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C-Phycocyanin, a Very Potent and Novel Platelet Aggregation Inhibitor from *Spirulina platensis*

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The aim of this study was to systematically examine the inhibitory mechanisms of C-phycocyanin (C-PC), one of the major phycobiliproteins of *Spirulina platensis* (a blue-green alga), in platelet activation. In this study, C-PC concentration-dependently (0.5−10 nM) inhibited platelet aggregation stimulated by agonists. C-PC (4 and 8 nM) inhibited intracellular Ca^{2+} mobilization and thromboxane A_2 formation but not phosphoinositide breakdown stimulated by collagen (1 μg/mL) in human platelets. In addition, C-PC (4 and 8 nM) markedly increased levels of cyclic GMP and cyclic GMP-induced vasodilator-stimulated phosphoprotein (VASP) Ser^{157} phosphorylation. Rapid phosphorylation of a platelet protein of M_w 47 000 (P47), a marker of protein kinase C activation, was triggered by phorbol-12,13-dibutyrate (150 nM). This phosphorylation was markedly inhibited by C-PC (4 and 8 nM). In addition, C-PC (4 and 8 nM) markedly reduced the electron spin resonance (ESR) signal intensity of hydroxyl radicals in collagen (1 μg/mL)-activated platelets. The present study reports on a novel and very potent (in nanomolar concentrations) antiplatelet agent, C-PC, which is involved in the following inhibitory pathways: (1) C-phycocyanin increases cyclic GMP/VASP Ser^{157} phosphorylation and subsequently inhibits protein kinase C activity, resulting in inhibition of both P47 phosphorylation and intracellular Ca^{2+} mobilization, and (2) C-PC may inhibit free radicals (such as hydroxyl radicals) released from activated platelets, which ultimately inhibits platelet aggregation. These results strongly indicate that C-PC appears to represent a novel and potential antiplatelet agent for treatment of arterial thromboembolism.

KEYWORDS: Blue-green alga; C-phycocyanin; thromboxane A_2; protein kinase C; cyclic GMP; vasodilator-stimulated phosphoprotein; hydroxyl radical

INTRODUCTION

The cyanobacterium (blue-green alga) *Spirulina platensis* has been commercialized in several countries for its use as a health food and for therapeutic purposes due to its valuable constituents, particularly proteins and vitamins (*1*). Cyanobacteria and algae possess a wide range of colored compounds, including carotenoids, chlorophyll, and phycobiliproteins (*2*). The principle phycobiliproteins are C-phycocyanin (C-PC), allo-phycocyanin, and phycoerythrin, which are made up of dissimilar α and β polypeptide subunits (*2*). C-PC has been shown to have hepatoprotective (*3*), antiinflammatory (*4*), and antioxidant properties, as well as being a free radical scavenger (*5*). Dietary foods that possess antioxidant activity may play a role in human health, particularly in diseases believed to be involved, at least in part, with oxidation, such as coronary heart disease, inflammation, and mutagenesis leading to carcinogenesis (*6*). On the other hand, it was also demonstrated that oral administration of C-PC exerted an antiinflammatory effect in arthritis induced by zymosan in mice (*7*). Owing to its fluorescence properties, it has gained importance in the development of phycofluor probes for immunodiagnostics (*8*).

Intravascular thrombosis is one generator of a wide variety of cardiovascular diseases. The initiation of an intraluminal thrombosis is believed to involve platelet adherence and aggregation. In normal circulation, platelets cannot aggregate by themselves. However, when a blood vessel is damaged, platelets adhere to the disrupted surface, and the adherent platelets release some biologically active constituents, which induce aggregation (*9*). Thus, platelet aggregation may play a crucial role in the atherothrombotic process. Indeed, antiplatelet agents (e.g., ticlopidine and aspirin) have been shown to reduce the incidence of stroke in high-risk patients (*10*). However, the pharmacological function of C-PC in platelets has not yet been studied, and no data are available concerning the detailed effects of C-PC in platelet aggregation. In the present study, we report for the first time that C-PC at nanomolar concentrations

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possesses very potent inhibitory activity against platelet aggregation.

MATERIALS AND METHODS

Materials. Collagen (type I, bovine Achilles' tendon), C-phycocyanin (C-PC), ADP, luciferin/luciferase, fluorescein sodium, Dowex-1 (100−200 mesh; X_8, chloride form), myoinositol, prostaglandin E_1 (PGE_1), arachidonic acid, phorbol-12,13-dibutyrate (PDBu), apyrase, bovine serum albumin, and thrombin were purchased from Sigma Chem. (St. Louis, MO). Fura 2-AM was purchased from Molecular Probe (Eugene, OR). *myo*-2-[^{3}H]Inositol was purchased from Amersham (Buckinghamshire, HP, UK). Thromboxane B_2, cyclic AMP, and cyclic GMP EIA kits were purchased from Cayman (Ann Arbor, MI). C-PC was dissolved in normal saline and stored at −4 °C until use.

