

# Cordycepin (3'-deoxyadenosine) inhibits human platelet aggregation in a cyclic AMP- and cyclic GMP-dependent manner

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## Abstract

Cordycepin (3'-deoxyadenosine) is isolated from *Cordyceps militaris*, a species of the fungal genus *Cordyceps*. Cordycepin is an ingredient used in traditional Chinese medicine and is prescribed for various diseases, such as cancer and chronic inflammation. In this study, we investigated the novel effect of cordycepin (3'-deoxyadenosine) on collagen-induced human platelet aggregation. Cordycepin inhibited dose-dependently collagen-induced platelet aggregation in the presence of various concentrations of exogenous CaCl<sub>2</sub>. Of two aggregation-inducing molecules, cytosolic free Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>i</sub>) and thromboxane A<sub>2</sub> (TXA<sub>2</sub>), cordycepin (500 μM) blocked the up-regulation of [Ca<sup>2+</sup>]<sub>i</sub>, by up to 74%, but suppressed TXA<sub>2</sub> production by 46%. Subsequently, Ca<sup>2+</sup>-dependent phosphorylation of both 47-kDa and 20-kDa proteins in collagen-treated platelets was potently diminished by cordycepin. However, upstream pathways for producing these two inducers, such as the activation of phospholipase C-γ2 (PLC-γ2) (assessed by the phosphotyrosine level) and the formation of inositol 1,4,5-trisphosphate (IP<sub>3</sub>), were not altered by cordycepin. Cordycepin increased the level of second messengers adenosine 3',5'-cyclic monophosphate (cAMP) and guanosine 3',5'-cyclic monophosphate (cGMP) in collagen-stimulated platelets. Whereas the NO-sensitive guanylyl cyclase inhibitor ODQ did not alter the cordycepin-induced up-regulation of cGMP, the adenylyl cyclase inhibitor SQ22536 completely blocked the cAMP enhancement mediated by cordycepin, indicating that cordycepin had different modes of action. Therefore, our data suggest that the inhibitory effect of cordycepin on platelet aggregation might be associated with the down-regulation of [Ca<sup>2+</sup>]<sub>i</sub> and the elevation of cAMP/cGMP production.

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**Keywords:** Human platelet aggregation; Cordycepin; cAMP; cGMP

## 1. Introduction

Platelet aggregation is absolutely essential to the formation of a hemostatic plug when normal blood vessels are injured. However, the interaction between platelets and collagen can also cause circulatory disorders, such as thrombosis, atherosclerosis, and myocardial infarction (Schwartz et al., 1990). Inhibition of the platelet-collagen interaction might be a promising approach to the prevention of thrombosis. An important role in the mechanism by which collagen induces platelet aggregation is played by thromboxane A<sub>2</sub> (TXA<sub>2</sub>) formation (Cattaneo et al., 1991), which also contributes to an

increase in cytosolic free Ca<sup>2+</sup> level ([Ca<sup>2+</sup>]<sub>i</sub>) in collagen-activated platelets. It is known that collagen and its related peptide-induced stimulation of platelets activates tyrosine kinase-dependent mechanisms that involve the tyrosine phosphorylation of Syk and phospholipase C-γ2 (PLC-γ2) (Wonerow et al., 2002). PLC-γ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) to inositol 1,4,5-trisphosphate (IP<sub>3</sub>) and diacylglycerol. Moreover, IP<sub>3</sub> causes Ca<sup>2+</sup> mobilization from the endoplasmic reticulum and diacylglycerol activates diacylglycerol-dependent-protein kinase C (C-kinase) (Berridge and Irvine, 1989). An increase in [Ca<sup>2+</sup>]<sub>i</sub> activates both the Ca<sup>2+</sup>/calmodulin-dependent phosphorylation of myosin light chain (20-kDa) and the diacylglycerol-dependent phosphorylation of cytosolic protein (40- or 47-kDa) to induce platelet aggregation (Nishikawa et al., 1980;

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Kaibuchi et al., 1982). In addition, diacylglycerol also can be hydrolyzed by diacylglycerol lipase to produce arachidonic acid (20:4), a precursor of TXA<sub>2</sub>, which is a potent platelet aggregation agent generated from 20:4 liberated when PIP<sub>2</sub> is broken down by collagen, thrombin and ADP (Nishikawa et al., 1980; Kaibuchi et al., 1982; Menshikov et al., 1993). Verapamil and theophylline have an antiplatelet function that elevates the adenosine 3',5'-cyclic monophosphate (cAMP) level, and then decreases the [Ca<sup>2+</sup>]<sub>i</sub>, an essential factor for platelet aggregation. Vasodilators (such as molsidomine and nitroprusside) and guanosine 3',5'-cyclic monophosphate (cGMP) phosphodiesterase (PDE) inhibitors (such as zaprinast and erythro-9-[2-hydroxy-3-nonyl]adenine) elevate cGMP levels in platelets (Menshikov et al., 1993). It is believed that cGMP is produced via the activation of guanylate cyclase in the presence or absence of nitric oxide (NO). NO, synthesized in platelets, decreases agonist-elevated [Ca<sup>2+</sup>]<sub>i</sub> (Pasqui et al., 1991) and has a role in inhibiting platelet activation (Rodomski et al., 1990).

Cordycepin (3'-deoxyadenosine) (Fig. 1) is a nucleoside derivative isolated from *Cordyceps militaris*, a species of the fungal genus *Cordyceps*, which is an ingredient of traditional Chinese medicine and is prescribed for inflammatory and cancer diseases (Cunningham et al., 1951; Ng and Wang, 2005). Cordycepin is indeed known to have anti-tumor effects on cancers of the bladder, colon, lung, and fibrosarcoma (Hubbell et al., 1985) and to inhibit the production of inflammatory mediators (Won and Park, 2005). Adenosine is known to elevate cAMP and to increase cGMP via a mechanism associated with NO synthesis when platelet aggregation is induced by ADP (Anfossi et al., 2002). However, cordycepin, 3'-deoxyadenosine, is known to inhibit adenylate cyclase activity in platelets (Haslam et al., 1978; Londos and Wolff, 1977), and thus the elevation of cAMP would not be expected. A cordycepin analogue, 2',5'-dideoxyadenosine, does not affect on the inhibition of platelet aggregation, and the production of cGMP or cAMP is not altered by this analogue during collagen-induced

platelet aggregation (Jang et al., 2002). Cordycepin has also been reported to inhibit cAMP-dependent protein kinase (PKA) in bovine heart as well as cGMP-dependent protein kinase (PKG) in fetal guinea pig *in vitro* (Glazer and Kuo, 1977). In general, the inhibition of PKA or PKG is closely associated with the stimulation of platelet aggregation.

