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Effect of Halothane on Large-conductance Calcium-dependent Potassium Channels in Cerebrovascular Smooth Muscle Cells of the Rat

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Background: **Patch-clamp methods were used to examine the effects of the volatile general anesthetic halothane on large-conductance calcium-dependent potassium channels (BK channels) in dispersed cerebrovascular smooth muscle cells of adult rats.**

Methods: **Inside-out membrane patches were used for recordings at 21–23°C. Halothane was administered at aqueous concentrations of 0.5–2.8 mM in conjunction with free cytoplasmic calcium concentrations of 1 or 100 μM and at a membrane potential of −60 mV or +60 mV.**

Results: **At a free cytoplasmic calcium concentration of 1 μM, the clinically relevant dose of 0.5 mM (2 MAC) halothane reduced the open probability of large-conductance calcium-dependent potassium channels without altering the single-channel conductance. This effect was blocked by increasing the concentration of cytoplasmic free calcium to 100 μM, but was not intrinsically voltage dependent.**

Conclusion: **The marked dilation of cerebral vessels seen during surgical anesthesia with halothane cannot be attributed to direct effects of the drug on large-conductance calcium-dependent potassium channels. The protective effect of calcium suggests that halothane exerts its effects at channel sites located within the cell membrane. (Key words: Anesthetic, volatile: halothane. Smooth muscle, cerebrovascular: potassium channel.)**

HALOTHANE anesthesia is associated with dilation of cerebral vessels, increased cerebral blood flow, and impairment of cerebral blood flow autoregulation.[1–3] The mechanisms by which halothane produces these cerebrovascular effects remain unclear. However, in clinically relevant concentrations (0.5–2 vol%, corresponding to 0.6–2.2 mM in plasma at 37°C), halothane causes electromechanical uncoupling in cerebrovascular smooth muscle cells (CVSMCs).[4] In this situation, dilation of the arterial wall is accompanied by a depolarization of the CVSMC membrane, which under normal circumstances would trigger calcium (Ca^{2+}) entry through voltage-operated Ca^{2+} channels and thereby promote contraction.[4–6]

Halothane suppresses both the Ca^{2+}[6,7] and the potassium (K^{+}) conductances of excitable cells.[8,9] In principle, electromechanical uncoupling in CVSMCs could result if the depressant effects of halothane on membrane Ca^{2+} fluxes normally predominate *in vivo*. A recent study suggests that this is indeed the case in some cerebral vessels, where blockers of membrane K^{+} conductance potentiate halothane-induced relaxation.[10]

Our laboratory has recently shown that rat CVSMCs possess a population of Ca^{2+}-dependent K^{+} channels that are activated by increases in the intracellular concentration of free Ca^{2+} ions.[11,12] These channels belong to the "big-conductance" (BK) class of Ca^{2+}-dependent K^{+} channel, as previously defined.[13,14] In other tissues, the function of BK channels is known to be inhibited by clinically relevant concentrations of halothane.[15–19] The aim of the current study was to determine whether halothane also alters the conductance or open probability (P_o) of the BK channels present in rat CVSMCs. This information is necessary for clarifying the mechanisms by which halothane causes electromechanical uncoupling and dilation of cerebral vessels.

Materials and Methods

CVSMCs were dissociated from basilar, middle and posterior cerebral arteries of adult Wistar rats, 250–300 g.[20] During pentobarbital anesthesia (30 mg/kg), rats were decapitated and the arteries removed with aseptic techniques. Arteries were cut into small pieces and incubated for 10 min at 37°C in 0.1% trypsin (type

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3, Sigma, St. Louis, MO) dissolved in potassium glutamate solution containing (millimolar): 140 potassium glutamate, 16 $NaHCO_3$, 16.5 dextrose, 0.5 NaH_2PO_4, and 25 hydroxyethylpiperazineethanesulfonic acid (HEPES), *p*H 7.4. Artery fragments were washed in potassium glutamate solution containing 0.5% trypsin inhibitor (Sigma) and incubated for 15 min at 37°C in potassium glutamate saline with 0.3% collagenase (type 1A, Sigma). On dispersion, cells were washed in Minimum Essential Medium (Gibco, Burlington, Ontario, Canada) and resuspended in a balanced salt solution containing (millimolar): 130 NaCl, 5 KCl, 0.8 $CaCl_2$, 1.3 $MgCl_2$, 5 glucose, and 10 HEPES, *p*H 7.4. Cells were plated onto glass coverslips precoated with poly-D-lysine and laminin (Sigma), kept at 4°C and used within 48 h of dispersion.

