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PKC independent inhibition of voltage gated calcium channels by volatile anesthetics in freshly isolated vascular myocytes from the aorta

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Abstract

In this study we used barium currents through voltage gated L-type calcium channels (recorded in freshly isolated cells with a conventional patch-clamp technique) to elucidate the cellular action mechanism for volatile anesthetics. It was found that halothane and isoflurane inhibited (dose-dependently and voltage independently) Ba²⁺ currents through voltage gated Ca²⁺ channels. Half maximal inhibitions occurred at 0.64 ± 0.07 mM and 0.86 ± 0.1 mM. The Hill slope value was 2 for both volatile anesthetics, suggesting the presence of more than one interaction site. Current inhibition by volatile anesthetics was prominent over the whole voltage range without changes in the peak of the current voltage relationship. Intracellular infusion of the GDPβS (100 μM) together with staurosporine (200 nM) did not prevent the inhibitory effect of volatile anesthetics. Unlike pharmacological Ca²⁺ channel blockers, volatile anesthetics blocked Ca²⁺ channel currents at resting membrane potentials. In other words, halothane and isoflurane induced an 'initial block'. After the first 4 to 7 control pulses, the cells were left unstimulated and anesthetics were applied. The first depolarization after the pause evoked a Ca²⁺ channel current whose amplitude was reduced to $41 \pm 3.4\%$ and to $57 \pm 4.2\%$ of control values. In an analysis of the steady-state inactivation curve for voltage dependence, volatile anesthetics induced a negative shift of the 50% inactivation of the calcium channels. By contrast, the steepness factor characterizing the voltage sensitivity of the channels was unaffected. Unitary L-type Ca²⁺ channels blockade occurred under cell-attached configuration, suggesting a possible action of volatile anesthetics from within the intracellular space or from the part of the channel inside the lipid bilayer.

Keywords

PKC; halothane; isoflurane; L-type Calcium channels

Introduction

The effects of anesthetics on ion channels have been the subject of intense research. Volatile anesthetics have been reported to constrict blood vessels [1–4]; relax blood vessels [5–9]; or

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produce both effects sequentially [10–11]. Voltage activated calcium channels (VOCC) may account for some aspects of the diverse vascular responses produced by volatile anesthetics.

Pharmacological and physiological experiments have differentiated multiple types of native high voltage activated (HVA) Ca^{2+} channels. HVA are products of different genes, which give rise to 1 subunits that form the pores of the Ca^{2+} channels: the CaV1 family (1S, 1C, 1D, 1F) encoding the L-type; CaV2.1 (1A) encoding the P/Q-type; CaV2.2 (1B) encoding the N-type; and CaV2.3 (1E) encoding the R-type. HVA, or dihydropyridine-sensitive L-type Ca^{2+} channels, are widely expressed in vascular beds. HVA Ca^{2+} channels expressed in *Xenopus* oocytes and in cultured cells have shown sensitivity to volatile anesthetics [12–13]. It has also been suggested that volatile anesthetics may regulate HVA Ca^{2+} channels open probability by the modulation of G-proteins [14].

To our knowledge, there has been no systematic study reporting the effects of volatile anesthetics on the HVA Ca^{2+} channels in freshly dissociated smooth muscle cells from large elastic arteries. The aorta, among elastic arteries, has special features that position the aorta as a unique organ within the cardiovascular system. It cooperates with the left ventricle in a “game of catch” with the stroke volume. In other words, the intrinsic relaxation and contraction of the aorta augment left ventricular hemodynamic function. In humans, the ratio of the flow pulse amplitude to the mean flow decreases from roughly 6 in the aortic arch to less than 2 in the femoral artery [15–16]. This mechanism, known as the Windkessel effect [17–18], prevents an abrupt fall of arterial pressure after the heart valves close and reduces the pulse pressure, the pressure wave velocity and the hydraulic impedance faced by the heart.

In this paper we report our study of the effects of volatile anesthetics on L-type Ca^{2+} channels in freshly dissociated cells from mouse aorta. The results show that halothane and isoflurane inhibit whole-cell HVA Ca^{2+} currents in a dose dependent manner. Volatile anesthetics decreased open probability of L-type Ca^{2+} channels recorded in cell attached patches. The analysis showed that blockade occurred largely in a state-independent manner.

