Inhibition of G Protein-Activated Inwardly Rectifying K⁺ Channels by Ifenprodil

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G protein-activated inwardly rectifying K⁺ channels (GIRK, also known as Kir3) are regulated by various G-protein-coupled receptors. Activation of GIRK channels plays an important role in reducing neuronal excitability in most brain regions and the heart rate. Ifenprodil, which is a clinically used cerebral vasodilator, interacts with several receptors, such as α_1 adrenergic, *N*-methyl-D-aspartate, serotonin and σ receptors. However, the molecular mechanisms underlying the various clinically related effects of ifenprodil remain to be clarified. Here, we examined the effects of ifenprodil on GIRK channels by using *Xenopus* oocyte expression assays. In oocytes injected with mRNAs for GIRK1/GIRK2, GIRK2 or GIRK1/GIRK4 subunits, ifenprodil reversibly reduced inward currents through the basal GIRK activity. The inhibition was concentration-dependent, but voltage- and time-independent, suggesting that ifenprodil may not act as an open channel blocker of the channels. In contrast, Kir1.1 and Kir2.1 channels in other Kir channel subfamilies were insensitive to ifenprodil. Furthermore, GIRK current responses activated by the cloned κ -opioid receptor were similarly inhibited by ifenprodil. The inhibitory effects of ifenprodil were not observed when ifenprodil was applied intracellularly, and were not affected by extracellular pH, which changed the proportion of the uncharged to protonated ifenprodil, suggesting its action from the extracellular side. The GIRK currents induced by ethanol were also attenuated in the presence of ifenprodil. Our results suggest that direct inhibition of GIRK channels by ifenprodil, at submicromolar concentrations or more, may contribute to some of its therapeutic effects and adverse side effects.

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INTRODUCTION

Ifenprodil was originally developed as an α_1 adrenergic receptor antagonist (Chenard *et al*, 1991), and has cerebral and peripheral vasodilator effects that stimulate blood circulation (Carron *et al*, 1971; Young *et al*, 1983). The drug has been clinically used in the treatment of cerebrovascular diseases and peripheral arterial obliterative disease (Carron *et al*, 1971; Young *et al*, 1983; Marquis *et al*, 1998). Furthermore, it is well known to be a new class of *N*-methyl-D-aspartate (NMDA) receptor antagonists that selectively inhibits NMDA receptors containing the NR2B subunit (Williams, 2001). Ifenprodil has been shown to have neuroprotective (Gotti *et al*, 1988), anticonvulsant (Thurgur and Church, 1998; Yourick *et al*, 1999) and antinociceptive effects (Chizh *et al*, 2001), and to have potential for the treatment of several neuropsychiatric disorders, such as Parkinson's disease (Nash *et al*, 2000), alcoholism

(Malinowska *et al*, 1999; Napiórkowska-Pawlak *et al*, 2000; Narita *et al*, 2000), and drug addiction (Witkin and Acri, 1995; Suzuki *et al*, 1999). Inhibition of NMDA receptor channels by ifenprodil is thought to have important implications in these therapeutic effects (Williams, 2001). It has also been shown that ifenprodil interacts with several other receptors, namely, serotonin (5-hydroxytryptamine; 5-HT) subtype 1A, 5-HT₂ and 5-HT₃ receptors (Chenard *et al*, 1991; McCool and Lovinger, 1995), and σ receptors (Karbon *et al*, 1990) at nanomolar concentrations and that it inhibits voltage-gated Ca²⁺ channels at micromolar concentrations (Church *et al*, 1994; Bath *et al*, 1996). These effects might also be involved in the molecular mechanisms underlying some of the therapeutic effects and side effects of ifenprodil.

G protein-activated inwardly rectifying K⁺ (GIRK) channels (also known as Kir3 channels) are members of a family of inwardly rectifying K⁺ (Kir) channels that includes seven subfamilies (Doupnik *et al*, 1995; Reimann and Ashcroft, 1999). Four GIRK channel subunits have been identified in mammals (Dascal *et al*, 1993; Kubo *et al*, 1993b; Lesage *et al*, 1995; Wickman *et al*, 1997). Neuronal GIRK channels are predominantly heteromultimers composed of GIRK1 and GIRK2 subunits in most brain regions (Kobayashi *et al*, 1995; Lesage *et al*, 1995; Karschin *et al*,

