

G-protein-coupled inwardly rectifying potassium channels are targets of alcohol action

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G-protein-coupled inwardly rectifying potassium channels (GIRKs) are important for regulation of synaptic transmission and neuronal firing rates. Because of their key role in brain function, we asked if these potassium channels are targets of alcohol action. Ethanol enhanced function of cerebellar granule cell GIRKs coupled to GABA_B receptors. Enhancement of GIRK function by ethanol was studied in detail using *Xenopus* oocytes expressing homomeric or heteromeric channels. Function of all GIRK channels was enhanced by intoxicating concentrations of ethanol, but other, related inwardly rectifying potassium channels were not affected. GIRK2/IRK1 chimeras and GIRK2 truncation mutants were used to identify a region of 43 amino acids in the carboxyl (C) terminus that is critical for the action of ethanol on these channels.

Potassium channels are a diverse family of ion channels with a wide variety of functions in the central nervous system. In neurons and other excitable cells, these channels set resting potential and modulate ability to generate and terminate action potentials^{1–3}, and some potassium channels have been implicated as targets of anesthetic action (reviewed in refs. 2, 4, 5 and 6).

Potassium channels are also a likely target for the action of alcohols in the central nervous system. Previous studies show that both the *Drosophila* Shaw2 channel and a twin-pore, open-rectifying, voltage-independent potassium channel are inhibited by ethanol^{7,8}, whereas the activity of large conductance, calcium-activated potassium channels is increased⁹.

Inwardly rectifying potassium channels are activated by neurotransmitters and hormones³ as well as other intracellular messengers such as kinases and G proteins (reviewed in ref. 10). In the central nervous system, the G-protein-dependent subtype of these channels, GIRK channels, may be activated by muscarinic m₂, α₂ adrenergic, D₂ dopaminergic, histamine, 5HT_{1A}, adenosine A₁, GABA_B, μ, κ and δ opioid and somatostatin receptors^{11,12}. Activation of these receptors by neurotransmitters leads to the liberation of Gβγ, which binds directly to the N and C termini of the GIRK channel^{13–19}. The resulting outward flow of potassium ions causes a decrease in neuronal excitability, and GIRK channels are important in regulating inhibitory responses in the central nervous system¹⁰. The activation of GIRKs by Gβγ is accelerated by some members of the RGS (regulators of G-protein signaling) family of proteins²⁰ and stabilized by direct interaction with phosphatidylinositol 4,5-bisphosphate (PIP₂)^{21,22}.

Five subunits of the GIRK channel have been identified and designated GIRK 1–5 in one system of nomenclature and

K_{ir} 3.1–3.5 in another system¹⁰. These subunits are differentially distributed in brain and are likely to have distinct functions in different neuronal populations²³. GIRK channels are widely distributed in brain and have an important function in regulating inhibitory responses in the nervous system. We investigated the role of these channels in the action of alcohols.

RESULTS

To assess the sensitivity of GIRK channels to ethanol in a native system, we studied GIRK function coupled to GABA_B receptors in cerebellar granule cells in culture. Increasing evidence suggests that GIRK activation via G-protein-coupled receptors mediates a hyperpolarization that is localized postsynaptically, whereas presynaptic G-protein-coupled receptors modulate neurotransmitter release via direct coupling to voltage-gated calcium channels²⁴. Cerebellar granule cell GABA_B-GIRK currents were elicited by rapid pressure ejection of baclofen during a voltage step from E_K (–80 mV) to –120 mV. Bath application of ethanol (35 mM, 161 mg per 100 ml; Fig. 1a) increased the cell's GABA_B-GIRK current from 20 to 40 pA, which subsequently returned to control levels upon ethanol washout. In the same cell, bath application of the selective GABA_B-receptor antagonist CGP 55845A reversibly and completely inhibited the baclofen-induced current. The current reversed about E_K and was also reversibly inhibited by bath application of 500 μM BaCl₂ (ref. 25). Ethanol (75 mM) potentiated GABA_B-GIRK current in a concentration-related manner in cerebellar granule cells (Fig. 1b; 135 ± 4% of control in 35 mM ethanol in 4 of 6 cells tested, *p* < 0.01, paired Student's *t*-test; 141 ± 4% of control current in 75 mM ethanol in 6 of 8 cells tested, *p* < 0.001, paired Student's *t*-test). The time course of ethanol potentiation of the

