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Abstract

Ethanol consumption has been considered to contribute to the occurrences of paroxysmal atrial fibrillation (AF). Pulmonary veins (PVs) are known to initiate AF. This study investigated whether ethanol may induce AF through increasing arrhythmogenic activity of PV cardiomyocytes. Using the whole-cell clamp technique, the action potential and ionic currents were investigated in rabbit single PV beating cardiomyocytes with and without (control) incubation of ethanol. Compared with control cardiomyocytes, PV cardiomyocytes receiving 0.3 mg/ml or 1 mg/ml ethanol had shorter action potential duration, but had similar beating rates (2.6±1.3 Hz, 2.7±1.2 Hz, 2.7±1.2 Hz) and incidences (45%, 41%, 32%) of delayed afterdepolarization. PV cardiomyocytes receiving ethanol had smaller L-type calcium currents and larger transient outward currents, but had similar transient inward, delayed rectified outward, inward rectified and pacemaker currents. These results suggest that ethanol did not change the arrhythmogenic potential of PV cardiomyocytes with little effect on automaticity and triggered activity.

Keywords: Action potential, Atrial fibrillation, Ethanol, Pulmonary vein

Introduction

Atrial fibrillation is the most common sustained cardiac rhythm disturbance and is responsible for substantial morbidity and mortality in the general population. Previous studies have indicated that alcohol consumption may trigger the occurrence of paroxysmal atrial fibrillation (Ettinger et al., 1978; Cohen et al., 1988; Koskinen et al., 1990). On the other hand, several studies also showed that ethanol was not associated with occurrences of atrial fibrillation (Psaty et al., 1997; Kannel et al., 1998) Ethanol is known to have several cardiovascular effects (Regan, 1990). Previous studies have indicated that ethanol could shorten the action potential (AP) duration in cardiac Purkinje fibers (Williams et al., 1980) and enhance the spontaneous activity of sinoatrial cells (Carpentier & Gallardo, 1987). In contrast, other studies have shown that ethanol has little effect on the spontaneous activity of Purkinje fiber or sinoatrial cells (Williams et al., 1980; Jain & Carpentier, 1998). However, only a few studies have evaluated the effects of ethanol on membrane currents of cardiomyocytes. Ethanol decreased calcium and sodium currents in ventricular cardiomyocytes (Habuchi et al., 1995), and inhibited electrically-induced calcium transients in isolated cardiomyocytes (Thomas et al., 1989). Knowledge about the effects of ethanol on ionic currents of pacemaker cells was limited.

Pulmonary veins (PVs) are important sources of ectopic beats for the initiation of paroxysmal atrial fibrillation and the foci of ectopic atrial tachycardia and focal atrial fibrillation (Walsh et al., 1992; Haissaguerre et al., 1998; Chen et al., 1999). They are also associated with the maintenance of atrial fibrillation (Pappone et al., 2000; Sueda et al., 2001). PVs were known to contain cardiomyocytes with electrical activity and suggested to be subsidiary pacemakers or possibly induce atrial arrhythmias (Cheung, 1981; Blom et al., 1999; Chen et al., 2000; Saito et al., 2000). Studies of single cells showed that PVs had cardiomyocytes with distinct electrophysiological characteristics (Chen et al., 2001; Chen et al., 2002a; Chen et al., 2002b).

Furthermore, PVs were found to have arrhythmogenic activity from the enhancement of automaticity or induction of triggered activity in PV cardiomyocytes with pacemaker activity (Chen et al., 2001; Chen et al., 2002a; Chen et al., 2002b). Long-term atrial pacing or administration of thyroid hormone was shown to increase PV arrhythmogenic activity (Chen et al., 2001; Chen et al., 2002a). All of these findings suggested that PVs play a critical role in the genesis of atrial fibrillation. Therefore, it is possible that ethanol could increase PV arrhythmogenic activity and trigger ectopic beats from PVs to induce atrial fibrillation. The purpose of this study was to investigate whether ethanol would change the electrophysiological characteristics, arrhythmogenic activity and membrane currents of PV cardiomyocytes.

