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## Direct Inhibitory Mechanisms of Halothane on Human Platelet Aggregation

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**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  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_i$ ) 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 ( $[\text{cAMP}]_i$ ).

**Methods:** Washed platelet suspensions, obtained from healthy volunteers, were preincubated with halothane (0-2 mM) for 2 min and then exposed to 0.02 units/ml thrombin for 3 min. The glycoprotein Ib bound to fluorescein-labeled antibody was measured by fluorescence flow cytometry.  $[\text{Ca}^{2+}]_i$  was measured, simultaneously with aggregation, in Fura-2 ( $\text{Ca}^{2+}$  indicator)-loaded platelets by use of a fluorometer. Inositol 1,4,5-triphosphate and  $[\text{cAMP}]_i$  were measured by radioimmunoassay.

**Results:** Halothane had no effect on glycoprotein Ib expression with or without thrombin. Halothane decreased the thrombin-stimulated  $[\text{Ca}^{2+}]_i$  transient and inhibited platelet aggregation in a dose-dependent manner, both in the presence and in the absence of external  $\text{Ca}^{2+}$ . Isoflurane had no apparent effect on either platelet aggregation or  $[\text{Ca}^{2+}]_i$  in the absence of external  $\text{Ca}^{2+}$ . Halothane inhibited the increase in inositol 1,4,5-triphosphate induced by thrombin. Halothane moderately but significantly increased  $[\text{cAMP}]_i$ , but the adenylate cyclase activator forskolin (which has the same inhibitory ability on aggregation as halothane) increased  $[\text{cAMP}]_i$  to a much greater extent than did halothane.

**Conclusions:** Halothane inhibits thrombin-induced human platelet aggregation by decreasing  $[\text{Ca}^{2+}]_i$  without inhibiting agonist-receptor binding; the inhibitory effect of halothane on  $[\text{Ca}^{2+}]_i$  might be mediated by a decrease in inositol 1,4,5-

triphosphate and in part by an increase in  $[\text{cAMP}]_i$ . (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 vivo*<sup>1,2</sup> and *in vitro*.<sup>1,3,4</sup> 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.<sup>5</sup> Walter *et al.*<sup>4</sup> 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 ( $[\text{cAMP}]_i$ ). 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  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_i$ ),<sup>6,7</sup> and (2) an inhibition of myosin light chain kinase activity by the activation of cAMP-dependent protein kinase<sup>8</sup> (fig. 1).

As in other tissues, platelet processes are regulated by  $\text{Ca}^{2+}$  (fig. 1).<sup>9</sup> Increasing the free  $\text{Ca}^{2+}$  concentration in its cytoplasm is a necessary and sufficient event in the cell's activation that involves a shape change and exocytotic processes leading to aggregation.<sup>10,11</sup> Conversely, many aggregating agonists (e.g., thrombin) increase the concentration of intracellular inositol 1,4,5-triphosphate ( $[\text{IP}_3]_i$ ) liberated from phosphatidylinositol 4,5-bisphosphate<sup>12,13</sup> (fig. 1) and intracellular  $\text{IP}_3$  is a major second messenger to regulate agonist-induced cytosolic  $\text{Ca}^{2+}$  responses in platelets through release of  $\text{Ca}^{2+}$  from dense tubular systems (DTS).<sup>14,15</sup> There is some evidence in other tissues that halothane regulates the concentration of this second messenger at clinically used concentrations.<sup>16,17</sup> Furthermore, it has been reported that halothane reduces the ligand-binding affinity of the platelet-surface thromboxane  $\text{A}_2$  receptor.<sup>18</sup> Thus, to study the inhibitory mechanisms of

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## EFFECTS OF HALOTHANE

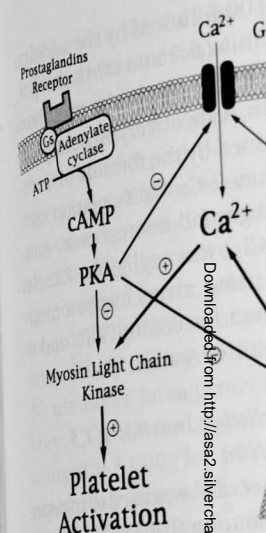


Fig. 1. Signal transduction and platelet activation. When thrombin binds to the thrombin receptor complex at the membrane, it is activated via G protein, resulting in the activation of phospholipase C- $\beta$ . Phospholipase C- $\beta$  cleaves phosphatidylinositol 4,5-bisphosphate and 1,2-diacylglycerol into inositol 1,4,5-triphosphate and diacylglycerol. Inositol 1,4,5-triphosphate mobilizes  $\text{Ca}^{2+}$  from dense tubular systems, resulting in an increase in intracellular free  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_i$ ). Diacylglycerol activates protein kinase C (PKC). PKC also activates phospholipase C- $\beta$ . PKC also activates myosin light chain kinase (MLCK). MLCK phosphorylates myosin, leading to platelet activation. cAMP also inhibits PKC and MLCK.

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 designed to clarify the inhibitory mechanisms of halothane on human platelet aggregation by measuring (1) glycoprotein Ib (GpIb) expression, (2) the concentration of intracellular free  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_i$ ) simultaneously with aggregation, (3) the concentration of inositol 1,4,5-triphosphate ( $[\text{IP}_3]_i$ ) during exposure to thrombin, and (4) measuring the concentration of cyclic 3',5'-adenosine monophosphate ( $[\text{cAMP}]_i$ ) during exposure to halothane or forskolin.

## Methods and Materials

### Preparation of Platelet Suspension

This study was approved by the University Ethical Committee.

## EFFECTS OF HALOTHANE ON PLATELET AGGREGATION

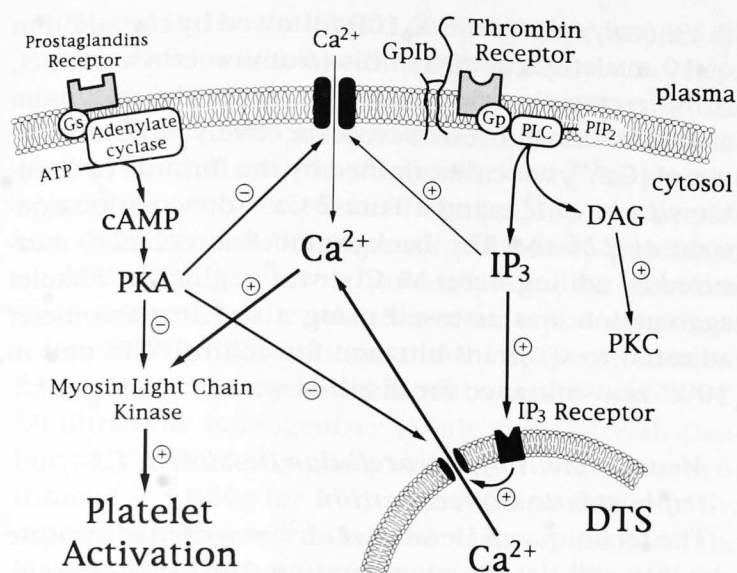


Fig. 1. Signal transduction and intracellular  $\text{Ca}^{2+}$  regulation in platelets. When thrombin binds to glycoprotein Ib-thrombin receptor complex at the membrane surface, phospholipase C is activated via G protein, resulting in the rapid hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-triphosphate and 1,2-diacylglycerol, both of which act as second messengers. While 1,2-diacylglycerol activates  $\text{Ca}^{2+}$ /phospholipid-dependent protein kinase at the membrane, inositol 1,4,5-triphosphate mobilizes  $\text{Ca}^{2+}$  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.

