Background: Ketamine has been shown to suppress platelet aggregation, but its mechanisms of action have not been defined. The purpose of the current study is to clarify the effects of ketamine on human platelet aggregation and to elucidate the underlying mechanisms of its action.

Methods: Platelet aggregation was measured using an eight-channel aggregometer, and cytosolic free calcium concentration was measured in Fura2/AM–loaded platelets using a fluorometer. Inositol 1,4,5-triphosphate (IP3) was measured with use of a commercially available IP3 assay kit. To estimate thromboxane A2 (TXA2) receptor binding affinity and expression, Scatchard analysis was performed using [3H]S145, a specific TXA2 receptor antagonist. TXA2 agonist binding assay was also performed. The membrane-bound guanosine 5′-triphosphatase activity was determined using [γ-32P]guanosine triphosphate by liquid scintillation analyzer.

Results: Ketamine (500 μM) suppressed aggregation induced by adenosine diphosphate (0.5 μM), epinephrine (1 μM), (-)-9,11-epithia-11,12-methano-TXA2 (STA2) (0.5 μM), and thrombin (0.02 U/ml) to 39.1 ± 10.9, 46.3 ± 4.3, 2.0 ± 1.9, and 16.8, and 86.6 ± 1.4% of zero-control, respectively. Ketamine (250 μM–1 mM) also suppressed thrombin- and STA2–induced cytosolic free calcium concentration increase dose dependently. Although ketamine (2 μM) had no effect on TXA2 receptor expression and its binding affinity, it (1 μM) suppressed intracellular peak IP3 concentrations induced by thrombin and STA2 from 6.60 ± 1.82 and 4.3 ± 2.41 to 2.41 ± 0.98 and 1.90 ± 0.86 pmol/109 platelets, respectively, and it suppressed guanosine triphosphate hydrolysis induced by thrombin (0.02 units/ml) and STA2 (0.5 μM) to 50.3 ± 3.2 and 67.5 ± 5.5% versus zero-control, respectively.

Conclusion: Ketamine inhibits human platelet aggregation possibly by suppressed IP3 formation and subsequent suppression of cytosolic free calcium concentration. The site of action of ketamine is neither TXA2 nor thrombin binding sites but possibly receptor-coupled mechanisms, including G-protein.

BECAUSE platelet function may directly affect hemostasis during perioperative periods, information regarding the effects of anesthetics on platelet function may be needed for clinical practice of anesthesia, especially for a case complicated with bleeding disorders. The inhibitory effects of halothane and sevoflurane on human platelet aggregation were shown in in vitro1–5 and in vitro6 studies. We have also shown the antiaggregatory effect of propofol in vitro using human platelets.7 An antiaggregatory effect of ketamine has been reported by Atkinson et al.8 They demonstrated that ketamine inhibits human platelet aggregation induced by adenosine diphosphate (ADP), epinephrine, collagen, and arachidonic acid. Because it reduced thromboxane B2 formation in their experiment, they ascribed the antiaggregatory effect of this anesthetic to the suppressed thromboxane synthetase activity. To our knowledge, the effect of ketamine on the function of thromboxane A2 (TXA2) in platelets has not been examined in the literature.

In the current study, we observed that ketamine, although at concentrations higher than clinically relevant ones, obviously suppressed thrombin- and TXA2–induced aggregation. This finding indicated that the suppressed formation of TXA2 is not the sole function of this anesthetic on platelets. In the vascular smooth muscle of rabbit mesenteric artery, Kanmura et al.9 demonstrated that ketamine inhibits agonist-induced Ca2+ release through its inhibitory action on agonist-induced synthesis of inositol triphosphate (IP3). It suggested to us the possibility of the inhibitory effects of ketamine on platelet second messenger systems10 including intracellular IP3 formation and membrane-bound guanosine 5′-triphosphatase (GTPase) activity. Therefore, we tested this possibility, attempting to clarify the mechanisms underlying the antiaggregatory effect of ketamine.

