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The Inhibitory Effects of Cordycepin (3'-deoxyadenosine) on Thapsigargin-enhanced Cytosolic Ca^{2+}-influx and -mobilization in Human Platelets

Hyun-Jeong Cho[1] and Hwa-Jin Park[2,†]

[1]Department of Biomedical Laboratory Science, College of Medical Science, Konyang University, 685, Gasuwon-dong, Seo-gu, Daejeon 302-718, Korea
[2]Department of Biomedical Laboratory Science, College of Biomedical Science and Engineering and Regional Research Center, Inje University, 607, Obang-dong, Gimhae, Gyungnam 621-749, Korea

Cordycepin (3'-deoxyadenosine) is an adenosine analogue isolated from *Cordyceps militaris*, and it has been used as an anti-cancer and anti-inflammation ingredient in traditional Chinese medicine. We investigated the effects of cordycepin on human platelet aggregation induced by thapsigargin, and determined the cytosolic free Ca^{2+} levels ($[Ca^{2+}]_i$), an aggregation-stimulating factor. Cordycepin significantly inhibited thapsigargin-induced platelet aggregation. Its inhibitory effect was continually sustained at the maximal aggregation concentration of thapsigargin. The thapsigargin-induced $[Ca^{2+}]_i$ were clearly reduced by cordycepin in the presence of exogenous $CaCl_2$ or extracellular Ca^{2+}-chelator (EDTA). These results suggest that cordycepin inhibited thapsigargin-induced Ca^{2+}-influx from extracellular domain and thapsigargin-induced Ca^{2+}-mobilization from intracellular Ca^{2+} storage. Accordingly, our data demonstrated that cordycepin may have a beneficial effect on platelet aggregation-mediated thrombotic diseases by inhibiting a $[Ca^{2+}]_i$-elevation.

Key Words: Cordycepin, Platelet aggregation, Intracellular Ca^{2+}, Anti-platelet activity, Thapsigargin

INTRODUCTION

Platelet aggregation is an essential part of the haemostatic process when blood vessels are injured. Platelet aggregation is brought about by many platelet activating factors such as thrombin, collagen, ADP, serotonin, and von Willebrand factor, which may be the cause of thrombosis in atherosclerosis and myocardial infarction (Schwartz et al., 1990; Son et al., 2005). In particular, cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) plays a central role in the activation of platelet aggregation (Sage et al., 1992). The $[Ca^{2+}]_i$ for platelet activation is increased by the inositol 1,4,5-trisphosphate (IP_3)-mediated Ca^{2+} release from internal stores *via* the IP_3 receptor (Furuichi et al., 1995). The increased $[Ca^{2+}]_i$ activates both the Ca^{2+}/calmodulin-dependent phosphorylation of a myosin light chain (20 kDa) and the 1,2-diacylglycerol (DG)-dependent phosphorylation of cytosolic pleckstrin (40 or 47 kDa) in order to induce platelet aggregation (Furuichi et al., 1995; Kaibuchi et al., 1982). In addition, DG is hydrolyzed by DG lipase and monoacylglycerol lipase to produce arachidonic acid, thromboxane A_2 (TXA_2) precursor (Ohkubo et al., 1996). TXA_2 is known to elevate $[Ca^{2+}]_i$ and activate platelets to induce their clustering, secretion, and shape change (Saitoh et al., 1986). Anti-platelet aggregating drugs, including verapamil, theophylline, molsidomine, and nitroprussside, elevate either cyclic-adenosine monophosphate (cAMP) or cyclic-guanosine monophosphate (cGMP) levels, thereby decreasing the collagen-, thrombin-, and ADP-elevated $[Ca^{2+}]_i$ (Jang et al., 2002; Menshikov et al., 1993). Cordycepin (3'-deoxyadenosine) is an adenosine analogue and was isolated from *Cordyceps militaris*

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†Corresponding author: Hwa-Jin Park, Department of Biomedical Laboratory Science, College of Biomedical Science and Engineering, Inje University, Gyungnam 621-749, Korea.
Tel: +82-55-320-3538, Fax: +82-55-334-3426
e-mail: mlsjpark@inje.ac.kr