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  $[\text{Ca}^{2+}]_i$  simultaneously with platelet aggregation during exposure to thrombin and halothane, (3) measuring  $[\text{IP}_3]_i$  during exposure to thrombin and halothane, and (4) measuring  $[\text{cAMP}]_i$  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 ( $\sim 15$  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-citrate-dextrose solution. The acid-citrate-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 17:3, and the final concentration of citrate in the whole blood was  $\sim 13$  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.<sup>19,20</sup> 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 Tyrode's solution. The Tyrode's solution contained (in mM) 140 NaCl, 2.7 KCl, 0.23  $\text{MgCl}_2$ , 0.4  $\text{NaH}_2\text{PO}_4$ , 12  $\text{NaHCO}_3$ , 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 ( $22\text{--}24^\circ\text{C}$ ). The experiments were started after preincubation with or without 1 mM  $\text{Ca}^{2+}$  for 1 min at  $37^\circ\text{C}$  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,<sup>21</sup> by fluorescence flow cytometry.<sup>22</sup> After the preincubation with 1 mM  $\text{Ca}^{2+}$  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  $37^\circ\text{C}$ . 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  $\text{Na}_2\text{HPO}_4$ , and 2  $\text{KH}_2\text{PO}_4$ ; pH 7.4). The fixed platelets were then incubated with an isothiocyanate fluorescein-labeled mouse monoclonal immune globulin G antibody (SZ-2-FITC, 2  $\mu\text{g}/5 \times 10^6$  platelets) for 30 min at room temperature, washed twice, and resuspended in phosphate-buffered saline at a final concentration of  $5 \times 10^7$  platelets/milliliter. The GpIb antibody used in this study (SZ-2) binds specifically to the human platelet GpIb  $\alpha$ -subunit with mean  $K_d = 6.6 \times 10^{-10}$  M.<sup>23</sup> The labeled platelets were analyzed with an argon ion laser fluorescence flow cytometer (FACScan 440; Becton Dickinson, Mountain View, CA) using a 488-nm wavelength at 300 mW. Fluorescence was detected through a  $530 \pm 15$  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  $10^4$  platelets, and the results are expressed as the fluorescein intensity ratio to the control without thrombin or halothane.

#### Measurements of Intracellular Free $\text{Ca}^{2+}$ Concentration and Aggregation

The method of Pollock and Rink<sup>24</sup> was followed. Platelet-rich plasma was incubated with Fura-2/AM (5  $\mu\text{M}$ ) for 30 min at room temperature. After the Fura-2 loading, WPS was obtained as described earlier. Approximately 1 ml of the WPS ( $\sim 1.5 \times 10^7$  platelets per cuvette) was added to the stirred (1,000 rpm) cuvette attachment of a fluorometer (CAF-110; JASCO, Tokyo, Japan). After the WPS was preincubated with 1 mM  $\text{Ca}^{2+}$  or 50  $\mu\text{M}$  EGTA for 1 min, the samples were incubated with various concentrations of halothane-containing solution (range of final concentration: 0–2.0 mM) for 2 min and then stimulated with 0.02 units/ml thrombin for 3 min. To further investigate the effect of other anesthetics on the increase of  $[\text{Ca}^{2+}]_i$  due to  $\text{Ca}^{2+}$  release from intracellular stores (DTS), we performed additional experiments using isoflurane (range 0–2.0 mM) in the absence of external  $\text{Ca}^{2+}$  as well.

The changes of  $[\text{Ca}^{2+}]_i$ , 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-( $\beta$ -amino ethylether)-N, N, N'-tetraacetic acid (EGTA) to obtain the maximum and minimum fluorescence, respectively. Absolute values of  $[\text{Ca}^{2+}]_i$  were determined by the formula of Grynkiewicz *et al.*<sup>25</sup> using a Fura-2- $\text{Ca}^{2+}$  dissociation constant of 224 nM. The background fluorescence, measured by adding 1 mM  $\text{MnCl}_2$ , 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-triphosphate Concentration

The technique of Uemura *et al.*<sup>26</sup> was used to measure the intracellular  $\text{IP}_3$  concentration ( $[\text{IP}_3]_i$ ). After preincubation with 1 mM  $\text{Ca}^{2+}$  for 1 min, WPS (0.5 ml,  $1 \times 10^9$  platelets/ml) was first incubated with halothane-containing solution (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 15 min to remove insoluble materials. The pH level of the supernatant was adjusted precisely to 7.5 with 10 N KOH and insoluble precipitates (primarily  $\text{KClO}_4$ ) were removed by centrifugation at 2,000g for 10 min. The resultant supernatant was lyophilized and stored at  $-20^\circ\text{C}$ . The lyophilized samples were dissolved in 100  $\mu\text{l}$  distilled water, and the amount of  $\text{IP}_3$  was measured using the Amersham  $\text{IP}_3$  assay system (code TRK 1000; Amersham Japan, Tokyo, Japan). This assay is based on competition between unlabeled  $\text{IP}_3$  in the sample and a fixed quantity of tritium-labeled  $\text{IP}_3$  for a limited number of high affinity binding sites on a specific  $\text{IP}_3$  binding protein.<sup>27</sup> The determinations were made in duplicate and the results are expressed as pmol/ $5 \times 10^8$  platelets.

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

Washed platelet suspension (1 ml,  $5 \times 10^8$  platelets/milliliter) was incubated with 1 mM  $\text{Ca}^{2+}$  for 1 min. Intracellular cAMP concentrations ( $[\text{cAMP}]_i$ ) were measured under four conditions 6 min after addition of  $\text{Ca}^{2+}$ : 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. After the addition of forskolin, an activator, instead of halothane, the drug were used that produced aggregation (1 and 2 mM) on the platelets. Measurement of  $[\text{cAMP}]_i$  was done by a modification of the methods.<sup>28</sup> At the end of the experiment, the platelets were stopped by the addition of ice-cold 0.1 N HCl solution and immediately homogenized in 50 ultrasonic homogenizer (Biosonik, CT). The homogenized platelets were centrifuged at 3,000g for 15 min and the supernatant was used for  $[\text{cAMP}]_i$  analysis. We analyzed  $[\text{cAMP}]_i$  by a radioimmunoassay method (Yamaoka, phosphate assay system; Yamaoka, determinations were made in duplicate and are expressed as pmol/ $10^8$  platelets).

