DUAL EFFECT OF ETHANOL ON INWARD RECTIFIER POTASSIUM CURRENT $I_{K1}$ IN RAT VENTRICULAR MYOCYTES

Alcohol consumption may result in electrocardiographic changes and arrhythmias. Important role of modifications of the inward rectifier potassium current $I_{K1}$ in arrhythmogenesis is well established. Considering lack of relevant data, we aimed at studying the effect of 0.2–200 mM ethanol on $I_{K1}$ in enzymatically isolated rat right ventricular myocytes using the whole cell patch-clamp technique at 23±1°C. Ethanol reversibly affected $I_{K1}$ in a dual way. At a very low concentration of 0.8 mM (~0.004%), ethanol significantly decreased $I_{K1}$ by 6.9±2.7%. However, at concentrations of ethanol ≥20 mM (~0.09%), $I_{K1}$ was conversely significantly increased (by 16.6±4.0% at 20 mM and 24.5±2.4% at 80 mM). The steady-state $I_{K1}$ increase was regularly preceded by its transient decrease at the beginning of ethanol application. Under 2 and 8 mM ethanol, $I_{K1}$ was decreased at the steady-state in some cells but increased in others. Both effects were voltage-independent. In agreement with the observed effects of ethanol on $I_{K1}$, a transient action potential (AP) prolongation followed by its final shortening were observed after the application of ethanol in a low concentration of 8 mM (~0.04%). Under the effect of 0.8 mM ethanol, only AP prolongation was apparent which agreed well with the above described $I_{K1}$ decrease. Other AP characteristics remained unaltered in both concentrations. These observations corresponded with the results of mathematical simulations in a model of the rat ventricular myocyte. To summarize, changes of the cardiac $I_{K1}$ under ethanol at concentrations relevant to the current alcohol consumption were first demonstrated in ventricular myocytes in this study. The observed dual ethanol effect suggests at least two underlying mechanisms that remain to be clarified. The ethanol-induced $I_{K1}$ changes might contribute to the reported alterations of cardiac electrophysiology related to alcohol consumption.

Key words: ethanol, arrhythmia, inward rectifier, dual effect, rat ventricular myocytes, rat ventricular action potential model

INTRODUCTION

Alcohol intoxication may induce arrhythmias (1-4), even the sudden cardiac death (5, 6). Changes of electrocardiographic parameters in human (namely a prolongation of the P wave, QRS complex, PR, QT and QTc intervals) were also reported, under moderate (7, 8) and even low (9) doses of alcohol.

Ethanol is known to alter the action potential (AP) configuration. Namely, AP shortening, deceleration of the upstroke velocity, and/or decrease of AP amplitude were observed (10-14). Ethanol was documented to inhibit some cardiac voltage-gated ionic currents: the sodium current ($I_{Na}$) (13, 15), the L-type calcium current ($I_{Ca}$) (13), and structural correlate of α-subunit of the fast delayed rectifier potassium current $I_{K1}$ (hERG, human ether-a-go-go-related gene channel) (16, 17). Laszlo et al. (18) revealed that a 120-hour intravenous ethanol infusion in rabbits reduced $I_{Na}$ and $I_{Ca}$ in subsequently isolated atrial cells but did not significantly alter the examined potassium currents, namely the transient outward current $I_{to}$, the sustained outward current $I_{so}$, and the inward rectifier current $I_{K1}$. In our recent study (19), ethanol inhibited $I_{to}$, $I_{Ca}$ and $I_{so}$ in rat ventricular myocytes; $I_{Na}$ was the most sensitive current to ethanol.

The impact of $I_{K1}$ on regulation of the resting membrane potential, AP configuration and excitability in cardiomyocytes is well known (20). Modifications of $I_{K1}$, (including those genetically-based) and $I_{K1}$ heterogeneity are known to play an important role in the pathogenesis of arrhythmias (21-26). The role of selective $I_{K1}$ inhibitors in the treatment of atrial fibrillation, the most common arrhythmia related to the alcohol consumption, has been analysed and discussed (27). Unfortunately, the current knowledge about changes of the cardiac $I_{K1}$ under ethanol is very limited. To our knowledge, only the above mentioned study by Laszlo et al. (18) includes measurements of the ethanol effect on $I_{K1}$ in cardiomyocytes. The authors concluded that the ethanol infusion (the blood alcohol levels 34-93 mM) did not significantly alter $I_{K1}$ current density in rabbit atrial myocytes. Other sporadic data related to $I_{K1}$ were obtained in particular Kir2.x subunits, namely in the GIRK1 (Kir2.1) expressed in Xenopus oocytes or human embryonic kidney (HEK) 293T cells, showing a resistance (28) or a decrease of the current (29). These studies were, however, primarily focused on the G-proteins-coupled inwardly rectifying (GIRK) channels and, thus, data related to the effect of ethanol on Kir2.1 are extremely marginal.
Considering the role of $I_{K1}$ in the cardiac electrophysiology and the limited knowledge of ethanol-induced changes of this current, we decided to examine the effect of ethanol on $I_{K1}$ in rat ventricular myocytes, at concentrations relevant to current alcohol consumption in humans.

MATERIALS AND METHODS

The experiments were carried out with respect to recommendations of the European Community Guide for the Care and Use of Laboratory Animals; the experimental protocol (No. 4-11-06-2012) was approved by the Local Committee for Animal Treatment at Masaryk University, Faculty of Medicine (permission No. 12175/2001-1020(A)).

