Mechanism underlying bupivacaine inhibition of G protein-gated inwardly rectifying K^+ channels

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Local anesthetics, commonly used for treating cardiac arrhythmias, pain, and seizures, are best known for their inhibitory effects on voltage-gated Na^+ channels. Cardiovascular and central nervous system toxicity are unwanted side-effects from local anesthetics that cannot be attributed to the inhibition of only Na^+ channels. Here, we report that extracellular application of the membrane-permeant local anesthetic bupivacaine selectively inhibited G protein-gated inwardly rectifying K^+ channels (GIRK:Kir3) but not other families of inwardly rectifying K^+ channels (ROMK:Kir1 and IRK:Kir2). Bupivacaine inhibited GIRK channels within seconds of application, regardless of whether channels were activated through the muscarinic receptor or directly via coexpressed G protein $G_{\beta\gamma}$ subunits. Bupivacaine also inhibited alcohol-induced GIRK currents in the absence of functional pertussis toxin-sensitive G proteins. The mutated GIRK1 and GIRK2 (GIRK1/2) channels containing the high-affinity phosphatidylinositol 4,5-bisphosphate (PIP_2) domain from IRK1, on the other hand, showed dramatically less inhibition with bupivacaine. Surprisingly, GIRK1/2 channels with high affinity for PIP_2 were inhibited by ethanol, like IRK1 channels. We propose that membrane-permeant local anesthetics inhibit GIRK channels by antagonizing the interaction of PIP_2 with the channel, which is essential for $G_{\beta\gamma}$ and ethanol activation of GIRK channels.

Local anesthetics are commonly used for treating cardiac arrhythmias, alleviating pain, and controlling seizures (1). Accidental overdose of local anesthetics, however, produces cardiovascular and central nervous system toxicity. The initial phase of bupivacaine overdose leads to tachycardia in the heart and convulsions in the brain (2). Local anesthetics are well known for their inhibitory effects on voltage-gated Na^+ channels (3). The suppression of voltage-gated Na^+ channels would be expected to reduce membrane excitability. The inhibition of K^+ channels, however, increases membrane excitability and could therefore contribute to bupivacaine-induced tachycardia and convulsions. Indeed, some local anesthetics inhibit voltage-gated K^+ channels (4–6). The effect of local anesthetics on native inwardly rectifying K^+ channels has been equivocal (5–8). Interestingly, several investigators have observed that a permanently charged derivative of lidocaine, QX-314, suppresses the activity of G protein-gated inwardly K^+ currents when introduced directly into the cytoplasm of neurons (9–11). QX-314 is not clinically useful, however, because it does not cross the cell membrane. We report here that the family of G protein-gated inwardly rectifying K^+ channels (GIRK), but not other families of inwardly rectifying K^+ channels, are inhibited by the clinically relevant membrane-permeant local anesthetics.

GIRK channels (also referred to as Kir3) are members of a family of inwardly rectifying K^+ channels that contain seven different groups (Kir1–7) (12). Like all inwardly rectifying K^+ channels, GIRK channels sustain a larger influx than efflux of K^+ ions. The small efflux of K^+ ions near the cell's resting membrane potential, however, is responsible for reducing membrane excitability (3). Many neurotransmitters exert their inhibitory actions, in part, by stimulating pertussis toxin (PTX)-sensitive G protein-coupled neurotransmitter receptors and activating GIRK channels (3). Mutant mice that lack GIRK2 channels and $GABA_B$ receptor-activated GIRK currents are more susceptible to seizures (13, 14). GIRK channel activity is also important for regulating the heart beat (15). Inhibiting GIRK channel activity therefore has the potential to affect cardiac and brain function.

GIRK channels contain cytoplasmic N- and C-terminal domains, two putative transmembrane domains (M1, M2), and a highly conserved pore–loop complex that is involved in ion selectivity (16). For brain and cardiac GIRK channels, considerable evidence indicates that the direct binding of G protein $G_{\beta\gamma}$ subunits to the channel protein opens GIRK channels (17–20). In addition to $G_{\beta\gamma}$ subunits, GIRK channels can also be activated by alcohol through a G protein-independent pathway (21, 22). Both $G_{\beta\gamma}$ subunits and alcohol seem to open GIRK channels by interacting with amino acids in the C-terminal domain of GIRK channels (17, 19, 21, 23). The activation by $G_{\beta\gamma}$ subunits is regulated by the levels of phosphatidylinositol 4,5-bisphosphate (PIP_2) in the membrane; $G_{\beta\gamma}$ subunits fail to activate GIRK channels in the absence of PIP_2 (24). Little is known about the molecular mechanism underlying alcohol activation of GIRK channels.

In this article, we report a specific inhibitory action of membrane-permeant local anesthetics on $G_{\beta\gamma}$ and ethanol activation of GIRK channels. Mutant GIRK1 and GIRK2 channels that contained the high-affinity PIP_2 domain from IRK1 (25) showed greatly reduced inhibition with local anesthetics and were not activated by ethanol. We propose that both bupivacaine and ethanol exert their actions by modulating the interaction of PIP_2 with GIRK channels.

