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Role of Intracellular Ca^{2+} Stores in the Inhibitory Effect of Halothane on Airway Smooth Muscle Contraction

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Background: **Halothane directly inhibits contraction of airway smooth muscle, mainly by decreasing the intracellular concentration of free Ca^{2+} ($[Ca^{2+}]_i$). The role of intracellular Ca^{2+} stores, sarcoplasmic reticulum, is still unclear. We investigated the role of sarcoplasmic reticulum in the inhibitory effect of halothane on contraction of airway smooth muscle by measuring $[Ca^{2+}]_i$ and intracellular concentration of inositol 1,4,5-triphosphate ($[IP_3]_i$), a second messenger for release of Ca^{2+} from sarcoplasmic reticulum.**

Methods: **$[Ca^{2+}]_i$ was monitored by measuring the 500-nm light emission ratio (F_{340}/F_{380}) of a Ca^{2+} indicator fura-2 with isometric tension of canine tracheal smooth muscle strip. During Ca^{2+}-free conditions, carbachol (10^{-5} M) was introduced with pretreatment of halothane (0–3%). During Ca^{2+}-free conditions, 20 mM caffeine, a Ca^{2+}-induced Ca^{2+} release channel opener, was introduced with or without halothane. We measured $[IP_3]_i$ during exposure to carbachol and halothane by radioimmunoassay technique.**

Results: **Pretreatment with halothane significantly diminished carbachol-induced increases in $[Ca^{2+}]_i$ by 77% and muscle tension by 83% in a dose-dependent manner. Simultaneous administration of halothane significantly enhanced caffeine-induced transient increases in $[Ca^{2+}]_i$ and muscle tension in a dose-dependent manner, by 97% and 69%, respectively. Pretreatment with halothane abolished these responses. Rapid increase in $[IP_3]_i$ produced by carbachol was significantly inhibited by 32% by halothane in a dose-dependent manner.**

Conclusions: **Halothane, during Ca^{2+}-free conditions, inhibits transient contraction of airway smooth muscle induced by muscarinic receptor stimulation, mainly by attenuating the increase in $[Ca^{2+}]_i$. Depletion of Ca^{2+} from sarcoplasmic reticulum *via* Ca^{2+}-induced Ca^{2+} release channels also may contribute to the attenuation of the increase in $[Ca^{2+}]_i$ by halothane. (Key words: Calcium; calcium-induced Ca^{2+} release (CICR) channel; inositol 1,4,5-triphosphate (IP_3); IP_3-induced Ca^{2+} release (IICR) channel; sarcoplasmic reticulum.)**

HALOTHANE has a potent and direct relaxing effect on airway smooth muscle.[1,2] Because the intracellular concentration of free Ca^{2+} ($[Ca^{2+}]_i$) plays a central role in the regulation of airway smooth muscle tone,[3,4] a possible mechanism for relaxation by this anesthetic agent is a decrease in $[Ca^{2+}]_i$. Yamakage[2] and Jones *et al.*,[5] using the Ca^{2+} indicator fura-2, demonstrated that relaxation of contracted canine tracheal smooth muscle by halothane at clinically relevant concentrations was associated with a decrease in $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ is regulated by influx of Ca^{2+} through membrane-associated Ca^{2+} channels (voltage-dependent and Ca^{2+} depletion-activated Ca^{2+} channels) and by release of Ca^{2+} from intracellular Ca^{2+} stores, especially from sarcoplasmic reticulum (SR) (fig. 1).[3] Entry of extracellular Ca^{2+} through voltage-dependent channels is necessary for maintenance of the contraction of airway smooth muscle.[2,6] Yamakage *et al.*,[7] using patch clamp techniques, demonstrated that halothane had an inhibitory effect on the voltage-dependent channels of porcine tracheal smooth muscle cells at clinically relevant concentrations. The role, however, of intracellular Ca^{2+} stores, called SR, in the inhibitory effect of halothane on airway smooth muscle contraction is still unclear.

Release of Ca^{2+} from SR in airway smooth muscle is regulated by two mechanisms: inositol 1,4,5-triphosphate (IP_3)-induced Ca^{2+} release (IICR)[8] and Ca^{2+}-induced Ca^{2+} release (CICR) channels (fig. 1).[9] The current study therefore was designed to clarify the role of SR in the inhibitory effect of halothane on contraction of airway smooth muscle (1) by measuring $[Ca^{2+}]_i$ simultaneously with muscle tension during exposure to a

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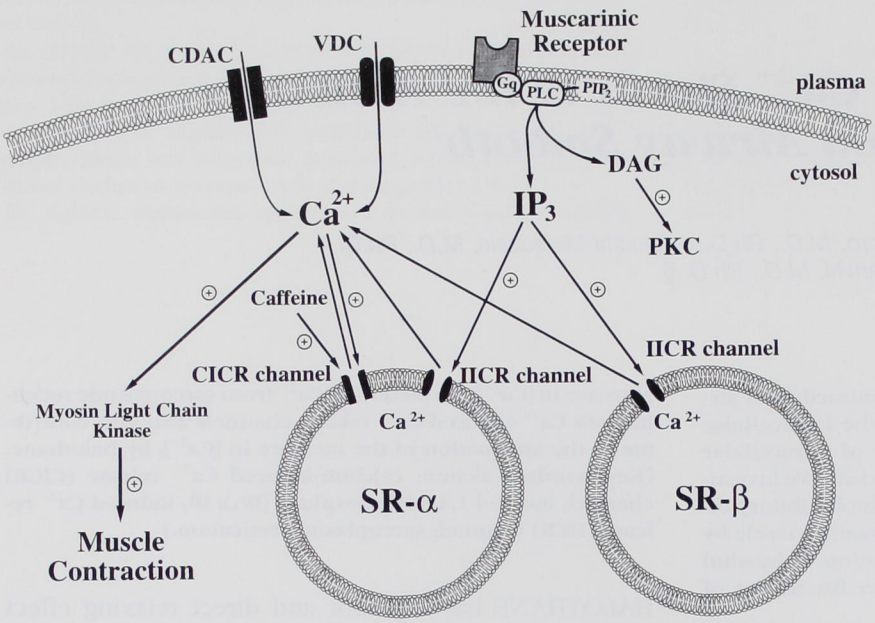

