The Sensitivity of G Protein-activated K⁺ Channels toward Halothane Is Essentially Determined by the C Terminus*

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G protein-activated K⁺ channels (GIRKs or Kir3.x) are targets for the volatile anesthetic, halothane. When coexpressed with the m₅ acetylcholine (ACh) receptor in Xenopus oocytes, agonist-activated GIRK₁F₁₃₇S, and GIRK₂-mediated currents are inhibited by halothane, whereas in the absence of ACh, high concentrations of halothane induce GIRK₁F₁₃₇S-mediated currents. To elucidate the molecular mechanism of halothane action on GIRK currents of different subunit compositions, we constructed deletion mutants of GIRK₁F₁₃₇S (GIRK₁Δ₁₃₆₇) and GIRK₂ (GIRK₂Δ₃₅₆₉) lacking the C-terminal ends, as well as chimeric GIRK channels. Mutated GIRK channels showed normal currents when activated by ACh but exhibited different pharmacological properties toward halothane. GIRK₂Δ₃₅₆₉ showed no sensitivity against the inhibitory action of halothane but was activated by halothane in the absence of an agonist. GIRK₁Δ₁₃₆₇ was activated by halothane more efficiently. Currents mediated by chimeric channels were inhibited by anesthetic concentrations that were at least 30-fold lower than those necessary to decrease GIRK₂ wild type currents. Glutathione S-transferase pulldown experiments did not show displacement of bound Gβγ by halothane, indicating that halothane does not interfere with Gβγ binding. Single channel experiments revealed an influence of halothane on the gating of the channels: The agonist-induced currents of GIRK₁ and GIRK₂ carried mostly by brief openings, were inhibited, whereas higher concentrations of the anesthetic promoted long openings of GIRK₁ channels. Because the C terminus is crucial for these effects, an interaction of halothane with the channel seems to be involved in the mechanism of current modulation.

The effects of anesthetics that induce general anesthesia are poorly understood. On the cellular level, the synaptic transmission of nerve impulses appears to be impaired by anesthetics (1). The underlying molecular targets for this effect are still a matter of debate. Besides ion channels, which have been shown to be modulated by anesthetics (2), other elements of signal transduction such as receptors and G proteins may also be involved in general anesthesia.

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¶ The abbreviations used are: GIRK, G protein-activated inwardly rectifying K⁺ channel; ACh, acetylcholine; GST, glutathione S-transferase; aa, amino acid; WT, wild type; HK, high K⁺ extracellular medium.

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The family of G protein-activated inwardly rectifying K⁺ (GIRK) channels is comprised of at least five isoforms, designated GIRK₁-GIRK₅ (Kir3.1–3.5) (3). There is strong evidence that functional GIRK channels are homotetrameric or heterotetrameric complexes with the mammalian GIRK₁-GIRK₄ isoforms being differentially expressed primarily in the central nervous system and cardiac tissue (4–6). Functionally, GIRK channels are activated by the binding of Gβγ subunits to the intracellular portions of the channel protein (7, 8). Gβγ is released from heterotrimeric, inactive Goβγ subunit complexes that have been activated by the binding of an agonist to a G protein-coupled receptor. On the cellular level, the opening of GIRK channels stabilizes the membrane potential at E₉⁺ and thus counteracts membrane excitability. As a result, the activation of GIRK channels leads to a decrease in heart rate after the release of acetylcholine from the vagus nerve, and in the central nervous system GIRK channels play an important role in the mediation of opioid- and ethanol-induced analgesia (9–12).

Pharmacologically, GIRK channels are modulated by volatile anesthetics (13). GIRK₁F₁₃₇S or GIRK₁ containing heteromeric channels such as GIRK₁/GIRK₄, but not homomeric GIRK₄ channels, are activated by high concentrations of halothane in the absence of an agonist (14). In contrast, low concentrations of halothane are able to inhibit the agonist-induced currents through GIRK channels when expressed in Xenopus oocytes. GIRK₂ channels are not activated by halothane at the basal level but are most sensitive to the inhibitory action of halothane when activated by an agonist. These effects are independent from the receptor coexpressed and specific for the GIRK channels (14). However, the mechanism of this dualistic modulation of GIRK channels is not clear. The existence of at least two independent mechanisms of halothane modulation of GIRK channels had to be assumed. From our previous experiments (14) we concluded that halothane was able to activate the GIRK₁ channel, probably by increasing its affinity to Gβγ, and that the inhibition of agonist-activated GIRK channels was because of the impairment of the G protein signaling cascade. This is consistent with the findings of various other laboratories that demonstrate the inhibition of signaling through Go₁ by halothane (15–18).

