Direct Inhibitory Mechanisms of Halothane on Human Platelet Aggregation

Shinji Kohro, M.D.,† Michiaki Yamakage, M.D., Ph.D.†

Background: Although halothane directly inhibits platelet aggregation, the mechanisms of this effect are still unknown. The current study aimed to clarify the inhibitory mechanisms of halothane on thrombin-induced human platelet aggregation by measuring (1) platelet surface glycoprotein Ib expression, (2) the concentration of intracellular free Ca²⁺ ([Ca²⁺]ᵢ) measured simultaneously with aggregation, (3) the concentration of intracellular inositol 1,4,5-triphosphate, and (4) the concentration of intracellular cyclic 3,5'-adenosine monophosphate ([cAMP]).

Methods: Washed platelet suspensions, obtained from healthy volunteers, were preincubated with halothane (0–2 ms) for 2 min and then exposed to 0.02 units/ml thrombin for 5 min. The glycoprotein Ib bound to fluorescent-labeled antibody was measured by fluorescence flow cytometry. [Ca²⁺]ᵢ was measured simultaneously with aggregation, in Fura-2 (Ca²⁺ indicator)-loaded platelets by use of a fluorometer. Inositol 1,4,5-triphosphate and [cAMP] were measured by radioimmunoassay.

Results: Halothane had no effect on glycoprotein Ib expression with or without thrombin. Halothane decreased the thrombin-stimulated [Ca²⁺]ᵢ, transient and inhibited platelet aggregation in a dose-dependent manner, both in the presence and in the absence of external Ca²⁺. Isoflurane had no apparent effect on either platelet aggregation or [Ca²⁺]ᵢ, in the absence of external Ca²⁺. Halothane increased the inositol 1,4,5-triphosphate induced by thrombin. Halothane moderately but significantly increased [cAMP], but the adenylate cyclase activator forskolin (which has the same inhibitory ability on aggregation as halothane) increased [cAMP] to a much greater extent than did halothane.

Conclusions: Halothane inhibits thrombin-induced human platelet aggregation by decreasing [Ca²⁺]ᵢ without inhibiting agonist-receptor binding; the inhibitory effect of halothane on [Ca²⁺]ᵢ might be mediated by a decrease in inositol 1,4,5-triphosphate and in part by an increase in [cAMP]. (Key words: Anesthetics, volatile; halothane. Blood, platelet; human. Ions, calcium. Receptors; glycoprotein Ib. Second messenger: cyclic 3,5'-adenosine monophosphate, inositol 1,4,5-triphosphate.)

HALOTHANE, in concentrations used clinically, directly inhibits platelet aggregation in vitro[1,2] and in vitro. However, because the aggregation pathways are complex and the platelet function per se is easily activated by many factors, the mechanism of this effect of halothane is still far from clear. Walter et al. demonstrated that halothane increased platelet adenylate cyclase activity with the inhibition of aggregation and concluded that the direct inhibitory effect of halothane could be mediated in part by a resultant increase in the concentration of intracellular cyclic 3,5'-adenosine monophosphate ([cAMP]). Cyclic 3,5'-adenosine monophosphate inhibits platelet aggregation and this inhibitory effect is thought to be mediated through two pathways: (1) a decrease in the concentration of intracellular free Ca²⁺ ([Ca²⁺]ᵢ)[4,5] and (2) an inhibition of myosin light chain kinase activity by the activation of CAMP-dependent protein kinase[6].

In other tissues, platelet processes are regulated by Ca²⁺[7,8]. Increasing the free Ca²⁺ concentration in its cytoplasm is a necessary and sufficient event in the cell's activation that involves a shape change and cytoytic processes leading to aggregation.[10,11] Conversely, many aggregating agonists (e.g., thrombin) increase the concentration of intracellular inositol 1,4,5-triphosphate ([IP₃]ᵢ) liberated from phosphatidylinositol 4,5-biphosphate[12,13] (fig. 1) and intracellular IP₃ is a major second messenger to regulate agonist-induced cytosolic Ca²⁺ responses in platelets through release of Ca²⁺ from dense tubular systems (DTS) [12,15]. There is some evidence in other tissues that halothane regulates the concentration of this second messenger at clinically used concentrations[16]. Furthermore, it has been reported that halothane reduces the ligand-binding affinity of the platelet-surface thromboxane A₂ receptor. Thus, to study the inhibitory mechanisms of HALOTHANE on platelet aggregation, we know the effect of halothane on the essential intracellular second messenger on the ligand-binding affinity.

The current study was conducted to determine whether halothane inhibits human platelet aggregation via the glycoprotein Ib (GpIb) receptor. To investigate the possibility of halothane to the receptor, halothane was administered simultaneously during exposure to thrombin. Under these conditions, the platelet aggregation decreased,

Methods and Material

Preparation of Platelet Suspension

This study was approved by the institutional review board of the University of Tokyo School of Medicine.