Methods

Molecular Biology. GIRK1 cDNA was in pBSK (26), GIRK2 cDNA was in pBTG (27), ROMK1 was in pSPORT (28), and GIRK4 cDNA was in pBSK (29). The GIRK4-GIRK1 dimer was provided by E. Reuveny. The construction of GIRK1–GIRK2 dimer and chimera I1G2(96–189) was described previously (14, 30). Chimera R1G2(97–168) contained amino acids Met-1–Thr-82 and Ala-156–Met-391 from ROMK1 and Ile-97–Glu-168 from GIRK2. Chimera R1G2(118–193) contained Met-1–Tyr-104 and Lys-181–Met-391 from ROMK1 and Val-118–Val-193 from GIRK2. The PIP_2 domain from IRK1 (Lys-207–Leu-245), as defined by Zhang *et al.* (25), was transferred into GIRK1 (Lys-208–Leu-246) and GIRK2 (Lys-219–Leu-257) by using overlapping PCR. *In vitro* methyl-capped cRNA was made from linearized cDNA with T3 or T7 RNA polymerase (Stratagene). The quality of cRNA was evaluated on an ethidium-stained formaldehyde gel and the concentration was measured by spectrophotometry. *Xenopus* oocytes were isolated as described previously (31). Oocytes were injected with a 46-nl solution

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Abbreviations: GIRK, G protein-gated inwardly rectifying K^+ channels; PTX, pertussis toxin; PIP_2, phosphatidylinositol 4,5-bisphosphate.

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containing cRNA for the G protein $G_{\beta 1}$ (≈1–8 ng) and $G_{\gamma 2}$ (≈1–8 ng) subunits or the human m2 muscarinic receptor (0.2–1 ng) and the GIRK channels (0.5 to 5 ng). In some experiments, PTX-S1 cRNA (≈2 ng) was coinjected with the cRNA for GIRK channels (32). Oocytes were incubated in ND96 (96 mM NaCl/2 mM KCl/1 mM $CaCl_2$/1 mM $MgCl_2$/5 mM Hepes, pH 7.6 with NaOH) for 1–7 days at 18°C.

Electrophysiology and Biochemistry. Macroscopic currents were recorded from oocytes with a two-electrode voltage-clamp amplifier (Geneclamp 500, Axon Instruments, Foster City, CA), filtered at 0.05–2 kHz, digitized (0.1–2 kHz) with a Digidata 1200 A/D interface (Axon Instruments), and stored on a laboratory computer. Electrodes were filled with 3 M KCl and had resistances of 0.6–1 MΩ. Oocytes were perfused continuously with a solution containing 90 mM XCl [X = K^+, Na^+, or *N*-methyl-D-glucamine (NMDG)], 2 mM $MgCl_2$, and 10 mM Hepes (pH 7.5 with ≈5 mM XOH or HCl for NMDG). Na/NMDG solutions were used to determine the leak current. The pH 6 and 9 solutions were made by adding HCl or NaOH, respectively. QX-314 (Research Biochemicals) and lidocaine (Sigma) were dissolved in nanopore deionized H_2O (dH_2O) at a concentration of 40–50 mM. Bupivacaine (100 mM, Sigma) was dissolved in 95K^+. Stock solutions were diluted in the extracellular solution before each experiment. For ethanol activation, 100% ethanol was added directly to the 95K^+ solution to give 100 mM ethanol (EtOH density = 0.7893 g/ml). A small chamber (0.125 × 0.600 inches) with fast perfusion was used to change the extracellular solutions and was connected to ground via a 3 M KCl/agarose bridge. For examining the effect of intracellular QX-314, we injected 32.2 nl of 20 mM QX-314 dissolved in dH_2O and waited 30–60 min before recording the currents for a second time. Assuming a volume of ≈1 μl in the oocyte (33), the intracellular concentration was ≈625 μM. $G_{\beta\gamma}$ binding to glutathione *S*-transferase fusion proteins was measured as described previously (17).

Analysis. For dose–response experiments, the data were normalized by dividing the current in the presence of the drug by the current in the absence of the drug (I/I_o) and fit with the Hill equation, $I/I_o = 1/(1 + ([X]/K_i)^h)$, where K_i = the concentration at which there is ½ inhibition and h = the Hill coefficient. The mean K_i and Hill coefficients were determined by fitting each set of data points individually. All values were reported as mean ± SEM. Data were analyzed for statistical significance (SIGMASTAT) by using Student's unpaired *t* test or one-way ANOVA followed by an appropriate post hoc test. Values of $P < 0.05$ were considered significant.

Results

Bupivacaine Inhibits GIRK Channels. GIRK channels exist primarily as heteromultimers of GIRK subunits in the brain and heart (29, 34) and homomultimers in the substantia nigra (35). Stimulation of neurotransmitter receptors coupled to $G_{\alpha i/o}$ (PTX-sensitive) G proteins leads to the release of $G_{\beta\gamma}$ subunits, which then open GIRK channels (36–38). To examine the effect of local anesthetics on GIRK channels, we coexpressed the m2 muscarinic receptor, which couples to $G_{\alpha i/o}$ G proteins, along with the GIRK1 and GIRK4 channel subunits in *Xenopus* oocytes. After GIRK1/4 channels were opened by stimulation of the m2 muscarinic receptor, extracellular application of the local anesthetic bupivacaine (500 μM; racemic mixture of *S*(−) and *R*(+) enantiomers) produced a rapid and complete inhibition of inward K^+ current through GIRK1/4 channels (Fig. 1*a*). Note that the rate of inhibition was much faster than the rate of deactivation after removal of the muscarinic agonist; deactivation is caused by reassociation of $G_{\beta\gamma}$ subunits with G_α-GDP into the inactive G protein heterotrimer ($G_{\alpha\beta\gamma}$). When coapplied

