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Cordycepin (3′-deoxyadenosine) inhibits human platelet aggregation induced by U46619, a TXA_2 analogue

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Abstract

Cordycepin (3′-deoxyadenosine), which comes from *Cordyceps militaris*, the Chinese medicinal fungal genus *Cordyceps*, is known to have anti-tumour activity. In this study, we investigated the novel effect of cordycepin on human platelet aggregation that was induced by U46619, a thromboxane A_2 (TXA_2) analogue. TXA_2 is an aggregation-inducing autacoidal molecule that is produced in various agonist-activated platelets. Cordycepin completely inhibited U46619-induced platelet aggregation and simultaneously reduced cytosolic free Ca^{2+} ($[Ca^{2+}]_i$), which was increased by U46619 (5 μM) up to 66%. Furthermore, the U46619-stimulated phosphorylation of Ca^{2+}-dependent proteins (20 kDa of a myosin light chain and 47 kDa of pleckstrin) was strongly inhibited by cordycepin. These results suggest that cordycepin may have a beneficial effect on autacoidal TXA_2-mediated thrombotic diseases by inhibiting TXA_2-induced platelet aggregation via suppression of the Ca^{2+} level.

Introduction

Platelet aggregation is essential for haemostatic processes when blood vessels are injured. Especially, cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) in platelets plays a central role in the activation of platelet aggregation. Thus, $[Ca^{2+}]_i$ is known to be increased from internal stores by inositol 1,4,5-trisphosphate (IP_3) via its receptor. Diacyglycerol (DG), generated by the breakdown of phophatidylinositiol 4,5-bisphosphate (PIP_2), and the increased $[Ca^{2+}]_i$ sequentially activates the Ca^{2+}/calmodulin-dependent phosphorylation of the myosin light chain (20 kDa) and the protein kinase C (PKC)-dependent phosphorylation of the cytosolic protein (40 or 47 kDa) to trigger cytoskeleton rearrangement, finally leading to platelet aggregation (Furuichi & Mikoshiba 1995). In addition, DG is hydrolysed by DG lipase and monoacylglycerol lipase to produce arachidonic acid and eventually thromboxane A_2 (TXA_2) to accelerate this event by increasing $[Ca^{2+}]_i$ level (Ohkubo et al 1996). TXA_2 alone is also known to activate platelets to induce their clustering, secretion and shape change (Saitoh et al 1986) and, indeed, TXA_2 analogue U46619 (9,11-dideoxy-9α,11α-methanoepoxyprostaglandin F2α) (Figure 1) itself is used as a good platelet aggregation-inducing agent, simultaneously elevating $[Ca^{2+}]_i$ and increasing the phosphorylation of myosin light chain and pleckstrin (Cattaneo et al 1991; Su et al 1999). Although these events are processes of normal blood responses, the excessive aggregation phenomenon is linked to various blood vessel diseases, such as arteriosclerosis. Therefore, the inhibition of severe platelet aggregation may be a promising approach to prevent these diseases.

Cordycepin (3′-deoxyadenosine) (Figure 1) is an adenosine analogue isolated from *Cordyceps militaris* (Cunningham et al 1951), a species of the fungal genus *Cordyceps*, which has been used as an ingredient for traditional Chinese medicine (Pegler et al 1994). Cordycepin is indeed known to have an anti-tumour effect on mouse melanoma and lung carcinoma (Nakamura et al 2006) and to possess anti-inflammatory effects on the production of inflammatory mediators (Won & Park 2005). Although it has been recently reviewed that cordycepin has many biological functions (Ng & Wang 2005), there are

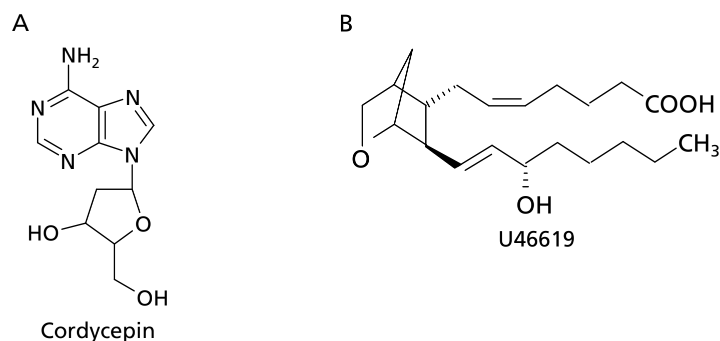

Figure 1 The structures of cordycepin (3′-deoxyadenosine) (A) and U46619 (9,11-dideoxy-9α,11α-methanoepoxyprostaglandin $F_{2\alpha}$) (B).