Materials and Methods
Platelet Preparation
The study was conducted in accordance with the human research standards of the Kyoto University Ethics Committee (Kyoto, Japan). Venous blood was obtained from nine healthy volunteers who had taken no drugs
known to affect platelet aggregation for at least 2 weeks. After adding a 10% volume of 3.8% (weight/volume) trisodium citrate, the blood was centrifuged at 160g for 10 min to prepare platelet-rich plasma (PRP) or at 1,600g for 30 min to prepare platelet-poor plasma (PPP). After 1 h of storage, PRP was used for the aggregation studies and Ca\(^{2+}\) studies. To prepare washed platelets, a 10% volume of 100 mM EDTA (pH 7.4) was added to PRP, and the mixture was centrifuged at 900g for 15 min. The platelet pellet was suspended in buffer A (8 mM Na\(_2\)HPO\(_4\), 2 mM NaH\(_2\)PO\(_4\), 10 mM EDTA, 5 mM KCl, and 135 mM NaCl [pH 7.2]) and was then recentrifuged at 900g for 15 min. Platelet pellets for the binding assay were resuspended in buffer B (25 mM Tris-HCl, 1 mM EGTA, 5 mM MgCl\(_2\), and 138 mM NaCl [pH 7.5]). The platelet counts were adjusted to 1 × 10\(^9\)/μl. Platelet pellets for IP\(_3\) measurement, and aggregation study were resuspended in HEPES buffer (10 mM HEPES, 145 mM NaCl, 5 mM KCl, 0.5 mM NaH\(_2\)PO\(_4\), 1 mM CaCl\(_2\), and 6 mM glucose [pH 7.5]) and were adjusted to 1 × 10\(^9\)/μl, 3 × 10\(^8\)/μl, and 3 × 10\(^7\)/μl respectively. Washed platelets were stored in Ca\(^{2+}\)-free buffer at 4°C for 1 h. A few minutes before use, the Ca\(^{2+}\) concentration of platelet suspension was adjusted to 1 mM by adding CaCl\(_2\), except for \(^{3}\)H]-labeled TXA\(_2\) receptor antagonist, 5Z-7-(3-endo-[\(^{3}\)H] phenyl) sulfonylamo-[2.2.1.] bicyclohept-2-exo-yl) heptenoic acid (\(^{3}\)H]SI45) binding assay, and (+)-9,11-epithia-11,12-methano-TXA\(_2\) (STA\(_2\)) binding assay.

**Aggregation Study**

Aliquots (200 μl) of PRP or platelet suspension in HEPES buffer, with or without ketamine (0.25–2.0 mM), were placed in siliconized glass tubes 2 min before each experiment and were kept at 37°C. PRP was used for STA\(_2\), ADP\(_{\text{a}}\), epinephrine\(_{\text{a}}\), and A23187-induced aggregation studies. Platelet suspension in HEPES buffer was used for thrombin-induced aggregation study to avoid coagulation. Platelet aggregation induced by STA\(_2\) (0.5 mM), ADP (0.5 μM), epinephrine (1.0 μM), A23187 (calcium ionophore; 0.5 μM), or thrombin (0.02 U/ml) was measured at 37°C by light transmission using an eight-channel aggregometer (MCM Hematracr 212; MC Medical Inc., Tokyo, Japan; n = 5 each). The light transmission of PPP or HEPES buffer was taken as 100%. The extent of aggregation in steady states (1 min after epinephrine stimulation and 3 min after stimulation with other stimulants) was compared between groups.

**Measurements of Intracellular Free Ca\(^{2+}\)**

Concentration

After loading of Fura-2/AM, washed platelets were prepared as described. For the study of intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) increase, Fura-2/AM-loaded PRP was washed twice and then resuspended in 1 ml HEPES buffer. After 1 h of incubation on ice, the platelet count was adjusted by adding PPP for STA\(_2\)-induced or HEPES buffer for thrombin-induced [Ca\(^{2+}\)]\(_i\) increase study. After addition of CaCl\(_2\) (final, 1 mM), an aliquot (800 μl) of washed platelets or PRP was placed in a siliconized glass cuvette and preincubated for 1 min in a fluorometer (CAF-110; Japan Spectroscopic, Tokyo, Japan). The samples were stimulated with thrombin (0.02 U/ml) or STA\(_2\) (0.5 μM) for 5 min with or without various concentrations of ketamine that had been added 3 min before stimulation. The intensities of 500-nm fluorescence induced by 340- and 380-nm excitation were monitored. To obtain the minimum and maximum fluorescences, the cells were treated with 1 mM EDTA or 2 μM ionomycin, respectively. The luminescent signal was converted to [Ca\(^{2+}\)]\(_i\) by using a Fura-2-Ca\(^{2+}\) dissociation constant of 224 nM as described by Grynkiewicz et al.\(^{11}\)

**Measurement of Intracellular Inositol 1,4,5-Triphosphate Formation**

Aliquots of washed platelets (120 μl; counts, 1,000,000/mm\(^3\)) were stimulated by STA\(_2\) (1 μM) or thrombin (0.02 U/ml) after preincubation at 37°C with or without ketamine (1 mM) for 3 min. Ketamine had been added 3 min before stimulation. At various time points after stimulation (0, 5, 10, and 60 s), the samples were added with 50 μl ice-cold perchloric acid (10%) to terminate the reaction, were kept in an ice bath for 30 min, and were then centrifuged at 1,800g, 4°C, for 10 min. After adjusting the pH to 7.4, the supernatants were placed in the ice bath for more than 30 min and were then centrifuged at 8,000g, 4°C, for 3 min. The IP\(_3\) concentrations of the supernatants were measured using a commercially available IP\(_3\) assay kit according to the manufacturer’s protocol.