However, in the present study we report that cordycepin strongly inhibits [Ca<sup>2+</sup>]<sub>i</sub> elevation and TXA<sub>2</sub> production, and simultaneously increases the intracellular levels of cAMP and cGMP without having an inhibitory effect on IP<sub>3</sub> production or PLC-γ2 activation in collagen-induced human platelet aggregation.

## 2. Materials and methods

### 2.1. Materials

Cordycepin (Fig. 1, which also shows other structurally related cGMP PDE inhibitor [EHNA: Erythro-9-(2-hydroxy-3-nonyl)adenine] and ADP receptor (P2Y1/P2Y12) antagonists [Dickinson et al., 1997; Xu et al., 2002]) from *C. militaris* was purchased from the Sigma Chemical Co. (St. Louis, USA), and collagen was obtained from the Chrono-Log Corporation (Havertown, PA). Protein molecular weight standards were obtained from Bio-Rad Laboratories (Richmond, CA). Anti-phospho-PLC-γ2 Ab and anti-phosphotyrosine mAb were acquired from Santa Cruz Biotechnology (Santa Cruz, CA). 9-(tetrahydro-2'-furyl)adenine (SQ22536) and 1H-[1,2,4]oxadiazolo[4,3a]quinoxaline-1-one (ODQ) were purchased from Calbiochem (La Jolla, CA). Fura 2-AM, zaprinast and other reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Carrier-free phosphorus-32 for protein phosphorylation, [<sup>3</sup>H]-D-*myo*-IP<sub>3</sub>-, [<sup>3</sup>H-cyclic AMP]-, [<sup>3</sup>H-cyclic GMP]-radioimmunoassay kits, and a TXB<sub>2</sub> EIA system were obtained from Amersham Bioscience (Buckinghamshire, UK).

### 2.2. Preparation of washed human platelets

Blood was drawn from the antecubital vein of normal healthy human volunteers and anticoagulated with ACD solution (0.8% citric acid, 2.2% sodium citrate, 2.45% glucose). Platelet-rich plasma was centrifuged at 125 ×g for 10 min to remove the red blood cells, and the platelets were washed twice with washing buffer (138 mM NaCl, 2.7 mM KCl, 12 mM NaHCO<sub>3</sub>, 0.36 mM NaH<sub>2</sub>PO<sub>4</sub>, 5.5 mM glucose, and 1 mM EDTA, pH 6.5). The washed platelets were then suspended in suspending buffer (138 mM NaCl, 2.7 mM KCl, 12 mM NaHCO<sub>3</sub>, 0.36 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.49 mM MgCl<sub>2</sub>, 5.5 mM glucose, 0.25% gelatin, pH 6.9) (Rittenhouse-Simmons and Deykin, 1976) to a final concentration of 5 × 10<sup>8</sup>/ml. All of the procedures above were carried out at 25 °C to avoid platelet aggregation on cooling.

### 2.3. Measurement of platelet aggregation

Washed platelets (10<sup>8</sup>/ml) were preincubated for 3 min at 37 °C in the presence of various concentrations of exogenous

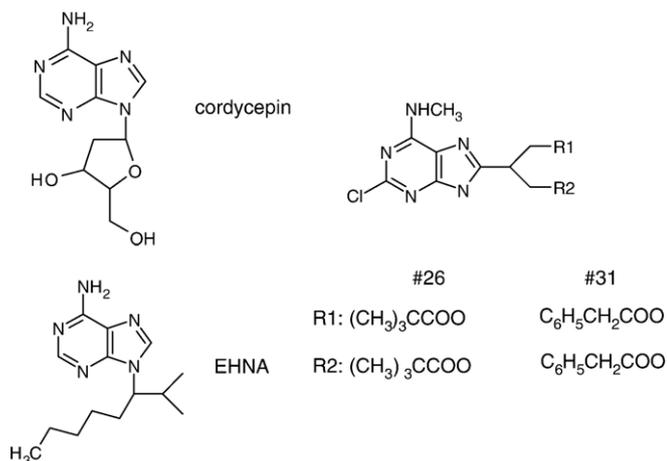


Fig. 1. Chemical structures of cordycepin and its structurally similar compounds derived from adenine. EHNA: Erythro-9-(2-hydroxy-3-nonyl) adenine; compound #26: 2,2-Dimethyl-propionic Acid 3-(2-chloro-6-methylamino-purin-9-yl)-2-(2,2-dimethyl-propionyloxymethyl)-propyl Ester; and compound #31: Phenylacetic Acid 3-(2-chloro-6-methylamino-purin-9-yl)-2-phenylacetoxymethyl-propyl Ester (Xu et al., 2002).

CaCl<sub>2</sub> with or without cordycepin and then stimulated with 10 µg of collagen/ml for 5 min. Aggregation was monitored using an aggregometer (Chrono-Log, Corp., Havertown, PA) at a constant stirring speed of 1000 rpm. Each aggregation rate was evaluated as an increase in light transmission. The suspending buffer was used as reference (transmission 0%). Cordycepin was dissolved in platelet-suspending buffer (pH 6.9).

#### 2.4. Determination of [Ca<sup>2+</sup>]<sub>i</sub>

Platelet-rich plasma was incubated with 5 µM fura 2-AM at 37 °C for 60 min. Because fura 2-AM is light-sensitive, the tube containing the platelet-rich plasma was covered with aluminum foil during loading. The fura 2-loaded washed platelets were also prepared using the procedure described above. Fura 2-loaded washed platelets (10<sup>8</sup>/ml) were preincubated for 3 min at 37 °C with various concentrations of cordycepin in the presence of 2 mM CaCl<sub>2</sub> and then stimulated with 10 µg of collagen/ml for 5 min for evaluation of [Ca<sup>2+</sup>]<sub>i</sub>. Fura 2 fluorescence was measured with a spectrofluorimeter (SFM 25, Bio-Tek Instrument, Italy) with an excitation wavelength that changed every 0.5 s from 340 nm to 380 nm; the emission wavelength was set at 510 nm. The [Ca<sup>2+</sup>]<sub>i</sub> values were calculated using the method of Schaeffer (Schaeffer and Blaustein, 1989).

#### 2.5. Measurement of cAMP and cGMP

Washed platelets (10<sup>9</sup>/ml) were preincubated for 3 min at 37 °C with various concentrations of cordycepin or other factors such as SQ22536, ODQ, and zaprinast in the presence of 2 mM CaCl<sub>2</sub>, and then stimulated with 10 µg of collagen/ml for 5 min for platelet aggregation. The aggregation was terminated by adding 80% ice-cold ethanol. cAMP and cGMP were measured using the respective radioimmunoassay kits of [<sup>3</sup>H-cAMP] and [<sup>3</sup>H-cGMP]. Cordycepin and SQ22536 were dissolved in platelet suspending buffer (pH 6.9). Because ODQ and zaprinast were dissolved in 0.007% dimethylsulfoxide (DMSO), the levels of cAMP and cGMP were calculated by subtracting the effect of DMSO (0.007%).

