Different Inhibitory Effects of Sevoflurane on Hyperreactive Airway Smooth Muscle Contractility in Ovalbumin-sensitized and Chronic Cigarette-smoking Guinea Pig Models

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Background: The authors hypothesized that sevoflurane had different inhibitory effects on hyperreactive airway smooth muscle contractility in different types of hyperreactive airway models.

Methods: The effects of sevoflurane on hyperreactive airways in ovalbumin-sensitized and chronic cigarette-smoking guinea pig models were investigated by measuring (1) total lung resistance, (2) smooth muscle tension and intracellular concentration of free Ca$^{2+}$, (3) voltage-dependent Ca$^{2+}$ channel activity, and (4) cyclic adenosine monophosphate levels.

Results: Ovalbumin and muscarinic airway hyperreactivity was seen in ovalbumin-sensitized animals. Enlarged alveolar ducts/alveoli and lesser muscarinic hyperreactivity were observed in chronic cigarette-smoke animals. Although sevoflurane inhibited the acetylcholine-induced increase in total lung resistance in the control and ovalbumin-sensitized models, the anesthetic had a smaller effect in the chronic cigarette-smoking model. Similarly, in the chronic cigarette-smoking model, sevoflurane had a smaller inhibitory effect on carbachol-induced muscle contraction and increase in intracellular concentration of free Ca$^{2+}$. Sevoflurane also had a smaller inhibitory effect on voltage-dependent Ca$^{2+}$ channel activity in the chronic cigarette-smoking group than in the other two groups. The sevoflurane-induced increase in cyclic adenosine monophosphate that was seen in the control and ovalbumin-sensitized groups was significantly suppressed in the chronic cigarette-smoking group.

Conclusions: Although sevoflurane potently inhibited airway contractility in control and ovalbumin-sensitized models, the anesthetic had a smaller effect in a chronic cigarette-smoking model. The different inhibitory effects of sevoflurane on airway contractility depend, at least in part, on different effects on voltage-dependent Ca$^{2+}$ channel activity and cyclic adenosine monophosphate level.

VOLATILE anesthetics are potent bronchodilators. Bronchodilation induced by volatile anesthetics occurs indirectly by inhibition of reflex neural pathways and directly by effects on airway smooth muscle cells. Some investigators have demonstrated, using the Ca$^{2+}$ indicator fura-2, that the direct relaxation of contracted airway smooth muscle by volatile anesthetics is associated with a decrease in the intracellular concentration of free Ca$^{2+}$ ([Ca$^{2+}$]), a key second messenger (fig. 1). The decrease in [Ca$^{2+}$], induced by volatile anesthetics is mainly due to inhibition of cell membrane-associated voltage-dependent Ca$^{2+}$ channels (VDCCs). An increase in the intracellular cyclic adenosine monophosphate (AMP) level caused by volatile anesthetics also contributes to a decrease in [Ca$^{2+}$] by stimulation of Ca$^{2+}$ efflux and of Ca$^{2+}$ uptake into the sarcoplasmic reticulum pool. Although the decrease in [Ca$^{2+}$] caused by volatile anesthetics seems to be the main mechanism of the muscle relaxation, a decrease in Ca$^{2+}$ sensitivity due to inhibition of protein kinase C (PKC) activity and inhibition of G-protein (G$_{q}$) function has also been reported.

Volatile anesthetic-induced bronchodilation has been demonstrated clinically in humans. Rooke et al. reported that sevoflurane decreased respiratory system resistance as much as or more than isoflurane or halothane after tracheal intubation in subjects without asthma. Volta et al. reported that isoflurane and sevoflurane both induced bronchodilation in patients with chronic obstructive pulmonary disease (COPD). Based on these findings, volatile anesthetics are preferentially used for the maintenance of general anesthesia in patients with hyperreactive airway disease and for the treatment of status asthmaticus.

In addition to the beneficial and clinical effects of volatile anesthetics in patients with hyperreactive airway disease, there is some evidence from basic research to demonstrate bronchodilatory effects of volatile anesthetics in hyperreactive airway models. Using Basenji-greyhound dogs as an allergic model, Hirshman et al. demonstrated that halothane specifically inhibited airway constriction of the models to ascaris challenge. On the other hand, Schütz et al. showed that volatile anesthetics induced only slight and transient inhibition of methacholine-induced airway constriction in an ovalbumin-sensitized guinea pig model. It has recently been demonstrated that the level of muscarinic receptor expression was increased and that the number of high-affinity $\beta$-adrenergic receptors was decreased in airway smooth muscles of the Basenji-greyhound dog model. Because we have also found that airway reactivity to the nonspecific $\beta$-adrenergic receptor antagonist proprano...
(2) muscle tension and \[Ca^{2+}\] in the hyperreactive airway in acute ovalbumin-sensitized guinea pigs. Sevoflurane, the most widely used volatile anesthetic, was used to investigate the inhibitory effects of volatile anesthetics on various hyperreactive airway models and to clarify the mechanism of the inhibitory effects of volatile anesthetics on Ca^{2+} sensitivity or contractile elements.