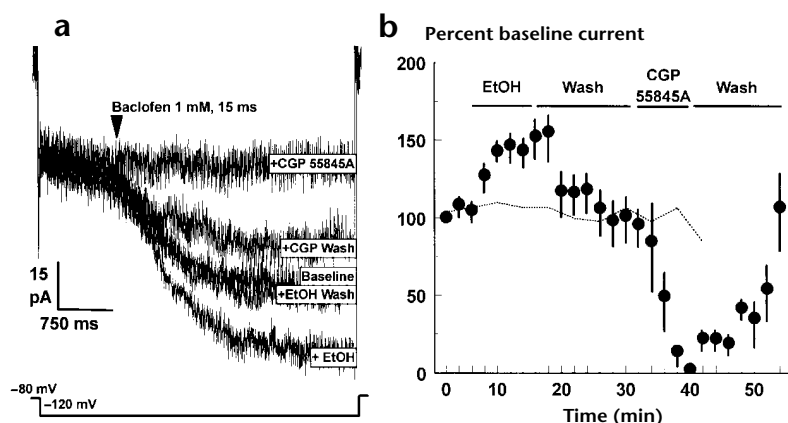


Fig. 1. Ethanol potentiates a potassium current induced by rapid activation of GABA_B receptors in cerebellar granule cells in culture. **(a)** An ensemble of baclofen evoked GIRK-current traces from a cerebellar granule cell in the absence and presence of ethanol and the GABA_B receptor antagonist CGP 55845A. Each current trace shown is an average of three determinations. **(b)** Cumulative data for cells exposed to 75 mM ethanol. Currents are normalized to the initial baseline current; drug applications are shown at instant of valve application and do not account for perfusion onset time (1.5 min). Both the ethanol potentiation of the GIRK current and the inhibition by CGP 55845A were reversible. The dotted line depicts a typical response of a control neuron and demonstrates the stability of GABA_B-GIRK currents in this preparation (symbols were omitted for clarity).

current is notable in that ethanol enhanced GIRK currents immediately upon wash-in and terminated this enhancement immediately upon wash-out. Furthermore, in several experiments, similar potentiation by ethanol (75 mM) was observed when baclofen application was titrated to elicit approximately 20% or 80% of maximal current levels. Taken together, these findings suggest that ethanol may affect GIRK channel function directly rather than via slower actions affecting G-protein coupling to the channel.

To assess ethanol effects on GIRKs coupled to different G-protein-coupled receptors, the GIRK 1 and 4 cRNAs were co-expressed with μ opioid receptor cRNA. Application of morphine (3 or 30 nM), an agonist at the opioid receptor, enhanced the basal GIRK current ($25 \pm 7\%$ above basal for 3 nM morphine; $110 \pm 20\%$ for 30 nM morphine), which was further enhanced by co-application of 100 mM ethanol ($56 \pm 9\%$ enhancement by 3 nM morphine with 100 mM ethanol; $157 \pm 30\%$ enhancement by 30 nM morphine with 100 mM ethanol). There was no difference in the component of the current due to ethanol alone ($23 \pm 3\%$ or $18 \pm 2\%$ enhancement, respectively). Hence, ethanol enhancement of GIRK channel function was found with two different G-protein-coupled receptors and seemed independent of the extent of receptor occupancy for both types of receptors. GIRK 1/4 channels expressed in *Xenopus* oocytes show basal activity that can be blocked by pertussis toxin¹⁰; thus, this basal activity is due to the inherent activity of G proteins. Subsequent experiments were carried out on the GIRK channel without a co-expressed neurotransmitter receptor.

In a physiological buffer (modified Barth's solution, MBS), application of 100 mM ethanol to *Xenopus* oocytes expressing GIRK1/4 channels resulted in an outward current. Because the basal current obtained in MBS is small, a high-potassium solution (hK) was used to reverse the driving force of the channel and provide a large inward current as previously reported²⁵. Application of the hK solution elicited a current that was blocked by

BaCl₂ (ref. 25); ethanol (100 mM) rapidly and reversibly enhanced this current (Fig. 2a). In addition, the effect of 100 mM ethanol on the GIRK1/4 channel was sustained and reversible with repeated testing of the same oocyte over a 20-minute period (Fig. 2b). The function of the GIRK1/4 channel was enhanced by ethanol in a concentration-dependent manner (Fig. 2c). GIRK channels are quite sensitive to ethanol at a concentration of 10 mM (about 44 mg per 100 ml, below the legal level for intoxication), resulting in significant enhancement of channel function (49 ± 13 nA, approximately 3%; $p < 0.01$). Enhancement of GIRK channel function may not have been maximal at 200 mM ethanol, but higher concentrations were not used because of their toxic effects on oocytes²⁶.