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1996; Liao *et al*, 1996) or homomultimers composed of GIRK2 subunits in the substantia nigra and ventral tegmental area (Inanobe *et al*, 1999), whereas atrial GIRK channels are heteromultimers composed of GIRK1 and GIRK4 subunits (Krapivinsky *et al*, 1995). A variety of G-protein-coupled receptors (GPCRs), such as M₂ muscarinic, α_2 adrenergic, D₂ dopaminergic, 5-HT_{1A}, μ -, δ - and κ -opioid, nociceptin/orphanin FQ and A₁ adenosine receptors, activate GIRK channels (North, 1989; Ikeda *et al*, 1995, 1996, 1997) through the direct action of G-protein $\beta\gamma$ -subunits released from pertussis toxin (PTX)-sensitive G_{i/o} proteins (Reuveny *et al*, 1994). On the other hand, GIRK channels are inhibited by PTX-insensitive G_qPCRs, such as substance P, M₁, and M₃ muscarinic, α_1 adrenergic, thyrotropin-releasing hormone, bombesin and endothelin receptors (Stanfield *et al*, 2002). In addition, ethanol activates GIRK channels independent of G-protein-coupled-signaling pathways (Kobayashi *et al*, 1999; Lewohl *et al*, 1999). Activation of GIRK channels causes membrane hyperpolarization (North, 1989). Also, GIRK2 knockout mice show spontaneous seizures (Signorini *et al*, 1997), whereas GIRK4 knockout mice show blunted heart rate regulation and mild tachycardia (Wickman *et al*, 1998; Bettahi *et al*, 2002). Thus the channels play an important role in the inhibitory regulation of neuronal excitability and heart rate. Therefore, modulators of GIRK channel activity may affect many brain and cardiac functions. Using the *Xenopus* oocyte expression system, we previously demonstrated that various antipsychotic drugs including haloperidol inhibited GIRK channels (Kobayashi *et al*, 1998, 2000, 2004a). Ifenprodil, a phenylethanolamine, is structurally related to the antipsychotic drug haloperidol (Williams, 2001), which weakly inhibits the channels (Kobayashi *et al*, 2000). Therefore, we hypothesized that ifenprodil may also interact with GIRK channels. In the present study, we examined the effects of ifenprodil on brain-type GIRK1/2 and GIRK2 channels and cardiac-type GIRK1/4 channels by using the *Xenopus* oocyte expression assay.

MATERIALS AND METHODS

Preparation of Specific mRNAs

Plasmids containing the entire coding sequences for the mouse GIRK1, GIRK2, and GIRK4 channel subunits and the κ -opioid receptor (κ OR) were obtained by using the polymerase chain reaction method as described previously (Ikeda *et al*, 1995; Kobayashi *et al*, 1995, 2000). In addition, cDNAs for rat Kir1.1 in pSPORT and mouse Kir2.1 in pcDNA1 were provided by Dr Steven C Hebert and Dr Lily Y Jan, respectively. These plasmids were linearized by digestion with the appropriate enzyme as described previously (Ho *et al*, 1993; Kubo *et al*, 1993a; Kobayashi *et al*, 2000); and the specific mRNAs were synthesized *in vitro* by using the mMESSAGE mMACHINE™ *In vitro* Transcription Kit (Ambion, Austin, TX, USA).

Electrophysiological Analyses

Adult female *Xenopus laevis* frogs were purchased from Copacetic (Soma, Aomori, Japan) and maintained in the laboratory until used. Frogs were anesthetized by immer-

sion in water containing 0.15% tricaine (Sigma Chemical Co., St Louis, MO, USA). A small incision was made in the abdomen to remove several ovarian lobes from the frogs, which were humanely killed after the final collection. Oocytes (Stages V and VI) were isolated manually from the ovary and maintained in Barth's solution (Kobayashi *et al*, 2002). *Xenopus laevis* oocytes were injected with mRNA(s) for GIRK1/GIRK2 or GIRK1/GIRK4 combinations (each ~0.4 ng), GIRK2 (~5 ng), Kir1.1 (~5 ng) or Kir2.1 (~0.5 ng), and/or κ OR (~10 ng). The oocytes were incubated at 19°C in Barth's solution, and defolliculated following treatment with 0.8 mg ml⁻¹ collagenase as described previously (Kobayashi *et al*, 2002). Whole-cell currents of the oocytes were recorded from 2 to 10 days after the injection with a conventional two-electrode voltage clamp (Kobayashi *et al*, 1999; Ikeda *et al*, 2003). The membrane potential was held at -70 mV, unless otherwise specified. Microelectrodes were filled with 3 M KCl. The oocytes were placed in a 0.05 ml narrow chamber and superfused continuously with a high-potassium (hK) solution (composition in mM: KCl 96, NaCl 2, MgCl₂ 1, CaCl₂ 1.5 and HEPES 5, pH 7.4 with KOH) or a K⁺-free high-sodium (ND98) solution (composition in mM: NaCl 98, MgCl₂ 1, CaCl₂ 1.5 and HEPES 5, pH 7.4 with NaOH) at a flow rate of 2.5 ml min⁻¹. Kir channels allow K⁺ ions to enter the cells much more readily than does K⁺ permeation in the outward direction (Kubo *et al*, 1993a). In the hK solution used to readily analyze Kir currents by enhancing the magnitude of currents, the K⁺ equilibrium potential (E_K) was close to 0 mV, and inward K⁺ current flow through GIRK channels was observed at negative holding potentials, as shown in previous studies (Dascal *et al*, 1993; Lewohl *et al*, 1999; Kobayashi *et al*, 2004b). For examining the effect of intracellular ifenprodil, 23 nl of 10 mM ifenprodil or 30 mM lidocaine *N*-ethyl bromide (QX-314) dissolved in distilled water was administered to an oocyte through an additional pipette by pressure injection using a Nanoliter injector (World Precision Instruments, Sarasota, FL, USA) as described previously (Kobayashi *et al*, 2003), and the oocyte currents were then continuously recorded for approximately 30–40 min. As the volume of a *Xenopus* oocyte used is ~1 μ l, the intracellular concentration of ifenprodil or QX-314 was presumed as ~225 or ~674 μ M, respectively. Data were fitted to a standard logistic equation by using KaleidaGraph (Synergy Software, Reading, PA, USA) for analysis of concentration–response relationships. The EC₅₀ value, which is the concentration of a drug that produces 50% of the maximal current response for that drug; the IC₂₅ and IC₅₀ values, which are the concentrations of a drug that reduces control current responses by 25 and 50%, respectively; and the Hill coefficient (n_H) were obtained from the concentration–response relationships.