Fig. 1. Regulation of signal transduction and intracellular Ca^{2+} in airway smooth muscle. When the muscarinic receptor is stimulated, voltage-dependent Ca^{2+} channels (VDC) and Ca^{2+} depletion-activated Ca^{2+} channels (CDAC) are activated. Ca^{2+} enters cytosol through these channels. Similarly, phospholipase C (PLC) is activated *via* the G proteins (Gq) linked to it, resulting in the rapid breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG), both of which act as second messengers. Whereas DAG activates Ca^{2+}/phospholipid-dependent protein kinase (PKC). IP_3 mobilizes Ca^{2+} from sarcoplasmic reticulum (SR) through IP_3-induced Ca^{2+} release (IICR) channels,[9] which are also regulated by Ca^{2+}. The SR can be functionally separated into two components: SR-α and SR-β. SR-α involves two types of channels, IICR and Ca^{2+}-induced Ca^{2+} release (CICR) channels,[8] whereas SR-β involves only IICR channels. CICR channels can be activated by caffeine[9] and Ca^{2+}.

muscarinic receptor agonist carbachol or a CICR channel opener caffeine, with or without halothane, during Ca^{2+}-free conditions; and (2) by measuring intracellular concentration of IP_3 ($[IP_3]_i$) during exposure to carbachol, with or without halothane.

Methods

Preparation of Muscle Strips

This study was approved by the Sapporo Medical University Ethical Committee on Animal Research. Adult mongrel dogs (weight, 9–12 kg) were anesthetized with intravenous thiamylal (20 mg/kg). After a surgical level of anesthesia was attained, the trachea was quickly excised and placed in physiologic salt solution (PSS) at room temperature. The PSS contained (in mM) NaCl 136.9, KCl 5.4, $CaCl_2$ 1.5, $MgCl_2$ 1.0, $NaHCO_3$ 23.9, glucose 5.5, and EDTA 0.01. The solution was aerated continuously with a 95% O_2/5% CO_2 gas mixture (*p*H 7.4). The smooth muscle was dissected free of epithelium, cartilage, and connective tissue and cut into small strips ≈1 mm wide and ≈8 mm long.

Measurement of $[Ca^{2+}]_i$ and Muscle Tension

Fura-2 loading was performed according to the previously described method.[2] The muscle strips were pretreated with a 5-μM acetoxymethyl ester of fura-2 (fura-2/AM), an indicator of Ca^{2+}, in PSS for ≈7 h at room temperature (22–24°C). Cremophor EL (0.02% vol/vol), a noncytotoxic detergent, was added to increase the solubility of fura-2/AM. After fura-2 loading, the muscle strip was held horizontally in a temperature-controlled (37°C) 5-ml organ bath. One end of the muscle strip was connected to a strain gauge transducer (120T-20B; Kyowa Co., Tokyo, Japan). The strip was then washed with PSS for 30 min to remove uncleaved fura-2/AM. Experiments were conducted within 60 min after washing.

Experiments used a fluorescence spectrometer (CAF-110; Japan Spectroscopic Co., Tokyo, Japan) specially designed to measure the surface fluorescence of living tissue. Excitation light obtained from a xenon high-pressure lamp (75 W) was passed through a rotating filter wheel (128 Hz) that contained 340 and 380 nm filters. The emitted light from the muscle strip at 500 nm was measured with a photomultiplier. The time constant of the optical measurements was 0.25 s. The ratio of the fluorescence from excitation at 340 nm to that at 380 nm (F_{340}/F_{380}) was calculated from successive illumination periods and used as an indicator of $[Ca^{2+}]_i$, as has been reported.[2,10]

The first contraction evoked by a 72.7 mM high K^+ solution served as a control (100%). The high K^+ solution was made by substituting NaCl in the PSS with equimolar KCl. After washing the muscle strip with PSS and determining the resting tension, the organ bath

solution was substituted transiently (for ≈5 s) with Ca^{2+}-free PSS (with 5 mM EGTA) to remove Ca^{2+} from the cell surface[11] and then substituted with Ca^{2+}-free PSS (with 50 μM EGTA) to maintain $[Ca^{2+}]_i$ at the normal resting level. During this condition, the muscle strip was stimulated with 10^{-5} M carbachol, a potent muscarinic receptor agonist. After this protocol, the muscle strip was reincubated with PSS, including 1.5 mM Ca^{2+}. A second contraction was evoked by 72.7 mM high K^+ solution to restore Ca^{2+} in SR.[11] The PSS was again substituted with Ca^{2+}-free PSS as described earlier. During this condition, halothane (1.0, 2.0, or 3.0% in the gas phase) was introduced into a bath solution for 3 min, and the muscle strip was stimulated with 10^{-5} M carbachol. The concentration of carbachol (10^{-5} M) used in this study could induce maximum contraction[12] and maximum increase in $[IP_3]_i$.[13] The order of these two protocols was randomized.