Materials and methods

Isolation of PV cardiomyocytes

The investigation conformed with the institutional *Guide for the Care and Use of Laboratory Animals.* As previously described (Chen et al., 2002a; Chen et al., 2002b), rabbits weighing 1-2 kg were anesthetized with intraperitoneal injection of sodium pentobarbital (40 mg/kg). A mid-line thoracotomy was then performed and the heart along with lung was quickly removed. The PVs were perfused in a retrograde manner via polyethylene tubing (outer diameter, 3.5 mm) cannulated through the aorta and left ventricle into the left atrium. The free end of the polyethylene tubing was connected to a Langendorff perfusion column for perfusion with oxygenated normal Tyrode solution (containing in mM: NaCl 137, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.5, HEPES 10 and glucose 11; the pH was adjusted to 7.4 by titrating with 1 N NaOH) at 37°C. The perfusate was replaced with oxygenated Ca^{2+}-free Tyrode solution containing 300 units/ml collagenase (Sigma, Type I) and 0.25 units/ml protease (Sigma, Type XIV) for 8-12 min, after which time the proximal PVs (8-12 mm) were cut away from the atrium and lung and placed in a dissection chamber containing Ca^{2+}-free oxygenated Tyrode

solution. The piece of tissue was cut into fine pieces and gently shaken in 5-10 ml of Ca^{2+}-free oxygenated Tyrode solution until single cardiomyocytes were obtained. The solution was then gradually changed to normal oxygenated Tyrode solution. Only cells with spontaneous activities and cross striations were used. Experiments were carried out at 35±1°C, and the cells were allowed to stabilize in the bath for at least 30 min before experiments.

Electrophysiological and pharmacological study

Whole-cell patch-clamp was performed in PV cardiomyocytes without (control) and with incubation of ethanol (0.3 mg/ml or 1 mg/ml) over 30 minutes by means of an Axopatch 1D amplifier (Axon Instruments, Union, Calif, USA). Borosilicate glass electrodes (o.d., 1.8 mm) were used, with tip resistances of 3-5 MΩ. Before formation of the membrane-pipette seal, tip potentials were zeroed in Tyrode solution. Junction potentials between the bath and pipette solution (9 mV) were corrected for action potential (AP) recording. AP and membrane currents were measured during superfusion with normal Tyrode solution with (control group) or without ethanol. The composition of pipette solution was as follows (in mM): KCl 20, K aspartate 110, $MgCl_2$ 1, Mg_2ATP 5, HEPES 10, EGTA 0.5, LiGTP 0.1, and Na_2 phosphocreatine 5, adjusted to pH 7.2 with 1 N KOH. To study the L-type calcium currents ($I_{Ca,L}$), micropipettes were filled with a solution containing (in mM) CsCl 130, $MgCl_2$ 1, Mg_2ATP 5, HEPES 10, EGTA 10, NaGTP 0.1, and Na_2 phosphocreatine 5 (at pH 7.2, adjusted with CsOH). NaCl and KCl in normal Tyrode solution were replaced by tetraethylammoniums choloride and CsCl, respectively. The APs were recorded in current-clamp mode and ionic currents in voltage-clamp mode as described previously (Chen et al., 2001; Chen et al., 2002a; Chen et al., 2002b). A small hyperpolarizing step from a holding potential of -50 mV to a testing potential of -55 mV for 80 ms was delivered at the beginning of each experiment. The area under the capacitative currents was divided by the applied voltage step to obtain the total cell capacitance. Normally 60% to 80% series resistance (R_s) was electronically compensated. After compensation, the average time constant was 114±8 μs (cell capacitance, 63.1±2.4 pF, n= 248),

and average R_s was 1.7±0.1 MΩ. Currents rarely exceeded 1.5 nA, and the maximal voltage error did not exceed 3 mV.

Voltage command pulses were generated by a 12-bit digital-to-analog converter controlled by pCLAMP software (Axon Instruments). AP measurements were begun 5 minutes after cell rupture. The steady-state AP duration at 20% (APD_{20}) and 50% (APD_{50}) of full repolarization were only measured during spontaneous beating within the rates 2 to 3.5 Hz to minimize the effects of beating rate on AP parameters. Recordings were low pass-filtered at half the sampling frequency. Data were sampled at rates varying from 2 to 25 kHz. Oscillatory potentials were defined as the APs spontaneously fluctuated at depolarized levels (Gonzalez ¡fi Vassalle, 1993).

