

Sevoflurane Does Not Inhibit Human Platelet Aggregation Induced by Thrombin

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Background: Sevoflurane reportedly inhibits adenosine diphosphate-induced platelet aggregation by suppressing thromboxane A₂ formation. The increase in intracellular calcium concentration that fosters platelet aggregation, however, is also induced by other cell signaling pathways, such as activation of the production of inositol 1,4,5-triphosphate by thrombin. The current study aimed to clarify the net influence of sevoflurane on thrombin-induced platelet aggregation.

Methods: Washed platelets were stimulated by thrombin after incubation with 0.5, 1.0, or 1.5 mM sevoflurane, halothane, or isoflurane. Aggregation curves were measured by an aggregometer. Intracellular calcium concentration was measured fluorometrically using fura-2. Calcium mobilization *via* plasma membrane calcium channels and the dense tubular system was assessed differentially. Intracellular inositol 1,4,5-triphosphate was measured by radioimmunoassay.

Results: Halothane significantly suppressed aggregation ratios at 5 min compared with those in controls (89 ± 7%) to 71 ± 10% (1.0 mM) and 60 ± 11% (1.5 mM) and the increase in intracellular calcium concentration (controls, 821 ± 95 nM *vs.* 440 ± 124 nM [1.0 mM] or 410 ± 74 nM [1.5 mM]). Halothane also significantly inhibited release of calcium from the dense tubular system (controls, 220 ± 48 nM *vs.* 142 ± 31 nM [1.0 mM]). Neither sevoflurane nor isoflurane produced a net change in aggregation ratios, intracellular calcium concentration, or calcium mobilization. Halothane (1 mM) significantly suppressed inositol 1,4,5-triphosphate concentrations, whereas neither 1 mM isoflurane nor 1 mM sevoflurane had any effect.

Conclusions: Although sevoflurane has been reported to inhibit human platelet aggregation induced by weak agonists such

as adenosine diphosphate, it does not inhibit human platelet aggregation induced by strong agonists such as thrombin. (Key words: Blood; halothane; isoflurane; second messenger; volatile.)

SEVERAL studies of the effects of inhalation anesthetic agents on platelet function have been reported since Ueda¹ demonstrated in 1971 that clinical concentrations of halothane inhibited adenosine diphosphate (ADP)-induced platelet aggregation. Although some reported findings remain controversial,²⁻⁵ halothane is considered to inhibit platelet aggregation⁶⁻⁸ whereas isoflurane is not.^{8,9} Recently, Hirakata *et al.*¹⁰ reported that sevoflurane inhibited platelet aggregation by suppressing the formation of thromboxane A₂, concluding that the inhibitory effect of sevoflurane could be mediated by a decrease in cyclooxygenase activity. This study, however, was performed on the platelet aggregation induced by weak agonists such as ADP and epinephrine. Aggregation agonists may be grouped as strong agonists, such as thrombin, and weak agonists, such as ADP.¹¹ Platelet stimulation by strong agonist is mediated by additional or distinct intracellular events compared with stimulation by weak agonists,¹¹⁻¹³ suggesting that the inhibition of one of these pathways may not necessarily inhibit platelet aggregation as observed clinically.

To clarify the influence of sevoflurane on thrombin-induced platelet aggregation, halothane was used as a positive control, and isoflurane was used as a negative control. We measured intracellular calcium concentration ($[Ca^{2+}]_i$) simultaneously with platelet aggregation; analyzed calcium mobilization by influx through calcium channels in the plasma membrane or the release from the dense tubular system using the divalent cations, Ni²⁺ and Mn²⁺; and measured intracellular inositol 1,4,5-triphosphate (IP₃) concentrations by radioimmunoassay.

Materials and Methods

This study was approved by the Ethical Committee on Human Research of Kyoto Prefectural University of Med-

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icine, and written informed consent was obtained from all participants.