(Cunningham et al., 1950; Yu et al., 2001), which has been used as an ingredient in traditional Chinese medicine (Yu et al., 2004). Cordycepin is known to have anti-tumor effects on cancers of the bladder, colon and lungs, as well as fibrosarcoma (Hubbell et al., 1985), and also possesses anti-inflammatory effects by producing inflammatory mediators (Yu et al., 2004; Won et al., 2005; Yang et al., 2000). It has been reported that an adenosine analogue, 2',5'-dideoxyadenosine, does not alter the levels of either cAMP or cGMP in collagen-induced platelet aggregation (Jang et al., 2002). Cordycepin elevated the levels of cAMP or cGMP to inhibit collagen-induced platelet aggregation, and decreased collagen-elevated $[Ca^{2+}]_i$ without the inhibitory effect on IP_3 production (Cho et al., 2007). Thapsigargin is known to influx and mobilize Ca^{2+} without having any direct interaction with the IP_3 receptor in endoplasmic reticulum, Ca^{2+}-storage (Hashimoto et al., 1993; Saxena et al., 1992; Vostal et al., 1996; Sargean et al., 1993). Accordingly, it is interesting to determine whether cordycepin inhibits $[Ca^{2+}]_i$ elevation by thapsigargin-stimulation in platelets. In this study, we have investigated the effects of cordycepin on thapsigargin-induced $[Ca^{2+}]_i$-influx from extracellular domain and thapsigargin-induced Ca^{2+}-mobilization from intracellular Ca^{2+}-storage.

MATERIALS AND METHODS

1. Materials

Cordycepin from *Cordyceps militaris* was purchased from the Sigma Chemical Co. (St. Louis, USA). Fura 2-AM, thapsigargin, and other reagents were obtained from the Sigma Chemical Co. (St. Louis, USA).

2. Preparation of the washed human platelets

Blood was drawn from the antecubital veins of normal healthy human volunteers and anticoagulated with ACD solution (0.8% citric acid, 2.2% sodium citrate, 2.45% glucose). The whole blood was centrifuged for 10 min at 125 × g to remove the red blood cells and platelet-rich plasma (PRP), which, in turn, centrifuged for 10 min at 1,300 × g to obtain the platelet pellets. The platelets were washed twice with washing buffer (138 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.36 mM NaH_2PO_4, 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 $NaHCO_3$, 0.36 mM NaH_2PO_4, 0.49 mM $MgCl_2$, 5.5 mM glucose, 0.25% gelatin, pH 6.9) to a concentration of 5×10^8/ml. All of the above the procedure were carried out at 25℃ to avoid platelet aggregation on cooling.

3. Measurement of platelet aggregation

Washed platelets (10^8/ml) were preincubated for 3 min at 37℃ in the presence of exogenous 2 mM $CaCl_2$, either with or without cordycepin and then stimulated with thapsigargin (10 μM) for 5 min. Aggregation was monitored by using an aggregometer (Chrono-Log, Corp., Havertown, PA, USA) at a constant stirring speed of 1,000 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 a platelet suspending buffer (pH 6.9). The light transmission (%) was not affected by 0.004% of ethanol, a vehicle of thapsigargin.

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

Platelet rich plasma (PRP) was incubated with 5 μM of fura 2-AM for 60 min at 37℃. As fura 2-AM is light-sensitive, PRP was covered with aluminium foil during the fura 2 loading. The fura 2-loaded washed platelets were also prepared using the procedure described above. The fura 2-loaded washed platelets (10^8/ml) were preincubated for 3 min at 37℃ with or without cordycepin in the presence of 2 mM $CaCl_2$ or 2 mM EDTA. Here, $CaCl_2$ was used as an exogenous Ca^{2+} source for investigating Ca^{2+}-influx from extracellular domain, and EDTA was used as an extracellular Ca^{2+}-blocker for investigating Ca^{2+}-mobilization from intracellular Ca^{2+}-storage. The platelets were then stimulated with thapsigargin (10 μM) for 5 min. Fura 2 fluorescence was measured in a spectrofluorimeter (SFM 25, Bio-Tek Instrument, Italy) with an excitation wavelength which ranged between 340 and 380 nm, altering every 0.5 sec; the wavelength of emission was at 510 nm. The $[Ca^{2+}]_i$ was calculated by the method of Schaeffer (Schaeffer et al., 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; F_{min} and F_{max} are the fluorescence intensity levels at very low and very high Ca^{2+} concentration amounts, respectively. In our experiment, F_{max} is the fluorescence intensity of 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%), and 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 is the fluorescence intensity of the fura 2-complex at 510 nm after platelet suspension was stimulated by thapsigargin, either with or without cordycepin, in the presence of $CaCl_2$ or EDTA. A vehicle (0.004% ethanol) of thapsigargin, did