**Materials and Methods**

**Animals**

The experimental protocols and the methods for animal care used in this study were approved by the Experimental Animal Research Committee at our institution (Sapporo, Hokkaido, Japan). Young (4-week-old), male, pathogen-free Hartley guinea pigs (Japan SLC, Hamamatsu, Japan), weighing approximately 250 g at the time of purchase, were housed for at least 2 weeks after purchase in an air-conditioned room at a temperature of 23° ± 1°C and 60 ± 10% humidity and illuminated from 08:00 to 20:00 h. The guinea pigs were fed a standard laboratory diet and given water ad libitum. The weight of the guinea pigs was determined on arrival and just before the experiments (16 and 151 days), and there were no differences in mean weight gains between the treatment groups (data not shown).

**Preparation of Acute Ovalbumin-sensitized and Chronic Cigarette-smoking Models**

After the conditioning period, the animals were divided into two groups: an acute ovalbumin-sensitized group (n = 108) and a chronic cigarette-smoking group (n = 104). Each group was subdivided into control and intervention groups. An experimental model of acute ovalbumin-sensitization (n = 56) was established by using egg white ovalbumin as an antigen. Briefly, the ovalbumin-sensitized group received ovalbumin (1 mg/2 ml sterilized 0.9% saline) intraperitoneally, and ovalbumin sensitization was boosted after 15 days by exposure to aerosolized ovalbumin (10 mg/1 ml saline) for 10 min.

A control group for this model (n = 52) received the same volume of sterilized saline instead of ovalbumin and remained nonsensitized to ovalbumin during preparation.

The animals in the chronic cigarette-smoking group (n = 52) were exposed to diluted mainstream cigarette smoke 1 h/day for 5 days/week over a period of 150 days via an automated cigarette smoking machine (model INH06-CIGR01; Medical Interface Project Station, Osaka, Japan). Each awake guinea pig was held in an exposure chamber that was connected to the smoking machine. A puff of mainstream cigarette smoke (35 ml) generated from Hi-lite cigarettes (1.4 mg nicotine and 17 mg tar per cigarette; Japan Tobacco, Tokyo, Japan) was diluted with 280 ml room air and delivered to the chamber. Each cigarette was puffed 40 times with a suction volume of 600 ml/min. A control group for this model (n = 52) was exposed to air instead of cigarette smoke for the same period.

At 24 h after the last exposure to ovalbumin or cigarette smoke, the animals were randomly selected and then used for the following experiments.

**Histologic Demonstration of Acute Ovalbumin-sensitized and Chronic Cigarette-smoking Models in This Study**

Four animals from each group were anesthetized with intraperitoneal urethane (1.5 g/kg) and killed by exsanguination. The trachea and lungs were immediately removed en bloc, and phosphate-buffered 10% formalin (pH 7.4) was instilled into the lungs through the trachea at a pressure of 20 cm H2O. After fixation for 48 h, tissues were mounted in paraffin, and 3-μm-thick sections were then stained with hematoxylin and eosin for light microscopic examinations.
Measurements of Total Lung Resistance and Dynamic Lung Compliance

The guinea pigs (n = 6 in each group) were anesthetized intraperitoneally with urethane (1.5 g/kg), and supplemental doses of the anesthetic were administered whenever necessary to maintain abolition of corneal and withdrawal reflexes. The trachea was cannulated with an endotracheal tube (50 cm length of polyethylene tubing: PE-240) just below the larynx via a tracheotomy. The animals were placed in the supine position and were ventilated with a respirator (Harvard model 683; South Natick, MA) at a constant rate of 45 breaths/min and a tidal volume of approximately 6–8 ml/kg; the latter was adjusted in each animal to maintain end-tidal carbon dioxide partial pressure (5250 RGM; Datex-Ohmeda Japan, Tokyo, Japan) at approximately 40 mmHg. The inspiratory fraction of oxygen was maintained at approximately 0.30–0.35. The right jugular vein and right carotid artery were cannulated for intravenous injections and arterial blood pressure/heart rate monitoring, respectively. The intrapleural pressure was measured via a water-filled cannula (PE-240) that was placed in the lower third of the esophagus and connected to one port of the differential pressure transducer. The transpulmonary pressure was determined by monitoring the difference between pressure in the external end of the tracheal cannula and the esophageal cannula using a Statham differential transducer (DP-45; Validyne Engineering, Northridge, CA). A Fleisch pneumotachograph and a differential transducer were used to monitor respiratory flow rate (PULMOS-II; Medical Interface Project Station, Osaka, Japan). All signals were recorded, and total lung resistance (R_L) and dynamic lung compliance (C_Dyn) were analyzed continuously by an on-line computer on a breath-by-breath basis. A heating pad was placed under each animal, and the rectal temperature was kept at approximately 37°C during the study period.

To verify airway hyperresponsiveness, dose-response curves of R_L and C_Dyn to bolus intravenous injections and arterial blood pressure/heart rate monitoring, respectively. The inspiratory fraction of oxygen was maintained at approximately 0.30–0.35. The right jugular vein and right carotid artery were cannulated for intravenous injections and arterial blood pressure/heart rate monitoring, respectively. The intrapleural pressure was measured via a water-filled cannula (PE-240) that was placed in the lower third of the esophagus and connected to one port of the differential pressure transducer. The transpulmonary pressure was determined by monitoring the difference between pressure in the external end of the tracheal cannula and the esophageal cannula using a Statham differential transducer (DP-45; Validyne Engineering, Northridge, CA). A Fleisch pneumotachograph and a differential transducer were used to monitor respiratory flow rate (PULMOS-II; Medical Interface Project Station, Osaka, Japan). All signals were recorded, and total lung resistance (R_L) and dynamic lung compliance (C_Dyn) were analyzed continuously by an on-line computer on a breath-by-breath basis. A heating pad was placed under each animal, and the rectal temperature was kept at approximately 37°C during the study period.