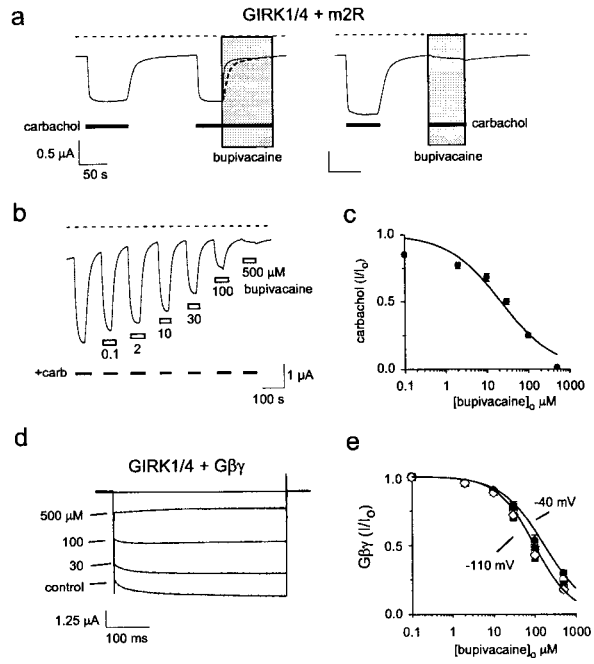

Fig. 1. Extracellular bupivacaine inhibits GIRK1/4 channels. Oocytes were injected with the cRNA for GIRK4-GIRK1 dimer plus the m2 muscarinic receptor. (*a*) Macroscopic current was recorded continuously by using two-electrode voltage clamp from an oocyte bathed in 95 mM KCl (pH 7.5) with the muscarinic agonist carbachol (0.3 μM; solid bar) and then with carbachol plus 500 μM bupivacaine (gray box). The holding potential (V_H) was −80 mV. Dashed line indicates the zero current level. (*Right*) Coapplication of carbachol and bupivacaine (500 μM). (*b*) Continuous current recording shows the effect of increasing concentrations of bupivacaine coapplied with carbachol. (*c*) The carbachol-induced current was normalized (carbachol, I/I_o) and plotted as a function of bupivacaine concentration. Smooth curve shows the best fit to the Hill equation, with an apparent K_i of 22 ± 4 μM and a Hill coefficient of 0.68 ± 0.06 ($n = 7$). (*d*) *Xenopus* oocytes injected with the cRNA for GIRK1, GIRK4, and the G protein $G_{\beta 1}$ and $G_{\gamma 2}$ subunits. Current responses were elicited by 500-ms voltage step to −80 mV (V_H = 0 mV) in 0, 30, 100, or 500 μM bupivacaine. (*e*) The normalized current ($G_{\beta\gamma}$, I/I_o) was plotted as a function of bupivacaine concentration. Smooth curves show the best fit to the Hill equation for −40 mV (K_i = 170 ± 12 μM; Hill = 0.83 ± 0.04) and −110 mV (K_i = 98 ± 3 μM; Hill = 0.96 ± 0.03).

with bupivacaine, carbachol induced little or no inward current (Fig. 1*a*). We quantified the sensitivity of GIRK1/4 channels to inhibition by extracellular bupivacaine by measuring the amplitude of the carbachol-induced current in different concentrations of bupivacaine (Fig. 1*b*). The Hill plot indicated an apparent inhibition constant (K_i) of 22 ± 4 μM. The Hill coefficient was near unity (0.68 ± 0.06, $n = 7$), indicating little cooperativity in the bupivacaine inhibition of GIRK1/4 channels (Fig. 1*c*). To the best of our knowledge, this is the first report that extracellular application of a local anesthetic inhibits GIRK channels.

To determine whether the inhibition produced by bupivacaine occurred through an alteration in the G protein coupling of the m2 muscarinic receptor (39), the G protein-coupled receptor was bypassed by injecting the cRNA for the G protein $G_{\beta\gamma}$ subunits ($G_{\beta 1}$ and $G_{\gamma 2}$) into *Xenopus* oocytes along with the cRNA for the GIRK1 and GIRK4 subunits. Under these conditions, oocytes express large inwardly rectifying K^+ currents that are produced by the persistent activation of GIRK channels by $G_{\beta\gamma}$ subunits (36). Like the carbachol-induced current, the large inward K^+ current was inhibited by bupivacaine in a dose-dependent manner (Fig. 1*d*). The inhibition of GIRK1/4

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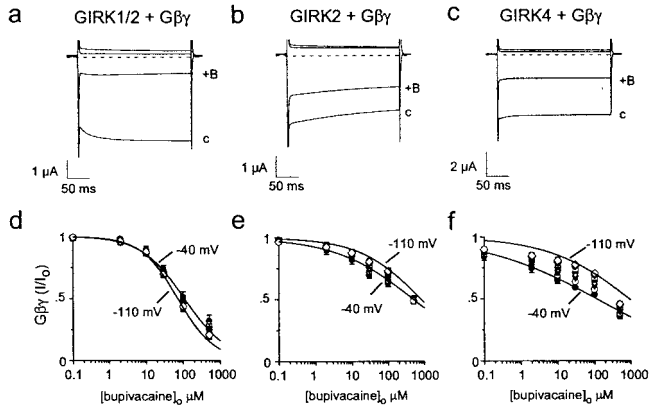

