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Ethanol inhibits Kv7.2/7.3 channel open probability by reducing the PI(4,5)P_2 sensitivity of Kv7.2 subunit

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Ethanol often causes critical health problems by altering the neuronal activities of the central and peripheral nerve systems. One of the cellular targets of ethanol is the plasma membrane proteins including ion channels and receptors. Recently, we reported that ethanol elevates membrane excitability in sympathetic neurons by inhibiting Kv7.2/7.3 channels in a cell type-specific manner. Even though our studies revealed that the inhibitory effects of ethanol on the Kv7.2/7.3 channel was diminished by the increase of plasma membrane phosphatidylinositol 4,5-bisphosphate (PI(4,5)P_2), the molecular mechanism of ethanol on Kv7.2/7.3 channel inhibition remains unclear. By investigating the kinetics of Kv7.2/7.3 current in high K^+ solution, we found that ethanol inhibited Kv7.2/7.3 channels through a mechanism distinct from that of tetraethylammonium (TEA) which enters into the pore and blocks the gate of the channels. Using a non-stationary noise analysis (NSNA), we demonstrated that the inhibitory effect of ethanol is the result of reduction of open probability (P_O) of the Kv7.2/7.3 channel, but not of a single channel current (i) or channel number (N). Finally, ethanol selectively facilitated the kinetics of Kv7.2 current suppression by voltage-sensing phosphatase (VSP)-induced PI(4,5)P_2 depletion, while it slowed down Kv7.2 current recovery from the VSP-induced inhibition. Together our results suggest that ethanol regulates neuronal activity through the reduction of open probability and PI(4,5)P_2 sensitivity of Kv7.2/7.3 channels. [BMB Reports 2021; 54(6): 311-316]

INTRODUCTION

Ethanol uptake acutely and chronically changes behavioral and psychological activities, such as mild behavior disinhibition, sedation, amnesia, and unconsciousness (1-3). Early studies focused on the effects of ethanol on the perturbation of membrane lipids in producing the physiological results at high concentrations (4). Recently, it has been revealed that ethanol regulates the activity of specific membrane proteins, such as receptors and voltage-gated and ligand-gated ion channels (4, 5). Ethanol treatment on the ventral tegmental neurons increased spontaneous firing frequency through the inhibition of Kv7 channels (6). On the contrary, ethanol enhances the current of G protein-gated inwardly rectifying potassium (GIRK) channels by directly binding the hydrophobic alcohol-binding pocket (7).

The heterotetrametric Kv7.2/7.3 channel is a voltage-gated potassium channel widely expressed in the central and peripheral nervous systems. It was originally termed M-channel since the currents were fully inhibited by the activation of M_1 muscarinic receptor (M_1R) (8). In neurons, the Kv7.2 and 7.3 channel subunits highly co-localize at axon initial segments (AIS) regions (9) and form a stable Kv7.2/7.3 channel to generate a slowly activating and non-inactivating outward current with subthreshold membrane voltage near −60 mV (10). Hence, the modulation of Kv7.2/7.3 channel gating plays a crucial role in controlling the membrane potential and neuronal excitability (9). Interestingly, it has been revealed that plasma membrane phosphatidylinositol 4,5-bisphosphate (PI(4,5)P_2) is absolutely required for activating Kv7 channels via enhancing the open probability (11). The Kv7.2/7.3 current is thus almost fully inhibited by depletion of plasma membrane PI(4,5)P_2 through the activation of M_1R or exogenously expressed voltage-sensing phosphatase (VSP) (12, 13). Several recent papers suggest that PI(4,5)P_2 controls the Kv7.2/7.3 channel by interacting with S2-S3 linker, S4-S5 linker, the proximal C-terminus, and the A-B helix linker on C-terminus (14, 15).