Preparation of Washed Platelet Suspension Loaded with Fura-2

Venous blood (32 ml) was obtained by antecubital venipuncture from healthy volunteers who had not taken any drug known to affect platelet aggregation for at least 2 weeks. These samples were collected into plastic tubes containing acid-citrate-dextrose solution consisting of 85 mM sodium citrate, 70 mM citric acid, and 110 mM glucose. The ratio of blood to acid-citrate-dextrose solution was 4:1. The samples were centrifuged at 110g for 15 min. The upper layer was withdrawn as platelet-rich plasma. These platelet-rich plasma samples were incubated immediately with 3 mM fura-2 acetoxymethyl ester for 15 min at 37°C and then centrifuged at 250g for 15 min in the presence of apyrase (0.3 U/ml). A washed platelet suspension (WPS) loaded with fura-2 was obtained after discarding the supernatant and resuspending the pellet in Tyrode-HEPES buffer at pH 7.4.¹⁴ Using a Coulter Counter Model MD II (Coulter Electronics, Hialeah, FL), numbers of platelets in the WPS were adjusted to 10^5 cells/ μ l for the investigation of aggregation curves and intracellular calcium mobilization, or alternatively to 10^6 cells/ μ l for the measurement of intracellular IP₃ concentration.

Assessment of Platelet Aggregation Curve

Platelet aggregation curves were investigated using the turbidimetric technique.

Aliquots of WPS (250 μ l, including 2.5×10^7 platelets per cuvette) were placed in a siliconized glass tube, maintained at 37°C, and stirred at 1,000 rpm throughout the experiments. After adjustment of the extracellular free calcium concentration to 1 mM using CaCl₂, platelet aggregation induced by thrombin (final concentration, 0.005–0.020 U/ml) was measured for 5 min using an aggregometer (HEMA TRACER 601; NIKOH Bioscience, Tokyo, Japan).

After incubation for 5 min with halothane, isoflurane, or sevoflurane (final concentration and percentages: for halothane, 0.5 mM [0.5%], 1.0 mM [1.1%], and 1.5 mM [1.6%]; for isoflurane, 0.5 mM [0.9%], 1.0 mM [1.8%], and 1.5 mM [2.7%]; for sevoflurane, 0.5 mM [1.9%], 1.0 mM [3.8%], and 1.5 mM [5.7%]), platelet aggregation was measured for 5 min after addition of thrombin (final concentration, 0.01 U/ml).

Measurement of Intracellular Free Ca²⁺ Concentration and Mobilization

Aliquots (500 μ l) of WPS loaded with fura-2, including 5×10^7 platelets per cuvette, were added to a fluorometric cuvette stirred at 1,000 rpm within a fluorometer (CAF-110; JASCO, Tokyo, Japan), which repeatedly subjected samples to alternating excitation wavelengths of 340 and 380 nm while measuring emission at 505 nm (sampling frequency, 2 Hz).¹⁵ Immediately after adjustment of extracellular free calcium concentration to 1 mM using CaCl₂ and incubation with each anesthetic agent (final concentrations, 0.0 [control], 0.5, 1.0, or 1.5 mM of halothane, isoflurane, or sevoflurane) for precisely 5 min at 37°C to record basal fluorescence intensity, samples were stimulated for 5 min with thrombin (final concentration, 0.01 U/ml). The ratio of fluorescence intensity at 340 nm relative to that of 380 nm (R340:380)^{16,17} was recorded at resting and peak phases. At each point, [Ca²⁺]_i was calculated according to the equation of Tsien *et al.*,¹⁸ using a dissociation constant for fura-2 and Ca²⁺ of 224 nM. After the end of each experiment, cells were treated with 0.3% Triton-X followed by the addition of 3 mM ethylene glycol-bis-(β -amino ethylether)-N,N,N'-tetraacetic acid to obtain maximum and minimum fluorescence, respectively. Data are expressed as the increase in [Ca²⁺]_i from its baseline levels.

Ni²⁺ (final concentration, 1 mM) was added to samples instead of CaCl₂ to block the influx of extracellular calcium. Therefore, the thrombin-induced release of calcium from the dense tubular system was observed.^{19,20}

Entry of Mn²⁺ was evaluated with a quenching technique to investigate the influx of calcium into the cytosol.^{19,20} MnCl₂ (final concentration, 1 mM) was added to the samples, and fluorescence from fura-2 was monitored at the calcium-insensitive excitation wavelength of 360 nm. Mn²⁺ entry, which represents calcium influx, was measured as the rate of decrease of fluorescence after stimulation by thrombin. Data are expressed as percent of initial fluorescence.