few reports on its anti-platelet effect. Previously, the fact that cordycepin displays an inhibitory effect on adenylate cyclase activity in either particulated platelet fraction or platelet membrane has rather suggested its negativity in regulating platelet activation (Londos & Wolff 1977; Haslam et al 1978). However, recent evidence, that, firstly, adenosine derivatives, structurally similar compounds to cordycepin, are being developed as potent anti-platelet agents (Boyer et al 2002; Xu et al 2002; Cattaneo et al 2004) and, secondly, cordycepin negatively modulates collagen-induced platelet aggregation (Cho et al 2004), led us to explore its therapeutic activity against the functional activation and aggregation of platelets in response to various platelet stimuli such as TXA_2. This study, therefore, examined the modulatory effect of cordycepin on U46619-mediated platelet aggregation and its critical molecular events, such as the elevation of $[Ca^{2+}]_i$ and the phosphorylation of 20 kDa and 47 kDa proteins.

Materials and Methods

Materials

Cordycepin (Figure 1), which was from *Cordyceps militaris*, was purchased from the Sigma Chemical Co. (St Louis, MO). U46619 (9,11-dideoxy-9α,11α-methanoepoxyprostaglandin $F_{2\alpha}$) was purchased from Calbiochem (a brand of CN Biosciences, Inc., an affiliate of Merck KgaA, Darmstadt, Germany). Fura-2/AM and other reagents were obtained from Sigma Chemical Co. (St Louis, MO).

Preparation of washed human platelets

Blood was obtained from the antecubital veins by venipuncture using a siliconized 18-gauge needle from healthy subjects who had abstained from any drugs for at least 1 week before sampling. Blood was collected at 10:00 h every day. The collected blood was anticoagulated with an ACD solution (0.8% citric acid, 2.2% sodium citrate, 2.45% glucose) during preparation. The platelet-rich plasma (PRP) was centrifuged at 125 *g* for 10 min to remove red blood cells and it was centrifuged again at 1300 *g* for 10 min to obtain platelet pellets. The platelets were washed twice with a washing buffer (composition in mM: 138 NaCl, 2.7 KCl, 12 $NaHCO_3$, 0.36 NaH_2PO_4, 5.5 glucose and 1 EDTA, pH 6.5) (Rittenhouse-Simmons & Deykin 1976). The washed platelets were then suspended in a suspending buffer (composition in mM: 138 NaCl, 2.7 KCl, $NaHCO_3$, 0.36 NaH_2PO_4, 0.49 $MgCl_2$, 5.5 glucose and 0.25% gelatin, pH 6.9) (Rittenhouse-Simmons & Deykin 1976). The platelet number was adjusted to 5×10^8 mL^{-1} in the suspending buffer. All procedures were carried out at 25°C so as to avoid platelet aggregation from any effect of low temperature and human platelets' use in this study was approved by the Ethics Committee of the College of Biomedical Science and Engineering, Inje University, Korea.

Measurement of platelet aggregation

The washed platelets (10^8 mL^{-1}) were pre-incubated for 3 min at 37°C in the presence of 2 mM $CaCl_2$ with or without cordycepin, and then the platelets were further stimulated with U46619 for 5 min (8 min total incubation time). The aggregation was monitored by using an aggregometer (Chrono-Log, Corp., Havertown, PA) with gentle stirring. Each aggregation rate was evaluated as a percent of light transmission. The suspending buffer was used as a reference (transmission 0%). Platelet aggregation was assessed with a ChronoLog aggregometer (ChronoLog, Corp., Havertown, PA) at 37°C by recording the increase in light transmission through a stirred suspension of PRP. Cordycepin and U46619 were dissolved in a platelet suspending buffer (pH 6.9) (Rittenhouse-Simmons & Deykin 1976).