Structurally, GIRK channels consist of a core region with two transmembrane domains (TM1 and TM2) with a reentrant P-loop in between (Fig. 1A, P). This core region is flanked by N- and C-terminal regions, which constitute the “cytoplasmic pore” as judged from crystal structure analysis (19, 20). This structure is exposed to the cytoplasm and is believed to encom-
pass the Gβγ binding sites (21–24). These parts of the proteins show a high degree of homology between the different isoforms. The most divergent regions are the distal N and C termini of the protein. The long distal C terminus of GIRK1 is unique among the GIRK channels and shows virtually no homology to the C termini of other GIRK channels. The role of the distal C terminus is not clear, yet it is not thought to take part in constitution of the cytoplasmic pore. Rather, it protrudes into the cytoplasm where it may function as an interaction domain for other proteins or the channel itself.

To elucidate the mechanism of halothane action on GIRK channels on the molecular level and to investigate the structural requirements for the modulation by halothane, we performed single channel experiments and used deletion mutants of GIRK1-3728 (the substitution of a serine for a phenylalanine in GIRK1 is necessary to get conductive homooligomeric GIRK1 channels; Ref. 25) and GIRK2 as well as chimeric proteins between these two channels. In addition, we tested whether halothane interacts with the binding of Gβγ to GST fusion proteins, which comprise the putative Gβγ binding sites of the N- and C termini of GIRK1.

MATERIALS AND METHODS

Oocyte Culture—Adult female Xenopus laevis were anesthetized by placing the frogs in 0.15% MS222 (pH 7.4). When narcosis was completed, the frogs were decapitated and ovaries were removed. Oocytes were prepared as described (26), and 50 nL of cRNA solutions were injected in ng/μL given in parenthesis (30), GIRK1-3728 (0.3), GIRK2-367 (0.3), GIRK2 WT (30), GIRK2367 (3) and GIRK2-356 (0.25-0.85), GIRK2/1/2 (250), GIRK2/1/2/1 (3). The expression of endogenous GIRK5 was suppressed by coinjection of 20 ng/μL of mRNA for the GIRK5 cRNA. Just after injection, the oocytes were washed and placed on a Winnie dish in 1 L of the solution (27). The temperature was 18°C, and for 5–9 days at 18°C (containing in mM: NaCl (96), KCl (2), MgCl2 (1), CaCl2 (1), HEPES (5), pyruvate (2.5), and penicillin, 100 units/mL streptomycin 100 μg/mL, adjusted with NaOH to pH 7.5).

Preparation of cRNAs—Plasmids were isolated from bacteria and linearized using standard procedures (28). The following DNA's were used: m2 receptor (29), rat GIRK1-3728 from atrium, and GIRK2 WT from mouse testis cloned into pBS-mxt. The truncated GIRK1-367 (aa 1–382) and GIRK2-356 (aa 1–356) channels were created by PCR via primers containing the appropriate restriction sites EcoRV/EcoRI and ClaI/SpeI, respectively, as well as a stop codon at the end of each coding region (LW5, 5'-CCCGATATCATGTCTGCACTCCGAAGG-3', LW6, 5'-GGGAACTTTCCATCCCTGCTGTTCG-3'). Analysis of current amplitudes relative to GST fusion proteins that contained GST and the N termini of GIRK1 (aa 84, CD1) or the C terminus of GIRK1 (aa 219–231) were synthesized in reticulocyte lysate (Promega) and diluted 1:2. The fusion proteins (4 μg/ml) were mixed with [halo] the concentration of halothane, h the Hill coefficient, and I the control value.