Materials and Methods

Animals were purchased and housed in accordance with institutional IACUC guidelines and requirements of the relevant regulatory agencies. C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). All rodents were maintained on a 12 hr light/12 hr dark cycle with free access to food and water. Treatment, care, and housing were carried out in accordance with the National Institutes of Health guidelines on animal care. Male C57BL/6 mice that were 4 weeks old were anesthetized with 2-bromo-2-chloro-1,1,1-trifluoroethane. The aorta was isolated as described previously [19–20]. The thoracic aorta was removed, cleaned of fat tissue and placed in a low Ca^{2+} solution containing (in mM): NaCl 137, KCl 5.4, K_2HPO_4 0.44, NaH_2PO_4 0.42, MgCl_2 2, NaHCO_3 4.17, CaCl_2 0.2, glucose 11, EGTA 0.05 and HEPES 10 (pH was adjusted to 7.4 with NaOH). The aorta was incubated for 40 min. at 37°C in a low Ca^{2+} solution containing 2 mg/mL elastase (type IV) and 2mg/mL collagenase (type II). Vascular smooth muscle cells were isolated by careful shaking of the tissue in a Ca^{2+} -free solution containing (mM): NaCl 137, KCl 5.4, K_2HPO_4 0.44, NaH_2PO_4 0.42, NaHCO_3 4.17, MgCl_2 2, EGTA 1.8 and glucose 11 (pH was adjusted to 7.2 with NaOH), and then placed on cover slips and stored at 4°C.

Patch-clamp recording

Step-pulse, linear ramps, steady state protocols and data acquisition were performed at room temperature (22–25°C). Currents were filtered at 1 kHz and digitized at 5 kHz. Membrane currents were recorded at room temperature (25°C) using a nystatin-perforated patch or a

whole cell configuration with a patch amplifier (Axopatch 200B, Axon Instruments Inc.). The experimental configuration used is indicated below. The patch electrodes were pulled from borosilicate capillary glass using a Sutter instrument (P-2000, Novato, CA, USA).

Ca^{2+} channel currents were recorded in the whole-cell or perforated patch configuration with nystatin. Currents were recorded from holding potentials of -80 mV during linear voltage ramps at 0.67 V/s from -100 mV to $+80$ mV or 300 ms-step pulses to different potentials with a pulse frequency of 0.2 Hz. Ba^{2+} was used as the charge carrier and K^{+} currents were blocked by Cs^{+} . In whole-cell experiments, the extracellular solution contained (in mM) NaCl 125, BaCl_2 10.8, CsCl 5.4, glucose 10, and Na-Hepes 10 (pH 7.4 at 37°C). The patch pipette was filled with a solution containing (in mM) CsCl 120, MgCl_2 1, Mg-ATP 3, EGTA 10, Cs-Hepes 10 (pH 7.4 at 22°C). For perforated patch experiments, the bath solution contained (in mM) NaCl 125, BaCl_2 10.8, CsCl 5.4, glucose 10, and Na-Hepes 10 (pH 7.4 at 22°C); the pipette was filled with a solution containing (in mM) CsCl 75, MgCl_2 5, Hepes 5, Aspartate 75 (pH 7.4 at 22°C). The resistance of the pipettes was 4 to 6 MOhm.

In cell-attached patch experiments, the pipette solution contained (in mM) BaCl_2 110, Hepes 5, buffered to pH 7.4 with tetraethylammonium hydroxide. Patch pipettes were made of borosilicate glass and coated with wax to minimize capacitative transients and noise; pipette resistance was $5 - 8\text{ M}\Omega$. The recordings were filtered (23 dB, 5-pole Tchebicheff filter) at 1 kHz, and sampled at 5 kHz. The bath solution contained (in mM) potassium aspartate 140, MgCl_2 5, EGTA 5, Hepes 10, buffered to pH 7.4 with KOH. The cells were continuously perfused during all recordings. Drugs were applied by changing the bath solution.

