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Inhibition of the human intermediate conductance Ca^{2+}-activated K^+ channel, hIK1, by volatile anesthetics

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Abstract

Ca^{2+}-activated K^+ channels (K_{Ca}) regulate a wide variety of cellular functions by coupling intracellular Ca^{2+} concentration to membrane potential. There are three major groups of K_{Ca} classified by their unit conductances: large (BK), intermediate (IK), and small (SK) conductance of channels. BK channel is gated by combined influences of Ca^{2+} and voltage, while IK and SK channels are gated solely by Ca^{2+}. Volatile anesthetics inhibit BK channel activity by interfering with the Ca^{2+} gating mechanism. However, the effects of anesthetics on IK and SK channels are unknown. Using cloned IK and SK channels, hIK1 and hSK1-3, respectively, we found that the currents of hIK1 were inhibited rapidly and reversibly by volatile anesthetics, whereas those of SK channels were not affected. The IC_{50} values of the volatile anesthetics, halothane, sevoflurane, enflurane, and isoflurane for hIK1 inhibition were 0.69, 0.42, 1.01 and 1.03 mM, respectively, and were in the clinically used concentration range. In contrast to BK channel, halothane inhibition of hIK1 currents was independent of Ca^{2+} concentration, suggesting that Ca^{2+} gating mechanism is not involved. These results demonstrate that volatile anesthetics, such as halothane, enflurane, isoflurane, and sevoflurane, affect BK, IK, and SK channels in distinct ways. © 2000 Published by Elsevier Science B.V. All rights reserved.

Keywords: Anesthetic; volatile; K^+ channel; Ca^{2+}-activated; *Xenopus* oocyte

1. Introduction

Volatile anesthetics are among the most commonly used drugs for clinical anesthesia. Most of them are halogenated hydrocarbons or their ethers. Their structural similarity promoted the researchers to investigate the target of these compounds for years. Although the mechanism of anesthesia still remains unclear, several ion channels are found to be modulated by volatile anesthetics. These include Na^+ and Ca^{2+} channels, GABA and glutamate receptors (for reviews see Franks and Leib, 1994, 1998).

Ca^{2+}-activated K^+ channels (K_{Ca}) regulate cellular functions in many cells (for review see Sah, 1996) and volatile anesthetics have been proposed to influence the cellular functions by modulating them. For example, in a rat hippocampal preparation, halothane, enflurane (MacIver and Kendig, 1991), and isoflurane (Berg-Johnsen and Langemon, 1990) induce hyperpolarization in CA1 neurons by activating K_{Ca} (Krnjevic, 1991). On the other hand, halothane was shown to reduce afterhyperpolarization by inhibiting K_{Ca} (Fujiwara et al., 1988; Pearce, 1996). In addition, Ca^{2+}-activated ^{86}Rb influx in rat glioma C6 cells was suppressed by halothane (Tas et al., 1989). Together, volatile anesthetics seem to have divergent effects on K_{Ca} depending on preparations.

K_{Ca} consists of three subclasses. Large conductance channel (BK) is gated by combined influences of Ca^{2+} and membrane potential, and has a large unitary conductance of 100–200 pS. Small (SK) and intermediate (IK) conductance channels are gated solely by Ca^{2+} and their unitary conductances are 2–20 and 20–85 pS, respectively. In addition, heterogeneity in SK channels has been further identified by cDNA cloning (Köhler et al., 1996). There-

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fore, variable effects of volatile anesthetics on K_{Ca} described above could be due to their divergent effects on different subclasses of K_{Ca}.

BK channel has been shown to be inhibited by volatile anesthetics, halothane, and isoflurane (Pancrazio et al., 1993). However, no study has been performed how anesthetics modulate SK and IK, partly because overlapping sensitivity to channel blockers (Ishii et al., 1997a,b; Vergara et al., 1998) and overlapping distribution (Köhler et al., 1996) would make the pharmacological isolation of each channel difficult.

Recently, hSK1, rSK2, rSK3, and hIK1 have been cloned as mammalian SK (Köhler et al., 1996) and IK (Ishii et al., 1997a,b) cDNAs, respectively. In this investigation, to characterize how SK and IK are modulated by volatile anesthetics, these cloned channels were expressed in *Xenopus laevis* oocytes and sensitivities of the channels to volatile anesthetics were examined. We show that IK (hIK1) currents are inhibited by volatile anesthetics at clinically relevant concentrations, whereas none of the SK subtypes are sensitive to the anesthetics. In addition, anesthetic inhibition of IK is Ca^{2+}-independent while that of BK channel is sensitive to Ca^{2+} concentration (Pancrazio et al., 1993). These results demonstrate that the volatile anesthetics, such as halothane, enflurane, isoflurane, and sevoflurane, modulate subclasses of K_{Ca} in distinct ways.