In another experiment, the first contraction was similarly evoked by a 72.7 mM high K^+ solution, which served as a control (100%). After washing the muscle strip with PSS, including 1.5 mM Ca^{2+}, the organ bath solution was substituted with Ca^{2+}-free PSS as described earlier. During this condition, the muscle strip was exposed to 20 mM caffeine, a CICR opener.[9] After this protocol, the same strip was similarly reincubated with PSS, including 1.5 mM Ca^{2+}, and the PSS was again substituted with Ca^{2+}-free PSS. Halothane (1.0, 2.0, or 3.0% in the gas phase) and caffeine (20 mM) were introduced simultaneously into the bath solution or halothane was preintroduced into the bath solution for 3 min. The muscle strip was then exposed to 20 mM caffeine during this condition. The order of these three protocols was randomized.

To further investigate the effect of other anesthetic agents on the increase of $[Ca^{2+}]_i$ attributable to release of Ca^{2+} from SR, we performed additional experiments using isoflurane (range, 0.0–4.5%) in the absence of external Ca^{2+}.

Measurement of $[IP_3]_i$

The muscle strips also were used for measuring $[IP_3]_i$. After preincubating three or four muscle strips for 30 min in PSS at 37°C, the muscle strips were first incubated with halothane-containing (0.0, 1.0, 2.0, or 3.0% in the gas phase) PSS for 2 min and then stimulated with 10^{-5} M carbachol. The reactions were terminated after 0, 5, 10, 15, 30, 60, or 120 s of stimulation with carbachol by freezing the tissue samples in liquid nitrogen.[14]

The technique of Uemura *et al.*[15] was used to measure the $[IP_3]_i$. The frozen tissue sample was homogenized with 2 ml of 10% (vol/vol) ice-cold $HClO_4$ for 20 min. A 200-μl aliquot of the homogenized solution was used to measure concentrations of protein.[16] The remaining aliquots were centrifuged at 2,000*g* for 15 min to remove insoluble materials. The *p*H of the supernatant was adjusted precisely to 7.5 with 10 N KOH/HEPES. Insoluble precipitates (primarily $KClO_4$) were removed by centrifugation at 2,000*g* for 10 min. The resultant supernatant was lyophilized and stored at −20°C. The lyophilized samples were dissolved in 100 μl distilled water, and the amount of IP_3 was measured using the Amersham IP_3 assay system (code TRK 1,000; Amersham Japan Co., Tokyo, Japan). This assay is based on competition between unlabeled IP_3 in the samples and a fixed quantity of tritium-labeled IP_3 for a limited number of high-affinity binding sites on a specific IP_3 binding protein.[17] The determinations were made in duplicate, and the results were expressed as pmoles per milligram of protein.

Determination of Concentrations of the Anesthetic Agent in the Bath Solution

The tissue samples in all experiments were quickly (within 10 s) exposed to a bath solution equilibrated with halothane (1.0, 2.0, or 3.0% in the gas phase) or isoflurane (1.5, 3.0, or 4.5% in the gas phase). The bath solution was continuously bubbled with the same concentration of the anesthetic agent. Concentrations of the anesthetic agents in bath solution samples were analyzed with a gas chromatograph (GC-12A; Shimadzu Co., Kyoto, Japan) equipped with a flame ionization detector (FTD-8; Shimadzu) and an integrator (Chromatopac C-R 3A; Shimadzu). The mean concentrations of halothane in the solution (1.0, 2.0, and 3.0% in the gas phase) were 0.33, 0.75, and 1.15 mM, respectively, whereas the mean concentrations of isoflurane in the solution (1.5, 3.0, and 4.5% in the gas phase) were 0.35, 0.79, and 1.21 mM, respectively. The concentration of the anesthetic agents in the bath solution had close linear correlation with the concentration in the gas phase, and the anesthetic potencies in dogs between these agents were comparable.[18,19]

Materials

With the exceptions noted later, reagents were obtained from Sigma Chemical Co. (St. Louis, MO) and Dojindo Co. (Kumamoto, Japan). Halothane, caffeine, and the IP_3 assay system were obtained from ICI Co.