Statistical Analysis of Results

The values obtained are expressed as the mean $\pm$ SEM, and n is the number of oocytes tested. Statistical analysis of differences between groups was carried out by using paired *t*-test, Student's *t*-test, one-way ANOVA or two-way factorial ANOVA followed by Bonferroni/Dunn *post hoc* test. A probability of 0.05 was taken as the level of statistical significance.

Compounds

Ifenprodil tartrate and trans-($\pm$)-3,4-dichloro-*N*-methyl-*N*-(2-[1-pyrrolidinyl]cyclohexyl)benzeneacetamide (U50488H), a selective κ -opioid-receptor agonist, were purchased from Research Biochemicals Inc. (Natick, MA, USA). Ifenprodil was dissolved in distilled water or dimethyl sulfoxide (DMSO), and U50488H was dissolved in distilled water. The stock solutions of all of the compounds were stored at -30°C until used. Ethanol was purchased from Wako Pure Chemical Industries (Osaka, Japan). Each compound was added to the perfusion solution in appropriate amounts immediately before the experiments.

RESULTS

Inhibition of GIRK Channels by Ifenprodil

To investigate whether ifenprodil interacts with brain-type GIRK1/2 and GIRK2 channels and cardiac-type GIRK1/4 channels, we conducted *Xenopus* oocyte expression assays. In oocytes co-injected with GIRK1 and GIRK2 mRNAs, basal GIRK currents (Kobayashi *et al*, 2003), which are known to depend on free G-protein $\beta\gamma$ -subunits present in the oocytes because of the inherent activity of G-proteins (Dascal, 1997), were observed under the conditions of a hK solution containing 96 mM K^+ and negative membrane potentials (1961.9 ± 248.6 nA at -70 mV, $n = 5$, Figure 1a). Application of 3 μM ifenprodil immediately and reversibly caused a reduction of the inward currents through the expressed GIRK channels in the hK solution (Figure 1a). The current responses were abolished in the presence of 3 mM Ba^{2+} , which blocks the Kir channel family including GIRK channels ($n = 3$). The 3 mM Ba^{2+} -sensitive current components in oocytes expressing GIRK channels are considered to correspond to the magnitudes of GIRK1/2 currents (Kobayashi *et al*, 2004b). In uninjected oocytes, ifenprodil, even at the highest concentration used, or 3 mM Ba^{2+} caused no significant response (Figure 1c; $n = 4$), suggesting no effect of ifenprodil or Ba^{2+} on intrinsic oocyte channels. The Ba^{2+} -insensitive current components in oocytes expressing GIRK channels were not significantly different from those in uninjected oocytes as previously shown (Kobayashi *et al*, 2000), indicating that the current components insensitive to 3 mM Ba^{2+} were composed of intrinsic oocyte currents independent of the GIRK currents. Moreover, in oocytes co-injected with GIRK1 and GIRK2 mRNAs, ifenprodil produced no significant response in the K^+ -free ND98 solution containing 98 mM Na^+ instead of the hK solution ($n = 3$; data not shown), suggesting that the ifenprodil-sensitive current components show K^+ selectivity. In addition, application of DMSO, the solvent vehicle, at the highest concentration (0.3%) used, had no significant effect on the current responses ($n = 4$; data not shown). These results suggest that ifenprodil inhibited GIRK1/2 channels. Similarly, in oocytes injected with either GIRK1 and GIRK4 mRNAs (Figure 1b) or GIRK2 mRNA, basal GIRK currents were observed under the same conditions; and the current components sensitive to 3 mM Ba^{2+} were 1197.7 ± 203.9 nA ($n = 9$) or 915.0 ± 223.4 nA ($n = 4$) at -70 mV, respectively. Ifenprodil inhibited basal GIRK1/4 and GIRK2 currents (Figures 1b and 3), suggesting that