Reagents

The reagents used were as follows:

- Halothane (Aldrich Chemical, Milwaukee, WI)
- Fura-2 AM (Molecular Probes, Eugene, OR)
- Inositol 1,4,5-triphosphate (Sigma, St. Louis, MO)
- Cyclic 3,5'-adenosine monophosphate (Sigma)
- Myosin light chain kinase (New England Biolabs, Beverly, MA)
- Cyclic 3,5'-adenosine monophosphate-stimulating G protein (Sigma)

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EFFECTS OF HALOTHANE ON PLATELET AGGREGATION

Fig. 1. Signal transduction and intracellular Ca²⁺ regulation in platelets. When thrombin binds to glycoprotein Ib-thrombin receptor complex at the membrane surface, phospholipase C is activated via G protein, resulting in the rapid hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-triphosphate and 1,2-diacylglycerol, both of which act as second messengers. While 1,2-diacylglycerol activates Ca²⁺/phospholipid-dependent protein kinase at the membrane, inositol 1,4,5-triphosphate mobilizes Ca²⁺ from intracellular stores, especially dense tubular systems. Adenylate cyclase is activated by some prostaglandins, resulting in the hydrolysis of adenosine triphosphate to cyclic 3',5'-adenosine monophosphate. Cyclic 3',5'-adenosine monophosphate activates cyclic 3',5'-adenosine monophosphate-dependent protein kinase. Gs: stimulating G protein.

Inhalation of halothane on platelet aggregation, it is important to know the effect of halothane on the concentrations of essential intracellular second messengers and its effect on the ligand-binding affinity of aggregating agonists.

The current study was designed to clarify the inhibitory mechanisms of halothane on thrombin-induced human platelet aggregation by: (1) measuring the glycoprotein Ib (Gpib) expression on the platelet surface to investigate the possible interruption of the binding of thrombin to the receptor by halothane, (2) measuring [Ca²⁺], simultaneously with platelet aggregation during exposure to thrombin and halothane, (3) measuring [IP₃], during exposure to thrombin and halothane, and (4) measuring [CAMP], in the presence of halothane or forskolin.

Methods and Materials

Preparation of Platelet-rich Plasma and Washed Platelet Suspension
This study was approved by the Sapporo Medical University Ethical Committee on Human Research. After informed consent was obtained, blood (~15 ml) was drawn by antecubital venipuncture from 80 healthy volunteers who had not taken any medications for at least 14 days before the donation. The mean age of the volunteers was 26.8 yr (range 22–30 yr). Blood was collected into plastic syringes containing acid-citratedextrose solution. The acid-citrat-dextrose solution contained 85 mm sodium citrate, 70 mm citric acid, and 110 mm glucose. The ratio of blood to acid-citrate-dextrose solution was 1:3, and the final concentration of citrate in the whole blood was ~15 mm. Processing of blood samples was begun within 20 min of venipuncture.

Platelet-rich plasma and washed platelet suspension (WPS) were prepared by a modification of previously published methods. Briefly, the whole blood was centrifuged at 150g for 10 min and the upper two thirds of the platelet-rich plasma was collected. The WPS was prepared by centrifuging the platelet-rich plasma at 600g for 10 min and resuspending the pellet in a modified Tyrod’s solution. The Tyrod’s solution contained (in mm) 137 NaCl, 2.7 KCl, 0.23 MgCl₂, 0.4 NaH₂PO₄, 12 NaHCO₃, 5 glucose, and 10 N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid; pH level was adjusted to 7.4 with NaOH. Platelets were counted by a Coulter Counter STKS (Coulter, Hialeah, FL) for adjustment of the concentrations of platelet-rich plasma and WPS. All procedures were performed at room temperature (22–24°C). The experiments were started after preincubation with or without 1 mm Ca²⁺ for 1 min at 37°C and were completed within 3 h after venipuncture. Siliconized glassware and plastic tools were used throughout.

Measurement of the Platelet surface Glycoprotein Ib Expression
To investigate the interruption of thrombin-receptor binding by halothane, we measured platelet-surface nonbinding Gpib, a part of the thrombin receptor, by fluorescence flow cytometry. After the preincubation with 1 mm Ca²⁺ for 1 min, samples of WPS were incubated with halothane-containing solution (final concentration: 0, 0.5, 1.0, 1.5, or 2.0 mm) for 2 min and were then stimulated with or without 0.02 units/ml thrombin for 3 min at 37°C. Platelet preparations were stirred at 1,000 rpm during this protocol. Each incubation was stopped by the addition of an equal volume of 1% (vol/vol) paraformaldehyde. After the fixation, the paraformaldehyde-treated platelets were washed twice by centrifugation (600g for 5 min) and
resuspension in phosphate-buffered saline (composition in mm: 120 NaCl, 2.7 KCl, 8 Na,HPO4, and 2 KH2PO4; pH 7.4). The fixed platelets were then incubated with an isothiocyanate fluorescent monoclonal antibody (2′,7′-dichloro-4,5-diamino-2-subunit) with medium K 0.6×10^11 M. The labeled platelets were analyzed with an argon ion laser fluorescence flow cytometer (FACSscan +; Becton Dickinson, Mountain View, CA) using a 488-nm wavelength at 300 mW. Fluorescence was detected through a 530 ± 15 nm band-pass filter. The fluorescence histograms were analyzed on an attached computer (Consort 30, Becton Dickinson). The determinations were made in duplicate of the channel-weighted mean fluorescence intensity for 10^5 platelets, and the results are expressed as the fluorescence intensity ratio to the control without thrombin or halothane.