#### 2.6. Determination of protein phosphorylation

Protein phosphorylation was carried out according to the method of Laemmli (Laemmli, 1970). Washed platelets (10<sup>8</sup>/ml) were suspended in Tris buffer (10 mM Tris-hydroxymethyl-aminomethane, 129 mM sodium chloride, 10.9 mM sodium citrate, tribasic, 8.9 mM sodium bicarbonate, 1 mg/ml dextrose, 2.8 mM potassium chloride, pH 6.5), and then incubated for 60 min at 37 °C with phosphorus-32 (0.5 mCi/ml). [<sup>32</sup>P]-labeled platelets (10<sup>8</sup>/ml) were preincubated with or without cordycepin in the presence of 2 mM CaCl<sub>2</sub> at 37 °C for 3 min, and then collagen (10 µg/ml) was added for 5 min to trigger protein kinase activation. Activation was terminated by adding an equal volume of 2 × sodium-dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) buffer (0.125 M Tris, 4% SDS, 20% glycerol, 5% mercaptoethanol, 0.01% bromphenol blue, pH

6.8). Samples were boiled to completely denature the proteins for 5 min, and then 50 µg proteins were taken from each reaction tube and subjected to SDS-PAGE (11%, 1.0 mM gel). The gels were then dried, and the relative intensity of the phosphoproteins was analyzed using a Storage Phospho System (Cyclone, A Packard Bioscience Company, USA).

#### 2.7. Measurement of TXB<sub>2</sub>

Washed platelets (10<sup>8</sup>/ml) were then preincubated with or without cordycepin for 3 min in the presence of 2 mM CaCl<sub>2</sub>, and activated for 5 min with 10 µg/ml of collagen. The reactions were terminated by adding ice-cold 5 mM EDTA and 0.2 mM indomethacin. The amount of TXB<sub>2</sub>, a stable metabolite of TXA<sub>2</sub>, was determined using a TXB<sub>2</sub> EIA kit. To determine the direct effects of cordycepin on 20:4 metabolism, the cells were first sonicated with a sonicator (Bandelin, HD2070, Germany) to obtain platelet lysates. The platelet lysates were incubated with various concentrations of cordycepin for 5 min, and then 100 pmol of 20:4 was added to 200 µl of the lysate. The lysate mixtures were incubated further for 10 min, and the amount of TXB<sub>2</sub> was determined as described above.

#### 2.8. Immunoprecipitation of PLC-γ2

Washed platelets (10<sup>8</sup>/ml) were preincubated for 3 min with or without various concentrations of cordycepin in the presence of 2 mM CaCl<sub>2</sub> and then were stimulated with 10 µg/ml of collagen for 5 min or different times (0.5, 1, 5 min) at 37 °C. The reactions were terminated by adding an equal volume of ice-cold lysis buffer (2% Triton X-100, 100 mM Tris-HCl, 2 mM EGTA, 2 mM vanadate, 1 mM PMSF, 100 µg/ml leupeptin, pH 7.2). The lysates were then sonicated with a sonicator (Bandelin, HD2070, Germany) and centrifuged at 16,000 ×g for 20 min. The soluble fraction was precleared with protein A-Sepharose beads for 30 min. The cleared supernatant was then incubated with 1 µg of polyclonal anti-PLC-γ2 antibody for 1 h, and the immune complex was precipitated with protein A-Sepharose beads for 1 h at 4 °C with 100 µl of protein A-Sepharose (50 mg/ml stock solution). After the mixture had been rotated for 1 h at 4 °C, the Sepharose beads were washed three times with lysis buffer and once with 10 mM Hepes buffer (pH 7.5). Finally, 50 µl of Hepes buffer and 25 µl of SDS-PAGE buffer (pH 6.8) were added to the beads, and the proteins were eluted by boiling for 3 min. The proteins were then separated by SDS-PAGE (8%) under reducing conditions and transferred onto a nitrocellulose membrane. Tyrosine phosphorylation of PLC-γ2 was detected by Western blotting, using an anti-phosphotyrosine mAb, 4G10.

#### 2.9. Measurement of IP<sub>3</sub>

Washed platelets (10<sup>8</sup>/ml) were preincubated with or without cordycepin in the presence of 2 mM CaCl<sub>2</sub>, and were activated with 10 µg/ml of collagen for 5 min at 37 °C, and an equal volume of ice-cold 15% (w/v) trichloroacetic acid (TCA) was added to the platelet suspension to terminate the reaction. The

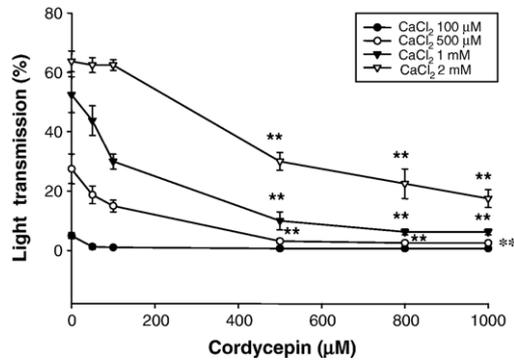


Fig. 2. Effects of cordycepin pretreatment on collagen-induced platelet aggregation by exogenous  $\text{CaCl}_2$ . Washed platelets ( $10^8/\text{ml}$ ) were preincubated with various concentrations of cordycepin in the presence of  $\text{CaCl}_2$  for 3 min at  $37^\circ\text{C}$ , and then stimulated with collagen ( $10\ \mu\text{g}/\text{ml}$ ) for 5 min. The dose responsive effects of cordycepin (0.1, 0.5, 1, and 2 mM) of  $\text{CaCl}_2$ . Platelet aggregation (%) was recorded as an increase in light transmission. Inhibition by cordycepin was recorded as percentage of the collagen-induced aggregation rate. Data are expressed as means  $\pm$  S.D. ( $n=4$ ). \*\*:  $p<0.01$  compared with collagen alone.

mixtures were then kept on ice for 30 min, centrifuged at  $2000 \times g$  for 15 min at  $4^\circ\text{C}$ , and the supernatant was treated five times with 5 ml of water-saturated diethyl ether to extract  $\text{IP}_3$ . Residual ether was removed under a flow of  $\text{N}_2$  flow. The samples were neutralized by titration with  $\text{NaHCO}_3$ , and the amount of  $\text{IP}_3$  was determined using a [ $^3\text{H}$ ]-D-*myo*- $\text{IP}_3$  assay kit.

### 2.10. Statistical analysis

All data are shown as means  $\pm$  S.D. Student's *t*-test was used for data analysis and paired or unpaired comparison was used where necessary.

