to arrhythmogenic vulnerability — especially in an *in vivo* environment of heightened compensatory sympathetic drive. The acute negative inotropic effects of ethanol have been shown to be additive with those of other socially accessed cardio-depressive agents [29]. The present study shows that, in such situations, even low-level alcohol consumption may contribute to the cardiotoxic actions of drug combinations.

In an experimental situation, these findings caution against the use of ethanol as a superfusion/perfusion additive to enhance the solubility of non-aqueous agents in the study of isolated cardiomyocyte function [30]. The
cardiomyocyte response to ethanol is dependent on the recording environment, and with physiological stimulus and temperature conditions the use of ethanol as a vehicle constituent at a concentration of 0.05% (v/v) or higher would be expected to have a significant functional effect. Interpretation of data would be further confounded by the instability associated with the rebound effect of ethanol removal if a crystalloid-only solution is utilized.

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REFERENCES


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