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Effects of Volatile Anesthetics on the G Protein-Regulated Muscarinic Potassium Channel

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SUMMARY

The muscarinic-activated K^+ channel $K_{(ACh)}$, a prototype of channels regulated by neuroendocrine agonists via G proteins, was used to investigate the mode of action of isoflurane and halothane on G protein-coupled signal transduction processes. The evolution of the muscarinic current $I_{K(ACh)}$ was characterized through rapid agonist application and washout. At physiologically relevant concentrations, halothane and isoflurane reduced the rate of $I_{K(ACh)}$ activation without comparable effects on deactivation. Furthermore, both anesthetics reduced or eliminated the spontaneous decay (rapid desensitization) typical of the muscarinic response. In contrast to these similarities of anesthetic action on the time course of the response, the magnitude of $I_{K(ACh)}$ was slowly reduced by isoflurane but rapidly augmented by halothane. Neither halothane nor isoflurane altered the conductance of single $I_{K(ACh)}$ channels, indicating that these volatile anesthetics act on channel open-close kinetics. The reduced $I_{K(ACh)}$ activation rates suggest that impaired receptor/G protein interactions are induced by both anesthetics. For halothane, the increased amplitude of the response, also seen for $I_{K(ACh)}$ activated in a receptor-independent manner by guanosine-5′-*O*-(3-thio)triphosphate, suggests a direct action on the channel. Alteration of signal transduction processes by halothane and isoflurane may underlie some anesthetic actions of these compounds as well as secondary effects on the cardiovascular system.

The mechanisms of action of volatile anesthetics are poorly understood. It is likely, however, that some of these involve altered function of ion conductive channels in excitable cell membranes (1). Direct action on the channel protein is an obvious way in which anesthetics may accomplish this. However, a less obvious but equally important possibility is that anesthetics act indirectly by modifying the function of those molecules that regulate channel function through signal transduction cascades. We attempted to evaluate the relative importance of these alternatives by examining the effects of isoflurane and halothane, two typical volatile anesthetics, on muscarinic-activated K^+ channels, a prototype of G protein-coupled, neuroendocrine-regulated ion-conductive channels.

The muscarinic K^+ channel $K_{(ACh)}$ is the most extensively studied example of G protein-coupled, hormonally regulated ion-conductive channels (1, 2). An increasing number of neurophysiologically important channels are believed to be regulated via this mechanism (for a recent review, see Ref. 3). In atrial myocytes, stimulation of muscarinic (m2) receptors by cholinergic agonists activates the inwardly rectifying K^+ current without involving any diffusible cytoplasmic factors (4). Activated receptors interact in the membrane with G_K, which on release of GDP and uptake of GTP, acts on the channel by increasing the likelihood of its opening (2). This system lends itself to precise determinations of the time course of $I_{K(ACh)}$ activation, which can be used as a rapid, sensitive, and selective indicator of receptor/G protein and G protein/effector interactions (5). In contrast to most previous studies of anesthetic effects on G protein function that use biochemical techniques in cell-free systems, muscarinic activation of $I_{K(ACh)}$ in patch-clamped cells allows examination of this system in its native environment with excellent time resolution.

Volatile anesthetics have complex effects on excitable cell membranes. It has been suggested that they act on membrane proteins directly and/or indirectly by altering the physical state of membrane lipids (6). Several reports used biochemical techniques to examine the effects of anesthetics on receptors with respect to agonist and antagonist binding to isolated membranes. Halothane was found to potentiate antagonist binding to muscarinic receptors without affecting agonist binding in rat heart and brain (7, 8). Furthermore, halothane was reported to prevent the shift from high affinity to low affinity for agonist binding induced by the hydrolysis-resistant GTP analog Gpp(NH)p in rat brain muscarinic

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ABBREVIATIONS: $I_{K(ACh)}$, ACh-activated K^+ current; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; ACh, acetylcholine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Gpp(NH)p, guanosine-5′-(β,γ-imido)triphosphate.

receptors (9); this shift is the hallmark of receptor/G protein interactions (10). In contrast to these findings, it has been reported that halothane has no effect on the ability of Gp-p(NH)p to reduce the affinity for agonists in human myocardium (11). Isoflurane and halothane were also found to decrease muscarinic inhibition of adenylate cyclase in rat heart, without an effect on the β-adrenergic stimulation of adenylate cyclase (12). Although somewhat inconsistent, these results indicate that disruption of receptor/G protein coupling may be a general feature of the action of volatile anesthetics.

The effects of halothane and isoflurane on $I_{K(ACh)}$ were characterized and compared in the current study. We found that both anesthetics slowed the rate of $I_{K(ACh)}$ activation with only minor effects on the rate of $I_{K(ACh)}$ deactivation. Furthermore, both anesthetics reduced the spontaneous decay (the so-called fast desensitization) of the response. In contrast to their similar effects on time course, isoflurane decreased but halothane increased the magnitude of the response. Halothane also increased the magnitude of $I_{K(ACh)}$ when it was activated in a receptor-independent manner by hydrolysis-resistant GTP analogs, suggesting a direct effect on channel open time, as confirmed with single-channel measurements. Some of these results have been presented in a preliminary form (13, 14).

Materials and Methods

Solutions. HEPES/Ringer's solution contained 90 mM NaCl, 2.5 mM KCl, 5 mM $MgCl_2$, 2.5 mM $CaCl_2$, and 20 mM HEPES, pH adjusted to 7.4 with NaOH. Dissociation medium contained 110 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, and 10 mM HEPES, supplemented with 0.9–1.5 mg/ml collagenase (Worthington Biochemicals, Freehold, NJ) and 0.3 mg/ml trypsin (Sigma Chemical, St. Louis, MO). For whole-cell voltage-clamp measurements, the composition of the internal (pipette) solution was 80 mM K-aspartate, 30 mM KCl, 1 mM EGTA, and 5 mM HEPES, pH adjusted to 7.4 with KOH. The internal solution also contained 0.2 mM Li_4GTP and 2.5 mM Mg·ATP unless indicated otherwise.

For single-channel measurements, pipettes were filled with a solution consisting of 100 mM KCl, 2.5 mM $CaCl_2$, 2 mM $MgCl_2$, and 20 mM HEPES, pH adjusted to 7.4 with KOH. The bath solution composition was 110 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA-KOH, and 20 mM HEPES, pH adjusted to 7.4 with KOH. The bath solution was supplemented with 2 mM Mg·ATP and 0.2 mM Li_4GTP or 0.05 mM $Li_4GTP\gamma S$ as indicated.

GTP, GTPγS, and GDP were obtained from Boehringer-Mannheim Biochemicals (Indianapolis, IN); Mg·ATP was from Sigma Chemical, and tetrodotoxin was from Calbiochem (San Diego, CA). Isoflurane (Forane) was from Ohmeda PPD (Liberty Corner, NJ) and halothane was from Halocarbon Laboratories (River Edge, NJ). Thymol-free halothane was a gift of Halocarbon Laboratories. All other chemicals were of the highest purity available. Experiments were carried out at room temperature (22–23°).

