Further Characterization of Galloyl Pedunculagin as an Effective Autophosphorylation Inhibitor of C-Kinase *in Vitro*

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The inhibitory effect of galloyl pedunculagin (GP) isolated from *Platycarya strobilacea* on the activity and autophosphorylation of Ca^{2+}- and phospholipid-dependent protein kinase (C-kinase) was examined *in vitro*. It was found that (i) GP inhibited the activity (phosphorylation of complement C3 from guinea pig) of C-kinaseα (rat brain) in a dose-dependent manner with an ID_{50} of approx. 0.12 μM; (ii) GP at lower doses (ID_{50}=approx. 6 nM) inhibited autophosphorylation of C-kinaseα; and (iii) the GP-induced inhibition of autophosphorylation of C-kinaseα and its enzyme activity was a manner non-competitive to ATP. Similar inhibitory effect of GP on autophosphorylation of recombinant human C-kinaseη (rhC-kinaseη) and its phosphorylating activity was observed. These results suggest that GP is an effective autophosphorylation inhibitor of these two C-kinase isoforms (α and η) *in vitro*. In addition, the CD analysis suggests that the proline-containing six amino acid residues (PVLTPP) including a threonine residue (autophosphorylation site) at the C-terminal region (positions 635—640) of C-kinaseα may be one of the GP-binding sites.

Key words galloyl pedunculagin; autophosphorylation; C-kinase; C-kinase inhibitor

Recently, we characterized (i) plant polyphenol-containing antioxidant compounds, such as quercetin, epigallocatechin gallate (EGCG) and 8-chloro-3′,4′,5,7-tetrahydroxyisoflavone (8C-3′,4′,5,7-THI)[1,2] as potent inhibitors of casein kinase II (CK-II)[2—7] *in vitro*; and (ii) ellagitannins, such as galloyl pedunculagin (GP; see Fig. 1) and eugeniin (a reductive form of GP) isolated from *Platycarya strobilacea*,[8] as selective autophosphorylation inhibitors (ID_{50}=approx. 6.6 nM) for the β-subunit of cAMP-dependent protein kinase (A-kinaseβ) containing a proline-rich cluster (positions 82—87 in bovine type I A-kinaseβ[9] and positions 83—88 in human type I A-kinaseβ[10]) that interacts with ellagitannins.[11] Kashiwada *et al.* evaluated 56 tannin derivatives, including ellagitannins, for their inhibitory effects on the activity (phosphorylation of histone) of Ca^{2+}- and phospholipid-dependent protein kinase (C-kinase), and reported that ellagitannins function as potent C-kinase inhibitors *in vitro*.[12] Furthermore, it has been reported that both A-kinase and C-kinase *in vitro* phosphorylate human complement C3, which plays an important role of many acute inflammatory processes.[13,14] Therefore, the present study was carried out to determine the inhibitory effect of GP on the autophosphorylation of two C-kinase isoforms (C-kinaseα, Ca^{2+}- and phospholipid-dependent protein kinase; and C-kinaseη, phospholipid-dependent protein kinase) and their enzyme activities using different protein substrates under *in vitro* experimental conditions. Here, we describe (i) further characterization of GP as an effective autophosphorylation inhibitor of these two C-kinase isoforms (α and η); (ii) the inhibitory kinetics of GP on the enzyme activities of these two C-kinases *in vitro*; and (iii) circular dichroism (CD) analysis of the GP-binding site on C-kinaseα using a synthetic proline-containing fragment (PVLTPP).

MATERIALS AND METHODS

Chemicals [γ-^{32}P]ATP (3000 Ci/mmol) was obtained from Amersham Pharmacia Biotech. (Arlington Heights, U.S.A.); and dithiothreitol (DTT), phosphatidylserine (PS), diacylglycerol (DAG) and histone IIIS (calf thymus) from Sigma Chemical (St. Louis, U.S.A.). Standard complement fraction of guinea pig was obtained from Cedarlane (Hornby, Ontario, Canada). Anti-serum to rat C3 was obtained from ICN Pharmaceutical Inc. (Aurora, Ohio, U.S.A.). A synthetic fragment (PVLTPP) was obtained from Tana Laboratories (Texas, U.S.A.). GP (Fig. 1) was isolated and purified from *Platycarya strobilacea*.[8]

Protein Kinases C-Kinaseα (approx. 80 kDa, specific activity: 1—2 μmol phosphate/min/mg protein transferred to histone) purified from rat brain was obtained from Molecular Probes (Oregon, U.S.A.). Recombinant human C-kinaseη [rhC-kinaseη (approx. 78 kDa), specific activity: 0.9 mmol phosphate/min/mg protein transferred to epsilon peptide substrate] from Biomol Research Laboratories Inc. (Plymouth Meeting, PA, U.S.A.).