Patch-clamp recordings were conducted at 21–23°C with an amplifier (EPC-5, List, Darmstadt, Germany) and patch electrodes (10–20 MΩ) containing (millimolar): 140 KCl and 10 HEPES, *p*H 7.4. Standard methods were used to obtain inside-out membrane patches from dispersed CVSMCs.[11,12,21] The cytoplasmic face of isolated membrane patches was exposed to a saline of composition (millimolar): 140 KCl, 10 HEPES, and 3 ethyleneglycol-bis-(β-aminoethyl ether)-tetraacetic acid, *p*H 7.4. Total calcium chloride concentration was adjusted to yield a free cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) of 1 μM or 100 μM.[22]

Patch current and voltage were recorded on frequency-modulation wideband tape, digitized at 8 kHz and subjected to a Gaussian digital filter of bandwidth DC-2 kHz. Off-line data analysis was performed on a computer (Mega 4, Atari, Sunnyvale, CA) with software devised by Instrutech (New York, NY). The threshold for event detection was set at 50% of the mean current in open BK channels. The probability P_o of a single BK channel being open during a recording of duration T_{tot} was calculated as $P_o = (T_1 + T_2 + \ldots + T_N)/NT_{tot}$, where N = the number of functional BK channels in the patch, and $T_1, T_2, \ldots T_N$ = the times for which at least 1, 2, . . . N channels were open. N was estimated by exposing the cytoplasmic membrane face to 100 μM free Ca^{2+} at a membrane potential of +60 mV. Under these conditions, an N value of 1 was observed in most patches studied. Results were expressed as mean ± SEM. All data were required to reach a level of significance of $P < 0.05$ by Student's *t* test for paired data or analysis of variance for unpaired data, as appropriate.

For patches containing a single BK channel, open- and closed-time distributions were plotted on a logarithmic time axis, transforming the exponential function $y = A \cdot e^{-t/\tau}$ into a curve with peak amplitude at the time constant τ.[23] These distributions were fitted by sums of exponential terms by using Simplex maximization of likelihood.[24] Computer-generated Gaussian terms were fitted by eye to frequency distributions of single-channel current amplitudes.

Halothane (Fluothane, Ayerst, Montreal, Canada) was equilibrated with experimental salines diluted in compressed air carrier gas (1 l/min) with a Fluotec 3 vaporizer (Cyprane, Keighley, Yorkshire, UK). The minimum alveolar concentration (MAC) for halothane in the rat is 1.0%, corresponding to an aqueous drug concentration of 0.29 mM at 37°C.[25] However, the water–gas partition coefficient for halothane increases with decreasing temperature, and the aqueous drug concentration that corresponds to 1 MAC is reduced to 0.23 mM at 22°C. Clinically relevant halothane concentrations therefore range from 0.1 to 0.5 mM under these conditions.[25]

After 30-min equilibration periods at various vaporizer settings, halothane concentrations present in experimental salines were measured[19] by fluorine nuclear magnetic resonance techniques.[17,25] Trifluoroacetic acid (3 mM) served as an external standard. The salines contained halothane concentrations of 0.5, 1.6, and 2.8 mM. Salines containing halothane were applied by rapid perfusion (5 ml/min) to the cytoplasmic face of membrane patches positioned within 200 μm of the solution inlet port.

Results

The effect of 2.8 mM halothane on single BK channel currents are shown in figure 1. These recordings were obtained at $[Ca^{2+}]_i$ = 100 μM and at a membrane potential of V = −60 mV. Inspection of these traces indicated that even high concentrations of halothane had no discernible effect on the amplitude of currents flowing in open BK channels. This impression was confirmed by comparison of the amplitude distributions of BK channel currents recorded in the presence and absence of the anesthetic (fig. 2A). In both cases, these distributions were well fit by single Gaussian terms, the modal value of which was not altered by halothane. Over the dose range tested, neither the slope of the current–voltage relation for BK channels (single-channel conductance, control value 203 ± 11 pS from 13 patches) nor the reversal potential of these single-