Preparation of Human Platelet Suspensions. Human platelet suspensions were prepared as previously described (*11*) with some modifications. In this study, human volunteers gave 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. After centrifugation at 120*g* for 10 min at room temperature, the supernatant (platelet-rich plasma; PRP) was supplemented with heparin (6.4 IU/mL), EDTA (2 mM), and apyrase (1 U/mL) and then incubated for 10 min at 37 °C and centrifuged at 500*g*. The washed platelets were finally suspended in Tyrode's solution containing bovine serum albumin (3.5 mg/ mL) and adjusted to about 4.5×10^8 platelets/mL. The final concentration of Ca^{2+} in Tyrode's solution was 1 mM.

Platelet Aggregation. The turbidimetric method was applied to measure platelet aggregation, using a Lumi-Aggregometer (Payton, Canada). Platelet suspensions (4.5×10^8 platelets/mL, 0.4 mL) were prewarmed to 37 °C for 2 min, and then C-PC (0.5−10 nM) was added 3 min before the addition of the agonists. The reaction was allowed to proceed for at least 6 min, and the extent of aggregation was expressed in light-transmission units. While measuring ATP release, 20 μL 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 (*12*). Fluorescence-conjugated triflavin was also prepared as previously described (*13*). 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 C-PC (4 and 8 nM) and FITC−triflavin (2 μg/mL) for 3 min, followed by the addition of collagen (1 μg/mL) to trigger platelet activation. Suspensions were then incubated for another 5 min, and the volume was adjusted to 1 mL/tube with Tyrode's solution. The suspensions were then assayed for fluorescein-labeled platelets using a flow cytometer (Becton Dickinson, FACScan Syst., San Jose, CA). Data were collected from 50 000 platelets per experimental group. All experiments were repeated at least four times to ensure reproducibility.

Measurement of Platelet $[Ca^{2+}]_i$ Mobilization by Fura 2-AM Fluorescence. Citrated whole blood was centrifuged at 120*g* for 10 min. The supernatant was incubated with Fura 2-AM (5 μM) for 1 h. Human platelets were then prepared as described above. Finally, the external Ca^{2+} concentration of the platelet suspensions was adjusted to 1 mM. The rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) 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. The $[Ca^{2+}]_i$ was calculated from the fluorescence measured using 224 nM as the Ca^{2+}-Fura 2 dissociation constant (*14*).

Labeling of Membrane Phospholipids and Measurement of the Production of [^{3}H]Inositol Phosphates. The method was carried out as previously described (*11*). Briefly, citrated human platelet-rich plasma was centrifuged, and pellets were suspended in Tyrode's solution containing [^{3}H]inositol (75 μCi/mL). Platelets were incubated for 2 h followed by centrifugation and were finally resuspended in Ca^{2+}-free Tyrode's solution (5×10^8/mL). C-PC (4 and 8 nM) was preincubated with 1 mL of loaded platelets for 3 min, and collagen (1 μg/mL) was then added to trigger aggregation. The reaction was stopped, and samples were centrifuged for 4 min. The inositol phosphates of the supernatants were separated in a Dowex-1 anion exchange column. Only [^{3}H]inositol monophosphate (IP) was measured as an index of total inositol phosphate formation.

Measurement of Thromboxane B_2, Cyclic AMP, and Cyclic GMP Formations. Platelet suspensions (4.5×10^8/mL) were preincubated for 3 min in the presence or absence of C-PC (4 and 8 nM) before the addition of collagen (1 μg/mL). Six minutes after the addition of agonists, 2 mM EDTA and 50 μM indomethacin were added to the reaction suspensions. The vials were then centrifuged, and the TxB_2 levels of the supernatants were measured using an EIA kit. In addition, platelet suspensions were incubated with nitroglycerin (10 μM), PGE_1 (10 μM), and C-PC (4 and 8 nM) for 6 min. The incubation was stopped, and the solution was immediately boiled for 5 min. Fifty microliters of the supernatant was used to determine the cyclic AMP and cyclic GMP contents with EIA kits following acetylation of the samples, as described by the manufacturer.

Measurement of Protein Kinase C Activity. Washed platelets (2×10^9/mL) were incubated for 60 min with phosphorus-32 (0.5 mCi/mL). The ^{32}P-labeled platelets were incubated with C-PC (4 and 8 nM) for 3 min, and then PDBu (60 nM) was added for 1 min to trigger protein kinase C (PKC) activation. Activation was terminated by the addition of Laemmli sample buffer and analyzed by electrophoresis (12.5%; wt/vol) as described previously (*15*). The gels were dried, and the relative intensities of the radioactive bands were analyzed using a bioimaging analyzer system (FAL2000, Fuji, Tokyo, Japan).