Partial Purification of Guinea Pig C3 Complement C3 in guinea pig complement fraction was partially purified by CM-sepharose and Mono Q HPLC column chromatographies according to a modification of a method described originally by Hammer *et al.*[15]

Assay for the Activities of C-Kinaseα and rhC-Kinaseη The activity (phosphorylation of guinea pig C3) of C-kinaseα (approx. 80 kDa) was assayed in reaction mixtures

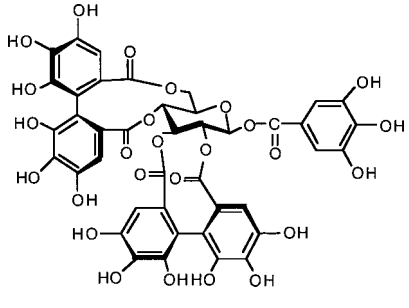

Fig. 1. The Chemical Structure of GP

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comprising 40 mM Tris–HCl (pH 7.4), C-kinaseα (approx. 10 ng), 20 μM [γ-^{32}P]ATP (500 cpm/pmol), 10 mM Mg^{2+}, C-kinase activators (1 mM Ca^{2+}, 25 μM PS and 3 μM DAG) and approx. 4 μg of protein substrate (purified guinea pig C3 or histone IIIS). The activity (phosphorylation of protamine-sulfate) of rhC-kinaseη (approx. 78 kDa, approx. 10 ng) was determined in the presence of 25 μM PS.[16] After incubation for 30 min at 30 °C, ^{32}P-labeled C3α (p115) or autophosphorylated C-kinase isoforms [α (p80) and η (p78)] in the reaction mixtures were detected directly by autoradiography after SDS–PAGE, as reported previously.[1—7,11]

RESULTS

Further Purification of C3 by Gel Filtration on a Superdex 200 pg HPLC Column When guinea pig C3 in the Mono Q fraction was further purified by gel filtration on a Superdex 200 pg HPLC column, a single major protein peak was observed (Fig. 2A). SDS–PAGE (Fig. 2B) detected two major polypeptides (p115 and p63), which were crossreacted with anti-C3 serum, in the indicated fractions (Fig. 2C). The two major polypeptides correspond to those reported for C3α (115 kDa) and C3β (65 kDa) purified from serum of guinea pig.[17] Therefore, this purified C3 (Superdex fraction 12) was used as a phosphate acceptor for C-kinaseα *in vitro.*

Inhibitory Effect of GP on the Activities of Two C-Kinase Isoforms (α and η) *in Vitro* To confirm the substrate activity of guinea pig C3 for C-kinaseα, purified C3 was incubated with C-kinaseα and [γ-^{32}P]ATP in the presence or absence of C-kinase activators (1 mM Ca^{2+}, 25 μM PS and 3 μM DAG) *in vitro*. As shown in Fig. 3A, (i) p115 (C3α) was slightly phosphorylated by C-kinaseα in the absence of C-kinase activators (lane 2); (ii) phosphorylation of C3α by C-kinaseα was greatly stimulated in the presence of C-kinase activators (lane 3); and additionally (iii) C-kinaseα (p80) was autophosphorylated (lanes 2, 4). From these results *in vitro*, it was confirmed that C3α functions as a phosphate acceptor for C-kinaseα, as has been reported for phosphorylation of human C3 by C-kinase *in vitro*.[13,14] Under our experimental conditions, we have confirmed that human C3α is phosphorylated by C-kinaseη (data not shown). Phosphorylation of C3α by C-kinaseα reached a plateau within 90 min (Fig. 3B). GP inhibited autophosphorylation of C-kinaseα in a dose-dependent manner with an ID_{50} of approx. 6 nM, but the ID_{50} of GP on autophosphorylation of rhC-kinaseη was approx. 10 nM (Fig. 4A). No difference of the GP sensitivities to autophosphorylation between these two C-kinase isoforms (α and η) in the presence or absence of their protein substrates (C3 and protamine sulfate) was observed.