Figure 1 Inhibition by ifenprodil of brain-type GIRK1/2 channels and cardiac-type GIRK1/4 channels expressed in *Xenopus* oocytes. (a) In an oocyte co-injected with GIRK1 and GIRK2 mRNAs, current responses to 3 μM ifenprodil and to 3 mM Ba^{2+} are shown. (b) In an oocyte co-injected with GIRK1 and GIRK4 mRNAs, current responses to 3 μM ifenprodil and to 3 μM ifenprodil in the presence of 3 mM Ba^{2+} are shown. (c) In an uninjected oocyte, no significant current responses to 300 μM ifenprodil or 3 mM Ba^{2+} are shown. Current responses were measured at a membrane potential of -70 mV in a hK solution containing 96 mM K^+ . Asterisks show the zero current level. Bars show the duration of application.

ifenprodil also inhibited GIRK1/4 channels and GIRK2 channels.

We further investigated the inhibitory effect of ifenprodil on GIRK channels in more detail. The instantaneous GIRK1/4 currents elicited by the voltage step to -100 mV from a holding potential of 0 mV were diminished in the presence of 3 μM ifenprodil (Figure 2a). The percentage inhibition of the steady-state GIRK current at the end of the voltage step by ifenprodil was not significantly different from that of the instantaneous current (paired *t*-test, $p > 0.05$; $n = 4$ at -60 , -80 , -100 , and -120 mV). These results suggest that the channels were inhibited by ifenprodil primarily at the holding potential of 0 mV and in a time-independent manner during each voltage pulse. Furthermore, similar results were obtained for GIRK1/2 channels ($n = 4$; data not shown).

Like 3 mM Ba^{2+} -sensitive currents corresponding to basal GIRK currents, ifenprodil-sensitive currents in oocytes expressing GIRK channels increased with negative membrane potentials, and the current-voltage relationships showed strong inward rectification (Figure 2b), a characteristic of GIRK currents.

The percentage inhibition of GIRK currents by 3 μM ifenprodil was measured at membrane potentials between -100 and -20 mV. For GIRK1/2 and GIRK1/4 channels, the percentage inhibition showed no significant difference across the voltages ($p > 0.05$, one-way ANOVA; Figure 2c), thus suggesting that the inhibition of GIRK channels by ifenprodil was voltage-independent.

Figure 2 Characteristics of the inhibitory effects of ifenprodil on GIRK currents. (a) Representative GIRK currents elicited by a voltage step to -100 mV for 2 s from a holding potential of 0 mV in the absence and presence of $3 \mu\text{M}$ ifenprodil in a *Xenopus* oocyte injected with GIRK1 and GIRK4 mRNAs. Current responses were recorded in a hK solution containing 96 mM K^+ . Arrow indicates the zero current level. (b) Current-voltage relationships of 3 mM Ba^{2+} -sensitive inward currents and $3 \mu\text{M}$ ifenprodil-sensitive inward currents in oocytes expressing GIRK1/2 channels or GIRK1/4 channels. Current responses were normalized to the 3 mM Ba^{2+} -sensitive current component measured at a membrane potential of -100 mV. The Ba^{2+} -sensitive current components were $1873.2 \pm 476.2 \text{ nA}$ ($n=5$) in oocytes expressing GIRK1/2 channels and $2110.5 \pm 377.3 \text{ nA}$ ($n=7$) in oocytes expressing GIRK1/4 channels. (c) Percentage inhibition of GIRK channels by ifenprodil over the voltage range of -100 to -20 mV. There was no significant interaction between the ifenprodil effect and the membrane potential effect ($p > 0.05$ for GIRK1/2, $n=5$, and $p > 0.05$ for GIRK1/4, $n=7$; one-way ANOVA). All values are the mean and SEM.

Ifenprodil Concentration-Dependently Inhibits GIRK Channels, but not Kir1.1 and Kir2.1 Channels

We next investigated the concentration-response relationship of the inhibitory effects of ifenprodil on GIRK channels expressed in *Xenopus* oocytes, compared with the current components sensitive to 3 mM Ba^{2+} , which fully blocks basal GIRK currents (Kobayashi *et al*, 2004b). Figure 3 shows that the inhibition of GIRK1/2, GIRK2, and GIRK1/4 channels by ifenprodil was concentration-dependent with distinctive potency and effectiveness at nanomolar concentrations or more. Table 1 shows the EC_{50} and n_{H} values obtained from the concentration-response relationships for ifenprodil and the percentage inhibition of the GIRK currents by ifenprodil at the highest concentrations tested.