2. Materials and methods

2.1. Materials

Clotrimazole and D-tubocurarine were obtained from Sigma (St. Louis, MO, USA). Halothane and sevoflurane were purchased from Takeda Pharmaceutical (Osaka, Japan) and Maruishi Pharmaceutical (Osaka, Japan), respectively. Enflurane and isoflurane were from Dainabot (Osaka, Japan). Female *Xenopus* were obtained from Hamamatsu Seibutsu Kyouzai (Hamamatsu, Japan).

2.2. Oocyte expression

A segment of *Xenopus* ovary was treated with 2% collagenase (Nitta Gelatin, Osaka, Japan) and then mature (stage V and VI) oocytes were defolliculated and isolated manually (Ishii et al., 1997a,b). hIK1 (Ishii et al., 1997a,b), hSK1, rSK2 and rSK3 cDNAs (Köhler et al., 1996) cloned in a oocyte expression vector, pBF (Fakler et al., 1994) were linearized by appropriate restriction endnucleases and were in vitro transcribed with SP6 RNA polymerase (Promega, Madison, WI, USA) in the presence of 3 mM m7G(5′)ppp(5′)G (Ambion, Austin, TX, USA). The capped RNA was dissolved in sterile water at $\sim 1\ \mu g/\mu l$. Fifty nl of the RNA solution was microinjected into an oocyte using a microinjector (Nanoject, Drummond, Broomall, PA, USA).

2.3. Voltage-clamp analysis

Injected oocytes were incubated for 48–96 h at 18°C in modified Barth's medium (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.3 mM $CaNO_3$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$) supplemented with 0.1 mg/ml gentamicin. Prior to experiments, vitelline membrane was removed after brief exposure to 200 mM mannitol in Barth's medium. Electrodes were pulled from thin-walled filamented glass capillaries (Narishige, Tokyo, Japan), and filled with a solution containing 116 mM K-gluconate, 4 mM KCl, and 10 mM HEPES pH 7.2. Resistance of the electrodes were 4–8 MΩ with this solution . Inside-out macropatches were excised into an intracellular solution containing 116 mM K-gluconate, 4 mM KClk, and 10 mM HEPES pH 7.2 supplemented with $CaCl_2$ to give a Ca^{2+} concentration of 10 μM; proportion of the calcium binding to gluconate was calculated, using the stability constant for Ca-gluconate of 15.9 M^{-1}. Membrane patches were voltage-clamped using Axoclamp2B (Axon Instruments, Foster City, CA, USA). The current was low pass filtered at 10 kHz, sampled at sampling frequency of 1 kHz, and stored through Powerlab 4/s and analyzed using Powerlab software (AD Instruments, Castle Hill, Australia). At the end of each experiment, 1 mM D-tubocurarine (for hSK1, rSK2, and rSK3) or 2 μM clotrimazole (for hIK1) was added to the bath to estimate the leak current and the value was subtracted from each measured current. The leak currents were typically between 0.05 and 0.1 nA at −100 mV. In data shown in Figs. 2A and 3, three or more measurements were performed using at least two oocytes for each experimental conditions and shown in mean ± S.D.

2.4. Application of anesthetics

Each volatile anesthetic was delivered through an agent specific vaporizer [Fluotech, Enfluratec, Fortec (these three vaporizers are from Datex-Ohmeda, Helsinki, Finland) and typeS MKII (Acoma, Tokyo, Japan) for halothane, enflurane, isoflurane, and sevoflurane, respectively] which was calibrated with a gas analyzer (Anesthetic gas monitor 303, Atom, Tokyo, Japan) and was equilibrated for at least 30 min in the intracellular solution at room temperature by bubbling. To calibrate the gas analyzer, concentrations of equilibrated anesthetics were measured by gas chromatography (Hewlett-Packard 5890A, Palo Alto, CA, USA). The intracellular solution was applied to the macropatches by the fast application method with Y-tube described elsewhere (Ogata and Tatebayashi, 1991; Takano and Noma, 1997). It took 20–50 ms to completely replace the solution surrounding the patch in application of anesthetics and wash out (Takano and Noma, 1997 and data not shown). In the experiment of Fig. 3, Ca^{2+} concentration was adjusted below 1 μM using 1 mM EGTA and an appropriate amount of $CaCl_2$ as calculated by CABUFFER pro-

gram (provided by Dr. Kleinschmidt through an internet ftp site).