Currents obtained with other GIRK subunits (1, 2 or 4), expressed as either homomeric or heteromeric channels, were also enhanced by ethanol, with GIRK2-containing channels showing the greatest enhancement (Fig. 3). The GIRK1 subunit does not form homomeric channels; rather, it is likely that this subunit forms heteromeric channels with the GIRK5 subunit, which is endogenously expressed in *Xenopus* oocytes¹⁰. For the purposes of this report, the channel will be designated GIRK1, although the

precise subunit composition was not determined. All GIRK-mediated potassium ion currents were significantly enhanced by 100 mM ethanol ($p < 0.0001$). GIRK1 and GIRK4 homomeric channels as well as GIRK1/4 heteromeric channels showed enhancement of approximately 15%, whereas homomeric GIRK2 channels and heteromeric GIRK1/2 and 2/4 channels were enhanced approximately 40% by 100 mM ethanol. Other inwardly rectifying potassium channels, IRK1, ROMK1, ROMK2 and ROMK3, showed no response to 100 mM ethanol.

The reversal potential of the GIRK 1/4 channel was found to be dependent on the extracellular potassium ion concentration, demonstrating that the current being studied is due to a potassium ion conductance (Fig. 4a). The actions of ethanol in changing membrane conductance were studied by comparing membrane currents measured in MBS with and without ethanol and in hK solution with and without ethanol (Fig. 4b). The potassium current was enhanced by ethanol, independent of the potassium ion concentration, with no change in the reversal potential, revealing that these drugs affect the potassium channel conductance with no contribution by endogenously expressed ion channels.

To investigate the mechanism of the action of ethanol at the GIRK channel, we tested several factors. Bathing the oocytes with hK may result in an increase in calcium ions within the cell as a result of inhibition of the Na⁺/Ca²⁺ exchanger²⁵. To address this possible complication, the hK solution was modified to remove calcium ions. However, this did not alter the enhancement of the GIRK channel by 100 mM ethanol (enhancement with calcium, $18 \pm 3\%$; enhancement without calcium, $19 \pm 6\%$), indicating that the action of ethanol on the channel is not due to calcium influx. Acute and chronic ethanol exposure are known to affect protein kinases²⁷, which could be responsible for the enhancement of GIRK channel function by ethanol. Protein kinase C (PKC) and protein kinase A are known to modulate some of the actions of GIRK channels (reviewed in ref. 10), so we

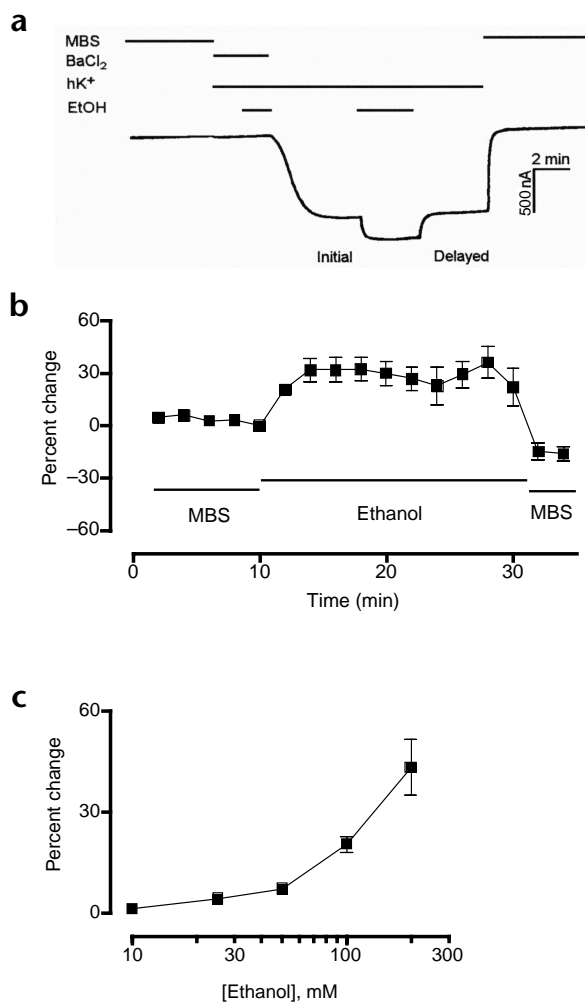


Fig. 2. Ethanol enhances function of GIRK channels expressed in *Xenopus* oocytes. **(a)** Representative tracing of ethanol-induced enhancement of GIRK1/4 channel-mediated K⁺ current in hK solution. After initial perfusion with MBS, elevated extracellular potassium (hK solution) was applied for 2 min; 100 mM ethanol (EtOH) was then applied in hK solution for 2 min before returning to the hK for an additional 2 min. The current elicited by hK solution was blocked by co-application of BaCl₂ (300 μM). **(b)** Time course of ethanol response on GIRK1/4 channels expressed in *Xenopus* oocytes. Oocytes were bathed in MBS with 100 mM ethanol and pulsed every 20 s with hK also containing 100 mM ethanol. Ethanol rapidly enhanced the function of the GIRK channel; this action was maintained over the 20-min period. After 20 min, ethanol was removed, and the response returned to baseline. **(c)** Concentration–response curve for ethanol-activated K⁺ currents of the GIRK1/4 channel expressed in *Xenopus* oocytes. The current was significantly enhanced by 10 mM ethanol, suggesting that the channel is particularly sensitive to ethanol's action ($p = 0.006$). Values are mean \pm s.e. of the percentage of hK current of ≥ 8 oocytes.