We recently reported that ethanol reduces the outward K^+ current through Kv7.2/7.3 channels and elevates the membrane excitability in superior cervical ganglion (SCG) neurons, which is dependent upon the expression amount of Kv7 channels (16). Although we showed that ethanol inhibition of Kv7.2/7.3 channels was antagonized by PI(4,5)P_2 elevation in a concentration-dependent manner, underlying mechanisms of ethanol regulation remains unclear. In the present study, we revealed that ethanol suppresses the open probability of Kv7.2/7.3 channels through regulating gating activity without binding the channel pores directly. We also provided evidence that ethanol reduces the PI(4,5)P_2 sensitivity of Kv7.2/7.3 channels with the inhibitory order of Kv7.2 > Kv7.2/7.3 > Kv7.3 channels. These findings

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enhance our understanding of the molecular mechanisms underlying the ethanol-induced inhibitory modulation of Kv7 channel gating and neuronal excitability.

RESULTS

Ethanol inhibition of Kv7.2/7.3 channels differs from block with internal TEA

We examined whether the inhibitory action of ethanol is due to the direct blocking Kv7.2/7.3 channel pores as with TEA. TEA binds reversibly and independently to the outer and inner mouths of the pore and inhibit the currents through K^+ channels (17, 18). By increasing extracellular K^+ concentration, we first confirmed that changing the direction of the current does not alter the inhibitory mechanism by extracellular ethanol and TEA (see Supplementary Text and Supplementary Fig. 1). Since ethanol inhibits Kv7 current dose-dependently from 30 mM to 400 mM in both primary SCG neurons and tsA201 cell line (16), here we applied 400 mM of ethanol to tsA201 cells for studying the inhibitory mechanism of ethanol.

As cells were dialyzed internally with TEA, there were changes both in current waveform and in rectification (19). When TEA is drawn into the inner mouth of the pore at positive potentials, it blocks outward flow of K^+. In contrast, when TEA is expelled from the inner mouth back into the cytoplasm at negative potentials, it allows inward flow of K^+ through the pores but generates a "hook" shape current because of the ratification. Therefore, we tested for any current modification due to pore blockage by ethanol. For this, cells were dialyzed internally with 400 mM ethanol in the high-K^+ solution. However, there was no difference in the kinetics of Kv7.2/7.3 traces (Fig. 1A, C). On the other hand, with 1 mM internal TEA, the kinetics of the Kv7.2/7.3 current were significantly changed. (Fig. 1B, C). For examining the action of intracellular ethanol, Fig. 1D shows that after 3-5 min of TEA dialysis, the inward tail current had developed a hook at −70 mV. However, we found that intercellular ethanol did not change the current waveforms at any voltage application, indicating that the inhibition mechanism of ethanol is not due to direct binding to and blocking the pore of channel.

Ethanol reduces the open probability of the Kv7.2/7.3 channel

To further understand the mechanism of ethanol inhibition of Kv7.2/7.3 channels, we investigated single channel properties using the non-stationary noise analysis (NSNA) in the absence and presence of ethanol. As shown in Fig. 2A, B, the saturated Kv7.2/7.3 current was inhibited by 37.6 ± 1.9% with ethanol treatment in normal Ringer's solution. For performing the NSNA, the Kv7.2/7.3 current was measured at −20 mV for 2 s without or with ethanol and its variance was analyzed under each condition (Fig. 2C). Then, the variance-open probability relationship was fitted to the noise parabola equation for estimation of the maximum open probability (P_Omax), the single channel current amplitude (i) and the channel numbers (N) (Fig. 2D). Our data showed that ethanol did not alter the single channel current or the numbers of Kv7.2/7.3 channel (Fig. 2E, F). However, ethanol significantly inhibited the maximum open probability of Kv7.2/7.3