Western Blot Analysis of Vasodilator-Stimulated Phosphoprotein (VASP) Phosphorylation. The method of Li et al. (*16*) was followed. In brief, platelet lysates were analyzed by SDS−PAGE gel (10%) and electrotransferred to PVDF membranes. The membranes were blocked with 5% nonfat dry milk in Tris (tris(hydroxymethyl)aminomethane)-buffered saline (TBS, pH7.5), incubated with the mAb 5C6 (CALBIOCHEM), specific for the phosphorylated Ser^{157} site of VASP (0.1 μg/mL). After three washes in TBS containing 0.05% Tween 20, the membrane was incubated with peroxidase-conjugated goat anti-mouse IgG (Amersham) for 2 h. The band with peroxidase activity was detected by enhanced chemiluminescence detection reagents (ECL^+ system, Amersham).

Measurement of Free Radicals in Platelet Suspensions by Electron Spin Resonance (ESR) Spectrometry. The ESR method used a Bruker EMX ESR spectrometer as described previously (*17*). In brief, platelet suspensions (4.5×10^8 platelets/mL, 0.4 mL) were prewarmed to 37 °C for 2 min, and then C-PC (4 and 8 nM) was added for 3 min before the addition of collagen (1 μg/mL). The reaction was allowed to proceed for 1 min, followed by the addition of 100 mM DEPMPO for the ESR study. ESR spectra were recorded on a Bruker EMX ESR spectrometer using a quartz flat cell designed for aqueous solutions. Conditions of ESR spectrometry were as follows: 20 mW power at 9.78 GHz, 1 G modulation, and 100 G scanning for 42 s, with 10 scans accumulated.

Statistical Analysis. The experimental results are expressed as the means ± SEM and are accompanied by the number of observations. Data were assessed using analysis of variance (ANOVA). If this analysis indicated significant differences among the group means, then each group was compared using the Newman−Keuls method. A p value of < 0.05 was considered statistically significant.

RESULTS

Effect of C-PC on Platelet Aggregation in Human Platelets. C-PC (0.5−10 nM) markedly inhibited platelet aggregation stimulated by collagen (1 μg/mL) and U46619 (1 μM), a thromboxane A_2 analogue in washed human platelets (**Figure 1**) and platelet-rich plasma (data not shown). Under the same concentrations (0.5−10 nM), C-PC only slightly inhibited thrombin (0.05 U/mL) but not arachidonic acid (60 μM) induced platelet aggregation (**Figure 1B**). However, at a higher concentration, C-PC (2 μM) markedly inhibited platelet aggregation

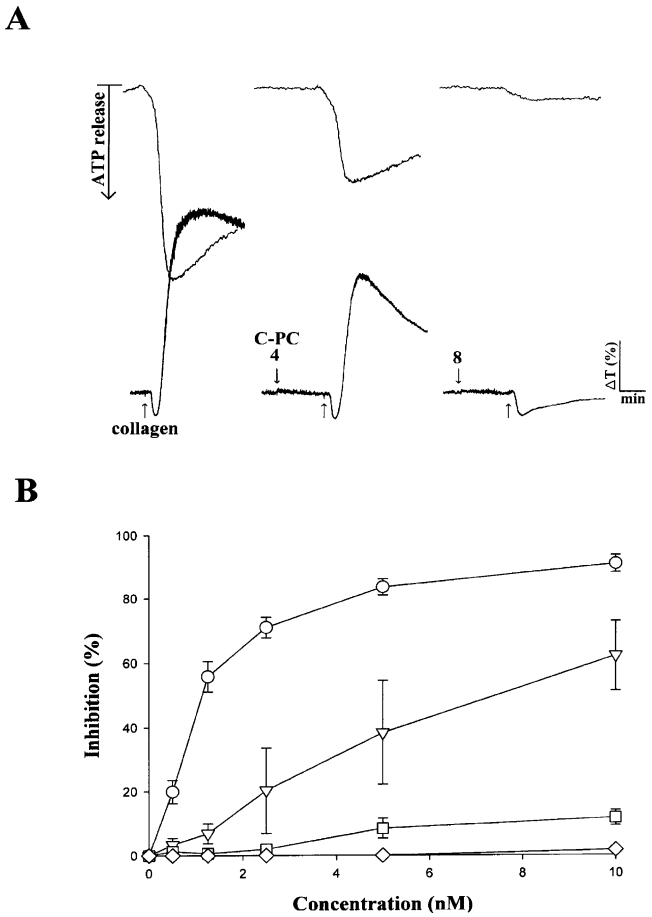

Figure 1. (**A**) Tracing curves and (**B**) concentration–inhibition curves of C-phycocyanin on collagen (1 μg/mL, ○), U46619 (1 μM, ▽), thrombin (0.05 U/mL, □), and arachidonic acid (60 μM, ◇) induced platelet aggregation in washed human platelets. Platelets were preincubated with C-phycocyanin (0.5–10 nM) for 3 min; agonists were then added to trigger aggregation (lower tracings) and ATP release (upper tracings) (**A**). Data are presented as a percentage of the control (means ± SEM, $n = 4$) (**B**).