Measurement of Intracellular Inositol 1,4,5-Triphosphate Concentration

Intracellular IP₃ concentration was measured using the technique of Uemura *et al.*²¹ After preincubation with 1 mM of Ca²⁺ for 1 min, WPS (120 μ l, including 1.2×10^8 platelets) first was incubated with halothane, isoflurane, or sevoflurane (final concentration, 1 mM), respectively, for 5 min and then was stimulated with thrombin (final concentration, 0.01 U/ml) for precisely 15, 30, and 60 s. The reactions were terminated by adding ice-cold 20%

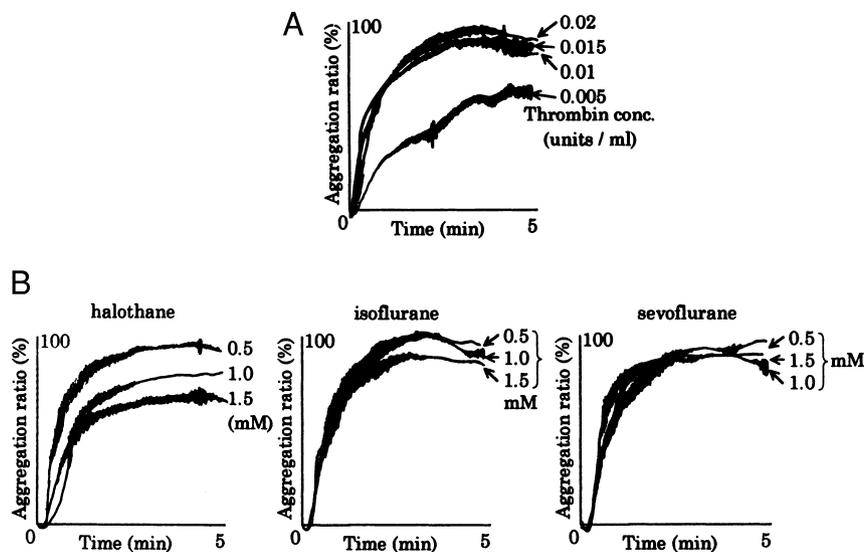


Fig. 1. Representative platelet aggregations. (A) Aggregation curves induced by four concentrations of thrombin (0.005, 0.010, 0.015, and 0.020 U/ml). The ordinate represents the optical density, defined as 0% for platelet-rich plasma and 100% for platelet-poor plasma. The abscissa represents time (0–5 min). (B) Aggregation curves induced by thrombin (0.01 U/ml). Samples were pretreated with halothane, isoflurane, or sevoflurane for 5 min. Concentrations for each anesthetic agent were 0.5, 1.0, and 1.5 mM. Both ordinate and abscissa are as in (A).

perchloric acid in an amount equal to one fifth of the original volume. The samples were kept in an ice bath for 20 min and were then centrifuged at 2,000g for 15 min. The pH of the supernatant was adjusted to 7.5 with 4 N KOH, and insoluble precipitates were removed by centrifugation at 2,000g 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 (TRK1,000; Amersham International, Buckinghamshire, United Kingdom). Determinations were made in duplicate, and the results are expressed as $\text{pmol}/10^8$ platelets.

Determination of Anesthetic Concentration

Immediately after an aliquot of each anesthetic agent was added directly to WPS-containing tubes, the tops were tightly sealed with parafilm to minimize evaporation. Anesthetic concentrations in the samples were analyzed using a gas chromatograph equipped with a flame ionization detector (GC-9A; Shimadzu, Kyoto, Japan), a headspace sampler (HSS-2A; Shimadzu), and an integrator (Chromatopac C-R4A; Shimadzu).

Materials

Sevoflurane was obtained from Hoechst Marion Roussel (Tokyo, Japan), halothane from Hoechst Japan (Tokyo, Japan), isoflurane from Dainabott (Tokyo, Japan), fura-2 acetoxymethyl ester from Dojindo Laboratories (Kumamoto, Japan), and apyrase from Sigma Chemical (St. Louis, MO). Thrombin was donated by Mochida

Pharmaceutical (Tokyo, Japan). All other compounds were purchased from commercial sources.

Statistical Analysis

Values in this study are expressed as mean \pm SD. Unpaired *t* tests were adopted for analysis of aggregation ratios. One-way analysis of variance followed by Bonferroni correction was adopted for analysis of the increase in $[\text{Ca}^{2+}]_i$ and release of calcium from the dense tubular system. Two-way analysis of variance followed by Bonferroni correction with repeated measures was used to analyze differences in the calcium influx and the IP_3 concentration. A *P* value < 0.05 was regarded as significant for all comparisons.