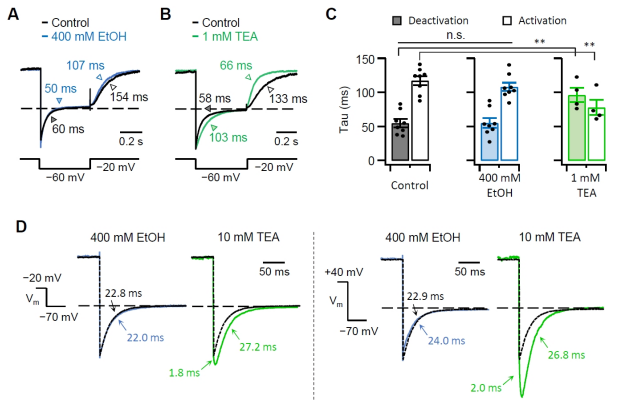

Fig. 1. Intracellular TEA and EtOH show differential effects on the activation and deactivation kinetics of Kv7.2/7.3 current in high-K^+ external solution. Comparison of Kv7.2/7.3 current traces obtained from cells intracellular perfused with vehicle (control) or 400 mM EtOH (A) and 1 mM TEA (B). (C) Summary of time constants for deactivation and activation in cells perfused with 400 mM EtOH or 1 mM TEA. (control, n = 8; 400 mM EtOH, n = 8; 1 mM TEA, n = 4). One-way analysis of variance followed by Sidak's posthoc test, **P < 0.01, as compared with control. n.s., non-significance. (D) Deactivation kinetics of inward current at −70 mV after −20 mV (left) or +40 mV (right) pulse step in high-K^+ external solution. Dotted line indicates Kv7.2/7.3 current in control cells. Dashed line is the zero-current level.

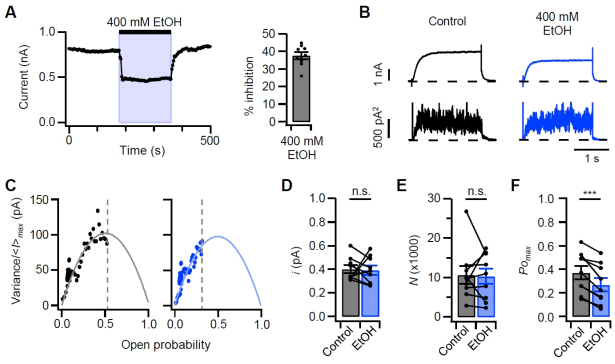

Fig. 2. Non-stationary noise analysis (NSNA) for whole cell configuration of Kv7.2/7.3 channel with or without EtOH treatment. (A) Left, time-dependent regulation of Kv7.2/7.3 current upon treatment of 400 mM EtOH for 150 s. Right, summary of current inhibition by 400 mM EtOH (n = 9). (B) Representative traces of Kv7.2/7.3 current (upper panel) and variance (under panel) elicited in the absence (black) and presence (blue) of 400 mM EtOH in normal Ringer's solution. (C) The variance/$<I>_{max}$ plotted against the open probability of Kv7.2/7.3 channel without (left) or with (right) 400 mM EtOH. Mean single channel current (i; D), channel number (N; E), and maximal open probability (P_Omax; F) were calculated by non-stationary noise analysis (n = 9). Paired Student's t-test were performed. ***P < 0.001.

channel (Fig. 2G). Together, the non-stationary noise analysis showed that ethanol inhibited the Kv7.2/7.3 current by reducing the open probability of the Kv7.2/7.3 channel.

Ethanol inhibition of the Kv7.2/7.3 current is the result of reduction in PI(4,5)P_2 binding sensitivity of the Kv7.2/7.3 channel

We previously showed that the inhibitory effect of ethanol on the Kv7.2/7.3 current is modulated by the concentration of the plasma membrane PI(4,5)P_2 by activating *Ciona intestinalis* voltage-sensing phosphatase (*Ci*-VSP) (16). However, the acute effects of ethanol on the kinetics of PI(4,5)P_2 degradation and resynthesis in the plasma membrane was not clearly solved. Here, in order to further understand how ethanol alters the kinetics of Kv7.2/7.3 current inhibition by VSP activation, whole-cell patch clamp and FRET imaging were simultaneously performed in the same living cell and the changes of Kv7.2/7.3 current and PI(4,5)P_2 concentration during and after VSP activation were measured at the same time. The perturbation of plasma membrane PI(4,5)P_2 level was detected using Förster resonance energy transfer (FRET) assay between two PI(4,5)P_2 probes, CFP-PH (PLCδ1) and YFP-PH(PLCδ1), as described previously (20, 21). The results showed that Kv7.2/7.3 current inhibition upon activation of *Danio rerio* VSP (*Dr*-VSP) by depolarization to +50 mV for 40 s was facilitated approximately 2-fold by ethanol treatment (Fig. 3A-top and 3B-left). However, FRET imaging in response to PI(4,5)P_2 degradation to PI(4)P by VSP activation was not significantly changed (Fig. 3A-bottom and 3B-right). Similarly, ethanol slowed Kv7.2/7.3 recovery after VSP activation, whereas the PI(4,5)P_2 resynthesis indicated by FRETr was not changed (Fig. 3C, D), suggesting that ethanol decreases the PI(4,5)P_2 sensitivity of the Kv7 channel as the inhibitory effects of ethanol are stronger at low PI(4,5)P_2 concentration in the plasma membrane.