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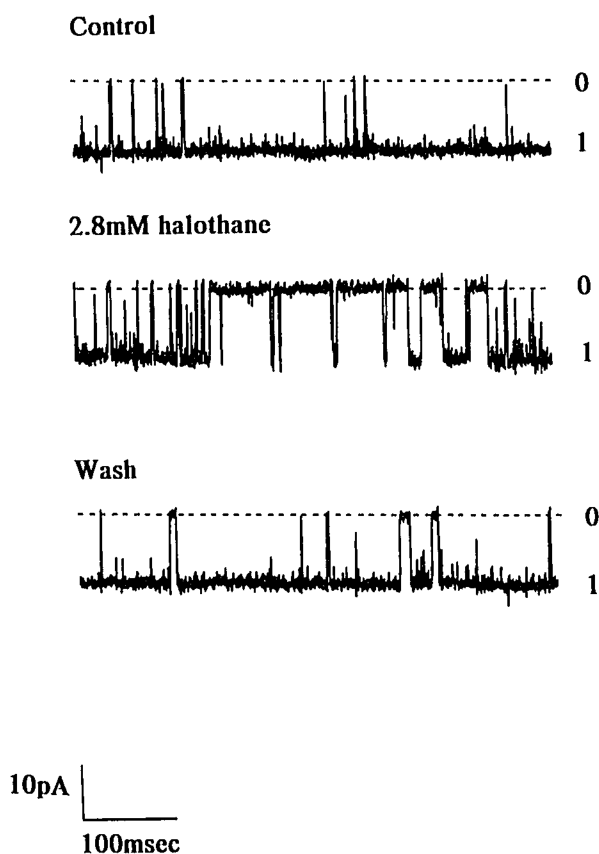

Fig. 1. The effect of 2.8 mM halothane on single large-conductance calcium-dependent potassium channel currents recorded from an inside-out patch of cerebrovascular smooth muscle cell membrane voltage-clamped to a potential of −60 mV. Halothane was applied by bath to the cytoplasmic membrane face. 0 = Closed-channel current; 1 = open-channel current; downward deflection from baseline = inward current. Temperature 22°C; bandwidth of recording DC-2 kHz. $[K^+]_o$ and cytoplasmic free potassium concentration = 140 mM; cytoplasmic free calcium concentration = 100 μM.

channel currents (0 mV in symmetrical 140 mM KCl salines) was altered by 2.8 mM halothane (fig. 2B).

Figure 1 further implied that halothane reduced the P_o of BK channels. This possibility was confirmed with data from patches at $[Ca^{2+}]_i$ = 100 μM and V = −60 mV (fig. 3). Under these conditions, control values of P_o averaged 0.83 ± 0.07 and application of 0.5 mM halothane did not significantly alter this value (n = 7 patches, $P > 0.05$, paired *t* test). Application of carrier gas alone also had no effect on P_o. However, application of 1.6 mM halothane significantly and reversibly reduced P_o by an average of 14 ± 3.3% (n = 11 patches, $P < 0.05$, paired *t* test). On increasing the halothane concentration to 2.8 mM, a larger decrease of 59 ± 7.4% was seen in P_o (n = 11, $P < 0.05$, paired *t* test). This effect also reversed on perfusion with drug-free saline.

The effect of membrane potential on the ability of halothane to reduce P_o in BK channels was studied in patches exposed to 1.6 mM halothane with $[Ca^{2+}]_i$ = 100 μM and voltage clamped to V = −60 mV or V = +60 mV. No significant difference was seen between data obtained at these two holding potentials (at V = −60 mV, mean reduction in P_o = 9 ± 1.0%, n = 7 patches; at V = +60 mV, mean reduction in P_o = 14 ± 3.3%, n = 11 patches, $P > 0.05$, analysis of variance).

Recent reports have indicated that the inhibitory effect of halothane on some BK channels is enhanced at low $[Ca^{2+}]_i$.[18,19] Therefore, the effects of halothane on BK channels were also examined in patches voltage clamped to V = −60 mV, but now exposed to saline containing $[Ca^{2+}]_i$ = 1 μM (figs. 3 and 4).