Determination of the $[Ca^{2+}]_i$

The PRP was incubated with 5 μM of fura-2/AM for 60 min at 37°C. As fura-2/AM is light-sensitive, the PRP was covered with aluminium foil during the fura-2 loading. The fura-2-loaded washed platelets were also prepared using the procedures described above. Since U46619 is known to activate platelets only when external Ca^{2+} is present (Ohkubo et al 1996), the platelets were activated by

U46619 in the presence of 2 mM of $CaCl_2$. The fura-2-loaded washed platelets (10^8 mL^{-1}) were pre-incubated for 3 min at 37°C with cordycepin in the presence of 2 mM of $CaCl_2$. Then, the platelets were stimulated with U46619 for 5 min. Fura-2 fluorescence was measured in a spectrofluorometer (SFM 25, Bio-Tek Instrument, Italy) with an excitation wavelength in the range 340–380 nm, altering every 0.5 s; the emission wavelength was at 510 nm. The $[Ca^{2+}]_i$ was calculated by the method of Schaeffer (Schaeffer & Blaustein 1989). $[Ca^{2+}]_i$ in cytosol = 224 nM × (F − F_{min})/(F_{max} − F) where 224 nM is the dissociation constant of the fura-2–Ca^{2+} complex and F_{min} and F_{max} represent the fluorescence intensity levels at very low and very high Ca^{2+} concentration, respectively. In our experiment, F_{max} is the fluorescence intensity of the fura-2–Ca^{2+} complex at 510 nm after the platelet suspension containing 2 mM of $CaCl_2$ had been solubilized by Triton X-100 (0.1%). F_{min} is the fluorescence intensity of the fura-2–Ca^{2+} complex at 510 nm after the platelet suspension containing 20 mM Tris/3 mM EGTA had been solubilized by Triton X-100 (0.1%); F represents the fluorescence intensity of the fura-2-complex at 510 nm after platelet suspension was stimulated by U46619 with and without cordycepin in the presence of 2 mM $CaCl_2$. U46619 was dissolved in a platelet suspending buffer (pH6.9) (Rittenhouse-Simmons & Deykin 1976).

Determination of protein phosphorylation

Protein phosphorylation was carried out according to the method of Laemmli. Washed platelets (10^8 mL^{-1}) were suspended in a 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^{-1} dextrose and 2.8 mM potassium chloride, pH 6.5). The platelets were incubated for 60 min at 37°C with phosphorus-32 (0.5 mCi mL^{-1}). [^{32}P]-Labelled platelets (10^8 mL^{-1}) were pre-incubated with or without cordycepin in the presence of 2 mM $CaCl_2$ at 37°C for 3 min and then U46619 or collagen was added. The reaction was terminated by the addition of an equal volume of a sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) buffer (0.125 M Tris, 4% SDS, 20% glycerol, 5% mercaptoethanol and 0.01% bromphenol blue, pH 6.8). Samples were boiled for 5 min to completely denature the proteins, then 50 μg of protein were subjected to SDS-PAGE (11%, 1.0 mm gel). The relative intensity levels of the phosphoproteins were analysed by using the Storage Phospho System (Cyclone, PackardBioscience Company, USA).

Statistical analysis

Unless otherwise stated, all measurements were made from four observations; similar results were obtained with other two separate experiments. Data are expressed as mean ± s.d. For statistical comparison, results were analysed with analysis of variance/Scheffe's post-hoc test and Kruskal–Wallis test using the computer program STATISTICA, version 4.5 (StatSoft Inc, Microsoft corporation, Oklahoma, OK). $P < 0.05$ was considered a statistically significant difference.

Results and Discussion

TXA_2 acts as a positive feedback promoter on activated platelets and is a strong agonist on resting platelets (Halushka et al 1995). TXA_2 is also a vasoconstrictor and a bronochoconstrictor (Hamberg et al 1975; FitzGerald 1991). Thus, a compound that can inhibit TXA_2 action or formation may have a potential application as an anti-thrombotic agent. Therefore, we investigated the effects of cordycepin on TXA_2 mimetic compound (U46619)-induced platelet aggregation. As shown in Figure 2, when human platelets (10^8 mL^{-1}) were stimulated by U46619 (5 μM), cordycepin inhibited U46619-induced platelet aggregation in a dose-dependent manner. Since a critical factor in U46619-induced platelet aggregation is known to be an increased $[Ca^{2+}]_i$, whether cordycepin had an inhibitory effect on U46619-mediated $[Ca^{2+}]_i$ was first examined. As Figure 3 indicates, U46619 elevated $[Ca^{2+}]_i$ from 134 ± 0.8 nM (basal level) to 524 ± 32 nM in the platelets. The up-regulation of $[Ca^{2+}]_i$ was similarly found with 2 mM of exogenous Ca^{2+}, comparable with previous findings (1 mM of Ca^{2+}) (Ohkubo et al 1996). When the platelets, however, were incubated in the presence of both cordycepin (500 μM) and U46619, the level of $[Ca^{2+}]_i$ was significantly decreased to 267 ± 35 nM (66% inhibition compared with U46619 alone), although the decreased $[Ca^{2+}]_i$ level was still 133 nM greater than that of basal level (Figure 3, Table 1).