stimulated by thrombin (0.05 U/mL) and arachidonic acid (60 μM) by about 78% and 92%, respectively (data not shown). C-PC also inhibited the ATP-release reaction when stimulated by agonists (i.e., collagen) (**Figure 1A**). IC_{50} values of C-PC for platelet aggregation induced by collagen and U46619 were estimated to be approximately 4.0 and 7.5 nM, respectively. When platelets were preincubated with a higher concentration of C-PC (2 μM) or normal saline for 10 min, followed by two washes with Tyrode's solution, we found that there were no significant differences between the aggregation curves of either platelet preparation stimulated by collagen (1 μg/ mL), indicating that the effect of C-PC on inhibition of platelet aggregation occurs in a reversible manner (data not shown). In subsequent experiments, we used collagen as an agonist to explore the inhibitory mechanisms of C-PC in platelet aggregation.

Effect of C-PC on Collagen-Induced Conformational Change of the Glycoprotein IIb/IIIa Complex in Human Platelets. Triflavin is an Arg-Gly-Asp-containing antiplatelet peptide purified from *Trimeresurus flavoviridis* snake venom (*12*). Triflavin inhibits platelet aggregation through direct interference with fibrinogen binding to the glycoprotein IIb/IIIa complex ($\alpha_{IIb}\beta_3$ integrin) (13). 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 aggregation. Therefore, we decided to further evaluate whether C-PC binds directly to the platelet

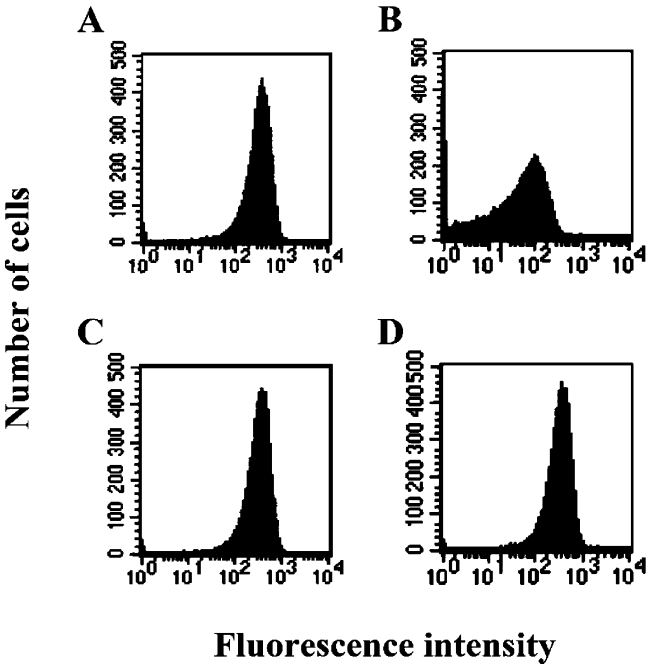

Figure 2. Flow cytometric analysis of FITC–triflavin binding to collagen-activated platelets in the absence or presence of C-phycocyanin. (**A**) The solid line represents the fluorescence profiles of FITC–triflavin (2 μg/mL) in the absence of C-phycocyanin as a positive control, (**B**) in the presence of EDTA (5 mM) as the negative control, or in the presence of C-phycocyanin (**C**) (4 nM) and (**D**) (8 nM). The profiles are representative examples of four similar experiments.

glycoprotein IIb/IIIa complex, leading to inhibition of platelet aggregation induced by agonists.

In this study, the relative intensity of the fluorescence of FITC−triflavin (2 μg/mL) bound directly to collagen (1 μg/ mL)-activated platelets was 319.8 ± 8.5 (**Figure 2A**), and it was markedly reduced in the presence of 5 mM EDTA (negative control, 80.1 ± 3.3) (**Figure 2B**). C-PC (4 and 8 nM) did not significantly inhibit FITC−triflavin binding to the glycoprotein IIb/IIIa complex in platelet suspensions (4 nM, 320.6 ± 9.0; 8 nM, 321.5 ± 9.9) (**Figure 2C,D**), indicating that the mechanism of C-PC's inhibitory effect on platelet aggregation does not involve binding to the glycoprotein IIb/IIIa complex.

Effect of C-PC on $[Ca^{2+}]_i$ Mobilization and Phosphoinositide Breakdown in Human Platelets. Free cytoplasmic Ca^{2+} concentrations in human platelets were measured by the Fura 2-AM loading method. As shown in **Figure 3A**, collagen (1 μg/mL) evoked a marked increase in $[Ca^{2+}]_i$, and this increase was markedly inhibited in the presence of C-PC (4 nM, 77.5 ± 5.0%; 8 nM, 84.8 ± 3.8%) (**Figure 3A**). Furthermore, phosphoinositide breakdown occurs in platelets activated by many different agonists. In this study, we found that collagen (1 μg/ mL) induced the rapid formation of radioactive IP, IP_2, and IP_3 in human platelets loaded with [^{3}H]inositol. We only measured [^{3}H]IP formation as an index of total inositol phosphate formation. As shown in **Figure 3B**, the addition of collagen (1 μg/mL) resulted in a rise of IP formation of about 1.8-fold compared to that in resting platelets [(10.8 ± 0.3 vs 5.9 ± 0.4) × 10^3 cpm]. However, in the presence of C-PC (4 and 8 nM), the radioactivity of IP formation in collagen-stimulated human platelets did not significantly decrease (**Figure 3B**).