Under these conditions, halothane once again failed to alter the conductance of BK channels (control mean, 187 ± 13 pS, mean for 1.6 mM halothane, 177 ± 10

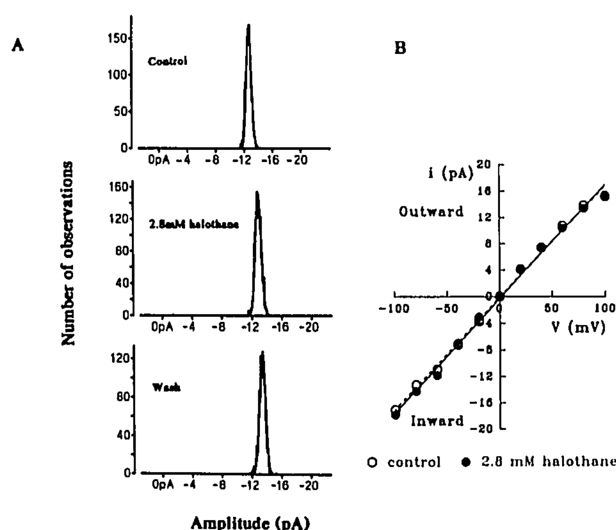

Fig. 2. (*A*) Lack of effect of 2.8 mM halothane on the amplitude distribution of single large-conductance calcium-dependent potassium channel currents recorded from a second inside-out patch voltage-clamped to −60 mV. All three distributions were well fitted by single Gaussian terms (smooth curves) with modal values at −12.9 pA (control), −13.0 pA (halothane) and −13.4 pA (wash). (*B*) Lack of effect of 2.8 mM halothane on the current–voltage relation of single BK channel currents recorded from a third inside-out patch. Both data sets were well fitted by least-squares regression to straight lines, which yielded a single-channel conductance of 178 pS for control data (solid line) and 177 pS for halothane data (dashed line). Corresponding reversal potentials were 2 mV (control) and 1 mV (halothane).

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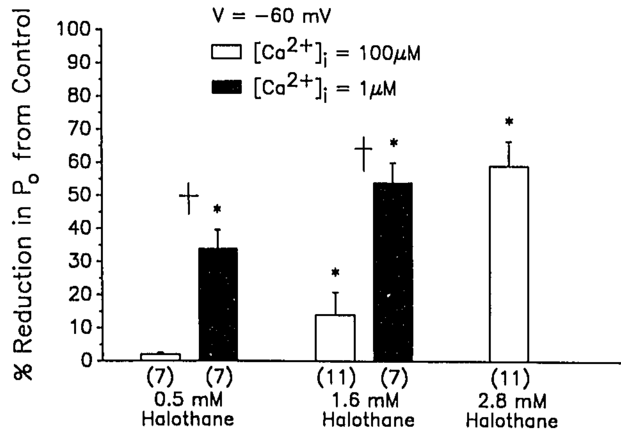

Fig. 3. Percentage reduction in the open probability (P_o) of large-conductance calcium-dependent potassium channels caused by 0.5, 1.6, and 2.8 mM halothane applied to inside-out patches of cerebrovascular smooth muscle cell membrane. Data were obtained at a membrane potential V = −60 mV and with cytoplasmic free calcium concentration ($[Ca^{2+}]_i$) = 100 μM (open bars) or 1 μM (filled bars). Numbers in parentheses = number of patches in each group. *Halothane significantly reduced P_o relative to control values in same patches, (P < 0.05, paired Student's *t* test); †data obtained with $[Ca^{2+}]_i$ = 100 μM and $[Ca^{2+}]_i$ = 1 μM significantly different (P < 0.05, analysis of variance).

pS, n = 7 patches, $P > 0.05$, paired *t* test). The mean control value of P_o was 0.32 ± 0.07 and 0.5 mM halothane was now effective in reducing this by an average of 34 ± 5.6% (7 patches, $P < 0.05$, paired *t* test, figs. 3 and 4). Application of 1.6 mM halothane resulted in a 54 ± 6.0% reduction in P_o (n = 7 patches, $P < 0.05$, paired *t* test, fig. 3). The reductions in P_o caused by 0.5 mM and 1.6 mM halothane were both significantly larger in the presence of saline containing $[Ca^{2+}]_i$ = 1 μM than were seen with solutions containing $[Ca^{2+}]_i$ = 100 μM ($P < 0.05$, analysis of variance).