Results

Thrombin-induced Aggregation Curves

Among four aggregation curves induced by thrombin at concentrations of 0.005, 0.010, 0.015, and 0.020 U/ml ($n = 8$ each), stable aggregation could not be obtained at a thrombin concentration of 0.005 U/ml (fig. 1A). Among the three stable aggregation curves induced by thrombin at 0.010, 0.015, and 0.020 U/ml, the aggregation ratio at 5 min ($89 \pm 7\%$, $86 \pm 7\%$, and $89 \pm 6\%$, respectively) did not differ significantly; typical curves are shown in figure 1A. Accordingly, 0.01 U/ml thrombin was the minimum concentration that induced stable platelet aggregation in this study.

We compared aggregation curves induced by 0.01

SEVOFLURANE AND PLATELET AGGREGATION

Table 1. Aggregation Ratios at 5 min after the Onset of Thrombin Stimulation

	Aggregation Ratio at 5 min (%)*		
	Concentration		
	0.5 mM	1.0 mM	1.5 mM
Halothane	87 ± 8	71 ± 10†	60 ± 11†
Isoflurane	83 ± 10	90 ± 7	89 ± 9
Sevoflurane	84 ± 7	89 ± 9	90 ± 11

Data are shown as mean percent aggregation ± SD (n = 8 per group).

* Value for control (no agent) is 89 ± 7.

† P < 0.05 compared with control value.

U/ml thrombin among 10 preparations (three concentrations of three anesthetic agents plus a control group) from each of the eight specimens.

Aggregation ratios at 5 min (table 1) with 1.0 and 1.5 mM halothane were significantly lower than in controls. Isoflurane and sevoflurane had no influence on the aggregation curves. Typical patterns of aggregation are shown in figure 1B.

Changes in Intracellular Calcium Concentration

We compared the increase in $[Ca^{2+}]_i$ induced by 0.01 U/ml thrombin among the 10 preparations described earlier for aggregation curves (n = 8 for each). Baseline $[Ca^{2+}]_i$ was 97 ± 11 nM in controls. With halothane, baseline $[Ca^{2+}]_i$ was 101 ± 9 nM at 0.5 mM; 96 ± 14 nM at 1.0 mM; and 97 ± 14 nM at 1.5 mM. With isoflurane, baseline $[Ca^{2+}]_i$ was 96 ± 9 nM at 0.5 mM; 92 ± 14 nM at 1.0 mM; and 96 ± 10 nM at 1.5 mM. With sevoflurane, baseline $[Ca^{2+}]_i$ was 88 ± 17 nM at 0.5 mM; 93 ± 11 nM at 1.0 mM; and 104 ± 9 nM at 1.5 mM. None of the three anesthetic agents significantly changed baseline intracellular calcium concentrations.

The thrombin-induced increases in $[Ca^{2+}]_i$ are shown in table 2. Values for halothane at 1.0 and 1.5 mM were significantly lower than in controls (P < 0.01). Typical patterns for the R340:380 value, indicating $[Ca^{2+}]_i$, are shown in figure 2.

Analysis of Intracellular Calcium Mobilization

When Ni^{2+} was added to the sample instead of $CaCl_2$, the increase in $[Ca^{2+}]_i$ was 220 ± 48 nM in controls; 142 ± 31 nM with halothane at 1.0 mM; 200 ± 58 nM with isoflurane at 1.0 mM; and 206 ± 64 nM with sevoflurane at 1.0 mM. The increase in $[Ca^{2+}]_i$ was significantly lower (P < 0.01) with halothane than with other agents. Typical patterns for aggregation and for changes in the

R340:380 value, indicating $[Ca^{2+}]_i$, are shown in figure 3.

Figure 4 shows Mn^{2+} entry, expressed as percent decrease of fluorescence at 360 nm. After stimulation by thrombin, fluorescence decreased rapidly for more than 20–30 s and then gradually decreased. The early phase represented Mn^{2+} entry (i.e., Ca^{2+} influx) caused by thrombin stimulation, and the delayed phase was attributable to passive leakage through the plasma membrane.^{19,20} Decreases in fluorescence showed no significant difference between control conditions and any of the anesthetic agents for either phase.