For patch clamp experiments the vitelline membrane of oocytes was removed with fine forceps after putting the cells in a hypertonic solution (PG2000Cs, in mM: glutamate (180), KCl (154), MgCl2 (1), Hepes (10), pH 7.5). Immediately after devitellinization the oocytes were transferred to the recording chamber filled with 500 μL of bath solution (in mM: NaCl (144), KCl (10), EDTA (1), MgCl2 (1), CaCl2 (1), HEPES (10), KOH to pH 7.5). Patch pipettes were pulled from borosilicate glass 1B150 (WPI), sylgard coated, fire polished, and back filled with pipette solution (PS, in mM: KCl (144), NaCl (2), MgCl2 (1), CaCl2 (1), MgCl2 (1), CaCl2 (1), EDTA (1), HEPES (10), KOH to pH 7.5). The tip was filled with the same solution without CdCl2. Measurements were performed in the cell-attached mode at a holding potential of −50 mV with a Heka EPC-9 amplifier and recorded with the Pulse program (HEKA, Germany) at a sampling rate of 15 kHz and a filter frequency of 5 kHz. Whole cell recordings was performed with the Fetchan 6.0 software (Axon Instruments).

For halothane sensitivity of GIRK channels, the most divergent regions are the distal N and C termini of other GIRK channels. The role of the distal C terminus is not clear, yet it is not thought to take part in constitution of the cytoplasmic pore. Rather, it protrudes into the cytoplasm where it may function as an interaction domain for other proteins or the channel itself.

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buffer. Bound proteins were eluted with glutathione buffer (in mM: glutathione (15), NaCl (120), Tween 20 (0.05%), Tris (100), pH 8.0) and analyzed on a 12% SDS-polyacrylamide gel followed by Coomassie Blue staining and autoradiography using phosphorimaging as described (31). All steps of the procedure, except the gel electrophoresis, were performed in the presence of the respective concentrations of halothane. The halothane concentration in the solutions was achieved by diluting a saturated stock solution as described above. Halothane-containing wash solutions were prepared directly before use to minimize loss caused by evaporation.

Data Presentation and Statistics—Experiments were repeated at least twice, and results are given as mean ± S.E. Tests for statistical significance were performed with the two-tailed Student’s t test according to Ref. 32 or performed with Sigmaplot 6.0 (SPSS Inc., Erkrath, Germany). In the figures, levels of significance are given by asterisks: *, p < 0.05; **, p < 0.01; ***, p < 0.001.

RESULTS

Current Characteristics of Mutated Channels—The expression of GIRK channels led to an increase in the background or basal currents when rinsed with HK solution (IHK) at a membrane potential of ~70 mV for all types tested. The application of 10 μM acetylcholine evoked additional inward currents (IACh) when the m2 ACh receptor was coexpressed. All GIRK constructs that we used (Fig. 1A) showed normal activation and desensitization properties when compared with GIRK wild type channels. However, the current response to HK and ACh was higher through truncated channels when compared with the respective wild type channels (Fig. 1B). The average ACh-induced current in oocytes injected with 150 pg of GIRK2Δ356 cRNA was 2.8 ± 0.4 μA (n = 11), whereas GIRK2 wild type-injected oocytes (1500 pg) reacted to ACh with currents of only 0.7 ± 0.2 μA. Even with low amounts of injected cRNAs, both the deletion mutant GIRK2Δ356 and the chimera GIRK2/1Δ356 showed, on the average, higher currents than GIRK2 wild type channels. An increased ratio of IACh/IHK from 3.3 to about 7.8 for the deletion mutant GIRK2Δ356 and 8.1 for the chimera GIRK2/1Δ356 was observed. The difference in currents was less pronounced for GIRK1 F137S and GIRK1Δ363* channels; the truncated channels showed an increase in the current amplitude by a factor of about 4.5 and no change in the current ratios (Fig. 1B) when the same amount of cRNA was injected. Injection of oocytes with even high amounts of chimeric GIRK2/1/2 RNA gave only small GIRK currents and reduced the survival rate of oocytes.