To verify airway hyperresponsiveness, dose-response curves of R_L and C_Dyn to bolus intravenous injections (0.2 ml) of acetylcholine (1.0–6.0 μg/kg) were determined in each animal by successively increasing the concentration of acetylcholine solution at 5-min intervals, with each injection followed by flushing with 0.05 ml saline (the dead space of the tubing was 0.02 ml). After completion of the first acetylcholine challenge, the guinea pigs were exposed to sevoflurane. When the end-tidal concentration of the volatile anesthetic had reached 0.5 minimum alveolar concentration (MAC) as determined by a calibrated gas monitor (5250 RGM; 0.5 MAC = 1.0% for sevoflurane in guinea pigs), the acetylcholine challenge was repeated as described above. Finally, the volatile anesthetic concentration was increased to 1.0, 1.5, and 2.0 MAC, and data were collected before and during acetylcholine infusion.

Four of the animals in the ovalbumin-sensitized group were exposed to ovalbumin (10 mg/ml saline for 10 min) by inhalation to demonstrate ovalbumin-induced airway hyperreactivity in this model.

Simultaneous Measurements of Muscle Tension and Intracellular Ca²⁺ Concentration

In another experiment, the animals in the acute ovalbumin-sensitized (n = 6) and chronic cigarette-smoking (n = 6) groups were anesthetized with intraperitoneal urethane and killed by exsanguination. The lungs and trachea were removed en bloc and placed immediately in ice-cold physiologic salt solution (136.9 mM NaCl, 5.4 mM KCl, 1.5 mM CaCl₂, 1.0 mM MgCl₂, 23.9 mM NaHCO₃, and 5.5 mM glucose) that was aerated continuously with 5% carbon dioxide in oxygen. Sections of the midportion of the trachea, three cartilage rings wide, were dissected free from the connective tissue and loaded with 5 μM acetoxyximethyl ester of fura-2 in a physiologic salt solution containing 0.02% (vol/vol) Cremophor EL (Sigma Chemical, St. Louis, MO) for 5 h at room temperature (approximately 22°–24°C). After the fura-2 loading, each tracheal ring was held in a temperature-controlled (37°C) tissue bath with an initial isometric tension of 0.5 g by a strain gauge transducer (LVS-20GA; Kyowa, Tokyo, Japan) for 60 min. After equilibration, basal muscle tone was slowly increased to an optimal tension of 1.5 g.21 Experiments were performed using a fluorescence spectrometer (CAF-100; Japan Spectroscopic Co., Tokyo, Japan). Excitation light was passed through a rotating filter wheel (48 Hz) that contained 340- and 380-nm filters. The light emitted from the tracheal smooth muscle side at 500 nm was measured using a photomultiplier. The ratio of the fluorescence resulting from excitation at 340 nm to that resulting from excitation at 380 nm (R_340/380) was calculated and used as an indicator of [Ca²⁺] as previously reported.3,22

In a preliminary study, a dose–response curve in each group was obtained in tracheal ring preparations without loading fura-2 (n = 6 each). Contractions were induced by carbachol (approximately 10⁻⁹ to 10⁻⁵ M), a potent muscarinic receptor agonist. After obtaining the concentration of ED₅₀ for each model, the fura-2-loaded muscle tissue was exposed to sevoflurane (0.5, 1.0, 1.5, and 2.0 MAC) via bubbling in the presence of carbachol.

Measurement of Voltage-dependent Ca²⁺ Channel Activity

The perforated whole cell patch clamp technique was used to observe inward currents through VDCCs,23 and Ba²⁺ was used for the charge carrier instead of Ca²⁺ to reduce Ca²⁺-mediated inactivation of VDCCs.24 Tracheal smooth muscle tissue was dissected and minced using a dissecting microscope. The minced tissue was then digested for 120 min at 37°C in Ca²⁺-free Tyrode solution, to which 0.1% (wt/vol) collagenase and 0.05% (wt/vol) trypsin inhibitor were added. This solution contained...
135 mM NaCl, 5.4 mM KCl, 1.0 mM MgCl₂, 5.0 mM glucose, 5.0 mM HEPES, and 0.1% (wt/vol) bovine serum albumin with pH adjusted to 7.4 with tris(hydroxymethyl)aminomethane. Cells were dispersed by trituration, filtered through nylon mesh, and centrifuged. The pellet was resuspended in a modified Krebs-Ringer solution and stored at 4°C for up to 5 h before use. The modified Krebs-Ringer solution contained the following: 85 mM KCl, 30 mM K₂HPO₄, 5.0 mM MgSO₄, 5.0 mM Na₂ATP, 5.0 mM pyruvic acid, 5.0 mM creatine, 20 mM taurine, 5.0 mM β-hydroxybutyrate, and 0.1% (wt/vol) fatty-acid free bovine serum albumin, with pH adjusted to 7.25 with tris(hydroxymethyl)aminomethane.