Effects of C-PC on Thromboxane B_2, Cyclic AMP, and Cyclic GMP Formations. As shown in **Table 1**, resting platelets produced less TxB_2 compared with collagen-activated platelets. PGE_1 (10 μM) inhibited TxB_2 formation in collagen-

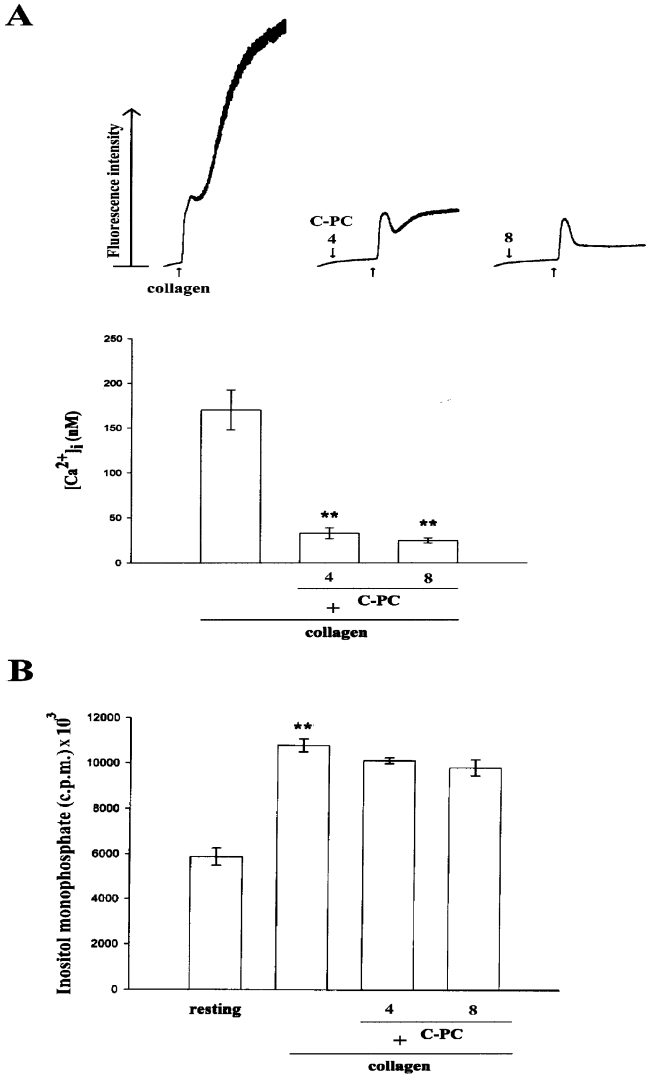

Figure 3. Effects of C-phycocyanin on collagen-induced (**A**) intracellular Ca^{2+} mobilization and (**B**) inositol monophosphate formation in human platelets. Labeled platelets were preincubated with C-phycocyanin (4 and 8 nM) followed by the addition of collagen (1 μg/mL) to trigger platelet activation, as described in Materials and Methods. (**A**) Profiles are representative examples of four similar experiments; data are presented as the means ± SEM ($n = 4$). $^{**}p < 0.001$ as compared with the collagen group; (**B**) data are presented as the means ± SEM ($n = 4$). $^{**}p < 0.001$ as compared with the resting group.

activated platelets by 82% (data not shown). Furthermore, results obtained using various concentrations of C-PC indicated that C-PC (4 and 8 nM) concentration-dependently inhibited TxB_2 formation in platelet suspensions stimulated by collagen (1 μg/mL). In addition, the level of cyclic AMP in unstimulated platelets was about 2.0 ± 0.2 pmol/mL. The addition of PGE_1 (10 μM) markedly increased the cyclic AMP level (**Table 1**). C-PC (4 and 8 nM) did not significantly increase cyclic AMP levels in human platelets (**Table 1**). We also performed a similar study measuring the cyclic GMP response. The level of cyclic GMP in unstimulated platelets was less, but when nitroglycerin (10 μM) was added to the platelet suspensions, the cyclic GMP level markedly increased from the resting level to 1.3 ± 0.2 pmol/mL (**Table 1**). The addition of C-PC (4 and 8 nM) resulted in significant increases in platelet cyclic GMP levels (**Table 1**).