Intracellular Inositol 1,4,5-Triphosphate Concentration

Time courses of intracellular IP_3 concentrations in thrombin-stimulated WPS in the absence (control) and in the presence of 1 mM halothane, isoflurane, or sevoflurane are shown in figure 5. After stimulation by thrombin, intracellular IP_3 concentrations increased instantly within 15 s and then returned rapidly to baseline levels. The IP_3 concentrations at rest (0 s) were (in pmol/ 10^8 platelets) 1.6 ± 1.2 in controls, 1.8 ± 1.4 with halothane, 1.3 ± 1.0 with isoflurane, and 1.7 ± 1.1 with sevoflurane. Halothane significantly suppressed the peak value (2.0 ± 1.5 pmol/ 10^8 platelets at 15 s) to lower than that seen in controls (3.9 ± 1.9 pmol/ 10^8 platelets). No significant differences in peak concentration of IP_3 were observed among control, isoflurane, and sevoflurane groups.

Discussion

Effects of anesthetic agents on platelet function, such as the aggregation curve, the increase in $[Ca^{2+}]_i$, and IP_3

Table 2. Changes in Intracellular Calcium Concentration

	Increase in $[Ca^{2+}]_i$ (nM)*		
	Concentration		
	0.5 mM	1.0 mM	1.5 mM
Halothane	755 ± 93	440 ± 124†	410 ± 74†
Isoflurane	800 ± 96	734 ± 124	762 ± 71
Sevoflurane	793 ± 124	729 ± 127	708 ± 92

Human-washed platelets were preincubated with each anesthetic at 37°C for 5 min, then thrombin (0.01 u/ml) was added. Increase in $[Ca^{2+}]_i$ was defined as the difference between peak $[Ca^{2+}]_i$ and its resting levels. Values are presented as mean ± SD.

* Value for control (no agent) is 821 ± 95.

† P < 0.01 compared with control value.

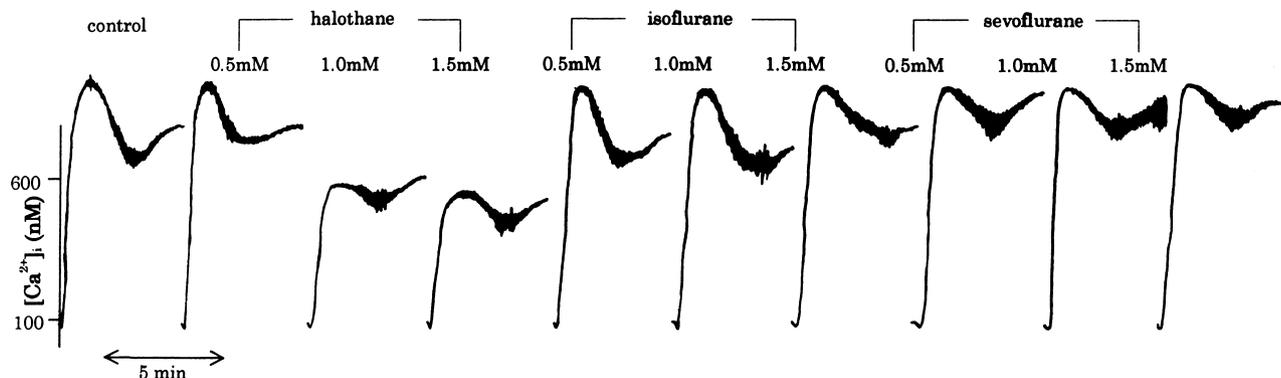


Fig. 2. Change in the intracellular calcium concentration ($[Ca^{2+}]_i$) induced by thrombin at 0.01 U/ml. Samples were pretreated with halothane, isoflurane, or sevoflurane for 5 min. Concentrations of each anesthetic agent were 0.5, 1.0, and 1.5 mM, respectively. The ordinate represents the R340:380 value, indicative of $[Ca^{2+}]_i$; the common abscissa represents time.

production, are measured using stimulated platelets by aggregation agonists. Aggregation agonists can be divided into strong agonists, such as thrombin, collagen, and thromboxane A_2 , and weak agonists, such as ADP, epinephrine, and serotonin, which depend on secretion to obtain a full response.¹¹ Intracellular signals elicited by strong agonists are different from those elicited by weak agonists.¹¹⁻¹³ These differences complicate comparison of platelet function induced by different agonists, which could account for several conflicting reports about the effects of anesthetic agents on platelet function. Further, determination of the appropriate concentration of aggregation agonists is critical, because agents do not inhibit platelet aggregation if stimulation is too intense.

