

Mechanisms Involved in the Antiplatelet Activity of Midazolam in Human Platelets

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Background: Midazolam is widely used as a sedative and anesthetic induction agent. The aim of this study was to systematically examine the inhibitory mechanisms of midazolam in platelet aggregation.

Methods: The inhibitory mechanisms of midazolam in platelet aggregation were explored by means of analysis of the platelet glycoprotein IIb-IIIa complex, phosphoinositide breakdown, intracellular Ca^{+2} mobilization, measurement of membrane fluidity, thromboxane B_2 formation, and protein kinase C activity.

Results: In this study, midazolam dose-dependently (6–26 μM) inhibited platelet aggregation in human platelets stimulated by agonists. Midazolam also dose-dependently inhibited phosphoinositide breakdown and intracellular Ca^{+2} mobilization in human platelets stimulated by collagen. Midazolam (6–26 μM) significantly inhibited thromboxane A_2 formation stimulated by collagen in human platelets. Moreover, midazolam (15 and 26 μM) dose-dependently decreased the fluorescence of platelet membranes tagged with diphenylhexatriene. Rapid phosphorylation of a platelet protein of Mr 47,000 (P47), a marker of protein kinase C activation, was triggered by collagen (2 $\mu g/ml$). This phosphorylation was markedly inhibited by midazolam (26 μM).

Conclusions: These results indicate that the antiplatelet activity of midazolam may be involved in the following pathways: the effects of midazolam may initially be caused by induction of conformational changes in platelet membrane, leading to a change in the activity of phospholipase C, and subsequent inhibition of phosphoinositide breakdown and thromboxane A_2 formation, thereby leading to inhibition of both intracellular Ca^{+2} mobilization and phosphorylation of P47 protein.

MIDAZOLAM is widely used as a sedative and anesthetic induction agent.¹ The pharmacologic actions of midazolam are identical to those of other benzodiazepines and include sleep induction, sedation, anxiolysis, and amnesia.² Its clinical use is primarily reserved for conscious sedation and induction of general anesthesia.¹

Benzodiazepines exert many pharmacologic activities at the periphery. Among these activities, some benzodiazepines inhibit platelet aggregation. Triazolam and al-

prazolam inhibit platelet-activating factor-induced aggregation,³ and the inhibition of platelet-activating factor binding to platelets has been proposed to explain this antiaggregatory effect.⁴ However, this is controversial, for diazepam has been shown both to inhibit arachidonate-induced aggregation⁵ and to have no effect.³ In addition, Karaseva *et al.*⁶ reported that gidazepam and phenazepam exert inhibitory properties of platelet aggregation, possibly through inhibition of lipid peroxidation in rat platelets. Choppin and Berry⁷ found that clonazepam, diazepam, and flumazenil inhibit arachidonic acid-induced platelet aggregation, possibly by mediating the inhibition of thromboxane synthesis in rabbit platelets. These reports indicate that the effect of benzodiazepine derivatives on platelet aggregation *in vitro* remains unclear, controversial, and species-specific.

On the other hand, studies of midazolam in platelets have relative rarely been compared with other derivatives of benzodiazepines. Lingjaerde⁸ presented data indicating that midazolam inhibits serotonin uptake in human platelets. However, the detailed mechanisms underlying the midazolam signaling pathways still remain obscure. We therefore systematically examined the influence of midazolam on washed human platelets in this study and used the findings to characterize the mechanisms involved in this influence.

Materials and Methods

Materials

Collagen (type I, bovine achilles tendon), adenosine 5'-diphosphate, midazolam, arachidonic acid, EDTA, luciferin-luciferase, Dowex-1 (100–200 mesh; X_8 , chloride form), prostaglandin E_1 , trichloroacetic acid, EGTA, bovine serum albumin, acrylamide, sodium pyruvate, β -NADH, diphenylhexatriene, apyrase, heparin, thrombin, and myoinositol were purchased from Sigma Chemical Co. (St. Louis, MO). Fura 2-AM and fluorescein isothiocyanate (FITC) were purchased from Molecular Probe Inc. (Eugene, OR). *Trimeresurus flavoviridis* venom was purchased from Latoxan (Rosans, France). Myo-2-[³H] inositol was purchased from Amersham (Buckinghamshire, United Kingdom). Thromboxane B_2 enzyme immunoassay kits were purchased from Cayman Co. (Ann Arbor, MI).

Preparation of Human Platelet Suspensions

Human platelet suspensions were prepared as previously described.⁹ In this study, human volunteers gave

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informed consent. In brief, blood was collected from healthy human volunteers who had taken no medicine during the preceding 2 weeks and was mixed with acid/citrate/glucose (9:1, vol/vol). After centrifugation at 120g for 10 min at room temperature, the supernatant (platelet-rich plasma) was supplemented with prostaglandin E₁ (0.5 μ M) and heparin (6.4 IU/ml), then incubated for 10 min at 37°C and centrifuged at 500g for 10 min. The washed platelets were finally suspended in Tyrode solution containing bovine serum albumin (3.5 mg/ml) and adjusted to approximately 4.5×10^8 platelets/ml. The final concentration of Ca²⁺ in Tyrode solution was 1 mM.

Platelet Aggregation

The turbidimetric method used a Lumi-Aggregometer (Payton, Canada) as described previously.¹⁰ In brief, platelet suspensions (0.4 ml) were prewarmed at 37°C for 2 min, and then midazolam was added for 3 min before the addition of platelet-aggregation inducers. The reaction was allowed to proceed for at least 6 min, and the extent of aggregation was expressed as a percentage of the control (in the absence of midazolam). The degree of aggregation was expressed in light-transmission units. While measuring adenosine triphosphate (ATP) release, 20 μ l of a luciferin-luciferase mixture was added 1 min before the addition of agonists, and ATP release was compared with that of the control.