**Thromboxane A\(_2\) Receptor Binding Assay**

Ketamine (0 or 2 mM) was added directly to test tubes containing 100 μl washed platelets (1,000,000/mm\(^3\)) and 700 μl buffer B for 10 min before addition of \(^{3}\)H]SI45. After incubation for 10 min at 37°C, [\(^{3}\)H]SI45 (1–5 nm) was added to the test tubes (n = 3 each) and was then incubated for 30 min. For analysis of STA\(_2\) binding affinity, a 100-μl platelet suspension was added to the test tube containing 1 mM ketamine, 1 mM [\(^{3}\)H]SI45, various concentrations of STA\(_2\) (1 mM–1 μM), and buffer B (total 200 μl) (n = 4 each) and was preincubated for 10 min at room temperature. After 30 min of incubation at 37°C, the samples were added to 5 ml ice-cold Tris-HCl buffer (5 mM; pH 7.4) to terminate the reaction, were filtered in vacuo through a Whatman GF/C filter, and were washed three times with 5 ml precooled Tris-HCl buffer. The radioactivity of each filter was determined using a liquid scintillation analyzer (Tri-Carb 1900 CA; Packard Instrument Co., Meriden, CT). Scatchard analysis studies were conducted, and equilibrium dissociation constant (K\(_d\)) and maximal concentra-
tion of binding sites (B_max) of each experiment were
calculated.

Measurement of Guanosine Triphosphate
Hydrolysis
Platelet pellets were suspended in 5 mM Tris-HCl, pH 7.4 at a density of 10,000,000 platelets/mm^3 and were homogenized by two strokes in a Potter-Elvehjem homogenizer to prepare the membrane fraction. The homogenate was centrifuged at 100,000 g for 30 min. Precipitates were suspended in 20 mM Tris-HCl, pH 7.4, at 5 mg protein/ml. Platelet high-affinity GTPase activity was assessed essentially by the method of Ushikubi et al. In brief, the reaction mixture contained 50 μg membrane protein, 5 mM MgCl_2, 1 mM EGTA, 0.2 mM ATP, 2 mM AMP-PNP, 1 mM phosphocreatine, 50 units creatine phosphokinase, 0.2 mM dithiothreitol, 100 mM NaCl, 0.1 mM [γ-32P]GTP (222 TBq/mM), 20 mM Tris-HCl (pH 7.4), and an agonist (0.02 U/ml thrombin or 300 nM STA_2i) in a final volume of 50 μl. The reaction mixture had been preincubated for 3 min before the agonist was added. High-affinity GTPase activity was defined as that inhibited by the addition of 1 mM GTP in the assay. The assay mixture was incubated for 10 min at 37°C and added to 1 ml ice-cold Norit A (5%) in 20 mM phosphate buffer (pH 7.4). After vortex and centrifugation (20,000g, 5 min), aliquots (500 μl) of the supernatant were counted for radioactivity by a liquid scintillation counter. A blank value was less than 5% of the total [γ-32P]GTP added.

Materials
The drugs used in this study were as follows: ketamine, thrombin, EGTA, ATP, AMP-PNP, phosphocreatine, dithiothreitol (Sigma Chemical Co., St. Louis, MO); acetoxymethyl ester of Fura-2 (Fura-2/AM; Dojindo, Kumamoto, Japan); and creatine phosphokinase (Nacalai tesque, Kyoto, Japan). [3H]S145 and STA_2 were gifts obtained from New England Nuclear (Boston, MA).

Statistics
The data were analyzed using an unpaired t test for aggregation study, measurements of [Ca^{2+}], IP_3 assay, and measurement of GTP hydrolysis. Data are expressed as mean ± SD. For TXA_2 receptor binding assay, data of the Scatchard analysis was analyzed using an unpaired Student t test, and data of the STA_2 binding affinity was analyzed using analysis of variance.