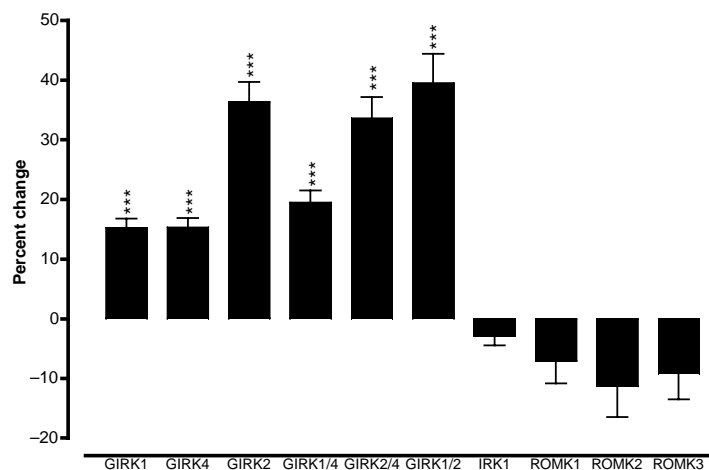
To identify the region of the GIRK channel that is important for the action of ethanol, two chimeras were constructed between GIRK2 and IRK1. GIRK182-IRK resulted from targeted (directed) chimeragenesis as outlined in Methods and consists of GIRK2 from amino acid 1 to 182 and IRK1 from 183 to 428. The junction at position 182 is approximately halfway through the putative second transmembrane region. GIRK223-IRK was constructed using random chimeragenesis and consists of amino acids 1–223 from GIRK2 and 224–428 from IRK1. The junction at position 223 is in the proximal C terminus of GIRK2 just beyond the putative G $\beta\gamma$ -binding site¹³. Electrophysiological recordings were made from oocytes expressing GIRK182-IRK and GIRK223-IRK chimeric subunits homomerically or in combination with wild-type GIRK1 subunits. Oocytes expressing wild-type GIRK2 and GIRK1/2 channels were also tested. Oocytes expressing homomeric GIRK182-IRK channels showed little or no response to hK or hK with 100 mM ethanol (Fig. 6). When expressed in combination with GIRK1, the response to hK was greater than for the homomeric channels, but there was no measurable response to hK with 100 mM ethanol. Hence, the GIRK182-IRK chimera is non-functional and has a dominant negative effect when expressed in combination with GIRK1. Oocytes expressing homomeric GIRK223-IRK channels and heteromeric GIRK223-IRK/GIRK1 channels responded to hK, but showed little or no response when perfused with hK with 100 mM ethanol (Fig. 6). These data concur with previous findings that the region of the proximal C terminus adjacent to the second transmembrane region is critical for G-protein binding¹³. These data also suggest that the region responsible for the enhancement of GIRK channels by ethanol is in the intracellular C terminus between amino acids 224 and 412, and that ethanol's effect is independent of G-protein activation.

To localize the region more closely, we constructed truncation mutants by introducing stop codons at positions 287, 331 and 374 of GIRK2 (GIRK2-286, GIRK2-330 and GIRK2-373). Application of hK to oocytes expressing these truncation mutants elicited a response equivalent to that observed for wild-type GIRK2 channels. Oocytes expressing homomeric GIRK2-286 and GIRK2-330 channels showed no response to hK with 100 mM ethanol, whereas oocytes expressing homomeric GIRK2-373 channels showed enhancement by $34 \pm 4\%$, equivalent to that observed for wild-type GIRK2 channels ($40 \pm 2\%$; Fig. 6). Hence, the region of GIRK2 important for the enhancement of GIRK channels by ethanol is between amino acids 331 and 373.

tested concentrations of two kinase inhibitors that inhibit the action of ethanol on metabotropic receptors expressed in *Xenopus* oocytes²⁸. The enhancement of GIRK function by 100-mM ethanol was not affected by either staurosporine, a nonspecific kinase inhibitor (800 nM; enhancement with staurosporine, $18 \pm 3\%$; control, $20 \pm 2\%$) or GF109203X, an inhibitor of PKC (800 nM ; enhancement with GF109203X, $17 \pm 3\%$; control, $18 \pm 3\%$).