#### Determination of Anesthetic Concentration

Because of the possibility of bubbling *per se* affects the measurement, we introduced a 50-ml microcuvette using a 50-ml microcuvette (Werk, Ilmenau, Germany) in the Tycode's anesthetic volatilization from the top of the cuvette with the expected concentration of the cuvette ( $\sim 1$  l/min). Anesthetics in the cuvette were analyzed in a gas chromatograph (GC-12A, Shimadzu) equipped with a flame ionization detector (FID) and an integrator (Shimadzu). Both halothane and isoflurane in the cuvette were constant for more than 5 min (data not shown). We measured the halothane and isoflurane concentrations of the cuvette using a calibrated infrared analyzer (RGM; Ohmeda, Madison, WI). The mean halothane concentrations were 2.0, 3.0, and 4.0% halothane, and the mean isoflurane concentrations were 0.8, 1.8, 2.5, and 3.5% isoflurane in the cuvette, respectively. The concentrations of the anesthetics in the cuvette were expressed as mmHg, respectively. The concentrations of the anesthetics in the cuvette were expressed as mmHg, respectively.

## EFFECTS OF HALOTHANE ON PLATELET AGGREGATION

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  $[cAMP]_i$  of platelets was done by a modification of previously published methods.<sup>28</sup> At the end of the incubations, the reactions were stopped by the addition of an equal volume of ice-cold 0.1 N 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  $\mu$ l of the supernatant was used for the measurement of the  $[cAMP]_i$ . We analyzed  $[cAMP]_i$  by a sensitive radioimmunoassay method (Yamasa cyclic adenosine monophosphate assay system; Yamasa, Chiba, Japan). The determinations were made in duplicate and the results are expressed as pmol/ $10^8$  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- $\mu$ l microsyringe (MS-50P4AF; Verwerk, 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 ( $\sim 1$  l/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); acetoxymethyl 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, Marseilles, France) and phosphate-buffered saline (Life Technologies, Grand Island, NY).

#### Statistical Analysis

Data are expressed in scatter diagrams or as means  $\pm$  SEM. The effects of halothane on the aggregation or on the  $[Ca^{2+}]_i$  of platelets were assessed by analysis of the variance of regression coefficients. Comparisons for all of the other data were analyzed with unpaired, two-tailed *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  $51.7 \pm 4.8/10^4$  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  $25 \pm 4\%$  ( $P < 0.01$ ). 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 ( $21 \pm 5\%$ ) but significant inhibitory effect on the GpIb-thrombin binding ( $P < 0.05$ , data not shown in table 1).



### Effects of Halothane and Isoflurane on Intracellular Free $\text{Ca}^{2+}$ Concentration and Aggregation

Figure 2 shows the effects of halothane on  $[\text{Ca}^{2+}]_i$  and on the aggregation of thrombin-stimulated human platelets in the presence of 1 mM extracellular  $\text{Ca}^{2+}$ . The resting  $[\text{Ca}^{2+}]_i$  was  $106 \pm 6$  nM ( $n = 5$ ), and halothane did not change  $[\text{Ca}^{2+}]_i$  when tested at concentrations up to 2 mM.  $[\text{Ca}^{2+}]_i$  was rapidly increased by 0.02 units/ml thrombin with a biphasic response: a first peak was seen at  $\sim 15$  s and a second at  $\sim 2$  min after exposure to thrombin. The  $[\text{Ca}^{2+}]_i$  at the first and second peaks were  $520 \pm 18$  and  $453 \pm 22$  nM, respectively ( $n = 5$ ). The thrombin-stimulated platelets were rapidly aggregated, reaching a peak ( $\sim 60\%$  aggregating ratio) at  $\sim 3$  min. We show representative effects of halothane at 0.9 and 1.8 mM on the platelet aggregation and  $[\text{Ca}^{2+}]_i$  in figure 2. In this case, both peaks of  $[\text{Ca}^{2+}]_i$  induced by thrombin were substantially decreased by halothane, but the time courses of changes in  $[\text{Ca}^{2+}]_i$  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)  $[\text{Ca}^{2+}]_i$  of the first peak in the presence of 1 mM external  $\text{Ca}^{2+}$  are shown in figure 3. Halothane (0–2 mM) significantly inhibited aggregation and caused a dose-dependent decrease in  $[\text{Ca}^{2+}]_i$ . Linear relationships were observed both between halothane concentrations and percent aggregation ( $r = -0.94$ ,  $n = 50$ ,  $P < 0.01$ ) and between halothane concentrations and  $[\text{Ca}^{2+}]_i$  ( $r = -0.92$ ,  $n = 50$ ,  $P < 0.01$ ).

We also investigated the effects of halothane and isoflurane on  $[\text{Ca}^{2+}]_i$  and the aggregation of thrombin-stimulated human platelets in the absence of external  $\text{Ca}^{2+}$  (solution containing 50  $\mu\text{M}$  EGTA instead). As shown in figure 4A, the resting  $[\text{Ca}^{2+}]_i$  was  $73 \pm 5$  nM ( $n = 5$ ) and was significantly less than that obtained in the presence of 1 mM external  $\text{Ca}^{2+}$  ( $P < 0.05$ ). Halothane did not change the resting  $[\text{Ca}^{2+}]_i$  in the concen-

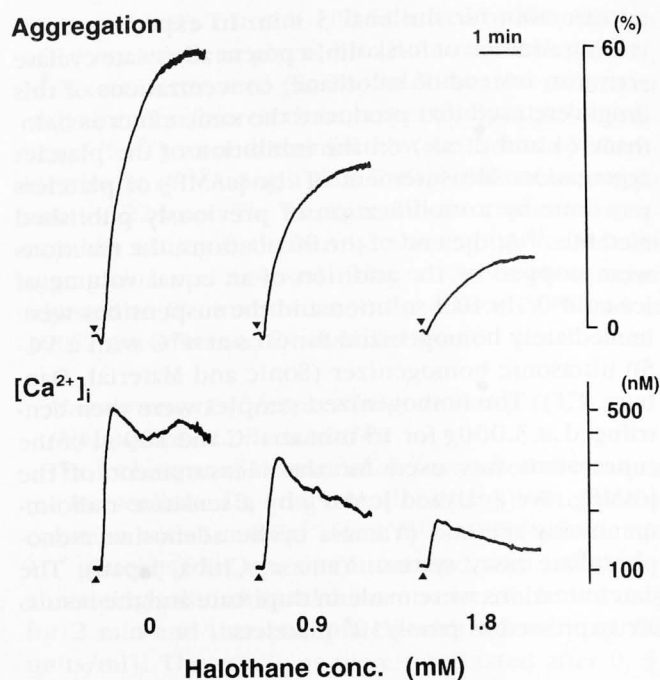


Fig. 2. Representative recordings of aggregation and intracellular free  $\text{Ca}^{2+}$  concentration of human platelets during exposure to thrombin (▲) and halothane in the presence of 1 mM external  $\text{Ca}^{2+}$ . Samples of washed platelet suspension were pretreated with halothane 2 min before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