Table 1. Effects of C-Phycocyanin on Thromboxane B_2, Cyclic AMP, and Cyclic GMP in Washed Platelet Suspensions[a]

	concn	TxB_2 (ng/mL), $n = 6$	cyclic AMP (pmol/mL), $n = 4$	cyclic GMP (pmol/mL), $n = 7$
resting		6.1 ± 1.8	2.0 ± 0.2	0.4 ± 0.1
collagen	1 μg/mL	54.4 ± 3.0**	–	–
+C-phycocyanin	4 nM	43.8 ± 3.0#	–	–
	8 nM	25.9 ± 7.5##	–	–
PGE1	10 μM	–	29.6 ± 3.1**	–
NTG	10 μM	–	–	1.3 ± 0.2*
C-phycocyanin	4 nM	–	1.4 ± 0.3	0.9 ± 0.1*
	8 nM	–	1.7 ± 0.2	1.2 ± 0.1*

[a] Human platelet suspensions were preincubated with C-phycocyanin (4 and 8 nM) for 3 min at 37 °C, and then collagen (1 μg/mL) was added to trigger thromboxane B_2 (TxB_2) formation. Addition of prostaglandin E_1 (PGE_1) and nitroglycerin (NTG) into the platelet suspensions served as positive controls of cyclic AMP and cyclic GMP formations, respectively. Data are presented as the means ± SEM (n= number). $^{*}p < 0.01$ and $^{**}p < 0.001$ as compared with the resting groups and $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ as compared with the collagen group.

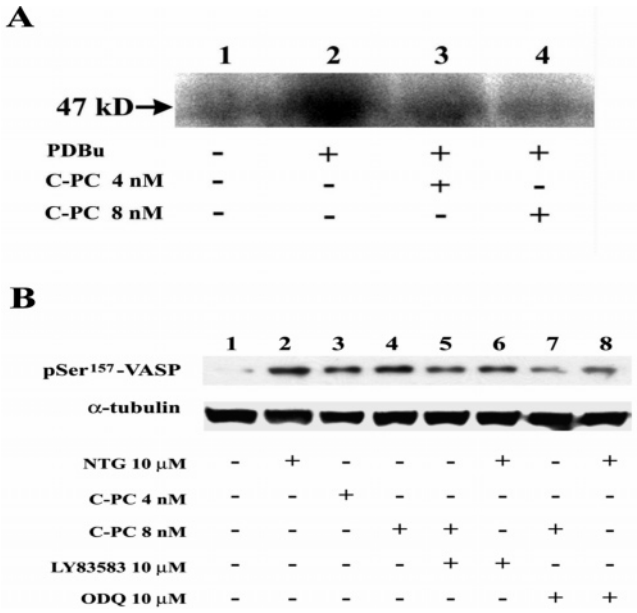

Figure 4. Effects of C-phycocyanin on (**A**) PDBu-stimulated phosphorylation of a protein of M_w 47 000 (P47) and (**B**) agonist-induced phosphorylation of vasodilator-stimulated phosphoprotein (VASP) at Ser^{157} in human platelets. (**A**) Platelets were preincubated with C-phycocyanin (4 and 8 nM) before challenge with PDBu (150 nM). Lane 1, platelets with Tyrode's solution only (resting group); lane 2, platelets activated by PDBu (150 nM) (control group); lane 3, platelets with C-phycocyanin (4 nM); and lane 4, platelets with C-phycocyanin (8 nM) for 3 min followed by the addition of PDBu (150 nM) to trigger protein kinase C activation. The arrow indicates a protein of M_w 47 000 (P47). (**B**) Platelets were incubated with nitroglycerin (NTG, 10 μM) and C-phycocyanin (C-PC, 4 and 8 nM) in the absence or presence of LY83583 (10 μM) or ODQ (20 μM), and solubilized directly in SDS–PAGE sample buffer. Phosphorylation of VASP at Ser^{157} was detected by immunoblotting with a monoclonal antibody specifically recognizing Ser^{157}-phosphorylated VASP. Profiles are representative examples of four similar experiments.

Effects of C-PC on PDBu-Stimulated Phosphorylation of the 47-kDa Protein and Vasodilator-Stimulated Phosphoprotein (VASP) Phosphorylation. Stimulation of platelets with a number of different agonists, PDBu in particular, induces activation of PKC, which then phosphorylates proteins of M_w 40 000−47 000 in addition to other proteins (*18*). In this study,