We tested a series of *n*-alcohols for the ability to enhance the function of the GIRK1/4 channel. The effectiveness of various *n*-alcohols in enhancing function of some ligand-gated ion channels shows a distinct cutoff, possibly indicating that certain alcohols interact with a hydrophobic pocket of defined dimensions^{29,30}. Methanol, ethanol and propanol enhanced channel function with potency that seemed to increase with carbon-chain length, but longer-chain alcohols did not enhance GIRK function. At concentrations corresponding to the anesthetic EC₅₀, butanol and octanol had no statistically significant effect on the channel, whereas higher concentrations (3–10 times the anesthetic EC₅₀) of butanol, octanol and all concentrations of pentanol and hexanol tested (1–10 times the anesthetic EC₅₀) inhibited the channel (Fig. 5). Co-application of 100 μM octanol and 1 mM hexanol with 100 mM ethanol had no effect on the enhancement of the channel by ethanol. Hence, it seems unlikely that these longer chain alcohols act at the same site as ethanol.

Fig. 3. Selective action of ethanol on GIRK, IRK and ROMK subunits expressed as homomeric or heteromeric channels in *Xenopus* oocytes. Values are mean \pm s.e. of the percentage of hK current of ≥ 8 oocytes. All GIRK channels tested showed a significant enhancement by ethanol (100 mM; *** $p < 0.0001$). Other inwardly rectifying potassium channels, IRK1, ROMK1, ROMK2 and ROMK3, were also tested for responses to 100 mM ethanol. The majority of oocytes expressing these potassium channels showed no statistically significant response to ethanol. However, when these channels did respond to ethanol, they were always inhibited, and the responses were highly variable. Hence, the means were not significantly different from zero for any of these channels (IRK1, $p = 0.07$; ROMK1, $p = 0.09$; ROMK2, $p = 0.06$; ROMK3, $p = 0.07$).



DISCUSSION

The results presented here demonstrate that ethanol enhances the function of native and recombinant GIRK channels, with GIRK2-containing receptors showing the greatest enhancement. Currents of inwardly rectifying potassium channels of the IRK and ROMK families were not enhanced by ethanol. These experiments identify a new class of ethanol-sensitive membrane channels that are potential *in-vivo* targets of ethanol. Enhancement of GIRK channel function by ethanol contrasts with effects of alcohol on other potassium channels of a wide variety of classes, which are either unaffected by ethanol or other alcohols³¹ or are inhibited by high concentrations of ethanol^{7,8}. Only one other potassium channel (the *mslo* α -subunit of the calcium activated BK channel) is known to be enhanced by ethanol³².

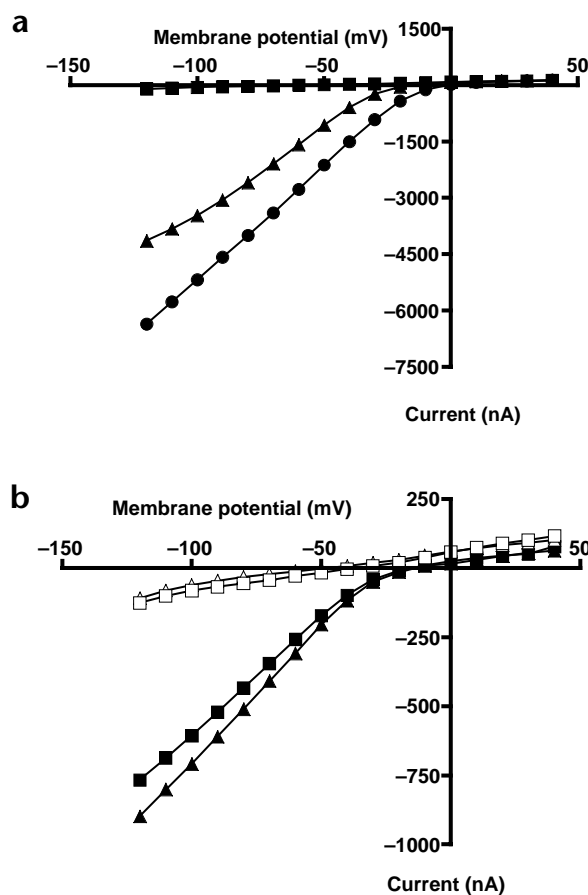
GIRK channels are significantly enhanced by 10 mM ethanol, which produces behavioral impairment *in vivo*. The increase in current produced by low concentrations of ethanol is small: 10 mM ethanol enhances current by only approximately 50 nA (or 3%). However, this relatively minor change in current may have a substantial effect on the membrane potential of a neuron. Another important feature of these findings is that the activation of GIRK channels by ethanol seems independent of the extent of receptor activation. Hence, *in vivo*, ethanol may act to exaggerate the effects of normal neurotransmitter release. When these receptors are activated by neurotransmitters, the channels open and the membrane potential is drawn closer to the equilibrium potential for potassium ions and farther from the firing threshold. Hence, even modest activation of GIRK channels produced by low ethanol concentrations can dampen excitation and can have an overall inhibitory effect^{1,12}. The critical role of K⁺ channels in brain excitability is demonstrated by the finding that a 25% loss of K⁺-channel function is sufficient to cause epilepsy³³.