2.5. Data analysis

Values are presented as mean ± S.D. except when the Hill equation ($I = 1/(1 + ([x]/IC_{50})^h)$) was fitted to concentration–response data. This was done using a weighted least-squares minimalization program (Kaleida graph, Synergy software, PA, USA). It provided with estimated values of IC_{50} and Hill coefficients ± approximate S.D. (for details see Colquhoun et al., 1974; Press et al., 1997).

3. Results

3.1. Halothane reversibly reduce currents of hIK1

Inside-out patches of oocytes expressing hIK1 displayed inwardly rectifying currents that were sensitive to 2 μM clotrimazole (Fig. 1A control and Ishii et al., (1997a,b)). Bath application of 1.2 mM halothane reduced both of the outward and inward currents by 62% and 67%, respectively, in the experiment shown in Fig. 1. The block was readily reversible with wash out (Fig. 1B). To examine time course of the inhibition, halothane was applied to the excised membrane patch through the tip of Y-tube while it was stimulated by a 20 ms test pulse of −100 mV from a holding potential of 0 mV every 100 ms. As shown in Fig. 1C, the inward current was reduced by 61.2 ± 3.2% ($n = 3$) within 200 ms after the application of halothane, and was returned to the initial current level within 2–3 s after termination of halothane. These results show that halothane inhibits IK rapidly and reversibly.

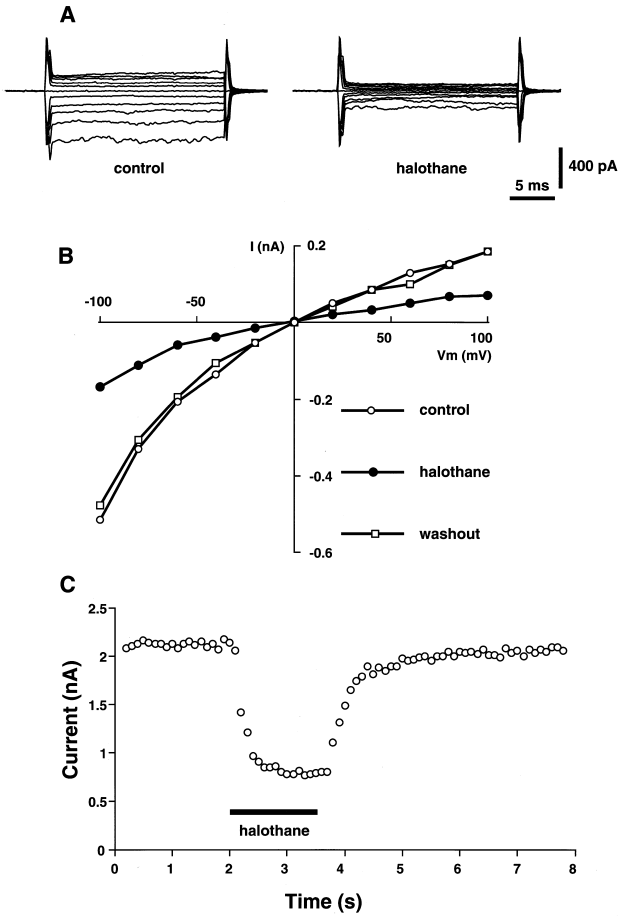

Fig. 1. Inhibition of IK current by halothane. (A) IK current trace: inside-out macropatch excised from oocytes expressing hIK1 was stepped from holding potential of 0 mV to test potentials between −100 and +100 mV at 20 mV intervals. Ten μM Ca^{2+} with and without 1.2 mM halothane was applied through Y-tube. (B) Current–voltage relationship: the membrane patch was stimulated as (A) (C) Time course : inside-out macropatch was stimulated every 100 ms by 20 ms test potential of −100 mV from holding potential of 0 mV. Halothane of 1.2 mM in 10 μM Ca^{2+} was applied through Y-tube (solid bar).