Cell isolation

Myocytes were isolated from right ventricles of adult male Wistar rats (250±50 g) anaesthetised by intramuscular administration of a mixture of tiletamin and zolazepam (65 mg kg$^{-1}$ in 5% v/v DMSO, Rometar® inj., Spofa, Czech Republic; 100 mg kg$^{-1}$; Zoletil® 100 inj., Virbac, France), and xylazine (20 mg kg$^{-1}$; Rompav® inj., Spofa, Czech Republic).

The dissociation procedure was previously described in detail (30). In brief, the heart was retrogradely perfused via aorta with 0.9 mM CaCl$_2$, Tyrode solution and then with nominally Ca-free Tyrode solution. During the first digestion step, the perfusion continued with nominally Ca-free Tyrode solution containing collagenase (type S, Yakult Pharmaceuticals, 0.2 mg mL$^{-1}$), protease (type XIV, Sigma-Aldrich, 0.053 mg mL$^{-1}$), and EGTA (Sigma-Aldrich; 34 µM). In the second digestion step, protease was omitted. The enzyme solution was then washed out in two steps by a perfusion with the low calcium Tyrode solutions (0.09 and 0.18 mM CaCl$_2$). All solutions were oxygenated with 100% O$_2$ at 37°C.

Solutions and chemicals

Tyrode solution with the following composition was used both during the dissociation procedure and to perfuse myocytes during the measurements (in mM): NaCl 135, KCl 5.4, MgCl$_2$ 0.9, HEPES 10, Na$_2$HPO$_4$ 0.33, CaCl$_2$ 0.9, glucose 10 (pH was adjusted to 7.4 with NaOH). To inhibit $I_{K1}$ and the delayed rectifier potassium current $I_{Kp}$ in the course of $I_{K1}$ measurements, CoCl$_2$ (2 mM) and tetraethyammonium chloride (TEA, 50 mM), respectively, were applied. Additionally, 1 µM atropine and 10 µM glibenclamide were administered to avoid a contribution of the acetylcholine-activated potassium current $I_{K1}$ and the ATP-sensitive potassium current $I_{KS}$ to the observed $I_{K1}$ changes despite it is unlikely under our experimental conditions (5 mM ATP in the pipette solution, isolated ventricular cells). In the case of measurements of $I_{K1}$ changes under 8 mM ethanol (Fig. 7), TEA (50 mM) and 4-aminoypyridine (4-AP, 3 mM) were applied to inhibit $I_{Kp}$ and $I_{KS}$, respectively.

The patch electrode filling solution contained (in mM): L-aspartic acid 130, KCl 25, MgCl$_2$ 1, K$_2$ATP 5, EGTA 1, HEPES 5, GTP 0.1, Na$_2$-phosphocreatine 3 (pH 7.35 adjusted with KOH).

CoCl$_2$ (Sigma-Aldrich), atropine (Sigma-Aldrich) and 4-AP (Sigma-Aldrich) were prepared as 1 M, 1 mM and 100 mM stock solutions, respectively, in the deionized water (in the case of 4-AP, pH adjusted to 7.4 with HCl). Glibenclamide (Sigma-Aldrich) was prepared as 100 mM stock solution in dimethyl sulfoxide (DMSO; AppliChem GmbH, Germany). The concentration of DMSO in the final control solution and test solutions was identical (0.01%), moreover, it is unlikely that this concentration of DMSO has any effect on the cardiac $I_{K1}$ (31, 32). To prepare the TEA-containing stock solution, NaCl in the used Tyrode solution (described above) was replaced equimolarly by TEACl (Sigma-Aldrich). Ethanol (99.5%, TAMDA, Czech Republic) was added to the Tyrode solution to obtain the final concentrations between 0.2 and 200 mM (corresponding to 0.0009 and 0.9%, respectively).

The solutions were applied in a close vicinity of the measured cell via a perfusion system; the time to change the solution was approximately 2 s.

Electrophysiological measurements and evaluation

Single rod-shaped cells with well visible striations were used for the current and AP recordings applying the whole cell patch-clamp technique in the voltage clamp and current clamp mode, respectively. The patch pipettes were pulled from borosilicate glass capillary tubes and heat polished on a programmable horizontal puller (Zeitz-Instrumente, Germany). The resistance of the filled glass electrodes was below 1.5 MΩ to keep the access resistance as low as possible. For generation of experimental protocols and data acquisition, the Axopatch 200B equipment and pCLAMP 9.2 software (Molecular Devices) were used. The series resistance was compensated up to 75%. The measured ionic currents were digitally sampled at 10 kHz and stored on the hard disc. Experiments were performed at room temperature (23±1°C). Experimental protocols are described in Results. The holding potential was -75 mV and the stimulation frequency was 0.2 Hz in all experiments except for data included in Fig. 6 and in Table 2 where the stimulation frequency was 1 Hz.

$I_{K1}$ was evaluated as the Ba$^{2+}$-sensitive current at the end of 500-ms pulse, either to -100 mV or to varying voltages between -120 and +10 mV. The evaluated current is expressed as the current density (in pA/pF) all over the manuscript to reduce differences among cells caused by their varying size.

The time course of the effect of 8 and 80 mM ethanol on $I_{K1}$ was fitted with a double-exponential function $y=A_1*exp(-t/t_1)+A_2*exp(-t/t_2)+A_0$. Time course of the wash-out from the effect of 8 mM ethanol was evaluated as the time to 95% recovery of the current to its control level.