$I_{Ca,L}$ were measured as inward currents during depolarization from -50 to +60 mV in 10 mV steps for 300 ms at a frequency of 0.1 Hz. In order to avoid the 'run-down' effects, $I_{Ca,L}$ were measured between 5 and 15 minutes after rupturing the membrane patch in each cardiomyocyte from control and ethanol groups. Transient outward currents (I_{to}) were studied with a double-pulse protocol. A 30 ms pre-pulse from -80 to -40 mV was used to inactivate sodium channel, followed by a 300 ms test pulse to +60 mV in 10 mV steps at a frequency of 0.1 Hz. 200 μM $CdCl_2$ was added to the bath solution to inhibit $I_{Ca,L}$. I_{to} were measured as the difference between peak outward current and the outward current at the end of the test pulse. Delayed rectified outward potassium currents (I_K) were measured from the peak outward current at the end of 1s depolarization from -40 to +60 mV during the infusion of 200 μM $CdCl_2$ in the bath solution to inhibit $I_{Ca,L}$.

Transient inward current was induced by clamped potentials from -40 to +40 mV for a duration of 3 sec and then repolarized to -40 mV. The amplitude

of transient inward current was measured as difference between the peak of the transient current and the mean of current just before and after the transient current (Chen et al., 2001; Chen et al., 2002a).

Hyperpolarization-activated membrane currents were activated from -40 mV to test potentials ranging from -20 to -120 mV in 10 mV steps for 1s at a frequency of 0.1 Hz during the infusion of 200 μM $CdCl_2$ in the bath solution. The amplitudes of inward rectified currents (I_{K1}) were measured as 1mM barium sensitive currents. A progressive large inward current developed with slow voltage-dependent kinetics and was not inactivated. It was suppressed by 5 mM cesium and was measured as pacemaker current, I_f.

Statistics

Continuous variables are expressed as mean ± SE. The differences among the PV cardiomyocytes with or without administration of ethanol were analyzed by one-way ANOVA test. Multiple comparisons were analyzed with the Fisher LSD test. Nominal variables were compared by Chi-square analysis with Yates correction or Fisher's exact test. A P value lower then 0.05 was considered to be statistically significant.

Results

Effects of ethanol on action potentials of PV cardiomyocytes

The beating rates were similar among the PV cardiomyocytes receiving 0.3 mg/ml ethanol (2.6 ± 1.3 Hz, n = 44) and 1 mg/ml ethanol (2.7 ± 1.2 Hz, n = 37) and control (2.7 ± 1.2 Hz, n = 47) ($P > 0.05$). However, the cells receiving 0.3 mg/ml or 1 mg/ml ethanol had shorter APD_{20} and APD_{50} than the cells without the administration of ethanol (Table 1). Figure 1 shows the example of PV cardiomyocytes with and without the administration of ethanol. The PV cardiomyocytes with ethanol administration had shorter AP duration

than control during similar spontaneous beating rates. In addition, oscillatory potentials occurred in 10 (25%) control PV cardiomyocytes, 11 (25%) PV cardiomyocytes receiving 0.3 mg/ml ethanol and 11 (30%) PV cardiomyocytes receiving 1 mg/ml ethanol ($P > 0.05$). Figure 2 shows the examples of oscillatory potentials generated before full repolarization in PV cardiomyocytes with or without the administration of ethanol. Moreover, 15 of 47 (32%) control PV cardiomyocytes, 20 of 44 (45%) PV cardiomyocytes receiving 0.3 mg/ml ethanol and 15 of 37 (41%) PV cardiomyocytes receiving 1 mg/ml ethanol had delayed afterdepolarization ($P > 0.05$).