## 3. Results

### 3.1. Associated effects of cordycepin and exogenous $\text{Ca}^{2+}$ on platelet aggregation

The concentration of collagen that induced maximal platelet aggregation was approximately  $10\ \mu\text{g}/\text{ml}$  (Cho et al., 2004). Therefore,  $10\ \mu\text{g}$  of collagen/ml was used as a platelet agonist in this study. When washed human platelets ( $10^8/\text{ml}$ ) were activated with  $10\ \mu\text{g}$  of collagen/ml, the light transmission, as an indication of the rate of aggregation, was found to be dependent on the addition of various concentrations of exogenous  $\text{CaCl}_2$  (100  $\mu\text{M}$ , 500  $\mu\text{M}$ , 1 mM, and 2 mM) (Fig. 2). This indicates that platelet aggregation by collagen required extracellular  $\text{Ca}^{2+}$ . However, various concentrations of cordycepin (50, 100, 500, 800, and 1000  $\mu\text{M}$ ) significantly reduced the  $10\ \mu\text{g}/\text{ml}$  collagen-induced platelet aggregation in a dose dependent manner. The extent of the inhibitory activity of cordycepin was dependent on the concentration of  $\text{CaCl}_2$  (0.1, 0.5, 1, and 2 mM) (Fig. 2). The half-maximal inhibitory concentration ( $\text{IC}_{50}$ ) of cordycepin in the presence of 2 mM  $\text{CaCl}_2$  was 500  $\mu\text{M}$ .

### 3.2. Effects of cordycepin on the up-regulation of aggregation-inducing molecules, $[\text{Ca}^{2+}]_i$ and $\text{TXA}_2$

The  $[\text{Ca}^{2+}]_i$  level in response to various concentrations of cordycepin (50, 100, and 500  $\mu\text{M}$ ) in intact platelets ( $10^8/\text{ml}$ ) was  $136.4 \pm 6.8$  nM (at 50  $\mu\text{M}$  of cordycepin),  $135.3 \pm 7.9$  nM (100  $\mu\text{M}$ ), and  $130.4 \pm 5.3$  nM (500  $\mu\text{M}$ ), respectively, which was not significantly different from that ( $134.1 \pm 8.6$  nM) in resting platelets. As shown in Fig. 3A, when the washed platelets ( $10^8/\text{ml}$ ) were stimulated by collagen ( $10\ \mu\text{g}/\text{ml}$ ), the level of  $[\text{Ca}^{2+}]_i$  increased from 134 to 771 nM. However, this was significantly reduced by various concentrations (50, 100, and 500  $\mu\text{M}$ ) of cordycepin in a dose-dependent manner (74% inhibition at 500  $\mu\text{M}$ ), suggesting that the inhibitory activity of cordycepin on collagen-induced platelet aggregation was due to lowering of the level of  $[\text{Ca}^{2+}]_i$ .

$\text{TXA}_2$  is a potent stimulus of platelet aggregation, and its receptor G-protein (Gq)-PLC- $\text{IP}_3$  signaling pathway is activated by collagen treatment (Wang et al., 1998). Therefore, we next

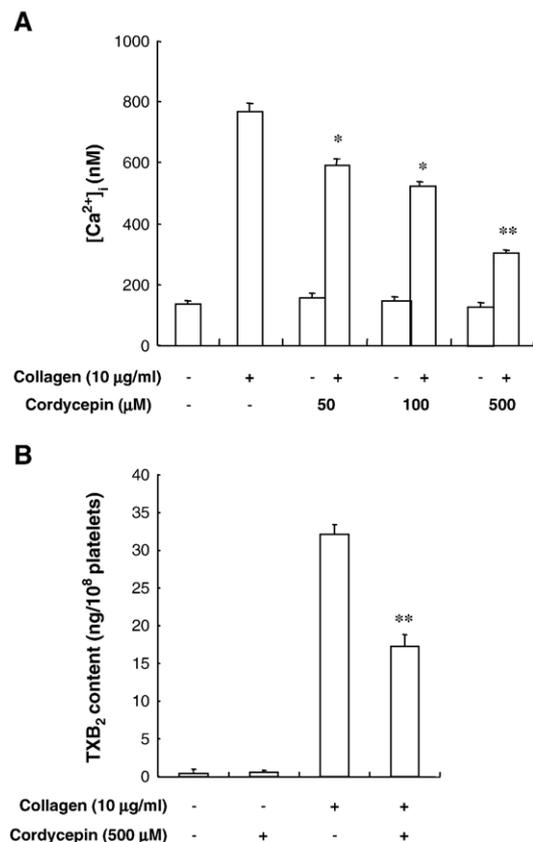


Fig. 3. Effects of cordycepin on collagen-induced  $\text{Ca}^{2+}$  mobilization and  $\text{TXB}_2$  production. (A) Effects of cordycepin on collagen-induced  $[\text{Ca}^{2+}]_i$  mobilization. Fura 2-loaded platelets ( $10^8/\text{ml}$ ) were preincubated with various concentrations of cordycepin in the presence of 2 mM  $\text{CaCl}_2$  for 3 min at  $37^\circ\text{C}$ , and then collagen ( $10\ \mu\text{g}/\text{ml}$ ) was added.  $[\text{Ca}^{2+}]_i$  was determined as described in "Materials and methods". (B) Effects of cordycepin (500  $\mu\text{M}$ ) on the production of  $\text{TXB}_2$  induced by collagen. Washed platelets ( $10^8/\text{ml}$ ) were preincubated with cordycepin (500  $\mu\text{M}$ ) for 3 min in the presence of 2 mM  $\text{CaCl}_2$ , and then stimulated with collagen ( $10\ \mu\text{g}/\text{ml}$ ). The content of  $\text{TXB}_2$  was measured using a  $\text{TXB}_2$  EIA kit. Data are expressed as means  $\pm$  S.D. ( $n=4$ ). \*:  $p<0.05$  and \*\*:  $p<0.01$  compared with collagen alone.

examined whether cordycepin blocked the production of TXA<sub>2</sub> under collagen exposure. The TXA<sub>2</sub> (determined as TXB<sub>2</sub>) level in intact platelets was 0.4 ng/10<sup>8</sup> platelets, and this was markedly increased to 32.1 ng/10<sup>8</sup> platelets in the collagen-stimulated platelets (Fig. 3B). However, cordycepin (500 μM) significantly reduced the production of TXA<sub>2</sub> to 17.3±1.5 ng/10<sup>8</sup> platelets (to 46%), although the level of TXA<sub>2</sub> was still up to 40-fold higher than the basal level (0.4±0.1 ng/10<sup>8</sup> platelets; Fig. 3B). To determine if the inhibitory effect on TXA<sub>2</sub> release of cordycepin was due to the direct suppression of cyclooxygenase-1 (COX-1) or TXA<sub>2</sub> synthetase, cell-free enzyme assay method was used. When platelet lysates were incubated with or without cordycepin for 5 min at 37 °C in the presence of AA (20:4), a substrate of COX-1, cordycepin (50 to 500 μM) had no significant effect on TXA<sub>2</sub> production (data not shown), suggesting that the decrease in TXA<sub>2</sub> production by cordycepin possibly is not directly related to inhibition of its metabolic enzyme, COX-1 or TXA<sub>2</sub> synthetase.