Preparation of myocytes. Bullfrog (*Rana catesbeiana*) atrial myocytes were obtained as described previously (18). Briefly, the heart was removed, washed in, and perfused with Ca^{2+}-free Ringer's solution for 50 min. Subsequently, the heart was perfused with dissociation medium for 25–30 min. The atrium was then separated from the ventricle and placed in 0.2 mM Ca^{2+}-containing Ringer's solution. Myocytes were obtained through agitation of the atrial tissue in this solution. One aliquot of the cells was stored at room temperature for immediate use. The other was kept in the refrigerator (4°) and used on the next day. Solutions used during cell isolation were bubbled with O_2 and held at 30°.

Anesthetic application. Solutions were equilibrated with inhalation anesthetics by being bubbled for ≥20 min before use with an anesthetic-containing O_2 stream generated by a vaporizer at room temperature. Control experiments were carried out to verify that bubbling with oxygen by itself has no effect on the muscarinic response (data not shown). The range of halothane and isoflurane content in the gas was nominally 0.5–5.0% v/v. To avoid escape of anesthetics from the superfusing solutions, Teflon tubing was used throughout. In addition, the space between solution reservoirs and the measuring chamber was reduced as much as possible. Furthermore, the tubing was flushed for ≥30 sec before directing the solution stream onto the patch-clamped cell. The actual concentration of anesthetics in the effluents was determined by gas chromatography. Solutions equilibrated with 0.5%, 2%, 3%, 4%. and 5% halothane/O_2 yielded actual concentrations of 0.2, 0.9, 1.3, 2.0, and 2.2 mM, respectively. For isoflurane/O_2, the corresponding concentrations were 0.1, 0.8, 1.2, 1.7, and 1.9 mM. These values give Bunsen water/gas partition coefficients of 1.04 and 0.92 for halothane and isoflurane, respectively. The corresponding partition coefficients given by Franks and Lieb (6) were 1.2 and 1.08 at 25°, respectively. The reasonable agreement between these partition coefficients indicates that the calibration of the vaporizers was accurate.

Control ACh applications were made at the beginning of each experiment. In experiments in which we studied the long-term effects of the anesthetics, a 30-sec control ACh application was made in the absence of anesthetic. The anesthetic was then applied, followed by test applications of ACh with anesthetic at 5-min intervals for as long as stable recordings were obtained. In experiments in which we examined the short-term effects of the anesthetics, two control applications of ACh were made, separated by a 5-min interval. This was followed by a test application of ACh plus anesthetic and, after a 5-min washout period, a final application of ACh alone. The two control applications were made to determine whether multiple ACh applications give essentially identical responses compared with that obtained in the presence of the anesthetic. The response after the anesthetic washout showed at least partial, and in most cases complete, recovery of the control response.

Experimental arrangement. Cells or excised patches were positioned in front of the outlet tube of a rapid solution changer device (RSC-100; Molecular Kinetics, Pullman, WA). The solution exchanger rotates under computer control (DAP 800; Microstar Laboratories, Bellevue, WA), placing different outlets in front of the cell, each of which contains different solutions that superfuse the cell. For whole-cell measurements, the timing and quality of the solution change were indicated by a change in the holding current generated by a slight difference in K^+ concentration between control (2.5 mM K^+) and ACh-containing Ringer's (7.5 mM K^+). Solution changes were usually complete within 20 msec.

For whole-cell measurements, pipettes were fabricated from square bore borosilicate capillaries (Glass Company of America, Millville, NJ) using a Sutter P-80 puller (Sutter Instruments, San Rafael, CA). The electrode resistance was 2–5 MΩ when filled with internal solution. All measurements were carried out in HEPES/Ringer's solution supplemented with 0.5 mM $CdCl_2$ to block calcium currents. For single-channel measurements, pipettes were pulled form quartz capillaries using a Sutter P-2000 puller (Sutter Instruments). The tip resistance was 6–10 MΩ.

Currents were recorded with an Axopatch 200A current amplifier (Axon Instruments, Foster City, CA). The data were low-pass filtered at 5 kHz and stored on videotape (VR-10, Instrutech, Great Neck, NY) for later analysis. The DAP 800 processor was used to digitize the data. The sampling rates were in the range of 1–4 kHz for the whole-cell measurements and 10 kHz for single-channel data. The DAP 800 processor was also used to generate the command potentials.

Results

Isoflurane and halothane alter the muscarinic response. Anesthetic effects on the muscarinic K^+ current were evaluated by comparing ionic currents elicited by rapid applications of ACh. With the exception of the periods of ACh application, cells were paced by applying alternating 250-msec pulses to −105 and −5 mV from holding potentials of −85 mV lasting 250 msec. Soon before ACh was applied, the membrane potential was set to −35 mV. Under these conditions, the outward current elicited by ACh is entirely due to activation of the muscarinic K^+ channel (15). For most experiments, the time course of $I_{K(ACh)}$ was recorded in response to rapid applications of 10 μM ACh for ~30 sec. Typically, a control application of the agonist was followed by test applications made in the presence of the anesthetic at 5-min intervals (see Materials and Methods). Fig. 1 illustrates the overall effects of 0.8 mM isoflurane (2 volume %) and 0.9 mM halothane (2 volume %) in a comparison of the control response (*thick trace*) to a family of test responses (*thin traces*) obtained from the same cell after the indicated times of anesthetic exposure. The control responses show that as reported previously, $I_{K(ACh)}$ rises rapidly and reaches a maximum within 0.2–0.3 sec of agonist application. This rising phase of the response represents the process of G_K activation. At longer times, the current decays spontaneously through a process termed fast desensitization to a steady state value. On washout of the agonist $I_{K(ACh)}$ fades with an exponential time course, representing the deactivation of the response (16).

Fig. 1A also shows that a 5-min exposure to 0.8 mM isoflurane reduced both the peak and the steady state amplitudes of $I_{K(ACh)}$. The longer isoflurane was administered, the more pronounced this blocking effect became, until after ~20 min of exposure, when no further decay was evident. Interestingly, isoflurane also had an immediate effect on $I_{K(ACh)}$. This is illustrated in Fig. 1B (*thin trace*) through the coapplication of ACh and 800 μM isoflurane. Compared with the control trace (*heavy trace*), a significant decrease in the initial peak response is evident.