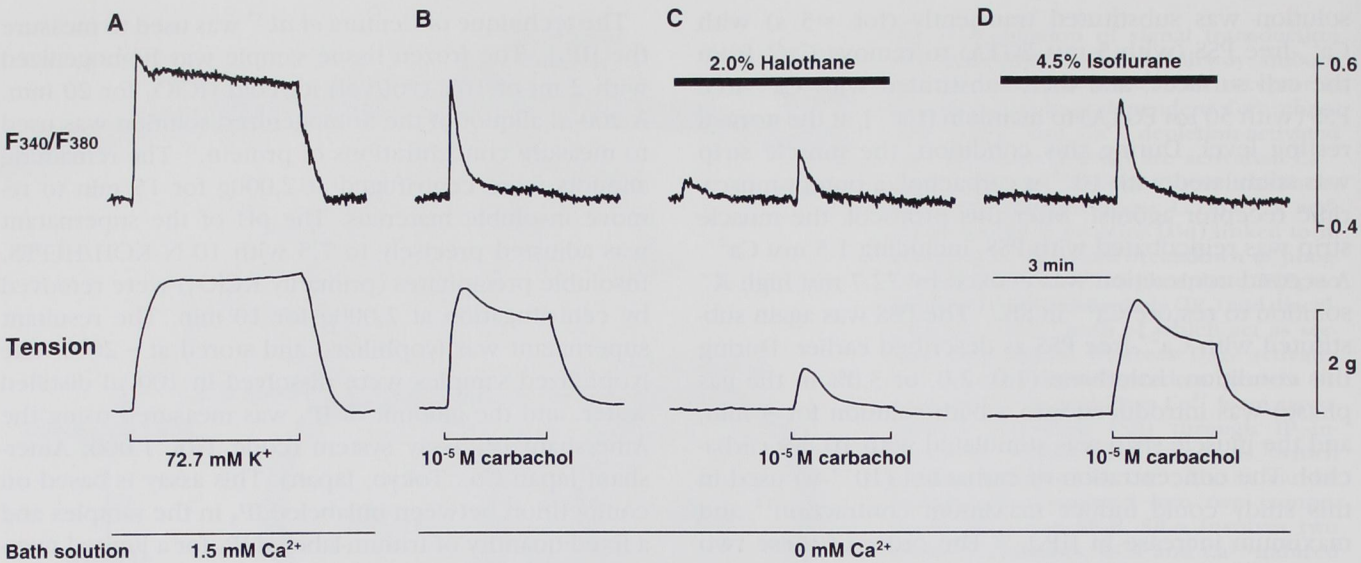

Fig. 2. Changes in intracellular concentration of free Ca^{2+} (indicated by F_{340}/F_{380} ratio) and muscle tension during contractions induced by high K^+ (72.7 mM) with 1.5 mM Ca^{2+} (*A*) and by carbachol (10^{-5} M) without (*B*) or with 2.0% halothane (*C*)/4.5% isoflurane (*D*) during a Ca^{2+}-free condition. (*C* and *D*) Carbachol (10^{-5} M) was introduced 3 min after the incubation of halothane or isoflurane.

(Dighton, MA), Wako Pure Chemical Co. (Osaka, Japan), and Amersham Japan Co. (Tokyo, Japan), respectively.

Statistical Analysis

All data are expressed as mean ± SD. For the measurement of $[Ca^{2+}]_i$ and muscle tension, high K^+-induced sustained changes in $[Ca^{2+}]_i$ (indicated by F_{340}/F_{380} ratio) and muscle tension were used as references (100%).[2,10] All data were analyzed using paired/unpaired two-tailed *t* test or one-factor analysis of variance with Fisher's *a posteriori* test. In all comparisons, a probability value <0.05 was considered significant.

Results

Effects of Halothane and Isoflurane on $[Ca^{2+}]_i$ and Muscle Tension

Figure 2 shows the effect of high K^+ (72.7 mM) with 1.5 mM Ca^{2+} and the effects of carbachol (10^{-5} M) with or without halothane 2.0%/isoflurane 4.5% during Ca^{2+}-free conditions on $[Ca^{2+}]_i$ and the tension of canine tracheal smooth muscle. The ratio F_{340}/F_{380}, an indicator of $[Ca^{2+}]_i$, was increased rapidly by high K^+ with a concomitant muscle contraction (fig. 2A). After washout with the Ca^{2+}-free PSS, the resting levels of $[Ca^{2+}]_i$ and muscle tension remained unchanged (fig. 2B). During this condition, carbachol (10^{-5} M) significantly increased muscle tension. This increased tension was followed by a slight decrease before the muscle tension reached a steady state. The peak and plateau levels of the muscle contraction were 71.2 ± 9.7 and 65.7 ± 8.6% of the contraction compared with the muscle tension induced by 72.7 mM high K^+ with 1.5 mM Ca^{2+}. In contrast, carbachol during Ca^{2+}-free conditions induced a transient increase of $[Ca^{2+}]_i$, followed by a substantial reduction. The percent peak of $[Ca^{2+}]_i$ was 68.4 ± 7.6% compared with the $[Ca^{2+}]_i$ induced by 72.7 mM high K^+ with 1.5 mM Ca^{2+}. $[Ca^{2+}]_i$ and muscle tension reached their respective peaks in 10–30 s. Pretreatment of halothane (2.0%) during Ca^{2+}-free conditions induced a slight and transient increase of $[Ca^{2+}]_i$, followed by a substantial reduction without change in the muscle tension (fig. 2C). During 2.0% halothane, carbachol (10^{-5} M) induced slight and transient increases of $[Ca^{2+}]_i$ and muscle tension, followed by substantial reductions (fig. 2C). These changes in $[Ca^{2+}]_i$ and muscle tension were smaller than those induced by carbachol without halothane. The order of the two protocols shown in figures 2B and 2C were randomized. There were no significant differences in the peak of $[Ca^{2+}]_i$ or muscle tension obtained by consecutive carbachol stimulation (data not shown). To determine whether these effects were induced by other anesthetic agents as well, we