### 3.3. Effects of cordycepin on Ca<sup>2+</sup>-dependent protein phosphorylation and upstream pathway (PLC-γ2 activation) for TXA<sub>2</sub>

To determine the mechanism underlying cordycepin-mediated inhibition of platelet aggregation, downstream or upstream pathways involving Ca<sup>2+</sup> and TXA<sub>2</sub> were investigated. It is known that 40-kDa (or 47-kDa) and 20-kDa polypeptides are phosphorylated by Ca<sup>2+</sup>-dependent protein kinase C (C-kinase) and Ca<sup>2+</sup>/calmodulin-dependent protein kinase (CaM-PK) during platelet aggregation (Nishikawa et al., 1980). The phosphorylation of these proteins participates in the release of platelet aggregation factors such as serotonin and ADP (Nishikawa et al., 1980). Under our conditions, collagen (10 μg/ml) significantly stimulated the phosphorylation of the 47-kDa protein *via* C-kinase activation as well as the 20-kDa protein *via* CaM-PK activation (Fig. 4A—lane 2). However, this phosphorylation was significantly inhibited by cordycepin (500 μM), as expected (Fig. 4A—lane 3).

Furthermore, TXA<sub>2</sub> production and Ca<sup>2+</sup> increase are initialized by the activity of Syk protein kinases and their substrate protein PLC-γ2 by increasing diacylglycerol and IP<sub>3</sub> levels when platelets are activated by collagen (Berridge and Irvine, 1989; Wonerow et al., 2002). To verify the involvement of the pathway, we further examined the regulatory effect of cordycepin on Syk-PLC-γ2 linked pathways. Since PLC-γ2 activation can be evaluated by determining its tyrosine phosphorylation level, we investigated whether cordycepin modified the collagen-induced tyrosine phosphorylation of PLC-γ2. When collagen-induced tyrosine phosphorylation of PLC-γ2 was evaluated, PLC-γ2 appeared to be tyrosine-phosphorylated 30 s after stimulation with collagen (data not shown). However, because the maximal level of platelet aggregation was evaluated 5 min after collagen was administered, the effect of cordycepin on the tyrosine phosphorylation of PLC-γ2 was also determined 5 min after collagen administration. Although cordycepin inhibited platelet aggregation, Ca<sup>2+</sup>-mobilization, and TXA<sub>2</sub> production in the

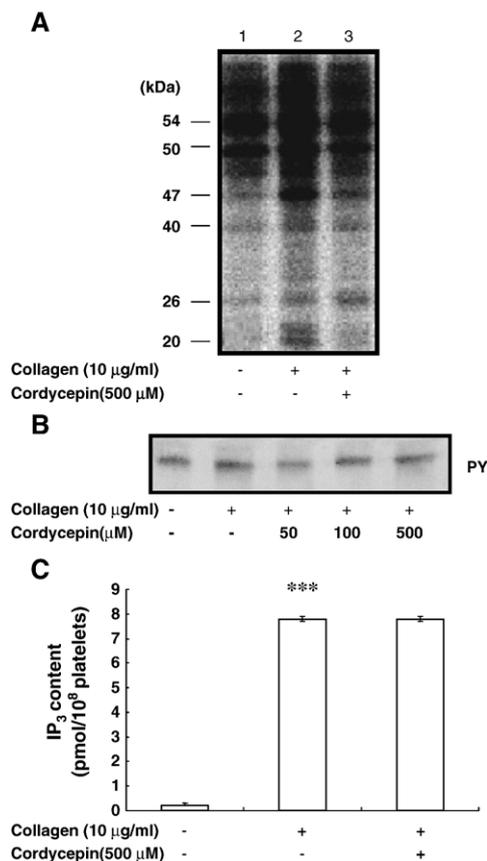


Fig. 4. Effects of cordycepin on upstream and downstream cellular events of Ca<sup>2+</sup> mobilization and TXB<sub>2</sub> production. (A) Effects of cordycepin on protein phosphorylation in collagen-induced platelets. Protein phosphorylation was performed as described in “Materials and methods”. <sup>32</sup>P-labeled platelets (10<sup>8</sup>/ml) were preincubated with cordycepin (500 μM) for 3 min in the presence of 2 mM CaCl<sub>2</sub> and then stimulated with collagen (10 μg/ml). Platelet proteins were analyzed by SDS-PAGE (11% polyacrylamide gel). Lane 1: Intact platelets as control, lane 2: collagen (10 μg/ml), lane 3: cordycepin (500 μM)+collagen (10 μg/ml). (B) Effects of cordycepin on PLC-γ2 tyrosine phosphorylation induced by collagen. Washed platelets (10<sup>8</sup>/ml) were preincubated with various concentrations of cordycepin for 3 min in the presence of 2 mM CaCl<sub>2</sub> and then stimulated with collagen (10 μg/ml). The reactions were terminated by adding lysis buffer, and the lysates were immunoprecipitated with anti-PLC-γ2 pAb and fractionated on 8% SDS-PAGE, followed by Western immunoblotting with anti-phosphotyrosine antibody. (C) Effects of cordycepin on IP<sub>3</sub> production induced by collagen. Washed platelets (10<sup>8</sup>/ml) were preincubated with cordycepin (500 μM) for 3 min in the presence of 2 mM CaCl<sub>2</sub> and then stimulated with collagen (10 μg/ml). The reactions were terminated by adding an equal volume of ice-cold 15% TCA. IP<sub>3</sub> content was measured using a [<sup>3</sup>H]-D-myo-IP<sub>3</sub> radioimmunoassay kit. Data are expressed as means±S.D. (n=4). \*\*\*: p<0.001 compared with normal.

presence of collagen, the tyrosine phosphorylation of PLC-γ2 was not affected by cordycepin (Fig. 4B). To confirm the activity of PLC-γ2 we also investigated whether cordycepin had an inhibitory effect on the production of IP<sub>3</sub> as a result of PLC-γ2 activation in collagen-induced platelet activation. The IP<sub>3</sub> level in intact platelets was 0.2 pmol/10<sup>8</sup> platelets. When platelets (10<sup>8</sup>/ml) were stimulated with collagen (10 μg/ml), IP<sub>3</sub> was potently up-regulated to 7.8 pmol/10<sup>8</sup> platelets (Fig. 4C). However, the IP<sub>3</sub> up-regulation by collagen was not altered by cordycepin (500 μM) (Fig. 4C). These results suggest that the cordycepin-sensitive targets in suppressing TXA<sub>2</sub> production

and  $[Ca^{2+}]_i$  increase are not included in their upstream pathways.