Analysis of the Platelet Surface Glycoprotein IIb-IIIa Complex by Flow Cytometry

Triflavin, a specific fibrinogen receptor (glycoprotein IIb-IIIa complex) antagonist, was prepared as previously described.¹¹ Fluorescence-conjugated triflavin was also prepared as previously described.¹² The final concentration of FITC-conjugated triflavin was adjusted to 1 mg/ml. Human platelet suspensions were prepared as described above. Aliquots of platelet suspensions (4.5×10^8 /ml) were preincubated with midazolam (15 and 26 μ M) for 3 min, followed by the addition of 2 μ l of FITC-triflavin. The suspensions were then incubated for another 5 min, and the volume was adjusted to 1 ml/tube with Tyrode solution. The suspensions were then assayed for fluorescein-labeled platelets with a flow cytometer (FACScan System; Becton Dickinson, San Jose, CA). Data were collected from 50,000 platelets per experimental group. All experiments were repeated at least five times to ensure reproducibility.

Labeling of Membrane Phospholipids and Measurement of the Production of [³H]-Inositol Phosphates

The method was conducted as previously described.¹³ Briefly, citrated human platelet-rich plasma was centrifuged, and the pellets were suspended in Tyrode solution containing [³H]-inositol (75 μ Ci/ml). Platelets were

incubated for 2 h followed by centrifugation, and finally resuspended in Ca²⁺-free Tyrode solution (5×10^8 platelets/ml). Midazolam was preincubated with 1 ml of loaded platelets at room temperature for 3 min, and collagen (2 μ g/ml) was then added to trigger aggregation. The reaction was stopped after 6 min, and the samples were centrifuged at 1,000g for 4 min. The inositol phosphates of the supernatants were separated in a Dowex-1 anion exchange column. Only [³H]-inositol monophosphate (IP) was measured as an index of total inositol phosphate formation.

Measurement of Platelet Intracellular Ca²⁺ Mobilization by Fura 2-AM Fluorescence

Citrated whole blood was centrifuged at 120g for 10 min. The supernatant was protected from light and incubated with Fura 2-AM (5 μ M) at 37°C for 1 h. Human platelet suspensions were then prepared as described above. Finally, the external Ca²⁺ concentration of the platelet suspensions was adjusted to 1 mM. The increase in intracellular Ca²⁺ concentration ([Ca²⁺]_i) was measured using a fluorescence spectrophotometer (CAF 110; Jasco, Tokyo, Japan) with excitation wavelengths of 340 and 380 nm and an emission wavelength of 500 nm, respectively. The [Ca²⁺]_i was calculated from the fluorescence measured using 224 nm as the Ca²⁺-Fura 2 dissociation constant.¹⁴

Measurement of Membrane Fluidity by Fluorescent Probe

The intensity of fluorescence in human platelets was measured as described previously.¹⁵ Briefly, platelets (4.5×10^8 /ml) were mixed with nine volumes of sodium-potassium-Tris medium. A solution of diphenylhexatriene in dimethyl formamide was added to the suspension at a final concentration of 0.5 μ M. Platelets were preincubated with various concentrations of midazolam (15 and 26 μ M) for 3 min followed by the addition of diphenylhexatriene at 37°C for another 6 min. The relative fluorescence intensity of platelets was measured in a fluorescence spectrophotometer (Hitachi F4500; Hitachi, Tokyo, Japan) at 37°C.

Measurement of Thromboxane B₂ Formation

Washed human platelet suspensions (4.5×10^8 /ml) were preincubated for 3 min in the presence or absence of midazolam (6–26 μ M) before the addition of collagen (2 μ g/ml). Six minutes after the addition of the agonist, 2 mM EDTA and 50 μ M indomethacin were added to the reaction suspensions. The vials were then centrifuged for 3 min at 14,000 rpm. The thromboxane B₂ concentrations of the supernatants were measured using an enzyme immunoassay kit according to the instructions of the manufacturer.

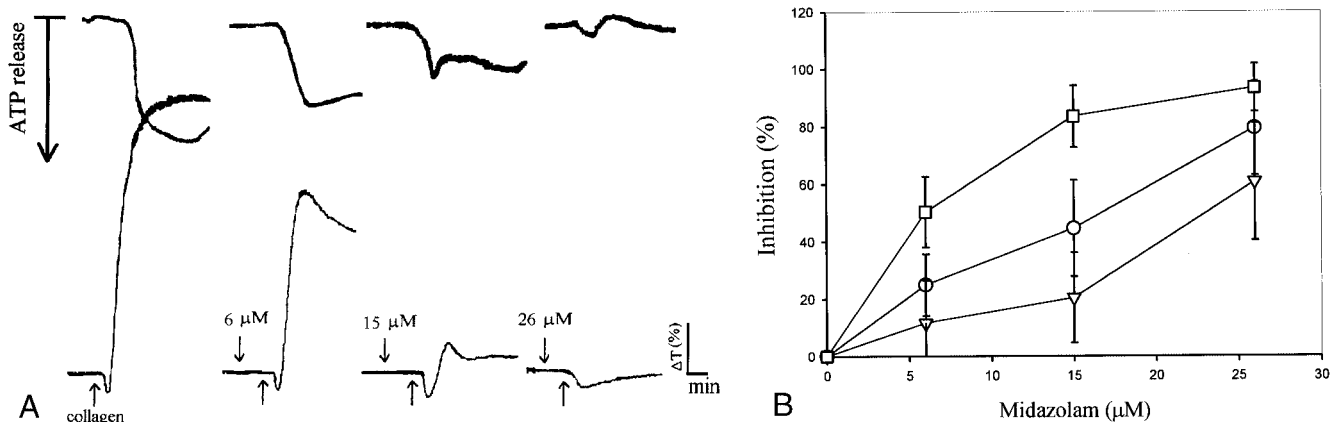


Fig. 1. (A) Tracing curves of midazolam on collagen (2 μg/ml)-induced aggregation and (B) concentration-inhibition curve of midazolam on collagen (2 μg/ml, circles)-, arachidonic acid (100 μM, triangles)-, and thrombin (0.5 U/ml, squares)-induced platelet aggregation in washed human platelet suspensions. Platelets were preincubated with midazolam (6–26 μM), and agonists were then added to trigger aggregation (lower tracings) and adenosine triphosphate release (upper tracings) (A). Data are presented as a percentage of the control (mean ± SD, n = 5).