Mathematical simulations

To simulate the impact of experimentally assessed changes of the main cardiac ionic currents including $I_{K1}$ under 0.8, 8 and 50 mM ethanol on AP configuration, a previously published mathematical model of the rat ventricular myocyte was used (33). The numerical solution was performed using the computational software MATLAB v.7.2 (MathWorks, Inc.). The code of the model is available at: http://www.it.cas.cz/en/cl3/1033/biophysics-cardiac-cells

Statistical analysis

The results are presented as means ± S.E.M. from $n$ cells (Origin, version 8.5.1, OriginLab Corporation). The curve fitting, paired and unpaired t-test, and one-way and repeated measures ANOVA with the Bonferroni post-test were performed using the GraphPad Prism, version 4.0 (GraphPad Software, Inc.); *P<0.05 was considered statistically significant.

RESULTS

Effect of ethanol on the cardiac $I_{K1}$ at a high concentration (80 mM)

Effect of 80 mM (~0.37%) ethanol is shown in Fig. 1. Superimposed representative steady-state recordings of the
membrane current (Fig. 1A) elicited by a 500-ms hyperpolarizing test pulse from the holding potential of -75 mV to -100 mV were recorded in control conditions and under the effect of ethanol (15-ms prepulse to -40 mV was applied to inactivate $I_{Na}$). The recordings were performed both in the absence and presence of 100 µM Ba$^{2+}$ to distinguish $I_{K1}$ from contaminating currents that, anyway, appeared to be insensitive to ethanol. It follows that ethanol-induced changes of the measured current are exclusively caused by changes of $I_{K1}$.

Fig. 1B illustrates running changes of the ionic current evaluated at the end of 500-ms hyperpolarizing pulse to -100 mV in the course of the above described experiment. The cell was successively exposed to 80 mM ethanol, 100 µM Ba$^{2+}$, and a combination of ethanol and Ba$^{2+}$. The current at -100 mV was significantly increased by $24.5\pm2.4\%$ at the steady-state application of ethanol (eth) compared to control (con; $n=8$, $P<0.01$) and the effect of ethanol was fully reversible during the wash-out period (wash; inset in Fig. 1B).

The average current-voltage relationship in control and under 80 mM ethanol, both in the presence and absence of 100 µM Ba$^{2+}$ ($n=8$) is shown in Fig. 1C. Compared to the above described steady-state changes of the current at -100 mV under ethanol (inset in Fig. 1B), comparable significant changes were observed at physiologically relevant voltages, e.g. the current at -50 mV was significantly increased by $15.5\pm2.3\%$ at the steady-state application of ethanol (Fig. 1B).

**Fig. 1.** Effect of 80 mM ethanol on the ionic current mainly composed by $I_{K1}$.

A: Superimposed current traces in control conditions and under the effect of 80 mM ethanol, both in absence and presence of 100 µM Ba$^{2+}$ to distinguish $I_{K1}$ from contaminating currents; the experimental protocol is shown above.

B: Changes of the current (expressed as the current density in pA/pF) measured at the end of 500-ms pulse to -100 mV during an experiment; arrows indicate the approximate time of analysis of the steady-state current as presented in the inset (an average from 5 consecutive traces preceding the change of a solution was evaluated). Inset: Averaged steady-state current at the end of 500-ms pulse to -100 mV in control conditions (con), under 80 mM ethanol (eth) and during the wash-out period (wash; $n=8$); ** and *** - statistical significance at $P<0.01$ and $P<0.001$, respectively; the current densities in control and wash-out were comparable ($P>0.05$).

C: Current-voltage relationship of the current measured at the end of 500-ms pulses in control and under 80 mM ethanol, both in the absence and presence of 100 µM Ba$^{2+}$ ($n=8$). The residual current in the presence of 100 µM Ba$^{2+}$ was not affected by ethanol. Experimental protocol: 500-ms pulses between -120 and +10 mV in 10-mV steps preceded by 15-ms prepulse to inactivate $I_{Na}$, the holding potential of -75 mV. Inset: Averaged steady-state current at the end of 500-ms pulse to -50 mV in control conditions (con), and under 80 mM ethanol (eth), both in the absence of Ba$^{2+}$ ("total" denotes the total current), and in its presence in the concentration of 100 µM ("Ba$^{2+}$" denotes the Ba$^{2+}$-insensitive current); **-statistical significance at $P<0.01$ ($n=8$).
significantly increased by 25.9±5.6% (n=8, P<0.01; inset in Fig. 1C). The voltage dependence of the residual current in the presence of Ba2+ was nearly linear and unaffected by ethanol (see also inset in Fig. 1C).

Fig. 2A shows representative traces of the current-voltage relationship of Ik1 evaluated as the Ba2+-sensitive current in control conditions and under 80 mM ethanol. Both the inward and outward components of Ik1 were significantly increased in the presence of 80 mM ethanol (Fig. 2B); the increase was voltage-independent (Fig. 2C).