The inhibitory effect of GP on the activities of two C-kinase isoforms (α and η) was determined. It was found that GP inhibits two kinase activities in a dose-dependent manner with an ID_{50} of approx. 0.12 μM (Fig. 4B). These results show that GP has a similar sensitivity against autophospho-

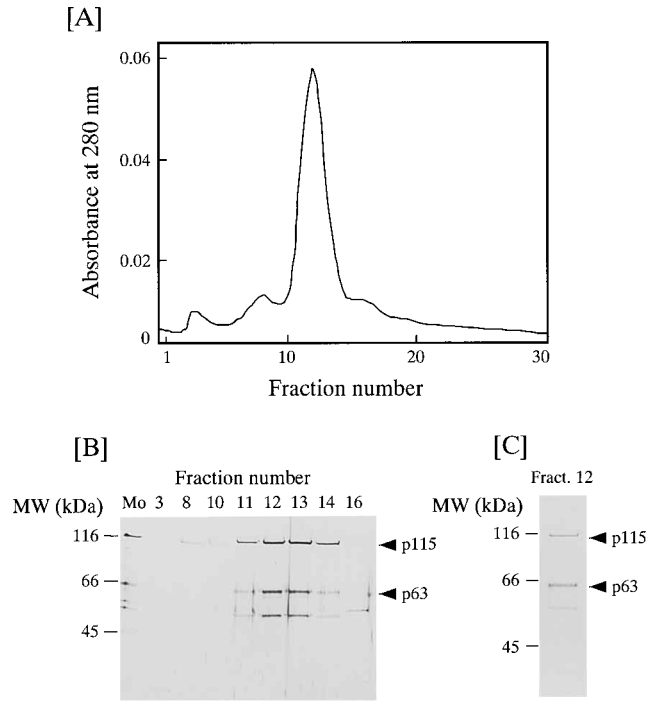

Fig. 2. Further Purification of Guinea Pig C3 in the Mono Q Fraction by Gel Filtration on a Superdex 200 pg Column (HPLC)

[A] The Mono Q fraction (approx. 3 mg protein) was applied to a Superdex 200 pg HPLC column, previously equilibrated with 40 mM Tris–HCl (pH 7.6) containing 0.1 mM PMSF, 1 mM EDTA, 0.5 mM EGTA and 1.0 M NaCl. Elution was carried out with the same buffer and 2.0 ml fractions were collected at a flow rate of 1.0 ml/min. (—), Absorbance at 280 nm. [B] Polypeptides in the indicated fractions were detected by silver staining after SDS–PAGE. Mo, Mono Q fraction. [C] C3 polypeptides (α=115 kDa and β=63 kDa) in Superdex fraction 12 were detected by Western blotting using anti-C3 serum.