Determination of Lactate Dehydrogenase

Lactate dehydrogenase was measured according to previously described methods.¹⁶ Platelets (4.5×10^8 /ml) were preincubated with various concentrations of midazolam for either 10 or 30 min, followed by centrifugation at 14,000 rpm for 5 min. An aliquot of supernatant was incubated with phosphate buffer containing 0.2 mg β-NADH for 20 min at room temperature. Thereafter, 100 μl of pyruvate solution was added, and the absorbance wavelength was read at 334 nm using an ultraviolet-visible recording spectrophotometer (UV-160; Shimadzu, Kyoto, Japan). A maximal value of lactate dehydrogenase was constructed from sonicated platelets.

Measurement of Protein Kinase C Activity

Washed human platelets (2×10^9 /ml) were incubated for 60 min at 37°C with phosphorus-32 (0.5 mCi/ml). Platelet suspensions were next washed twice with Tris-saline buffer. The [³²P]-labeled platelets were preincubated with midazolam (26 μM) in an aggregometer at 37°C for 3 min, then collagen (2 μg/ml) was added for 1 min to trigger protein kinase C activation. Activation was terminated by the addition of Laemmli sample buffer and analyzed by electrophoresis (12.5%, wt/vol) as described previously.¹⁷ The gels were dried, and the relative intensities of the radioactive bands were analyzed using a Bio-imaging analyzer system (FAL2000; Fuji, Tokyo, Japan), expressed as photostimulated luminescence per squared millimeters.

Statistical Analysis

Experimental results are expressed as the mean ± SD and are accompanied by the number of observations. Data were assessed by Student *t* test, and *P* < 0.05 was considered statistically significant.

Results

Effect of Midazolam on Platelet Aggregation in Human Platelet Suspensions

Midazolam dose-dependently inhibited platelet aggregation stimulated by collagen (2 μg/ml), arachidonic acid (100 μM), and thrombin (0.5 U/ml) in human platelet suspensions (figs. 1A and B). Furthermore, midazolam also inhibited the ATP-release reaction when stimulated by agonists (*i.e.*, collagen; fig. 1A). The IC₅₀ values of midazolam for platelet aggregation induced by collagen, arachidonic acid, and thrombin were estimated to be approximately 16.1, 23, and 9.7 μM, respectively. In addition, midazolam (26 and 50 μM) did not significantly induce platelet aggregation in the absence of agonists (data not shown). Furthermore, platelets were preincubated with midazolam (26 μM) or normal saline for 10 min followed by two washes with Tyrode solution, and we found that there are no significant differences between the aggregation curves of both platelet preparations stimulated by collagen (2 μg/ml), indicating that the effect of midazolam on inhibition of platelet aggregation occurs in a reversible manner (data not shown). These results also indicate that midazolam significantly inhibits agonist-induced human platelet aggregation.

Effect of Midazolam on Collagen-induced Glycoprotein IIb-IIIa Complex Exposure in Human Platelets

Triflavin is an Arg-Gly-Asp-containing antiplatelet peptide, purified from *Trimeresurus flavoviridis* snake venom.^{11,18} Triflavin inhibits platelet aggregation through direct interference with fibrinogen binding to the glycoprotein IIb-IIIa complex.¹¹ There is now a multitude of evidence suggesting that the binding of fibrinogen to the glycoprotein IIb-IIIa complex is the final common pathway for agonist-induced platelet ag-

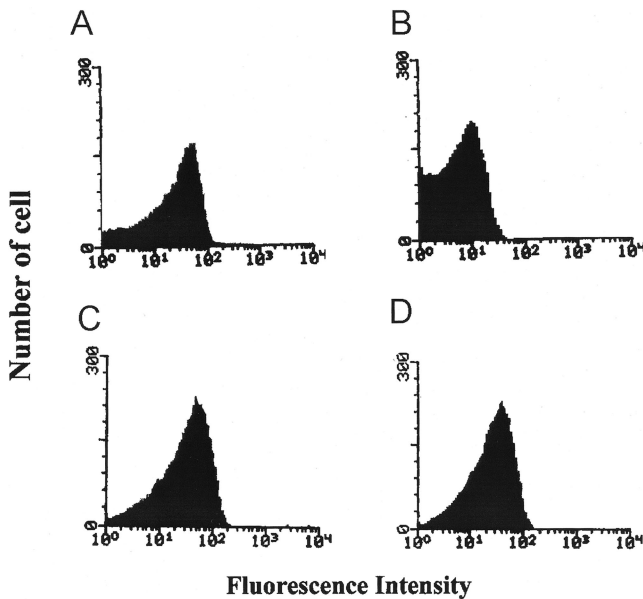


Fig. 2. Flow cytometric analysis of fluorescein isothiocyanate (FITC)-triflavin binding to human platelets in the absence or presence of various concentrations of midazolam (15 and 26 μM). (A) The solid line represents the fluorescence profiles of FITC-triflavin (2 $\mu\text{g}/\text{ml}$) in the absence of midazolam as a positive control; (B) in the presence of EDTA (5 mM) as the negative control; or in the presence of (C) 15 μM and (D) 26 μM midazolam. The profiles are representative examples of five similar experiments.

gregation. Therefore, we decided to further evaluate whether midazolam binds directly to the platelet glycoprotein IIb-IIIa complex, leading to inhibition of platelet aggregation induced by agonists.