Current densities (pA/pF) were obtained for each cell by normalization of whole cell current to cell capacitance in order to account for differences in cell membrane surface area. Capacity currents were measured for each cell during 10 ms pulses from a holding potential of -80 mV to a test potential of -70 mV. Currents were digitized and recorded at 5 kHz without filtering. Cell capacitance was calculated from the capacitative current transient recorded at the beginning of a 10 mV depolarizing voltage-clamp pulse. Total charge movement (Q) during the 10 mV step was obtained by integrating the area defined by the capacitative transient. Cell capacitance (C) was then obtained from the equation $C = Q/V$.

A solution containing halothane (Sigma) and isoflurane (Aerrane; Pharmacia Laboratories) was prepared from a 0.5 M stock solution made up in dimethyl sulfoxide (DMSO), as reported previously [21]. Solutions containing anesthetic were formulated with 0.12% dimethyl sulfoxide, a concentration that had no significant effect on calcium channel currents or on the electrical activity of the myocytes. For our experiments, five different dilutions of the saturated DMSO solution were freshly prepared. Our standard perfusion apparatus for electrophysiological experiments was equipped with 30 mL ground-glass syringes to serve as sealed reservoirs for the perfusate solutions. The reservoirs were connected to the bath via Teflon tubing. The reservoirs were filled completely to eliminate a headspace gas compartment within the sealed syringe. Reservoir syringes were mounted vertically in the perfusion apparatus with weights fixed onto the glass syringe plungers to provide a steady head pressure and facilitate flow. The 0.6 mM concentration of volatile anesthetics equates to approximately 2 times the minimum alveolar concentration for the rat [22] and therefore represents physiologically relevant and approximately equianesthetic concentrations. In previous studies [23] concentrations of the anesthetics, prepared by this method, in the superfusate were verified by gas liquid chromatography. It was found that the aqueous concentration was as calculated and was maintained over time.

Drugs

TEA (tetraethylammonium chloride), ethylene glycol-bis (2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), collagenase (II), elastase (type IV from porcine pancreas), papain, nifedipine, guanosine 5'-[γ -thio]diphosphate (GDP β S), Phorbol 12,13 dibutyrate (PDBu), staurosporine were obtained from Sigma Chemical (USA).

Data analysis and statistics

Patch-clamp data were processed using Clampfit 9.0 (Molecular Devices) and then analyzed in Origin 7 (Origin Lab, Northampton, MA, USA). Numerical data in the text and error bars in the figures are expressed as mean $\pm$ the standard error of the mean (SEM). Whenever possible, differences in I-V plots and dose response relationships were analyzed using two-way analysis of variance (ANOVA). Otherwise, data were analyzed by t-tests. P values of 0.05 were considered to be significant.

Results

Inhibition of L-type Ca²⁺ channel currents by halothane and isoflurane

Whole-cell calcium channel currents in freshly isolated cells from the aorta were characterized using 10.8 mM Ba²⁺ as a divalent charge carrier. Currents were elicited by voltage steps from a holding potential of -80 mV to 0 mV for 300 milliseconds. The inward currents were reversibly blocked by $1 \mu\text{M}$ nifedipine or $100 \mu\text{M}$ Cd²⁺, and were reversibly increased by $1 \mu\text{M}$ Bay K 8644 (not shown in the figures that follow). A fast inactivating (T-type) current was not observed in our preparation ($n=47$). Fig. 1A shows the average dose-response curve for inhibition of Ca²⁺ channel currents by halothane and isoflurane at different concentrations in myocytes freshly isolated from the aorta. The current in the presence of anesthetics, expressed as a fraction of its control value (I/I_{max}) at 0 mV, was mathematically characterized by the following equation:

$$I/I_{\text{max}} = A_2 + (A_1 - A_2) / (1 + ([\text{anesthetic}] / IC_{50})^n) \quad (\text{Eq. 1})$$

where [anesthetic] is the concentration of halothane or isoflurane, IC_{50} is the concentration of an anesthetic yielding half maximal inhibition, and n is the slope. Fitting the data points revealed that the IC_{50} occurred at 0.64 ± 0.07 mM ($n=5$) and 0.86 ± 0.13 mM ($n=5$) for halothane and isoflurane respectively. The Hill coefficient was 2.06 ± 0.44 and 2.05 ± 0.51 for halothane and isoflurane respectively. At a test potential of 0 mV, halothane (1 mM) induced an average inhibition of 63.9 ± 6.2 % ($n=7$) and isoflurane (1 mM) induced an average inhibition of 42.7 ± 5.3 % ($n=5$). This concentration was routinely applied in further experiments. The effect of halothane and isoflurane was reversible upon washout (Fig. 1).