Fig. 2. GIRK2 and GIRK4 homomultimers are less sensitive than GIRK1/2 or GIRK1/4 to bupivacaine inhibition. Oocytes were injected with cRNA for $G_{\beta 1\gamma 2}$ and the cRNA for GIRK1-GIRK2 dimer, GIRK2, or GIRK4. (*a–c*), Current responses were elicited by voltage steps to +50 and −100 mV in the absence and then presence of 500 μM bupivacaine (pH 7.5). I/I_o is plotted as a function of bupivacaine concentration for GIRK1/2 (*d*), GIRK2 (e), and GIRK4 (*f*) at voltages ranging from −110 mV to −40 mV (V_H = 0 mV). The smooth curves show the best fit to the Hill equation for −110 and −40 mV. For GIRK1/2, the K_i = 107 ± 14 μM (Hill coefficient h = 0.75 ± 0.06) at −40 mV and K_i = 71 ± 7 μM (h = 0.88 ± 0.03) at −110 mV. For GIRK2, the K_i = 500 ± 128 μM (h = 0.40 ± 0.05) and at −110 mV, the K_i = 825 ± 297 μM (h = 0.51 ± 0.07). For GIRK4, the K_i = 114 ± 26 μM (h = 0.28 ± 0.07) at −40 mV and K_i = 914 ± 394 μM (h = 0.39 ± 0.10) at −110 mV.

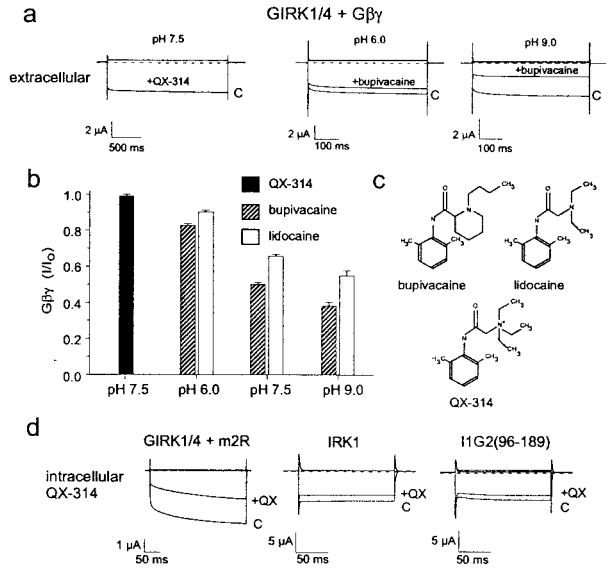

Fig. 3. Effect of neutral and charged forms of local anesthetics on GIRK channels. (*a*) Oocytes were injected with the cRNA for GIRK1 and GIRK4 subunits plus $G_{\beta 1\gamma 2}$ subunits. Current responses elicited by voltage steps to +50 and −100 mV are shown for extracellular QX-314 (500 μM) and bupivacaine (100 μM) at pH 6.0 and 9.0. The K^+ current changed less than 10% when switching among different pH solutions. (*b*) Fractional current remaining ($G_{\beta\gamma}$, I/I_o) is shown for 100 μM QX-314 (pH 7.5, n = 5), 100 μM bupivacaine (pH = 6, 7.5, and 9; n = 6), and 100 μM lidocaine (pH = 6, 7.5, and 9; n = 6). (*c*) Chemical structures for bupivacaine, lidocaine, and QX-314. (*d*) Intracellular QX-314 inhibits G protein activation of GIRK channels but not I1G2(96–189). Oocytes were injected with the cRNA for GIRK1, GIRK4, and m2 muscarinic receptor, IRK1, or chimera I1G2(96–189). Current responses were elicited by voltage steps from +50 and −100 mV (V_H = 0 mV) before and then 30–60 min following the injection of QX-314 into the same oocyte (n = 6). The carbachol-induced (+carb-basal) current is shown for GIRK1/4.

channels stimulated by $G_{\beta\gamma}$ had a K_i of 106 ± 5 μM and a Hill coefficient of 0.94 ± 0.03 at −80 mV (Fig. 1*e*), which was 5.4-fold less inhibition than the K_i for inhibition of muscarinic-activated GIRK1/4 channels. Bupivacaine did not appear to exert its actions by interfering with coupling of the G protein-coupled receptor but was less effective with high levels of $G_{\beta\gamma}$.

The inhibition of $G_{\beta\gamma}$-stimulated GIRK1/4 channels developed instantaneously with 30, 100, and 500 μM bupivacaine (Fig. 1*d*), suggesting that the channels were inhibited at the holding potential of 0 mV or the inhibition was too fast to be resolved with the perfusion system. The inhibition produced by bupivacaine showed weak voltage dependence over voltages of −40 to −110 mV (Fig. 1 *d* and *e*). By contrast, the inhibition of nonselective GIRK channels with permanently charged local anesthetics showed strong voltage dependence (δ = ≈0.7), suggesting the neutral form of bupivacaine may mediate some of the inhibition (30).

We compared the sensitivity of different GIRK channel heteromultimers and homomultimers to inhibition by extracellular bupivacaine. Bupivacaine inhibited the inward current through $G_{\beta\gamma}$-stimulated GIRK1/4 or GIRK1/2 heteromultimers to nearly the same extent (Fig. 2). By contrast, homomultimers composed of GIRK2 or GIRK4 subunits coexpressed with $G_{\beta\gamma}$ subunits were ≈5 times less sensitive to inhibition than heteromultimers containing the GIRK1 subunits (Figs. 1*e* and 2). The K_i at −80 mV was 748 ± 261 and 542 ± 213 μM for GIRK2 and GIRK4 homomultimers, respectively. The bupivacaine sensitivity (I/I_o) of the carbachol-induced current for GIRK2 homomultimers (0.18 ± 0.03, n = 10), however, was similar to that of heteromultimers of GIRK1/2 (0.07 ± 0.02, n = 5). Like GIRK1/4 or GIRK1/2 heteromultimers, the K_i for bupivacaine inhibition of GIRK2 or GIRK4 homomultimers showed little change with voltage but tended to increase with hyperpolarization (Figs. 1*e* and 2). This effect of voltage was not investigated further.