**Measurements of Intracellular Free Ca**2+ **Concentration and Aggregation**

The method of Pollock and Rink.24 was followed. Platelet-rich plasma was incubated with Fura 2/AM (5 μM) for 30 min at room temperature. After the Fura-2 loading, WPS was obtained as described earlier. Approximately 1 ml of the WPS (1.5×10^8 platelets per cuvette) was added to the stirred (1,000 rpm) cuvette attached to a fluorometer (Cetac 110; Jasco, Tokyo, Japan). After the WPS was preincubated with 1 mm CaCl2 or 50 μM EGTA for 1 min, the samples were incubated with various concentrations of halothane-containing solution (range of final concentration: 0–20 mM) for 2 min and then stimulated with 0.02 μM/ml thrombin for 3 min. To further investigate the effect of other anesthetics on the increase of [Ca**2+**], due to Ca**2+ release from intracellular stores (DTS), we performed additional experiments using isoflurane (range 0–2.0 mM) in the absence of external Ca**2+** as well.

The changes of [Ca**2+**], indicated by Fura-2 fluorescence, and of platelet aggregation were simultaneously measured with the CAF110 fluorometer. The platelet samples were illuminated alternately (128 Hz) at the excitation wavelengths of 340 and 380 nm. The intensities of 500-nm fluorescence induced by 340-nm excitation and by 380-nm excitation were monitored. At the end of each experiment, the cells were treated with 0.1% (vol/vol) Triton X-100 followed by the addition of 10 mm ethylene glycol-bis-(β-amino ethylether)-N,N,N′,N′-tetraacetic acid (EGTA) to obtain the maximum and minimum fluorescence, respectively. Absolute values of [Ca**2+**] were determined by the formula of Grynkiewicz et al.25 using a Fura-2-Ca**2+** dissociation constant of 224 nM. The background fluorescence, measured by adding 1 mm MnCl2, was negligible. Platelet aggregation was assessed using a spectrophotometer adjusted to 0% transmittance for control WPS and to 100% transmittance for distilled water.

**Measurement of Intracellular Inositol 1,4,5-tribisphosphate Concentration**

The technique of Uemura et al.26 was used to measure the intracellular IP3 concentration ([IP3]). Before preincubulation with 1 mm CaCl2 for 1 min, WPS (0.5 ml, 1×10^8 platelets/ml) was first incubated with halothane-containing medium (final concentration: 0, 1, or 2 mM) for 2 min and then stimulated with thrombin (0.02 units/ml). The reactions were terminated after 0, 5, 10, 15, 30, 60, or 120 s of thrombin stimulation by the addition of 0.2 volume of ice-cold 20% (vol/vol) perchloric acid solution. The samples were kept in an ice bath for 20 min and were then centrifuged at 2,000g for 5 min to obtain insoluble materials. The pH level of the supernatant was adjusted precisely to 7.5 with 10 n KOH and insoluble precipitates (primarily KClO4) 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 IP3 was measured using the Amersham IP3 assay system (code TRK 1000; Amersham Japan, Tokyo, Japan). This assay is based on competition between unlabeled IP3, in the sample and a fixed quantity of tritium-labeled IP3, for a limited number of high affinity binding sites on a specific IP3 binding protein.27 The determinations were made in duplicate and the results are expressed as pmol/5×10^8 platelets.

**Measurement of Intracellular Cyclic 3',5'-adenosine Monophosphate Concentration**

Washed platelet suspension (1 ml, 5×10^8 platelets/ml) was incubated with 1 mm CaCl2 for 1 min. Intracellular cAMP concentrations ([cAMP]) were measured under four conditions 6 min after addition of CaCl2: without exposure to halothane or thrombin; or with 5 min exposure to halothane (0, 1, and 2 mM, respectively) with additional exposure to 0.02 units/ml thrombin for the final 3 min; and with preincubation of forskolin with halothane (1 and 2 mM) on the platelet aggregation. Measurement of cAMP was performed by a modification of the method.28 At the end of the aggregation, platelet aggregation was assessed using a spectrophotometer adjusted to 0% transmittance for control WPS and to 100% transmittance for distilled water.

**Effects of Halothane**

Halothane in the presence of thrombin for the final 3 min, together with forskolin, activated thrombin-dependent aggregation. Thrombin was used at maximum concentrations and with preincubation of forskolin with halothane (1 and 2 mM) on the platelet aggregation. Measurement of cAMP was performed by a modification of the method.28 At the end of the aggregation, platelet aggregation was assessed using a spectrophotometer adjusted to 0% transmittance for control WPS and to 100% transmittance for distilled water.