Results

Platelet Aggregation Study
Figure 1 shows typical recordings of aggregation, in the absence and presence of ketamine (500 μM), in response to ADP (0.5 μM), epinephrine (1.0 μM), STA_2 (0.5 μM), thrombin (0.02 U/ml), and A23187 (0.5 μM). Ketamine (up to 1 mM) did not affect primary aggregations induced by ADP and epinephrine. Ketamine (150–500 μM) suppressed aggregation of platelet after 3 min of stimulation with ADP (0.5 μM; fig. 2A), thrombin (0.02 U/ml; fig. 2B), and STA_2 (0.5 μM; fig. 2D). Ketamine (150–1,000 μM) suppressed the aggregation after 3 min of stimulation with epinephrine (1.0 μM; fig. 2C) in a dose-dependent manner, but only 1 mM ketamine affected aggregation after 3 min of epinephrine stimulation, suggesting that ketamine delayed the onset of epinephrine-induced secondary aggregation. Platelet aggregation induced by A23187 (0.5 μM) was not altered by ketamine (up to 1 mM; fig. 1).

Effects of Ketamine on Cytosolic Free Ca^{2+}
Concentration
Ketamine (250 μM, 500 μM, and 1 mM) inhibited [Ca^{2+}]_i increase induced by thrombin (0.02 U/ml) and STA_2 (0.5 μM) significantly and in a dose-dependent manner (fig. 3).

Effect of Ketamine on Thromboxane A_2 Receptor Expression and Binding Affinity
Scatchard analysis of [3H]S145 binding showed that ketamine (2 mM) did not affect B_max or K_d (fig. 4A). Figure 4B shows that the STA_2 (agonist) binding affinity

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was not changed by ketamine. These results suggested that the inhibitory effect of ketamine on STA₂-induced platelet aggregation was not due to the inhibition of the receptor expression or binding affinity.

**Effect of Ketamine on Intracellular Inositol 1,4,5-Triphosphate Concentration**

Figure 5 shows the time course and the effects of 1 mM ketamine on intracellular IP₃ formation in human platelets stimulated by STA₂ (1 μM) or thrombin (0.02 U/ml). The intracellular IP₃ concentration ([IP₃]ᵢ) at time 0 was 0.394 ± 0.369 pmol/10⁹ platelets (n = 5) and was not significantly changed with ketamine (0.450 ± 0.522 pmol/10⁹ platelets). STA₂ (1 μM) induced a rapid increase of [IP₃]ᵢ. [IP₃]ᵢ peaked at 5 s after stimulation, and the mean peak value was 4.39 ± 2.41 pmol/10⁹ platelets. Ketamine (1 mM) inhibited the rapid increase of [IP₃]ᵢ (1.90 ± 0.86 pmol/10⁹ platelets, P < 0.05; fig. 5A). In the same manner, thrombin (0.02 U/ml) induced an increase (n = 4 each). The maximum [IP₃]ᵢ was at 10 s after stimulation, and the mean value was 6.60 ± 1.819 pmol/10⁹ platelets. Ketamine (1 mM) also reduced the [IP₃]ᵢ increase induced by thrombin (2.41 ± 0.98 pmol/10⁹ platelets, P < 0.05; fig. 5B).

**Effect of Ketamine on Guanosine Triphosphate Hydrolysis**

Figure 6 shows the effect of ketamine on GTP hydrolysis. Ketamine (≤ 125 μM) dose dependently suppressed platelet membrane-bound GTPase activity in STA₂ or thrombin-stimulated conditions; GTPase activities in the presence of ketamine (1 mM) were 50.3 ± 3.2% and 67.5 ± 5.5%, respectively (n = 4 each), of those in the absence of ketamine.

**Discussion**

Several anesthetic agents, including volatile and intravenous anesthetics, were reported to affect human platelet aggregation through different mechanisms. Kohro and Yamakage demonstrated that halothane strongly suppresses thrombin-induced human platelet aggregation by suppressing [Ca²⁺]ᵢ increase without affecting agonist receptor binding. They concluded that this suppressing effect of halothane on [Ca²⁺]ᵢ was likely to be mediated by decreased IP₃ formation, by increased cytosolic free cyclic adenosine monophosphate, or by both. We previously reported that propofol, although it...
KETAMINE SUPPRESSES AGGREGATION BY Ca\(^{2+}\) SUPPRESSION

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[Image 53x116 to 293x230]

Enhances STA2-induced IP3 formation,7 inhibits both ADP- and epinephrine-induced secondary aggregation by suppressing cyclooxygenase-1 activity.7

As for the effect of ketamine on platelet aggregation, Atkinson et al.13 demonstrated the inhibitory effects on thromboxane synthesis. In the current study, ketamine suppressed secondary aggregation induced by ADP and epinephrine without altering primary aggregation. Furthermore, ketamine suppressed thrombin- and STA2-induced platelet aggregation as well. Obviously, the suppression of the effects of thrombin and a TXA2 analog shown in the current study cannot be ascribed to the suppression of TXA2 formation. The results of our aggregation study thus strongly suggested that ketamine affects the binding of TXA2 and thrombin to their receptors or mechanisms that follow the receptor activation.