Neutral Form of Bupivacaine Is Required for Inhibition of GIRK Channels. Ionizable local anesthetics, such as bupivacaine, exist in a protonated or neutral form, depending on the pH and pK_a of the local anesthetic. We examined the effect of pH on bupivacaine (pK_a = 8.1) inhibition of GIRK channels (Fig. 3). An acidic pH of 6.0 reduced the inhibition, whereas a basic pH of 9.0 enhanced the inhibition by 100 μM bupivacaine (Fig. 3 *a* and *b*). We also examined the effect of pH on the inhibition of GIRK channels by lidocaine (pK_a = 7.7), a related but smaller local anesthetic (Fig. 3 *b* and *c*). Like bupivacaine, lidocaine inhibited a larger fraction of current at basic pH (Fig. 3*b*). The permanently charged derivative of lidocaine, QX-314 (extracellular), produced no significant decrease in the inward K^+ current (Fig. 3 *a–c*). Similar results were obtained with local anesthetic inhibition of voltage-gated muscle Na^+ channels (33, 40).

To see whether the protonated form of bupivacaine might interact with the channel, we examined the effect of intracellularly applied QX-314. We compared the carbachol-induced GIRK currents before and after injecting QX-314 directly into the oocyte. Although QX-314 (≈625 μM) inhibited the basal current of IRK1 (0.71 ± 0.05 of control, n = 8), in contrast to the effect of bupivacaine on IRK1, QX-314 inhibited significantly more of the carbachol-induced GIRK1/4 current (0.51 ± 0.03 of control, n = 7; see Fig. 3*d*). Taken together, the results suggest that the neutral form of bupivacaine permeates the membrane, whereupon it may interfere with GIRK channel activity in its neutral and/or protonated form.

Bupivacaine Inhibits Ethanol-Induced GIRK Currents. GIRK channels are activated by ethanol via a mechanism that does not require G proteins (21, 22). We postulated that if bupivacaine simply antagonized $G_{\beta\gamma}$ activation of GIRK channels, then bupivacaine would not suppress ethanol-induced currents. We first examined the effect of 100 mM ethanol on oocytes expressing the m2

muscarinic receptor with GIRK1/4 channels in the absence and then presence of bupivacaine. Surprisingly, bupivacaine reduced (I/I_o) the ethanol-induced GIRK1/4 current to 0.19 + 0.02 of control (Fig. 4 *a* and *c*). To examine the effect of ethanol and bupivacaine in the absence of G protein activation, we coexpressed the catalytic subunit (PTX-S1) of PTX (32), which uncouples $G_{i/o}$ G proteins by ADP-ribosylating the $G_{\alpha i/o}$ subunits. In oocytes coexpressing PTX, the carbachol-induced current was completely suppressed (Fig. 4*b*), indicating that the endogenous $G_{i/o}$ G proteins were uncoupled from the m2 receptor. In the absence of functional PTX-sensitive G proteins, bupivacaine inhibited the ethanol-induced GIRK1/4 current to 0.16 ± 0.05 of control (Fig. 4 *a* and *c*). These results suggest that bupivacaine inhibits GIRK1/4 channels by interfering with activation steps common to both $G_{\beta\gamma}$ subunits and ethanol.

G Protein-Insensitive Inward Rectifiers Are Insensitive to Bupivacaine. We next examined the ability of external bupivacaine to inhibit current through the G protein-*insensitive* inward rectifiers, such as IRK1 (Kir2.1) and ROMK1 (Kir1.1). Unlike GIRK heteromultimers, the current through IRK1 or ROMK1 channels was not inhibited by 500 μM bupivacaine (Fig. 5 *a*, *b*, and *e*). We investigated whether the G protein sensitivity of GIRK channels was an essential component to the inhibition produced by bupivacaine. To determine whether the bupivacaine interfered with the binding of $G_{\beta\gamma}$ to the channel, we examined the effect of bupivacaine (500 μM) on $G_{\beta 1\gamma 7}$ binding measured biochemically *in vitro* (17). $G_{\beta 1\gamma 7}$ coprecipitated with a glutathione *S*-transferase fusion protein that contained either the N- or C-terminal domain from GIRK1 (Fig. 5*f*); 500 μM bupivacaine had no detectable effect on $G_{\beta\gamma}$ binding. Thus, bupivacaine did not inhibit GIRK channels by antagonizing the binding of $G_{\beta\gamma}$ to the channel.