### 3.4. Effects of cordycepin on the formation of cAMP and cGMP

On the basis of previous reports in which structurally similar compounds (Fig. 1) were reported to increase intracellular levels of cAMP/cGMP, endogenous negative regulators of platelet functions, *via* the suppression of cGMP PDE and the interaction of  $G_i$  with ADP receptor (P2Y1/P2Y12) (Dickinson et al., 1997; Xu et al., 2002; Dorsam and Kunapuli, 2004), we next investigated whether cordycepin up-regulated the cellular level of cAMP/cGMP. As shown in Fig. 5A, cordycepin dose dependently increased the cAMP level in the presence of collagen, to 2.2-fold from basal ( $3.4 \text{ pmol}/10^9$  platelets), but not the absence of collagen. The increased level of cAMP was obliterated by treatment with the adenylate cyclase inhibitor SQ22536 ( $10 \mu\text{M}$ ) (Fig. 5B), suggesting that cordycepin-mediated cAMP enhancement might be mediated by the activity of adenylate cyclase. In contrast with the change in cAMP level, cordycepin alone increased the level of cGMP by  $2.1 \pm 0.2$  (at  $50 \mu\text{M}$  of cordycepin),  $2.6 \pm 0.7$  ( $100 \mu\text{M}$ ), and  $3.8 \pm 0.1 \text{ pmol}/10^9$  platelets ( $500 \mu\text{M}$ ), respectively. In collagen-activated

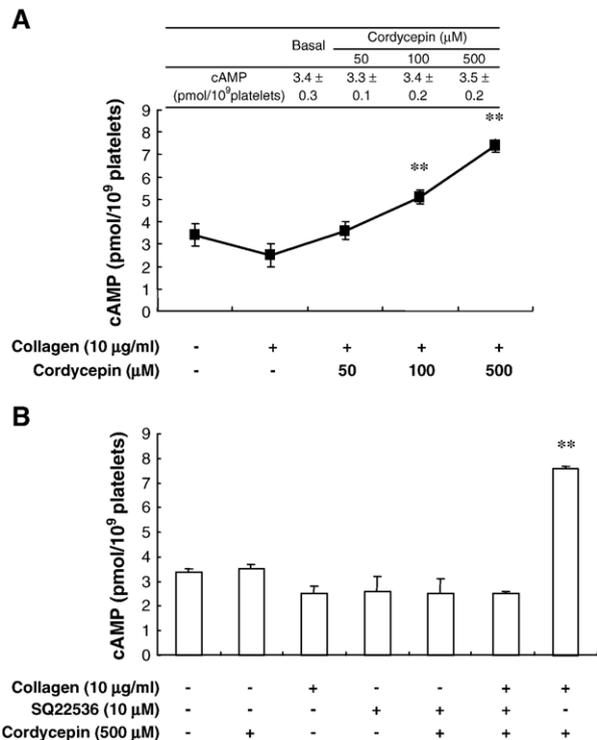


Fig. 5. Effects of cordycepin on cAMP production in resting (table insert) and collagen-stimulated platelets. Washed platelets ( $10^9/\text{ml}$ ) were preincubated with or without cordycepin or other factors for 3 min in the presence of  $2 \text{ mM CaCl}_2$  and then stimulated with collagen ( $10 \mu\text{g}/\text{ml}$ ) for 5 min at  $37^\circ\text{C}$ . The reactions were terminated by adding 80% ice-cold ethanol. cAMP content was measured using  $[^3\text{H-cAMP}]$  radioimmunoassay kits. (A) Effects of cordycepin on cAMP production in resting or collagen-stimulated platelets. (B) Effects of cordycepin with SQ22536 on cAMP production in collagen-stimulated platelets. SQ22536, adenylate cyclase inhibitor. Data are expressed as means  $\pm$  S.D. ( $n=4$ ). \*\*:  $p < 0.01$  compared with normal.

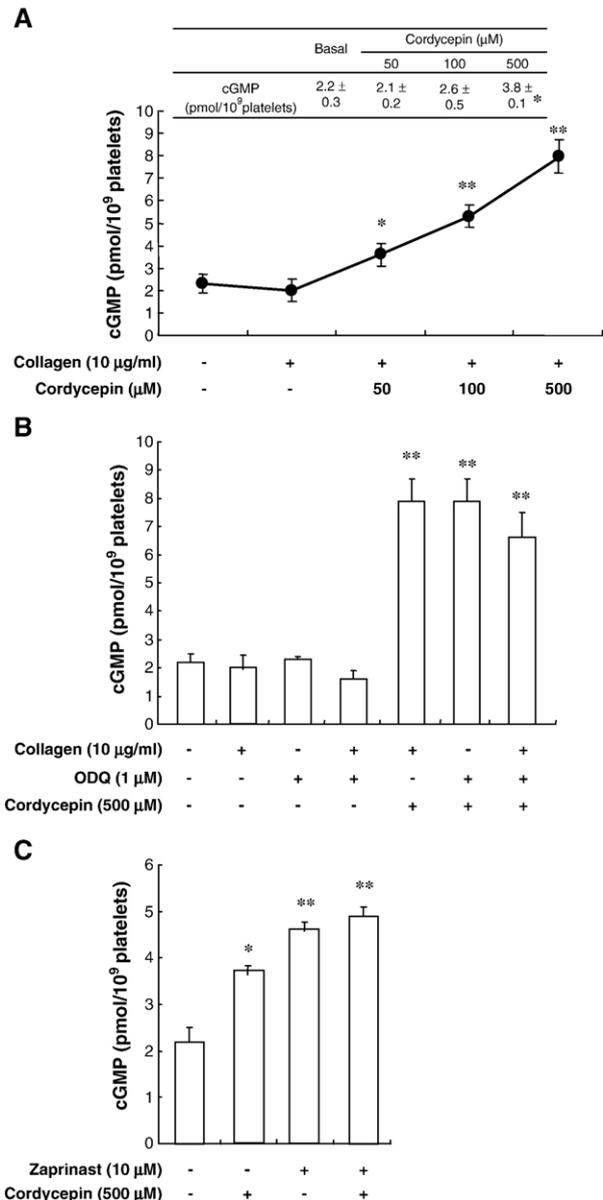


Fig. 6. Effects of cordycepin on cGMP production in resting (table insert) and collagen-stimulated platelets. Washed platelets ( $10^9/\text{ml}$ ) were preincubated with or without cordycepin or other factors for 3 min in the presence of  $2 \text{ mM CaCl}_2$  and then stimulated with collagen ( $10 \mu\text{g}/\text{ml}$ ) for 5 min at  $37^\circ\text{C}$ . The reactions were terminated by adding 80% ice-cold ethanol. cGMP content were measured using  $[^3\text{H-cGMP}]$  radioimmunoassay kits. (A) Effects of cordycepin only on cGMP production in resting or collagen-stimulated platelets. (B) Effects of cordycepin with NO-sensitive guanylate cyclase inhibitor ODQ on cGMP production in collagen-stimulated platelets. (C) Effects of cordycepin with PDE5 inhibitor zaprinast on cGMP production in collagen-stimulated platelets. Data are expressed as means  $\pm$  S.D. ( $n=4$ ). \*:  $p < 0.05$  and \*\*:  $p < 0.01$  compared with normal.