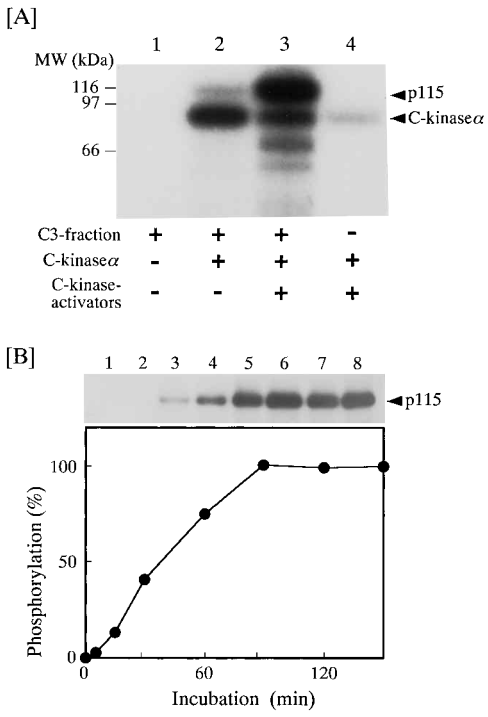

Fig. 3. The C-Kinaseα-Mediated Phosphorylation of p115 (C3α) and Its Kinetics

[A] Purified C3 (approx. 4 μg) was incubated for 60 min at 30 °C with C-kinaseα (approx. 10 ng) and 20 μM [γ-^{32}P]ATP (500 cpm/pmol) in the presence or absence of C-kinase activators (1 mM Ca^{2+}, 25 μM PS and 3 μM DAG). Phosphorylated proteins [p115 (C3α) and p80 (an autophosphorylated form of C-kinaseα)] in the reaction mixtures were detected by SDS–PAGE followed by autoradiography. Purified C3 fraction alone (lane 1); lane 2, lane 1+C-kinaseα; lane 3, lane 2+C-kinase activators; and lane 4, C-kinaseα+C-kinase activators. [B] Purified C3 (approx. 4 μg) was separately incubated for the indicated periods at 30 °C with C-kinaseα and 20 μM [γ-^{32}P]ATP (500 cpm/pmol) in the presence of C-kinase activators. The degree of phosphorylation of p115 (C3α) was measured by densitometry following standard exposure of X-ray film. 100% represents maximum phosphorylation of p115 by C-kinaseα after incubation for 120 min at 30 °C.

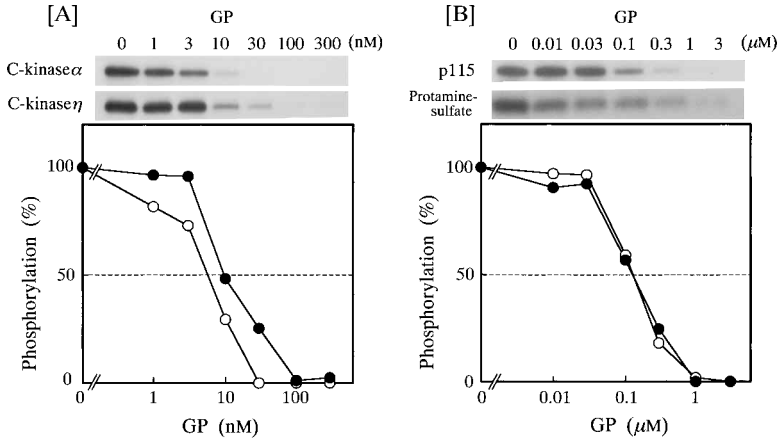

Fig. 4. The Effect of GP on Autophosphorylation of Two C-Kinase Isoforms (α and η) and Their Enzyme Activities

Two C-kinase isoforms (α and η, each approx. 10 ng) were separately incubated for 10 min in an ice bath with the indicated concentrations of GP in the standard reaction mixtures comprising 50 mM Tris–HCl (pH 7.4), 10 mM Mg^{2+} and 2 mM DTT. The activity (phosphorylation of histone IIIS) of C-kinaseα was determined in the presence of C-kinaseα activators (1 mM Ca^{2+}, 25 μM PS and 3 μM DAG). rhC-kinaseη was assayed in the presence of 25 μM PS using protamine sulfate as a substrate, as described in Materials and Methods. Phosphorylation was initiated by the addition of 20 μM [γ-^{32}P]ATP to the reaction mixtures and incubated for 30 min at 30 °C. ^{32}P-Labled proteins C3α (p115), C-kinaseα (p80) or rhC-kinaseη (p78) in the reaction mixtures were detected by autoradiography after SDS–PAGE. The autoradiogram was scanned with a spectrophotometer. 100% represents autophosphorylation of two C-kinases and their enzyme activities in the absence of GP. [A]: Effect of GP on autophosphorylation of C-kinaseα (○) and rhC-kinaseη (●). [B]: Effect of GP on the activities of C-kinaseα (○) and rhC-kinaseη (●).