The effects of halothane (0.9 mM) were similar to those of isoflurane in that initially the peak of the response was reduced (Fig. 1C). However, longer exposure to halothane gradually restored the peak as well as the steady state response. Thus, after 25 min of superfusion with halothane, the peak $I_{K(ACh)}$ was as large as that measured during the control ACh response. In contrast to these biphasic changes in the peak currents, halothane increased the steady state current monotonically. The immediate effect of 0.9 mM halothane (Fig. 1D) was similar to that of isoflurane: the peak amplitude of the response decreased, and the fast desensitization was almost completely abolished.

The effects of isoflurane and halothane (Fig. 1) were observed consistently in all atrial myocytes examined. This is summarized for the peak and steady state magnitudes of the $I_{K(ACh)}$ response in Fig. 2. The control ACh application made in the absence of anesthetic was used to normalize the test responses obtained at various times during the application of 0.8 mM isoflurane or 0.9 mM halothane. Note that the response at time = 0 was obtained through the simultaneous application of anesthetic and ACh, whereas the following responses were obtained in the continued presence of anesthetic.

Fig. 2A shows that the peak of the response decreased progressively due to the action of isoflurane. Even the immediate application of isoflurane reduced the peak current (25%) in a significant manner (t test, $p < 0.01$). The reduction in the peak currents seemed to stabilize after 20 min at 18% of control. Fig. 2B shows that in contrast to isoflurane, halo-

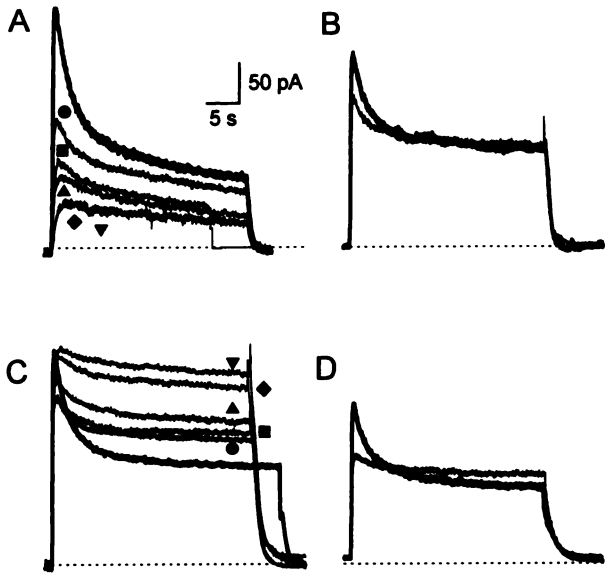

Fig. 1. Effect of isoflurane and halothane on $I_{K(ACh)}$. Muscarinic K^+ currents were elicited by rapid applications of 10 μM ACh to atrial myocytes. Whole-cell currents are shown for cells held at −35 mV. The superimposed current traces were recorded (*thick lines*) before and (*thin lines*) in the presence of anesthetics. A and C, Responses obtained after (●) 5-, (■) 10-, (▲) 15-, (◆) 20-, and (▼) 25-min exposure to (A) 0.8 mM isoflurane or (C) 0.9 mM halothane. B and D, Responses obtained when ACh and (B) 0.8 mM isoflurane or (D) 0.9 mM halothane were coapplied. *Dashed lines*, zero current.

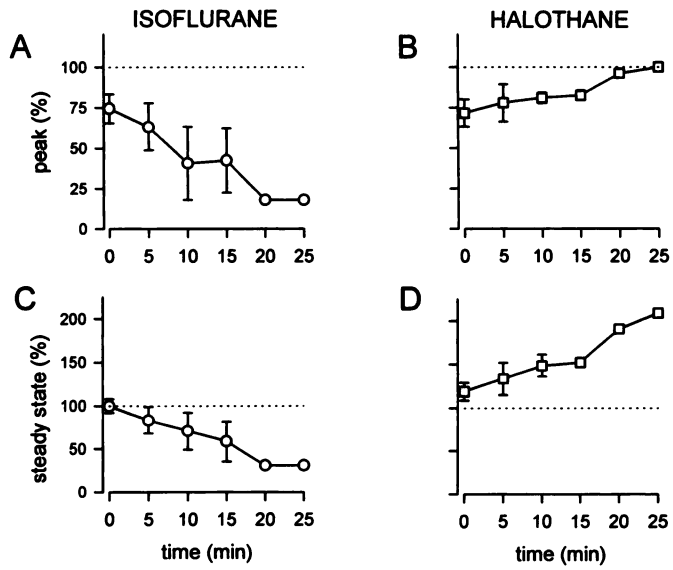

Fig. 2. Sensitivity of the peak and steady state values of $I_{K(ACh)}$ to isoflurane and halothane. The (A and B) relative peak and (C and D) steady state amplitudes of $I_{K(ACh)}$ are plotted as a function of the duration of (A and C) 0.8 mM isoflurane or (B and D) 0.9 mM halothane exposures. The amplitudes were determined as peak or steady state deflections from the current measured before the application of 10 μM ACh and were normalized to the amplitudes of the preanesthetic (control) responses.

thane modified the peak response in a more complex manner. Although we observed an immediate reduction in the peak to 72 ± 8.2% of the control, prolonged exposure to halothane gradually increased the peak current toward its control value.

Isoflurane and halothane consistently modified the steady state muscarinic response in opposite ways. Isoflurane (Fig. 2C) gradually decreased steady state currents without any discernible initial effect, an observation also made with 1.9 mM isoflurane (data not shown). In contrast, halothane (Fig. 2D) increased the steady state $I_{K(ACh)}$ immediately in a significant manner (t test, $p < 0.03$). The increase seemed to continue for ≤25 min without any indication of leveling off.

Anesthetic effects on the fast desensitization. Muscarinic activation of K^+ channels is characterized by a fast desensitization. In this process, after rising to a maximum, $I_{K(ACh)}$ decays spontaneously by ~70% even though the agonist is continually present (17, 18). Although this phenomenon has been studied extensively, its molecular origins remain poorly understood (19, 20). As Fig. 1 indicates, fast desensitization is affected both by isoflurane and halothane. To characterize the anesthetic effect on rapid desensitization, the spontaneously declining phase of $I_{K(ACh)}$ was fit by a sum of two exponentials:

$$I = A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2} + A_3 \quad (1)$$

which, as illustrated in Fig. 3, is well fit to the time course of the decaying phase of $I_{K(ACh)}$ under a variety of experimental conditions. A_1 and τ_1 are the amplitude and time constant of the rapid component of decay, A_2 and τ_2 are corresponding values for the slow component, and A_3 is the steady state current. The relative effects of 0.8 mM isoflurane and 0.9 mm halothane on the relaxation amplitudes A_1 and A_2 are shown in Fig. 4 as a function of anesthetic exposure.