tration range of 0–2 mM. As observed in the presence of external  $\text{Ca}^{2+}$ ,  $[\text{Ca}^{2+}]_i$  was rapidly increased by exposure to 0.02 units/ml thrombin. However, in contrast to the observation with external  $\text{Ca}^{2+}$ , this initial increase was followed by a substantial reduction and there was no second peak. The time course of the initial change in  $[\text{Ca}^{2+}]_i$  was similar to that seen in the presence of 1 mM external  $\text{Ca}^{2+}$  with a peak occurring  $\sim 15$  s after exposure to thrombin. However, the peak  $[\text{Ca}^{2+}]_i$  ( $293 \pm 14$  nM) was significantly less than that of the first peak obtained with extracellular  $\text{Ca}^{2+}$  ( $P < 0.01$ ,  $n = 5$ ). Platelets smoothly aggregated, reaching their

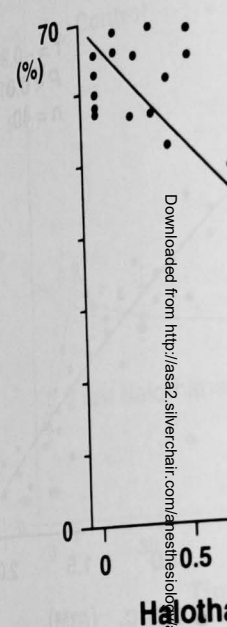
Table 1. Glycoprotein Ib Expression in Thrombin-stimulated Platelets Pretreated with Halothane

Thrombin (U/ml)	Halothane (mM)				
	0	0.5	1.0	1.5	2.0
0 ( $n = 7$ )	1.00	$1.03 \pm 0.06$	$0.97 \pm 0.08$	$1.01 \pm 0.05$	$1.05 \pm 0.10$
0.02 ( $n = 7$ )	$0.75 \pm 0.10^*$	$0.77 \pm 0.09^*$	$0.72 \pm 0.12^*$	$0.78 \pm 0.08^*$	$0.77 \pm 0.07^*$

Values are mean fluorescence intensity ratios  $\pm$  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.

### A. Aggregation



### B. $[\text{Ca}^{2+}]_i$

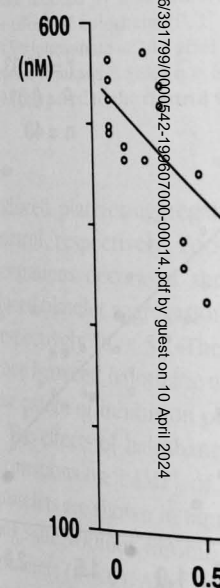


Fig. 3. Relations between halothane concentration and changes in (A) percentage of aggregation at 3 min after exposure to thrombin and (B)  $[\text{Ca}^{2+}]_i$  of the first peak in the presence of 1 mM external  $\text{Ca}^{2+}$ . Regression line for (A)  $r = -0.94$ ,  $n = 50$  (three or four points)

## EFFECTS OF HALOTHANE ON PLATELET AGGREGATION

## A. Aggregation

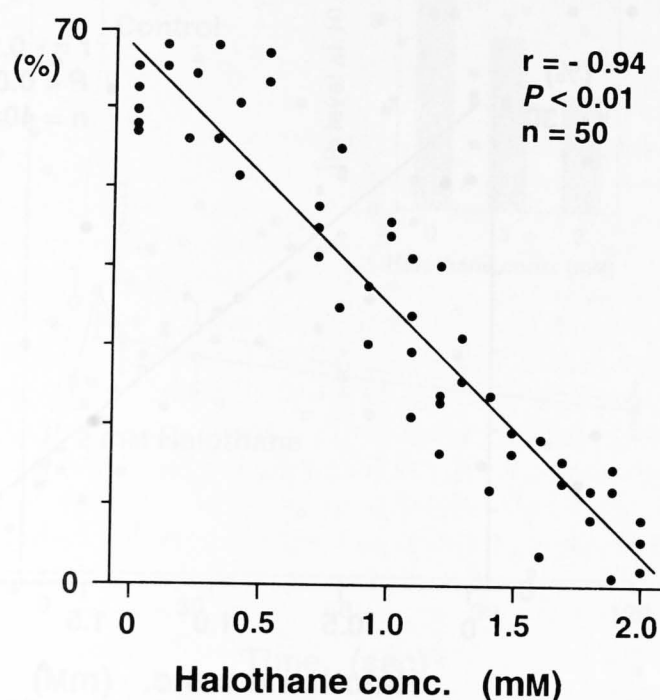
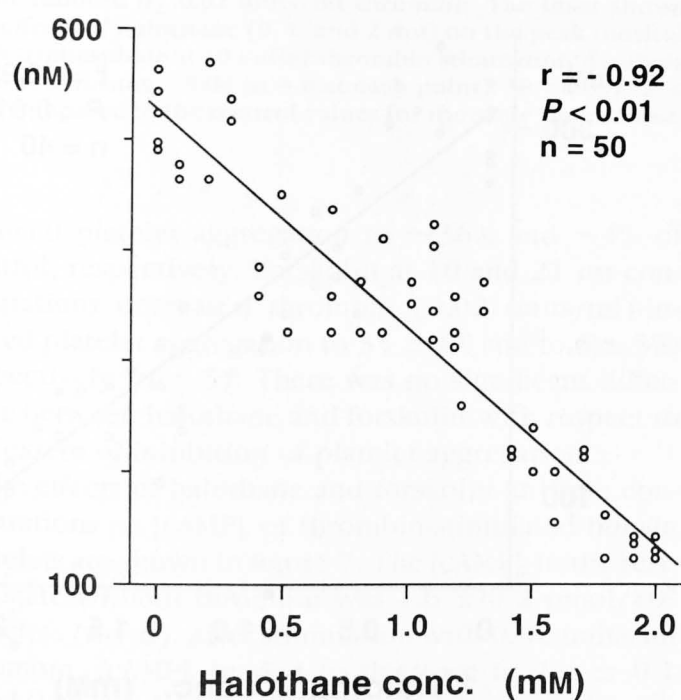
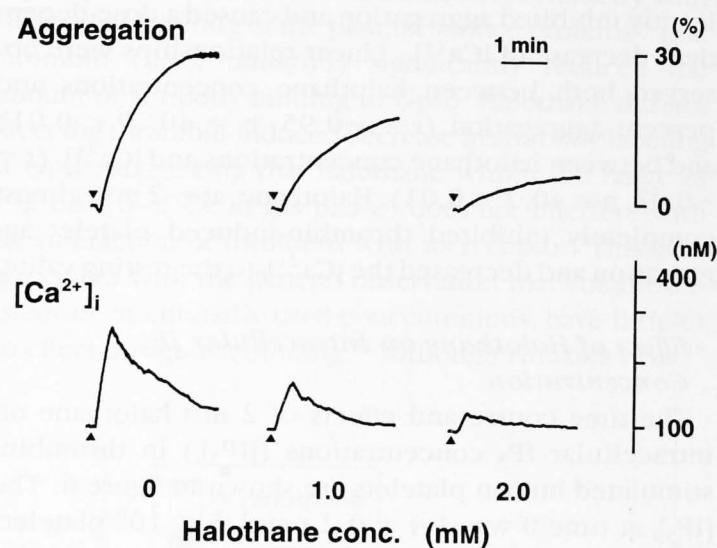
B.  $[Ca^{2+}]_i$ 