In this study, the relative intensity of fluorescence of FITC-triflavin (2 $\mu\text{g}/\text{ml}$) bound directly to collagen (2 $\mu\text{g}/\text{ml}$)-activated platelets was 89.2 ± 10.7 (fig. 2A), and it was markedly reduced in the presence of 5 mM EDTA (negative control, 6.6 ± 2.5 ; fig. 2B). At a concentration of 15 μM , midazolam did not significantly inhibit FITC-triflavin binding to glycoprotein IIb-IIIa in platelet suspensions (90.6 ± 17.4), or even at a higher concentration (26 μM ; 88.3 ± 14.5 ; figs. 2C and D), indicating that the mechanism of midazolam's inhibitory effect on platelet aggregation does not involve binding to the glycoprotein IIb-IIIa complex.

Effect of Midazolam on Phosphoinositide Breakdown in Human Platelet Suspensions

Phosphoinositide breakdown occurs in platelets activated by many different agonists.¹⁹ In this study, we found that collagen (2 $\mu\text{g}/\text{ml}$) induces the rapid formation of radioactive IP, IP₂, and IP₃ in human platelets loaded with [³H]-inositol. We only measured [³H]-IP formation as an index of total inositol phosphate formation. As shown in figure 3, the addition of collagen (2 $\mu\text{g}/\text{ml}$) resulted in an increase of IP formation of approximately 2.1-fold compared with that in resting platelets ($[1.6 \pm 0.6 \text{ vs. } 3.4 \pm 0.4] \times 10^{-3}$ cpm). In the presence of

midazolam (15 and 26 μM), the radioactivity of IP formation in collagen-stimulated human platelets markedly decreased. These results indicate that midazolam interferes with phosphoinositide breakdown in human platelets stimulated by collagen.

Effect of Midazolam on Intracellular Ca^{2+} Concentration Mobilization

Free cytoplasmic Ca^{2+} concentrations in human platelets were measured by the Fura 2-AM loading method. As shown in figure 4, collagen (2 $\mu\text{g}/\text{ml}$) evoked an increase of [Ca^{2+}]_i from 36.6 ± 5.0 to 201.6 ± 36.6 nM. This collagen-evoked increase in [Ca^{2+}]_i was markedly inhibited in the presence of midazolam (15 μM , $74.6 \pm 8.4\%$; 26 μM , $89.5 \pm 12.6\%$; n = 4). This suggests that midazolam exerts an inhibitory effect on [Ca^{2+}]_i mobilization in human platelets stimulated by collagen.

Effect of Midazolam on Thromboxane B₂ Formation

As shown in table 1, resting platelets produced relatively little thromboxane B₂ compared with collagen-activated platelets. Prostaglandin E₁ (10 μM) inhibited thromboxane B₂ formation in collagen-activated platelets by 81% (data not shown). Furthermore, results obtained using various concentrations of midazolam indicated that midazolam (6–26 μM) dose-dependently inhibited thromboxane B₂ formation in platelets stimulated by collagen (2 $\mu\text{g}/\text{ml}$; table 1). These results suggest that midazolam exerts an inhibitory effect on thromboxane A₂ formation.

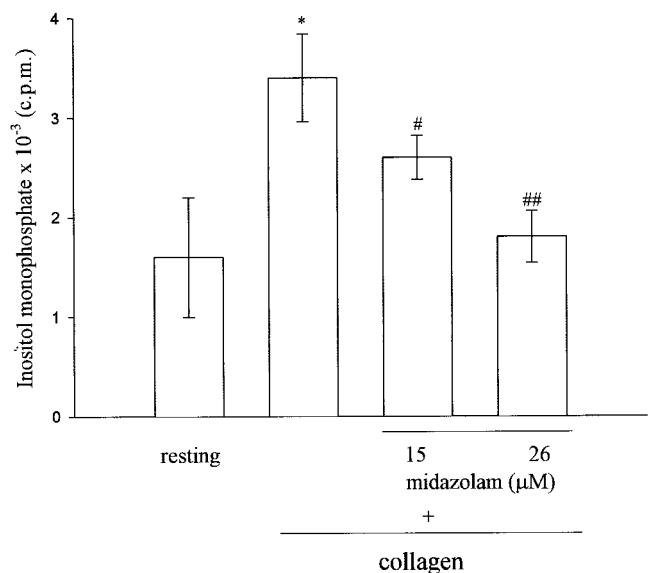
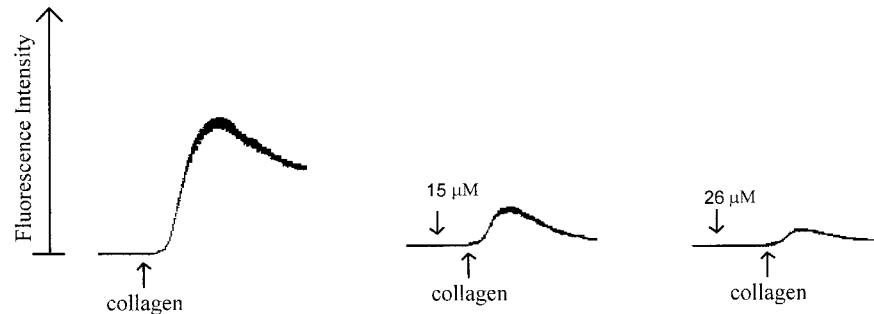


Fig. 3. Effect of midazolam on collagen-induced inositol monophosphate formation in human platelet suspensions. Platelets were labeled with [³H]-inositol and stimulated with or without collagen (2 $\mu\text{g}/\text{ml}$) in the presence of midazolam (15 and 26 μM). Data are presented as mean \pm SD (n = 4). **P* < 0.001 as compared with the resting group; #*P* < 0.01 and ##*P* < 0.001 as compared with the collagen group.