Dual effect of ethanol on Ik1 manifested in the time course of its action

Fig. 3A illustrates in detail the time course of effect of 80 mM ethanol on Ik1, evaluated at the end of 500-ms hyperpolarizing pulse to -100 mV in a representative cell (for the experimental protocol see Fig. 1A). Interestingly, the application of 80 mM ethanol regularly induced a distinct transient decrease of Ik1, by 9.4±2.1% on average, followed by its increase by 24.5±2.4% (n=8; P<0.05 and 0.001, respectively; visible also in Fig. 1B). This time course of Ik1 response to ethanol was approximated by a double-exponential function (for a representative fit see Fig. 3A) with the resulting average time constants τ1 = 13.9±1.7 s (related to the initial decrease) and τ2 = 50.3±5.4 s (related to the following increase; Table 1). The unusual mode of ethanol effect on Ik1 (the dual effect) points to two underlying mechanisms (for details see Discussion).

Dual effect of ethanol on Ik1, manifested in the concentration-dependence

The concentration-dependence of the effect of ethanol on Ik1 was studied in the concentration range between 0.2 and 200 mM (Fig. 3B; the experimental protocol - inset in Fig. 1A). Changes of Ik1 were evaluated after reaching the steady state. Surprisingly, the dual effect of ethanol on Ik1 appeared also in the concentration-dependence. At very low concentrations ≤0.8 mM (≤-0.004%), ethanol decreased Ik1 (the decrease was statistically significant at 0.8 mM ethanol; n=5; P<0.05). However, in the ethanol concentrations ≥20 mM (≥-0.09%), Ik1 was conversely significantly increased. Within the concentration range of a transition between the decrease and increase of Ik1, i.e. in response to the application of 2 mM (≤-0.009%) and 8 mM (≤-0.04%) ethanol, a decrease of Ik1
appeared in some cells but an increase in others (at 8 mM: \( I_{K1} \) decrease by 12.1±1.4% in 9 cells, but \( I_{K1} \) increase by 31.5±6.2% in other 9 cells). Both effects of ethanol were fully recovered during the subsequent wash-out (Figs. 4A and 4B).

### Table 1. Time constants characterizing biphasic time course of the ethanol effect and its wash-out.

<table>
<thead>
<tr>
<th>Ethanol effect</th>
<th>( \tau_1 ) (s)</th>
<th>( \tau_2 ) (s)</th>
<th>( t_{95} ) (s)</th>
<th>( n )</th>
</tr>
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<tbody>
<tr>
<td>80 mM ethanol - final ( I_{K1} ) increase</td>
<td>13.9±1.7</td>
<td>50.3±5.4</td>
<td>***</td>
<td>8</td>
</tr>
<tr>
<td>8 mM ethanol - final ( I_{K1} ) increase</td>
<td>13.2±3.2</td>
<td>54.4±6.2</td>
<td>***</td>
<td>9</td>
</tr>
<tr>
<td>8 mM ethanol - final ( I_{K1} ) decrease</td>
<td>22.0±2.5</td>
<td>42.4±6.7</td>
<td>*</td>
<td>8</td>
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</table>

\( \tau_1 \) and \( \tau_2 \) - time constants of double-exponential fits of time course of the ethanol effect. * and *** - statistical significance of difference between \( \tau_1 \) and \( \tau_2 \) at \( P<0.05 \) and \( P<0.001 \), respectively. \( t_{95} \) - the time to 95% recovery of the current to its control level during the subsequent wash-out period.

Fig. 3. Time- and concentration-dependence of ethanol effect on \( I_{K1} \).

A: Transient changes of \( I_{K1} \) during application of 80 mM ethanol in a representative cell. Red line represents a double-exponential approximation of the time course of \( I_{K1} \) changes, vertical arrows indicate the start and the end of the fit. Note similar transient changes under ethanol in Fig. 1B.

B: Pooled data of the concentration dependence of ethanol effect on \( I_{K1} \). Within the investigated concentration range (0.2 and 200 mM), a dual effect of ethanol was observed, proceeding from \( I_{K1} \) decrease (significant at a low concentration of 0.8 mM) to \( I_{K1} \) increase (at concentrations ≥20 mM); \( n=3-18 \) in particular concentrations. Red crosses represent the mean data ± S.E.M. in individual concentrations; *, ** and *** - statistical significance of the ethanol effect vs. the respective control at \( P<0.05 \), \( P<0.01 \) and \( P<0.001 \), respectively.

Effect of ethanol on the cardiac \( I_{K1} \) at a low concentration (8 mM)

Effect of 8 mM (~0.04%) ethanol is shown in Fig. 4. The time courses of \( I_{K1} \) changes in two representative cells responding respectively by the steady-state increase or decrease of the current are illustrated in Fig. 4A (the experimental protocol - inset in Fig. 1A). In accordance with the previous results obtained at 80 mM ethanol (Figs. 1B and 3A), the steady-state increase of \( I_{K1} \) under 8 mM ethanol by 30.2±6.9% was preceded by a transient \( I_{K1} \) decrease by 12.8±2.0% at the beginning of the application (\( n=9 \); Fig. 4A, upper panel). Parameters describing the time course of these biphasic changes in the presence of 8 mM ethanol were not significantly different from those obtained under 80 mM ethanol (Table 1).

In the cells showing a persistent decrease of \( I_{K1} \) in the presence of 8 mM ethanol (after ~400 s by 11.8±2.1%; \( n=9 \)), the course of \( I_{K1} \) changes was also biphasic (Fig. 4A, lower panel; Table 1). The inhibition transiently deepened by 17.2±5.1% (\( n=9 \)) at the beginning of ethanol application.