3.2. hIK1 inhibition by various volatile anesthetics

To clarify whether the inhibition is specific to halothane, several widely used halogenated anesthetic agents were also studied. As shown in Fig. 2, halothane, sevoflurane, enflurane, and isoflurane all reduced IK currents in a concentration-dependent manner. The concentration–response curves were fitted with Hill equation and the calculated IC_{50} values were 0.69 ± 0.09, 1.01 ± 0.09, 1.03 ± 0.24 and 0.42 ± 0.05 mM for halothane, enflurane, isoflurane, and sevoflurane, respectively. These values are the clinically relevant concentrations for human anesthesia (Wrigley and Jones, 1992). The estimated Hill coefficients for halothane, enflurane, isoflurane, and sevoflurane were all close to 1 (1.29 ± 0.16, 0.90 ± 0.10, 0.84 ± 0.21 and 1.07 ± 0.23, respectively), which is consistent with a notion that the inhibitory mechanisms of these anesthetics are similar.

3.3. Ca^{2+} dependency

BK channels are inhibited by halothane, an effect that is suppressed by high concentrations of Ca^{2+}, suggesting that anesthetics interfere with Ca^{2+} gating mechanism of BK channel (Pancrazio et al., 1993). To test whether anesthetics also inhibit the Ca^{2+} gating mechanism of hIK1, we examined the effect of halothane as a function of intracellular Ca^{2+} concentration. As shown in Fig. 3, Ca^{2+} activated hIK1 with EC_{50} of 0.37 ± 0.08 μM, consistent with the previous report (Ishii et al., 1997a,b). This hIK1 current was reduced by halothane at every Ca^{2+} concentration in a similar ratio up to 10 μM, demonstrating that the inhibition is Ca^{2+}-independent. Consistently, the EC_{50} value in the presence of halothane was 0.41 ± 0.15 μM, and was not significantly different from the control value.

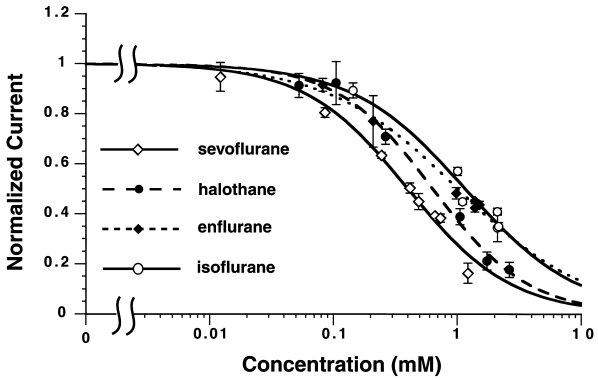

Fig. 2. Inhibition of IK channel by various volatile anesthetics. (A) Concentration–response relationships of IK channel inhibition by volatile anesthetics. Each anesthetic was applied through Y-tube while inside-out macropatch from oocytes expressing hIK1 was stimulated every 100 ms by 20 ms test potential of −100 mV from holding potential of 0 mV. The measured current was normalized by the current in 10 μM Ca^{2+} without anesthetics. Mean and S.D. values (vertical lines) are shown. The data were fitted with the Hill equation. (B) Correlation between IC_{50} value for IK inhibition and EC_{50} value for anesthesia. EC_{50} value for each anesthetic was calculated using minimal alveolar concentration and water/gas partition coefficient (Wrigley and Jones, 1992; Hönemann et al., 1998).

3.4. SK group of channels are insensitive to volatile anesthetics

The amino acid sequence of hIK1 is highly homologous with that of the cloned SK and shows identity of 42–44% (Ishii et al., 1997a,b). This suggests that volatile anesthetics may also affect SK currents. Oocytes expressing hSK1, rSK2 and rSK3 exhibited D-tubocurarine-sensitive inwardly rectifying currents (Köhler et al., 1996 and data not shown). In contrast to hIK1, halothane did not significantly inhibit any of the currents as shown in Fig. 4. Sevoflurane