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**Effects of Halothane**

Because of the possibility of hemolysis, the samples were incubated in cuvette using a 50-μl microfuge tube (Sarstedt, Ilmenau, Germany) and then measured with a spectrophotometer. The spectrophotometer was used with a cuvette with a 300-μm homogenizing chamber. The homogenization of the suspension was performed by using the 1,000g for 15 min; the supernatant was used for analysis. The suspension was prepared for analysis with a homogenizer (Vitrocom, Munich, Germany) and was measured with a spectrophotometer. The suspension was prepared for analysis with a homogenizer (Vitrocom, Munich, Germany) and was measured with a spectrophotometer. The suspension was prepared for analysis with a homogenizer (Vitrocom, Munich, Germany) and was measured with a spectrophotometer.
ml thrombin for the final 3 min. In experiments involving addition of forskolin, a potent adenylate cyclase activator, instead of halothane, concentrations of this drug were used that produced the same effect as halothane (1 and 2 mM) on the inhibition of the platelet aggregation.

Measurement of the [cAMP] of platelets was done by a modification of previously published methods. At the end of the incubations, the reactions were stopped by the addition of an equal volume of ice-cold 0.1 M HCl solution and the suspensions were immediately homogenized for 60 s at 4°C with a VC-50 ultrasonic homogenizer (Sonic and Material, Danbury, CT). The homogenized samples were then centrifuged at 3,000g for 15 min at 4°C and 100 µl of the supernatant was used for the measurement of the [cAMP]. We analyzed [cAMP] by a sensitive radioimmunoassay method (Yamasaki cyclic adenosine monophosphate assay system; Yamasa, Chiba, Japan). The determinations were made in duplicate and the results are expressed as pmol/10⁶ platelets.

Determination of Anesthetic Concentration

Because of the possibility that halothane or isoflurane bubbling per se affects platelet aggregation and its measurement, we introduced the anesthetic into the cuvette using a 50-µl microsyringe (MS-50P4AF, Ver Werk, Ilmenau, Germany) to deliver preconcentrated anesthetic in the Tyrode’s solution. To prevent anesthetic volatilization from WPS in the cuvette, we sealed the top of the cuvette with mineral oil and circulated the expected concentration of the anesthetic over the cuvette (~1/1 min). Anesthetic concentrations in the cuvette were analyzed in each experiment using a gas chromatograph (GC-12A; Shimadzu, Kyoto, Japan) equipped with a flame ionization detector (FTD-8; Shimadzu) and an integrator (Chromatopac C-R 3A; Shimadzu). Both halothane and isoflurane concentrations in the cuvette were constant during the experiment for more than 5 min (data not shown). In addition, we measured the halothane and isoflurane concentrations in WPS, which were bubbled extensively with known gas concentrations of the anesthetic, determined with a calibrated infrared anesthetic gas monitor (5250 RGM; Ohmeda, Madison, WI). After equilibration, the mean halothane concentrations in the solutions (1.0, 2.0, 3.0, and 4.0% halothane in the gas phase) were 0.8, 1.8, 2.5, and 3.5 mM, respectively; whereas the mean isoflurane concentrations in the solutions (2.0 and 4.0% isoflurane in the gas phase) were 1.2 and 2.2 mM, respectively. The concentration of the anesthetics in the solution had close linear correlation with the concentration of the agent in the gas phase.

Materials

The following drugs and chemicals were used in this study: halothane (ICI, Dighton, MA); isoflurane (Abbott Laboratories, North Chicago, IL); acetylmethyl ester of Fura-2 (Fura-2/AM; Dojindo, Kumamoto, Japan); Triton X-100 and sodium citrate (Katayama Chemical, Tokyo, Japan); citric acid (Kanto Chemical, Tokyo, Japan); EGTA, thrombin, paraformaldehyde, Triton X-100 and mineral oil (Sigma Chemical, St. Louis, MO); isothiocyanate fluorescein-labeled mouse monoclonal IgG antibody (SZ-2-FITC; Immunotech, Marseille, France) and phosphate-buffered saline (Life Technologies, Grand Island, NY).

Statistical Analysis

Data are expressed in scatter diagrams or as means ± SEM. The effects of halothane on the aggregation or on the [Ca²⁺]; of platelets were assessed by analysis of the variance of regression coefficients. Comparisons for all other data were analyzed using unpaired, twotailed t test or one-factor analysis of variance with Fisher’s a posteriori test. In all comparisons, P < 0.05 was considered significant.

Results

Effect of Halothane on Platelet-surface Glycoprotein Ib Expression

We measured the level of thrombin-receptor binding indirectly by estimating the presence of platelet-surface nonbinding GpIb with a fluorescence technique. The control fluorescence intensity of isothiocyanate-labeled platelets without thrombin or halothane was 51.7 ± 4.8/10⁶ platelets (n = 7). Table 1 shows the effects of thrombin and halothane on the GpIb expression. Halothane (0–2 mM) did not alter the binding between GpIb and the fluorescein-labeled antibody SZ-2-FITC in the absence of thrombin (P = 0.6). Thrombin (0.02 units/ml) significantly decreased labeled nonbinding GpIb by 25 ± 4% (P < 0.01). Halothane did not interfere with the binding between GpIb and thrombin in the range 0–2 mM (P = 0.7), but at a high concentration (5.5 mM), the anesthetic exhibited a small (21 ± 5%) but significant inhibitory effect on the GpIb-thrombin binding (P < 0.05, data not shown in table 1).