platelets, cordycepin ( $50$  to  $500 \mu\text{M}$ ) progressively increased the cGMP level from  $3.5 \pm 0.1$  to  $7.9 \pm 0.7 \text{ pmol}/10^9$  platelets in comparison with the control levels ( $2.1 \pm 0.2 \text{ pmol}/10^9$  platelets) (Fig. 6A). Because platelet guanylate cyclase is activated by NO (Homer and Wanstall, 2002), we investigated whether the NO-sensitive guanylate cyclase inhibitor ODQ ( $1 \mu\text{M}$ ) was able to block the up-regulation of intracellular cGMP. Fig. 6B,

however, shows that ODQ did not block the cGMP increase, indicating that cordycepin-induced cGMP enhancement might be independent of NO-sensitive guanylate cyclase activity. Moreover, there was no drastic increase in NO level following cordycepin exposure either (data not shown). Finally, the possibility that cordycepin can act as a cGMP PDE inhibitor was explored, according to a method outlined in a previous report (Dickinson et al., 1997). As Fig. 6C depicts, cotreatment with cordycepin and a higher concentration (10  $\mu$ M) of zaprinast, a cGMP-specific PDE V inhibitor, did not cause a remarkable up-regulation of cGMP level (4.8 pmol) compared with zaprinast alone (4.6 pmol) or the combination of 3  $\mu$ M of zaprinast and cordycepin (500  $\mu$ M) (data not shown). Considering that the maximum level of cGMP in response to zaprinast is 6.9 pmol of cGMP at the micromolar level in a cellular system (data not shown), our data suggest that these two inhibitors might share the same inhibitor binding pocket of PDE (Yoo et al., 2004). However, because using enzymatic inhibitors in cellular system does not give us precise information, we cannot exclude the possibility that zaprinast and cordycepin might have different binding pockets. Therefore, to verify the exact pharmacological features of cordycepin with regard to cGMP PDE, a direct competitive binding assay using purified enzyme will be used in a future experiment.

#### 4. Discussion

Of several aggregation-inducing molecules,  $\text{Ca}^{2+}$  and  $\text{TXA}_2$  are known to be essential for platelet aggregation (Charo et al., 1977). Cordycepin significantly blocked the action of these two molecules in collagen-activated platelets and the blocking of  $\text{Ca}^{2+}$  release seemed to be critical to the cordycepin-mediated inhibition of platelet aggregation (Fig. 3). Collagen-activated platelets require an adequate concentration of intracellular  $\text{Ca}^{2+}$  for aggregation, because the formation of platelet is accompanied by the migration of platelets and their adhesion. Aggregation events are reported to be critically regulated by cytoskeleton rearrangement, for which an increased activity of C-kinase and CaM-PK is essential (Waldmann et al., 1987; Eigenthaler et al., 1992). Indeed, exposure of platelets to collagen up-regulated the phosphorylation level of 40-kDa (or 47-kDa) and 20-kDa proteins (Fig. 4A—lane 2), which are substrate proteins of  $\text{Ca}^{2+}$ -dependent kinases, and of course this up-regulation was strongly diminished by cordycepin treatment (Fig. 4A—lane 3).

In the case of  $\text{TXA}_2$ , cordycepin suppressed collagen-induced production by 46% at 500  $\mu$ M, and by contrast, it blocked  $\text{Ca}^{2+}$  release by 74%. Additionally, the decreased level of  $\text{TXA}_2$  was 40-fold higher than under the basal level, suggesting that  $\text{TXA}_2$  inhibition itself might not be a critical cordycepin-inhibitory parameter. This suggestion is supported by several lines of evidence that 1] cordycepin did not affect the activation of PLC- $\gamma$ 2 (Fig. 4B), 2] there was no significant alteration of  $\text{IP}_3$  or diacylglycerol production by cordycepin (Fig. 4C), and 3] cordycepin did not block  $\text{TXA}_2$  formation from 20:4 *via*  $\text{TXA}_2$  synthetase/COX-1 in a cell-free system (data not shown). However, we cannot exclude the possibility that cordycepin can affect PLC $\gamma$ 2-independent (*e.g.*, PLC- $\beta$ )

$\text{TXA}_2$  biosynthesis pathways, as suggested in a previous study (Brass et al., 1997). Otherwise, 20:4 generated by the diacylglycerol lipase/monoacylglycerol lipase pathway is potentially the indirect target enzyme, since the enzymes of that pathway require cytosolic-free  $\text{Ca}^{2+}$  for activity (Rodomski et al., 1990). In particular, the fact that the  $\text{IP}_3$  level was not changed by cordycepin treatment led us to the assumption that there might be a significant interruption of the  $\text{Ca}^{2+}$  release pathway followed by an increased  $\text{IP}_3$  level. The most likely candidate seems to be the  $\text{IP}_3$  receptor level, according to previous studies in which several compounds blocked the function of the  $\text{IP}_3$  receptor *via* PKA/PKG-mediated phosphorylation (Homer and Wanstall, 2002; Park et al., 2004). In fact, compounds (such as cilostazol, EHNA [Fig. 1] and dipyridamole) with this pharmacological property have been shown to increase intracellular cAMP and cGMP levels (Sudo et al., 2003; Park et al., 2004).