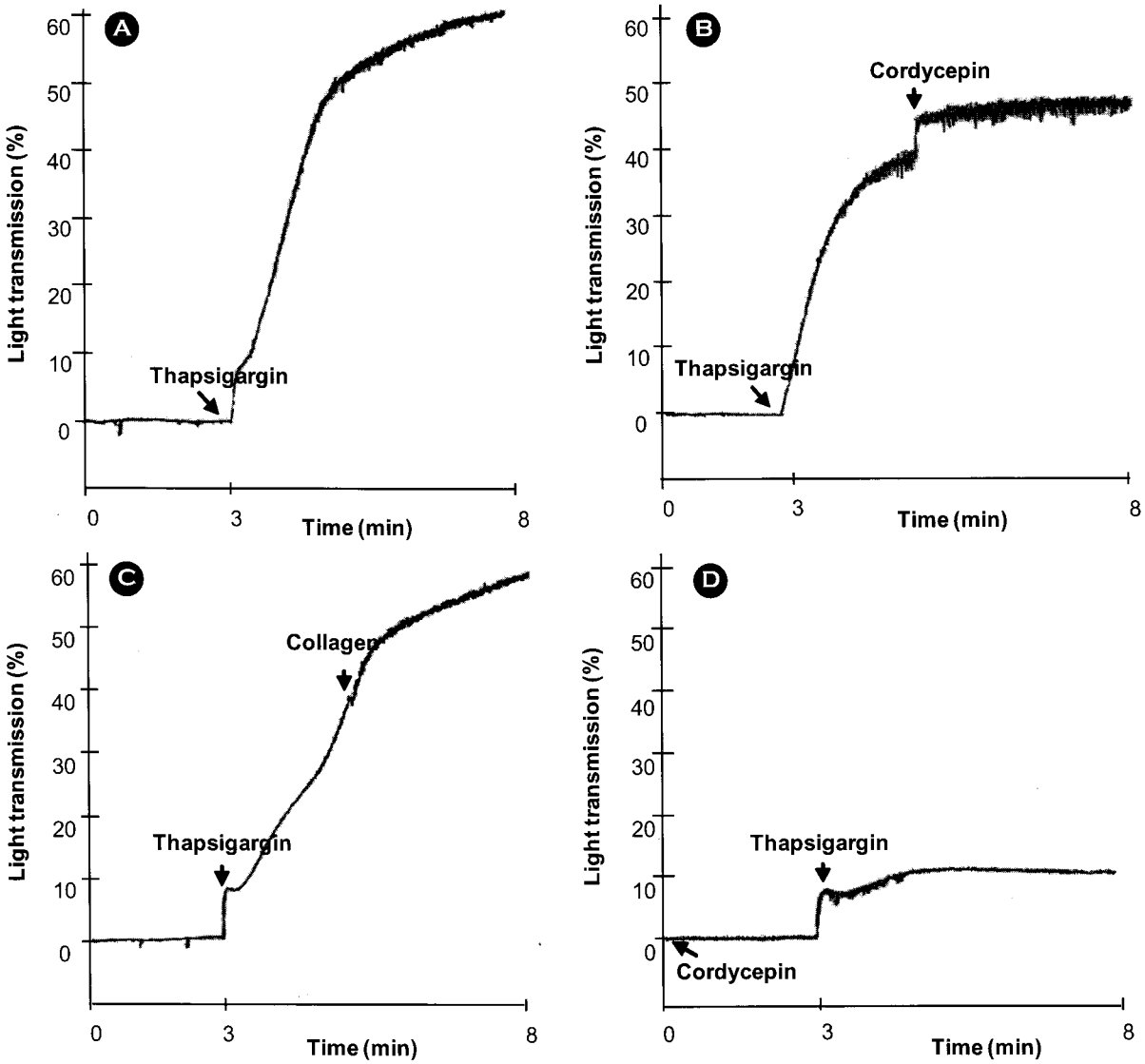

Fig. 1. The effects of cordycepin on thapsigargin-induced platelet aggregation. **(A)** The patterns of thapsigargin-induced platelet aggregation. The washed platelets (10^8/ml) were preincubated in the presence of 2 mM of $CaCl_2$ for 3 min at 37℃. The platelets were stimulated with thapsigargin (10 μM) for 5 min. **(B)** Effects of cordycepin post-treatment on thapsigargin-induced platelet aggregation. Washed platelets (10^8/ml) were preincubated in the presence of 2 mM $CaCl_2$ for 3 min at 37℃. The platelets were stimulated with thapsigargin (10 μM), and then cordycepin (500 μM) was added after 2 min of thapsigargin-stimulation. **(C)** Effects of collagen post-treatment on thapsigargin-induced platelet aggregation. Washed platelets (10^8/ml) were preincubated in the presence of $CaCl_2$ (2 mM) for 3 min at 37℃. The platelets were stimulated with thapsigargin (10 μM), and then collagen (10 μg/ml) was added after 2 min of thapsigargin-stimulation. **(D)** Effects of cordycepin pre-treatment on thapsigargin-induced platelet aggregation. Washed platelets (10^8/ml) were preincubated with cordycepin (500 μM) in the presence of $CaCl_2$ (2 mM) for 3 min at 37℃. The platelets were stimulated with thapsigargin (10 μM) for 5 min. Platelet aggregation (%) was recorded as an increase in light transmission.

not have an effect on the level of intracellular Ca^{2+}. Net mobilized-$[Ca^{2+}]_i$ was calculated by subtracting basal $[Ca^{2+}]_i$ from thapsigargin-increased $[Ca^{2+}]_i$ with or without cordycepin in the presence of EDTA. Net influxed-$[Ca^{2+}]_i$ was calculated by subtracting thapsigargin-induced $[Ca^{2+}]_i$, with EDTA, from thapsigargin-induced $[Ca^{2+}]_i$ with or without cordycepin in the presence of $CaCl_2$.

5. Statistical analysis

The experimental results are expressed as the means ± S. E. M. and are accompanied by the number of observations. Data were analyzed by a one-way analysis of variance