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Effects of Halothane and Isoflurane on Intracellular Free Ca\(^{2+}\) Concentration and Aggregation

Figure 2 shows the effects of halothane on [Ca\(^{2+}\)], and on the aggregation of thrombin-stimulated human platelets in the presence of 1 mM extracellular Ca\(^{2+}\). The resting [Ca\(^{2+}\)], was 106 ± 5 nm (n = 5), and halothane did not change [Ca\(^{2+}\)], when tested at concentrations up to 2 mM. [Ca\(^{2+}\)], was rapidly increased by 0.02 units/ml thrombin with a biphasic response: a first peak was seen at ~15 s and a second at ~2 min after exposure to thrombin. The [Ca\(^{2+}\)], at the first and second peaks were 520 ± 18 and 453 ± 22 nm, respectively (n = 5). The thrombin-stimulated platelets were rapidly aggregated, reaching a peak (~60% aggregating ratio) at ~3 min. We show representative effects of halothane at 0.9 and 1.8 mM on the platelet aggregation and [Ca\(^{2+}\)], in figure 2. In this case, both peaks of [Ca\(^{2+}\)], induced by thrombin were substantially decreased by halothane, but the time courses of changes in [Ca\(^{2+}\)], and of aggregation did not appear to change.

The relationships between halothane concentration (mM) and changes in (A) percentage of aggregation at 3 min after exposure to thrombin and (B) [Ca\(^{2+}\)], of the first peak in the presence of 1 mM external Ca\(^{2+}\) are shown in figure 3. Halothane (0–2 mM) significantly inhibited aggregation and caused a dose-dependent decrease in [Ca\(^{2+}\)]. Linear relationships were observed both between halothane concentrations and percent aggregation (r = −0.94, n = 50, P < 0.01) and between halothane concentrations and [Ca\(^{2+}\)], (r = −0.92, n = 50, P < 0.01).

We also investigated the effects of halothane and isoflurane on [Ca\(^{2+}\)], and the aggregation of thrombin-stimulated human platelets in the absence of external Ca\(^{2+}\) (solution containing 50 μM EGTA instead). As shown in figure 4A, the resting [Ca\(^{2+}\)], was 73 ± 5 nm (n = 5) and was significantly less than that obtained in the presence of 1 mM external Ca\(^{2+}\) (P < 0.05). Halothane did not change the resting [Ca\(^{2+}\)], in the concentration range of 0–2 mM. As observed in the presence of external Ca\(^{2+}\), [Ca\(^{2+}\)], was rapidly increased by exposure to 0.02 units/ml thrombin. However, in contrast to the observation with external Ca\(^{2+}\), this initial increase was followed by a substantial reduction and there was no second peak. The time course of the initial change in [Ca\(^{2+}\)], was similar to that seen in the presence of 1 mM external Ca\(^{2+}\) with a peak occurring ~15 s after exposure to thrombin. However, the peak [Ca\(^{2+}\)], (293 ± 14 nm) was significantly less than that of the first peak obtained with extracellular Ca\(^{2+}\) (P < 0.01, n = 5). Platelets smoothly aggregated, reaching their

![Fig. 2. Representative recordings of aggregation and intracellular free Ca\(^{2+}\) concentration of human platelets during exposure to thrombin (A) and halothane in the presence of 1 mM external Ca\(^{2+}\). Samples of washed platelet suspension were pretreated with halothane 2 min before exposure to 0.02 units/ml thrombin.](image)

Table 1. Glycoprotein Ib Expression in Thrombin-stimulated Platelets Pretreated with Halothane

<table>
<thead>
<tr>
<th>Thrombin (U/ml)</th>
<th>0</th>
<th>0.5</th>
<th>1.0</th>
<th>1.5</th>
<th>2.0</th>
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<tr>
<td>Halothane (mM)</td>
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<tr>
<td>0 (n = 7)</td>
<td>1.00</td>
<td>1.03 ± 0.06</td>
<td>0.97 ± 0.08</td>
<td>1.01 ± 0.05</td>
<td>1.05 ± 0.10</td>
</tr>
<tr>
<td>0.02 (n = 7)</td>
<td>0.75 ± 0.10*</td>
<td>0.77 ± 0.09*</td>
<td>0.72 ± 0.12*</td>
<td>0.78 ± 0.08*</td>
<td>0.77 ± 0.07*</td>
</tr>
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</table>

Values are mean fluorescence intensity ratios ± SEM, with 1.00 as the reference for platelets incubated without thrombin or halothane.