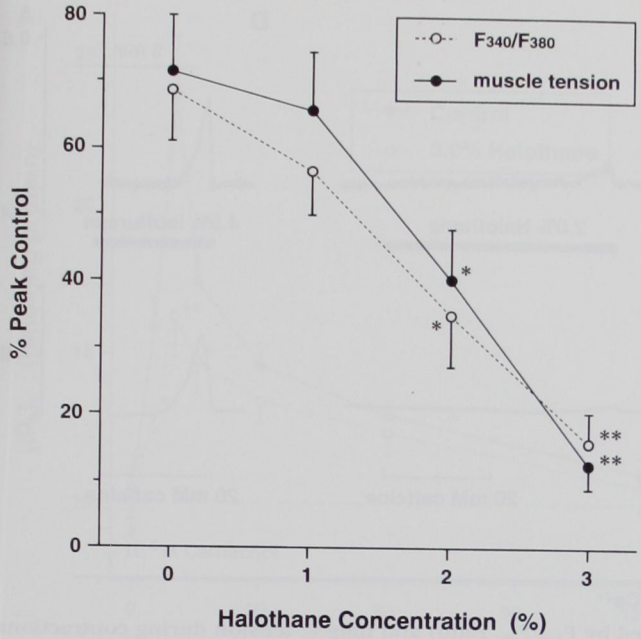

Fig. 3. Relation between concentrations of halothane in the gas phase and percent peak response of intracellular concentration of free Ca^{2+} (indicated by F_{340}/F_{380} ratio) or muscle tension stimulated by 10^{-5} M carbachol during a Ca^{2+}-free condition. Symbols represent mean ± SD (n = 8 at each point). *$P < 0.05$; **$P < 0.01$ compared with the control value without halothane.

performed additional experiments using isoflurane in the absence of external Ca^{2+} (fig. 2D). Isoflurane at concentrations of up to 4.5% in the gas phase had no apparent effect on either muscle contraction (inhibited by ≈9 ± 4% at 4.5% isoflurane) or the increase in $[Ca^{2+}]_i$ (inhibited by ≈7 ± 2% at 4.5% isoflurane) induced by carbachol (n = 8 at each point).

Figure 3 shows the relation between concentrations of halothane and the percent response of $[Ca^{2+}]_i$ or muscle tension. Halothane significantly decreased the peaks of $[Ca^{2+}]_i$ by ≈77% and muscle tension by ≈83% in a dose-dependent manner.

Figure 4 shows the effects of caffeine (20 mM) and halothane (2.0%) or isoflurane (4.5%) during Ca^{2+}-free conditions on $[Ca^{2+}]_i$ and the tension of canine tracheal smooth muscle. Caffeine (20 mM) induced increases in $[Ca^{2+}]_i$ and muscle tension during the Ca^{2+}-free condition. These increases were transient (fig. 4A) and similar to those obtained by carbachol stimulation during a Ca^{2+}-free condition (fig. 2B). $[Ca^{2+}]_i$ and muscle tension induced by caffeine reached their respective peaks in 10–30 s. Simultaneous administration of 2.0% halothane augmented the transient increases in $[Ca^{2+}]_i$ and muscle tension (fig. 4B). Figure 5 shows the relation between concentrations of halothane and the percent response of $[Ca^{2+}]_i$ or muscle tension. Halothane significantly increased the peaks of $[Ca^{2+}]_i$ by ≈97% and muscle tension by ≈69% in a dose-dependent manner. As shown in figure 2C, the pretreatment with halothane (2.0%) during the Ca^{2+}-free condition induced a slight and transient increase in $[Ca^{2+}]_i$ without changing the muscle tension (fig. 4C). During this condition, caffeine (20 mM) exerted almost no effect on either $[Ca^{2+}]_i$ or muscle tension during any concentration of halothane up to 3.0%. The order of these three protocols was randomized, and there were no significant differences in the peaks of either $[Ca^{2+}]_i$ or muscle tension obtained by consecutive carbachol stimulation (data not shown).

We performed additional experiments using isoflurane in the absence of external Ca^{2+} as well (fig. 4D). Isoflurane at concentrations up to 4.5% in the gas phase had no apparent effect on either muscle contraction (increased by ≈6 ± 2% at 4.5% isoflurane) or the increase in $[Ca^{2+}]_i$ (increased by ≈3 ± 2% at 4.5% isoflurane) induced by caffeine (n = 8 at each point).

Effect of Halothane on $[IP_3]_i$

Figure 6A shows the time course and effects of 3.0% halothane on $[IP_3]_i$ in carbachol-stimulated canine tracheal smooth muscle. The $[IP_3]_i$ at time 0 was 10.6 ± 0.8 pmol/mg protein (n = 8) and failed to change with the addition of halothane (10.2 ± 0.9, 10.6 ± 0.8, and 9.9 ± 0.7 pmol/mg protein at 1.0, 2.0, and 3.0% halothane, respectively). Carbachol (10^{-5} M) produced a rapid increase in the $[IP_3]_i$, which reached maximum (23.8 ± 2.1 pmol/mg protein) 10 s after the stimulation. The rapid increase in $[IP_3]_i$ induced by carbachol was followed by a rapid and substantial decrease to a concentration of ≈10 pmol/mg protein. Halothane (3.0%) significantly inhibited the increase in $[IP_3]_i$ induced by carbachol 5–15 s after stimulation with carbachol with no apparent change in the time course of $[IP_3]_i$. Figure 6B summarizes the effects of various concentrations of halothane (0.0, 1.0, 2.0, and 3.0%) on the peak $[IP_3]_i$ 10 s after stimulation with carbachol. Halothane significantly inhibited in a dose-dependent manner the increase in $[IP_3]_i$ by ≈32% induced by carbachol.