The reduced P_o seen in the presence of halothane could result from a decreased mean channel open time, an increased mean channel shut time, or both. This was determined by analyzing the effects of halothane on the kinetic properties of BK channels. In these experiments, patches contained one functional BK channel. Patches were voltage-clamped clamped to −60 mV and exposed to saline containing $[Ca^{2+}]_i$ = 1 μM.

Figure 5 shows the influence of 1.6 mM halothane on the open-time distribution of BK channels. In both the absence and presence of halothane, these distributions were well fit by the sum of two exponential terms, $y = N_f \cdot e^{-t/\tau_{of}} + N_s \cdot e^{-t/\tau_{os}}$. Here τ_{of} and τ_{os} were the time constants of the fast- and slow-fit components, displaying zero-time amplitudes of N_f and N_s, respectively. The areas of the fit components corresponding to τ_{of} and τ_{os} were denoted A_{of} and A_{os}, respectively. This result indicated that BK channels typically exhibit two kinetically resolvable open states, as shown in previous studies,[11,12] and that this behavior was not altered by halothane. Halothane had no significant effect on the mean value of τ_{of} (0.7 ± 0.15 ms, n = 7) at any of the doses tested. However, 1.6 mM halothane reversibly reduced the mean value of τ_{os} from 30 ± 11.0 to 15 ± 5.7 ms (n = 7, $P < 0.05$).

The mean open time of BK channels was calculated from the fitted open-time distributions by using the relation $\tau_{open} = A_{of}/(A_{of} + A_{os}) \cdot \tau_{of} + A_{os}/(A_{of} + A_{os}) \cdot \tau_{os}$. As shown in table 1, 1.6 mM halothane significantly reduced τ_{open} in a reversible manner.

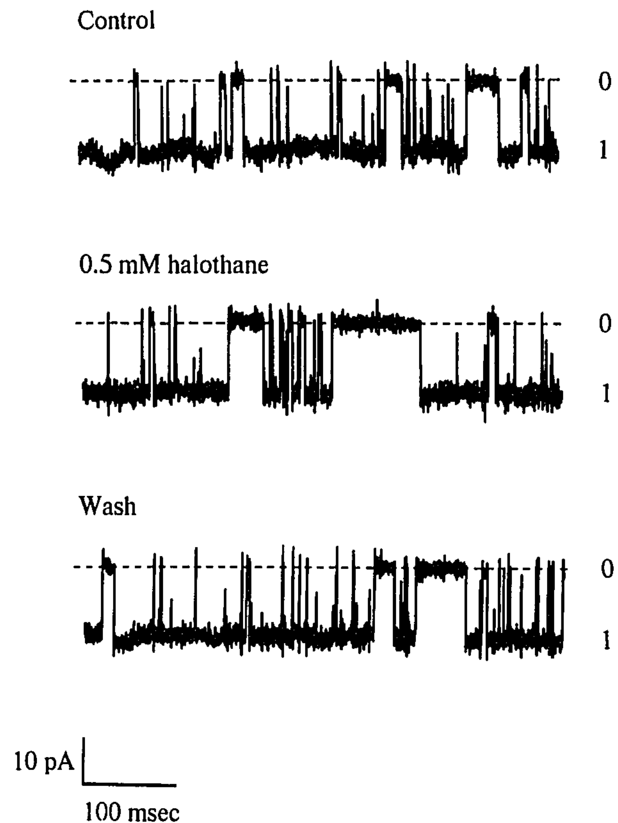

Fig. 4. Effect of 0.5 mM halothane on single large-conductance calcium-dependent potassium channel currents recorded from an inside-out patch voltage-clamped to V = −60 mV. Halothane was applied to the cytoplasmic membrane face, which was exposed to saline in which cytoplasmic free calcium concentration = 1 μM. 0 = Closed-channel current; 1 = open-channel current. Bandwidth of recording DC-2 kHz.