To localize the region required for bupivacaine inhibition of GIRK channels, we constructed chimeras of GIRK2 and IRK1 or ROMK1. We used GIRK2 for these chimeric studies because GIRK2 channels readily form K^+ selective homomultimers in *Xenopus* oocytes, unlike GIRK1 chimeras (30). A chimeric channel in which the N- and C-terminal domains of GIRK2, which are responsible for $G_{\beta\gamma}$ binding and gating (17–20, 41), were replaced with the homologous regions from IRK1 [chimera I1G2(96–189)] was insensitive to bupivacaine (Fig. 5 *d* and *e*) and not regulated by G proteins (data not shown). Chimera I1G2(96–189) also displayed reduced sensitivity to inhibition with internal QX-314 (0.80 ± 0.07 of control, $n = 8$; Fig. 3*d*), like that of IRK1. Two ROMK1/GIRK2 chimeras, containing the pore–loop complex and either the M1 [R1G2(97–168)] or M2 [R1G2(118–193)] transmembrane domain of GIRK2, were also insensitive to bupivacaine (Fig. 5 *c* and *e*). Thus, the N- and/or C-terminal domains of IRK1 or ROMK1 seem to confer bupivacaine insensitivity.

GIRK channels have lower affinity for PIP_2 than IRK1 and ROMK1 channels (24, 25). We hypothesized that the strength of interaction with PIP_2 might be important for bupivacaine inhibition. To test this directly, we replaced the PIP_2 domain in GIRK1 and GIRK2 with the homologous region from IRK1 to create GIRK channels with "high" affinity for PIP_2 (25). Heteromultimers composed of GIRK1(PIP_2) and GIRK2(PIP_2) exhibited large basal currents (Fig. 6 *b* and *e*) and could not be activated with stimulation of the muscarinic receptor. In addition, 200 mM ethanol inhibited, not activated, GIRK1(PIP_2) and GIRK2(PIP_2), similar to its effect on IRK1 channels (21). Remarkably, these high-affinity PIP_2 GIRK channels showed dramatically less sensitivity to inhibition with bupivacaine (Fig. 6 *b* and *f*). Similar results were obtained with GIRK2(PIP_2) homomultimers (Fig. 6 *c*, *e*, and *f*). We also examined the effect of a point mutation in the PIP_2 binding domain that reportedly increases the affinity for PIP_2 (25). These point mutations alone were less effective at reducing the bupivacaine inhibition than transferring the entire PIP_2 domain (Fig. 6 *d–f*). The results

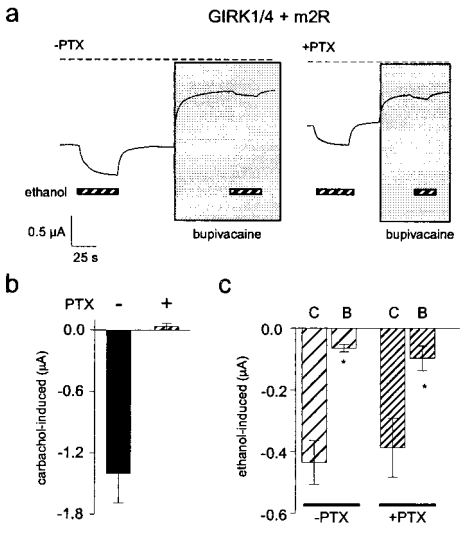

Fig. 4. Bupivacaine inhibits ethanol-induced GIRK currents in absence of functional PTX-sensitive G proteins. (*a*) Oocytes were injected with the cRNAs for GIRK1, GIRK4, and m2 muscarinic receptor alone or with the cRNA for PTX-S1. Continuous current recordings show the response to 100 mM ethanol in the absence and then presence of 500 μM bupivacaine ($V_H = -80$ mV). Note that bupivacaine also inhibited the PTX-resistant basal current. (*b*) Bar graph shows the average carbachol-induced currents in oocytes with or without coexpressed PTX-S1 ($n = 5$). (*c*) Bar graph shows the effect of 500 μM bupivacaine on ethanol-induced currents in the absence or presence of coexpressed PTX-S1 ($n = 5$). Asterisk indicates statistically significant difference ($P < 0.05$) between ethanol-induced current in the absence (*c*) and presence (*b*) of bupivacaine, using unpaired *t* test.

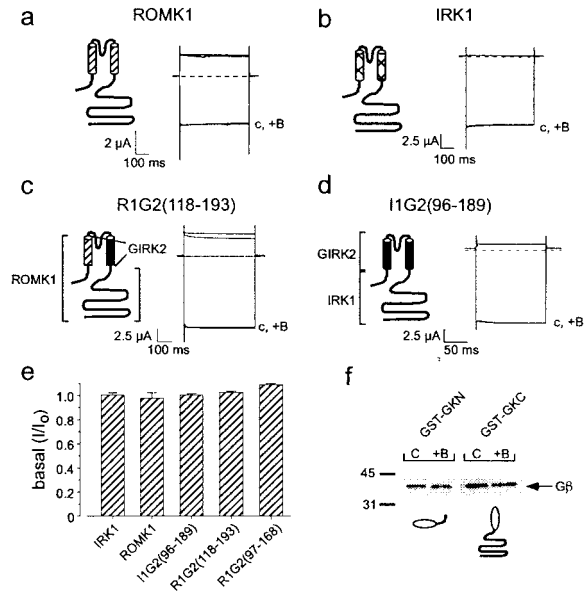

Fig. 5. G protein-insensitive inwardly rectifying K^+ channels are resistant to bupivacaine inhibition. Oocytes were injected with the cRNA for ROMK1, IRK1, R1G2(118–193), R1G2(97–168), or I1G2(96–189). (*a–d*) Current responses were elicited by voltage steps to +50 and −100 mV ($V_H = 0$ mV) in the absence and then presence of extracellular bupivacaine (500 μM, pH 7.5). (*e*) Fractional current remaining (I/I_o) shown for IRK1 ($n = 4$), ROMK1 ($n = 5$), I1G2(96–189) ($n = 5$), R1G2(118–193) ($n = 5$), and R1G2(97–168) ($n = 5$). (*f*) Western blot stained with anti-G_β G protein antibody. Pull-down assay was used to measure the $G_{\beta 1\gamma 7}$ binding to a glutathione *S*-transferase fusion protein containing the N- or C-terminal domain of GIRK1 in the absence (*c*) or presence (*b*) of 500 μM bupivacaine.