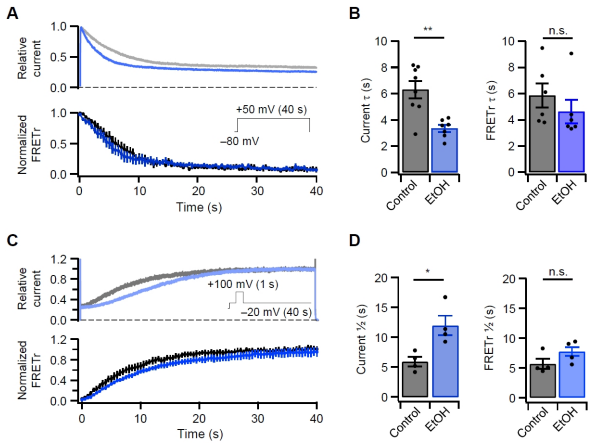

Fig. 3. Simultaneous measurement of EtOH effects on the regulation of Kv7.2/7.3 current and membrane PI(4,5)P_2 by *Danio rerio* voltage-sensing phosphatase (*Dr*-VSP) in a single cell. (A) Representative current traces (top) and FRETr (bottom) without (grey or black lines) or with (blue lines) 400 mM EtOH during PI(4,5)P_2 degradation by Dr-VSP. (B) Time constants (τ) for Kv7.2/7.3 current inhibition (left) and FRETr changes (right) caused by activation of Dr-VSP in the absence (grey bars) or presence (blue bars) of 400 mM EtOH. Current: control, n = 8; ethanol, n = 7; FRETr: control, n = 6; ethanol, n = 6. (C) Representative current traces (top) and FRETr (bottom) measured at −20 mV for 40 s after depolarization to +100 mV for 1 s to deplete membrane PI(4,5)P_2 in cells expressing Dr-VSP. (D) Half recovery (1/2) for Kv7.2/7.3 current (left) and FRETr changes (right) after activation of Dr-VSP in the absence (grey bars) and presence (blue bars) of 400 mM EtOH, n = 4 each. Mann-Whitney u-test, *P < 0.05, **P < 0.01.

Ethanol inhibition of current differs in each subtype of Kv7 channel and is inversely correlated with their PI(4,5)P_2 binding affinity (16). By using this characteristic, we further examined if ethanol differentially regulates the VSP-mediated current inhibition in Kv7 channel subtypes depending on their PI(4,5)P_2 binding affinity. As shown in Fig. 4, ethanol hastened the kinetics of VSP-induced current inhibition in Kv7.2 (Fig. 4A, D), but not in Kv7.3 channels (Fig. 4C, F). Heteromeric Kv7.2/7.3 channels had a significant reaction by ethanol (Fig. 4B, E). However, ethanol did not change the voltage-dependent activation of homomeric Kv7.2 and Kv7.3 channels (Supplementary Fig. 2), suggesting that ethanol selectively facilitated the inhibition of Kv7.2 channels with low apparent PI(4,5)P_2 binding affinity, but not Kv7.3 channels with high PI(4,5)P_2 binding affinity. Taken together, our results further indicated that ethanol inhibits Kv7.2/7.3 currents through the reduction of PI(4,5)P_2 sensitivity of the Kv7.2 subtype, which might be the molecular mechanism responsible for the ethanol-mediated K^+ current suppression through Kv7.2/7.3 channels.