Fig. 3. Relations between halothane concentration (mM) and changes in (A) percentage of aggregation and (B) intracellular free  $Ca^{2+}$  concentration ( $[Ca^{2+}]_i$ ) 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  $[Ca^{2+}]_i$  (Y; nM) is:  $Y = 523 - 192X$ ;  $r = -0.92$ .  $n = 50$  (three or four points from one subject).

respective peaks ( $\sim 30\%$  aggregating ratio) at  $\sim 3$  min. Halothane (1.0 and 2.0 mM) decreased the induced changes in  $[Ca^{2+}]_i$  and inhibited aggregation in the absence of external  $Ca^{2+}$ . To determine whether these effects were induced by other anesthetics as well, we performed additional experiments using isoflurane in the absence of external  $Ca^{2+}$  (fig. 4B). Isoflurane at

## A. Halothane



## B. Isoflurane

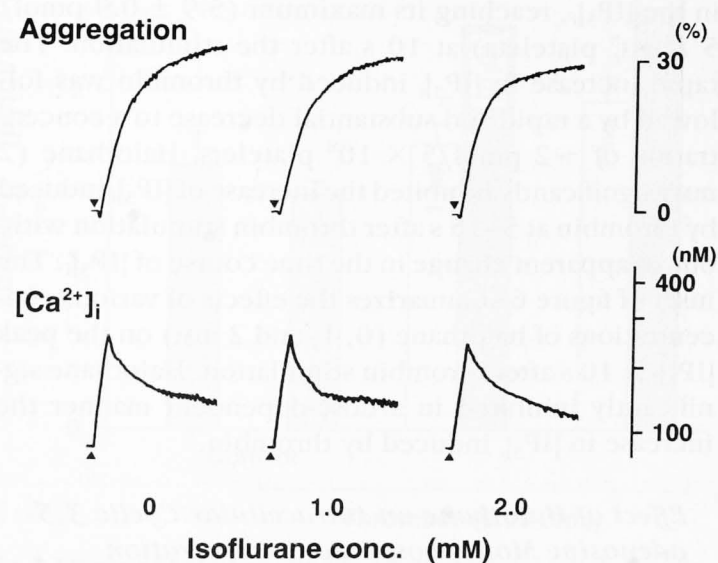


Fig. 4. Representative recordings of aggregation and intracellular free  $Ca^{2+}$  concentration of human platelets during exposure to thrombin ( $\Delta$ ) and (A) halothane or (B) isoflurane in the absence of external  $Ca^{2+}$  (with  $50 \mu M$  EGTA). Samples of washed platelet suspension were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

concentrations up to  $\sim 2$  mM ( $\sim 3.6\%$  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  $\sim 2$  mM, almost completely inhibited thrombin-induced platelet aggregation and decreased the  $[\text{Ca}^{2+}]_i$  to the resting value.

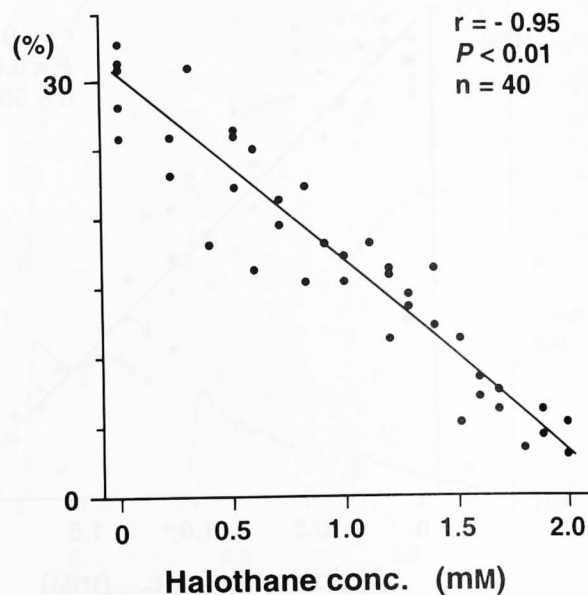
#### Effect of Halothane on Intracellular $\text{IP}_3$ Concentration

The time course and effects of 2 mM halothane on intracellular  $\text{IP}_3$  concentrations ( $[\text{IP}_3]_i$ ) in thrombin-stimulated human platelets are shown in figure 6. The  $[\text{IP}_3]_i$  at time 0 was  $1.4 \pm 0.2$  pmol/ $5 \times 10^8$  platelets ( $n = 6$ ) and did not change with the addition of halothane ( $1.3 \pm 0.3$  pmol/ $5 \times 10^8$  platelets at 1 mM and  $1.2 \pm 0.2$  pmol/ $5 \times 10^8$  platelets at 2 mM halothane). Thrombin (0.02 units/ml) produced a rapid increase in the  $[\text{IP}_3]_i$ , reaching its maximum ( $5.9 \pm 0.9$  pmol/ $5 \times 10^8$  platelets) at 10 s after the stimulation. The rapid increase in  $[\text{IP}_3]_i$  induced by thrombin was followed by a rapid and substantial decrease to a concentration of  $\sim 2$  pmol/ $5 \times 10^8$  platelets. Halothane (2 mM) significantly inhibited the increase of  $[\text{IP}_3]_i$  induced by thrombin at 5–15 s after thrombin stimulation without an apparent change in the time course of  $[\text{IP}_3]_i$ . The inset of figure 6 summarizes the effects of various concentrations of halothane (0, 1, and 2 mM) on the peak  $[\text{IP}_3]_i$  at 10 s after thrombin stimulation. Halothane significantly inhibited in a dose-dependent manner the increase in  $[\text{IP}_3]_i$  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 ( $[\text{cAMP}]_i$ ) were compared with those of halothane. As shown in figure 3A, halothane at 1 and 2 mM decreased thrombin-

