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Biochemical Characterization of Galloyl Pedunculagin (Ellagitannin) as a Selective Inhibitor of the β-Regulatory Subunit of A-Kinase *In Vitro*[1]

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The inhibitory effects of galloyl pedunculagin (GP) and eugeniin on the phosphorylation of histone H2B by cAMP-dependent protein kinase (A-kinase) and autophosphorylation of its β-regulatory subunit (A-kinase β) were examined *in vitro*. It was found that (i) GP (ID_{50} = approx. 50 nM) effectively inhibits the activity of A-kinase (heterodimer), but high doses are required to inhibit the activities of the α-catalytic subunit (ID_{50} = approx. 0.25 μM) and casein kinase II (CK-II, ID_{50} = approx. 0.6 μM); (ii) GP inhibits the autophosphorylation of A-kinase β in a dose-dependent manner with an ID_{50} of approx. 6.6 nM, which is about 30-fold lower than that observed with CK-IIβ; and (iii) GP reduces the suppressive effect of the β-subunit on the activity of the α-subunit. In addition, purified bovine heart A-kinase precipitates when incubated with excess GP at pH 5.0. A similar precipitation of A-kinase was observed with eugeniin. These results show that the direct binding of GP to the β-subunit prevents the physiological interaction between the β- and α-subunits of A-kinase *in vitro*. This conclusion is presumably consistent with the binding affinity of proline-rich proteins with tannins, since A-kinase β contains a proline-rich domain that interacts with GP or eugeniin. Therefore, GP will serve as a powerful inhibitor for *in vitro* and *in vivo* cellular studies of A-kinase β–mediated signal transduction.

Key words: cAMP-dependent protein kinase, casein kinase II, galloyl pedunculagin, inhibitor of A-kinase β, proline-rich protein.

Recently, we reported that, *in vitro*, 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*), (i) are potent inhibitors of casein kinase II (CK-II); and (ii) selectively inhibit the CK-II–mediated phosphorylation of glycyrrhizin (GL)-binding proteins, such as 60S acidic ribosomal P protein (*2*), lipoxygenase 3 (*3*), phospholipase A_2 (*4*), glucocorticoid receptor (5), and human immunodeficiency virus type 1 (HIV-1) gene products [reverse transcriptase (*1*, *6*) and protease (*7*)].

Kashiwada *et al.* reported the inhibitory effects of 56 tannin derivatives on the activities of Ca^{2+}- and phospholipid-dependent protein kinase (C-kinase) and cAMP-dependent protein kinase (A-kinase) *in vitro*, and they found that ellagitannins and complex tannins function as potent inhibitors of C-kinase, but do not significantly affect the activity (phosphorylation of histone) of A-kinase (*8*). Plant tannins are a class of polyphenols that bind to certain proteins (*9*), and it has been reported that they bind preferentially to proline residues in gramicidin S, a simple peptide with a rigid β-turn structure (*10*). Also, proline-rich salivary proteins have very high affinities for tannins under acidic pH conditions (*11*, *12*). Since the β-regulatory subunit of A-kinase (A-kinase β) includes a proline-rich domain [positions 82–87 in bovine type I A-kinase β (*13*) and positions 83–88 in human type I A-kinase β (*14*)], it is of interest to examine the inhibitory effects of ellagitannins [galloyl pedunculagin (GP) and eugeniin (a reductive form of GP); Fig. 1] isolated from *Platycarya strobilacea* (*15*), and of proanthocyanidins [procyanidin B2 3,3′-digallate (PB-2) and AC trimer] isolated from *Paeoniae radix* (*16*, *17*) on the activity (phosphorylation of histone H2B) of purified A-kinase and the autophosphorylation of A-kinase β *in vitro*.

In this paper, we describe (i) the characterization of GP and its related compounds (eugeniin and sanguiin H-6) as selective inhibitors of the autophosphorylation of A-kinase β; (ii) determination of their inhibitory kinetics on the auto-

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Abbreviations: A-kinase, cAMP-dependent protein kinase; A-kinase α, α-catalytic subunit of A-kinase; A-kinase β, β-regulatory subunit of A-kinase; bhA-kinase, bovine heart A-kinase; C-kinase, Ca^{2+}- and phospholipid-dependent protein kinase; CK-II, casein kinase II; DTT, dithiothreitol; EGCG, epigallocatechin gallate; GP, galloyl pedunculagin; HPLC, high-performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

phosphorylation of A-kinase β and CK-IIβ; (iii) determination of the direct binding of GP to A-kinase β and the inhibitory effect of GP on the phosphorylating activity of A-kinase α; and (iv) selective precipitation of purified A-kinase with GP under acidic pH conditions.

MATERIALS AND METHODS

Chemicals—[γ-^{32}P]ATP (3,000 Ci/mmol) was obtained from Amersham Pharmacia Biotech. (Arlington Heights, USA); and catechin, dithiothreitol (DTT), EGCG, genistin and quercetin (3,3′,4′,5,7-pentahydroxyflavone) from Sigma Chemical (St. Louis, USA). oGA [olean-11,13(18)-diene-3β,30-diol 3β,30-di-*O*-hemiphthalate 2Na] (*1*, *2*) was kindly supplied by Minophagen Pharmaceutical (Zama).