Figure 2 shows the effect of halothane (Panel A) and isoflurane (Panel C) on Ca²⁺ channel currents evoked by voltage steps to test potentials between -70 and 40 mV from a holding potential of -80 mV in a representative cell. Both anesthetics reduced the Ca²⁺ channel current. The corresponding IV curves were determined from peak Ca²⁺ channel currents recorded before and after application of the volatile anesthetics.

To substantiate this point, we recorded Ca²⁺ channel currents using linear voltage ramps varying from -100 mV to $+100$ mV, from a holding potential of -80 mV. As can be seen from the data in Fig. 2D (current voltage relationship is shown from -75 mV to 75 mV and is normalized to maximum), volatile anesthetics reduced the current without affecting the potential at which the peak Ca²⁺ channel currents occurred. The current was proportionally inhibited at all potentials where Ca²⁺ channel currents could be evoked; the apparent threshold and the reversal potential were not affected by volatile anesthetics. The current

voltage relationships recorded during voltage ramp pulses were U-shaped and exhibited a maximum at 7 ± 2 mV ($n=47$) (Fig. 2). The apparent threshold occurred at -27 ± 4 mV and the reversal potential at 51 ± 7 mV ($n = 47$). Both protocols showed current voltage relations with similar apparent thresholds and reversal potentials. Thus, the data demonstrate that both halothane and isoflurane induce a voltage-independent inhibition of L-type Ca^{2+} channels in myocytes isolated from the aorta.

The effect of anesthetics on steady-state inactivation

The effect of anesthetics on steady-state inactivation was also tested (Fig. 3, Panels A and B). Anesthetics indeed affected the voltage dependence of calcium channel inactivation in myocytes isolated from the aorta. The Ca^{2+} channel availability was determined by measuring currents during test pulses to 0 mV after 3 second conditioning voltage steps. The data were fitted with a Boltzmann equation of the form:

$$I/I_{\text{max}} = 1 / [1 + \exp((V - V_h)/k)] \quad (\text{Eq. 2})$$

where I is the amplitude of inward Ca^{2+} channel current elicited by a test pulse following the conditioning pre-pulse (V) with a duration of 3 s between -80 mV and $+40$ mV; I_{max} is the amplitude of inward Ca^{2+} current elicited by test pulse from a conditioning pre-pulse of -80 mV; V_h is the value of the conditioning potential causing 50% inactivation of the Ca^{2+} channel current; and k is the steepness factor characterizing the voltage-sensitivity of the channels. Halothane and isoflurane shifted the midpoint (V_h) of the normalized steady-state inactivation curve, by 7.5 mV and 6.2 mV respectively, to more negative membrane potentials compared with control conditions ($n=6$) ($P<.001$), (Fig. 3A). The slope factor, k , was not significantly altered; k values were 7.1 ± 0.7 , 6.7 ± 0.6 and 6.2 ± 0.5 for control, halothane, and isoflurane treated cells respectively.

Initial block of Ca^{2+} channel currents by anesthetics

The following experiments were designed to determine if halothane and isoflurane induced inhibition of Ca^{2+} currents occurred at resting potentials, i.e., if anesthetics could induce an 'initial block'. Myocytes were patched and held at -80 mV. Control currents were recorded during 300-ms pulses to 0 mV (at a frequency of 0.2 Hz). After approximately 4 pulses, cells were left unstimulated at the resting potential (-80 mV). Halothane (1 mM) or isoflurane (1 mM) was applied to the patched cell. After 3 to 5 minutes, pulsing was resumed at 0.2 Hz. The first depolarization evoked a Ca^{2+} channel current whose amplitude was reduced to $41 \pm 3.4\%$ ($n=6$) of the control values before application of halothane, and to $57 \pm 4.2\%$ ($n=5$) of the control values before application of isoflurane. Subsequent pulsing tended to further reduce calcium currents. This effect of anesthetics on initial block is illustrated in Fig. 3 for a representative myocyte.