Micropipettes were pulled from soda lime “hematocrit” tubing (GC-1.5; Narishige, Tokyo, Japan) using a brown-flaming horizontal puller (model P-97; Sutter Instruments, Novato, CA). Resistances were approximately 3–5 MΩ if filled with solution. The pipette solution contained the following: 50 mM CsCl, 90 mM CsCH₃SO₄, 1.0 mM MgCl₂, 1.0 mM EGTA, and 12 mM HEPES, with pH adjusted to 7.2 with tris(hydroxymethyl)aminomethane. The bath solution contained the following: 130 mM tetraethylammonium chloride, 1.0 mM MgCl₂, 5.0 mM BaCl₂, 10 mM glucose, and 10 mM HEPES, with pH adjusted to 7.4 with tris(hydroxymethyl)aminomethane.

Experiments were performed by a modification of the nystatin technique. Briefly, nystatin was prepared as a stock solution (40 mg/ml in dimethyl sulfoxide) and frozen in small aliquots for up to 5 days. Fresh working solution was prepared hourly by diluting the stock to a final concentration of 100 μg/ml in the pipette solution and sonicating. An aliquot (approximately 0.5 ml) of the cell suspension was placed in a chamber on the stage of an inverted microscope. A micromanipulator was used to position the patch pipette against the membrane of a tracheal smooth muscle cell. After obtaining a high-resistance seal (> 5 GΩ) with slight suction (approximately 10–30 cm H₂O), suction was released, and approximately 5–15 min was allowed for nystatin to be incorporated into the membrane patch, as indicated by a decrease in input resistance to less than 30 MΩ. The resistance then remained stable or slowly decreased during the course of the experiment (up to 60 min). Membrane currents were monitored using a CEZ-2400 patch clamp amplifier (Nihon Kohden, Tokyo, Japan), and the amplifier output was low-pass filtered at 2,000 Hz. Bath temperature during the patch clamp recording was controlled at approximately 37°C using a circular thermofoil heater of 115 mm in diameter (MT-1; Narishige) with a 35-mm central opening.

Inward Ba²⁺ currents (I₅₀) were elicited by 100 ms depolarizing pulses (−50 to +40 mV) from a holding potential of −80 mV. Leak currents were subtracted, and membrane capacitance and series resistance were compensated. After a stable baseline of peak J₅₀ had been obtained, cells were exposed to carbachol at a concentration of ED₈₀. The cells under this condition were then exposed to the bath solution equilibrated with sevoflurane (approximately 0.5–2.0 MAC). After exposure for 5 min, the perfusate was switched to the control solution to evaluate reversibility of the effects.

**Measurement of Intracellular Cyclic AMP Levels**

Levels of cyclic AMP, another important second messenger, were measured with and without sevoflurane. Tracheal smooth muscle tissue (n = 6 in each group) was dissected free from connective tissue and cartilage using a dissecting microscope. During stimulation by carbachol at a concentration of ED₈₀ for each model, the tracheal smooth muscle tissue was exposed to sevoflurane (1.0 or 2.0 MAC) via bubbling into physiologic salt solution at 37°C. After 5 min of the bubbling action, the muscle tissue was immediately removed and plunged into liquid nitrogen and stored at −80°C until the assay was performed. The tracheal smooth muscle tissues were homogenized in Hanks balanced salt solution, and 200 μl of the homogenized solution was used for measurement of protein concentrations. The homogenized solution was then centrifuged at 1,000g for 10 min at 4°C. Extraction of cyclic AMP from the supernatant was performed by the solid phase extraction method using Amprep SAX minicolumns (code RPN 1908; Biotrak, Del Mar, CA). Cyclic AMP levels in the tracheal smooth muscle tissues were measured using a Biotrak cyclic AMP enzyme immunoassay system (cAMP Biotrak EIA).

**Measurement of Sevoflurane Concentrations in the Gas Phase and in a Bath Solution**

Sevoflurane concentrations were measured according to a previously described method. Briefly, the vaporizer for the anesthetic tested was calibrated with an infrared anesthetic gas monitor (5250 RGM). Concentrations of the anesthetic agent in bath solution samples were analyzed using a gas chromatograph (GC-17A; Shimadzu, Kyoto, Japan). The mean concentrations of sevoflurane in the solutions in each experimental protocol were similar to those reported previously. Bath concentrations of the anesthetic in the experimental protocols were indistinguishable (n = 4 in each group, data not shown).

**Materials**

The following drugs and chemicals were used: ovalbumin, acetylcholine chloride, urethane, carbachol chloride, Cremophor EL, pyruvic acid, creatine, taurine, dimethyl sulfoxide, nystatin, tris(hydroxymethyl)aminomethane, β-hydroxybutyrate, fatty-acid free bovine serum albumin, HEPES, nifedipine, EGTA, type Iς trypsin inhibitor (Sigma Chemical, St. Louis, MO), sevoflurane (Sevofrane; Maruishi Pharmaceutical, Osaka, Japan), acetoxymethyl ester of fura-2 (Dojindo, Kumamoto, Japan), and type I collagenase. It was noted that the solution was prepared by the solid phase extraction method using Amprep SAX minicolumns (code RPN 1908; Biotrak, Del Mar, CA). Cyclic AMP levels in the tracheal smooth muscle tissues were measured using a Biotrak cyclic AMP enzyme immunoassay system (cAMP Biotrak EIA).
Nifedipine was dissolved in ethanol (0.01% final concentration).

