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 ++ - 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-kinase (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) as potent inhibitors of casein kinase II (CK-II) 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 and positions 83—88 in human type I A-kinaseβ) 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 ++ - 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 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 ++ - 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-32P]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. 5

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 nmol 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-kinase (approx. 10 ng), 20 μM [γ-32P]ATP (500 cpm/pmol), 10 mM Mg2+, C-kinase activators (1 mM Ca2+, 25 mM PS and 3 mM DAG) and approx. 4 μg of protein substrate (purified guinea pig C3 or histone H1S). The activity (phosphorylation of protamine-sulfate) of rhC-kinase η (approx. 78 kDa, approx. 10 ng) was determined in the presence of 25 μM PS. After incubation for 30 min at 30 °C, 32P-labeled C3 (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.

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 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 C3 (115 kDa) and C3β (65 kDa) purified from serum of guinea pig. Therefore, this purified C3 (Superdex fraction 12) was used as a phosphate acceptor for C-kinase 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-kinase α, purified C3 was incubated with C-kinase α and [γ-32P]ATP (500 cpm/pmol) in the presence or absence of C-kinase activators (1 mM Ca2+, 25 μM PS and 3 μM DAG) in vitro. As shown in Fig. 3A, (i) p115 (C3α) was slightly phosphorylated by C-kinase α in the absence of C-kinase activators (lane 2); (ii) phosphorylation of C3α by C-kinase α was greatly stimulated in the presence of C-kinase activators (lane 3); and additionally (iii) C-kinase α (p80) was autophosphorylated (lanes 2, 4). From these results, it was confirmed that C3α functions as a phosphate acceptor for C-kinase α, as has been reported for phosphorylation of human C3 by C-kinase in vitro. Under our experimental conditions, we have confirmed that human C3α is phosphorylated by C-kinase η (data not shown). Phosphorylation of C3α by C-kinase α reached a plateau within 90 min (Fig. 3B). GP inhibited autophosphorylation of C-kinase α in a dose-dependent manner with an ID50 of approx. 6 nM, but the ID50 of GP on autophosphorylation of rhC-kinase η 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 ID50 of approx. 0.12 μM (Fig. 4B). These results show that GP has a similar sensitivity against autophospho-
The Direct Binding of GP to a Synthetic Proline-Containing Fragment, no change of the C-Kinase GP may bind directly to these two isoforms.

Inhibitory Kinetics of GP on Autophosphorylation of C-Kinase and Its Enzyme Activity in Vitro The inhibitory kinetics of GP on autophosphorylation of C-kinaseα and its enzyme activity (phosphorylation of histone III S) were determined in vitro. In autophosphorylation of C-kinaseα, the \( V_{\text{max}} \) was shifted to 0.27 from 0.59 nmol/mg/min when GP (0.12 \( \mu \)M) was added to the reaction mixtures (Fig. 5A). In contrast, the activity (phosphorylation of histone III S) of C-kinaseα for the \( V_{\text{max}} \) was shifted to 0.14 from 0.22 nmol/mg/min in the presence of 0.45 \( \mu \)M GP (Fig. 5B). However, no change of the \( K_m \) (1.3 \( \mu \)M) 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 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-kinase activity (ID₅₀ ≈ approx. 0.12 μM) was about 60-fold more sensitive than the ID₅₀ dose (approx. 7 μM) reported by Kashiwada et al. 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, because (i) the ID₅₀ dose (approx. 6.6 μM) of GP on autophosphorylation of C-kinaseα in vitro (Fig. 4) is similar to that of ID₅₀ (approx. 6.6 μM) 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.

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. This conclusion is consistent with the observations reported for the binding ability of proline-rich salivary proteins with high affinity of ellagittannins under acidic pH conditions. Because the A-kinaseβ has a proline-rich cluster that interacts with tannins. Although any C-kinase isoform does not have such a proline-rich domain as in the A-kinaseβ, 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) and isoformη (682 amino acids containing 29 proline residues). Interestingly, there is a unique sequence comprising six amino acid residues (PVLTPP) in the catalytic domain of the C-terminal regions of three C-kinase isoforms (α, βII and γ) and C-kinase isoform η includes a similar six amino acid residues (PVLTPP). 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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