Both effects induced by 8 mM ethanol were reversible during the subsequent wash-out (Figs. 4A and 4B) within a comparable time (Table 1, \( P>0.05 \)). Intriguingly, the character of ethanol effect seemed to be dependent on the density of \( I_{K1} \) in control conditions. Of all the cells measured under 8 mM ethanol, the final increase of \( I_{K1} \) was
Fig. 4. Dual effect of 8 mM ethanol on $I_{K1}$.

A: Time course of changes of $I_{K1}$ evaluated at the end of 500-ms pulse to -100 mV (the experimental protocol - inset in Fig. 1A) under the effect of ethanol (full circles) and the subsequent wash-out in two representative cells showing either the final increase of $I_{K1}$ (upper panel) or the final decrease of $I_{K1}$ (lower panel); con, the control conditions; eth - p, the peak effect of ethanol at the beginning of its application; eth - s, the steady-state effect of ethanol at the end of its application; wash, the wash-out.

B: Averaged steady-state $I_{K1}$ at the end of 500-ms pulse to -100 mV (for abbreviations see A) in cells with the final $I_{K1}$ increase (left panel; $n=9$) or the final $I_{K1}$ decrease (right panel; $n=9$); *, ** and *** - statistical significance at $P<0.05$, $P<0.01$ and $P<0.001$, respectively; $I_{K1}$ densities in control and wash-out were comparable within the respective groups ($P>0.05$) but significantly different between the two groups of cells (# - statistical significance at $P<0.05$).

C, D: Current-voltage relationships of $I_{K1}$ in control conditions (open circles) and under the effect of 8 mM ethanol (full circles) in the case of final $I_{K1}$ increase (C; $n=5$) and final $I_{K1}$ decrease (D; $n=5$); the experimental protocol described in legend to Fig. 1C; insets: details of changes of the outward component of $I_{K1}$; *, ** and *** - statistical significance of the difference between $I_{K1}$ under ethanol vs. in control at $P<0.05$, $P<0.01$ and $P<0.001$, respectively.

E, F: The relative changes of $I_{K1}$ under 8 mM ethanol did not show any statistically significant voltage dependence.
present rather in the cells showing a smaller control $I_{K1}$ density (-3.77±0.60 pA/pF; $n=9$; Fig. 4B, left panel) whereas the final decrease of $I_{K1}$ was observed in cells with the higher control $I_{K1}$ density (-5.42±0.49 pA/pF; $n=9$; Fig. 4B, right panel). The difference of the control current density in cells showing the opposite final changes of $I_{K1}$ was statistically significant ($P<0.05$).

Figs. 4C and 4D show the current-voltage relationship of $I_{K1}$ in control conditions and under 8 mM ethanol in cells with the final $I_{K1}$ increase (C) or $I_{K1}$ decrease (D; the experimental protocol - legend to Fig. 1C). Both the inward and outward components of $I_{K1}$ were significantly changed. The dual effect of 8 mM ethanol on $I_{K1}$ was virtually voltage-independent in both directions of the changed current (Figs. 4E and 4F). For instance, the inward current at -100 and the outward current at -50 mV were either comparably increased in a part of cells (by 43.4±11.2% and 42.3±10.3%, respectively; $n=5$, $P>0.05$), or decreased in others (by 10.2±2.5% and 15.4±5.2%, respectively; $n=5$, $P>0.05$).

Effect of ethanol in clinically relevant concentrations on AP configuration

The recorded alterations of AP configuration induced by ethanol in a moderate concentration of 50 mM (~0.24%) were supplemented by mathematical simulations. APs were elicited by 0.5-ms suprathreshold current pulses of 4-10 nA at the stimulation frequency of 0.2 Hz; $V_m$ - the membrane voltage.

Fig. 5. Impact of $I_{K1}$ changes on the action potential (AP) configuration under 50 mM (~0.24%) ethanol.

A: Representative original APs in control conditions and under 50 mM ethanol recorded in a rat right ventricular myocyte (APs were elicited by 0.5-ms suprathreshold current pulses of 4-10 nA at the stimulation frequency of 0.2 Hz); $V_m$ - the membrane voltage.

B: Simulated AP waveforms (upper panel) were generated by a previously published model of the rat ventricular myocyte (33; parameters of the stimulation were set in agreement with experiments). Ethanol effect at 50 mM was simulated by implementing experimentally assessed inhibitions of $I_{Na}$ by 5.6%, $I_{Ca}$ by 8.35% and $I_{to}$ by 2% (19) along with the activation of $I_{K1}$ by 20% (Fig. 7).

Note that AP shortening in the final repolarization remained nearly unaltered when the impact of $I_{K1}$ change alone was simulated (see the blue dashed AP waveform). The lower panel illustrates simulated traces of $I_{K1}$ in control and under 50 mM ethanol. For details see the related text.
repolarization under the effect of 50 mM ethanol is predominantly caused by the ethanol-induced changes of $I_{K1}$.

Fig. 6 shows the effect of ethanol on AP configuration at low concentrations (the stimulation frequency was 1 Hz). During application of ethanol at 8 mM (~0.04%), $APD_{90}$, $APD_{50}$ (APD at 50% repolarization) and $APD_{at-50\,mV}$ (APD at -50 mV) were first prolonged and, subsequently, gradually shortened reaching a steady-state (Fig. 6A, left panel; see also Table 2) in agreement

Table 2. Action potential characteristics in control conditions and under 8 mM ethanol.