*P < 0.05 versus values without thrombin at the same concentrations of halothane.
EFFECTS OF HALOTHANE ON PLATELET AGGREGATION

A. Aggregation

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\begin{align*}
\text{Halothane conc. (mM)} & \quad r = -0.94 \\
& \quad P < 0.01 \\
& \quad n = 50
\end{align*}
\]

B. \([\text{Ca}^{2+}]_i\)

\[
\begin{align*}
\text{Halothane conc. (mM)} & \quad r = -0.92 \\
& \quad P < 0.01 \\
& \quad n = 50
\end{align*}
\]

Fig. 3. Relations between halothane concentration (mM) and changes in (A) percentage of aggregation and (B) intracellular free \(\text{Ca}^{2+}\) concentration ([\(\text{Ca}^{2+}\)]) in the presence of 1 mM external Ca\(^{2+}\). Regression line for the relation between halothane concentration (X; mM) and aggregation (Y; %) is: \(Y = 67 - 32X; r = -0.94\), and for the relation between halothane concentration (X; mM) and [\(\text{Ca}^{2+}\]) (Y; mM) is: \(Y = 523 - 192X; r = -0.92\). n = 50 (three or four points from one subject).

A. Halothane

B. Isoflurane

Fig. 4. Representative recordings of aggregation and intracellular free \(\text{Ca}^{2+}\) concentration of human platelets during exposure to thrombin (A) and (A) halothane or (B) isoflurane in the absence of external \(\text{Ca}^{2+}\) (with 50 \(\mu\)M EGTA). Samples of washed platelet suspension were pretreated with halothane or isoflurane 2 min before exposure to 0.02 units/ml thrombin.

Fig. 5. Effects of halothane on platelet aggregation and internal calcium concentration in the absence of external \(\text{Ca}^{2+}\). Aggregation and change in internal calcium concentration as a function of halothane concentration. Halothane (1.0 and 2.0 mM) decreased the induced changes in [\(\text{Ca}^{2+}\)] and inhibited aggregation in the absence of external \(\text{Ca}^{2+}\). To determine whether these effects were induced by other anesthetics as well, we performed additional experiments using isoflurane in the absence of external \(\text{Ca}^{2+}\) (fig. 4B). Isoflurane at respective peaks (~30% aggregating ratio) at ~3 min.
concentrations up to \( \sim 2 \text{ mm} (\sim 3.6\% \text{ in gas phase}) \) had no apparent effect on either platelet aggregation (inhibited by \( \sim 5 \pm 5\% \) at 1 mm and \( \sim 8 \pm 6\% \) at 2 mm, respectively) or the increase in \([\text{Ca}^{2+}]_i\) (inhibited by \( \sim 3 \pm 5\% \) at 1 mm and \( \sim 6 \pm 8\% \) at 2 mm, respectively) induced by thrombin (n = 5 at each point).

Figure 5 shows the relationships obtained in the absence of external \([\text{Ca}^{2+}] \) between halothane concentration and changes in (A) percentage of aggregation at 3 min and (B) peak \([\text{Ca}^{2+}]_i\). Halothane (0–2 mm) significantly inhibited aggregation and caused a dose-dependent decrease of \([\text{Ca}^{2+}]_i\). Linear relationships were observed both between halothane concentrations and percent aggregation (\( r = -0.95, n = 40, P < 0.01 \)) and between halothane concentrations and \([\text{Ca}^{2+}]_i\) (\( r = -0.93, n = 40, P < 0.01 \)). Halothane, at \( \sim 2 \text{ mm} \), almost completely inhibited thrombin-induced platelet aggregation and decreased the \([\text{Ca}^{2+}]_i\) to the resting value.

**Effect of Halothane on Intracellular \( \text{IP}_3 \) Concentration**

The time course and effects of 2 mm halothane on intracellular \( \text{IP}_3 \) concentrations ([\(\text{IP}_3\)]) in thrombin-stimulated human platelets are shown in figure 6. The \([\text{IP}_3]\) at time 0 was \(1.4 \pm 0.2 \text{ pmol/5 \times 10^6 \text{ platelets}} \) (n = 6) and did not change with the addition of halothane (1.5 \pm 0.3 pmol/5 \times 10^6 platelets at 1 mm and 1.2 \pm 0.2 pmol/5 \times 10^6 platelets at 2 mm halothane). Thrombin (0.2 units/ml) produced a rapid increase in the \([\text{IP}_3]\), reaching its maximum (5.9 \pm 0.9 pmol/5 \times 10^6 platelets) at 10 s after the stimulation. The rapid increase in \([\text{IP}_3]\), induced by thrombin was followed by a rapid and substantial decrease to a concentration of \( \sim 2 \text{ pmol/5 \times 10^6 \text{ platelets}} \). Halothane (2 mm) significantly inhibited the increase of \([\text{IP}_3]\), induced by thrombin at 5–15 s after thrombin stimulation without an apparent change in the time course of \([\text{IP}_3]\). The inset of figure 6 summarizes the effects of various concentrations of halothane (0, 1, and 2 mm) on the peak \([\text{IP}_3]\) at 10 s after thrombin stimulation. Halothane significantly inhibited in a dose-dependent manner the increase in \([\text{IP}_3]\) induced by thrombin.