Neither isoflurane nor halothane altered the time course of the fast desensitization (i.e., the spontaneous decay of the response). Thus, the rapid time constant τ_1 was 1.8 ± 0.1 and 1.7 ± 0.2 sec before and after 10 min of 0.8 mm isoflurane application, respectively; the corresponding data for 0.9 mM halothane were 1.6 ± 0.1 and 1.8 ± 0.1 sec. When allowed to vary, τ_2 values ranged between 6 and 12 sec. Note, however, that for traces recorded in the presence of anesthetics, A_2 was reduced to the point that τ_2 was not well constrained by curve fitting, which prompted us to change its value to that of control. Regardless, τ_2 values are likely to be underestimates due to the intentionally short duration of the ACh applications in our experiments (19).

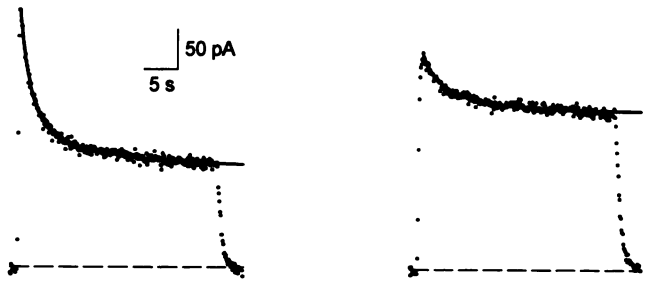

Fig. 3. Characterization of rapid desensitization with and without anesthetics. The spontaneously declining phase of $I_{K(ACh)}$ was fitted by eq. 1. *Left*, control application of 10 μM ACh. *Solid line*, drawn to eq. 1 using the best-fitting parameters: τ_1 = 1.37 sec, τ_2 = 7.83 sec, A_1 = 109 pA, A_2 = 45.2 pA, and A_3 = 95.3 pA. *Right*, response after 5-min exposure to 3% halothane. *Solid line*, drawn to eq. 1 using the best-fitting parameters: τ_1 = 1.25 sec, τ_2 = 7.83 sec, A_1 = 8.66 pA, A_2 = 31.7 pA, and A_3 = 150 pA.

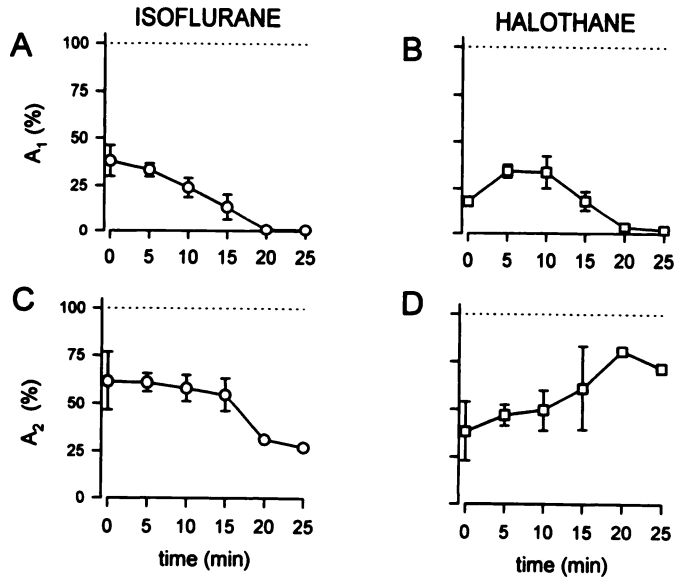

Fig. 4. Anesthetic sensitivity of $I_{K(ACh)}$ desensitization. Relaxation amplitudes A_1 and A_2 were obtained by fitting eq. 1 to the decaying phase of $I_{K(ACh)}$. Values of the (A and B) fast and (C and D) slow components normalized to the preanesthetic (control) responses are plotted as a function of exposure time to (A and C) 0.8 mM isoflurane or (B and D) 0.9 mM halothane.

As illustrated in Fig. 4, both isoflurane and halothane decreased the amplitude of the rapidly decaying component, A_1. The corresponding reduction in peak current measured in the presence of anesthetics is chiefly due to this effect. Isoflurane also reduced the amplitude of the slower component A_2 but to a much lesser extent than that seen for A_1. Thus, for example, A_2 was 62 ± 15% of control after 5 min in 0.8 mM isoflurane compared with 33 ± 3.6% for A_1. Note that within this period, the steady state component A_3 remained practically unaltered (see Fig. 2, C and D). For longer periods of isoflurane application, however, all three components were reduced by >70%. The initial decrease in A_2 was more pronounced in halothane than in isoflurane. However, A_2 started to increase after a 5-min halothane application but remained at 80% of control even after 25 min of exposure to halothane.

Isoflurane and halothane reduce the rate of $I_{K(ACh)}$ activation. The effects of anesthetics on the rise of $I_{K(ACh)}$ are shown in Fig. 5 with the use of expanded time scales. Note that on rapid applications of the agonist, $I_{K(ACh)}$ develops only after a characteristic delay representing molecular events that precede channel opening (15). To estimate accurately the latency of $I_{K(ACh)}$ activation, it was important to mark the exact time of ACh arrival at the cell membrane. This was done by having a higher (7.5 mM) K^+ concentration in the ACh-containing solution (see Materials and Methods), which on reaching the cell membrane, generates an essentially instantaneous outward shift of the holding current due to a shift in the K^+ equilibrium potential. After the latent phase, currents start to rise due to $K_{(ACh)}$ channel activation. Fig. 5 (*top*) shows that both isoflurane and halothane increased this latency of response and slowed $I_{K(ACh)}$ activation.

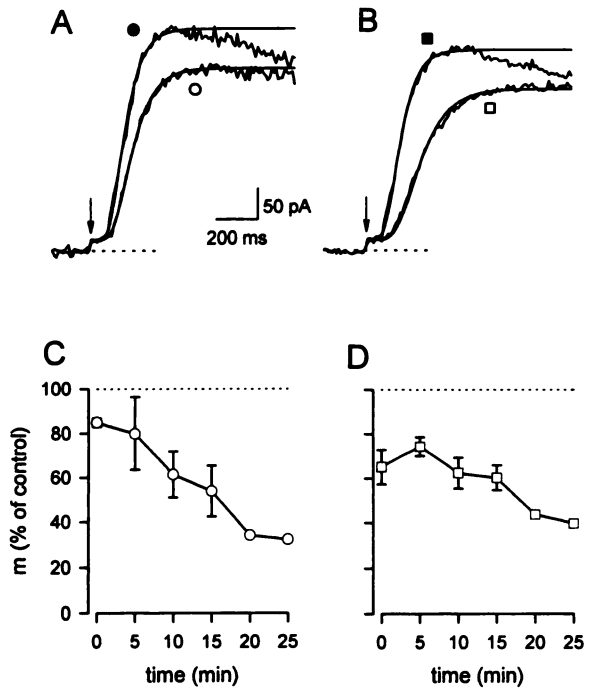

Fig. 5. Isoflurane and halothane slow the rate of $I_{K(ACh)}$ activation. A and B, Rising phase of $I_{K(ACh)}$ is plotted with the use of expanded time scales. Conditions were identical to those for Fig. 1. ● and ■, Control responses. ○ and □, Test responses obtained after 5-min exposure to (A) 0.8 mM isoflurane or (B) 0.9 mM halothane. *Smooth lines*, drawn to eq. 2 using best-fitting activation rates (m) of (●) 17.1 sec^{-1}, (○) 13.9 sec^{-1}, (■) 17.5 sec^{-1}, and (□) 10.9 sec^{-1}. *Arrows*, time origin. C and D, Activation rates (m) are plotted as a function of the duration of (C) isoflurane or (D) halothane application. Data were obtained by fitting of eq. 2. The m values of the control ACh application were used to normalize the data. Data plotted at time = 0 represent normalized activation rates for responses obtained when the anesthetic and ACh were applied simultaneously.