How the incomplete inhibition of Ca^{2+} level was able to make a perfect blockade of platelet aggregation is not fully elucidated in this study. This seems to suggest either that cordycepin may activate another pathway involved in

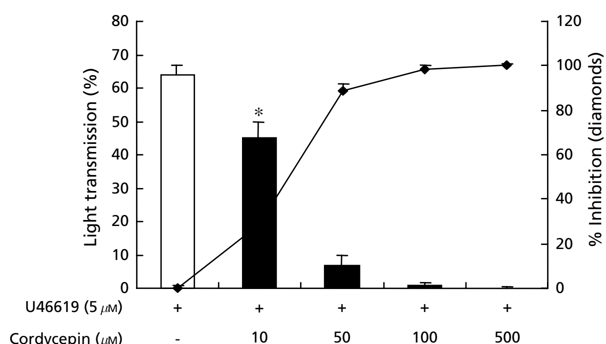

Figure 2 The effects of cordycepin pretreatment on U46619-induced platelet aggregation. Washed human platelets (10^8 mL^{-1}) were pre-incubated with or without cordycepin in the presence of 2 mM $CaCl_2$ for 3 min at 37°C. Then, the platelets were stimulated with U46619 for 5 min. Platelet aggregation (%) was recorded as an increase in light transmission. Inhibition by cordycepin was recorded as a percentage of the U46619-induced aggregation rate. Data are given as the mean ± s.d., n = 4. *$P < 0.05$ vs U46619.

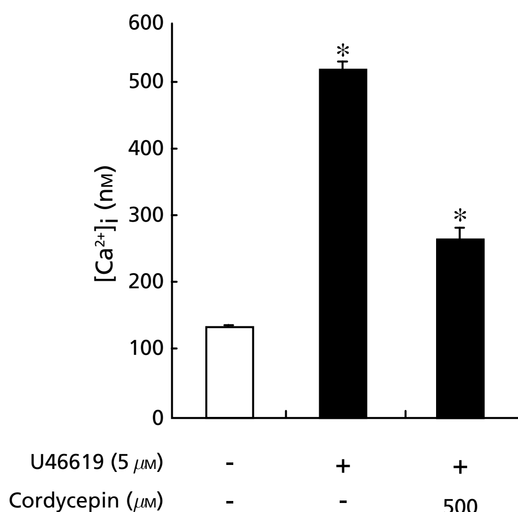

Figure 3 The effects of cordycepin on U46619-induced increase of cytosolic free Ca^{2+}. Fura-2-loaded platelets (10^8 mL^{-1}) were pre-incubated with or without cordycepin in the presence of 2 mM $CaCl_2$ for 3 min at 37°C, then U46619 was added. Cytosolic free Ca^{2+} levels were determined as described in Materials and Methods. Data are given as the mean ± s.d., n = 4. *$P < 0.05$, compared with control.

Table 1 Inhibitory degree of cordycepin on U46619-elevated $[Ca^{2+}]_i$

	$[Ca^{2+}]_i$ (nM)	Inhibition (%)
Basal	134 ± 0.8	—
U46619 (5 μM)	524 ± 32*	0
U46619 (5 μM) + cordycepin (500 μM)	267 ± 35**	66

*$P < 0.05$ vs basal $[Ca^{2+}]_i$. **$P < 0.05$ vs U46619 (5 μM)-induced $[Ca^{2+}]_i$.

negatively regulating platelet aggregation (such as a generating pathway of cAMP/cGMP, negative regulators of functional activation of platelets) regardless of increased Ca^{2+} level, or that the concentration of Ca^{2+} obtained by cordycepin may not be enough to carry on its role in triggering platelet aggregation. In fact, cordycepin has been found to up-regulate cAMP/cGMP under collagen treatment (Cho et al unpublished data), but the effect seemed not drastic enough to give a complete inhibition, suggesting a weak involvement as a major inhibitory mechanism, as shown in the cases of other strong anti-platelet aggregation inhibitors such as KR32560 and sanguinarine (Jeng et al 2006; Lee et al 2006). Hence, we assumed that the latter may be a more favourable mode of inhibitory action by cordycepin.