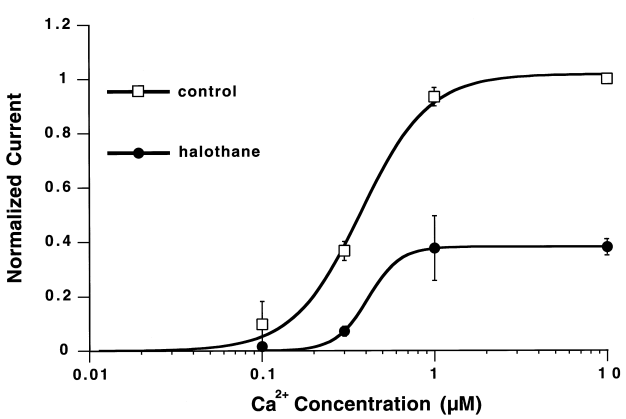

Fig. 3. Ca^{2+} dependency. The currents of inside-out patches from oocytes expressing hIK1 were determined under various concentrations of Ca^{2+} with or without 0.65 mM halothane. The currents were measured while the patch was stimulated every 100 ms by 20 ms test potential of −100 mV from holding potential of 0 mV and was normalized with current in 100 μM Ca^{2+} without any anesthetics. Mean and S.D. values (vertical lines) are shown.

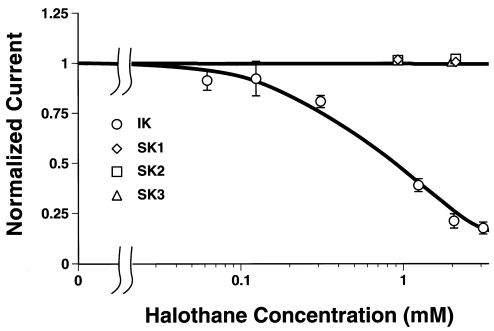

Fig. 4. Effect of halothane on SK channels. The currents of inside-out patches from oocytes expressing hIK1, hSK1, rSK2, and rSK3 were determined under various concentrations of halothane. The currents were measured while the patch was stimulated every 100 ms by 20 ms test potential of −100 mV from holding potential of 0 mV and was normalized with current without halothane. Mean and S.D. values (vertical lines) are shown.

was also unable to inhibit hIK1: normalized current with 0.96 mM sevoflurane were 0.99 ± 0.01, 1.02 ± 0.01 and 1.06 ± 0.02 ($n = 3$) for SK1, SK2 and SK3, respectively. Enflurane and isoflurane were also unable to modify the SK currents at clinically used concentrations (data not shown).

4. Discussion

4.1. Differential modulation of K_{Ca} by volatile anesthetics

Our results show that a cloned K_{Ca}, hIK1, was rapidly and reversibly inhibited by widely used volatile anesthetics including halothane, sevoflurane, isoflurane, and enflurane. The inhibition was concentration-dependent. In contrast, structurally related SK groups of K_{Ca} channels were insensitive to volatile anesthetics. The inhibition of hIK1 was not affected by cytoplasmic Ca^{2+} concentration, suggesting that Ca^{2+} gating mechanism is not involved. This is different from the case of BK channel where halothane and isoflurane apparently inhibit the current by interacting with Ca^{2+} gating mechanism (Pancrazio et al., 1993). These results indicate that BK, IK, and SK channels are differentially affected by volatile anesthetics.

4.2. Mechanism of inhibition of IK with volatile anesthetics

The fact that anesthetics mediated inhibition of BK channel and IK are different in terms of Ca^{2+}-dependency suggests a mechanism of the inhibition. The mechanism through which Ca^{2+} affects BK channel gating has not been clearly established yet, although aspartate rich ''calcium bowl'' domain in the intracellular C-terminal domain has been implicated as a site of Ca^{2+} sensing (Schreiber and Salkoff, 1997). Alternatively, some of the

BK channel associtaed beta-subunits might be sensitive to Ca^{2+} and modulate the gating as well as the pharmacology of channel. On the other hand, distinct mechanism for Ca^{2+} gating mechanism for IK and SK has been described. In these channels, calmodulin binds to a structurally conserved domain in the intracellular C-terminal domain and binding of Ca^{2+} to calmodulin transduces a conformational alteration to the channel protein resulting in channel gating (Xia et al., 1998). Therefore, it is possible that BK channels are influenced by interaction of anesthetics with the Ca^{2+} bowl or the beta subunits, structures not present in IK channel, while they interact with a domain unrelated to the calmodulin interaction site in IK channel. The lack of anesthetic effects on Ca^{2+} gating in IK channel is consistent with the finding that volatile anesthetics do not interfere with the interaction between Ca^{2+} and calmodulin (Levin and Blanck, 1995).