Discussion

The major findings of this study are that, in canine tracheal smooth muscle *in vitro*, clinically relevant con-

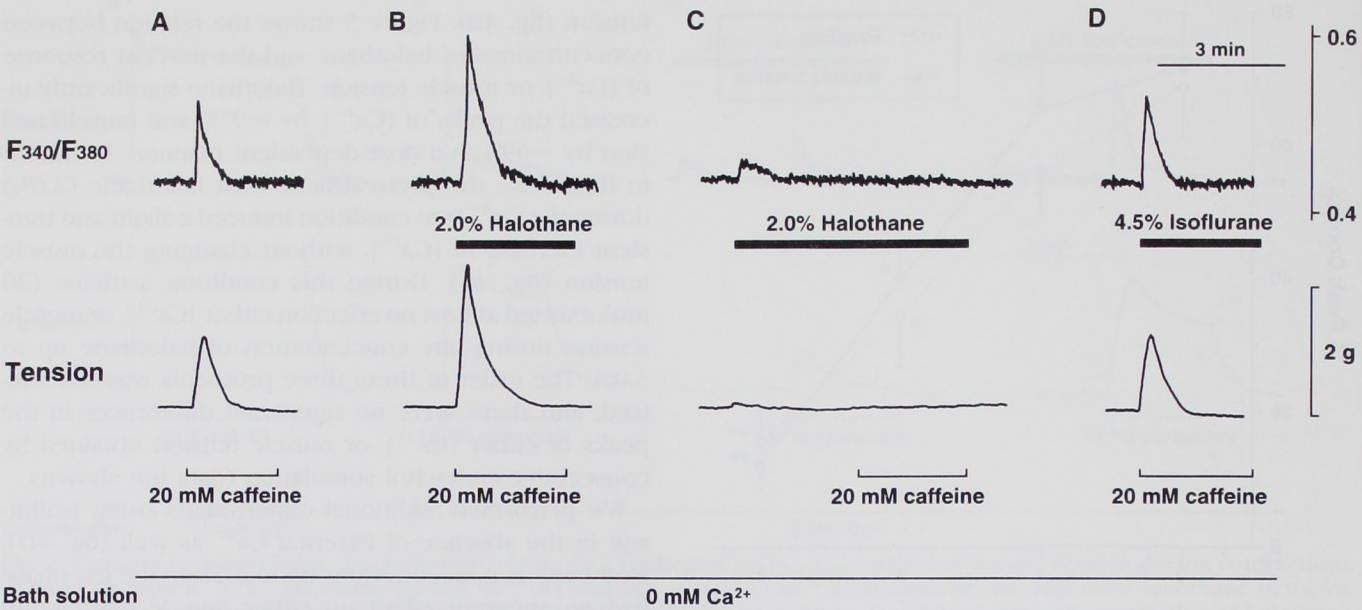

Fig. 4. Changes in intracellular concentration of free Ca^{2+} (indicated by F_{340}/F_{380} ratio) and muscle tension during contractions induced by caffeine (20 mM) without (*A*) or with 2.0% halothane (*B, C*)/4.5% isoflurane (*D*) during a Ca^{2+}-free condition. Caffeine (20 mM) was introduced alone (*A*), with 2.0% halothane (*B*) or 4.5% isoflurane (*D*) simultaneously and with the preincubation of 2.0% halothane (*C*).

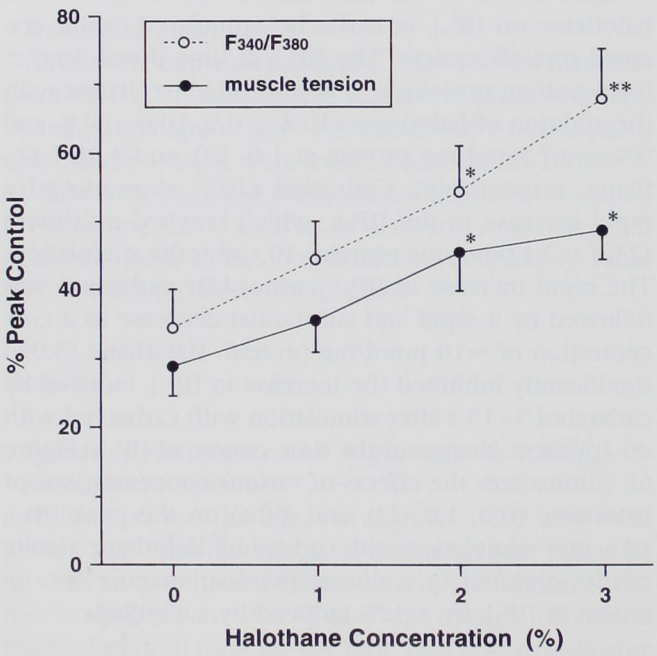

Fig. 5. Relation between concentrations of halothane in the gas phase and percent peak response of intracellular concentration of free Ca^{2+} (indicated by F_{340}/F_{380} ratio) or muscle tension stimulated by caffeine (20 mM). Symbols represent mean ± SD (n = 8 at each point). *$P < 0.05$; **$P < 0.01$ compared with the control value without halothane.

centrations of halothane altered the intracellular free Ca^{2+} transient and attenuated the muscle contraction induced by muscarinic receptor stimulation even without external Ca^{2+}. These effects were dose-dependent at the concentrations of halothane studied and are consistent with the previous study that used the luminescent Ca^{2+} indicator aequorin.[20]