Fig. 4. Effect of midazolam on collagen-induced intracellular Ca^{2+} mobilization in Fura 2-AM-loaded human platelets. Platelet suspensions were preincubated with Fura 2-AM ($5 \mu\text{M}$), followed by the addition of collagen ($2 \mu\text{g/ml}$) in the absence or presence of midazolam (15 and $26 \mu\text{M}$), which was added 3 min before the addition of collagen. The profiles are representative examples of four similar experiments.



Effect of Midazolam on Platelet Membrane Fluidity
Platelet membrane fluidity was measured in diphenylhexatriene-labeled human platelets. Measurements using the fluorescent probe technique demonstrated that midazolam is capable of direct interaction with platelet membranes (fig. 5). Addition of midazolam (15 and $26 \mu\text{M}$) to platelet preparations for 3 min resulted in a dose-dependent decrease in the diphenylhexatriene-related fluorescence intensity. This result implies that the inhibitory effect of midazolam on platelet aggregation may be a result, at least in part, of the results of its effects on platelet membrane fluidity.

Effect of Midazolam on Lactate Dehydrogenase Released from Platelet Cytosol

In this study, midazolam ($26 \mu\text{M}$) treatment of human platelets for 10 min did not significantly increase lactate dehydrogenase activity compared with resting platelets (resting platelets, 35.6 ± 5.6 units *vs.* midazolam-treated platelets, 37.5 ± 9.8 units; $n = 4$), even when the incubation time of midazolam with platelets was prolonged to 30 min (34.6 ± 8.6 units; $n = 4$; data not shown). This result indicates that although midazolam (15 and $26 \mu\text{M}$) significantly changes the fluidity of platelet membranes, by itself, it does not affect platelet permeabilization or induce platelet cytolysis under this range of concentrations.

Effect of Midazolam on Collagen-stimulated Phosphorylation of the 47-kd Protein

Stimulation of platelets with a number of different agonists induces activation of protein kinase C, which

then phosphorylates proteins of Mr 40,000–47,000 in addition to other proteins.²⁰ In this study, phosphorylation experiments were conducted to examine the role of midazolam in activation of protein kinase C in human platelets. When collagen ($2 \mu\text{g/ml}$) was added to human platelets prelabeled with $^{32}\text{PO}_4$ for 2 min, a protein with an apparent Mr of 47,000 (P47) was predominately phosphorylated as compared with resting platelets (figs. 6A and B). On the other hand, midazolam ($26 \mu\text{M}$) markedly inhibited the phosphorylation of P47 in human platelets stimulated by collagen ($2 \mu\text{g/ml}$) when preincubated with midazolam for 3 min. In this study, the extent of radioactivity in P47 was expressed as a relative detection density (photostimulated luminescence per squared millimeters) of the radioactive bands.

Discussion

The principal objective of this study was to describe the detailed mechanisms involved in the inhibition of agonist-induced human platelet aggregation by midazolam. Midazolam is a familiar agent commonly used to provide sedation and for behavioral emergencies.¹ The results demonstrate that midazolam exerts inhibitory activity on human platelet aggregation, a finding that has not been described previously. This inhibitory effect of midazolam was demonstrable with the use of various agonists: collagen, arachidonic acid, and thrombin. The inhibition was directly proportional to the concentration of midazolam used. Ramoska *et al.*²¹ demonstrated adequate sedation with effective intravenous doses of 1–3 mg midazolam. Harper *et al.*²² reported that intravenous doses of 0.3 mg/kg may be required when midazolam is used for sedation in surgical patients. In this study, midazolam was used at concentrations (approximately 6–26 μM) that inhibited platelet aggregation induced by agonists. These results indicate that the concentrations of midazolam used to inhibit platelet aggregation *in vitro* are reasonably close to those of blood concentrations obtained during midazolam-induced sedation *in vivo*.²³ In this study, both platelet aggregation and the ATP-release reaction induced by these agonists (*i.e.*, collagen) appeared to be affected by the presence of midazolam. Therefore, this partly infers that midazolam may affect Ca^{2+} release from intracellu-

Table 1. Effect of Midazolam on Thromboxane B_2 Formation Induced by Collagen in Washed Human Platelets

	Dose	Thromboxane B_2 (ng/ml)
Resting	—	45.8 ± 13.4
Collagen ($\mu\text{g/ml}$)	2	$256.6 \pm 42.4^*$
+ Midazolam (μM)	6	$176.5 \pm 22.8^\dagger$
	15	$119.7 \pm 17.8^\ddagger$
	26	$72.0 \pm 13.0^\ddagger$

Platelet suspensions were preincubated with midazolam (6 – $26 \mu\text{M}$) for 3 min at 37°C , and then collagen ($2 \mu\text{g/ml}$) was added to trigger thromboxane B_2 formation. Data are presented as mean \pm SD ($n = 4$).

* $P < 0.001$ as compared with the resting group; $^\dagger P < 0.05$ and $^\ddagger P < 0.001$ as compared with the collagen group.