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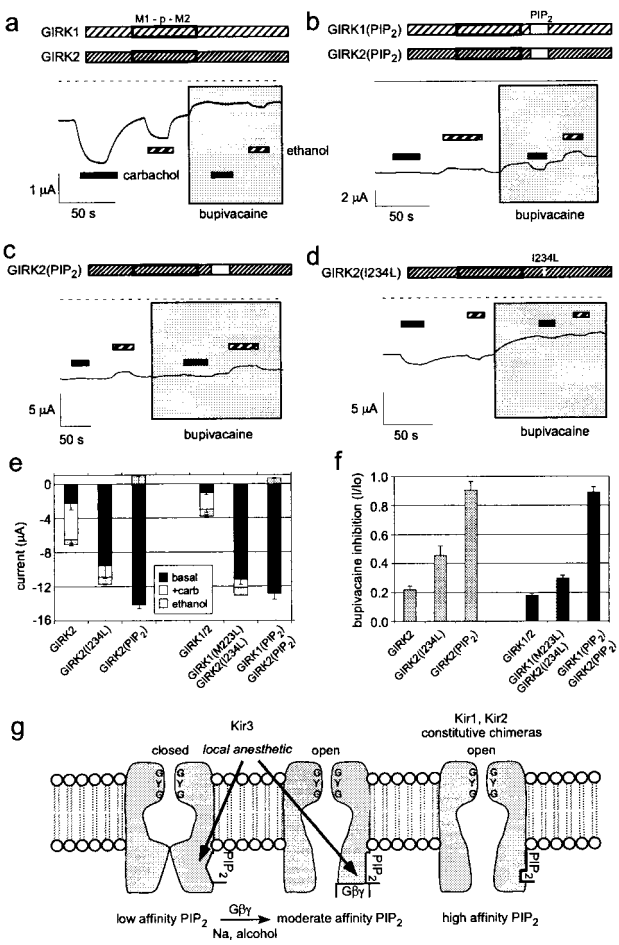

Fig. 6. Increasing PIP_2 affinity dramatically alters the inhibition by bupivacaine and activation by ethanol. (*a–f*) *Xenopus* oocytes were injected with the cRNA for GIRK1/2, GIRK1(PIP_2)/GIRK2(PIP_2), GIRK2(PIP_2), GIRK1(M223L)/GIRK2(I234L), or GIRK2(I234L), along with the m2 muscarinic receptor. (*a–d*) Continuous current recordings show the response to 10 μM carbachol or 200 mM ethanol in the absence and then presence of 500 μM bupivacaine (shaded box). The extracellular solution was 95 mM KCl (pH 7.5). (*e*) Bar graph shows the amplitude of the basal (agonist-independent), carbachol-stimulated (+carb-basal), and ethanol-stimulated (+ethanol-basal), $n = 5–12$. (*f*) Bar graph shows the fractional current (I/I_o) remaining with 500 μM bupivacaine. The total current (basal+carb) was measured. (*g*) Working model proposes that local anesthetic inhibition and ethanol activation result from regulating the interaction of PIP_2 with GIRK channels. Both $G_{\beta\gamma}$ and Na^+ help stabilize PIP_2, making GIRK channels more resistant to inhibition with local anesthetics. Ethanol enhances the interaction of PIP_2 with GIRK channels or increases the levels of PIP_2. The model predicts that some local anesthetics might inhibit IRK1 or ROMK1 channels.

suggest that bupivacaine exerts its action by regulating the interaction of PIP_2 with the proximal C-terminal domain of GIRK channels (Fig. 6*g*).

Discussion

Local anesthetics are best known for their actions on voltage-gated Na^+ channels (3). We report here that GIRK (Kir3) channels, but not ROMK1 (Kir1) or IRK1 (Kir2) channels, are also targets for the membrane-permeant local anesthetics. Similar to the effect of local anesthetics on some types of voltage-gated Na^+ channels (33, 40), extracellular ionizable local anesthetics but not the permanently charged QX-314 inhibited GIRK channels. In contrast to voltage-gated Na^+ channels, the inhibition of GIRK channels was not use- or frequency-dependent. In fact, bupivacaine inhibited GIRK channels regardless of whether the channels were in the agonist-independent basal state or constitutively open ($G_{\beta\gamma}$ stimulated) state. In addition, mutagenesis studies of voltage-gated Na^+ channels implicated both the pore–loop complex and the S6 transmembrane domain, which form a majority of the pore, in the inhibition by local anesthetics (33, 40). The pore–loop complex and M2 transmembrane domain of GIRK2 channels, however, did not confer bupivacaine sensitivity. Rather, the interaction with PIP_2 seemed to determine the extent of inhibition (see below).