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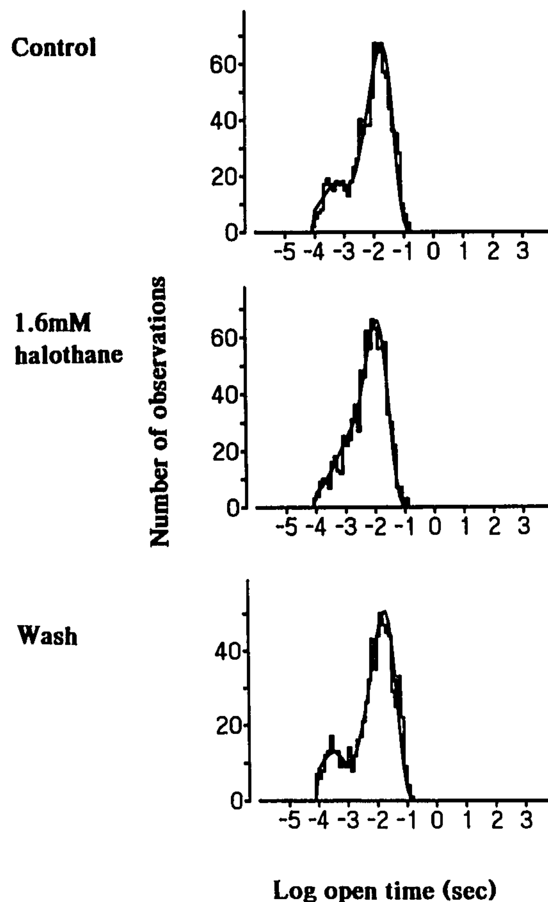

Fig. 5. Effect of 1.6 mM halothane on the open-time distribution of single large-conductance calcium-dependent potassium channel currents recorded from an inside-out membrane patch voltage-clamped to −60 mV with cytoplasmic free calcium concentration = 1 μM. This patch contained only one functional channel. Time scale is logarithmic. These distributions were constructed from 700 to 989 events and were well fitted, by maximization of likelihood, by the sum of two exponential terms (smooth curves). In the control plot, the fast-fit component had a time constant τ_{of} = 0.46 ms, and this component made up 16% of the total openings. The slow-fit component had a time constant τ_{os} = 20 ms and made up the remaining 84% of openings. Corresponding values for the other two distributions were as follows. Halothane: τ_{of} = 0.79 ms, 13% of openings; τ_{os} = 13 ms, 87% of openings. Wash: τ_{of} = 0.29 ms, 18% of openings; τ_{os} = 21 ms, 82% of openings. The mean channel open times (τ_{open}) calculated from these parameter fits were 17 (control), 11 (halothane), and 17 (wash) ms.

In both the absence and presence of halothane, closed-time distributions for BK channels were well fit by the sum of three exponential terms, $y = N_f \cdot e^{-t/\tau_{cf}} + N_m \cdot e^{-t/\tau_{cm}} + N_s \cdot e^{-t/\tau_{cs}}$, where τ_{cf}, τ_{cm}, and τ_{cs} = the time constants of the fast-, medium-, and slow-fit components, respectively (fig. 6). The areas of these three components were designated A_{cf}, A_{cm}, and A_{cs}, respectively. This result indicated that BK channels normally exhibit three kinetically resolvable closed states, and that this behavior was not modified by halothane.

At a concentration of 1.6 mM, halothane did not significantly alter the mean value of τ_{cf}, which averaged 0.5 ± 0.1 ms in 7 control measurements ($P > 0.05$). Similarly, τ_{cm} (38 ± 13.0 ms, n = 7) was unaltered by the presence of 1.6 mM halothane ($P > 0.05$). However, this concentration of halothane did reversibly increase the mean value of τ_{cs} from 180 ± 32 to 301 ± 64 ms (n = 7, $P < 0.05$).

The mean channel closed time, τ_{closed} was calculated from the fit parameters of closed-time distributions by using the equation $\tau_{closed} = A_{cf}/(A_{cf} + A_{cm} + A_{cs}) \cdot \tau_{cf} + A_{cm}/(A_{cf} + A_{cm} + A_{cs}) \cdot \tau_{cm} + A_{cs}/(A_{cf} + A_{cm} + A_{cs}) \cdot \tau_{cs}$. Application of 1.6 mM halothane significantly and reversibly increased τ_{closed}, as shown in table 1.

Discussion

The current study shows that an aqueous solution of 0.5 mM halothane (equivalent to 2 MAC in the rat[25–28]) significantly reduced the P_o of BK channels in cerebrovascular smooth muscle. This effect was seen when $[Ca^{2+}]_i$ was buffered to 1 μM, a concentration characteristic of smooth muscle cells during contractile activity.[20] When $[Ca^{2+}]_i$ was increased to 100 μM, the inhibitory effect of 0.5 mM halothane of BK channel function was suppressed.