Like such compounds, cordycepin acted as a strong intracellular inducer of platelet cAMP/cGMP, endogenous negative regulators of platelet aggregation (Qi et al., 1996; Homer and Wanstall, 2002; Park et al., 2004), in the presence of collagen (Figs. 5A and 6A). The levels of intracellular cAMP/cGMP are regulated by the balance between cyclic nucleotide-producing enzymes, adenylate/guanylate cyclases, and hydrolyzing enzymes, cAMP/cGMP PDEs, to regulate their downstream enzyme (PKA/PKG) activity. The next question therefore arose: what is the mechanism by which cordycepin caused up-regulation of the levels of intracellular cAMP/cGMP. To answer this question, two cyclic nucleotide-producing cyclase inhibitors (SQ22536, an adenylate cyclase inhibitor, and ODQ, a soluble guanylate cyclase inhibitor) were used. Figs. 5B and 6B show that the cordycepin-mediated cAMP enhancement might be adenylate cyclase-dependent, whereas that of cGMP is not. Thus, SQ22536 (but not ODQ) prevented the increase in intracellular cAMP (but not cGMP). Furthermore, there was no remarkable production of NO, a known activator of soluble guanylate cyclase in platelets, by cordycepin (data not shown), although the cAMP level was increased and consequently activation of PKA-dependent NO synthase was to be expected (Russo et al., 2004). These findings therefore seem to suggest that cGMP is up-regulated by cordycepin in a hydrolyzing-enzyme (PDE) dependent manner. Indeed, structurally related compounds (Fig. 1) are known to be cGMP PDE inhibitors and co-treatment with cordycepin and the cGMP PDE inhibitor zaprinast appeared to be effective in the inhibitor binding pocket of the PDE, so that the increasing effect of cGMP was not altered (Fig. 6C), as shown to be the case with other PDE IV inhibitors (Yoo et al., 2004). In this regard, the possibility that cordycepin affects cAMP PDE activity cannot be excluded. However, considering that PDE inhibitors or cyclic nucleotide cyclase activators generally are capable of enhancing basal cyclic nucleotide levels (Yu et al., 1996), cordycepin might act not as a cAMP-PDE inhibitor or cyclase activator, but as another agonist or antagonist involved in the regulation of the cAMP level, unlike the case of cGMP.

Recently the Jacobson group published interesting data for numerous derivatives synthesized from compounds structurally

similar to cordycepin. They found that these compounds (#29 and #31 in Fig. 1) very strongly blocked platelet aggregation by blocking the function of ADP receptors (P2Y) (Xu et al., 2002), which are highly distributed in platelets (Hechler et al., 2005). Furthermore, they also found that these compounds did not increase the cAMP level in the absence of an aggregation inducer (Xu et al., 2002), suggesting that this class of antagonists might not directly affect the activity of adenylate cyclase and cAMP PDE, as cordycepin did. ADP receptors are activated by ADP released from activated platelets after TXA<sub>2</sub> release and are associated with an inhibitory G protein, the G<sub>i</sub> protein, which blocks the activation of adenylate cyclase (Hechler et al., 2005). Under these conditions, activated platelets are found to lower the negative endogenous cAMP level, evoking activation pathways involved in platelet aggregation. The increased level of cAMP (as well as cGMP) participates in activating PKA (and PKG) and consequently this enzyme phosphorylates its substrate proteins, resulting in negative regulation of platelet aggregation. The negatively regulated substrate proteins include vasodilator-stimulated phosphoprotein, a regulator of actin dynamics (Sudo et al., 2003), IP<sub>3</sub> receptor (Komalavilas and Lincoln, 1994) and TXA<sub>2</sub> receptor (Kinsella et al., 1994). Thus, the increased cAMP level is linked to the activation of PKA and in turn, according to its negative regulation, down-regulation of cellular events (cytoskeleton rearrangement, migration and cluster formation of platelet clusters) that are necessary for platelet aggregation. Therefore, cordycepin might block these cellular events *via* enhanced levels of cAMP/cGMP and its linked PKA/PKG activity.

The inhibitory effect of cordycepin on platelet aggregation seems to be closely related to both the decrease in [Ca<sup>2+</sup>]<sub>i</sub> and the reduction of 47-kDa and 20-kDa protein phosphorylation, but not to TXA<sub>2</sub>-mediated Ca<sup>2+</sup> mobilization (Kinsella et al., 1994). Thus, after cordycepin treatment at a 50% inhibitory concentration the level of TXA<sub>2</sub> (17.3 ± 1.5 ng/10<sup>8</sup> platelets) was still 40-fold higher than basal level (Figs. 2 and 3B). Under these conditions, it is assumed that the produced TXA<sub>2</sub> leads to the phosphorylation of both 47-kDa and 24-kDa proteins by increasing [Ca<sup>2+</sup>]<sub>i</sub> *via* the TXA<sub>2</sub> receptor. This possibility might be related to a slightly increased level of [Ca<sup>2+</sup>]<sub>i</sub> (169 nM) compared with the basal level (134 ± 1.2 nM) after exposure to cordycepin (500 μM). Nevertheless, the collagen-induced phosphorylation of 47-kDa and 20-kDa (Fig. 4A—lanes 2 and 3) proteins was completely reduced. This strong inhibition might be a result of the 169 nM Ca<sup>2+</sup> level not being sufficient for the phosphorylation of 47-kDa and 24-kDa proteins, because the level of [Ca<sup>2+</sup>]<sub>i</sub> required for phosphorylation is at least 1 μM (Adelstein et al., 1978). Therefore, cordycepin exerts an inhibitory effect on platelet aggregation by suppressing the phosphorylation of 47-kDa and 20-kDa proteins, a final response in agonist-mediated Ca<sup>2+</sup> signaling, *via* the elevation of cAMP and cGMP (Waldmann et al., 1987; Eigenthaler et al., 1992).

The occurrence of thrombosis mainly results from irreversible aggregation is intimately related to serotonin release (Charo et al., 1977). Serotonin release from platelets is stimulated *via*

Ca<sup>2+</sup>/calmodulin-dependent protein phosphorylation by elevated [Ca<sup>2+</sup>]<sub>i</sub> (Nishikawa et al., 1980) and is inhibited by a drug that increases the amount of cGMP/cAMP (Charo et al., 1977). We found that preincubation with cordycepin of collagen (20 μg/ml)-stimulated platelets reduced the serotonin release (10.5%, *n* = 3), compared with that of collagen-stimulated platelets (25.1%, *n* = 3) (data not shown). Accordingly, [Ca<sup>2+</sup>]<sub>i</sub> reduction and cAMP/cGMP elevation (Figs. 5A and 6A) by cordycepin might also be connected with the inhibition of serotonin release. This suggestion is supported by the result that cordycepin inhibited 20-kDa CaM-PK-dependent phosphorylation induced by collagen (Fig. 4A—lanes 2 and 3), providing us with a possible application for cordycepin in severe thrombosis-mediated diseases.

In conclusion, we found that cordycepin dose dependently inhibited collagen-induced platelet aggregation in the presence of various concentrations of exogenous CaCl<sub>2</sub>. This inhibitory effect by cordycepin seems to be due to lowering of [Ca<sup>2+</sup>]<sub>i</sub> and TXA<sub>2</sub> and the up-regulation of intracellular levels of cAMP and cGMP in collagen-stimulated platelets. These results suggest, therefore, that cordycepin may be effective in inhibiting platelet aggregation.

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