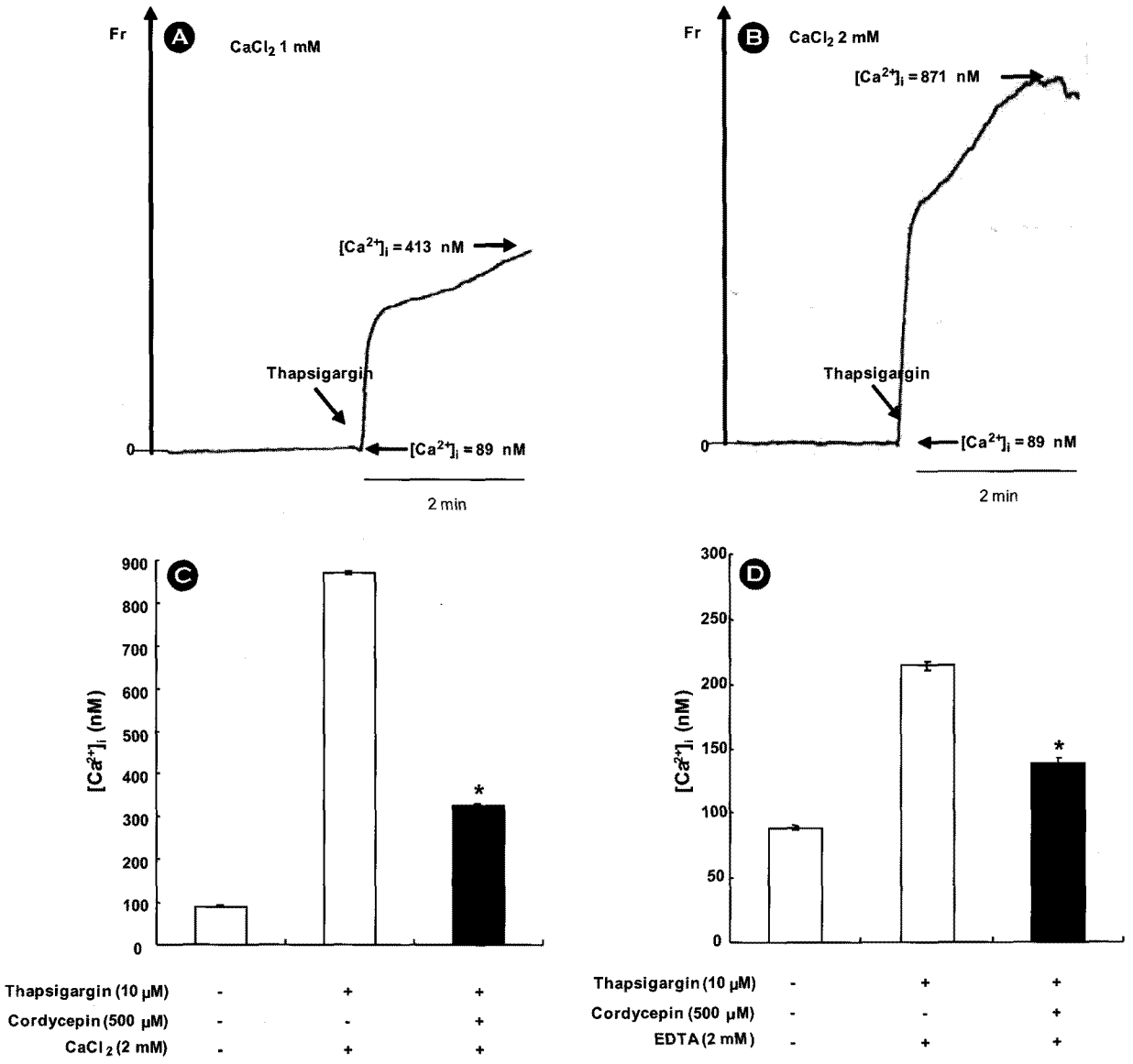

Fig. 2. The effects of cordycepin $[Ca^{2+}]_i$-influx and $[Ca^{2+}]_i$-mobilization by thapsigargin stimulation. **(A)** Increase of $[Ca^{2+}]_i$-influx by thapsigargin in the presence of exogenous $CaCl_2$ (1 mM). **(B)** Increase of $[Ca^{2+}]_i$-influx by thapsigargin in the presence of exogenous $CaCl_2$ (2 mM). Fura 2-loaded platelets (10^8/ml) were preincubated in the presence of 1 mM $CaCl_2$ or 2 mM $CaCl_2$ for 3 min at 37℃, and then thapsigargin (10 μM) was added. The fluorescence intensity of fura 2-Ca^{2+} complex was monitored for 2 min with spectrofluorimeter. **(C)** Effects of cordycepin on thapsigargin-induced $[Ca^{2+}]_i$-influx. Fura 2-loaded platelets (10^8/ml) were preincubated with or without cordycepin (500 μM) in the presence of exogenous $CaCl_2$ (2 mM) for 3 min at 37℃. Then, thapsigargin (10 μM) was added. **(D)** Effects of cordycepin on thapsigargin-induced $[Ca^{2+}]_i$-mobilization. Fura 2-loaded platelets (10^8/ml) were preincubated with or without cordycepin (500 μM) in the presence of exogenous $CaCl_2$-chelator EDTA (2 mM) for 3 min at 37℃. Then, thapsigargin (10 μM) was added. $[Ca^{2+}]_i$ was determined as described in "Materials and Methods". Data are given as means ± S. E. M. (n = 4). *P<0.001 compared with the thapsigargin-induced platelets.

(ANOVA). If this analysis indicated significant differences among the group means, then each group was compared by the Newman-Keuls method. A P value less than 0.05 was considered statistically significant.