<table>
<thead>
<tr>
<th></th>
<th>con</th>
<th>8 mM eth - peak</th>
<th>8 mM eth - s-s</th>
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<tbody>
<tr>
<td>RMP (mV)</td>
<td>-76.7±1.8</td>
<td>-76.8±1.7</td>
<td>-76.9±1.8</td>
</tr>
<tr>
<td>APA (mV)</td>
<td>107.7±6.1</td>
<td>107.9±6.0</td>
<td>107.2±6.0</td>
</tr>
<tr>
<td>$(dV/dt)_{max}$ (V/s)</td>
<td>164.1±12.5</td>
<td>164.8±12.1</td>
<td>164.4±11.9</td>
</tr>
<tr>
<td>$APD_{10}$ (ms)</td>
<td>1.3±0.6</td>
<td>1.3±0.6</td>
<td>1.3±0.5</td>
</tr>
<tr>
<td>$APD_{50}$ (ms)</td>
<td>23.9±11.6</td>
<td>24.5±11.9</td>
<td>22.1±10.8</td>
</tr>
<tr>
<td>$APD_{90}$ (ms)</td>
<td>45.5±12.7</td>
<td>46.4±13.0*</td>
<td>43.3±12.3*</td>
</tr>
<tr>
<td>$APD_{at-50,mV}$ (ms)</td>
<td>35.0±13.0</td>
<td>35.7±13.2</td>
<td>32.5±12.2*</td>
</tr>
<tr>
<td>$APD_{90}$ (%)</td>
<td>100</td>
<td>102.3±0.7*</td>
<td>93.2±1.6**</td>
</tr>
<tr>
<td>$APD_{50}$ (%)</td>
<td>100</td>
<td>101.9±0.1***</td>
<td>95.2±1.4*</td>
</tr>
<tr>
<td>$APD_{at-50,mV}$ (%)</td>
<td>100</td>
<td>101.7±0.4**</td>
<td>93.3±1.1***</td>
</tr>
</tbody>
</table>

* and ** - significant changes under 8 mM ethanol (eth) vs. control (con) at P 0.05 and 0.01, respectively ($n=6$); RMP - the resting membrane potential; APA - the action potential amplitude; $(dV/dt)_{max}$ - the maximum upstroke velocity; $APD_{10}$, $APD_{50}$, $APD_{90}$ and $APD_{at-50\,mV}$ - the action potential duration at 10%, 50%, 90% repolarization and at -50 mV, respectively; peak - values at the maximum prolongation of $APD_{90}$; s-s - values at the steady-state application of ethanol.

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**Fig. 6.** Impact of $I_{K1}$ changes on the action potential (AP) configuration under 8 and 0.8 mM ethanol at the stimulation frequency of 1 Hz.

A: Application of 8 mM (~0.04%) ethanol resulted in a transient AP prolongation (the green line) followed by its shortening reaching a steady-state (the red line), both in experiments and in the model of rat ventricular myocyte (33). Other AP characteristics remained unaltered. $V_m$ - the membrane voltage.

B: Under the effect of 0.8 mM (~0.004%) ethanol, APs were prolonged both in experiments and in the model.

C: The simulated course of changes of AP duration at 90% repolarization ($APD_{90}$) during the first 300 s of application of 0.8 and 8 mM ethanol.
with the previously described effects of 8 mM ethanol on \( I_{K1} \). The modelled APs showed similar changes (Fig. 6A, right panel). Other AP characteristics remained unaltered (Table 2) which confirms the unique effects of 8 mM ethanol on \( I_{K1} \). Under the effect of 0.8 mM (-0.004%) ethanol, we observed only a prolongation of APs both in experiments and in the model (Fig. 6B). Fig. 6C demonstrates the course of \( A_{DP} \) changes during application of 0.8 and 8 mM till reaching the steady-state as simulated in the model.

**DISCUSSION**

In an effort to reveal mechanisms underlying effects of the alcohol consumption on the cardiac electrophysiology, a series of detailed studies has appeared, focused on the effect of ethanol on individual current components in mammalian cardiac cells (13, 15-19). However, a systematic study aimed at the ethanol effect on \( I_{K1} \), one of the principle potassium currents in cardiomyocytes, has been missing so far. The present study performed on rat ventricular cells showed for the first time that:

1) the sensitivity of \( I_{K1} \) to ethanol appears to exceed that of the majority of other currents explored so far (Fig. 7); and ethanol affects the cardiac \( I_{K1} \) in a dual way, manifested both in the time course and concentration dependence of its action in voltage clamp experiments. These effects became apparent also in corresponding changes of AP configuration as recorded in experiments and simulated on a computer model (Figs. 5 and 6).

**Fig. 7.** Concentration dependence of the ethanol effect on \( I_{K1} \) in comparison with that on several other cardiac ionic currents. The mean ± S.E.M. values of relative changes of \( I_{K1} \) (red crosses; presented also in Fig. 3B) were fitted by a biphasic sigmoidal curve (the red line); for details see the related text; red dotted lines - the two components of the biphasic fit according to eq. (1). Effects of ethanol on sodium current \( I_{Na} \), L-type calcium current \( I_{Ca} \), and transient outward potassium current \( I_{to} \) as obtained in our previous study (19) are plotted for comparison.

Under 8 mM ethanol, two populations of the used ventricular myocytes were distinguishable according to their response to the ethanol effect, one with the resulting activation of \( I_{K1} \) and the other with its inhibition. The final response seems to be dependent on the current density in the control conditions; the cells with significantly lower control \( I_{K1} \) density showed the final activation and vice versa (Fig. 4B-4D). This dual effect of ethanol on \( I_{K1} \) points to at least two underlying mechanisms.