Our results agree well with data previously reported for the BK channels of bovine adrenal chromaffin cells,[18] transverse tubules of skeletal muscle,[19] rat glioma cells,[16] and human red blood cells.[29] Despite their diverse origins, the ED_{50} for halothane action on all of these BK channels is approximately 0.5 mM, provided that the $[Ca^{2+}]_i$ is buffered to low concentrations.[16,18,19,29]

Table 1. Effects of 1.6 mM Halothane on the Mean Open Time, TAU_{Open} and Mean Closed Time, TAU_{Closed} of BK Channels, as Calculated From the Fit Parameters of the Open and Closed Time Distributions

	Control	Halothane	Wash
TAU_{open} (msec) (n = 7)	14 ± 3.5	7 ± 1.2*	11 ± 1.8
TAU_{closed} (msec) (n = 7)	61 ± 17	112 ± 36*	40 ± 12

Recording conditions were V = −60 mV, $[Ca^{2+}]_i$ = 1 μM, temperature 21–23°C.
* Significantly different from control and from post-halothane wash, $P < 0.05$, ANOVA. *n* is the number of patches in each group.

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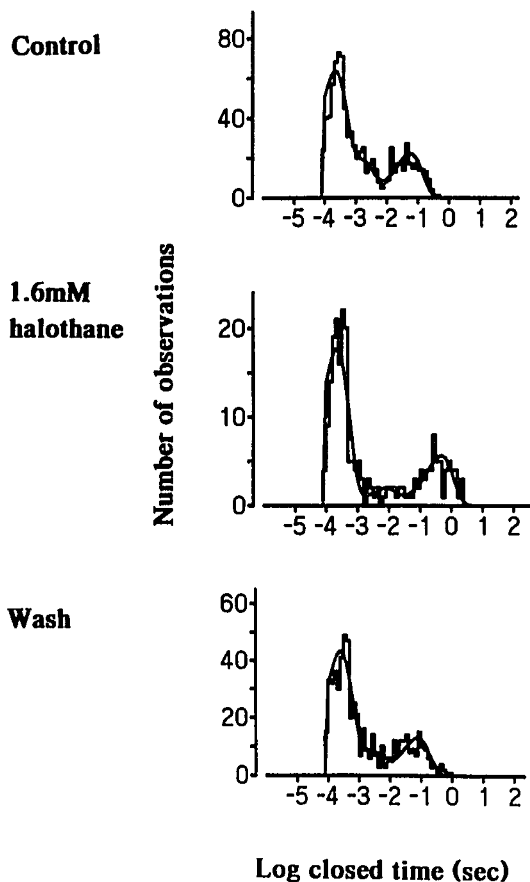
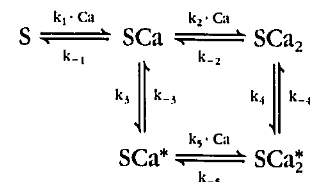


Fig. 6. The effect of 1.6 mM halothane on the closed-time distributions of single large-conductance calcium-dependent potassium channel currents recorded from an inside-out membrane patch voltage-clamped to −60 mV with cytoplasmic free calcium concentration = 1 μM. This patch contained only one functional channel. Time axis is logarithmic. These distributions were constructed from 590–800 events and were fitted by maximum likelihood by the sum of three exponential terms (smooth curves). In the control distribution, the fast-fit component had a time constant τ_{cf} = 0.23 ms and made up 58% of closings. The medium-fit component had a time constant τ_{cm} = 1.6 ms and comprised 17% of closings. The slow-fit component had time constant τ_{cs} = 64 ms (25% of closings). Corresponding values of these parameters in the other two distributions were as follows. Halothane: τ_{cf} = 0.25 ms, 70% of closings; τ_{cm} = 7.6 ms, 7% of closings; τ_{cs} = 532 ms, 23% of closings. Wash: τ_{cf} = 0.27 ms, 67% of closings; τ_{cm} = 2.5 ms, 12% of closings; τ_{cs} = 86 ms, 21% of closings. The mean closed times of this channel, calculated from these parameter fits, were 16 (control), 123 (halothane), and 19 (wash) ms.