Effect of Halothane on Currents through Homooligomeric Wild Type GIRK Channels—As exemplified in Fig. 2A, halothane was able to modulate the current through homooligomeric GIRK1F137S channels in Xenopus oocytes, corroborating our previous results (14). Background currents were augmented by about 90% in the presence of 1 mM halothane, but the current induced by 10 μM acetylcholine (IACh) was inhibited by low concentrations of halothane with maximum attenuation of about 50% at a concentration of 100 μM halothane. When the halothane concentration was increased, the effect reversed, and rinsing the oocytes with 1 mM halothane led to an increase in IACh (14).

To test whether the inhibition of ACh-induced currents by halothane is because of the disturbance of the binding of Gβγ to
Halothane activates basal currents but inhibits $I_{ACh}$ through GIRK1$^{F137S}$ channels. A, time course of a GIRK1$^{F137S}$-mediated current expressed in a Xenopus oocyte measured with the two-electrode voltage clamp technique. The application of HK solution induced the background current ($I_{HK}$), and 10 μM acetylcholine was used to evoke the agonist-induced current ($I_{ACh}$). Low concentrations of halothane (100 μM) led to the inhibition of $I_{ACh}$, whereas high concentrations of the anesthetic augmented the background current. B, pull-down experiments of in vitro synthesized $^{35}$S methionine-labeled Gβγ with a fusion protein of GST and the C terminus of GIRK1 (CD2, aa 183–501 from GIRK1). The upper panel shows a Coomassie Blue-stained gel. The lower panel is a scan of the radioactive signals detected by phorimaging. C, quantitative analysis of the pulldown experiments did not show an influence of halothane on the binding of Gβγ to the fusion protein. The control value is the amount of Gβγ bound to CD2 in the absence of halothane. D, channel activity in a multichannel patch. Unstimulated channels showed low activity. Addition of halothane increased the number of open events and, as shown in panel F, the mean open time of channels. Control traces and traces after addition of halothane are from the same cell. E, the voltage ramp shows inward rectifying channels to be activated by halothane. H, single channel conductivity was not affected by halothane. G, the relative change in open probability of the channels due to the application of 1 mM halothane. In the absence of an agonist an increase was observed. The control values are normalized to 100%. Test for statistical significance was done against the respective control values with *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Results are mean ± S.E.

the channel, we performed pulldown experiments with GST-GIRK1 fusion proteins. These fusion proteins contained the N terminus (aa 1–84, CD1) or the C-terminal part of the channel (aa 183–501), thus including the putative Gβγ binding sites that are believed to be responsible for the agonist-induced current activation (24). With CD1 the amount of pulled down $^{35}$S Gβγ was not sufficient to perform a quantitative analysis. With CD2 the amount of Gβγ that was pulled down was less than 10% of the total labeled Gβγ by the fusion protein. The control value is the amount of Gβγ bound to CD2 in the absence of halothane. Halothane (100 μM, n = 7; 300 μM, n = 6; and 1 mM, n = 5) did not change the amount of bound Gβγ (Fig. 2B and C), thus giving no evidence for inhibition of Gβγ binding to the channel by halothane.

Patch clamp experiments showed low basal activity of the channels in the absence of an agonist with a relative open probability of 4.0 · 10$^{-4}$ ± 1.2 · 10$^{-4}$ (n = 5). However, the open time distribution of detected events showed the existence of at least two populations of channel openings with a long and a short dwell time (Table I). The greater part (93 ± 1.0%) of openings was short ($\tau_1 = 0.66 ± 0.10$ ms), whereas only 7% showed a time constant of 14.2 ± 0.57 ms ($\tau_2$). Halothane increased the open channel probability by propagation of channel openings with longer open times ($\tau_2 = 22.5 ± 9.66$ ms) from a fraction of 7% to 44 ± 15% without changing the time constants of the openings (n = 5). However, this shift led to an increase in the mean open time by a factor of 3.6 (Fig. 2F). In addition the number of openings increased (Fig. 2D), which led to an increase in open probability by 852 ± 368% (Fig. 2G). The single channel conductivity of 15.7 ± 0.5 picoamperes as well as the inward rectification properties of the channel remained unchanged by halothane (Fig. 2E and H). Addition of 50 nM ACh to the pipette solution activated GIRK1$^{F137S}$ channels in cell-attached patches. The relative open probability was increased more than 20-fold compared with channels in the absence of ACh (relative open channel probability 0.080 ± 0.029, n = 5). Again the current was mainly caused by brief openings with a duration of less than one ms (Table I and Fig. 2A and B). Longer lasting events contributed to only 17 ± 8% of the total number of events. The addition of 1 mM halothane decreased the total number of events and decreased the open probability to 39 ± 23% of control (n = 4). Short events were more affected, so that a shift to longer openings was observed. Longer openings contributed to 61 ± 10% of the number of events in the presence of halothane.
Changes in open time distribution caused by halothane for different GIRK channels