In airway smooth muscle, muscarinic receptor stimulation activates the plasma membrane-bound phospholipase C *via* G proteins (fig. 1).[21] Phospholipase C subsequently catalyzes the hydrolysis of membrane-associated phosphatidylinositol 4,5-bisphosphate to IP_3 and diacylglycerol. A rapid increase in $[IP_3]_i$ induces release of Ca^{2+} from the SR *via* IICR channels.[8,22] Diacylglycerol activates Ca^{2+}/phospholipid-dependent protein kinase at its membrane site, resulting in sensitization of the contractile elements to intracellular Ca^{2+}.[2,5,12] Stimulation of muscarinic receptors also increases the slow influx of extracellular Ca^{2+} across the plasma membrane.[3,23] An additive increase in $[Ca^{2+}]_i$ activates the Ca^{2+}- and calmodulin-dependent myosin light chain kinase, resulting in the contraction of the muscle cells.[3] Release of Ca^{2+} from the SR is therefore important to initiate the muscle contraction.[2-4,12,20]

This study shows that the airway smooth muscle-sustained contraction can be obtained during Ca^{2+}-free

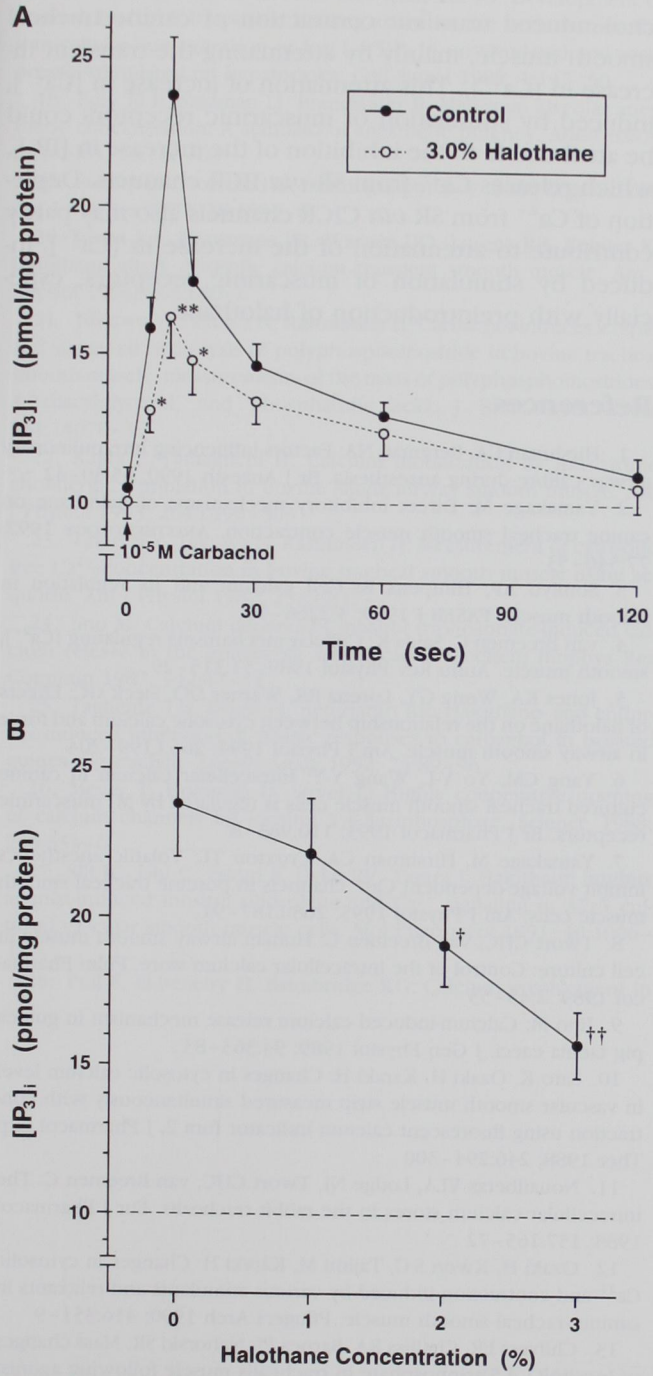

Fig. 6. Effects of halothane on intracellular concentration of inositol 1,4,5-triphosphate ($[IP_3]_i$) of carbachol-stimulated canine tracheal smooth muscle. (*A*) Effects of 3.0% halothane on time-dependent changes in the $[IP_3]_i$ induced by 10^{-5} M carbachol. (*B*) Effect of halothane (0.0, 1.0, 2.0, and 3.0%) on the peak $[IP_3]_i$ 10 s after stimulation with carbachol. Symbols represent mean ± SD (n = 8 at each point). *$P < 0.05$; **$P < 0.01$ compared with the control values for the same time course. †$P < 0.05$; ††$P < 0.01$ compared with the control value without halothane.