Effects of ethanol on membrane currents of PV cardiomyocytes

Depolarization-induced currents. Figure 3A shows the recordings of $I_{Ca,L}$ from the cells with or without the administrations of ethanol. PV cardiomyocytes receiving 1 mg/ml ethanol (n = 10) but not PV cardiomyocytes receiving 0.3 mg/ml ethanol (n = 9) have smaller current density of $I_{Ca,L}$ than control (n = 9) PV cardiomyocytes (Figure 3B).

Figure 4A shows the recordings of I_{to} from the cells with or without the administrations of ethanol. I_{to} were larger in cardiomyocytes receiving 0.3 mg/ml (n = 14) or 1 mg/ml (n = 13) ethanol than in control (n = 9) PV cardiomyocytes (Figure 4B). However, the current densities of I_K were similar among control (n = 13) and PV cardiomyocytes receiving 0.3 mg/ml (n = 21) or 1 mg/ml (n = 16) ethanol (Figure 5A). Figure 5B shows the recordings of I_K from the cells with or without the administrations of ethanol.

Hyperpolarization-induced currents. Figures 6A shows the recording of I_{K1} in the cells with or without the administration of ethanol. There were similar I_{K1} among control (n = 10) and PV cardiomyocytes receiving 0.3 mg/ml (n = 6) or 1 mg/ml (n = 7) ethanol (Figure 6B). In addition, 36% of the 14 control cells,

43% of the 14 cells receiving 0.3 mg/ml ethanol and 50% of the 14 cells receiving 1 mg/ml ethanol had I_f ($P > 0.05$). The peak I_f density was similar among the cells with or without the administration of ethanol (Figure 7).

As the example shown in Figure 8A, transient inward currents were present in 32 of 34 (94%) control PV cardiomyocytes, 21 of 21 (100%) PV cardiomyocytes receiving 0.3 mg/ml ethanol, and 33 of 37 (89%) PV cardiomyocytes receiving 1 mg/ml ethanol ($P > 0.05$). There were similar transient inward currents among these cells with or without the administration of ethanol (Figure 8B).

Discussion

Major findings

This study demonstrated that ethanol shortened the AP duration, but did not alter the automaticity or triggered activity of PV cardiomyocytes. Ethanol decreased $I_{Ca,L}$ and increased I_{to}, but did not change transient inward currents, I_K, I_{K1}, and I_f.

Effect of ethanol on arrhythmogenic activity of PV cardiomyocytes

Although previous studies have suggested that alcohol consumption may induce the occurrence of atrial fibrillation with the frequently used term "Holiday heart syndrome" (Ettinger et al., 1978; Cohen et al., 1988; Koskinen et al., 1990), the role of ethanol in the genesis of atrial fibrillation is still uncertain. Ethanol was shown to shorten the atrial AP duration which would decrease atrial refractoriness and may facilitate the maintenance of atrial fibrillation (Regan, 1990). Increased circulating catecholamines, enhancement of sympathetic reaction, myocardial damage and altered myocardial metabolism have been suggested to be potential mechanisms underlying ethanol-induced atrial fibrillation (Mathews et al., 1981; Tiina et al., 1998). In

contrast, ethanol was also found to have little effects on the occurrence of atrial fibrillation (Psaty et al., 1997; Kannel et al., 1998). In this study, as in our previous studies, we demonstrated the presence of spontaneous activity and triggered activitity in PV cardiomyocytes (Chen et al., 2001; Chen et al., 2002a; Chen et al., 2002b). The administration of ethanol shortened the AP duration in PV cardiomyocytes, which was similar to the known effects of ethanol on ventricular myocytes. However, unlike the findings in PVs after long-term atrial pacing or administration of thyroid hormone (Chen et al., 2001; Chen et al., 2002a), there were similar automaticity and triggered activity between PV cardiomyocytes with or without the administration of ethanol. These results may suggest that ethanol has little arrhythmogenic effects on PV cardiomyocytes, although, this in vitro experiment still does not completely exclude the possibility that ethanol may induce atrial fibrillation through its systemic effects or arising from the squeal of myocardial damage by ethanol (Mathews et al., 1981; Tiina et al., 1998).