G protein and PKC independent Ca^{2+} channel inhibition

Volatile anesthetics have been reported to alter protein kinase C activity or to affect sites of phosphorylation in G-proteins [24–26]. The GDP analog, GDP β S, stabilizes G proteins in the inactive GDP-bound form. GDP β S was intracellularly applied via the patch pipette at a concentration of 100 μM in whole-cell patch clamp recordings. Calculations of the diffusion velocity [27] revealed that the cytoplasmic concentration of GDP β S (M.W. 477) and of staurosporine (M.W. 466) should reach 50% of the concentration in the pipette solution within about 2 minutes after disruption of the membrane patch, and more than 80% within 5 minutes after membrane disruption. The effect of GDP β S and staurosporine on the halothane- and isoflurane-induced inhibition is shown in Fig. 4. After 5 minutes of intracellular infusion of GDP β S and staurosporine, 1 mM of halothane or 1 mM of isoflurane inhibited the peak Ca^{2+} channel current by $47 \pm 4.1\%$ ($n=6$) and by $38 \pm 5.8\%$

(n=5) respectively. These values were not significantly different from the values obtained in control experiments (dialysis of the cell with standard pipette solution). This result indicates that G proteins are not involved in the inhibitory effect of halothane on Ca^{2+} channels.

To substantiate this point, we studied the effect of PKC activator PDBu (1 μM) on barium currents. PKC has been reported to activate [28,29,30], and to inhibit $\text{CaV}1.2\text{a}$ channels in heterologous systems [31,30]. Some studies in native cells have reported PKC-mediated stimulation of $\text{CaV}1.2\text{a}$ [32], whereas others report inhibition [33]. PKC has been reported to enhance $\text{CaV}1.2\text{b}$ channel currents in smooth muscle preparations [34,35,36]. We tested regulation of barium currents through L-type calcium channels by PKC. Currents were elicited by voltage steps from a holding potential of -80 mV to 0 mV for 300 milliseconds. PDBu (1 μM) gave rise to a significant increase in Ba^{2+} -current amplitude to $132 \pm 4\%$ in 14 and had no significant effect in 9 tested cells (Fig. 4 Panel from C to F). To find if volatile anesthetics and PKC have independent or occlusive action on barium currents PDBu with halothane or isoflurane were co-applied simultaneously to cells pretreated with PDBu. Halothane and isoflurane inhibited Ba^{2+} -currents in all PDBu-pretreated cells independently from the effect produced by PDBu on Ca^{2+} channel current. Halothane (1mM) or isoflurane (1mM) inhibited the peak Ca^{2+} channel current by $62 \pm 3.8\%$ (n = 14) and by $49 \pm 5.2\%$ (n = 9) respectively. This result suggests that there are different sites on the L-type Ca^{2+} channel affected by volatile anesthetics and PKC.

Effect of Halothane on L-type Ca^{2+} Channel Activity

Single openings of voltage gated Ca^{2+} channels were studied in a cell-attached configuration. The pipette solution contained 110 mM of Ba^{2+} . The effect of volatile anesthetics on a single L-type Ca^{2+} channel within the patch was determined. Barium currents (I_{Ba}) were evoked every 4s by either 500-ms pulses to 0 mV (from holding potential of -50 mV) or steady state recordings at 0 mV (Fig. 5). The I_{Ba} open probability was augmented by application of 1 μM (-)-Bay K 8644 to the bath solution and decreased by addition of 1 μM nimodipine, thus indicating the presence of functional L-type Ca^{2+} channels (not shown). Control conditions contained 200 nM (-)-Bay K 8644 in the bath solution to promote mode-2 gating and prolong openings. Typical control recordings are shown in Fig. 5 with the corresponding histogram analysis. After control currents were recorded, halothane (1 mM) was perfused over the cells. As shown in Fig. 5, the open probability was dramatically reduced in the presence of halothane. Maximum, steady state inhibition was reached after approximately 2 to 4 minutes. No change in leak current was observed during the halothane application. The effect of halothane was reversible as evidenced by recovery of unitary currents within 3 to 5 min after washout (not shown).