Regarding the mechanism of action, two lines of evidence suggest that ethanol interacts with a protein site rather than via

nonspecific interactions. Firstly, the effect of *n*-alcohols on the GIRK1/4 channel is critically dependent on chain length. Because there is no correlation between increasing chain length or lipid solubility and potency for altering channel function, the data suggests that these alcohols interact with a protein site of defined dimensions. The putative hydrophobic pocket would be smaller than that of some ligand-gated ion channels (GABA_A, glycine, 5-HT₃, NMDA, AMPA)^{34,35}, but similar to that of an ATP-gated ion channel³⁰ and that of the enzyme alcohol dehydrogenase³⁶.

Secondly, chimeras constructed between sensitive and insensitive inward rectifiers (GIRK2 and IRK1) and GIRK2 trunca-

Fig. 4. (a) Current–voltage plot of changes in membrane conductance in response to extracellular potassium concentration. Membrane currents were measured during superfusion with 1 mM KCl (MBS, closed squares), 50 mM KCl (closed triangles) or 96 mM KCl (hK, closed circles). (b) I–V plot of membrane conductance changes evoked by ethanol in MBS and hK solution. Steady-state currents were measured in response to a series of voltage jumps in a single voltage-clamped oocyte (see Methods). Membrane currents were measured during superfusion with MBS (open square), MBS plus 100 mM ethanol (open triangle), hK (closed square) or hK plus 100 mM ethanol (closed triangle).



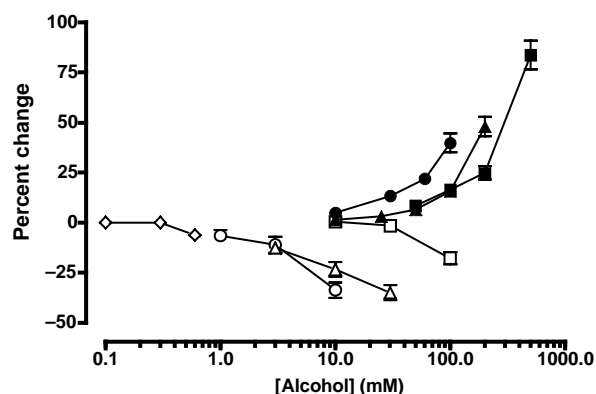


Fig. 5. Concentration–response curves for *n*-alcohols on the GIRK1/4 channel expressed in *Xenopus* oocytes. Values are mean \pm s.e. of the percentage of hK current of ≥ 8 oocytes. Methanol (filled squares), ethanol (filled triangles) and propanol (filled circles) all enhanced the function of GIRK1/4 channels. The lower concentrations of butanol (open squares, 10 and 30 mM), and octanol (open diamonds, 100 and 300 μ M) had no statistically significant effect on the channel. The channel was inhibited by higher concentrations of butanol (100 mM, $p < 0.001$) and octanol (600 μ M, $p < 0.001$), as well as all concentrations of pentanol tested (open triangles; 3 mM, $p < 0.0001$; 10 mM, $p < 0.001$, 30 mM, $p < 0.0001$) and hexanol (open circles; 1 mM, $p < 0.05$; 3 mM, $p < 0.05$; 10 mM, $p < 0.0001$). For comparison, the anesthetic EC₅₀s are 590 mM for methanol; 190 mM for ethanol, 73 mM for propanol, 11 mM for butanol, 2.9 mM for pentanol, 570 μ M for hexanol and 57 μ M for octanol (ref. 46).

tion mutants identified a region of 43 amino acids that is required for the action of ethanol on the GIRK2 channel. Ethanol may interact directly with a binding pocket in the C terminus of the channel; however, the possibility that ethanol modulates the GIRK channel indirectly via an intracellular messenger cannot be ruled out. Acute or chronic ethanol exposure affect intracellular signaling pathways, particularly those involving G proteins and protein kinases²⁷, which could be responsible for the enhancement of GIRK channel function by ethanol. The data presented here demonstrate that the actions of ethanol on these channels are independent of G-protein activation and are not mediated by PKA or PKC or calcium ion influx, but this does not preclude the involvement of another as-yet-unknown GIRK channel activator.

TASK and TREK-1, members of the outwardly rectifying two-pore-domain K⁺ channels, are directly activated by volatile anesthetics, independently of a second messenger³⁷. The C-terminal region, and specifically, the last 48 amino acids in each protein are critical for this activation. TASK and TREK-1 are structurally related to the inward rectifiers, and that finding, along with the data presented here, indicates the possibility of a common mechanism of action of alcohols and anesthetics on K⁺ channels.