### Statistical Analysis

All data are presented as mean ± SD. For the measurements of $[Ca^{2+}]_i$, and muscle tension, carbachol-induced sustained changes in $[Ca^{2+}]_i$, (indicated by $R_{460/380}$) and muscle tension were used as references (100%). For the determination of $ED_{50}$ of carbachol in each group, a sigmoidal function with variable slope was used (Prism 4; GraphPad Software, San Diego, CA). The significant and different effects of contractile agonists and sevoflurane on measured parameters were determined by two-way analysis of variance with repeated measures, followed by the Scheffé $F$ test for multiple comparisons. In all comparisons, a $P$ value $< 0.05$ was considered to be significant.

### Results

#### Histologic Demonstration of Acute Ovalbumin-sensitized and Chronic Cigarette-smoking Models in This Study

Representative photomicrographs of histologic preparations of control (cigarette-sham), acute ovalbumin-sensitized, and chronic cigarette-smoking guinea pig models are shown in figures 2A–C. Acute ovalbumin-sensitized animals as well as control animals showed normal lung structures. Large numbers of inflammatory cells such as eosinophils and macrophages were not observed in the ovalbumin-sensitized animal model or in the chronic cigarette-smoking model, but enlarged alveolar ducts and alveoli, as seen in a human emphysematous lung, were observed in the chronic cigarette-smoking model. Randomized sampling of four animals of this model showed similar emphysematous structures.

#### Measurement of Total Lung Resistance and Dynamic Lung Compliance

Figures 3A–C show the effects of acetylcholine alone and with 2.0% sevoflurane on changes in total $R_L$ and $C_{dy}$ in the control, acute ovalbumin-sensitized, and chronic cigarette-smoking groups. In the control group (cigarette-sham), acetylcholine significantly increased $R_L$ and decreased $C_{dy}$. Two percent sevoflurane significantly decreased $R_L$ and increased $C_{dy}$. There were no significant differences in the values of these parameters between the two control groups (cigarette-sham and ovalbumin-sham groups).

In the acute ovalbumin-sensitized group, inhaled ovalbumin elicited a transient but significant increase in $R_L$ of approximately 280% and a decrease in $C_{dy}$ of approximately 185% ($n = 4$, data not shown). These transient changes in $R_L$ and $C_{dy}$ seem to be regarded as an early asthmatic response. Ovalbumin sensitization significantly decreased the $ED_{200}$ of acetylcholine from 2.8 ± 0.4 µg/kg to 1.5 ± 0.3 µg/kg ($P < 0.05$) and, similar to the data obtained in control nonsensitized animals (ovalbumin-sham), sevoflurane significantly decreased $R_L$ and increased $C_{dy}$.

Figure 3C shows the effect of acetylcholine and 2.0% sevoflurane on these parameters in the chronic cigarette-smoking group. $R_L$ at any concentration of acetylcholine was significantly higher than that in the control group.
whereas values of $C_{\text{Dyn}}$ in this model were not significantly different from those in the control group. Sevoflurane had a smaller effect on these parameters in the cigarette-smoking model.

Figures 4A–C summarize the effect of sevoflurane in the presence of 6.0 $\mu$g/kg acetylcholine on percent changes in $R_L$ and $C_{\text{Dyn}}$ in the control (cigarette-sham), acute ovalbumin-sensitized, and chronic cigarette-smoking groups. The volatile anesthetic significantly decreased $R_L$ and increased $C_{\text{Dyn}}$ in both the control and ovalbumin-sensitized models. The anesthetic had a smaller effect on these parameters in the chronic cigarette-smoking model.

Fig. 3. Effects of acetylcholine alone and 2.0% sevoflurane in the presence of acetylcholine on changes in total lung resistance ($R_L$) and dynamic lung compliance ($C_{\text{Dyn}}$) in control (cigarette-sham; A), acute ovalbumin-sensitized (B), and chronic cigarette-smoking (C) groups. Data are expressed as mean ± SD (n = 6 each). * $P < 0.05$ versus control values in each model. † $P < 0.05$ versus control values in each sham model. Two percent sevoflurane similarly and significantly decreased $R_L$ and increased $C_{\text{Dyn}}$ in both the control and ovalbumin-sensitized models. The anesthetic had a smaller effect on these parameters in the chronic cigarette-smoking model.

Fig. 4. Effects of sevoflurane in the presence of 6.0 $\mu$g/kg acetylcholine on percentage changes in total lung resistance ($R_L$) and dynamic lung compliance ($C_{\text{Dyn}}$) in control (cigarette-sham; A), acute ovalbumin-sensitized (B), and chronic cigarette-smoking (C) groups. Data are expressed as mean ± SD (n = 6 each). * $P < 0.05$ versus each control value without sevoflurane. MAC = minimum alveolar concentration. Sevoflurane significantly and substantially decreased $R_L$ and increased $C_{\text{Dyn}}$ in the control (cigarette-sham), acute ovalbumin-sensitized, and chronic cigarette-smoking groups. The volatile anesthetic significantly decreased $R_L$ and increased $C_{\text{Dyn}}$ in a dose-dependent manner in both the control and acute ovalbumin-sensitized groups. Sevoflurane inhibiting increases in $R_L$ produced
by muscarinic receptor stimulation but was less potent in the chronic cigarette-smoking group. Sevoflurane responses on these parameters at lower doses of acetylcholine were similar to the responses at 6 μg/kg (data not shown).