To test this hypothesis, ^{32}P-labelled platelets were stimulated by U46619 or collagen (10 μg mL^{-1}, as a positive control) and the 20 kDa and 47 kDa proteins were markedly found to be phosphorylated up to 160–200% (Figure 4A, lanes 2 and 4). It is known that the U46619-mediated generation of the phosphoproteins was due to activation of a series of signalling pathways, such as binding TXA_2 to its receptor, increasing in $[Ca^{2+}]_i$, activating phospholipase C-β (Baldassare et al 1993), generating IP_3 and DG and activating protein kinase C (Arita et al 1989). Interestingly, cordycepin treatment completely blocked the Ca^{2+}-dependent phosphorylation, suggesting the importance of Ca^{2+} level. Thus, the cordycepin-suppressed level of $[Ca^{2+}]_i$ perfectly blocked the 47 kDa (pleckstrin) and 20 kDa (myosin light chain) phosphorylation (Figure 4), indicating that this Ca^{2+} level may be not enough to activate Ca^{2+}/calmodulin-dependent kinases such as Ca^{2+}/DG-dependent protein kinase or Ca^{2+}/calmodulin dependent protein kinase (Rink & Sage 1990), leading to no further cytoskeleton rearrangement and aggregation events. To activate Ca^{2+}/calmodulin-dependent enzyme ternary complex, one mole of calmodulin is reported to require 3 moles of Ca^{2+} (Blumenthal & Stull 1980). This suggests that the amount of intracellular Ca^{2+} should be at least three times more than in inactive conditions. A detailed study to exactly explain this discrepancy, however, will be further explored in terms of the relationship between Ca^{2+} level and platelet aggregation.

Precisely what is the target molecule(s) of cordycepin in U46619-induced platelet aggregation is not clearly elucidated in this study. Several potential targets, such as receptor proteins (e.g. TXA_2 receptor or Ca^{2+} receptor) and signalling proteins (e.g. PKC), can be considered. However, the fact that TXA_2 receptor binding compounds, TXA_2 receptor function blockers and TXA_2 synthase inhibitors (such as U46619, S18886, picotamide, 2NTX-99, 2-[(4-acetylphenyl)amino]-3-chloro-1,4-naphthalenedione, terbogrel and NQ301) are structurally distinct (Berrettini et al 1990; Chang et al 1997; Guth et al 2004; Jin et al 2005; Buccellati et al 2006) seems to suggest that TXA_2 receptor and its directed pathway may not be a target molecule. Instead, it is notable that adenine nucleotide derivatives structurally related to cordycepin diminished platelet aggregation by blocking adenosine diphosphate (ADP) receptor (i.e. P_2Y_2, P_2Y_{12}), which are linked to both Gq and $G_{12/13}$ proteins (Boyer et al 2002; Xu et al 2002; Cattaneo et al 2004; Hechler et al 2005). This suggests that ADP receptor itself or a series of ADP receptor-mediated pathways may be involved in cordycepin-mediated inhibition of platelet activation as a potential target protein(s) or pathway. To identify the exact target molecule(s), however, more detailed pharmacodynamic studies, including a receptor binding assay, should be carried out as future work.

Conclusion

We investigated whether cordycepin (Figure 1) has an inhibitory effect on platelet activating molecules such as $[Ca^{2+}]_i$ and phosphoproteins (20 kDa of myosin light chain and 47 kDa of pleckstrin). Cordycepin inhibited the mobilization of $[Ca^{2+}]_i$ and the phosphorylation of Ca^{2+}-dependent proteins (20 kDa and 47 kDa) induced by U46619, a TXA_2 analogue. Accordingly, cordycepin may be an interesting candidate regulating the TXA_2-mediated platelet aggregation system. Considering that TXA_2 is a potent stimulator of platelet aggregation and it is a vasoconstrictor, cordycepin may have an inhibitory effect on platelet aggregation-mediated thrombotic diseases such as cardiovascular disease.