Table 1 Inhibitory Effects of Ifenprodil on GIRK Channels

	GIRK1/2	GIRK2	GIRK1/4
EC_{50}	5.47 ± 1.21	9.53 ± 1.27	1.00 ± 0.15
IC_{25}	1.48 ± 0.16	1.96 ± 0.18	0.40 ± 0.04
IC_{50}	7.01 ± 0.92	8.76 ± 1.26	2.83 ± 0.69
% max	87.5 ± 2.8	97.8 ± 3.7	83.0 ± 5.9
(μM ; n)	(300; 5)	(300; 4)	(300; 9)
n_{H}	0.76 ± 0.03	0.72 ± 0.01	0.83 ± 0.07

The mean $\pm$ SEM of the EC_{50} values and the concentrations required to reduce basal GIRK currents by 50 and 25% (IC_{50} and IC_{25}) are shown in μM . The values of % max indicate the mean $\pm$ SEM % inhibition of basal GIRK currents by ifenprodil at the highest concentrations tested. The highest concentrations tested (μM) and the number of oocytes tested (n) are indicated in parentheses. The n_{H} values indicate the mean $\pm$ SEM of Hill coefficients.

To further compare the effects of ifenprodil on GIRK channels, we also calculated the drug concentrations required to inhibit the GIRK currents by 25 or 50% (Table 1). The rank order of the inhibition of GIRK channels by ifenprodil at 0.3 – $3 \mu\text{M}$ was as follows: GIRK1/4 $>$ GIRK1/2 $\geq$ GIRK2 channels ($p < 0.05$, significant interaction between the channel effect and the effect of ifenprodil, two-way factorial ANOVA; and $p < 0.05$, significant differences between the effects of ifenprodil on GIRK1/2 and GIRK2 channels and those on GIRK1/4 channels at 0.3 – $3 \mu\text{M}$, Bonferroni/Dunn *post hoc* test). However, there were no significant differences in the inhibitory effects on these GIRK channels by ifenprodil at each concentration from 10 to $300 \mu\text{M}$ ($p > 0.05$, Bonferroni/Dunn *post hoc* test).

Furthermore, we examined whether ifenprodil could interact with Kir1.1, an ATP-regulated Kir channel, and Kir2.1, a constitutively active Kir channel, in other Kir channel subfamilies. In oocytes expressing Kir1.1 or Kir2.1 channels, application of ifenprodil at $100 \mu\text{M}$ had no significant effect on the inward currents through the channels in the hK solution (Ba^{2+} -sensitive current components at -70 mV: $503.8 \pm 62.0 \text{ nA}$ for Kir1.1 and $936.6 \pm 213.6 \text{ nA}$ for Kir2.1, $n=8$; Figure 3).

Similar Inhibition of GIRK Channels by Ifenprodil at pH 7.4 and 9

At physiological pH or below, ifenprodil exists mainly in a protonated form, and the proportion of the uncharged form increases with an increase in pH, because ifenprodil has two pK_{a} values of 9.05 and 9.66. We examined whether changes in extracellular pH affect the inhibition by ifenprodil of GIRK channels expressed in oocytes prepared from the same donor. No significant effect of pH on the inhibition was observed in the concentration-response relationships for ifenprodil in oocytes expressing GIRK1/2 channels ($p > 0.5$, two-way factorial ANOVA; $p > 0.05$ at each concentration, Bonferroni/Dunn *post hoc* test, Figure 4). These results suggest that the inhibition may be mediated by both forms of ifenprodil with almost the same effectiveness. It also appears unlikely that the inhibition

by ifenprodil was caused by hydrophobic interactions with GIRK channels within the membrane bilayer.

Effect of Ifenprodil on GIRK Channels Activated by a G-Protein-Coupled Receptor or Ethanol

Moreover, we examined the effects of ifenprodil on GIRK channels activated by a G-protein-coupled receptor. In oocytes co-expressing GIRK1/2 channels and κ ORs (Kobayashi *et al*, 2004c), application of 100 nM U50488H,

Figure 3 Concentration-response relationships for the inhibitory effects of ifenprodil on GIRK1/2, GIRK2, and GIRK1/4 channels. The magnitudes of inhibition of Kir currents by ifenprodil were compared with the 3 mM Ba^{2+} -sensitive current components in oocytes expressing Kir1.1, Kir2.1, GIRK1/2 channels, GIRK2 channels, or GIRK1/4 channels (503.8 ± 62.0 nA, $n = 8$; 936.6 ± 213.6 nA, $n = 8$; 1961.9 ± 248.6 nA, $n = 5$; 915.0 ± 222.4 nA, $n = 4$; 1197.7 ± 203.9 nA, $n = 9$, respectively). Current responses were measured at a membrane potential of -70 mV in a hK solution containing 96 mM K^+ . Each point and error bar represent the mean and SEM of the percentage responses. Data points were fitted by using a logistic equation.