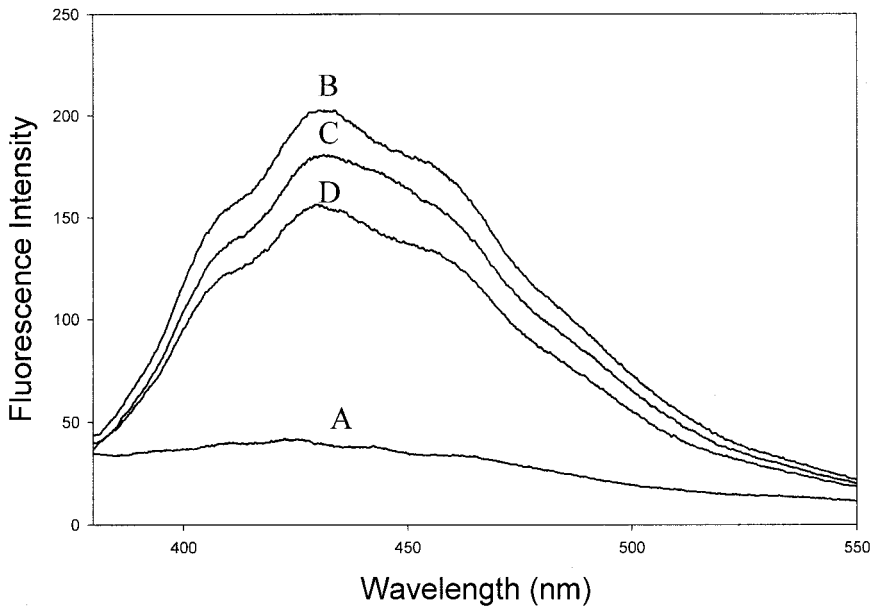


Fig. 5. Fluorescence emission spectra of platelet membranes in the (A) absence or (B) presence of diphenylhexatriene ($1 \mu\text{M}$). (C and D) Curves are the emission spectra of membranes labeled with diphenylhexatriene in the presence of $15 \mu\text{M}$ (C) and $26 \mu\text{M}$ (D) midazolam for 3 min. The profiles are representative examples of four similar experiments.

lar Ca^{2+} -storage sites (*i.e.*, dense tubular systems or dense bodies; fig. 4), and this is in accord with the concept that intracellular Ca^{2+} release is responsible for the ATP-release reaction.²⁴

Although the action mechanisms of various platelet aggregation agonists, such as collagen, arachidonic acid, and thrombin, differ (fig. 7), midazolam significantly

inhibited platelet aggregation stimulated by all of them. This implies that midazolam may block a common step shared by these inducers. These results also indicate that the site of action of midazolam is not at the receptor level of individual agonists. Platelet membrane plays a dominant role in regulating and executing platelet functions, particularly platelet aggregation. Triflavin acts by

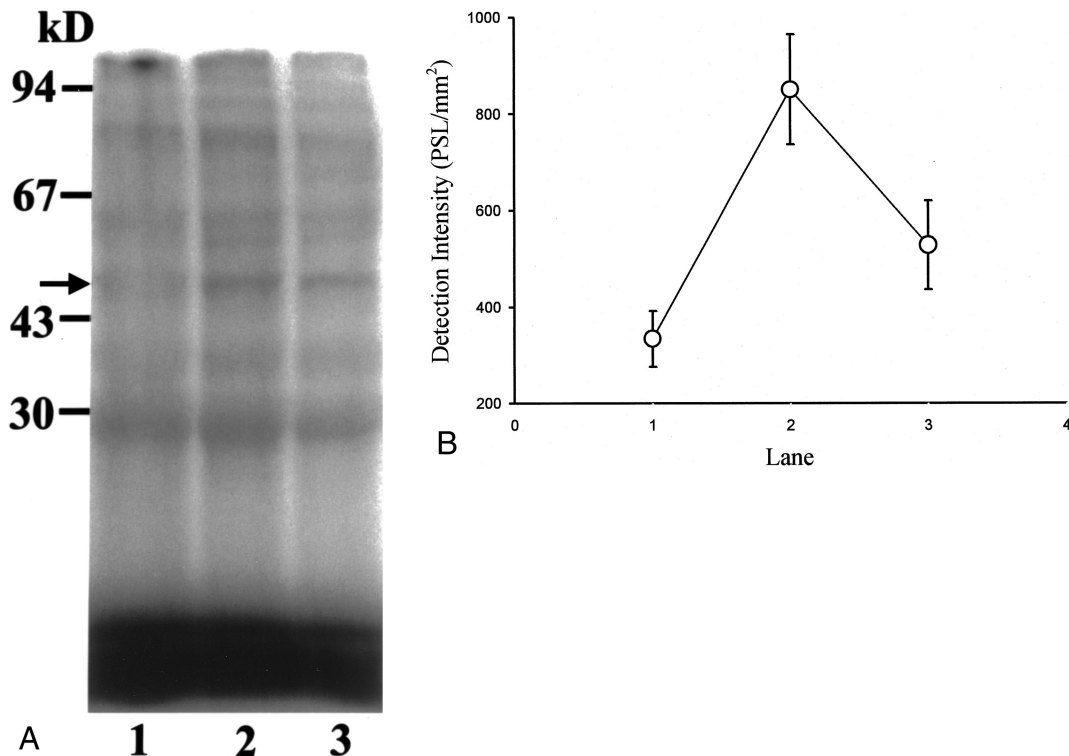
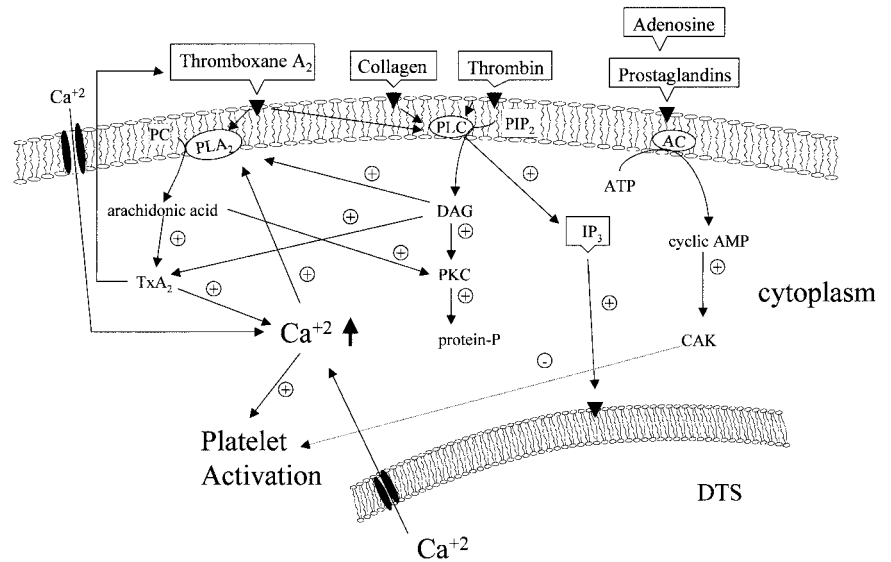