RESULTS

1. Effects of cordycepin on thapsigargin-induced platelet aggregation

As shown in Fig. 1A, when washed platelets (10^8/ml) were preincubated in the presence of $CaCl_2$ (2 mM), and were incubated with thapsigargin for 5 min at 37℃, thapsigargin (10 μM) strongly induced the aggregation of human platelets up to approximately 60%. However, when cordycepin (500 μM) is added after 2 min of thapsigargin-addition, the light transmission was increased no longer (Fig. 1B), suggesting that thapsigargin-induced platelet aggregation is not occurred subsequently in the presence of cordycepin. In another our experiment, because the half inhibitory concentration (IC_{50}) of cordycepin was 500 μM in collagen (10 μg/ml)-induced platelet aggregation (Cho et al., 2007), we used this concentration in this study. On the other hand, when collagen as a positive control is added after 2 min of thapsigargin-stimulation, unlike the case of cordycepin-addition, the light transmission was increased continually, suggesting that platelet aggregation occurs in the presence of both thapsigargin and collagen (Fig. 1C). When platelets were preincubated with cordycepin for 3 min at 37℃ in the presence of $CaCl_2$, and then incubated with thapsigargin, light transmission was decreased up to 10% (Fig. 1D). This result suggests that cordycepin inhibits thapsigargin-induced platelet aggregation.