conditions although influx of Ca^{2+} is important to maintain the muscle contraction.[2-4,12] The muscle contraction seen during the Ca^{2+}-free condition should be divided into two parts: (1) initial transient contraction with a concomitant increase in $[Ca^{2+}]_i$; and (2) decreased but sustained contraction with a substantial decrease in $[Ca^{2+}]_i$. Because in airway smooth muscle IP_3 is the primary regulator for release of Ca^{2+} from SR,[8] and because the time course of the increase in $[IP_3]_i$ induced by carbachol was very similar to that of the change in $[Ca^{2+}]_i$ (figs. 2 and 6A), we suggest that IP_3 is an important determinant of $[Ca^{2+}]_i$ during agonist stimulation during Ca^{2+}-free conditions, whereas IICR is also regulated by $[Ca^{2+}]_i$.[9,24] Accordingly, our results that halothane significantly attenuated the increase in $[IP_3]_i$ induced by muscarinic receptor stimulation (fig. 6) supported the results wherein the initial transient increase in muscle tension was significantly inhibited by halothane with a concomitant reduction of increase in $[Ca^{2+}]_i$ (figs. 2 and 3).[25] Because the release of Ca^{2+} from SR depends on the cube of $[IP_3]_i$,[26] and the resting level of $[IP_3]_i$ is ≈10 pmol/mg protein (fig. 6), it seems reasonable that the changes in carbachol-induced $[IP_3]_i$ produced by halothane do not linearly parallel the halothane-induced changes in carbachol-induced Ca^{2+}/tension changes (fig. 3).

Our results are in general agreement with studies in a variety of cell types, in which treatment with halothane has been associated with inhibition of the increase in $[Ca^{2+}]_i$ mediated by IP_3.[27-30] These studies have demonstrated that halothane alters Ca^{2+} homeostasis, an action that underlies the *in vivo* effect of the anesthetic agent. Smart *et al.*[31] and Rooney *et al.*,[32] however, showed that halothane induced formation of IP_3 in SH-SY5Y neuroblastoma cells and turkey erythrocytes, respectively. These discrepancies may result from the differences in cell types and species or in the selective effects of halothane on certain receptors, G proteins, or phospholipase C isozymes.[33]

The latter part of the sustained contraction obtained by stimulation of muscarinic receptors during a Ca^{2+}-free condition could be in part attributable to the protein kinase C-induced sensitization of contractile elements to Ca^{2+}.[2,12] Accordingly, the inhibition of the latter portion of the sustained contraction by halothane might partly be explained by the previously reported evidence that activity of protein kinase C is attenuated by halothane[2] and by the finding that the increase in $[IP_3]_i$ induced by carbachol was inhibited by halothane in this study (fig. 6).

Another kind of Ca^{2+} release channel, CICR channels, also exist in the SR membrane.[9] The SR has been functionally separated into two components: SR-α and SR-β.[9] SR-α involves two types of channels, IICR and CICR channels, whereas SR-β involves only IICR channels (fig. 1).[9,24,34] Because evidence shows that an increase in $[Ca^{2+}]_i$ *per se* induces release of Ca^{2+} from the SR *via* CICR channels,[9] there is a possibility that release of Ca^{2+} *via* the CICR channels partly involves the increase in $[Ca^{2+}]_i$ induced by stimulation of muscarinic receptors during the Ca^{2+}-free condition. We conducted another experiment on the effect of halothane on the CICR channels using the CICR opener[9] caffeine. As shown in figures 4 and 5, simultaneous administration of halothane in a dose-dependent manner significantly enhanced the release of Ca^{2+} by stimulation with caffeine. Conversely, the pretreatment with halothane apparently abolished the effect of caffeine on $[Ca^{2+}]_i$. Because the sole administration of halothane as shown in figures 2 and 4 transiently increased $[Ca^{2+}]_i$, we conclude that halothane settles the CICR channels into an open state. This results in depletion of Ca^{2+} from the SR-α and attenuation of the increase in $[Ca^{2+}]_i$ induced by caffeine. The preintroduction of halothane therefore could have a partial role in the inhibition of the initial increase in $[Ca^{2+}]_i$ induced by stimulation of muscarinic receptors. These results are consistent with some other investigations. In unstimulated cardiac,[35-37] skeletal,[38] and vascular smooth[27,39] muscles, halothane causes depletion of Ca^{2+} from the SR either by attenuating uptake of Ca^{2+} from the cytosol or by release of Ca^{2+} from the SR *via* CICR channels. Recently, Warner *et al.*[40] showed that halothane decreased $[Ca^{2+}]_i$ and muscle force in canine tracheal smooth muscle, only when they used submaximum stimulation and not maximum stimulation. This discrepancy may result from the differences in types and concentrations of agonists and from differences in experimental techniques we used. Further, the role of attenuation of uptake of Ca^{2+} into SR by halothane is unknown in airway smooth muscle.

It is noteworthy that isoflurane had little effect on the muscle contraction and increase in $[Ca^{2+}]_i$ induced either by carbachol or by caffeine during Ca^{2+}-free conditions (figs. 2 and 4). This observation parallels the clinical observation that halothane is more effective than other anesthetic agents at inhibiting airway smooth muscle contraction at clinical concentrations.[1] In addition, isoflurane does not activate release and depletion of Ca^{2+} from the SR *via* CICR as does halothane.

Halothane, during Ca^{2+}-free conditions, inhibits carbachol-induced transient contraction of canine tracheal smooth muscle, mainly by attenuating the transient increase in $[Ca^{2+}]_i$. This attenuation of increase in $[Ca^{2+}]_i$ induced by stimulation of muscarinic receptors could be attributable to the inhibition of the increase in $[IP_3]_i$, which releases Ca^{2+} from SR *via* IICR channels. Depletion of Ca^{2+} from SR *via* CICR channels also may partly contribute to attenuation of the increase in $[Ca^{2+}]_i$ induced by stimulation of muscarinic receptors, especially with preintroduction of halothane.

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