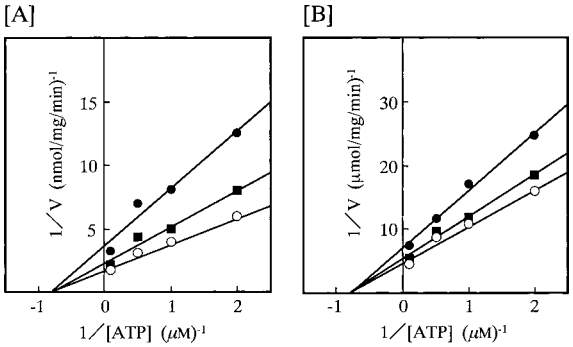

Fig. 5. The Inhibitory Kinetics of GP on Autophosphorylation of C-Kinaseα and the C-Kinaseα-Mediated Phosphorylation of Histone IIIS

After incubation (30 min for C-kinaseα at 30 °C), the radioactivity of ^{32}P-labeled C-kinaseα (p80) or histone IIIS on a glass filter was measured with a scintillation spectrometer, as reported previously.[11] The K_m and V_{max} values are represented on Lineweaver–Burk plots. [A]: Autophosphorylation of C-kinaseα: control, absence of GP (○); 40 nM GP (■); and 0.12 μM GP (●). [B]: Phosphorylation of histone IIIS by C-kinaseα: control, absence of GP (○); 0.15 μM GP (■); and 0.45 μM GP (●).

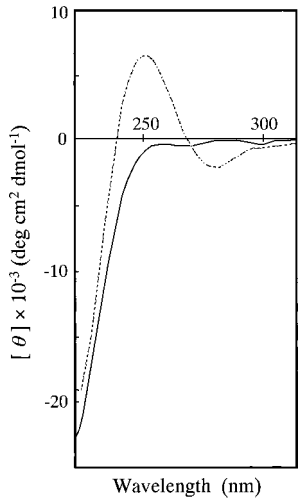

Fig. 6. The Direct Binding of GP to a Synthetic Proline-Containing Fragment

The far-UV CD spectra of synthetic fragment (PVLTPP, approx. 4 μM) incubated with 3 μM GP in 20 mM MES–NaOH (pH 6.8) were measured at 25 °C in the wavelength ranges of 210—300 nm at a recording rate of 50 nm/min using a 10 mm cell and a J-720 CD spectropolarimeter (JASCO). The spectra are presented as the average of three repeat scans. Synthetic fragment alone (——); incubated with 3 μM GP (.....).

rylation of two C-kinase isoforms (α and η) as well as their enzyme activities *in vitro*. The fact that GP has more sensitive effects on the autophosphorylation of these two C-kinase isoforms than their enzyme activities *in vitro* suggests that GP may bind directly to these two isoforms.

Inhibitory Kinetics of GP on Autophosphorylation of C-Kinaseα and Its Enzyme Activity *in Vitro* The inhibitory kinetics of GP on autophosphorylation of C-kinaseα and its enzyme activity (phosphorylation of histone IIIS) were determined *in vitro*. In autophosphorylation of C-kinaseα, the V_{max} was shifted to 0.27 from 0.59 nmol/mg/min when GP (0.12 μM) was added to the reaction mixtures (Fig. 5A). In contrast, the activity (phosphorylation of histone IIIS) of C-kinaseα for the V_{max} was shifted to 0.14 from 0.22 μmol/mg/min in the presence of 0.45 μM GP (Fig. 5B). However, no change of the K_m (1.3 μM) for the substrate was detected under the given experimental conditions. Similar inhibitory kinetics of GP on autophosphorylation and the enzyme activity of rhC-kinaseη were also obtained (data not shown). These results show that GP inhibits autophosphorylation of two C-kinase isoforms (α and η) as well as their enzyme activities in a manner noncompetitive to ATP.