Discussion

The important finding of this study is that volatile anesthetics inhibit L-type Ca^{2+} channels in myocytes freshly dissociated from the aorta independently from G-proteins and protein kinase C. Volatile anesthetics at the macroscopic level decreased the magnitude of whole cell L-type calcium channel currents, shifted the midpoint of the steady-state inactivation curve, and did not change the reversal potential, suggesting that they did not affect ionic selectivity. At the single-channel level, volatile anesthetics decreased the open lifetimes, without changing the single-channel conductance and increased the number of blank traces.

Halothane and isoflurane produced reversible, concentration-dependent depression of the peak barium current. Halothane (0.5 mM) depressed the peak by 35% in our preparation, which is comparable to the 25–45% peak reduction reported for Ba^{2+} currents through L-type Ca^{2+} channels: The peak barium current through calcium channels was reduced by 25% with the addition of 0.45 mM of halothane in guinea pig ventricular myocytes [37]; by 35%

with 0.4 mM of halothane in canine ventricular myocytes [38]; and by 35% with 0.45 mM of halothane in canine cardiac Purkinje cells [39]. Similar results have been reported with recombinant channels expressed in *Xenopus* oocytes and in HEK cells [12,40].

To compare the blocking mechanism produced by volatile anesthetics with those of synthetic L-type Ca^{2+} channel blockers, we analyzed channel activation, channel inactivation, and the 'initial channel block'. Halothane and isoflurane did not alter the voltage dependence of the activation process. This characteristic is similar to that of phenylalkylamines and benzothiazepines [41–43]. However, it is in contrast to the action of dihydropyridines, as these compounds shifted the current voltage relation to the left [44–45]. Halothane and isoflurane were found to shift the steady-state inactivation curves of Ca^{2+} channels to more negative membrane potentials. Similar effects are characteristic of dihydropyridines benzothiazepines, and verapamil-type Ca^{2+} antagonists [46–48].

Tested volatile anesthetics induced an 'initial block' when the membrane potential was held at resting potential. The data suggest that halothane and isoflurane are capable of inducing calcium channel blockade without the need for channel opening or inactivation. Phenylalkylamines, like verapamil and D-600, have a greater affinity for the open state of the L-type channel [49–50], whereas dihydropyridines preferentially bind to the inactivated state of the Ca^{2+} channels [51–52]. Blocking by these drugs is characterized by a low 'initial block' if the membrane potential is more negative than -45 mV. The efficacy of a halothane block at negative membrane potentials (-80 mV), as demonstrated in our study, suggests the possibility of a different binding site on the Ca^{2+} channel, perhaps at a point less affected by the membrane potential. The results of the anesthetics-induced 'initial blocks' suggest that both halothane and isoflurane depress the peak current through the reduction of activatable channels at resting membrane potentials. The underlying mechanisms remain unclear but may be explained by a hyperpolarizing shift in the steady state inactivation.

The reported data from other preparations of the steady state inactivation curve are contradictory. Volatile anesthetics have been reported to produce a negative shift, (such shifts have not been reported in expression systems [12], and were found in cardiac and nervous tissue [53–54,13]). This variability may point to possible divergent effects of these volatile agents on L-type Ca^{2+} -channel function [40]. The question of whether a 4 to 7 mV shift of the steady state inactivation is sufficient to reduce the number of available channels was beyond the scope of the current study and remains open for further research.

It has been proposed that halothane binds directly to the L-type Ca^{2+} channel because it shifts the binding curve of the dihydropyridines to the right. It has also been proposed that halothane interacts with G-proteins or protein kinase C and thereby modulates the activity of Ca^{2+} -channels [9,24,25,55,56]. Both mechanisms were suggested to be activated by halothane in pulmonary myocytes, expression systems, and in various cell lines. Our experiments suggest that PKC and G-proteins should not be employed by volatile anesthetics to inhibit L-type Ca^{2+} -channels in vascular myocytes isolated from the aorta. Eleven isoforms of PKC have been identified to date. They have been divided into three groups: 1) classic or conventional PKCs (cPKC) that are activated by diacylglycerol or phorbol ester and are Ca^{2+} sensitive; 2) novel or new PKCs (nPKC) that are activated by diacylglycerol or phorbol ester but are not Ca^{2+} dependent; and 3) atypical PKCs (aPKC) that are not activated by diacylglycerol, phorbol ester or Ca^{2+} [57]. Our experiments do not exclude the possibility that volatile anesthetics may stimulate atypical PKCs and thereby inhibit calcium channels. This possibility is supported by the fact that the PKC activator PDBu did not increase the calcium channel current in all tested cells. Alternatively, phorbol esters may inhibit Ca^{2+} channel currents independently from protein kinase C [58,59]. The inhibition of the calcium channel current was reported to be voltage independent and