Effect of ethanol on membrane currents of PV cardiomyocytes

This study demonstrated that high-concentration but not low-concentration ethanol would suppress $I_{Ca,L}$ in PV cardiomyocytes. Previous study also has shown that ethanol decreases $I_{Ca,L}$ in ventricular cardiomyocytes (Jain & Carpentier, 1998). However, little information was available about the effects of ethanol on potassium currents. This study showed that ethanol increased I_{to}, but not I_K or I_{K1} in PV cardiomyocytes. These effects of ethanol on $I_{Ca,L}$ and I_{to} would contribute to the shortening of AP duration in these cells.

I_f has been suggested to contribute to the automaticity of cardiomyocytes and may result in cardiac arrhythmia (DiFrancesco, 1986; Hoppe et al., 1998). Similarly, transient inward currents were also known to play a role in the

genesis of delayed afterdepolarization and have been shown to induce arrhythmia (Tseng & Wit, 1987). It is not clear whether ethanol has a role in these currents. This study showed that there were similar I_f and transient inward currents between PV cardiomyocytes with and without ethanol administration. Our previous studies also have shown that enhancement of I_f or transient inward currents play a role in the arrhythmogenic activity of PV cardiomyocytes (Chen et al., 2001; Chen et al., 2002a) Therefore, the small effects of ethanol on I_f and transient inward currents may account for the similar automaticity and triggered activity between PV cardiomyocytes with or without ethanol administration.

Conclusions

Ethanol shortened the AP duration of PV cardiomyocytes with pacemaker activity but did not change the automaticity or triggered activity of these cells. These findings suggest that ethanol has little directly arrhythmogenic effects on PV cardiomyocytes.

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Figure Legends

Figure 1. Action potentials of PV cardiomyocytes during spontaneous beating. PV cardiomyocytes without ethanol administration (panel A) had longer 20% or 50% of AP duration than PV cardiomyocytes receiving 0.3 mg/ml (panel B) and 1 mg/ml ethanol (panel C).

Figure 2. Oscillatory potentials (asterisk) occurred in PV cardiomyocytes without (panel A) or with (panel B) 1 mg/ml ethanol administration.

Figure 3. Current traces (panel A) and I-V relationship (panel B) of $I_{Ca,L}$ in PV cardiomyocytes with or without the administration of ethanol. Insets of current traces show the various clamp protocols. *P < 0.05, **P < 0.01, versus PV cardiomyocytes without administration of ethanol.

Figure 4. Current traces (panel A) and I-V relationship (panel B) of I_{to} in PV cardiomyocytes with or without the administration of ethanol. Insets of current traces show the various clamp protocols. *P < 0.05 versus PV cardiomyocytes without administration of ethanol.

Figure 5. Current traces (panel A) and I-V relationship (panel B) of I_K in PV cardiomyocytes with or without the administration of ethanol. Insets of current traces show the various clamp protocols.

Figure 6. Current traces (panel A) and I-V relationship (panel B) of I_{K1} in PV cardiomyocytes with or without the administration of ethanol. Insets of current traces show the various clamp protocols. I_{K1} were similar among control and PV cardiomyocytes receiving 0.3 or 1 mg/ml ethanol.

Figure 7. Effects of ethanol on I_f of PV cardiomyocytes. There was similar I_f among PV cardiomyocytes with or without administration of ethanol.

Figure 8. Current traces (Panel A) and densities (Panel B) of I_{ti} from PV cardiomyocytes with and without administration of ethanol. The arrows indicate the I_{ti} on repolarization to -40 mV after a depolarizing pulse from -40 to +40 mV for 3 s. Insets of current traces show the clamp protocols.

Table 1. The action potential parameters of PV cardiomyocytes with or without (Control) the administration of ethanol

	MDP (mV)	APA (mV)	APD_{20} (ms)	APD_{50} (ms)
Control (n=20)	-58±2	84±1	65±6	105±9
0.3 mg/ml ethanol (n=12)	-61±2	84±2	43±5*	77±8*
1 mg/ml ethanol (n=13)	-57±3	81±3	41±5**	75±8*

APA, amplitude of AP; MDP, maximal diastolic potential. Values are means ± SE. *P <0.05, **P <0.005 versus the same parameter of the control group.