Direct Binding of GP to a Synthetic Proline-Containing Fragment *in Vitro* Previously, we reported that GP preferentially binds to the β-subunit of A-kinase (A-kinaseβ) containing a proline-rich cluster (positions 82—87) in bovine type I A-kinaseβ.[11] Although C-kinaseα has not such a proline-rich domain as in the A-kinaseβ, there is a proline-containing sequence comprising six amino acid residues (PVLTPP) including a threonine residue (autophosphorylation site) at the C-terminal region (positions 635—640) of C-kinaseα. To confirm the direct binding of GP to the synthetic fragment of six amino acid residues (PVLTPP), the CD spec-

tra were determined after incubation of the synthetic fragment for 10 min at room temperature with or without GP. A positive peak at 250 nm clearly appeared when the synthetic fragment was incubated with 3 μM GP (Fig. 6). This result suggests that GP has a binding ability to the synthetic fragment (PVLTPP) *in vitro.*

DISCUSSION

In the present study, it was confirmed that GP inhibits the C-kinaseα-mediated phosphorylation of guinea pig C3 as well as the rhC-kinaseη-mediated phosphorylation of protamine-sulfate in a dose-dependent manner (Fig. 4). The inhibitory effect of GP on C-kinaseα activity (ID_{50}=approx. 0.12 μM) was about 60-fold more sensitive than the ID_{50} dose (approx. 7 μM) reported by Kashiwada *et al.*[12] This high discrepancy might be due to differences in the enzyme assay conditions of C-kinaseα, because the effective inhibition of the C-kinaseα activity by GP occurs when the compound is preincubated with C-kinaseα before the addition of its protein substrates. GP at lower doses effectively inhibited autophosphorylation of two C-kinase isoforms (α and η) (Fig. 4A). These results suggest that (i) GP is an effective autophosphorylation inhibitor of these two C-kinase isoforms *in vitro*; and (ii) the GP-induced inhibition of their autophosphorylation may result in the inhibition of their enzyme activities *in vitro*. This conclusion basically corresponds to our previous report concerning the inhibitory effect of GP on autophosphorylation of A-kinaseβ *in vitro*,[11] because (i) the ID_{50} dose (approx. 6 nM) of GP on autophosphorylation of C-kinaseα *in vitro* (Fig. 4) is similar to that (ID_{50}=approx. 6.6 nM) observed for autophosphorylation of A-kinaseβ; and (ii) the GP-induced inhibition of protein phosphorylation by two C-kinase isoforms (α and η) is typically noncompetitive to ATP (Fig. 5), as shown in a manner similar to the GP effect on autophosphorylation of A-kinaseβ.[11]

The GP-induced inhibition of A-kinase activity (phosphorylation of histone H2B) is due to the preferential binding of GP to the A-kinaseβ (heterodimer), thus preventing physiological interaction between the β-subunit and α-subunit of A-kinase *in vitro*.[11] This conclusion is consistent with the observations reported for the binding ability of proline-rich salivary proteins with high affinity of ellagitannins under acidic pH conditions,[18—20] because the A-kinaseβ has a proline-rich cluster that interacts with tannins. Although any C-kinase isoform has not such a proline-rich domain as in the A-kinaseβ,[11] autophosphorylation of two C-kinase isoforms (α and η) shows similar sensitivity to GP *in vitro*. The amino acid sequences of these two C-kinase isoforms among human, rat and mouse show 99.4% homology. However, there is only 34.4% homology of amino acid sequences between isoformα (672 amino acids containing 39 proline residues[21]) and isoformη (682 amino acids containing 29 proline residues[22]). Interestingly, there is a unique sequence comprising six amino acid residues (PVLTPP) including a threonine residue (autophosphorylation site) at the C-terminal regions of three C-kinase isoforms (α, βII and γ) and C-kinase isoformη includes a similar six amino acid residues (PVLTPI). As expected, the CD analysis showed that GP directly binds to the synthetic fragment comprising six amino acid residues (PVLTPP) (Fig. 6). Therefore, it seems that the common six amino acid residues (PVLTPP) including an autophosphorylation site (threonine residue) at the C-terminal regions of at least three C-kinase isoforms (α, βII and γ) may be one of the GP-binding sites.

To clearly understand the inhibitory action of GP on the physiological activities of C-kinases in metabolic alteration and the C-kinase-mediated regulation of functional mediators involved in signal transduction at the cellular level, further analytical studies will be required (i) to determine the other GP-binding sites of the GP-sensitive C-kinase isoforms (α, βII and γ); and (ii) to characterize GP as a potent selective inhibitor of the GP-sensitive C-kinase-mediated protein phosphorylation during cell proliferation, inflammation and immunological response.

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