Table 1. Effect of cordycepin on net Ca^{2+}-influx and inhibitory degree of net Ca^{2+}-influx

	$[Ca^{2+}]_i$ (nM)	Net Ca^{2+}-influx (nM)	Inhibition (%)
Basal[a]	89±2	–	–
$CaCl_2$ (2 mM) + Tg (10 μM)[b]	871±3	$657^{\Delta1}$	0
$CaCl_2$ + Tg + Cordycepin (500 μM)[c]	329±3*	$115^{\Delta2}$	82
EDTA (2 mM) + Tg[d]	214±4	–	–

Data are expressed as means ± S. E. M. (n=4). *P<0.05 compared with $CaCl_2$ (2 mM) + Tg (10 μM). Tg, thapsigargin. Net $[Ca^{2+}]_i$ influx$^{\Delta1}$ = (b-a) - (d-a) = b-d. Net $[Ca^{2+}]_i$-influx$^{\Delta2}$ = (c-a) - (d-a) = c-d. Inhibition (%) = (Δ1-Δ2)/Δ1 × 100

2. Effects of cordycepin on $[Ca^{2+}]_i$-elevation induced by thapsigargin

When human platelets were stimulated with thapsigargin in the presence of exogenous $CaCl_2$ 1 mM, fluorescence intensity (Fr) of fura 2-Ca^{2+} complex was increased, and $[Ca^{2+}]_i$ was calculated as 413±3 nM (Fig. 2A). On the other hand, Fr was increased more in the presence of exogenous $CaCl_2$ 2 mM than in the presence of exogenous $CaCl_2$ 1 mM, its $[Ca^{2+}]_i$ was increased up to 871±3 nM (Fig. 2B). These results suggest that thapsigargin increases Ca^{2+}-influx from extracellular domain into intracellular domain, cytosol, in response to elevation of exogenous $CaCl_2$ concentration (Hashimoto et al., 1993; Vostal et al., 1996). However, when human platelets were preincubated with cordycepin and $CaCl_2$ 2 mM for 3 min at 37℃, and then were stimulated with thapsigargin, $[Ca^{2+}]_i$ was decreased by 329±3 nM (Fig. 2C). These results suggest that cordycepin inhibited thapsigargin-elevated $[Ca^{2+}]_i$ from extracellular domain to cytosol. When human platelets were preincubated for 3 min at 37℃ with extracellular Ca^{2+} chelator EDTA, and then incubated with thapsigargin, $[Ca^{2+}]_i$ was increased from 89±2 nM to 214±4 nM (Fig. 2C). However, when human platelets were preincubated for 3 min at 37℃ with EDTA and cordycepin, and then incubated with thapsigargin, $[Ca^{2+}]_i$ was 139±3 nM (Fig. 2D). These results suggest that cordycepin inhibits thapsigargin-elevated $[Ca^{2+}]_i$ from endoplasmic reticulum, intracellular Ca^{2+}-storage, into the cytosol.

Table 2. Effect of cordycepin on net Ca^{2+}-mobilization and inhibitory degree of net Ca^{2+}-mobilization

	$[Ca^{2+}]_i$ (nM)	Net Ca^{2+}-mobilization (nM)	Inhibition (%)
Basal[a]	89±2	–	–
EDTA (2 mM) + Tg (10 μM)[b]	214±4	$125^{\Delta1}$	0
EDTA + Tg + Cordycepin (500 μM)[c]	139±3*	$50^{\Delta2}$	60

Data are expressed as means ± S. E. M. (n=4). *P<0.05 compared with EDTA (2 mM) + Tg (10 μM). Tg, thapsigargin. Net $[Ca^{2+}]_i$-mobilization$^{\Delta1}$ = b-a. Net $[Ca^{2+}]_i$-mobilization$^{\Delta2}$ = c-a. Inhibition (%) = (Δ1-Δ2)/Δ1 × 100