The effect of halothane on BK channel activity was suppressed in the presence of high $[Ca^{2+}]_i$, as noted in previous studies.[18,19] The Ca^{2+} binding sites of BK channels are located on protein sites accessible from the cytoplasmic side of the membrane.[13,14] In addition, these sites probably lie within the intramembrane domain of the channel, because Ca^{2+} binding to the channel is sensitive to changes in the transmembrane electric field.[13,14] These considerations suggest that halothane interacts with BK channels from within the lipid bilayer, as opposed to binding to extramembranous channel domains. A similar conclusion has been reached from study of halothane action on mutant K^+ channels with large deletions in their extramembranous segments.[30]

In this and previous studies,[18,19] effective doses of halothane decreased the mean open time and increased the mean shut time of BK channels while leaving channel conductance unchanged. In our study, halothane reduced the slow time constant for open-to-closed transitions of BK channels, as reported for the BK channels of muscle transverse tubules.[19] However, during comparable studies performed on bovine adrenal chromaffin cells,[18] halothane altered only the relative amplitude of this kinetic parameter, not its characteristic time constant. Discrepancies of this kind suggest that small structural differences may exist between the BK channel proteins of various cell types. This has also been inferred from the variable sensitivity of BK channels to Ca^{2+}[13,14] and to tetraethylammonium ions.[11,12,14,16,31] In the current study, as in two others performed on different preparations,[18,19] the number of kinetically resolvable open and closed states of the BK channel was not altered by halothane. Furthermore, the kinetic changes we observed on halothane exposure were similar to those seen in our preparation when $[Ca^{2+}]_i$ is decreased.[12] These results suggest that halothane may act relatively simply, by reducing the binding of Ca^{2+} to the channel protein, as shown in the following state diagram



This model has three closed states (S, SCa, and SCa_2) and two open states (SCa^* and SCa_2^*), as required by the observed distributions of channel dwell times. S is the channel state in the absence of bound calcium ions. The loss or gain of Ca^{2+} ions (Ca) allows direct transitions between SCa^*, a uniliganded open state of brief duration, and SCa_2^*, a biliganded state of longer average lifetime. This feature has been incorporated to account

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for the dependence of the slow time constant of open-time distributions on $[Ca^{2+}]_i$.[12] Halothane may alter BK channel kinetics by inhibiting the binding of Ca^{2+} to the channel protein, thereby reducing the values of the $[Ca^{2+}]_i$-dependent rate constants k_1, k_2 and k_5. The $[Ca^{2+}]_i$-independent rate constants k_3 and k_4 would be unaltered by halothane.

In the presence of 100 μM $[Ca^{2+}]_i$, the inhibitory effect of halothane on BK channel function was unaltered by a large change in the resting potential. This indicates that the interaction of halothane with its membrane binding sites is not intrinsically voltage dependent. However, when the value of $[Ca^{2+}]_i$ is low, membrane depolarization strongly promotes the binding of Ca^{2+} to the BK channel.[12–14] A decrease in the effectiveness of halothane can therefore be observed at positive membrane potentials.[19]

Inhibition of BK channel function by halothane would be expected to oppose repolarization of the smooth muscle membrane during action potentials,[32] thereby promoting the entry of Ca^{2+} through voltage-sensitive Ca^{2+} channels and markedly enhancing contraction of the vessel wall.[33] Under *in vivo* conditions however, vasodilatory actions of halothane on cerebrovascular smooth muscle must predominate, because marked dilation of cerebral vessels is seen during halothane anesthesia.[2,3] Vasodilatory actions of halothane probably include a marked reduction in membrane Ca^{2+} currents, as demonstrated for the related volatile anesthetic isoflurane in canine CVSMCs,[34] and shown for halothane itself in coronary artery smooth muscle cells[35] and in bovine adrenal chromaffin cells.[7] Other possible vasodilatory actions of halothane include decreased intracellular A accumulation,[36] lowered Ca^{2+} sensitivity of the contractile proteins,[37] activation of adenosine triphosphate–sensitive K^+ channels,[33] and modulation of endothelium-dependent vasoconstriction.[38]

It has been suggested that BK channels may play a role in the autoregulation of cerebral blood flow, by opening in response to Ca^{2+} entry triggered by membrane stretch.[39] Inhibition of BK channel function by halothane would impair this vasodilatory response. Disordered autoregulation of cerebral blood flow is indeed observed, especially during deep halothane anesthesia.[40]

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