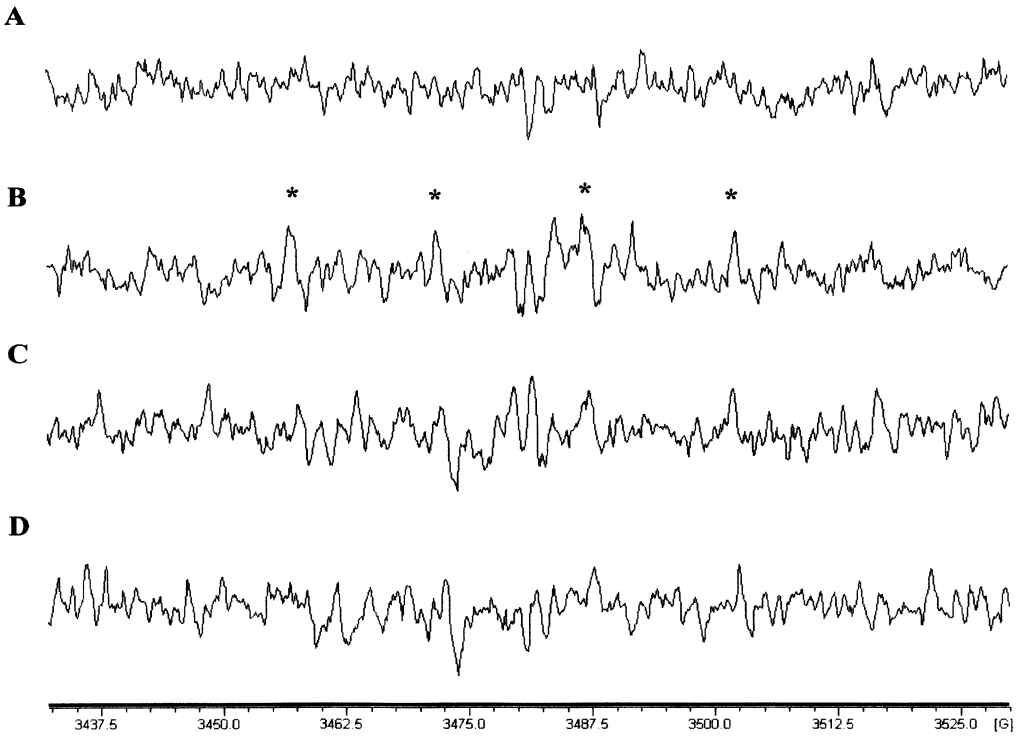

Figure 5. ESR spectra of the effect of C-phycocyanin on hydroxyl radical formation in collagen-activated platelets. (**A**) Platelet suspensions were preincubated with Tyrode's solution only (resting), or platelet suspensions were preincubated with (**B**) Tyrode's solution (control) or C-phycocyanin at (**C**) 4 nM and (**D**) 8 nM for 3 min, and then collagen was added (1 μg/mL) to trigger platelet activation. The reaction was allowed to proceed for 5 min, followed by the addition of DEPMPO (100 mM) for ESR experiments. The spectra are representative examples of four similar experiments.

phosphorylation experiments were performed to examine the role of C-PC in the activation of PKC in human platelets. When PDBu (150 nM) was added to human platelets prelabeled with $^{32}PO_4$, a protein with an apparent M_w of 47 000 (P47) was predominately phosphorylated as compared with resting platelets (**Figure 4A**). C-PC (4 and 8 nM) markedly inhibited the phosphorylation of P47 stimulated by PDBu (**Figure 4A**). However, C-PC (8 nM) did not significantly inhibit collagen (1 μg/mL)-induced phosphorylation of P47 in human platelets (data not shown). These results indicate that C-PC can directly interfere with the activation of PKC in human platelets.

Furthermore, it is presumed that cyclic GMP can induce VASP Ser^{157} phosphorylation in human platelets (*16*). In this study, nitroglycerin (10 μM) markedly induced VASP Ser^{157} phosphorylation, and this phosphorylation was significantly inhibited by the guanylate cyclase inhibitors LY83583 (10 μM) (*19*) and ODQ (10 μM) (*20*) (**Figure 4B**). C-PC (4 and 8 nM) concentration-dependently triggered VASP Ser^{157} phosphorylation, and this phosphorylation was also inhibited in the presence of LY83583 (10 μM) or ODQ (10 μM) (**Figure 4B**).

Effect of C-PC on the Free-Radical-Scavenging Activity in Collagen-Activated Platelets. The rate of free-radical-scavenging activity is defined by the following equation: inhibition rate = 1 − signal height (C-PC + collagen)/signal height (collagen) (*21*). In this study, a typical ESR signal of the hydroxyl radical (OH•) in collagen-activated platelets was observed as shown in **Figure 5**. C-PC (4 and 8 nM) significantly suppressed hydroxyl radical formation in collagen-activated platelets by about 51.7 ± 6.9% (n = 4) and 85.1 ± 7.6% (n = 4), respectively (data not shown).

DISCUSSION

This study demonstrates for the first time that C-PC in nanomolar concentrations possesses very potent antiplatelet activity in human platelets. The principal objective of this study was to describe the inhibitory mechanisms of C-PC in platelet activation. In this study, platelet aggregation induced by these agonists (i.e., collagen) appeared to be affected in the presence of C-PC. Therefore, this partly infers that C-PC may affect Ca^{2+} release from intracellular Ca^{2+}-storage sites (i.e., dense tubular systems or dense bodies), and this is in accord with the concept that intracellular Ca^{2+} release is responsible for platelet aggregation.