Figure 4 Concentration-response relationships for inhibition of GIRK channels by ifenprodil at different pH values. The magnitudes of inhibition by ifenprodil of GIRK current in oocytes expressing GIRK1/2 channels were compared with the 3 mM Ba^{2+} -sensitive current components, which were 1592.6 ± 181.3 nA at pH 6.0 ($n = 10$), 1961.9 ± 248.6 nA at pH 7.4 ($n = 5$), and 924.4 ± 141.2 nA at pH 9.0 ($n = 5$). Current responses were measured at a membrane potential of -70 mV in a hK solution. Each point and error bar represents the mean and SEM of the percentage responses obtained. Data points were fitted by using a logistic equation.

a selective κ -opioid-receptor agonist, induced inward GIRK currents, and application of ifenprodil alone inhibited basal GIRK currents consistently at the concentrations tested (Figure 5a). The effects of ifenprodil on GIRK channels activated by the κ OR were evaluated by measuring the amplitude of the U50488H-induced current response during application of ifenprodil at different concentrations. The current responses to 100 nM U50488H were reversibly inhibited by ifenprodil with an IC_{50} value of 3.4 ± 1.2 μM and an n_{H} value of 1.03 ± 0.09 ($n = 5$, Figure 5a and b). The percentage inhibition by ifenprodil was similar to that of basally active GIRK1/2 channels ($p > 0.05$ at each concentration, Student's *t*-test), suggesting interaction of ifenprodil with GIRK channels but not the κ OR. In addition, the U50488H-induced GIRK currents were not significantly affected by intracellularly applied ifenprodil ($90.4 \pm 12.7\%$ of untreated control current, paired *t*-test, $p > 0.1$, $n = 4$, Figure 5c), whereas such GIRK currents were significantly inhibited by intracellularly applied QX-314 as reported previously (Zhou *et al*, 2001; Kobayashi *et al*, 2003). The results indicate that intracellular ifenprodil could not inhibit GIRK channels and G-proteins mediated by κ OR activation. Taken together, it is suggested that extracellular ifenprodil directly inhibits GIRK channels activated by the κ OR.

a κ OR + GIRK

b

c

Figure 5 Inhibitory effect of ifenprodil on GIRK channels activated by a G-protein-coupled receptor. (a) In an oocyte co-injected with mRNAs for GIRK1 and GIRK2 subunits and the κ -opioid receptor (κ OR), current responses to 100 nM U50488H, 100 nM U50488H in the presence of 10 μM ifenprodil, and 100 nM U50488H are shown. Bars show the duration of application. Asterisk indicates the zero current level. (b) Concentration-dependent inhibition by ifenprodil of U50488H-induced GIRK currents. I_{control} is the amplitude of GIRK currents induced by 100 nM U50488H (538.0 ± 83.4 nA, $n = 5$), and I is the current amplitude in the presence of ifenprodil. Data points were fitted by using a logistic equation. (c) Lack of effect of intracellular ifenprodil on U50488H-induced GIRK currents. There was no significant difference in U50488H-induced GIRK currents between the groups before and after intracellular injection of ifenprodil ($p > 0.05$, $n = 4$, paired *t*-test). Current responses were measured at a membrane potential of -70 mV in a hK solution containing 96 mM K^+ . All values are the mean and SEM.

GIRK channels are also shown to be activated by ethanol independently of PTX-sensitive G-proteins (Kobayashi *et al*, 1999; Lewohl *et al*, 1999). Results of previous single-channel analyses with excised outside-out and cell-attached patch-clamp configurations suggested that ethanol activates GIRK channels directly without interacting with G-protein-signaling pathways and intracellular second messengers (Kobayashi *et al*, 1999). So we next examined the effect of ifenprodil on GIRK channel activation by ethanol. In oocytes expressing GIRK1/2 channels, the GIRK currents induced by ethanol were attenuated in the presence of ifenprodil, with an IC_{50} value of $6.1 \pm 1.8 \mu M$ and an n_H value of 0.69 ± 0.09 , in a reversible manner ($88.9 \pm 2.7\%$ inhibition at $100 \mu M$, $n = 6$; Figure 6). In addition, since the carboxyl terminal domains of GIRK channels are crucial for the ethanol sensitivity of the channel (Lewohl *et al*, 1999; Zhou *et al*, 2001), we examined whether intracellular ifenprodil affects ethanol activation of GIRK channels. However, the ethanol-induced GIRK currents were not significantly affected by intracellularly applied ifenprodil ($91.7 \pm 8.0\%$ of untreated control current, paired *t*-test, $p > 0.1$, $n = 4$, Figure 6c). These results, therefore, suggest that extracellular ifenprodil inhibits GIRK channels activated by ethanol.