In an attempt to formulate this idea in mathematical terms we tentatively fitted the ethanol-induced concentration dependence changes of \( I_{K1} \) observed in the current study to a biphasic sigmoidal curve of the form (expressed in the logarithmic scale).

\[
100 \cdot \frac{I_{K1,\text{eth}}}{I_{K1,\text{lim}}} = 100 - \frac{A_i}{1 + 10^{(\log(K_{ha}) - \log(1/A_i))}} - \frac{A_i}{1 + 10^{(\log(K_{hi}) - \log(1/A_i))}}.
\]

**IC_{50}** = 0.263 mM and **EC_{50}** = 4.365 mM are values of partial inhibition component and activation component, respectively; \( c \) denotes concentration of ethanol in mM, \( A_i (=8%) \) and \( A_i (=29%) \) are the maximal values of inhibition and activation components, \( h_i (=3) \) and \( h_i (=1.8) \) are the Hill coefficients. In Fig. 7, the concentration dependence of inhibition and activation terms appearing in eq. (1) are plotted as red dotted lines. It follows that the reducing and enhancing effects of ethanol on the rat ventricular \( I_{K1} \) appears to be saturated at concentrations above 1 and 20 mM, respectively.

Under 8 mM ethanol, two populations of the used ventricular myocytes were distinguishable according to their response to the ethanol effect, one with the resulting activation of \( I_{K1} \) and the other with its inhibition. The final response seems to be dependent on the current density in the control conditions; the cells with significantly lower control \( I_{K1} \) density showed the final activation and vice versa (Fig. 4B-4D). This dual population did not differ in the morphology and in the membrane capacitance (160±5.6 pF and 153.6±10.0 pF, respectively; \( n=9, P>0.05 \)), thus, a contamination by other cell types can be excluded.

The dual effect of ethanol on \( I_{K1} \) was also manifested in the development of \( I_{K1} \) changes during the ethanol application. At low concentrations of ethanol (<2 mM, \( I_{K1} \) regularly decreased monotonously (not shown). Surprisingly, 8 mM ethanol induced an initial decrease of \( I_{K1} \), followed by its final steady-state increase.
or decrease (Fig. 4). At concentrations \( \geq 20 \text{ mM} \), the initial \( I_{K1} \) decrease was always followed by its gradual increase leading to the steady-state increase (Fig. 3A). Fig. 3A also shows that the time course of running \( I_{K1} \) changes under ethanol could be approximated by a double-exponential function with significantly different time constants of particular phases (Table 1). The faster rate of the inhibition compared to the activation further supports the idea of two underlying mechanisms. The faster initial inhibition may be overcompensated (Fig. 4A, upper panel) or partially compensated (Fig. 4A, lower panel) by a slower developed activation resulting in the final steady-state increase or decrease of \( I_{K1} \).

Mechanisms underlying the effect of alcohols on inward rectifier K⁺ (Kir) channels have been mostly studied in the expressed G-proteins-coupled Kir (GIRK) channels, playing an important role in the central nervous system, less frequently in the G-proteins-independent Kir2.1 (IRK1) channels (29, 35-38). However, distinct similarities between GIRK and IRK channels in the structure of intracellular domain has been described including the possible binding site for alcohols (29, 36). On the other hand, the response of co-expressed subunits cannot be regarded as a simple sum of the individual subunit responses (39). Effects of a substance on an ionic current in cardiac cells may differ from its effects on subunits forming the respective ionic channel heterogeneously expressed in a cell line as have been recently documented by Szentandrassy et al. (40). Hence, analysis of ethanol effects in cardiomyocytes is crucial for the proper understanding of its action.

**Fig. 8.** Simulated features of the effect of ethanol on \( I_{K1} \).

A: Concentration dependence of the ethanol effect. Upper panel: differences between cells varying in fractions of channels responding to ethanol by inhibition (\( f_i \)). The curves intersect and, at concentrations below 2 mM, the cells with prevailing ethanol-induced activation (\( f_i = 0.4 \)) exhibit lower currents (red line) in comparison with the cells with prevailing ethanol-induced inhibition (\( f_i = 0.6 \); blue line). Lower panel: differences between cells varying in \( f_i \) after normalization to the control currents (in the absence of ethanol) in agreement with experimental results (circles). The difference between concentration-dependent current curves from the cells with different \( f_i \) is shifted toward higher concentrations (double arrow) revealing a range of concentrations where variations in \( f_i \) may induce inhibition or activation in different cells.