Fig. 6. (A) Effect of midazolam on phosphorylation of a protein of M_r 47,000 (P47) in human platelets challenged with collagen. Platelets were preincubated with midazolam ($26 \mu\text{M}$) before challenge with collagen ($2 \mu\text{g/ml}$). Lane 1, platelets with Tyrode solution; lane 2, platelets with collagen ($2 \mu\text{g/ml}$); lane 3, platelets with midazolam ($26 \mu\text{M}$) and collagen ($2 \mu\text{g/ml}$). The arrow indicates a protein of M_r 47,000 (P47). Data are representative examples of four similar experiments. (B) The relative detection densities of the radioactive bands are expressed as photostimulated luminescence (PSL) per squared millimeter. Data are presented as mean \pm SD ($n = 4$).

Fig. 7. Signal transductions of platelet aggregation. Agonists can activate several phospholipase, including phospholipase C (PLC) and phospholipase A₂ (PLA₂). The products of the action of phospholipase C on phosphatidylinositol 4,5-bisphosphate (PIP₂) include 1,2-diaclyglycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃). DAG stimulates protein kinase C (PKC), followed by phosphorylation of a 47-kd protein. IP₃ induces the release of Ca²⁺ from dense tubular systems (DTS). The major metabolite of arachidonic acid (AA) in platelets is thromboxane A₂ (TxA₂). Cyclic AMP = cyclic 3'-5'-adenosine monophosphate; PC = phosphatidylcholine; AC = adenylate cyclase; ATP = adenosine triphosphate; CAK = cyclic AMP-activated protein kinase.



binding to the glycoprotein IIb-IIIa complex on the platelet surface membrane, resulting in interference with the interaction of fibrinogen with its specific receptor.^{11,12,18} In this study, we found that midazolam did not significantly inhibit FITC-triflavin binding to the glycoprotein IIb-IIIa complex, indicating that the antiplatelet effect of midazolam might not be directly caused by interference with the binding of fibrinogen to its specific receptor on the platelet membrane. Conformational changes in the plasma membrane or changes in membrane fluidity represent a generally accepted mechanism for the antiplatelet effect of numerous drugs, including local anesthetics, chlorpromazine, and β blockers. Therefore, we wondered whether midazolam might also inhibit platelet aggregation by influencing membrane fluidity. To test this hypothesis, the fluorescent probe, diphenylhexatriene, was used to label biologic membranes. In this study, midazolam (15 and 26 μM) dose-dependently decreased the diphenylhexatriene relative fluorescence intensity of platelet membranes. These findings suggest that changes in platelet membrane fluidity may be the primary mechanism responsible for the antiplatelet effect of midazolam *in vitro*.

Furthermore, stimulation of platelets by agonists (*i.e.*, collagen) results in phospholipase C-catalyzed hydrolysis of the minor plasma membrane phospholipid, phosphatidylinositol 4,5-bisphosphate, with concomitant formation of inositol 1,4,5-trisphosphate and diacylglycerol (fig. 7).²⁵ There is strong evidence that inositol 1,4,5-trisphosphate induces the release of Ca²⁺ from intracellular stores.²⁶ Diacylglycerol activates protein kinase C, inducing protein phosphorylation and a release reaction (fig. 7). In this study, phosphoinositide breakdown of collagen-activated platelets was inhibited by midazolam, suggesting that inhibition of platelet aggregation by midazolam is related to inhibition of phosphoinositide breakdown. Thromboxane A₂ is an important mediator

of the release reaction and aggregation of platelets.²⁷ Collagen-induced formation of thromboxane B₂, a stable metabolite of thromboxane A₂, was markedly inhibited by midazolam (6–26 μM ; table 1). It has been demonstrated that phosphoinositide breakdown can induce thromboxane A₂ formation *via* free arachidonic acid release by diglyceride lipase or by endogenous phospholipase A₂ from membrane phospholipids (fig. 7).²⁸ Thus, it seems likely that thromboxane B₂ formation plays a role in the mediation of the inhibitory effect of midazolam on human platelets.

Furthermore, in this study, activation of protein kinase C in human platelets occurred in response to collagen. The important finding in our studies is that midazolam inhibits collagen-induced activation of protein kinase C. In addition, it has been reported that increased nitric oxide-cyclic guanosine monophosphate formation negatively affects agonist-induced protein kinase C activation.²⁹ However, midazolam (15 and 26 μM) did not significantly increase the concentration of nitric oxide or cyclic guanosine monophosphate in human platelets (data not shown). Therefore, based on the aforementioned observations, we suggest that the inhibitory effect of midazolam in collagen-induced activation of protein kinase C may be a result, at least in part, of its involvement in changing platelet membrane fluidity. This change in membrane fluidity influences the activation of phospholipase C, which is incorporated into plasma membranes, resulting in inhibition of phosphoinositide breakdown and subsequent inhibition of the activation of protein kinase C.

In conclusion, the observations of this study suggest that midazolam inhibits agonist-induced human platelet aggregation. This inhibitory effect may involve the following mechanisms: the antiplatelet activity of midazolam may be caused by the induction of conformational changes in platelet membrane, with a resulting influence

on membrane fluidity followed by inhibition of the activation of phospholipase C and subsequent inhibition of phosphoinositide breakdown and thromboxane A₂ formation, thereby leading to inhibition of both intracellular Ca²⁺ mobilization and phosphorylation of P47.