reached up to 30% of the control. Thus, the cell did not show a current increase because PDBu may inhibit channel activity at the same time PKC activated the calcium channels. Variations of the responses to PDBu may also arise from the genetic regulation of the Ca^{2+} channel subunits via tissue-specific alternative splicing [60,61]. The L-type Ca^{2+} channel is subject to complex genetic regulation that gives rise to a variety of channel subtypes. Alternative splicing of the α_1 subunit gene was reported to affect regulation of the Cav1.2 channels by protein kinase A. A similar mechanism may contribute to the variation of responses in smooth muscle cells of the aorta.

Cell-attached experiments were conducted to provide further insight on this issue. Our results indicate that volatile anesthetics inhibit the Ca^{2+} channels present within the patch. One of the explanations for this effect is that anesthetics first diffuse intracellularly through the plasma membrane, and then reach the ligand-binding site within the patch from the cytosolic side of the membrane. Such a mechanism has been proposed for the phenylalkylamine class of the Ca^{2+} channel blockers [62]. An alternative suggestion could be made—a lateral diffusion of halothane and isoflurane in the lipids of the plasma membrane, followed by binding to an intra-membrane site of the channel—as proposed for benzothiazepine- Ca^{2+} channel blockers [41]. The dose-response curves of halothane and isoflurane had Hill slope values of 2, which suggest the presence of at least two sites for interaction. The presence of hyperpolarizing shifts of steady state inactivation and single channel results, in combination with unaltered macroscopic activation, suggest that halothane may depress the peak current by promoting transitions from resting to drug-induced non-activatable states, as proposed previously [37]. These findings support a previously suggested model of the channel with distinct but overlapping mechanisms of L-type Ca^{2+} -channel inhibition by halothane and isoflurane [63–64]. Elucidation of the mechanisms of the action of halothane and isoflurane on L-type calcium channels may provide new insight into the control of the blood pressure, and may also have therapeutic and pharmacological implications.

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Fig. 1.

Dose-response curves of Ca^{2+} channel current amplitude normalized to maximum and volatile anesthetics concentration in vascular smooth muscle cells freshly isolated from the aorta. **Panel A** shows the dose-response curve of halothane-induced inhibition (filled squares, $n=5$) and of isoflurane-induced inhibition (filled circles, $n=5$) of inward Ba^{2+} currents through Ca^{2+} -channels. Inward Ca^{2+} channel currents were recorded as peak currents at test potentials of 0 mV. The symbols represent mean values. Data points were fitted by a logistic function. **Panels B** and **C** show representative traces of the recorded I_{Ba} currents in the presence of halothane (Panel B) and isoflurane (Panel C). Currents were recorded before (con.), and after application of 1mM of halothane or isoflurane, and after the washout (w.o.).

Fig. 2.

Effect of volatile anesthetics on inward Ba^{2+} currents through voltage-gated Ca^{2+} channels.

Panel A: Current-voltage relation of peak Ca^{2+} channel currents ($I_{\text{Ba}^{2+}}$) normalized to the membrane capacitance and recorded during step pulses (panel B) before (closed circles) and after application of 1mM halothane (closed squares) ($n = 5$). **Panel B:** Superimposed currents of a representative cell recorded at different test potentials before (Control) and after application of 1mM halothane (Halothane). Ca^{2+} channel currents were evoked by 300-ms depolarizing voltage step pulses to the test potentials. The holding potential was -80 mV. The dotted lines represent the zero current levels. **Panel C:** Current-voltage relation of peak Ca^{2+} channel currents normalized to the membrane capacitance and recorded during step pulses before (closed circles) and after application of 1mM isoflurane (closed squares) ($n = 5$). **Panel D:** Four superimposed current records of a representative cell before and after application of halothane and isoflurane, respectively. Calcium channel currents were normalized to membrane capacitance and to maximum amplitude.