First, we established an optimal concentration of thrombin for studies; 0.01 U/ml thrombin was the minimum stimulation needed to induce stable platelet aggregation (fig. 1A). At this concentration of thrombin, we demonstrated dose-dependent inhibition by halothane of the induced increase of $[Ca^{2+}]_i$ and of platelet aggrega-

tion (a positive control for investigation of sevoflurane) and no effect of isoflurane (a negative control for sevoflurane). These findings for halothane and isoflurane are consistent with those of previous studies.^{4,8-10}

Hirakata *et al.*¹⁰ have reported that sevoflurane inhibited ADP-induced platelet aggregation and formation of thromboxane A_2 by suppression of cyclooxygenase activity, concluding that sevoflurane inhibited human platelet aggregation. However, their study was performed on the platelet aggregation induced by weak agonists, such as ADP or epinephrine. We demonstrated here that sevoflurane does not affect the platelet aggregation induced by the strong agonist, thrombin, as there were no changes in the aggregation curve, increase in $[Ca^{2+}]_i$, calcium influx, release from the dense tubular system, or intracellular IP_3 concentration. Platelet stimulation by strong agonists is mediated by different intracellular events than is stimulation by weak agonists.¹¹⁻¹³ For example, the phospholipase C/protein kinase C pathway is involved in human platelet activation induced by thrombin,^{22,23} although it has little involve-

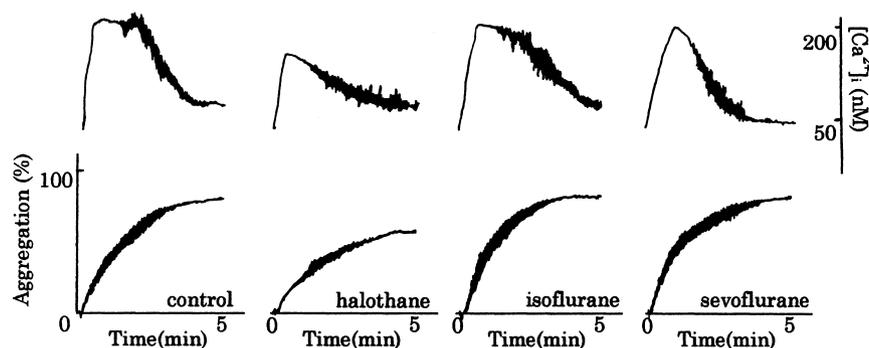


Fig. 3. Effects of each inhalation anesthetic agent (1.0 mM) on the increase in intracellular calcium concentration ($[Ca^{2+}]_i$) caused by stimulation with thrombin (0.01 U/ml) with a 1 mM extracellular concentration of Ni^{2+} instead of Ca^{2+} . The changes in $[Ca^{2+}]_i$ and the platelet aggregation curve more than 5 min in one representative sample in each group are shown ($n = 7$). The ordinate for the lower panels represents the optical density defined as 0% for platelet-rich plasma and 100% for platelet-poor plasma; the ordinate for the upper panels represents the R340:380 value, indicating $[Ca^{2+}]_i$. The common abscissa represents time (0–5 min).

SEVOFLURANE AND PLATELET AGGREGATION

ment in ADP-induced aggregation.^{24,25} Clinical platelet aggregation is thought to be induced by combined activation of multiple pathways mediated by thromboxane A₂ and IP₃ formation, accompanied by the increase in the [Ca²⁺]_i.²⁶ The inhibition of a single pathway may not result in the net inhibition of platelet aggregation. Platelet aggregation and calcium release induced by strong agonists such as thrombin reportedly are not inhibited by cyclooxygenase inhibitors²⁷ or thromboxane A₂ receptor blockers.²⁸ Further, amounts of thromboxane A₂ formed by thrombin-induced platelet aggregation are approximately 10 times greater than those formed in weak agonist-induced platelet aggregation.²⁹ These data strongly suggest that any decrease in calcium mobilization caused by sevoflurane-induced suppression of the formation of thromboxane A₂ could be compensated for by the activation of the thrombin-mediated pathway, maintaining normal platelet aggregation.