DISCUSSION

Various agonists-induced platelet aggregation is mediated by Ca^{2+} (Sage et al., 1992). Accordingly, it is suggested that thapsigargin-induced platelet aggregation (Fig. 1A) is due to Ca^{2+} level elevated by thapsigargin (Fig. 2). However, cordycepin inhibited thapsigargin-induced platelet aggregation (Fig. 1D). This is contributed to the inhibitory effect of cordycepin on thapsigargin-elevated $[Ca^{2+}]_i$ (Fig. 2). Thapsigargin is known to induce Ca^{2+}-mobilization (Vostal et al., 1996; Hough et al., 1999; Rosado et al., 2000; Tao et al., 1992). As shown in Table 1, thapsigargin elevated the $[Ca^{2+}]_i$ from 89±2 nM in intact cells to 871±3 nM in the presence of $CaCl_2$ (2 mM) as an exogenous source of Ca^{2+}, and elevated the $[Ca^{2+}]_i$ from 89±2 nM in intact cells to 214±4 nM in the presence of EDTA as influx-blocker of exogenous Ca^{2+} (Table 1). From these results, the level of net Ca^{2+}-influx by thapsigargin was calculated as 657 nM (Table 1). This suggests that thapsigargin influxed 657 nM of Ca^{2+} from extracellular domain (Hashimoto et al., 1993; Vostal et al., 1996). However, cordycepin inhibited the influxed-$[Ca^{2+}]_i$ from 657 nM to 115 nM (Table 1). This implies that cordycepin inhibited the Ca^{2+}-influx by thapsigargin.

As shown in Table 2, thapsigargin increased the $[Ca^{2+}]_i$ from 89±2 nM in intact cells to 214±4 nM in the presence of EDTA as an influx-blocker of exogenous Ca^{2+} (Table 2). Because this $[Ca^{2+}]_i$ increased in the presence of EDTA, an extracellular Ca^{2+} influx-blocker, the elevated $[Ca^{2+}]_i$ (214± 4 nM) seems to be contributed to Ca^{2+}-mobilization released out of Ca^{2+}-storage (i.e. dense tubular system, endoplasmic reticulum) by thapsigargin. In fact, the level of net Ca^{2+}-mobilization by thapsigargin was calculated as 125 nM (Table 2). However, cordycepin inhibited the mobilized-$[Ca^{2+}]_i$ from 125 nM to 50 nM (Table 2). This means that cordycepin inhibited the Ca^{2+}-mobilization by thapsigargin. From above results, we suggest that cordycepin have an inhibitory effect on both thapsigargin-induced Ca^{2+}-influx and thapsigargin-induced Ca^{2+}-mobilization in platelets. Thapsigargin is known to stimulate the formation of TXA_2 *via* the increase of $[Ca^{2+}]_i$, and the resulting TXA_2 also increases $[Ca^{2+}]_i$ in an action of its autacoids (Hashimoto et al., 1993). If cordycepin reduces the $[Ca^{2+}]_i$ which were increased by TXA_2, cordycepin may be considered as a good candidate for inhibiting thrombosis. We previously reported that cordycepin reduced $[Ca^{2+}]_i$ which was increased by U46619, a TXA_2 analogue (Cho et al., 2006). Furthermore, the U46619-stimulated phosphorylation of Ca^{2+}-dependent proteins such as myosin light chin and pleckstrin was strongly inhibited by cordycepin. In our other experiments, we reported that cordycepin increased cGMP level reduced by thapsigargin (Cho et al., 2007). In special, it is interesting to note that cordycepin modulated the production of cGMP in both intact platelets and thapsigargin-stimulated platelets (Cho et al., 2007). These results suggest that cordycepin-mediated inhibition of $[Ca^{2+}]_i$ (Table 1, 2) might be also associated with the enhancement of cGMP by cordycepin (Cho et al., 2007).

In conclusion, cordycepin potently inhibited thapsigargin-induced platelet aggregation (Fig. 1D). It was observed that cordycepin had potent inhibitory effects on both thapsigargin-elevated $[Ca^{2+}]_i$-influx and thapsigargin-elevated $[Ca^{2+}]_i$-mobilization (Fig. 2, Table 1, 2). Therefore, our results suggest that cordycepin itself may be developed as a novel therapeutic agent modulating platelet-mediated thrombotic diseases by regulating the $[Ca^{2+}]_i$.

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