Volatile anesthetics inhibit nicotinic acetylcholine receptor currents with a Hill coefficient around 1, suggesting that one molecule of anesthetic interacts with the channel which is formed by pentamer of subunits (Barann et al., 1998). Point mutation study implicated the M2 region of a subunit (Forman et al., 1995), indicating that one anesthetic molecule interact with the channel pore. Like many K^+ channels, IK channel is believed to be tetramer. As in the case of acetylcholine receptor, Hill coefficients for hIK1 were close to 1. Therefore, it is likely that volatile anesthetics may also bind to the pore of IK channel. In some of the neurotransmitter receptors, amino acid residues, which are important for effects of volatile anesthetics, has been reported. These are Ser in acetylcholine receptor α subunit (Forman et al., 1995), Ser and Ala in $GABA_A$ and glycine receptor α subunit (Mihic et al., 1997) and Gly in kainate receptor (Minami et al., 1998). There are several such smaller amino acid residues also found in the pore region of hIK1, suggesting that some of these are also important in IK inhibition. The high degree of sequence conservation between anesthetic-sensitive IK channel and insensitive SK channels provides an opportunity to delineate the regions responsible for IK inhibition using chimeric IK–SK channels.

4.3. Inhibition of IK channel with anesthetics in vivo

Human erythrocytes express IK channels, Gárdos channel (Gárdos, 1958) with biopysical and pharmacological properties (Christophersen, 1991) indistinguishable from cloned hIK1 (Ishii et al., 1997a,b). Caldwell and Harris (1985) studied Ca^{2+}-dependent ^{86}Rb efflux from human erythrocytes and found that halothane and enflurane inhibited the efflux in a concentration-dependent manner, at concentrations higher than 0.1 mM. This result strongly suggests that volatile anesthetics inhibit IK channel in vivo at similar concentrations as in the hetrologous expression system.

4.4. Anesthetic effects through inhibition of IK channel

IK is expressed in brain capillary endothelial cells where its activity shunts excess K^+ released by neurons into blood (Renterghem et al., 1995). Therefore, by inhibiting the IK channel, volatile anesthetics may cause increase in extracellular K^+ concentration, which may modify excitablitiy of neuronal cells. For example, excitability of parallel fibers of rat cerebellum (Malenka et al., 1981) and CA1 afferents of rat hipocampus (Poolos et al., 1987) decreases by accumulation of extracellular K^+ more than 6 mM. Although it is unlikely, this is the only mechanism of general anesthesia, such as modulation of neuronal function, that deserves further study.

Other than CNS, IK channel is expressed abundantly in erythrocytes (Christophersen, 1991), lymphocytes (Lewis, 1995), granulocytes (Krause and Welsh, 1990), macrophages (Gallin, 1989), platelets (De-Silva et al., 1997), and vascular endothelial cells (Ohlmann et al., 1997). Charybdotoxin and clotrimazole, IK channel blockers, inhibit T cell proliferation and IL-2 production (Price et al., 1989; Freedman et al., 1992; Varnai et al., 1993; Jensen et al., 1999). Likewise, halothane inhibits T cell proliferation and IL-2 receptor expression (Hamra and Yaksh, 1996). Chemotaxsis and phagocytosis, in which IK channel plays a role (Varnai et al., 1993), are transiently impaired during and after halothane anesthesia (Ciepichal and Kubler, 1998). Vascular endothelium produces endothelium-derived hyperpolarizing factor (EDHF) when stimulated by acetylcholine or bradykinin (Garland et al., 1995), and IK channel has been implicated in the effects of EDHF (Rapacon et al., 1996). Consistently, halothane, isoflurane, and sevoflurane inhibit the effects of EDHF in mesenteric artery preparations (Akata et al., 1995; Iranami et al., 1997). Although the mechanisms through which volatile anesthetics induced these functional modulation in vivo are not fully understood, our results suggest that the effects are mediated, at least in part, by altering IK channel activity.

In summary, we demonstrate here a rapid, potent, and specific inhibitory action of volatile anesthetics, such as halothane, enflurane, isoflurane, and sevoflurane, on cloned IK, hIK1, while not affecting cloned SK. In contrast to BK channel, the mechanism of IK inhibition does not involve the Ca^{2+} gating machinery. The differential modification of subclasses of K_{Ca} by volatile anesthetics may be useful for elucidating the mechanisms of the modification as well as understanding the pharmacological actions of anesthetics in various IK channel, expressing cells in vivo.

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