Simultaneous Measurements of Intracellular Ca$^{2+}$ Concentration and Muscle Tension

To determine why the volatile anesthetic had a smaller effect on enhanced lung resistance in the chronic cigarette-smoking model (figs. 3C and 4), we investigated the in vitro effects of the anesthetic on tracheal muscle tension and intracellular free Ca$^{2+}$, an important second messenger. In a preliminary study, dose–response curves for carbachol both in the acute ovalbumin-sensitized and chronic cigarette-smoking models were significantly shifted to the left (ED$^{\text{90}} = 2.2 \times 10^{-7}$ and $2.5 \times 10^{-7}$ M, respectively; $8.2 \times 10^{-7}$ and $8.4 \times 10^{-7}$ M in the control groups, respectively), indicating that these models in the current study exhibited in vitro airway hyperresponsiveness.

The effects of sevoflurane on the ED$^{\text{90}}$ carbachol-enhanced airway muscle tone and increased [Ca$^{2+}$]$_i$ are summarized in figure 5. Similar to the data obtained for respiratory function parameters, in both the control (cigarette-sham) and ovalbumin-sensitized groups, sevoflurane significantly and substantially inhibited muscle tension, with a concomitant decrease in [Ca$^{2+}$]$_i$ in a dose-dependent manner. Sevoflurane had a rather smaller but significant inhibitory effect on both muscle tension and [Ca$^{2+}$]$_i$ in the chronic cigarette-smoking group. The relation between muscle tension and [Ca$^{2+}$]$_i$ did not change in any groups.

Measurement of Voltage-dependent Ca$^{2+}$ Channel Activity

In the chronic cigarette-smoking group, depolarization-induced inward $I_{\text{Ba}}$ obtained with nystatin-perforated patches and 5 mM Ba$^{2+}$ peaked at approximately 15 ms (fig. 6A). In 24 cells, maximum $I_{\text{Ba}}$ was $-320 \pm 24$ pA (range, $-225$ to $410$ pA), and there was no significant difference in maximum $I_{\text{Ba}}$ among the four groups (data not shown). Similar to the data obtained by using the conventional whole cell patch clamp technique with external Ca$^{2+}$, threshold activation of $I_{\text{Ba}}$ occurred at approximately $-20$ mV, and maximum peak current amplitude was obtained $+10$ mV (fig. 6B). Nifedipine (1 μM) almost completely blocked $I_{\text{Ba}}$ (n = 3, data not shown), indicating that tracheal smooth muscle in guinea pigs has only L-type VDCCs, as in humans.

As shown in representative traces for depolarization from $-80$ to $+10$ mV (fig. 6A), carbachol at a concentration of ED$^{\text{90}}$ for the chronic cigarette-smoking group reduced the magnitude of $I_{\text{Ba}}$ but did not cause an apparent change in the time course of the currents. There was no difference between the degree of inhibition by carbachol in the groups (by approximately 22–26%, data not shown). Under this condition, 2.0% sevoflurane further and significantly inhibited the channel activity, with no apparent shift by carbachol and sevoflurane in the voltage dependence of induced $I_{\text{Ba}}$ (fig. 6B). Figure 6C summarizes the inhibitory effects of sevoflurane on $I_{\text{Ba}}$ in each group. Sevoflurane significantly and dose-dependently inhibited $I_{\text{Ba}}$ in all groups, but the degree of inhibition was significantly smaller in the chronic cigarette-smoking group than in the other groups.

Measurement of Intracellular Cyclic AMP Levels

Finally, we measured the level of cyclic AMP, another important second messenger, during exposure to carbachol and sevoflurane (fig. 7). Intracellular cyclic AMP level at steady state was approximately 2.2 pmol/mg protein in all groups, and the cyclic AMP level in guinea pigs was similar to that measured in canine tracheal smooth muscles. Stimulation by carbachol at a concentration of ED$^{\text{90}}$ significantly decreased the cyclic AMP levels in all groups, and there were no significant between-group differences in cyclic AMP levels. Sevoflurane significantly and dose-dependently increased cyclic AMP levels in all groups; however, the level in the...
chronic cigarette-smoking group was significantly less than levels in the other groups.