Figure 6 Inhibitory effect of ifenprodil on the ethanol-induced GIRK currents in *Xenopus* oocytes expressing GIRK1/2 channels. (a) In an oocyte co-injected with GIRK1 and GIRK2 mRNAs, current responses to 100 mM ethanol, 100 mM ethanol in the presence of $3 \mu M$ ifenprodil, and 100 mM ethanol are shown. Asterisk indicates the zero current level. Bars show the duration of application. (b) Concentration-dependent inhibition by ifenprodil of ethanol-induced GIRK currents. $I_{control}$ is the amplitude of GIRK currents induced by 100 mM ethanol (316.5 ± 81.4 nA, $n = 6$), and I is the current amplitude in the presence of ifenprodil. Data points were fitted by using a logistic equation. (c) Lack of effect of intracellular ifenprodil on ethanol-induced GIRK currents. There was no significant difference in ethanol-induced GIRK currents between the groups before and after intracellular injection of ifenprodil ($p > 0.05$, $n = 4$, paired *t*-test). Current responses were measured at a membrane potential of -70 mV in a hK solution containing 96 mM K^+ . All values represent the mean and SEM.

DISCUSSION

Characteristics of GIRK Channel Inhibition by Ifenprodil

The present study demonstrated that ifenprodil inhibited brain-type GIRK1/2 and GIRK2 channels and cardiac-type GIRK1/4 channels at nanomolar concentrations or more in a distinctive manner. The inhibition of GIRK channels by ifenprodil was concentration-dependent, but voltage-independent and time-independent with a primarily significant effect on the instantaneous current and a steady percentage inhibition during each voltage pulse. Our results also suggest that ifenprodil acted at the channels from the extracellular side of the cell membrane. On the other hand, blockade by extracellular Ba^{2+} and Cs^+ , typical of Kir channel blockers that occlude the pore of the open channel, shows a concentration-dependence, a strong voltage-dependence, and a time-dependence with a comparatively small effect on the instantaneous current but a marked inhibition on the steady-state current at the end of voltage pulses (Lesage *et al*, 1995). These observations suggest that ifenprodil probably causes a conformational change in the GIRK channels, but does not act as an open channel blocker of the channels, as Ba^{2+} and Cs^+ do. The action mechanism may also be involved in the incomplete blockade of GIRK currents by ifenprodil. In the present study, ifenprodil similarly inhibited GIRK currents induced by basally free G-protein $\beta\gamma$ subunits present in oocytes, by G-proteins mediated by κOR activation, or by ethanol. Further studies using single channel experiments may be useful for understanding the mechanism of the action of ifenprodil on GIRK channels.

In addition, the potency of inhibition by ifenprodil of GIRK1/4 channels was higher than that of GIRK1/2 and GIRK2 channels. Although the rank order of the effectiveness by ifenprodil at the highest concentrations tested was $GIRK2 > GIRK1/2 \geq GIRK1/4$ channels, the differences were not statistically significant. Moreover, Kir1.1 and Kir2.1 channels in other Kir channel subfamilies were insensitive to ifenprodil. Further studies using GIRK/Kir1.1 and GIRK/Kir2.1 chimeric channels and mutant GIRK channels may clarify the critical sites mediating the effects of ifenprodil on GIRK channels. Furthermore, high-resolution structure analysis of GIRK channels may allow characterization of the binding sites. Additionally, although haloperidol is structurally related to ifenprodil (Williams, 2001), haloperidol weakly inhibits GIRK1/2 and GIRK1/4 channels in a similar manner (Kobayashi *et al*, 2000). The different effectiveness of these drugs on GIRK channels may be due to the different chemical structures between them or to their different binding sites on GIRK channels. Studies on the relationship between the structures of GIRK channels and the structure of ifenprodil may provide the basis for designing candidates for potent GIRK inhibitors.

Clinical and Pharmacological Implications

The human plasma concentrations of ifenprodil are reported to be approximately $0.1 \mu M$ after a single administration of its clinical dosage (Aventis Pharma's data). In animals, the radioactive ifenprodil in the brain and heart after its intramuscular administration was approxi-

mately 5–8 times and 5–10 times higher, respectively, than that in blood (Nakagawa *et al*, 1975). Therefore, the present findings suggest that GIRK channels in the brain and heart may be inhibited by ifenprodil at clinically relevant concentrations in these tissues. Activation of GIRK channels in physiological conditions induces K^+ efflux, leading to membrane hyperpolarization (North, 1989), whereas inhibition of GIRK channels leads to a depolarization of the membrane potential, resulting in an increase in cell excitability (Kuzhikandathil and Oxford, 2002). Therefore, in clinical use ifenprodil might affect various brain and heart functions via the inhibition of GIRK channels, which are expressed widely in the nervous system and the atrium (Kobayashi *et al*, 1995; Karschin *et al*, 1996).

GIRK2 knockout mice show spontaneous seizures and are more susceptible to seizures induced by pentylenetetrazol, a GABA_A receptor antagonist, than wild-type mice (Signorini *et al*, 1997). In addition, the resting membrane potentials of neurons in GIRK knockout mice were depolarized compared to those in wild-type mice (Lüscher *et al*, 1997; Torrecilla *et al*, 2002). High doses of ifenprodil potentiated seizures induced by some convulsants including pentylenetetrazol (Mizusawa *et al*, 1976), although ifenprodil has been shown to have anticonvulsant effects (Thurgur and Church, 1998; Yourick *et al*, 1999), probably due to inhibition of NMDA receptor channels (Williams, 2001) and Ca^{2+} channels (Church *et al*, 1994; Bath *et al*, 1996). In spite of its anticonvulsant property, potent blockade of neuronal GIRK channels by ifenprodil may contribute to the increased susceptibility to seizure by causing an increase in neuronal excitability.