Open times were fitted by two exponential equations. The time constants are given as \( \tau_1 \) and \( \tau_2 \), and the distribution is indicated by the calculated percentages of \( f_1 \) and \( f_2 \) (the fraction of channels with a time constant of \( \tau_1 \) or \( \tau_2 \), respectively). ND, not determined because of insufficient number of opening events.

|          | Control, 50 nM ACh | 1 mM halothane, 50 nM ACh |
|----------|--------------------|--------------------------|
|          | \( \tau_1 \) | \( \tau_2 \) | \( f_1 \) | \( f_2 \) | \( \tau_1 \) | \( \tau_2 \) | \( f_1 \) | \( f_2 \) |
| GIRK1F137S (n = 4) | 83 ± 8 | 0.71 ± 0.23 | 17 ± 8 | 4.78 ± 2.87 | 39 ± 10\( ^a \) | 1.41 ± 0.31 | 61 ± 10\( ^a \) | 9.0 ± 2.8 |
| GIRK1363 (n = 7) | 86 ± 4 | 0.50 ± 0.05 | 14 ± 4 | 10.5 ± 2.7 | 59 ± 11\( ^a \) | 1.3 ± 0.3 | 41 ± 11\( ^a \) | 28 ± 18 |
| GIRK2 (n = 3) | 58 ± 3 | 0.22 ± 0.04 | 42 ± 3 | 1.11 ± 0.06 | ND | ND | ND | ND |
| GIRK25156 (n = 5) | 59 ± 10 | 0.24 ± 0.05 | 41 ± 10 | 2.6 ± 0.3 | 65 ± 5 | 0.20 ± 0.03 | 37 ± 5 | 1.6 ± 0.4 |

\( ^a \) Indicates a statistically significant difference (\( p < 0.05 \)) when compared to the controls (i.e. without halothane).

Fig. 3. The effects of halothane on the agonist-induced activity of GIRK1F137S channels. A, ACh activated GIRK channels by inducing short open events. B, open time histogram of ACh-evoked channel activity. C, halothane reduced the number of short events but promoted longer openings. D, open time histogram of opening events in the presence of 50 nM ACh and 1 mM halothane. E, the open probability of activated GIRK channels was reduced by halothane, although the mean open time was prolonged by halothane as shown in panel F.

GIRK2 WT channels have been shown to be most sensitive to the inhibitory action of halothane; activation of the basal current was never observed (13, 14). Concentrations of more than 100 \( \mu M \) halothane significantly reduced the current (Fig. 4A). In two-electrode voltage clamp experiments, the least square fitting of normalized current values to the Hill equation yielded a half-maximum effect of 143 \( \mu M \) halothane on ACh-induced currents and a Hill coefficient of 1.3. In one of three single channel experiments, 1 mM halothane completely blocked channel openings induced by 50 nM ACh. In two more experiments the number of events was reduced to an extent that prevented meaningful statistical analysis. The open channel probability was reduced to 6.5 ± 4.2% of control (\( n = 3 \), Fig. 4C).

**Effects of Halothane on Deletion Mutants of GIRK Channels**—Because GIRK1 and GIRK2 channels reacted differently to halothane, we constructed deletion mutants that lacked the unique C terminus of GIRK1 and the corresponding C terminus of GIRK2 to investigate whether the respective pharmacological properties reside on the channel itself.