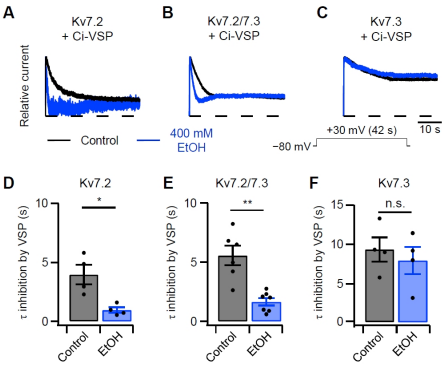

Fig. 4. Kinetics of Kv7 subtype current inhibition facilitated by VSP activation. (A-C) Cells were cells transfected with *Ciona intestinalis* VSP (*Ci*-VSP) plus Kv7.2 (A), both Kv7.2 and Kv7.3 (B), or Kv7.3 alone (C) and depolarized to +30 mV for 42 s to deplete PI(4,5)P_2 through Ci-VSP activation in the absence (black) or presence (blue) of EtOH. Relative current traces of the Kv7 channels subtracted to endogenous potassium current with Ci-VSP transfected are superimposed. (D-F) Time constants (τ) of Kv7 current inhibition by the activation of Ci-VSP in cells expressing Kv7.2 (n = 4 each) (D), both Kv7.2 and Kv7.3 (n = 6 for control, 7 for EtOH) (E), or Kv7.3 alone (n = 4 each) (F) without (black bars) and with (blue bars) 400 mM EtOH. Mann-Whitney u-test, *P < 0.05, **P < 0.01.

DISCUSSION

In this study, we demonstrated that ethanol inhibits Kv7.2/7.3 channels through a mechanism different from that of TEA. The kinetics analysis of current deactivation and activation in the presence of internal ethanol indicated that ethanol does not block the pore directly unlike TEA. We further confirmed that the ethanol inhibition of the Kv7.2/7.3 channel was caused by reduction of open probability of the Kv7.2/7.3 channel. We also obtained more definitive results showing that ethanol regulated the binding affinity between Kv7.2/7.3 channel and $PI(4,5)P_2$. Our data further show how ethanol modulates the gating of the Kv7 channels in a subtype-specific and phospholipid-dependent manner.

In Kv7.2/7.3 channels, extracellular TEA inhibits currents by binding the tyrosine residue of 323 of the pore of Kv7.2, but not the Kv7.3 channel because it has a threonine at the corresponding position (17). Thus, the inhibitory effects of TEA are different on different Kv7 subtypes. The kinetics of internal TEA inhibition has been reported for the prokaryotic KcsA channel through the internal quaternary ammonium ions located at the internal water cavity and one of the ethyl groups inserted into the selectivity filter (22, 23). Moreover, the mechanism of ethanol regulation has been studied for several potassium channels including GIRK2 channel and Kv3.4 channel. There are conserved hydrophobic alcohol-binding pockets exist in the cytoplasmic domain of GIRK2 channel (24, 25). Studies of the Kv3.4 channel indicate that the substitution of glycine at 371 of the S4-S5 linker to isoleucine alters the alcohol-sensitive channel (26). For these reasons, the ethanol binding sites of Kv7.2/7.3 channel might not be related to the pore region where TEA binds to block the current.

The properties of single channel appeared to be consistent with the literature under similar conditions of membrane potential (27). There are different results for single channel current of Kv7.2/7.3 channel, such as ~0.21 pA at −33 mV using NSNA or ~0.5 pA using the single channel recording technique (11, 27, 28). According to our data, the single channel current and the open probability were calculated for ~0.4 pA and 0.4 at −20 mV of membrane potential, respectively. The diversity in single channel amplitude indicates a result of the heterogeneous Kv7.2 and Kv7.3 channel complex (29, 30). The correlation between the regulation of amplitude of Kv7.2/7.3 currents and open probability by several drugs has been investigated. The single channel patch clamp technique showed that the open probability of Kv7.2/7.3 channel was increased in the presence of the positive allosteric modulator retigabine, a water soluble $PI(4,5)P_2$ analog, DiC_8-$PI(4,5)P_2$, or intracellular zinc (11, 28, 31). According to our data, ethanol inhibited the open probability of the Kv7.2/7.3 channel rather than regulating single channel current or channel numbers.