Discussion

Acute Ovalbumin-sensitized and Chronic Cigarette-smoking Models in This Study

First, we made an acute ovalbumin-sensitized model by short-duration exposure to ovalbumin as an antigen. This model showed a transient but significant increase in total lung resistance and a decrease in dynamic lung compliance upon exposure to the antigen, and it also showed hyperresponsiveness to a muscarinic receptor agonist (fig. 3). On the other hand, chronic bronchial asthma is an atopic disease characterized by bronchoconstriction, bronchial hyperreactivity, and an influx of inflammatory cells, especially eosinophils, into the airway.33 It has been demonstrated that after allergen provocation, atopic asthmatic subjects show an early airway response maximal approximately 15–30 min after provocation, returning to the baseline within 2 h, frequently followed by a late airway response occurring approximately 4–12 h after provocation, with the late airway response seen in approximately 60% of cases.30 Unfortunately, the acute ovalbumin-sensitized model in this study did not show any significant histologic changes and a late airway response. Although there has been much interest regarding the late airway response, the early airway response seems to be more important for perioperative airway management. The main reasons are as follows: (1) The most important risk factors for the development of bronchospasm during anesthesia are age (< 10 yr), perioperative respiratory infection, and COPD, implying hyperreactive airway34; and (2) tracheal intubation per se is a major trigger of bronchospasm during general anesthesia, implying reflex bronchospasm.1,35 The acute ovalbumin-sensitized model used in this study did not show any significant histologic changes and a late airway response. Although there has been much interest regarding the late airway response, the early airway response seems to be more important for perioperative airway management. The main reasons are as follows: (1) The most important risk factors for the development of bronchospasm during anesthesia are age (< 10 yr), perioperative respiratory infection, and COPD, implying hyperreactive airway34; and (2) tracheal intubation per se is a major trigger of bronchospasm during general anesthesia, implying reflex bronchospasm.1,35 The acute ovalbumin-sensitized model used in this study, therefore, seems appropriate as a model of airway hyperreactivity.

Chronic exposure to cigarette smoke has been shown to induce chronic bronchitis or emphysema in rats,18 mice,36 and guinea pigs.37 Although the guinea pig has long been recognized as a useful model in investigations...
related to asthma, it is only in the last few years that it has been used extensively in research related to COPD, especially emphysema. We therefore selected the guinea pig as a model in which to investigate the effects of chronic exposure to cigarette smoke on lung structure and function. Chronic exposure to cigarette smoke for periods of more than 4 months has been shown to cause emphysematous airspace enlargement. The mean chord lengths of alveoli and alveolar ducts were both increased, as can be seen in photomicrographs of histologic preparations shown in figure 2. Emphysematous changes in this model were also observed in pulmonary function tests. As shown in figure 3, the chronic cigarette-smoking model showed significantly higher $R_L$ at the steady state, indicating the existence of peripheral airway narrowing. This model showed rather smaller airway hyperresponsiveness to muscarinic receptor stimulation, comparing with the ovalbumin-sensitized model. In a clinical situation, we usually find an increase in "static" lung compliance in patients with severe emphysema due to destruction of the elastic components of the lung. However, "dynamic" lung compliance in the chronic cigarette-smoking model in this study was inconsistent with that in humans. This could be, in part, due to (1) the difference between static and dynamic lung compliance, (2) the high frequency of respiratory rates in this study, and/or (3) an increase in airway resistance in this model. In sum, there is a possibility that the high frequency of respiratory rates and increase in airway resistance could have increased or modified dynamic lung compliance.

Effects of Sevoflurane on Total Lung Resistance and Dynamic Lung Compliance

In the control and acute ovalbumin-sensitized groups, sevoflurane significantly inhibited the acetylcholine-induced increase in $R_L$ concomitant with an increase in $C_{Dyn}$. These results are consistent with the results of previous studies without airway hyperreactivity. Schütz et al. reported that isoflurane and sevoflurane showed a significant but transient inhibition of methacholine-induced bronchoconstriction in ovalbumin-sensitized guinea pigs. It is not currently known, however, why these results are inconsistent with our results. This discrepancy may be, at least in part, due to differences in sensitization, because in the study by Schütz et al., the guinea pigs were exposed to aerosolized ovalbumin every day for 1 week, and the late anesthetic-resistant airway reactivity would have meant a late airway response. The late airway response induces inflammatory cells and edema in the airway and only responds to steroids. 40, 41, 42

and our study, global parameters, such as "total" lung resistance ($R_L$), were determined to characterize the mechanical status of the lungs. Therefore, the relative contributions of airways and tissues to the increase in $R_L$ remain unknown. Habre et al. separated $R_L$ into its airway and parenchymal components to identify airway responses and found that sevoflurane as well as other volatile anesthetics exerted a marked protective effect against bronchoconstriction. On the whole, volatile anesthetics are preferable for the protection of tracheal intubation-induced or hyperreactive airway−induced (e.g., that caused by smoking, respiratory infection, or asthma) bronchoconstriction.

However, we found that $R_L$ without acetylcholine was higher in our chronic cigarette-smoking model than in the control group. Destruction and narrowing of the peripheral airway in this model might be responsible for this result. We also found that sevoflurane had a smaller effect on the acetylcholine-induced changes in respiratory parameters measured in this model. It is reasonable to assume that morphologic changes in the peripheral airway in this emphysematous model render these airways less able to respond to the anesthetic. It is premature, however, to conclude that this is the only reason for the resistance to the anesthetic before examining the direct effect of the anesthetic on airway smooth muscle in this model. Volta et al. nicely demonstrated that sevoflurane as well as isoflurane decreased respiratory system resistance in patients with COPD. The different reactivity of sevoflurane between these studies could be, in part, due to the methodology as well as different species. In brief, we used exogenous agonists to stimulate the airway, whereas they measured airway resistance in intubated and anesthetized COPD patients.