To estimate the rates of G_K activation, the following empirical equation was fit to the rising phase of the K^+ current:

$$I = I_{max}(1 - e^{-f(t)})^4 \quad (2)$$

where $f(t) = mt - (1 - e^{-mt})$, I_{max} is the maximum current at a given ACh concentration, and m is a parameter that is used to measure the rate of development of the response. We find that eq. 2 describes well the time course of $I_{K(ACh)}$ activation, including the apparent delay with which the response arises (13–15). Fig. 5 (*solid line*) represents the best fit of the rising phase. The m values obtained for 10 μM ACh are plotted in Fig. 5, C and D, respectively, for 0.8 mm isoflurane and 0.9 mm halothane as a function of the duration of anesthetic application. As indicated by the decreasing m values, the time course of G_K activation is slowed progressively by the anesthetics. Although both anesthetics reduce m, they do so with different time courses. The immediate application of isoflurane (i.e., coapplication of the anesthetic and ACh indicated as time = 0 on Fig. 5C) decreased m only slightly, to 82% of control. After 5 min of anesthetic exposure to isoflurane, m was still 84% of control, although it decreased to 32% of control by 25 min. In contrast, halothane decreased m to 65% of control immediately, but 20 min of exposure was required to further decrease m to 41% of control.

We considered the possibility that the anesthetic-induced slowing of $I_{K(ACh)}$ activation may be due to a decrease in the potency of the agonist because lower agonist concentrations also give rise to slower responses (15). This issue was addressed by measuring m as a function of the ACh concentration in the range of 50 nM to 1 mM, with and without 0.8 mM isoflurane or 0.9 mM halothane. The dose-response curve was determined after halothane or isoflurane was applied for 5 min. Because $I_{K(ACh)}$ changes slowly but continually in the presence of the anesthetics, it was important to minimize the time taken to determine the ACh concentration dependence of m. For this reason, we focused on the rising phase of $I_{K(ACh)}$ by applying ACh for 0.2–0.4 sec instead of 30 sec. Under these circumstances, the recovery of $I_{K(ACh)}$ from desensitization takes <20 sec, so a full dose-response curve can be obtained well within 5 min. The activation rates m measured in this way were normalized to the values obtained with 100 μM ACh alone and were fit by the Hill equation. The best-fitting parameters show a slight but significant change in the potency of the agonist, with values decreasing from 0.42 μM in controls to 0.10 and 0.19 μM for isoflurane and halothane, respectively. However, these slight changes cannot account for the anesthetic-induced decreases seen in the rate of $I_{K(ACh)}$ activation at 10 μM ACh.

Isoflurane and halothane slow somewhat the deactivation of the muscarinic response. On washout of the agonist, $I_{K(ACh)}$ decays and follows an exponential time course. A typical example is shown in Fig. 6. The time course of the decay is independent of the type of agonist used or its concentration, although it depends on the duration of the agonist application (16). This process seems to represent the deactivation of G_K due to GTP hydrolysis.

To determine the effect of isoflurane and halothane on the deactivation process, the decay of $I_{K(ACh)}$ after the washout of ACh was fit by a single exponential:

$$B = B_{max}e^{-t/\tau} + B_{min} \quad (3)$$

where B_{max} is the steady state $I_{K(ACh)}$ current, B_{min} is the steady state current after ACh washout, and τ is the time constant of $I_{K(ACh)}$ decay. Deactivation remained monoexponential in the presence of anesthetics (Fig. 6, A and B). Furthermore, as shown in Fig. 6C, the τ values remained unchanged during the immediate application of 0.8 mM isoflurane but rose consistently on continuing isoflurane exposure, although the increases were not statistically significant (t test, 95% confidence level). When 0.9 mM halothane was present, the trend toward an increased τ was more obvious and statistically significant for the 10-min data point (t test, $p < 0.05$). Thus, although both anesthetics slowed the deactivation process, the increase in τ was rather small: <45% in all cases.

Effects as a function of anesthetic concentration. Wee also explored how the muscarinic response is affected at different concentrations of anesthetics. Isoflurane and halothane in the 0.1–2.2 mm range were coapplied with 1 μM ACh to individual myocytes, and the responses were compared with control responses obtained from the same myocyte 5 min before the test application. Activation rates, peak response, and steady state response were measured and expressed as percentage of control for each myocyte. We found that even the lowest concentrations tested (0.1 mM isoflurane and 0.2 mM halothane) had an immediate effect on the muscarinic response. Thus, for example, the rate of activation was reduced to 85% and 83% of control,

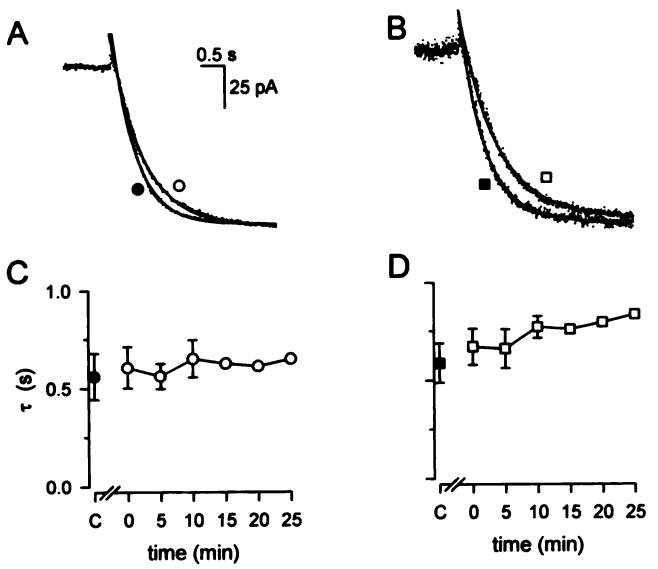

Fig. 6. Anesthetic dependence of $I_{K(ACh)}$ deactivation rates. A and B, Deactivation of $I_{K(ACh)}$ on agonist washout (● and ■) before and (○ and □) after 5-min exposure to (A) 0.8 mM isoflurane or (B) 0.9 mM halothane. The magnitude of the test traces was scaled to that of the control traces. *Smooth lines*, drawn to eq. 3 using best-fitting time constants of (●) 0.50 sec, (○) 0.54 sec, (■) 0.68 sec, and (□) 0.79 sec. C and D, Deactivation time constants τ as a function of the length of (C) 0.8 mM isoflurane or (D) 0.9 mM halothane exposure. C, τ values for control responses. The time = 0 point shows τ values measured when the anesthetic and ACh were applied simultaneously and ACh was washed out 30 sec later in the presence of anesthetic. The ACh concentration was 10 μM.