**Effect of Halothane on Intracellular Cyclic 3,5-adenosine Monophosphate Concentration**

The effect of various concentrations of forskolin on thrombin (0.02 units/ml)-induced platelet aggregation and on intracellular cAMP concentrations ([cAMP]) were compared with those of halothane. As shown in figure 3A, halothane at 1 and 2 mm decreased thrombin-

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**Fig. 5.** Relations between halothane concentration (mm) and changes in (A) percentage of aggregation and (B) intracellular free \([\text{Ca}^{2+}]_i\) concentration ([\(\text{Ca}^{2+}]_i\)) in the absence of external \([\text{Ca}^{2+}]_i\) . Regression line for the relation between halothane concentration (X mm) and aggregation (Yi, %) is: Yi = 30 − 13X; r = 0.95, and for the relation between halothane concentration (X mm) and \([\text{Ca}^{2+}]_i\) (Yi, nM) is: Yi = 299 − 102X; r = 0.93, n = 40 (three or four points from one subject).

**Fig. 4.** Effects of halothane on phosphatase concentrations of platelets. The main figure shows the time-dependent changes in platelet induced by 0.02 units/ml of the effects of halothane (0, 1, and 2 mm) on phosphatase at 10 s after stimulation. Each data point represents mean ± SEM (n = 6, 10, and 10), respectively, compared to the control value.
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![Graph showing effects of halothane on intracellular inositol 1,4,5-triphosphate concentrations](image)

**Discussion**

**Ligand-receptor Interaction**

Physiologic platelet activation or inhibition involves interaction of an extracellular signaling molecule with the platelet surface via ligand-receptor coupling. Based on the interactions of thrombin with the glycoprotein component of GpIIb, GpIIb is proposed to be a functionally significant thrombin receptor (fig. 1). As reported previously, we found using flow cytometry with fluorescent labeling of the platelet surface proteins that thrombin (0.02 units/ml) significantly reduced the amount of antibody binding to GpIIb. Halothane did not affect the thrombin-induced decrease in antibody labeling of GpIIb, suggesting that halothane within the range of 0–2 mm (0–2.4% in gas phase) does not interfere with the interaction of thrombin with its receptor. This is in agreement with the general observation that volatile anesthetics, at clinically used concentrations, have little or no effect on agonist binding.

Although Hirakata et al. 18 induced platelet aggregation to ~36% and ~4% of control, respectively. Forskolin at 10 and 21 μM concentrations decreased thrombin (0.02 units/ml)-induced platelet aggregation to 35 ± 4% and to 6 ± 3%, respectively (n = 5). There was no significant difference between halothane and forskolin with respect to the extent of inhibition of platelet aggregation.

The effects of halothane and forskolin at these concentrations on [cAMP], of thrombin-stimulated human platelets are shown in figure 7. The [cAMP], in the resting state without thrombin was 1.6 ± 0.2 pmol/10^8 platelets (n = 6). After stimulation with 0.02 units/ml thrombin, [cAMP] increased by 0.32 pmol/10^8 platelets, but this change was not significant.

Halothane at 1 and 2 mm moderately but significantly increased [cAMP], by ~20% and ~40%, respectively. In contrast, forskolin at 10 and 21 μM caused significantly greater increases in [cAMP], (P < 0.05, n = 6) than did halothane at concentrations equieffective for inhibition of aggregation.

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demonstrated that halothane reduced the receptor-binding affinity of a potent aggregating agonist thromboxane A2 in human platelets, this effect likely required the extremely high concentration of halothane (14.4 mm ~16% in gas phase) that they used.

**Effects of Halothane on Activating Second Messengers: Ca2+ and Inositol 1,4,5-triphosphate**

As is established in other tissues, Ca2+ is thought to be an important regulator of platelet activation (fig. 1).3,32 Results from the current study also support a central role for Ca2+ in platelet aggregation (figs. 2 and 4). Our results are similar to those observed by others with platelet stimulation by thrombin,1,8,31 collagen,33 or adenosine diphosphate.34 An increase in [Ca2+]i activates myosin light chain kinase, which initiates platelet aggregation through increased phosphorylation of myosin light chain.10,51 In platelets, the increase in [Ca2+]i involves the release of Ca2+ from intracellular stores, especially DTS, and later Ca2+ influx from the extracellular space.35 As shown in figure 2, [Ca2+]i-induced by aggregating agonists (e.g., thrombin) usually induces a biphasic response. The first peak of [Ca2+]i is thought to result mainly from Ca2+ release from DTS and the second peak of [Ca2+]i is thought to reflect Ca2+ influx from the extracellular space.24,49 Our measurement of [Ca2+]i with or without external Ca2+ support this model of Ca2+ regulation and its role as a regulator of platelet activation.