Interestingly, GIRK2 knockout mice show reduced anxiety with an increase in motor activity in three tests for anxiety: the elevated plus-maze, light/dark box, and canopy test (Blednov *et al*, 2001). Ifenprodil had an anxiolytic property with an increase in locomotion in MF1 mice in the elevated plus-maze test (Fraser *et al*, 1996), although it had no anxiolytic effect in the light/dark exploratory test and caused no change in locomotor activity in Wistar rats (Mikolajczak *et al*, 2003). This discrepancy might have been caused by differences in the behavioral tests including difference in the ratio of the two light/dark compartments in the apparatus and/or in animal species. A clinical study showed that ifenprodil improved anxiety, a decrease in spontaneity, and melancholy in patients with sequelae of cerebrovascular diseases (Otomo *et al*, 1976). Therefore, inhibition of neuronal GIRK channels by ifenprodil might partly contribute to the clinical effects on anxiety and decreased activity, which are observed in some neuropsychiatric disorders as well.

In the heart, acetylcholine opens atrial GIRK channels via activation of the M_2 muscarinic acetylcholine receptor, and ultimately causes slowing of the heart rate (Brown and Birnbaumer, 1990). Sinus tachycardia during treatment with ifenprodil is observed along with its hypotensive effect (Carron *et al*, 1971; Young *et al*, 1983; Yajima *et al*, 1987). Ifenprodil exhibits no significant affinity for the muscarinic acetylcholine receptor (Chenard *et al*, 1991). The present study demonstrated that ifenprodil, at submicromolar concentrations or more, inhibited cardiac-type GIRK1/4 channels, which are abundantly present in the atrium of the heart (Krapivinsky *et al*, 1995). Therefore, atrial GIRK

channels may also be inhibited by ifenprodil in clinical practice. GIRK1 or GIRK4 knockout mice show mild tachycardia (Bettahi *et al*, 2002). Additionally, the hypotensive effect of ifenprodil may induce compensational activation of the sympathetic nervous system, which plays an important role in the stimulatory regulation of the heart rate. Taken together, our data suggest that sinus tachycardia during treatment with ifenprodil may be partly related to inhibition of atrial GIRK channels.

Ifenprodil influenced ethanol-related behavioral changes in animals, such as suppression of amnesic effects and withdrawal signs including convulsions (Malinowska *et al*, 1999; Napiórkowska-Pawlak *et al*, 2000; Narita *et al*, 2000). Ethanol activates GIRK channels (Kobayashi *et al*, 1999; Lewohl *et al*, 1999). The present study demonstrated that ifenprodil inhibited GIRK1/2 currents induced by ethanol. Interestingly, GIRK2 knockout mice show reduced ethanol-induced conditioned taste aversion and conditioned place preference (Hill *et al*, 2003), and are less sensitive to some of acute ethanol effects, including anxiolysis, habituated locomotor stimulation and handling-induced convulsions after an acute administration of ethanol, than wild-type mice (Blednov *et al*, 2001). Taken together, ifenprodil might suppress GIRK-related ethanol effects.

Morphine, a commonly used potent analgesic, preferentially binds to the μ -opioid receptor, and exerts various pharmacological effects, including analgesia, euphoria, and dependence (Gutstein and Akil, 2001). The μ -opioid receptor is coupled to G-protein-mediated signal transductions involving GIRK channels, adenylyl cyclase, Ca^{2+} channels, and phospholipase C (Ikeda *et al*, 2002). Although morphine produces a conditioned place preference in animals, indicating its rewarding effect, pretreatment with ifenprodil suppresses the rewarding effect produced by morphine (Suzuki *et al*, 1999). However, ifenprodil exhibits no significant affinity for the opioid receptors (Chenard *et al*, 1991). The present study demonstrated that ifenprodil inhibited G-protein-mediated GIRK currents. It may be important to determine whether GIRK channel function contributes to the rewarding effect of morphine. Interestingly, GIRK knockout mice show reduced self-administration of cocaine (Morgan *et al*, 2003). In a clinical report, desipramine, which acts as an inhibitor of GIRK channels as well as of norepinephrine transporters (Kobayashi *et al*, 2004b), facilitated initial abstinence from cocaine (Gawin *et al*, 1989). Thus, selective GIRK inhibitors might be potential agents for the treatment of abusers of cocaine. Further studies on the effects of ifenprodil on GIRK knockout mice might clarify the roles of the GIRK-mediated effects of ifenprodil in addiction to morphine and cocaine.

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