The halothane-induced activation of background currents that were mediated by truncated GIRK1363 currents was more pronounced than with GIRK1F137S currents (Fig. 5, A and B). The background currents of GIRK1363 were activated by 514 ± 75% (\( n = 21 \), \( p < 0.001 \)) with 1 mM halothane. Lower concentrations were less effective, but still 300 \( \mu M \) halothane...
induced $104 \pm 14\%$ ($n = 17, p < 0.001$) augmentation of the current, a value reached with GIRK1<sup>F137S</sup>-mediated currents only at concentrations of more than 1 mM.

The agonist-induced currents of GIRK1<sup>3363</sup> were similarly sensitive to inhibition by halothane when compared with GIRK1<sup>F137S</sup> channels. The inhibition was most pronounced at 30 μM halothane with a decrease in the current of 59 ± 3% ($n = 4, p < 0.001$) and less at higher concentrations of the anesthetic, where activation of the channel obviously overrides the inhibition (see Fig. 5A, application of 1 mM halothane, and 5C).

Single channel experiments of the halothane effect on GIRK1<sup>3363</sup> yielded similar results compared with GIRK1<sup>F137S</sup> channels. The basal current was stimulated by 1 mM halothane because of an increase in the number of channel openings and open time (data not shown). The ACh-induced current was mainly carried by brief channel openings. Only 14 ± 10% of the opening events were characterized by a long time constant of 10.5 ± 2.7 ms ($n = 7$) (Fig. 5, D and E and Table I). The single channel conductivity was determined to be 14.5 ± 0.18 picrosiemens and, therefore, the same, as for GIRK1<sup>F137S</sup> channels. As expected from the two-electrode voltage clamp experiments, halothane at a concentration of 1 mM had no effect on the open probability of ACh-activated GIRK1<sup>3363</sup> channels, but similar to GIRK1<sup>F137S</sup> channels, it propagated channel openings with longer open times (Table I and Fig. 5, F and G).

Wild type GIRK2 channels never responded to halothane with activation. The truncation of the C terminus of GIRK2 at aa 356 (GIRK2<sup>A356</sup>) resulted in a channel protein that was activated by halothane in two-electrode voltage clamp experiments. Concentrations as low as 100 μM halothane induced an increase in basal currents (Fig. 6, A and B). Higher concentrations of halothane were more effective, and 1 mM of the anesthetic augmented the background current by 178 ± 39% ($n = 16, p < 0.001$). In contrast to the wild type GIRK2 channels, I<sub>ACh</sub> mediated by truncated GIRK2<sup>A356</sup> channels proved resistant against halothane inhibition. No reduction of the agonist-induced current was observed by halothane in concentrations of 0.01-1 mM when compared with control values (Fig. 6, C and D).

As expected, patch clamp experiments showed no influence of halothane on ACh-evoked channel activity. The single channel conductivity was 30.0 ± 1.0 picrosiemens and therefore higher compared with the GIRK2 wild type channel with 21.7 ± 3.9 picrosiemens. Both channel types showed frequent openings that obviously did not reach the full open level (Figs. 4B and 6E). The open time characteristic of both channel types was also very similar. Although both channel types showed long and short open times, the difference between both was not as pronounced as was the case for GIRK1 channels. The time constant for longer open events was 2.6 ± 0.3 ms, and it counted for 41 ± 10% of the opening events of the channel.

Halothane did not change the open time constants or influence the distribution of long and short events. The open probability and single channel conductivity remained unchanged by halothane. We failed to detect significant changes in single channel behavior in the absence of ACh. A small increase in the channel open probability was observed because of a slight increase in mean open time and in the number of opening events (data not shown).

**Effect of Halothane on Chimeric GIRK Channels**—To further investigate the role of the GIRK C terminus in halothane-induced channel modulation, we transfected the unique GIRK1 C terminus to the GIRK2<sup>A356</sup> channel. The sensitivity of the chimera against halothane was increased ~25-fold when compared with GIRK2 WT channels. I<sub>ACh</sub> through GIRK2<sup>1/1056</sup> channels was nearly completely inhibited by 100 μM halothane (Fig. 7, A and B), and the IC<sub>50</sub> value was found to be 5.5 μM halothane with a Hill coefficient of 0.7. In contrast to GIRK1<sup>F137S</sup> or GIRK2<sup>A356</sup> channels, the basal current of the chimera was inhibited instead of being activated by halothane.
Halothane at concentrations of 300 μM and 1 mM reduced I_{HK} by 34 ± 13% (n = 3) and 71 ± 7% (n = 3, p < 0.05), respectively.