Although the action mechanisms of various platelet aggregation agonists, such as collagen, U46619, and thrombin, differ, C-PC significantly inhibited platelet aggregation stimulated by all of them. This implies that C-PC may block a common step shared by these inducers. These results also indicate that the site of action of C-PC is not at the receptor level of individual agonists. Triflavin acts by binding to the glycoprotein IIb/IIIa complex on the platelet surface membrane, resulting in interference with the interaction of fibrinogen with its specific receptor (*12*). In this study, we found that C-PC did not significantly affect FITC−triflavin binding to the glycoprotein IIb/IIIa complex, indicating that the antiplatelet activity of C-PC is possibly not directly due to interference with the binding of fibrinogen to its specific receptor on the platelet membrane.

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 (IP_3) and diacylglycerol (*22*). There is strong evidence that IP_3 induces the release of Ca^{2+} from intracellular stores (*22*). Diacylglycerol activates PKC, inducing protein phosphorylation and a release reaction. In this study, neither phosphoinositide breakdown nor P47 phosphorylation of collagen-activated platelets was significantly inhibited by C-PC, suggesting that inhibition of

platelet aggregation by C-PC might not be mediated by inhibition of phospholipase C activation.

TxA_2 is an important mediator of the release reaction and aggregation of platelets (*23*). Collagen-induced TxB_2 formation, a stable metabolite of TxA_2, was markedly inhibited by C-PC. It has been demonstrated that TxA_2 formation can be induced by free arachidonic acid release by diglyceride lipase or by endogenous phospholipase A_2 from membrane phospholipids (*24*). Thus, it seems likely that TxB_2 formation plays a role in mediating the inhibitory effect of C-PC in human platelets.

Furthermore, C-PC significantly inhibited PDBu-induced PKC activation. PDBu is known to intercalate with membrane phospholipids and form a complex with PKC translocated to the membrane (*25*). Moreover, increased cyclic GMP can negatively affect agonist-induced PKC activation (*26*). Signaling by cyclic GMP somehow interferes with the agonist-stimulated phosphoinositide turnover that creates Ca^{2+}-mobilizing second messengers (*27*). In addition, the VASP is associated with actin filaments and focal adhesions, which form the interface between the cytoskeleton and the extracellular matrix. VASP is phosphorylated by both cyclic GMP- and cyclic AMP-dependent protein kinases in a variety of cells, including smooth muscle cells and platelets. Since both the cyclic GMP and cyclic AMP signaling cascades relax smooth muscle cells and inhibit platelet activation, it was speculated that VASP plays an important role in modulating actin filament dynamics and integrin activation (*28*). C-PC increased both cyclic GMP- and cyclic GMP-induced VASP Ser^{157} phosphorylation in human platelets; therefore, the inhibitory effect of C-PC in PDBu-induced PKC activation may be due, at least partly, to mediating the increase in cyclic GMP.

Reactive oxygen species (i.e., hydrogen peroxide and hydroxyl radicals) derived from platelet activation might affect cells with which they come into intimate contact, such as the endothelium, and this could result in an amplification of platelet reactivity during thrombus formation. Furthermore, reactive oxygen species act as second messengers during the initial phase of platelet activation processes (*29*). Mirabelli et al. (*30*) showed an increase in cytosolic Ca^{2+} concentration upon platelet exposure to oxidative stress. Platelets primed by exposure to subthreshold concentrations of AA or collagen are known to be activated by nanomolar levels of hydrogen peroxide, and this effect is mediated by hydroxyl radicals formed in an extracellular Fenton-like reaction (*31*). It is also evident that some of the hydrogen peroxide produced by platelets is converted into hydroxyl radicals, as platelet aggregation can be inhibited by hydroxyl radical scavengers (*32*). In this study, we found that C-PC inhibited platelet aggregation possibly, at least partially, through inhibition of free radical formation in activated platelets.

In conclusion, the most important findings of this study suggest that, at nanomolar concentrations (4−8 nM), C-PC exhibits very potent activity at inhibiting agonist-induced platelet aggregation. This inhibitory effect may possibly involve the following two mechanisms. (1) C-PC increases cyclic GMP/VASP Ser^{157} phosphorylation and subsequently inhibits PKC activity, ultimately resulting in inhibition of both the phosphorylation of P47 and intracellular Ca^{2+} mobilization; and (2) C-PC may inhibit free radicals (such as hydroxyl radicals) released from activated platelets, with a concomitant lowering of intracellular Ca^{2+} mobilization, followed by inhibition of TxA_2 formation, and finally inhibition of platelet aggregation. Platelet aggregation plays a pathophysiological role in a variety of thromboembolic disorders. Therefore, inhibition of platelet aggregation by drugs may represent an increased therapeutic possibility for such diseases. The important findings of this study suggest that C-PC may represent a potent and novel antiplatelet agent for treatment of arterial thromboembolism.

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