The predominant channels in brain are probably heteromeric channels composed of GIRK1, GIRK2 or GIRK3 subunits³⁸. Only one subunit, GIRK2, has been identified in some nuclei in the brainstem such as the ventral tegmentum and the dorsal raphe³⁸. It is possible that different brain areas are differentially affected by ethanol because they express distinct types of GIRK channels. For example, ethanol enhances the firing rate of dopaminergic neurons in the ventral tegmental region and, additionally, enhances function of an inwardly rectifying channel³² that may be a GIRK channel. Because GIRK channels are widely

distributed in brain and have a principle role in determining neuronal excitability, modulation of these channels by ethanol may have profound physiological consequences.

METHODS

Primary cultures of cerebellar granule (CBG) neurons were prepared from 4–7 day-old Sprague-Dawley rats. Whole-cell voltage-clamp electrophysiology in perforated-patch mode was used to analyze GABA_B-GIRK currents in CBG neurons over 4–10 days *in vitro*. The extracellular recording solution contained 165 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 0.9 mM MgSO₄, 10 mM glucose and 10 mM HEPES (pH 7.2–7.3) delivered at a flow rate of 1.5 ml per min. GABA_B-GIRK currents were elicited by pressure ejection of a 1 mM baclofen solution delivered from a patch pipet placed within 25 μ m of the CBG neuron. All other drugs were bath applied. Tight-seal whole-cell perforated-patch recordings were made at room temperature as described^{39,40}. Recording electrodes (3.5–5.5 M Ω) were fabricated from thin-wall borosilicate glass (World Precision Instruments, Sarasota, Florida) and were not fire polished or treated with Sylgard. The internal recording solution consisted of 12 mM NaCl, 135 mM KCH₃SO₄, 0.5 mM EGTA, 10 mM HEPES, 2 mM MgATP and 0.3 mM TrisGTP. Electrode tips were filled with this solution; the remainder of the electrode was filled with this solution with the addition of amphotericin B (200 μ g per ml with 0.1% DMSO). Access resistance, which normally dropped below 50 M Ω within 10 min of obtaining the patch, was monitored throughout the experiment. The cell was rejected if R_a changed by $> 10\%$. Cells were voltage clamped with an Axon Instruments Axopatch 200B amplifier under control of pCLAMP v7.0 software running on a Dell Dimension 400 MHz Pentium II-Digidata 1200B data acquisition system (Axon Instruments, Foster City, California). Cells were normally clamped around E_K (–80 mV); to resolve GABA_B-GIRK currents, a 4-s step to –120 mV was applied immediately before baclofen application (15–60 ms duration at 15–20 psi). Currents were Bessel filtered at 2 kHz and digitized at 8 kHz for off-line analysis. GABA_B-GIRK currents were measured as the difference between the current levels before and following baclofen pressure ejection during the step to –120 mV. All responses were elicited at least three times per cell, and all drug effects were rejected if responses did not return to at least 50% of pre-drug levels within 5 min. Statistical analyses by Student's *t*-test to compare means and by ANOVA to compare populations were considered significant at $p < 0.01$. GABA_B-GIRK currents that seemed insensitive to ethanol ($< 5\%$ change in baclofen current) were observed in some neurons. Such cells were omitted from statistical analysis. Responses for all cells (ethanol sensitive and insensitive) increased from 26 ± 4 pA for baseline currents to 32 ± 8 pA following application of 35 mM ethanol ($n = 6$), and from 33 ± 5 pA to 44 ± 8 pA following 75 mM ethanol application ($n = 8$).

Electrophysiological recordings of GIRK channels in *Xenopus* oocytes. Inwardly rectifying potassium-channel cDNAs were kindly provided by several investigators: GIRK1 was provided by Cesar Labarca, California

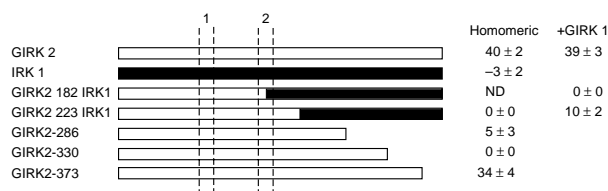


Fig. 6. Effect of 100 mM ethanol on wild-type GIRK2, IRK1, chimeras and truncation mutants. Ethanol enhanced the wild-type GIRK2 homomeric and GIRK1/2 heteromeric channels (white bars) and had no effect on wild-type IRK1 channels (black bars). The values on the right represent the percentage change due to 100 mM ethanol of each channel tested. Values are mean \pm s.e. of the percentage of hK current of ≥ 8 oocytes. Linear schematic representations of each chimera and truncation mutant are shown. The transmembrane regions are indicated by dashed lines. Interfaces between white and black bars indicate chimera junction sites. ND, no measurable current.