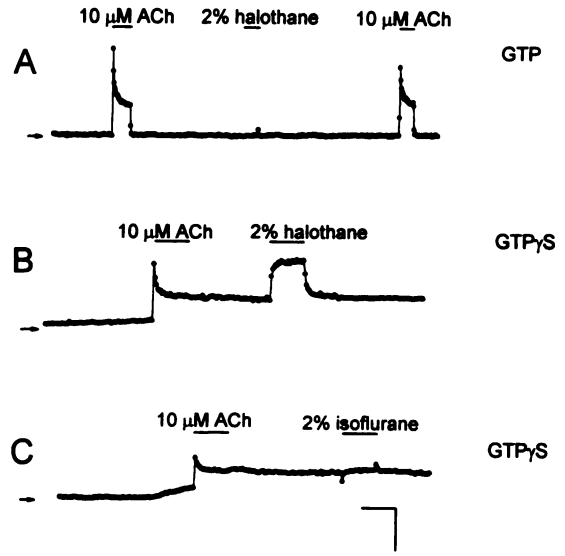

Fig. 7. Effects of halothane and isoflurane on the muscarinic K^+ channel. Whole-cell currents measured at the end of 250-msec pulses to −5 mV from a holding potential of −85 mV are plotted as a function of time. A, A 30-sec application of 0.9 mM halothane alone does not produce a current o alter subsequent muscarinic responses. B, When 0.5 mM GTPγS was included in the pipette solution, a spontaneously activated $I_{K(ACh)}$ was produced. Application of ACh induced a further, persistent increase in the current. Current through the persistently activated channels was enhanced by the application of 0.9 mM halothane. C, Like in B, showing that 0.8 mM isoflurane had no effect. *Horizontal bar*, 60 sec for trace A and 30 sec for traces B and C. *Vertical bar*, 0.2 nA.

whereas the peak response was reduced to 87% and 75% of control for isoflurane and halothane, respectively. The magnitude of these effects increased as the concentration of the anesthetic increased. For isoflurane, in contrast, the steady state response was not altered at any concentration, whereas for halothane it actually increased. We have also observed, as the above results imply, that rapid desensitization was reduced even at the lowest concentrations of both anesthetics; this effect became more pronounced at increased anesthetic concentrations.

In general, it seems that the immediate anesthetic effects are similar to those obtained when lower concentrations of the anesthetics are applied for longer periods of time.

The effect of isoflurane and halothane on persistently activated $I_{K(ACh)}$. Intracellular application of hydrolysis-resistant GTP analogs [GTPγS, Gpp(NH)p] induces persistent activation of $K_{(ACh)}$ because deactivation of the system through the GTPase activity of G_K cannot occur. Under these conditions, the system can be activated even in the absence of agonists via a basal, receptor-independent nucleotide exchange. This process is rather slow but can be greatly accelerated by agonist (18).

To examine whether anesthetics can affect the $I_{K(ACh)}$ channel directly, we used persistently activated $I_{K(ACh)}$ channels to minimize the influence of receptor/G protein interactions. Persistent $I_{K(ACh)}$ activation was produced by including 500 μM GTPγS in the pipette solution and applying a 30-sec pulse of 10 μM ACh to speed up activation. Note that $I_{K(ACh)}$, which was monitored in these experiments by measuring membrane currents at the end of 250-msec depolarizing pulses to −5 mV, remained fully activated despite the complete washout of ACh (Fig. 7, B and C). Under such conditions, 0.9 mM halothane rapidly increased the magnitude of $I_{K(ACh)}$ (Fig. 7B). The effect was reversible; on removal of halothane, the current returned to its prehalothane level. The current/voltage relationship of the halothane-induced current was similar to that of $I_{K(ACh)}$ (data not shown), indicating that halothane increased the probability of the channel being open. In contrast, 0.8 mM isoflurane did not alter persistently activated $I_{K(ACh)}$ (Fig. 7C). These results are in good agreement with the data of Figs. 1 and 2 that show that the steady current increased only in the presence of halothane.

Halothane (Fig. 7A) or isoflurane (data not shown) alone had no effect on the basal membrane currents. Furthermore, the signal transduction system remained functional after the anesthetic application; thus, as shown in Fig. 7A, when 10 μM ACh was applied 1.5 min after halothane washout, the response was essentially identical to that measured before anesthetic application.

Effects of halothane and isoflurane on single-channel properties. Anesthetic effects on persistently activated channels (Fig. 7) suggest that halothane, but not isoflurane, alters the properties of the activated muscarinic K^+ channel. In principle, changes in channel conductance, lifetime, or probability of opening alone or in combination must underlie altered macroscopic currents. To elucidate how these parameters contribute to the enhanced steady state current in the presence of halothane, we examined the single-channel properties of $I_{K(ACh)}$ in excised membrane patches exposed to halothane or isoflurane. Patches were excised into 110 mM KCl containing bath solution, and $K_{(ACh)}$ channels were persistently activated by the addition of 50 μM GTPγS to the bath solution. Fig. 8 shows representative current traces from single $I_{K(ACh)}$ channels recorded before and in the pres-