Halothane has been reported to prolong bleeding time³⁰ and to inhibit thrombin-induced platelet aggregation by decreasing [Ca²⁺]_i via a decrease in IP₃. Halothane also inhibits ADP-induced platelet aggregation by suppression of formation of thromboxane A₂ and binding to its receptors.^{8,9} We also demonstrated that halothane suppressed production of IP₃ and inhibited the increase in [Ca²⁺]_i in a dose-dependent manner by suppressing the release of calcium from the dense tubular system in thrombin-induced platelet aggregation, a finding that is in agreement with

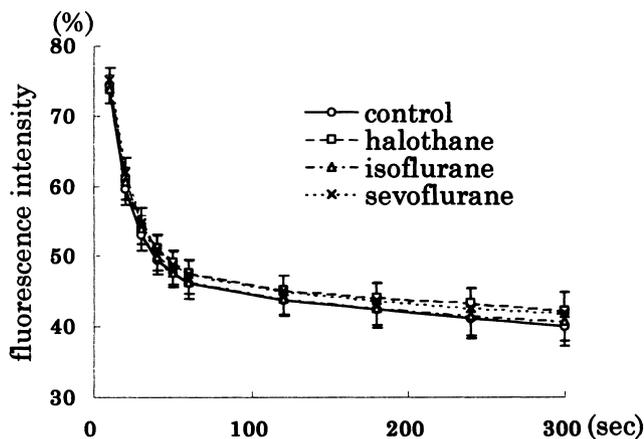


Fig. 4. Effects of inhalation anesthetic agents on fluorescence intensity at 360 nm after stimulation with thrombin (0.01 U/ml) in the presence of a 1 mM extracellular concentration of Mn²⁺. The ordinate represents fluorescence intensity expressed as percent of the initial fluorescence. The abscissa represents the time in seconds. The results from control incubations (open circles) and those with halothane (open squares), isoflurane (open triangles), and sevoflurane (crosses) are expressed as mean \pm SD (n = 7 per group).

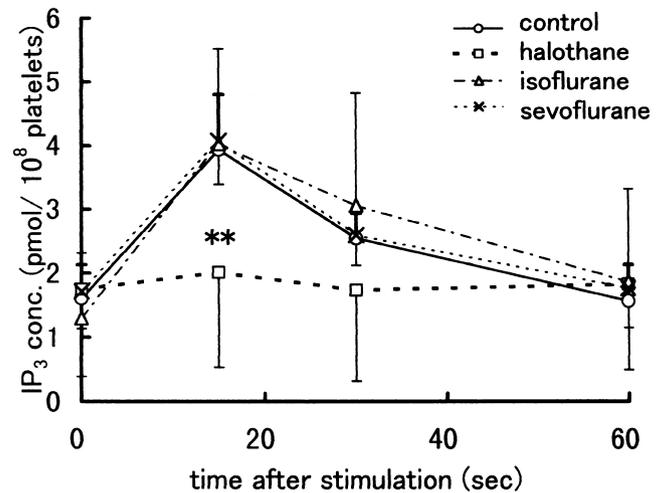


Fig. 5. Time course of inositol 1,4,5-triphosphate (IP₃) concentration in platelets, in the absence (control) and presence of 1 mM of the anesthetic agents, stimulated by 0.01 U/ml thrombin. The results from control incubations (open circles, n = 8) and those with halothane (open squares, n = 6), isoflurane (open triangles, n = 6), and sevoflurane (crosses, n = 7) are expressed as the mean \pm SD. The ordinate represents IP₃ concentration in pmol/10⁸ platelets. The abscissa represents time course in seconds. **P < 0.01 compared with the control value.

previous reports.^{8,9} These data suggest that an inhibitory effect on platelet aggregation can be observed clinically when the thrombin-induced and the ADP-induced platelet aggregation are suppressed, a situation not present with sevoflurane. In our study, isoflurane also did not inhibit thrombin-induced platelet aggregation, which is consistent with previous observations.^{4,8-10} Isoflurane suppressed neither production of IP₃ nor the increase in [Ca²⁺]_i in thrombin-induced platelet aggregation, producing no change in influx of calcium or release of calcium from the dense tubular system.

We demonstrated that a high clinical concentration of sevoflurane (1.5 mM corresponds to approximately 5.7%) does not affect thrombin-induced platelet aggregation, although it is reported to inhibit ADP-induced platelet aggregation. Clinically, platelet aggregation is thought to be induced by combined activation of multiple pathways involved in strong agonists and weak agonists separately. Therefore, any inhibition of platelet aggregation by sevoflurane through the ADP-involved pathway does not mean the net inhibition of clinical platelet aggregation.

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