Because of the lipophilic nature of halothane, the site of interaction with integral membrane proteins might lie within the transmembrane-spanning regions of the channel (33). We sought to test whether the halothane-induced activation of GIRK1 channels is a property that can be traced to a structure within these transmembrane domains or the pore region of the channel. Therefore, we substituted the region between aa 88 and aa 202 of the GIRK2 channel and the GIRK2/1356 channel for the homologous region of GIRK1 F137S. This part of the channel comprises the transmembrane and the pore-forming domains of the channel. Both chimeras showed a clear increase in sensitivity against the inhibitory action of halothane (Fig. 7, C and E). In neither case was activation of GIRK currents observed with halothane.

It has been postulated that halothane increases the affinity between G_{α} and G_{βγ} (16). Thus, the inhibition of activated GIRK channels by halothane might be the result of a shift in the equilibrium distribution of G_{βγ} from the effector (the activated channel) to the G protein α subunit. If this is the case, the rate of current decay because of halothane inhibition should be comparable with the rate of current decay because of the deactivation by the wash out of the agonist. However, the time course of halothane-induced inhibition of I_{ACh} was rapid compared with the decrease of the agonist-induced current due to the wash out of ACh. Fitting of the current decays to a single exponential equation gave time constants for the inhibition by 100 μM halothane of 3.0 ± 0.6 s (n = 6) and a time constant for the deactivation of ACh-induced currents of 19.0 ± 2.5 s (n = 6) for GIRK2/1356-mediated currents. The inhibitory effect of halothane on GIRK channel activity was not observed with either of the chimeric channels.
othane is thus about 6-fold more rapid than the deactivation of the current due to deprivation of the agonist.

DISCUSSION

From our previous work (14) it was not clear how halothane was able to inhibit agonist-activated GIRK currents with high potency but activate the background current with lower potency. A mechanism with two independent targets seemed most probable. An increase in $G/\beta\gamma$ affinity to the channel could have explained the activation by halothane, whereas disruption of the $G$ protein-signaling cascade downstream of the receptor could have been a reason for the inhibition of agonist-induced currents. However, in light of our new findings, the existence of two such independent mechanisms for modulation of GIRK currents by halothane, i.e. one on the $G$ protein and the other on the channel, seems unlikely. The inhibition of open channels by halothane cannot be assigned to the inhibition of the $G$ protein, because it can be abolished by deletion of the C terminus of the channel and therefore has to be a property of the channel itself. Halothane does not physically block the pore, but it seems to interact with the gating mechanism of the channel. Obviously there are at least two gates that are affected: a fast one is inhibited; it promotes flickering like channel openings due to the activation of the $G$-protein, whereas another slow one is responsible for longer openings and can be activated by halothane.

In the absence of an agonist, halothane induced activity of GIRK1F137S and GIRK1363* channels. The number of opening events and the duration of the openings was increased (Figs. 2, D and F). An increase in the number of long events was also seen when halothane was applied to channels that had been activated by ACh. In addition, halothane decreased the number of short openings in the presence of the agonist (Fig. 3C), thus explaining how the anesthetic was able to reduce the agonist-induced current but activate the background current.