### A. Aggregation



### B. $[\text{Ca}^{2+}]_i$

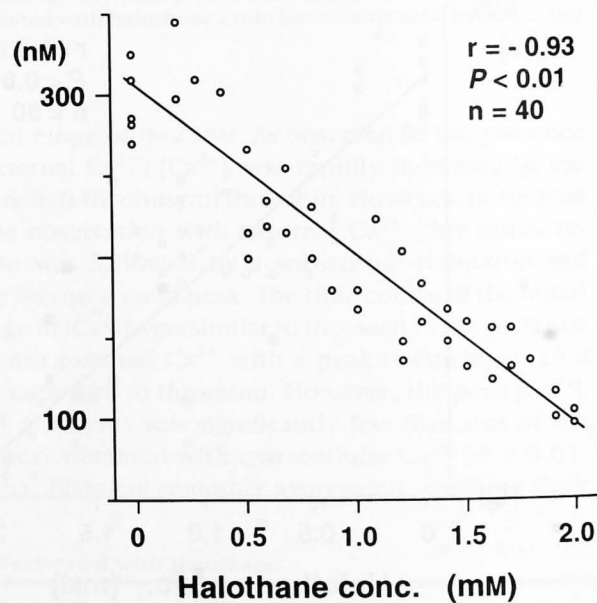


Fig. 5. Relations between halothane concentration (mM) and changes in (A) percentage of aggregation and (B) intracellular free  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) in the absence of external  $\text{Ca}^{2+}$ . Regression line for the relation between halothane concentration (X;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

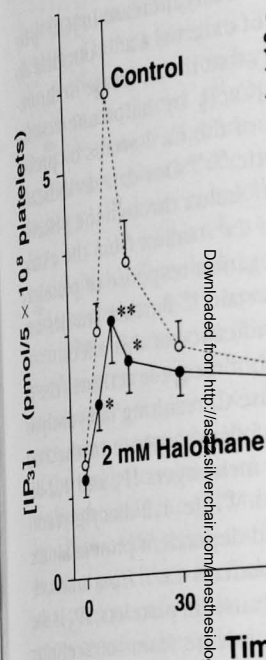


Fig. 6. Effects of halothane on intracellular  $\text{IP}_3$  concentrations of platelets. The main figure shows the time-dependent change in  $[\text{IP}_3]_i$  induced by 0.02 units/ml thrombin in the presence of halothane (0, 1, and 2 mM). The inset shows the effects of halothane (0, 1, and 2 mM) on the peak  $[\text{IP}_3]_i$  at 10 s after thrombin stimulation. Values represent means  $\pm$  SEM ( $n = 6$ ). \* $P < 0.05$ , \*\* $P < 0.01$  compared to the control value.

induced platelet aggregation. In the control, respectively. Forskolin at concentrations decreased the thrombin-induced platelet aggregation (Fig. 3A). The effects of halothane on the extent of inhibition of aggregation were compared with those of forskolin. The effects of halothane on  $[\text{cAMP}]_i$  of platelets are shown in figure 3B. After stimulation with thrombin,  $[\text{cAMP}]_i$  tended to increase in platelets ( $n = 6$ ). After stimulation with thrombin,  $[\text{cAMP}]_i$  tended to increase in platelets, but the increase was significantly inhibited by halothane at 1 and 2 mM. Halothane at 1 and 2 mM increased  $[\text{cAMP}]_i$  by  $\sim 20\%$ . In contrast, forskolin at 1 mM caused a significantly greater increase in  $[\text{cAMP}]_i$  than did halothane at concentrations that inhibited aggregation.



## EFFECTS OF HALOTHANE ON PLATELET AGGREGATION

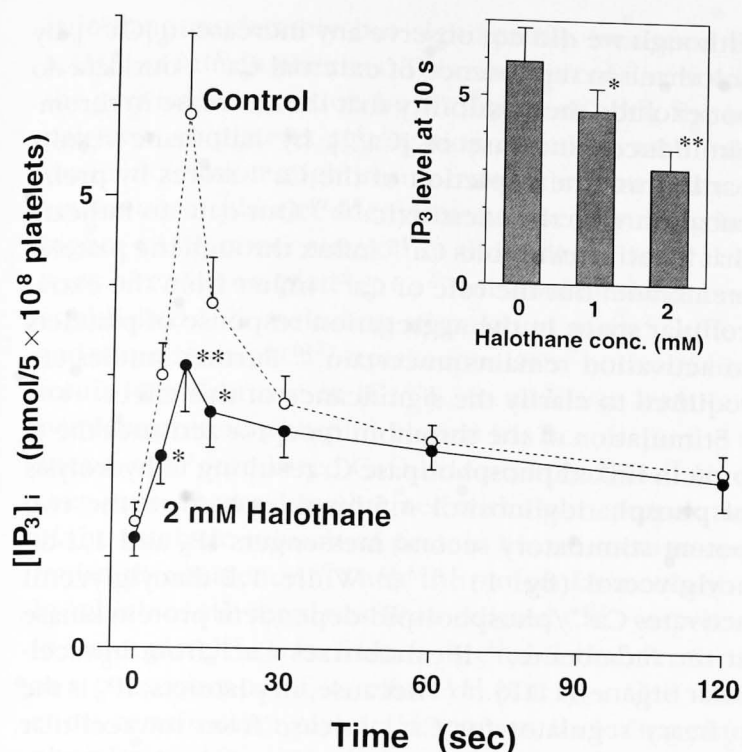


Fig. 6. Effects of halothane on intracellular inositol 1,4,5-triphosphate concentrations of thrombin-stimulated human platelets. The main figure shows the effect of 2 mM halothane on the time-dependent changes of the inositol 1,4,5-triphosphate induced by 0.02 units/ml thrombin. The inset shows the effects of halothane (0, 1, and 2 mM) on the peak inositol 1,4,5-triphosphate at 10 s after thrombin stimulation. Symbols represent means  $\pm$  SEM ( $n = 6$  at each point). \* $P < 0.05$ , \*\* $P < 0.01$  compared to the control values for the same time course.

induced platelet aggregation to  $\sim 36\%$  and  $\sim 4\%$  of control, respectively. Forskolin at 10 and 21  $\mu\text{M}$  concentrations decreased thrombin (0.02 units/ml)-induced platelet aggregation to  $35 \pm 4\%$  and to  $6 \pm 3\%$ , 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  $[\text{cAMP}]_i$  of thrombin-stimulated human platelets are shown in figure 7. The  $[\text{cAMP}]_i$  in the resting state without thrombin was  $1.6 \pm 0.2$  pmol/ $10^8$  platelets ( $n = 6$ ). After stimulation with 0.02 units/ml thrombin,  $[\text{cAMP}]_i$  tended to decrease to  $1.4 \pm 0.1$  pmol/ $10^8$  platelets, but this change was not significant. Halothane at 1 and 2 mM moderately but significantly increased  $[\text{cAMP}]_i$  by  $\sim 20\%$  and  $\sim 40\%$ , respectively. In contrast, forskolin at 10 and 21  $\mu\text{M}$  caused significantly greater increases in  $[\text{cAMP}]_i$  ( $P < 0.05$ ,  $n = 6$ ) than did halothane at concentrations equieffective for inhibition of aggregation.