Halothane decreased [Ca2+]i in parallel with its inhibition of platelet aggregation in the presence of external Ca2+ (figs. 2 and 3). Our data support the hypothesis that halothane inhibits platelet aggregation mainly by suppressing the increase of [Ca2+]i, induced by aggregating agonists. Halothane also decreased the peak [Ca2+]i in the absence of external Ca2+. There is some evidence that Ca2+ release from intracellular stores may be more essential for platelet activation than is Ca2+ influx from the extracellular space.20,21,46 Therefore, it is possible that the inhibition of the increase in [Ca2+]i due to intracellular stores is the main mechanism by which halothane inhibits platelet aggregation. Interestingly, isoflurane had little effect on the [Ca2+]i in the absence of external Ca2+. This observation parallels the clinical observation that halothane is more effective than other anesthetics in inhibiting platelet aggregation at clinically used concentrations.5

Hossein and Evers37 reported that in clonal (GH2) pituitary cells halothane increased the resting [Ca2+]i, through Ca2+ release from IP3-gated intracellular stores.

Although we did not observe any increase in [Ca2+]i by halothane in the absence of external Ca2+, our data do not exclude the possibility that the decrease in thrombin-induced increase of [Ca2+]i, by halothane results partly from the depletion of the Ca2+ stores by preincubation with the anesthetic.58,59 Our data do indicate that halothane inhibits Ca2+ influx through the platelet membrane, but the role of Ca2+ influx from the extracellular space in the aggregation response of platelets to activation remains uncertain.60 Further studies are required to clarify the significance of this effect.

Stimulation of the thrombin receptor activates the G protein-linked phospholipase C, resulting in hydrolysis of phosphatidylinositol 4,5-bisphosphate to the two potent stimulatory second messengers IP3 and 1,2-diacetylglycerol (fig. 1).12,13,14 While 1,2-diacetylglycerol activates Ca2+/phospholipid-dependent protein kinase at the membrane,53 IP3 mobilizes Ca2+ from intracellular organella DTSs.14,54 Because in platelets IP, is the primary regulator for Ca2+ release from intracellular stores and because the time course of the increase in [IP3], induced by thrombin was very similar to that of the change in [Ca2+]i (figs. 2 and 4), we suggest that IP3 is an important determinant of [Ca2+]i, during agonist stimulation. Furthermore, there is evidence that IP3 can directly enhance Ca2+ influx from the extracellular space.26,27 Therefore, inhibition of increases in [IP3], by halothane might itself account for the observed effects on both [Ca2+]i and aggregation.

Our results are in general agreement with studies in a variety of cell types, in which halothane treatment has been associated with inhibition of the increase in [Ca2+]i, mediated by IP3,6,13,22,23 These studies have demonstrated that halothane alters Ca2+ homeostasis, an action that underlies the in vivo effect of the anesthetic. However, Smart et al.61 and Rooney et al.62 showed that halothane induced IP3 formation in SH-SY5Y human neuroblastoma cells and turkey erythrocytes, respectively. Rooney and colleagues also showed activation of phospholipase C activity by halothane.63 These apparent discrepancies may result from the differences of cell types and species and/or the selective effects of halothane on certain receptors, G-proteins, or phospholipase C isoforms.

**Effects of Halothane on Inhibitory Second Messengers: Cyclic 3',5'-adenosine Monophosphate**

Cyclic 3',5'-adenosine monophosphate is another mechanism known to regulate [Ca2+]i. In this study,
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halothane moderately but significantly increased the [cAMP] of human platelets. This result is consistent with the previous suggestion by Walter et al.4 that the impairment of platelet aggregation observed with halothane-induced activation of platelet adenylate cyclase, resulting in a higher cAMP concentration in the cytosol. Several effects of cAMP on platelet Ca2+ metabolism have been established, including stimulation of Ca2+ efflux and stimulation of Ca2+ uptake into DTS, both of which result in a decrease in [Ca2+]i. In addition, cAMP-dependent protein phosphorylation has been reported to attenuate IP3-mediated Ca2+ release from DTS. However, more recent data obtained with a pure preparation of the catalytic subunit of protein kinase C suggest that there is no increase in intracellular Ca2+ in response to that of cAMP.5,6 In our study, agonist-induced IP3, which is not IP3, can activate a cAMP-dependent protein kinase, which phosphorylates myosin light chain kinase. A cAMP-dependent protein kinase phosphorylates myosin light chain kinase and inhibits its activity in vitro via a mechanism independent of Ca2+ (fig. 1).

In this study, forskolin was used to investigate the effect of cAMP in the inhibition by halothane of platelet aggregation. When forskolin was used at concentrations that inhibited platelet aggregation to the same extent as halothane (1 and 2 mm), the [cAMP] increased to a much greater extent than with halothane (fig. 7). Thus, the inhibitory effect of halothane on platelet aggregation appears to involve additional mechanisms other than an increase in [cAMP].

In summary, halothane in clinical concentrations inhibits thrombin-induced human platelet aggregation mainly by decreasing [Ca2+], without inhibiting agonist-receptor binding. The inhibitory effect of halothane on [Ca2+]i may be mediated both by a decrease in [IP3] and by an increase in [cAMP]. We suggest that, while cAMP both suppresses [Ca2+]i and acts via a Ca2+-independent pathway to inhibit platelet aggregation, IP3 is a crucial regulator of aggregation in platelets and that the inhibitory action of halothane on aggregation is explicable by its effects on IP3.

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