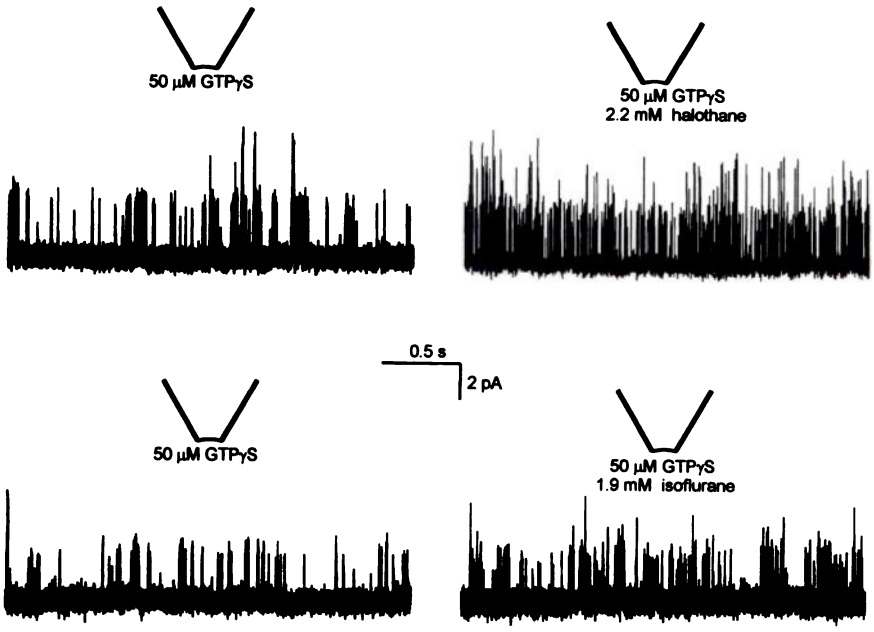

Fig. 8. Isoflurane and halothane alter single-channel properties differently. Single-channel currents were recorded in excised, inside-out atrial cell membrane patches. The intracellular compartment (bath) was held at −85 mV. Muscarinic K^+ channels were activated by applying 50 μM GTPγS in the bath solution. Recordings were obtained from the same patch of membrane before and after anesthetic was applied, as indicated on the figure. *Upward deflections*, channel opening.

ence of 2.2 mM halothane or 1.9 mM isoflurane in the bath solution.

The switch to halothane containing bath solution immediately changed the pattern of channel activity. As it is evident from Fig. 8, halothane did not alter the single-channel current. Data from four different patches were analyzed; the control periods contained 848, 616, 1726, and 447 openings, whereas the periods after the application of 2.2 mM halothane contained 4016, 3087, 6916, and 4819 openings, yielding single-channel currents of 2.5 ± 0.15 and 2.5 ± 0.16 pA without and with halothane, respectively. In contrast, a marked increase in the frequency of channel openings was evident. The mean open probability of the channels in each of the four patches, estimated as the ratio of total open times to total recording times, was 0.071 ± 0.05 and 0.20 ± 0.07 before and in the presence of halothane, respectively. Thus, halothane increases channel open probability without altering single-channel conductance. The 2–3-fold increase in mean open probability accounts for the increase of the steady state current observed in whole-cell recordings.

We also detected more openings in the presence of 1.9 mM isoflurane than in control, although the change is far less dramatic than it was for halothane. Although isoflurane slightly decreased the single-channel currents, from 2.30 ± 0.12 pA (three different control patches contained 2601, 1569, and 978 openings) to 2.15 ± 0.09 pA (corresponding patches in the presence of isoflurane, containing 7471, 4028, and 3495 openings), this difference was not significant ($p <$ 0.05). The overall channel open probability did not change (0.11 ± 0.08 and 0.11 ± 0.09 without and with isoflurane, respectively).

Controls for thymol, a preservative of commercial halothane. Thymol is added to halothane to retard its spontaneous oxidative decomposition. In addition to being an antioxidant, thymol is well known as a fungicide and antihelmintic (21). Thymol may not be an inert ingredient; it has been implicated, for example, in producing hepatotoxicity during halothane anesthesia. (22). Because the vapor pressure of thymol is 1 mm Hg at 64° (23) and that of halothane is 242 mm Hg at 20° (24), thymol is expected to remain mostly in the vaporizer unless the vaporizer is nearly depleted. Because we avoided the latter condition, thymol should not to be a significant contaminant in our experiments.

The possibility of interference by thymol was addressed in control experiments examining the effects of 0.01% thymol on $I_{K(ACh)}$. This is the maximal concentration of thymol that would be present in our test solution if we dissolved the equivalent of 2.2 mM halothane directly instead of using a vaporizer. We found that thymol by itself blocks $I_{K(ACh)}$ significantly.[1] Thus, in experiments in which halothane is not administered by vaporizer but the liquid halothane is added directly to the superfusing solution, thymol may contribute to the effects observed.

Control experiments using pure, thymol-free halothane were carried out to verify the lack of any thymol effects in our data. The results obtained with thymol-free halothane were indistinguishable from those obtained with commercial halothane delivered by vaporizer.

Discussion

Isoflurane and halothane alter $I_{K(ACh)}$. Our data show that both halothane and isoflurane significantly alter the muscarinic activation of K^+ channels even at clinically relevant concentrations, in the range of 0.1–0.9 mM. Phenomenologically, there are both similarities and differences in the ways in which these two anesthetics act. Halothane and isoflurane significantly reduced the rate of $I_{K(ACh)}$ activation and, to a minor extent, deactivation. Furthermore, both anesthetics reduced or eliminated the spontaneous decay (rapid desensitization) typical of the muscarinic response. In contrast to these parallel effects on the time course, the magnitude of the $I_{K(ACh)}$ response was slowly reduced by isoflurane but rapidly augmented by halothane. Neither halothane nor isoflurane altered the conductance of single $I_{K(ACh)}$ channels,

[1] J. Magyar and G. Szabo, manuscript in preparation.

indicating that their action must be via altered channel open-close kinetics.

It is interesting to note that both isoflurane and halothane had immediate effects on $I_{K(ACh)}$ as judged by test responses in which anesthetic and ACh were coapplied. The immediate effects were most prominent as a reduction in the peak response and the rate of channel activation. Other effects (e.g., the reduction in the steady state current by isoflurane), required considerable time to develop. A simple calculation taking into account diffusion through unstirred layers, with the assumptions that membrane permeability of the anesthetic is high and that at short times the cell acts as an anesthetic sink) shows that the initial anesthetic concentration at the cell membrane should be near 50% of the superfusing concentration. Therefore, rapid effects probably reflect direct actions of the anesthetics on components of the signal transduction system located in the cell membrane. In contrast, slowly developing effects may indicate indirect actions (e.g., altered phosphorylation or dephosphorylation) of some components of the signaling pathway that may require extended presence of the anesthetics intracellularly.

Muscarinic activation of K^+ channels comprises several steps, including activation of receptor by agonist, receptor/G protein interaction, nucleotide exchange on the G protein α subunit, and activation of the channel by activated G protein. In principle, each one of these steps may be affected by anesthetics. We used kinetic measurements and hydrolysis-resistant GTP analogs to determine how isoflurane and halothane affect these steps.

Anesthetic effects on receptor function. We found previously and confirm here that the rate of activation of $I_{K(ACh)}$ depends on the concentration of ACh in a manner that can be described by the Hill equation with an EC_{50} of 0.42 μM and a Hill coefficient of 0.6 (15). This dependency presumably reflects the fraction of receptors activated by ACh. Isoflurane and halothane reduced the EC_{50} from 0.42 to 0.10 and 0.19 μM, respectively, suggesting a small increase in receptor affinity due to the anesthetics. In contrast, the maximal attainable activation rate was reduced form 22 to 12.2 and 12.1 sec^{-1}, respectively, for isoflurane and halothane. Note that most rate measurements were carried out at saturating ACh concentrations (1 or 10 μM) at which the shift in receptor affinity would have no effect.