## 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 GpIb,<sup>29</sup> GpIb is proposed to be a functionally significant thrombin receptor (fig. 1).<sup>21</sup> As reported previously,<sup>30</sup> we found using flow cytometry with fluorescent labeling of the platelet surface proteins<sup>22</sup> that thrombin (0.02 units/ml) significantly reduced the amount of antibody binding to GpIb. Halothane did not affect the thrombin-induced decrease in antibody labeling of GpIb, 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.<sup>31</sup> Although Hirakata *et al.*<sup>18</sup>

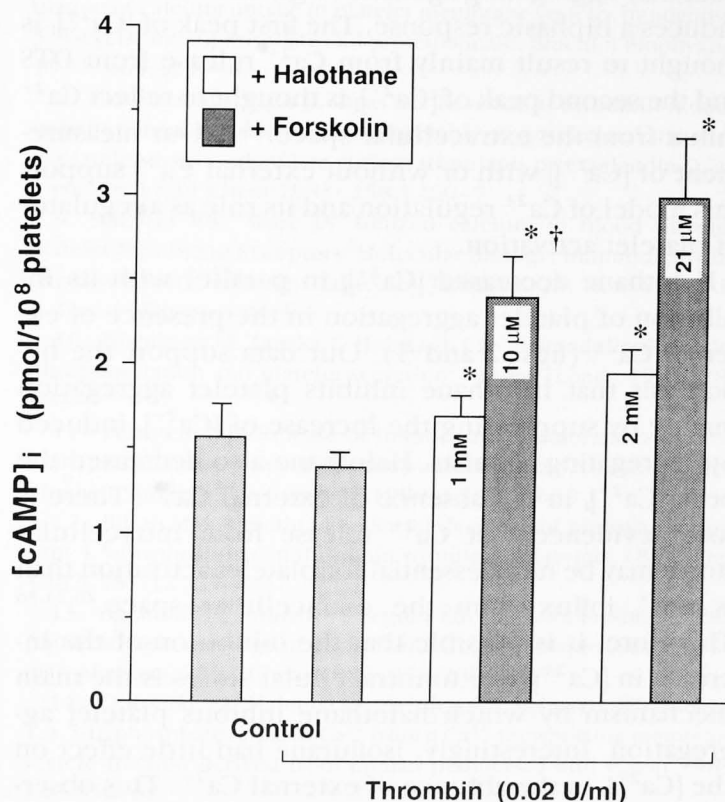


Fig. 7. Effects of halothane and forskolin on intracellular cyclic 3',5'-adenosine monophosphate concentrations of thrombin-stimulated human platelets. Symbols represent means  $\pm$  SEM ( $n = 6$  at each point). \* $P < 0.05$  compared to the control value with 0.02 units/ml thrombin. † $P < 0.05$  for comparisons between 1 mM halothane and 10  $\mu\text{M}$  forskolin and between 2 mM halothane and 21  $\mu\text{M}$  forskolin in the presence of thrombin.



demonstrated that halothane reduced the receptor-binding affinity of a potent aggregating agonist thromboxane  $A_2$  in human platelets, this effect likely required the extremely high concentration of halothane (14 mM  $\approx$  16% in gas phase) that they used.

*Effects of Halothane on Activating Second Messengers:  $Ca^{2+}$  and Inositol 1,4,5-triphosphate*

As is established in other tissues,  $Ca^{2+}$  is thought to be an important regulator of platelet activation (fig. 1).<sup>9,32</sup> Results from the current study also support a central role for  $Ca^{2+}$  in platelet aggregation (figs. 2 and 4). Our results are similar to those observed by others with platelet stimulation by thrombin,<sup>19,33</sup> collagen,<sup>33</sup> or adenosine diphosphate.<sup>34</sup> An increase in  $[Ca^{2+}]_i$  activates myosin light chain kinase, which initiates platelet aggregation through increased phosphorylation of myosin light chain.<sup>10,34</sup> In platelets, the increase in  $[Ca^{2+}]_i$  involves the release of  $Ca^{2+}$  from intracellular stores, especially DTS, and later  $Ca^{2+}$  influx from the extracellular space.<sup>35</sup> As shown in figure 2,  $[Ca^{2+}]_i$  induced by aggregating agonists (e.g., thrombin) usually induces a biphasic response. The first peak of  $[Ca^{2+}]_i$  is thought to result mainly from  $Ca^{2+}$  release from DTS and the second peak of  $[Ca^{2+}]_i$  is thought to reflect  $Ca^{2+}$  influx from the extracellular space.<sup>24,36</sup> Our measurement of  $[Ca^{2+}]_i$  with or without external  $Ca^{2+}$  support this model of  $Ca^{2+}$  regulation and its role as a regulator of platelet activation.

Halothane decreased  $[Ca^{2+}]_i$  in parallel with its inhibition of platelet aggregation in the presence of external  $Ca^{2+}$  (figs. 2 and 3). Our data support the hypothesis that halothane inhibits platelet aggregation mainly by suppressing the increase of  $[Ca^{2+}]_i$  induced by aggregating agonists. Halothane also decreased the peak  $[Ca^{2+}]_i$  in the absence of external  $Ca^{2+}$ . There is some evidence that  $Ca^{2+}$  release from intracellular stores may be more essential for platelet activation than is  $Ca^{2+}$  influx from the extracellular space.<sup>20,24,36</sup> Therefore, it is possible that the inhibition of the increase in  $[Ca^{2+}]_i$  due to intracellular stores is the main mechanism by which halothane inhibits platelet aggregation. Interestingly, isoflurane had little effect on the  $[Ca^{2+}]_i$  in the absence of external  $Ca^{2+}$ . This observation parallels the clinical observation that halothane is more effective than other anesthetics at inhibiting platelet aggregation at clinically used concentrations.<sup>5</sup>

Hossain and Evers<sup>37</sup> reported that in clonal (GH<sub>3</sub>) pituitary cells halothane increased the resting  $[Ca^{2+}]_i$  through  $Ca^{2+}$  release from IP<sub>3</sub>-gated intracellular stores.