The selective inhibition of GIRK channels by local anesthetics is notable for two reasons. First, no pharmacological tools are available to distinguish GIRK channels from ROMK1 or IRK1 channels. Inwardly rectifying K^+ channels are inhibited by cationic channel blockers (e.g., Cs^+, tetraethylammonium$^+$, and Ba^{2+}), but these ions discriminate poorly among the different types of inwardly rectifying K^+ channels (3). A limited number of peptide toxins inhibit inwardly rectifying K^+ channels, yet none has been isolated that selectively inhibits GIRK channels (42–44). Second, accidental overdose of local anesthetics produces cardiovascular and central nervous system toxicity. The levels of bupivacaine in blood (34–83 μM) that produce seizures in sheep (45) are close to the K_i determined for bupivacaine inhibition of GIRK channels ($\approx$20 μM). Some types of voltage-gated K^+ channels are also inhibited by bupivacaine (4–6). Thus, the suppression of GIRK channel activity together with other K^+ channels may contribute to local anesthetic toxicity.

Model for Regulation of GIRK Channels by Local Anesthetics and Ethanol. Bupivacaine inhibited GIRK channels within seconds of application. In studies of other ion channels, bupivacaine inhibition took several minutes (4, 5, 46, 47), leading to the proposition that the neutral form crosses the cell membrane and acts at an intracellular site. Lipophilic local anesthetics permeate the membrane very rapidly, however, as was shown for voltage-gated Na^+ channels (48) and some K^+ channels (6). The enhanced inhibition of bupivacaine at basic pH and the lack of effect with permanently charged QX-314 (extracellular) suggest that the neutral form of the local anesthetic partitions into the membrane, whereupon the neutral and/or protonated form acts on the ion channel. The inhibition of carbachol-activated GIRK currents with intracellular QX-314 indicated the protonated form of bupivacaine could produce some inhibition. The mechanism of inhibition with intracellular QX-314 may be similar to that produced with extracellular bupivacaine, as the bupivacaine-insensitive chimera I1G2(96–189) showed little inhibition with intracellular QX-314 or extracellular bupivacaine. Further studies are needed to determine the extent of inhibition produced by the neutral or charged form of bupivacaine.

How are GIRK channels but not other inwardly rectifying K^+ channels inhibited by local anesthetics? There are several sites of action that seem unlikely. First, local anesthetics have been reported to interfere with G protein signaling of neurotransmitter receptors (39, 49). In this study, bupivacaine inhibited GIRK channels that were constitutively activated by $G_{\beta\gamma}$ subunits, bypassing the G protein-coupled receptor. Bupivacaine inhibition of GIRK channels was not therefore caused by interference with G protein signaling. Second, bupivacaine inhibition did not show a time-dependent change in inward current or strong voltage dependence that is typical of cationic channel inhibitors that directly occlude the channel pore (50). Third, bupivacaine had no effect on the biochemical binding of $G_{\beta\gamma}$ to a fusion protein containing the N- or C-terminal domain of GIRK1, the two regions implicated in $G_{\beta\gamma}$ binding and activation (17–20). Moreover, bupivacaine dramatically suppressed ethanol-induced inward GIRK currents under conditions in which endogenous $G_{\alpha i/o}$ G proteins were uncoupled with PTX. These findings suggest that bupivacaine interferes with an activation mechanism that is common to both $G_{\beta\gamma}$ and ethanol.

Recently, PIP_2 has been identified as an important regulator in $G_{\beta\gamma}$ activation of GIRK channels (24, 25). Although PIP_2 is required for the activity of all inward rectifiers (24), GIRK channels have lower affinity for PIP_2 than IRK1 or ROMK1. The presence of $G_{\beta\gamma}$, however, seems to stabilize the interaction with PIP_2 (24). We propose that local anesthetics antagonize the interaction of PIP_2 with GIRK channels, thereby decreasing the current (Fig. 6*g*). Channels with high affinity for PIP_2, such as IRK1 and ROMK1, are resistant to inhibition with bupivacaine. Consistent with this model, mutations in GIRK1 and GIRK2 that are expected to increase the affinity for PIP_2 (25) dramatically attenuated the inhibition with bupivacaine. In addition, a GIRK2 chimera containing the N- and C-terminal domains of IRK1 was insensitive to bupivacaine. Taken together, these results suggest the local anesthetics either bind directly to the channel segment that interacts with PIP_2 or alter the local membrane structure, allowing PIP_2 to dissociate from the channel. Local anesthetics can perturb the membrane lipid structure (51). The stabilization of PIP_2 by $G_{\beta\gamma}$ subunits can then explain the 5-fold shift in K_i for $G_{\beta\gamma}$-stimulated GIRK channels as compared with carbachol-stimulated channels. Other factors may also contribute to the bupivacaine inhibition, however, because GIRK2 and GIRK1/2 channels have similar PIP_2 affinity (24) but differ in their sensitivity to bupivacaine.

The mechanism underlying ethanol activation of GIRK channels but not IRK1 or ROMK1 channels is not well understood. An analysis of GIRK2 channels with C-terminal deletions suggested that the distal C-terminal domain was involved in ethanol activation (21). Our results with GIRK1/2 channels that have high affinity for PIP_2 implicate the proximal C-terminal domain of GIRK channels. Ethanol may enhance the interaction of PIP_2 with GIRK channels. Alternatively, ethanol has been reported to increase the activity of phosphatidylinositol transfer protein, which could increase the levels of PIP_2 (52). Ethanol may not activate ROMK (Kir1) and IRK (Kir2) channels because of their high affinity for PIP_2. The model may also explain the ethanol insensitivity of GIRK2^{wv} channels (22); the enhanced Na^+ activation of GIRK2^{wv} channels may stabilize PIP_2, thereby decreasing ethanol activation (53–55).

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