B: Transient changes of \( I_{K1} \) following application of ethanol at concentrations 0.8 mM (upper panel) and 80 mM (lower panel). All values are normalized to the current in the absence of ethanol and equal abundance ratio of channels responding to ethanol by inhibition and by activation (\( f_i = 0.5 \)). The normalized values are plotted with negative sign for better comparison with experimental results.
We faced with the question whether all aspects of the complex manifestation of ethanol effect on $I_{K1}$ observed in this study could be explicated by an acceptable biophysically based model. Such a model must be compatible with actual findings obtained in expressed channel studies: (i) $I_{K1}$ channels in cardiac cells are constituted as heterogenic heterotramerms of different pore-forming $\alpha$-subunits, in the case of ventricles mainly of Kir2.1, less of Kir2.2 and Kir2.3 (41-44) with different sensitivities to blockers or activators (39, 45). (ii) The common important regulator of the channel activity is a membrane anchored anionic phospholipid, phosphatidylinositol-4,5-biphosphate (PIP2) that affects open channel probability (46-48). (iii) Two types of Kir channels have been reported with different response to ethanol. Besides other regulatory pathways which may affect their activity (49), GIRK channels appeared to be directly activated (in G-proteins-independent way) by primary alcohols up to the size of butanol in a PIP2-dependent manner (29, 38). A hydrophobic alcohol-bound "pocket" in the cytoplasmic domain of GIRK channels has been regarded a putative site for the activation by alcohols stabilizing the open conformation of these channels (29, 36, 38, 50). In contrast to GIRK channels, IRK1 (Kir2.1) channels were shown to be inhibited by short chain alcohols including ethanol (29). Aryal et al. (29) also showed that mutations in the alcohol-binding pocket of IRK1 channels had no effect on the alcohol-dependent inhibition, suggesting an alternate site involved in the inhibition.

A relatively simple model that is able to simulate all the described unusual features of the effect of ethanol on $I_{K1}$ (Fig. 8) is based on the following assumptions:

1. The dual effect of ethanol manifested in steady-state concentration responses (Fig. 3B) results from the presence of at least two populations of channels underlying ventricular $I_{K1}$, one responding to ethanol by the activation, another one by the inhibition. $EC_{50}$ for the inhibition is less than $EC_{50}$ for the activation, thus, only inhibition was observed at low ethanol concentrations in all cells (0.8 mM in Fig. 3B).

2. The both putative populations of channels are assumed to contribute differently to the total conductance of $I_{K1}$. Since the control conductance of channels responding to ethanol by the inhibition is higher, their contribution to $I_{K1}$ prevails. However, the ratio of amounts of the channels of both types may differ in different cells, which is described in the model by variations of their fractions $f$, stands for the fraction of channels responding to ethanol by the inhibition. This concept can explain why the cells that show the decrease at moderate concentration exhibit greater current under control conditions (~in the absence of ethanol). Fig. 8A (upper panel) shows simulated concentration responses in cells with different $f$. It is apparent that the curves intersect and cells showing the higher ethanol-induced activation exhibit the low current in control and vice versa. The concentration dependent effect illustrated in Fig. 3B summarizes experimental results obtained at individual concentrations from different cells so that all values are normalized to currents measured at the given cells in their control. Applying this normalization in the model (Fig. 8A, lower panel), the difference between currents from the cells with different fraction $f$ is shifted toward higher concentrations. This might explain our observations that at a certain range of ethanol concentrations some cells show increase and others decrease of the current at the steady state as indicated by the double arrow.

3. Kinetics of the transient changes following ethanol application is assumed to be governed by an interaction of the channel with PIP2. The response to ethanol bond is regarded several times faster in the channels that are activated in agreement with the time constants presented in Table 1. Interestingly, An et al. (51) described that changes of PIP2 concentrations evoked changes of the current with time constants around 16 and 55 s in Kir2.1 and Kir2.2 channels, respectively, which resembles the time constants of initial decrease and following increase of $I_{K1}$ after the ethanol application observed in this study. Results from the model (Fig. 8B) show that the cells respond to ethanol by a simple inhibition at very low concentration (0.8 mM). At 80 mM, the transient inhibition is followed by the gradual activation. Again, the cells showing the greatest ethanol-induced steady-state current $I_{K1}$ are those with the smallest current in control.

The observed changes of the cardiac $I_{K1}$ under ethanol within concentrations relevant to the current alcohol consumption may be significant considering the important role of $I_{K1}$ in control of the cardiac excitability and arrhythmogenesis (20, 22, 24). There are several clinical studies describing significant ECG changes in healthy volunteers at various ethanol plasma concentrations, Rossinen et al. (7) at moderate ethanol concentration of 26.1 mM (~0.12%), Camel et al. (9) even at low ethanol concentration of 0.05%, Lorsheyd et al. (52) at high and very high ethanol concentrations (0.4% and 0.8%, respectively). Surprisingly, more than 20% of volunteers in the latter study did not show presumptive ECG changes even at the very high ethanol level of 0.8%. These facts imply that the occurrence of ECG changes induced by ethanol in healthy persons has to be regarded as highly individual. The risk of ethanol-induced ECG changes and arrhythmias related to $I_{K1}$ changes may be increased under conditions affecting the cardiac $I_{K1}$, particularly under an increased sympathetic tone, coexisting heart diseases or usage of drugs (21, 53-58), or in the presence of clinically latent inherited syndromes with gain-/loss-of-function mutations in the gene encoding Kir2.1 (23, 25, 48, 59) or other potassium channels (60).

To summarize, this is the first study of the effect of ethanol on $I_{K1}$ in ventricular myocytes. Ethanol affected $I_{K1}$ within concentrations considerably lower than in the case of the majority of other cardiac ionic currents studied so far. The $I_{K1}$ was increased or decreased depending on the applied ethanol concentration. Both effects were voltage-independent and exhibited different kinetics, the inhibition proceeded faster than the activation. Changes of AP configuration studied under clinically relevant concentrations of ethanol agreed well with the data acquired in the voltage clamp experiments and with the simulated AP waveforms. The results suggest at least two different underlying mechanisms that remain to be clarified.

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