Although we did not observe any increase in  $[Ca^{2+}]_i$  by halothane in the absence of external  $Ca^{2+}$ , our data do not exclude the possibility that the decrease in thrombin-induced increase of  $[Ca^{2+}]_i$  by halothane results partly from the depletion of the  $Ca^{2+}$  stores by preincubation with the anesthetic.<sup>38,39</sup> Our data do indicate that halothane inhibits  $Ca^{2+}$  influx through the platelet membrane, but the role of  $Ca^{2+}$  influx from the extracellular space in the aggregation response of platelets to activation remains uncertain.<sup>40</sup> 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 IP<sub>3</sub> and 1,2-diacylglycerol (fig. 1).<sup>12,13,41</sup> While 1,2-diacylglycerol activates  $Ca^{2+}$ /phospholipid-dependent protein kinase at the membrane,<sup>41</sup> IP<sub>3</sub> mobilizes  $Ca^{2+}$  from intracellular organella DTS.<sup>14,15</sup> Because, in platelets, IP<sub>3</sub> is the primary regulator for  $Ca^{2+}$  release from intracellular stores and because the time course of the increase in  $[IP_3]_i$  induced by thrombin was very similar to that of the change in  $[Ca^{2+}]_i$  (figs. 2 and 4), we suggest that IP<sub>3</sub> is an important determinant of  $[Ca^{2+}]_i$  during agonist stimulation. Furthermore, there is evidence that IP<sub>3</sub> can directly enhance  $Ca^{2+}$  influx from the extracellular space.<sup>26</sup> Therefore, inhibition of increases in  $[IP_3]_i$  by halothane might in itself account for the observed effects on both  $[Ca^{2+}]_i$  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  $[Ca^{2+}]_i$  mediated by IP<sub>3</sub>.<sup>16,42,43</sup> These studies have demonstrated that halothane alters  $Ca^{2+}$  homeostasis, an action that underlies the *in vivo* effect of the anesthetic. However, Smart *et al.*<sup>17</sup> and Rooney *et al.*<sup>44</sup> showed that halothane induced IP<sub>3</sub> formation in SH-SY5Y human neuroblastoma cells and turkey erythrocytes, respectively. Rooney and colleagues also showed activation of phospholipase C activity by halothane.<sup>44</sup> 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 isozymes.

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

Cyclic 3',5'-adenosine monophosphate is another mechanism known to regulate  $[Ca^{2+}]_i$ . In this study,

halothane moderately but significantly decreased  $[Ca^{2+}]_i$  of human platelets. This result is in contrast to the previous suggestion by others that the impairment of platelet aggregation by halothane incubation might result from the inhibition of platelet adenylylation and subsequent activation of platelet adenylylation. Higher cAMP concentration is known to inhibit the effects of cAMP on platelet activation, including the stimulation of  $Ca^{2+}$  uptake and stimulation of  $Ca^{2+}$  release. This result in a decrease in  $[Ca^{2+}]_i$  is independent of protein phosphorylation and attenuates IP<sub>3</sub>-mediated  $Ca^{2+}$  release. However, more recent data obtained from intact platelets contradict this evidence. Thus, by which cAMP regulates  $Ca^{2+}$  release in intact platelets is far from clear as a consequence of its effects on certain other platelet proteins, including phosphorylation of myosin light chain kinase and inhibition of the mechanism independent of cAMP.

In this study, forskolin, a potent activator of cAMP, inhibited platelet aggregation. When forskolin was added to platelets that had been incubated with halothane (1 and 2 mM), the inhibitory effect of halothane on aggregation appeared to be partially reversed. This suggests that the inhibitory effect of halothane on aggregation is mediated by a decrease in cAMP. In summary, halothane inhibits thrombin-induced platelet aggregation mainly by decreasing  $[Ca^{2+}]_i$ . The inhibition of  $[Ca^{2+}]_i$  may be mediated by an increase in cAMP, which both suppresses  $[Ca^{2+}]_i$  and inhibits the pathway to inhibit platelet aggregation. The inhibitory action of halothane on aggregation is mediated by its effects on IP<sub>3</sub>.

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## EFFECTS OF HALOTHANE ON PLATELET AGGREGATION

halothane moderately but significantly increased the  $[cAMP]_i$  of human platelets. This result is consistent with the previous suggestion by Walter *et al.*<sup>4</sup> that the impairment of platelet aggregation observed with halothane incubation might result from halothane-induced activation of platelet adenylate cyclase, resulting in a higher cAMP concentration in the cytosol. Several effects of cAMP on platelet  $Ca^{2+}$  metabolism have been established,<sup>45,46</sup> including stimulation of  $Ca^{2+}$  efflux<sup>6</sup> and stimulation of  $Ca^{2+}$  uptake into DTS,<sup>7</sup> both of which result in a decrease in  $[Ca^{2+}]_i$ . In addition, cAMP-dependent protein phosphorylation has been reported to attenuate  $IP_3$ -mediated  $Ca^{2+}$  release from DTS.<sup>47</sup> However, more recent data obtained with a pure preparation of the catalytic subunit of protein kinase seem to contradict this evidence.<sup>48</sup> Thus, the molecular mechanism by which cAMP regulates  $IP_3$ -mediated  $Ca^{2+}$  responses in intact platelets is far from fully established. Primarily as a consequence of its effect on  $[Ca^{2+}]_i$ , cAMP influences certain other platelet responses, including phosphorylation of myosin light chain kinase<sup>10</sup>; cAMP-dependent protein kinase phosphorylates myosin light chain kinase and inhibits its activity *in vitro*<sup>49</sup> via a mechanism independent of  $Ca^{2+}$  (fig. 1).

In this study, forskolin was used to investigate the role 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  $[cAMP]_i$  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 of  $[cAMP]_i$ .

In summary, halothane in clinical concentrations inhibits thrombin-induced human platelet aggregation mainly by decreasing  $[Ca^{2+}]_i$  without inhibiting agonist-receptor binding. The inhibitory effect of halothane on  $[Ca^{2+}]_i$  may be mediated both by a decrease in  $[IP_3]_i$  and by an increase in  $[cAMP]_i$ . We suggest that, while cAMP both suppresses  $[Ca^{2+}]_i$  and acts *via* a  $Ca^{2+}$ -independent pathway to inhibit platelet aggregation,  $IP_3$  is a crucial regulator of aggregation in platelets and that the inhibitory action of halothane on aggregation is explicable by its effects on  $IP_3$ .

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