Institute of Technology, Pasadena, California, GIRK2 by Michel Lazdunski, Institut de Pharmacologie Moléculaire et Cellulaire, Valbonne France, GIRK4 by David Clapham, Harvard Medical School, IRK1 by L. Philipson, D. Hanck and J. W. Kyle, Northwestern University Medical School, Chicago, Illinois and ROMK1, 2 and 3 by Michael Bienkowski, Pharmacia and Upjohn, Kalamazoo, Michigan, cRNA was prepared using the mCAPTM RNA Capping Kit (Stratagene, La Jolla, California) according to the manufacturer's instructions.

Stage V and VI oocytes were isolated from female *Xenopus laevis* frogs as described⁴¹ and placed in MBS containing 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 10 mM HEPES, 0.82 mM MgSO₄, 0.33 mM Ca(NO₃)₂ and 0.91 mM CaCl₂ at pH 7.5 for microinjection. Ten ng of cRNA from each subunit was injected into the vegetal pole of the oocyte near the equator. Injected oocytes were incubated in MBS containing 10 mg of streptomycin and 10,000 U of penicillin G, 50 mg gentamycin per liter, 0.5 mM theophylline and 2 mM sodium pyruvate for 2–3 days before being used in electrophysiological recordings⁴².

The effect of ethanol and other drugs on the GIRK channel was studied using a two-electrode voltage-clamp system. Initial experiments were carried out in physiological solution (MBS). However, as GIRK channel activation results in a very small outward current in MBS, most experiments were carried out using a high potassium solution (hK) containing 2 mM NaCl, 96 mM KCl, 1.5 mM CaCl₂, 1 mM MgCl₂ and 5 mM HEPES at pH 7.5. Oocytes were bathed in the hK solution for 2 min until a stable response was seen. Then, varying concentrations of ethanol and other drugs were applied in the hK solution for 2 min before changing back to the hK solution for an additional 2 min. The oocytes were allowed to recover between each drug application by perfusing in MBS for 5 min.

Current–voltage (*I*–*V*) analysis was carried out essentially as described⁴³. The voltage dependence of GIRK1/4-mediated currents was studied by measuring steady-state currents at 10 mV intervals at potentials between –120 mV and +40 mV. Membrane potentials were clamped at –60 mV, then instantaneously stepped to each test potential (duration 250–400 ms). Before each drug application, two voltage-jump series were executed to measure the control currents and establish their stability. Current values were measured and averaged during the final 80 ms of the test interval, when they had reached steady state.

Construction of chimeras and truncation mutants. GIRK182-IRK was constructed using directed chimeragenesis. The Quick ChangeTM Site-directed Mutagenesis Kit (Stratagene) was used to introduce two mutations in GIRK2 (C to G at position 543 and T to C at position 546) and IRK 1 (A to G at position 495 and T to C at position 498) creating a *Pvu*I restriction enzyme site. GIRK2 and IRK1 were subcloned into the pBK-CMV vector, which has a *Pvu*I site downstream of the multiple cloning site at position 852. The plasmids were digested with *Pvu*I, and the resulting fragments were gel-purified. The C-terminal portion of IRK1 was ligated to the N-terminal portion of GIRK2, generating a chimeric construct. This construct was sequenced (Applied Biosystems, Foster City, California). The mCAPTM RNA Capping Kit (Stratagene) was used to prepare cRNA.

GIRK223-IRK was constructed using random chimeragenesis^{44,45}. GIRK2 was cloned into the *Hind* III and *Pst* I sites of pBluescript SK⁻. IRK1 was cloned into the *Sac* II and *Sac* I sites of this plasmid resulting in a tandem construct. The resulting plasmid was linearized between GIRK2 and IRK1 with *Sma* I and *Sac* II and transfected into HB101 or DH5 α cells (Life Technologies, Gaithersburg, Maryland). Chimeric constructs were confirmed by sequencing (Applied Biosystems). cRNA was prepared using the mCAPTM RNA Capping Kit (Stratagene).

Truncation mutants were constructed by introducing a stop codon into the sequence of GIRK2 at amino acid position 287, 331 or 374 using the Quick ChangeTM Site-directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. The sequence of each truncation mutant was confirmed by sequencing (Applied Biosystems), and cRNA was prepared using the mCAPTM RNA Capping Kit.

Statistical analysis. Most results are expressed as percentage of the current in high potassium. The control responses were measured before and after drug application. Each drug concentration or was tested in at least eight oocytes from three different frogs. The data was analyzed by analysis of variance (ANOVA) or Student's *t*-tests.

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