The fact that the sensitivity of GIRK channels to halothane was determined by the C terminus indicates that this part of the protein interacts with the $G/\beta\gamma$ binding sites or directly modulates the gating of the channel. Deleting this part of the
channel abolished the halothane sensitivity of GIRK2 channels (Fig. 6C). On the other hand, introduction of the C terminus of GIRK1 into GIRK2 further increased its sensitivity against halothane (Fig. 7A). The functional role of the C terminus in GIRK channels is still not clear. Although crystallography studies revealed the three-dimensional structure of inwardly rectifying K⁺/H11001 channels (19, 20, 34), the distal C terminus downstream residue 371 could not be incorporated into the three-dimensional structure. However, it is assumed that this part of the molecule is not part of the cytoplasmic pore but protrudes into the cytoplasm and probably mediates protein-protein interactions. From functional studies we know that the C terminus of GIRK1 may physically block the channel because treatment with trypsin from the cytosolic side activates GIRK in a manner similar to ACh (35). The coexpression of the C-terminal tail of GIRK1 effectively inhibits channel activity in Xenopus oocytes (36); furthermore, a peptide of 20 amino acid residues derived from the very C-terminal end of GIRK1 is a potent and reversible blocker of GIRK channels (37). Interestingly, the deletion mutants of GIRK1 as well as GIRK2 lacking the C-terminal end that we used in this study did not show any obvious gating abnormalities. This would be expected, if this very part of the C terminus directly took part in the binding of Gβγ and gating of the channel. The C-terminal deletion mutants were readily activated by ACh via the coexpressed m2 receptors and deactivated rapidly after washout of the agonist. The only difference seen was a general higher level of currents in oocytes injected with the cRNA of the deletion mutants (Fig. 1B). However, in patch clamp experiments we did not observe a general higher open probability of single mutated channels.

In vitro synthesized, metabolically labeled Gβγ has been successfully used in the past to identify specific Gβγ-binding sites of GIRK channels (24) or voltage-dependent Ca2⁺ channels (31). Our experiments showed no displacement of bound Gβγ to the GST-GIRK1 fusion protein in concentrations of up to 1 mM halothane. The fusion proteins were incubated in a solution with saturating Gβγ concentrations (about 10-fold excess); thus, with this method it is not possible to detect a potential

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**Fig. 7.** Chimeric GIRK channels developed increased sensitivities against halothane. A, the time course of GIRK2/C356-mediated currents measured with the two-electrode voltage clamp technique. B, agonist-induced currents were effectively inhibited by halothane. Sensitivity against halothane was increased about 30-fold in the chimeric channel when compared with GIRK2 channels, the most sensitive of the wild type channels. C, GIRK2/C2 channels were also inhibited by low concentrations of halothane. D, the IC50 value was estimated to be about 10 µM, 15 times less than for the GIRK2 WT channel. E, exchange of the C terminus of GIRK2/C2 against that of GIRK1 yielded the GIRK2/C2/1 channel. The current mediated by this channel showed further increased sensitivity against halothane. A concentration of 300 nM halothane was sufficient to reduce the current by about 50% as shown in panel F.
increase in $\text{G} \beta \gamma$ affinity to the channel protein. In contrast, a potential decrease in $\text{G} \beta \gamma$ affinity would have been detected because the bound complexes were washed with halothane-containing solutions in the absence of $\text{G} \beta \gamma$. Any decrease in affinity because of halothane would have reduced the amount of bound $\text{G} \beta \gamma$. Even if we take into account that there is more than one binding site for $\text{G} \beta \gamma$ in the C terminus of GIRK1 that could obscure a possible halothane-induced shift of binding from one site to another, it seems unlikely that washing the complexes in the absence of free $\text{G} \beta \gamma$ would not reduce the total amount of bound protein. Therefore, we conclude that halothane does not interrupt the $\text{G}$ protein-signaling on the level of $\text{G} \beta \gamma$ binding to the channel.

The $\text{C}$ terminus of GIRK, which plays a crucial role in mediating the anesthetic effect of halothane, is rather hydrophilic and therefore thought to protrude into the cytoplasm (19). Judged from this property one would not expect strong interaction of hydrophobic substances like halothane with the $\text{C}$ terminus of GIRK channels directly. However, it is impossible to predict binding of anesthetics to proteins from the amino acid sequence alone (38). Our findings show a strong dependence of the halothane action on the $\text{C}$ terminus of the GIRK channel, and therefore a direct interaction of halothane with this part of the protein seems possible. Still, other mechanisms, such as so-far unknown interactions with other proteins mediated by the $\text{C}$ terminus, may play a role in the observed halothane effects.

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The Sensitivity of G Protein-activated K⁺ Channels toward Halothane Is Essentially Determined by the C Terminus
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Page 34240: An error appeared in the funding footnote. The funding footnote should read as follows:

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