Fig. 3.

Effects of halothane (1 mM) and isoflurane (1 mM) on steady-state inactivation of Ca^{2+} channel currents and comparison of anesthetics-induced 'initial blocks' of Ca^{2+} channel currents in myocytes freshly isolated from the aorta. **Panel A:** Corresponding voltage-dependence of steady-state inactivation of Ca^{2+} channels averaged from 5 cells, in the absence (filled squares) and in the presence (filled circles) of 1 mM halothane, and of 1 mM isoflurane (filled triangles). Normalized peak Ca^{2+} channel currents (I/I_{max}) are plotted versus the voltage of the conditioning prepulses. Solid lines are drawn according to Eq. 2 for the data points. **Panel B:** Superimposed recordings of Ca^{2+} channel currents of a representative cell in the absence (Con.) and in the presence (Hal.) of 1 mM halothane. The currents were recorded during a 300 ms test pulse to 0 mV after a 3 s conditioning pre-pulse to voltages between -80 mV and 0 mV. The holding potential was -80 mV. **Panel C:** Ca^{2+} channel currents elicited by applying 4 test pulses to 0 mV from a holding potential of -80 mV. For a period of 3 min, 1 mM halothane was extracellularly applied to the unstimulated cell, i.e. clamped at a holding potential of -80 mV. After 3 minutes, pulsing was resumed. Further pulsing did not significantly decrease the initial block. **Panel D:** Ca^{2+} channel currents evoked by the same initial-block protocol as shown in Panel C. In this cell, isoflurane induced an initial block of 39%.

Fig. 4.

G protein and PKC independent inhibition of L-type Ca^{2+} channel currents. Ca^{2+} channel currents were evoked by linear voltage ramps with a pulse frequency of 0.2 Hz. Panel A and B: The pipette solution was supplemented in these experiments with 100 μM GDP βS and staurosporin (200 nM). Currents were recorded after 4 to 6 min of intracellular infusion with GDP βS and staurosporin. Effect of GDP βS and staurosporine on the inhibition of Ca^{2+} channel currents by 1 mM of isoflurane (Panel A) and by 1 mM halothane (Panel B). Control (Con.); isoflurane (Iso.) halothane (Hal.) ; and current after washout of (w.o.). Panels C,D,E and F show the effects of PDBu (1 μM) and halothane or isoflurane on Ca^{2+} channel currents. PDBu with halothane or isoflurane were co-applied simultaneously to cells pretreated with PDBu. PDBu inhibited (Panel E and F) and had no effect (Panel C and D) on Ca^{2+} channel currents. Halothane and isoflurane inhibited Ca^{2+} channel currents in both types of cells. Control (Con.); PDBu (PDBu); halothane+ PDBu (PDBu+hal.); isoflurane +PDBU (PDBu+iso.) and after washout (w.o.).

Fig. 5.

Effect of halothane on cell-attached unitary barium currents through L-type Ca^{2+} channels in a freshly isolated vascular myocyte. **Panel A:** Single-channel current-voltage relationship in the absence and presence of 1 mM halothane. Single-channel current measurements were performed before (closed circles), and after (closed squares), the addition of 1 mM of halothane. The conductance was 25.7 ± 0.8 pS for the control, and 27.1 ± 1.2 pS for 1 mM halothane ($n = 4$ for control, and $n=5$ for halothane). Panels B, C and D show currents recorded during step pulses applied from -60 to 0 mV for 3s intervals. The histogram plots show the summed activity from 5 sweeps. Representative sweeps are shown for each condition in the insets. Control sweeps (**Panel A**) were recorded in the presence of a 200 nM concentration of Bay K 8644, and show both prolonged openings and short, transient openings. Histogram analysis shows peaks corresponding to 1 and 2 simultaneous openings at 1.43 pA and 2.72 pA, respectively. **Panel B** shows unitary Ba^{2+} currents after 3 min perfusion with 1 mM halothane and 200 nM Bay K 8644. The histogram analysis shows no significant Ca^{2+} channel activity and shows a peak at 1.37 pA, corresponding to one channel opening. Panel D shows washout. The histogram analysis shows Ca^{2+} channel activity and shows a peak at 1.4 pA.