References

1. Yuan R, Flockhart DA, Balian JD: Pharmacokinetic and pharmacodynamic consequences of metabolism-based drug interactions with alprazolam, midazolam, and triazolam. *J Clin Pharmacol* 1999; 39:1109-25
2. Kupietzky A, Hout MI: Midazolam: A review of its use for conscious sedation in children. *Pediatr Dent* 1993; 15:237-41
3. Kordecki E, Ehrlich YH, Lenox RH: Platelet-activating factor-induced aggregation of human platelets specifically inhibited by triazolobenzodiazepines. *Science* 1984; 226:1454-6
4. Chesney CM, Pifer DD, Cagen LM: Triazolobenzodiazepines competitively inhibit the binding of platelet activating factor to human platelets. *Biochem Biophys Res Commun* 1987; 144:359-66
5. Romstedt K, Huzoor A: Benzodiazepines inhibit human platelet activation: Comparison of the mechanism of anti-platelet actions of flurazepam and diazepam. *Thromb Res* 1985; 38:361-74
6. Karaseva TL, Belikova MV, Pavlovskii VI, Andronati KS, Kabanova TA: In vitro effect of 1,2-dihydro-3H-1,4-benzodiazepine-2-one derivatives on the platelet aggregation and lipid peroxidation in rats. *Ukrain Biokhim Zhurn* 1998; 70:81-5
7. Choppin A, Berry CN: Peripheral benzodiazepine ligands inhibit aggregation and thromboxane synthesis induced by arachidonic acid in rabbit platelet in vitro. *Thromb Res* 1995; 78:293-303
8. Lingjaerde O: Effects of the benzodiazepine receptor ligands midazolam, RO15-1788, and RO 5-4864, alone or in combinations, on platelet serotonin uptake. *Pharmacopsychiatry* 1986; 19:15-8
9. Huang TF, Sheu JR, Teng CM: A potent antiplatelet peptide, triflavin, from *Trimeresurus flavoviridis* snake venom. *Biochem J* 1991; 277:351-7
10. Born GVR, Cross MJ: The aggregation of blood platelets. *J Physiol* 1963; 168:178-95
11. Sheu JR, Teng CM, Huang TF: Triflavin, an RGD-containing antiplatelet peptide, binds to GP IIIa of ADP-stimulated platelets. *Biochem Biophys Res Commun* 1992; 189:1236-42
12. Sheu JR, Lin CH, Peng CH, Huang TF: Triflavin, an Arg-Gly-Asp-containing peptide, inhibits the adhesion of tumor cells to matrix protein via binding to multiple integrin receptors expressed on human hepatoma cells. *Proc Soc Exp Biol Med* 1996; 213:71-9
13. Huang TF, Sheu JR, Teng CM: Mechanism of action of a potent antiplatelet peptide, triflavin, from *Trimeresurus flavoviridis* snake venom. *Thromb Haemostas* 1991; 66:489-93
14. Gryniewicz G, Poenie M, Tsien RY: A new generation of Ca²⁺ inducers with greatly improved fluorescence properties. *J Biol Chem* 1985; 260:3440-50
15. Kitagawa S, Shinohara T, Kametani F: Effects of alcohols on ADP-induced aggregation and membrane fluidity of gel-filtered bovine blood platelets. *J Membrane Biol* 1984; 79:97-102
16. Wroblewski F, Ladue JS: Lactic dehydrogenase activity in blood. *Proc Soc Exp Biol Med* 1955; 90:210-5
17. Grabarek J, Raychowdhury M, Ravid K, Kent KC, Newman PJ, Ware JA: Identification and functional characterization of protein kinase C isozymes in platelets and HEL cells. *J Biol Chem* 1992; 267:10011-7
18. Sheu JR, Hung WC, Wu CH, Ma MC, Kan YC, Lin CH, Lin MS, Luk HN, Yen MH: Reduction in lipopolysaccharide-induced thrombocytopenia by triflavin in a rat model of septicemia. *Circulation* 1999; 99:3056-62
19. Broekman MJ, Ward JW, Marcus AJ: Phospholipid metabolism in phosphatidyl inositol, phosphatic acid and lysophospholipids. *J Clin Invest* 1980; 66:275-83
20. Siess W, Lapetina EG: Platelet aggregation induced by alpha 2-adrenoceptor and protein kinase C activation: A novel synergism. *Biochem J* 1989; 263:377-85
21. Ramoska EA, Linkenheimer R, Glasgow C: Midazolam use in the emergency department. *J Emerg Med* 1991; 9:247-51
22. Harper KW, Collier PS, Dundee JW, Elliot P, Halliday NJ: Age and nature of operation influence the pharmacokinetic of midazolam. *Br J Anaesthesiol* 1985; 57:866-71
23. Garzone PD, Kroboth PD: Pharmacokinetics of the newer benzodiazepines. *Clin Pharmacokinet* 1989; 16:337-64
24. Charo IF, Feinman RD, Detwiler TC: Inhibition of platelet secretion by an antagonist of intracellular calcium. *Biochem Biophys Res Commun* 1976; 72:1462-7
25. Kirk CJ, Creba JA, Downes CP, Michell RH: Hormone-stimulated metabolism of inositol lipids and its relationship to hepatic receptor function. *Biochem Soc Transact* 1981; 9:377-9
26. Berridge MJ: Rapid accumulation of inositol trisphosphate reveals that agonists hydrolyse polyphosphoinositides instead of phosphatidylinositol. *Biochem J* 1983; 212:249-58
27. Hornby EJ: Evidence that prostaglandin endoperoxides can induce platelet aggregation in the absence of thromboxane A₂ production. *Biochem Pharmacol* 1982; 31:1158-60
28. McKean ML, Smith JB, Silver WJ: Formation of lysophosphatidylcholine in human platelets in response to thrombin. *J Biol Chem* 1981; 256:1522-4
29. Murohara T, Parkinson SJ, Waldman SA, Lefer AM: Inhibition of nitric oxide biosynthesis promotes P-selectin expression in platelets: Role of protein kinase C. *Arterioscler Thromb Vas Biol* 1995; 15:2068-75