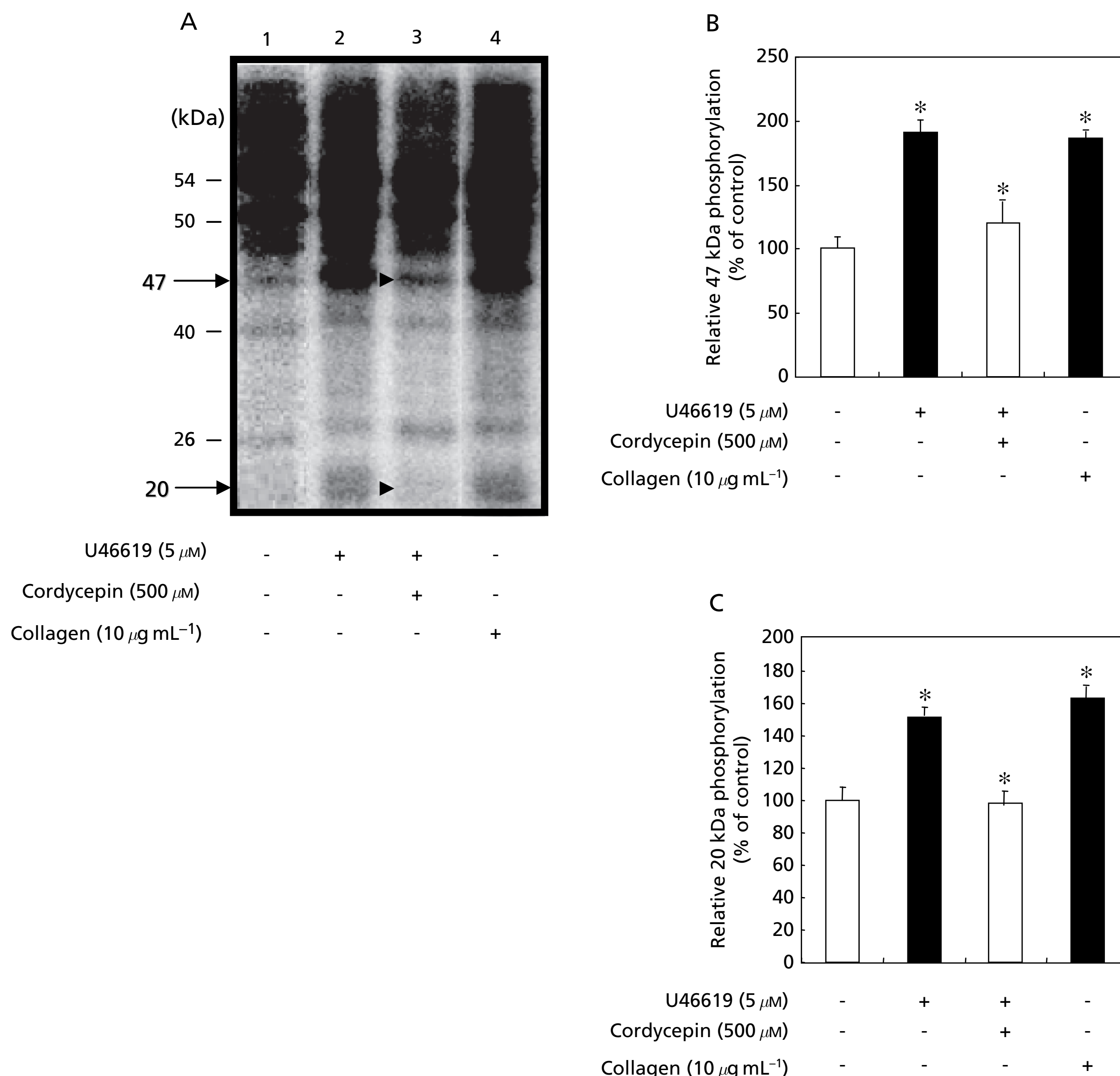

Figure 4 The effects of cordycepin on protein phosphorylation. Protein phosphorylation processes were performed as described in Materials and Methods. ^{32}P-labelled platelets (10^8 mL^{-1}) were pre-incubated with or without cordycepin (500 μM) for 3 min in the presence of 2 mM $CaCl_2$, and then the platelets were stimulated with U46619 (5 μM), or collagen (10 μg mL^{-1}) for 5 min. Platelet protein phosphorylation was analysed by SDS-PAGE (11% polyacrylamide gel). A. The pattern of protein phosphorylation: lane 1, intact platelets as control; lane 2, U46619 (5 μM); lane 3, cordycepin (500 μM) + U46619 (5 μM); lane 4, collagen (10 μg mL^{-1}). B. The inhibition of 47 kDa phosphorylation by cordycepin. C. The inhibition of 24 kDa phosphorylation by cordycepin. U46619, collagen and cordycepin were dissolved in a platelet suspending buffer (pH 6.9) control. *$P < 0.05$ vs U46619.

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