Through simultaneous measurement of the Kv7 current and $PI(4,5)P_2$ changes using patch clamp and FRET imaging, we further confirmed that the facilitatory effects of ethanol are not due to the elevation of VSP activity or inhibition of enzymes involved in the $PI(4,5)P_2$ metabolism pathways in the cell membrane. Rather, our results suggest that the ethanol effects on the Kv channel under the VSP activation are the result of reducing the $PI(4,5)P_2$ binding affinity for the Kv7 channels. This was further confirmed with ethanol experiments with Kv7 subtypes. Usually Kv7.3 has a more than 20-fold higher apparent affinity for $PI(4,5)P_2$ than Kv7.2 (32). Consistently, we found that the ethanol facilitation of K^+ current inhibition by VSP was much stronger in Kv7.2. The basic residues on the S2-S3 linker, the S4-S5 linker, and the C-terminus region of Kv7.2/7.3 channel have been suggested as the sites for the interaction with $PI(4,5)P_2$ by charge neutralization or deletion using a mutagenesis technique (11, 15, 32). However, it is still unclear how ethanol regulates the interaction of $PI(4,5)P_2$ with Kv7.2 subtypes.

In summary, we showed here that ethanol suppresses Kv7.2/7.3 channel activity by reducing the open probability and $PI(4,5)P_2$ sensitivity of Kv7.2/7.3 channels. Because ethanol affects acute and chronic physiological functions in excitable cells, including hormone secretion from glands, neural firing and neurotransmitter release, and vascular systems, determining the regulatory mechanism of Kv7 channel suppression by ethanol will provide an important clue to understanding such diverse effects of ethanol in physiology and pathophysiology.

MATERIALS AND METHODS

Additional materials and methods are included in the Supplementary Materials.

Electrophysiological recording

The whole cell patch clamp technique was performed at room temperature using an EPC-10 patch clamp amplifier (HEKA Elektronik, Germany). The electrodes were pulled from glass micropipette capillaries using a P-97 micropipette puller (Sutter Instrument, USA) with a resistance of 2-4 MΩ. Series-resistance errors were compensated by 60%. The average cell capacitance used in this study was 17.2 ± 0.65 pF. The Kv7 current was measured at −20 mV and in a 500 ms hyperpolarizing step to −60 mV every 2 or 4 s, as described previously (16).

Förster resonance energy transfer (FRET)

The FRET experiments were simultaneously performed while measuring the Kv7.2/7.3 current in a single cell. The FRET signals were acquired, and real time was calculated by a homemade program as previously described (33). The FRET calculated ratio (cFactor = CFP/YFP = 0.55) was used to adjust the raw YFP emission signal. The signal of the FRET ratio was calculated as follows: $FRETr = (YFPc - cFactor \times CFPc)/CFPc$, where YFPc is the signal from YFP excited from the FRET signal (YFP emission by CFP excitation), YFP emission is detected by the long wavelength photomultiplier (YFPc), and CFP emission is detected by the short wavelength photomultiplier.

Non-stationary noise analysis

Non-stationary noise analysis (NSNA) was performed as described (34, 35). Kv7.2/7.3 channels were depolarized every 3 s with −20 mV pulse during 2 s for channel activation from −80 mV holding potential. Twenty to forty traces of Kv7 current were collected when fully stabilized in the absence or presence of ethanol. The Kv7 currents were low-pass filtered at 10 kHz and digitized with a sampling rate of 100 kHz for acquiring sufficient data. The mean current versus variance was binned into 30 bins, and data were fit with the following equation: $\sigma^2 = iI - I^2/N + \sigma_b^2$ (σ^2: variance, i: single channel current, I: mean current, N: total number of channels, σ_b^2: background variance). The maximum open probability, $P_O max$, of the peak current can be calculated from the following equation: $P_O max = I_{max} / (i \times N)$, where I_{max} is the maximum value of mean current. For presenting plots of variance/$<I>_{max}$ versus open probability, the background variance (σ_b^2) is subtracted from variance and each individual trace was divided by the estimated $<I>_{max}$ to represent the open probability and variance/$<I>_{max}$ (36).

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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