However, the radioligand binding assay showed the absence of the effect of ketamine on the Kd and Bmax of STA2. Furthermore, STA2 binding assay showed that ketamine had no effect on the STA2-TXA2 receptor binding affinity. These results indicated that, at least in the platelet TXA2 signaling pathway, the inhibitory effects of ketamine are not due to suppression of TXA2 receptor binding.

The current [Ca\(^{2+}\)]i study showed clearly that ketamine suppresses thrombin- and STA2-induced [Ca\(^{2+}\)]i increase, concomitant with the suppression of aggregation. Moreover, the finding that ketamine did not affect calcium ionophore (A23187)-induced platelet aggregation indicates that ketamine does not affect the pathway that follows the cytosolic free calcium increase.

Thromboxane A2 receptors on platelets, as well as thrombin receptors, are known to be members of the seven transmembrane domain family that interacts with G-protein.14-17 The membrane-bound G-proteins coupled with these receptors are known to have α subunits belonging to the Gq family.18,19 This Gq activates platelet phospholipase Cβ.20 Phospholipase C hydrolyzes phosphatidyl inositol 4,5-bisphosphate and produces IP3 and diacylglycerol.21 IP3 is known to release Ca\(^{2+}\) from the dense tubular system of platelets,22-24 which is noted as the first peak of [Ca\(^{2+}\)].25 In an attempt to elucidate the mechanism by which ketamine suppresses [Ca\(^{2+}\)]i increase, we tested and demonstrated the suppressive effect of ketamine on IP3 formation. We further demonstrated suppression of GTP hydrolysis by ketamine in homogenized platelet membrane stimulated with thrombin and STA2. These results may suggest that ketamine suppresses receptor-coupled mechanisms, including G-protein, to decrease the activity of phospholipase Cβ.

An intravenous dose of 2 mg/kg ketamine in clinical use was reported to cause a peak plasma concentration of approximately 60 μM.26 In the current study, 150 μM
ketamine significantly suppressed epinephrine-induced platelet aggregation, and 250 μM suppressed thrombin-induced platelet aggregation and [Ca\textsuperscript{2+}] increase. Our experiments using higher concentrations of ketamine were performed mainly in an attempt to elucidate the underlying mechanism for pharmacologic interests. One may think that these high concentrations of ketamine could be cytotopic, exerting irreversible effects on platelets. However, Akata \textit{et al.}\textsuperscript{27} reported the reversibility of the inhibitory effect of ketamine (up to 3 mM) on vascular smooth muscle contraction. We also confirmed, in a preliminary study, the reversibility of the effects of ketamine on platelets. In brief, platelets were incubated with 2 mM ketamine for 15 min and were then washed by centrifugation. These platelets were aggregated normally by STA\textsubscript{2}, clearly denying the irreversible or cytotoxic effect of ketamine on platelets in our experiments.

In contrast to our study, Atkinson \textit{et al.}\textsuperscript{8} used clinically relevant concentrations of ketamine in their \textit{ex vivo} experiments with baboons and showed that ketamine, even at clinical concentrations, clearly suppresses platelet aggregation. One may argue that anesthesiologists do not notice bleeding tendency in a clinical situation using ketamine. It is possibly because in most situations, bleeding during surgery is more dependent on surgical technique than on the pharmacologic action of anesthetics. Furthermore, ketamine is known to affect plasma concentration of catecholamines by suppression of released catecholamine uptake,\textsuperscript{28,29} and therefore, it is also possible that this effect of ketamine ameliorates the antiaggregatory effect of ketamine observed \textit{in vivo}. Therefore, we speculate that the antiaggregatory effect of ketamine may not have clinical importance in most cases. In spite of these facts, we still consider that the antiaggregatory effect of ketamine may have clinical importance in critically ill patients or patients with hematoletic disorders.

In conclusion, ketamine has inhibitory effects on the thrombin- and TXA\textsubscript{2}-related Ca\textsuperscript{2+} release system of platelets. It was strongly suggested that the antiaggregatory effects of ketamine are at least partly ascribable to the inhibition of thrombin and TXA\textsubscript{2}-induced GTPase activation.

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