Isoflurane and halothane have been reported to have no effect on the agonist binding in rat heart and rat brain, whereas these anesthetics increased the antagonist binding to muscarinic ACh receptor by decreasing the dissociation at room temperature (8, 9). Bazil and Minneman (25) reported that at 37°, 1.25% halothane modified neither the agonist nor the antagonist binding to muscarinic or α- and β-adrenergic receptors in rat brain. In human myocardium, halothane consistently decreased the antagonist binding to muscarinic ACh receptor, whereas it had no effect on binding to β-adrenergic receptors. Halothane did not influence the antagonist affinity for muscarinic or β-adrenergic receptors in these experiments (11). Note, however, that these findings may not be relevant to our results because we measured very rapid effects of ACh, whereas binding studies involve long exposures to agonist so that long term effects (e.g., "desensitized" receptor conformational states due to long exposures to agonist or antagonist) may become a significant component of the measurement.

Anesthetic effects on receptor/G protein interactions. Formation of a stable complex of receptor and G protein in the absence of GTP or GDP, as demonstrated by a high affinity shift of agonist binding, has been used to study receptor/G protein coupling under a variety of circumstances (10). This system was used by Aronstam *et al.* (9), who found that halothane prevented the decrease in muscarinic agonist binding affinity induced by Gpp(NH)p in rat brain, implying that the anesthetic interfered with the formation of receptor/G protein complexes and therefore the signal transduction process. In contrast, Böhm *et al.* (11) reported that halothane had no effect on the ability of Gpp(NH)p to lower the agonist binding in human myocardium. We found that the rate of activation of $I_{K(ACh)}$ was reduced by both isoflurane and halothane. Because the activation rate at limiting ACh concentrations is a characteristic of receptor/G protein interactions, our data imply that the coupling process is slowed but not eliminated by these anesthetics. This impaired activation process may result from the effect of anesthetics on the receptor, G protein, or both; the data presented here does not distinguish between these possibilities. An alternate possibility (i.e., that the anesthetics decrease the rate of diffusion of receptors to G proteins by altering the state of the membrane lipids) is unlikely because anesthetics are expected to render the membrane less, not more viscous, although more complex mechanisms of this type (e.g., altered lipid domains or boundary lipid structures) cannot be excluded.

We found that both isoflurane and halothane reduced the rates of $I_{K(ACh)}$ activation. Given that the rates of deactivation were practically unaltered, this would imply a decrease in the magnitude of the response. A simple calculation using a two-state model indicates, however, that the expected reduction in the response is less than what is observed. Thus, for example, a 3% decrease is calculated but a 25% decrease is observed when 0.8 mM isoflurane is applied initially (Fig. 2), suggesting the additional mechanisms influence the magnitude of the response. Direct effects of the anesthetics on the channel, G protein, or their interactions are likely candidates.

Direct effects of isoflurane and halothane on the channel. Anesthetic effects on ion channels have been reported previously. In guinea pig heart cells, 2 volume % halothane depresses the calcium current and the delayed rectifier K^+ current but does not alter the inward rectifier K^+ current (26). In bullfrog atrial cells, halothane depresses the delayed rectifier K^+ current, increasing thereby action potential duration, but has no effect on the inward rectifier K^+ current (27). It has been reported that ether, isoflurane, and propofol modify the nicotinic ACh channel function in different ways (28). In these experiments, ether reduced the current amplitude, isoflurane caused flickering channel activity, and propofol only decreased the open time of the channel. The authors concluded that these diverse patterns can be explained by the direct action of the volatile anesthetics on the channel protein. Volatile anesthetics may not only block channel function but also enhance it. Thus, for example, halothane was found to markedly enhanced the γ-aminobutyric acid-activated Cl^- currents in cultured dorsal root ganglion neurons (29). In guinea pig ventricular cells, halothane increased the mean open probability of the large-conduc-

tance, calcium-activated K^+ channel, whereas isoflurane had no effect (30).

We find that halothane, but not isoflurane, increased the magnitude of $I_{K(ACh)}$. The extent of the enhancement depends on the concentration and duration of the halothane applied. Note that neither isoflurane nor halothane produced a detectable current when applied alone. In contrast, when $I_{K(ACh)}$ was activated persistently by GTPγS, halothane produced a large increase in $I_{K(ACh)}$, whereas isoflurane had no effect (Fig. 7). These results at the macroscopic level were corroborated by single-channel measurements. The primary effect of 0.8 mM halothane on excised patches of membrane containing $I_{K(ACh)}$ channels persistently activated by GTPγS was to increase the frequency of channel openings. The single-channel conductance was not altered and the mean channel lifetime was only marginally affected by the anesthetic. The consequent increase in the mean open time of the channel accounts for the increased amplitude of $I_{K(ACh)}$ seen in the whole-cell experiments. Clearly, halothane does not block the channel but rather changes its gating properties, presumably by altering the interactions of the channel with activated G protein.

It may be noted that our finding that halothane increased the magnitude of the muscarinic response differs from that of Zang *et al.* (19). Using guinea pig atrial cells, these authors observed a decreased amplitude of the muscarinic response after 9-min applications of 0.5 mM halothane. The difference may be ascribed to the use of different species. However, if the halothane used by these authors contained thymol (see Results) and the solutions were prepared by the addition of the halothane to the test solutions, then it is possible that the observed decrease was produced by thymol.

Isoflurane had less pronounced effects on $I_{K(ACh)}$. The single-channel conductance was not altered, indicating again that the anesthetic has little action on the open channel. We detected a small increase in the frequency of channel opening, but this seems to be counterbalanced by reduced channel lifetimes, so the mean open time τ remained approximately the same.

Rapid desensitization. The muscarinic response to application of a fixed concentration of ACh is characterized by a spontaneous decay of the current, the rapid desensitization (18). As illustrated in Fig. 1, both isoflurane and halothane gradually eliminated this feature of the response. A smaller decrease is seen for the amplitude of the slow component (A_2). Although the precise mechanisms of rapid desensitization are not known, it has been suggested that the slow and fast components represent different processes (19), with the former involving the receptor and the later involving phosphorylation or dephosphorylation of the G protein or the channel (20). Our observation of a preferential anesthetic sensitivity of the fast component is in agreement with this hypothesis. It will be interesting to determine the precise mechanisms by which anesthetics abolish these processes.

In summary, we find that isoflurane and halothane have similar effects on the time course of $I_{K(ACh)}$ but alter the magnitude of the response differently. The parallel effects of these volatile compounds may be related to their anesthetic action, whereas the differences could account for specific side effects of clinical importance.

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