Effects of Sevoflurane on In Vitro Muscle Tension with $[Ca^{2+}]_i$, $Ca^{2+}$ Channel Activity, and Cyclic AMP Levels in Tracheal Smooth Muscles

To determine why the chronic cigarette-smoking model in this study showed less sensitivity to the volatile anesthetic sevoflurane, we first measured tracheal muscle tension with $[Ca^{2+}]_i$. Although the main reactive airway is a peripheral airway in COPD, the use of tracheal smooth muscle in this study seems reasonable because hyperresponsive changes to constrictive agonists also occur in tracheal smooth muscle and because $M_2/M_3$ receptor distribution in guinea pig tracheal smooth muscle is similar to that in human peripheral airway smooth muscle. Similar to the results obtained in our respiratory function studies, sevoflurane had a smaller effect on carbachol-induced muscle contraction and increased $[Ca^{2+}]_i$. These results suggest that the insensitivity to sevoflurane seen in the chronic cigarette-smoking model also occurs at the in vitro smooth muscle level, and this is the first study to show the in vitro anesthetic insensitivity of airway tone in a hyperreactive
airway model. It was difficult to demonstrate simultaneous measurement of muscle tension and \([\text{Ca}^{2+}]_i\) in the guinea pig trachea, and therefore, we did not further investigate the \([\text{Ca}^{2+}]_i\) regulation in this experimental model.

We used a patch clamp technique to observe VDCC activity and an enzyme immunoassay to measure intracellular cyclic AMP levels, both of which are important for regulation of \([\text{Ca}^{2+}]_i\) in the conventional whole cell patch clamp technique, soluble intracellular contents, such as second messengers, may be lost by diffusion into the relatively large volume of the pipette solution. Therefore, inclusion of a high concentration of EGTA in the pipette solution may inhibit certain cholinergic responses, because \(\text{Ca}^{2+}\) is required for optimal activity of certain PKC isoforms. Therefore, use of the conventional technique does not rule out the possibility of an effect of sevoflurane via intracellular chemical mediators, and we therefore decided to use the perforated patch clamp technique in this experiment. As shown in figure 6A, carbachol significantly inhibited VDCC activity. Our result is in agreement with the results of previous studies in which smooth muscle cells were used.

Stimulation of \(M_3\) muscarinic cholinergic receptors causes \(\text{Ca}^{2+}\) release from the sarcoplasmic reticulum and activation of PKC via inositol triphosphate and diacylglycerol, respectively (fig. 1). In addition, stimulation of \(M_3\) muscarinic receptors reduces protein kinase A activity via an inhibitory G-protein (G_i) effect on adenylyl cyclase. Therefore, there is a possibility that these second messengers, especially PKC, could modify VDCC activity. We also found in this study that sevoflurane had a smaller inhibitory effect on VDCC activity in the chronic cigarette-smoking group compared with its effect in the other groups. Because we had demonstrated in previous studies that PKC regulates VDCC activity and that the volatile anesthetic halothane inhibits carbachol-induced PKC activity, it is reasonable to assume that the \(M_3\) muscarinic cholinergic pathway could have been modified in the chronic cigarette-smoking model. At least, \(\text{Ca}^{2+}\) sensitivity during exposure to sevoflurane in chronic cigarette-smoking model seems to be similar to those in the other groups (fig. 5), and the role of inositol triphosphate-induced \(\text{Ca}^{2+}\) release from sarcoplasmic reticulum is small because the increase in \([\text{Ca}^{2+}]_i\) due to inositol triphosphate is transient. Further studies are needed to clarify the role of \(M_3\) muscarinic cholinergic pathway in this model.

However, it was also shown in this study that the sevoflurane-induced increase in cyclic AMP levels seen in the control and acute ovalbumin-sensitized groups was significantly suppressed in the chronic cigarette-smoking group. This result is consistent with the results of a previous study showing that \(\beta_2\)-adrenoceptor function was suppressed in a chronic asthmatic model. A decrease in cyclic AMP levels in airway smooth muscle could result in an increase in \([\text{Ca}^{2+}]_i\), resulting in the anesthetic-insensitive airway seen in the chronic cigarette-smoking model. On the other hand, another group demonstrated that in canine tracheal smooth muscle the increase in cyclic AMP level produced by halothane was not responsible for the relaxation or the decrease in \([\text{Ca}^{2+}]_i\). Therefore, there is another possibility that the cyclic AMP pathway modifies the VDCC activity or that VDCC activity per se changes in the chronic cigarette-smoking model. Emala et al. also found selective impairment of isoproterenol stimulation of adenylate cyclase in membranes prepared from mononuclear leukocytes of Basenji-greyhound dogs used as a chronic asthmatic model despite the fact that there was no decrease in the number of \(\beta\)-adrenergic receptors. Although we did not investigate the \(\beta\)-adrenergic receptor pathway in our cigarette-smoking model, similar phenomena seem to have occurred in our model. It is conceivable that these in vivo and in vitro changes observed in the chronic cigarette-smoking model are, at least in part, due to the remodelled airway including main tracheal smooth muscle tissue.

In summary, we have succeeded in making different kinds of hyperreactive airway models by short-duration exposure to ovalbumin and long-term exposure to cigarette smoke, respectively. Sevoflurane inhibiting increases in total lung resistance produced by muscarinic receptor stimulus but was less potent in the chronic cigarette-smoking model. The anesthetic also showed a smaller inhibitory effect on tracheal smooth muscle contractility in the chronic cigarette-smoking model, and the in vitro mechanisms of this less sensitivity seem to be, at least in part, due to the remodelled airway in which inhibition of VDCC activity and induction of cyclic AMP production by the anesthetic are both suppressed.

References

EFFECTS OF SEVOFLURANE ON HYPERREACTIVE AIRWAY