Purification of Polyphenolic Tannins—Ellagitannins [GP (MW = 936) and eugeniin (MW = 938)] were isolated from *Platycarya strobilacea* (*15*). Arecatannin A-1, AC trimer and procyanidin B-2,3,3′-digallate (*16*, *17*) were extracted and purified from *Paeoniae radix*. Theaflavin was prepared from (−)-epicatechin and (−)-epigallocate, as previously reported (*18*).

Protein Kinases—Purified A-kinases (from porcine and bovine hearts), α-catalytic subunit (specific activity: 83 units/μg protein) and β-regulatory subunit (inhibitory activity: 83 units/μg protein) of A-kinase from bovine heart were obtained from Sigma Chemical (St. Louis, USA). Recombinant human CK-II (rhCK-II) [a heterodimer of $\alpha_2\beta_2$

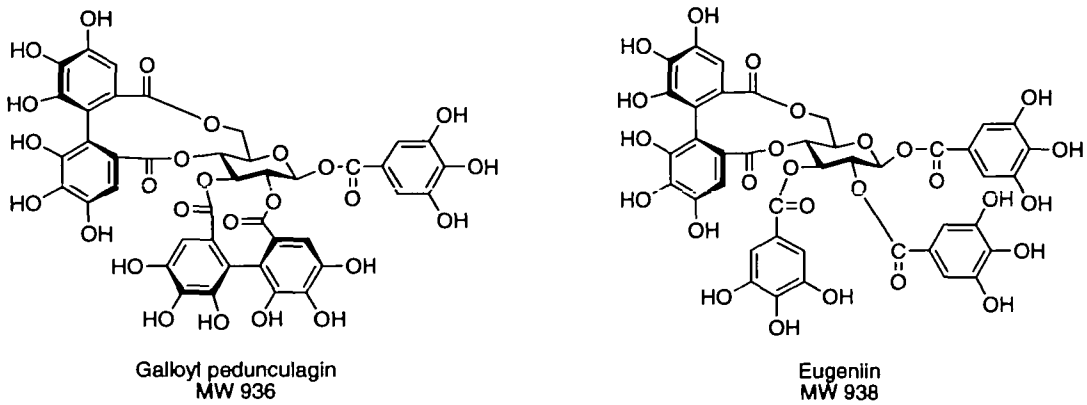

Fig. 1. **The chemical structures of galloyl pedunculagin (GP) and eugeniin.**

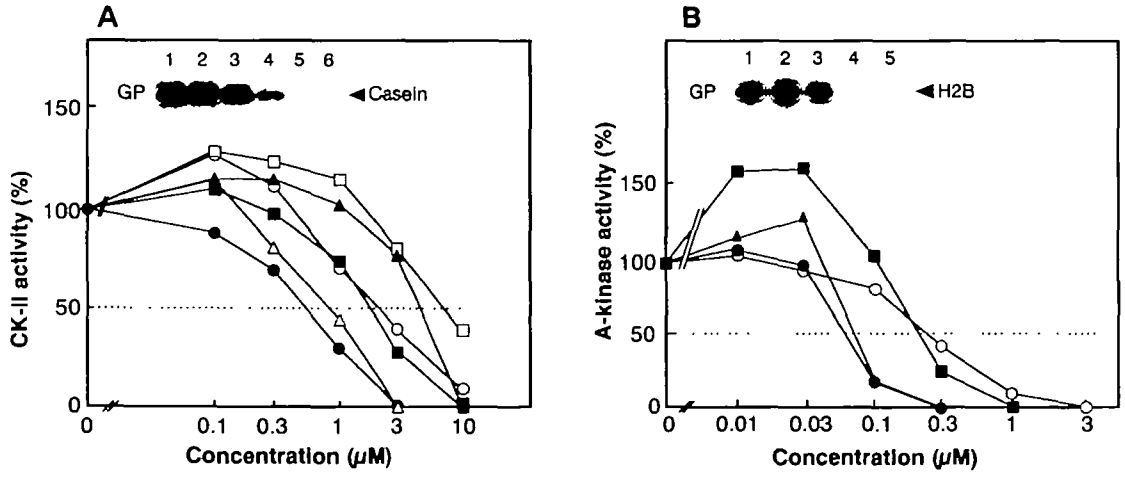

Fig. 2. **A: The inhibitory effects of EGCG, quercetin, and four tannins on CK-II activity *in vitro*.** The inhibitory effects of six polyphenolic compounds on CK-II activity were determined using α-casein as a substrate. After incubation (30 min at 30°C) in the presence of various doses of these compounds, ^{32}P-labeled α-casein in the reaction mixtures was determined by autoradiography after SDS-PAGE. The autoradiogram was scanned with a spectrophotometer. 100% represents the CK-II-mediated phosphorylation of α-casein determined in the absence of these compounds. ●, GP; ■, AC-trimer; ▲, theaflavin; ○, PB-2; □, EGCG; and △, quercetin. Insert: lane 1, absence of GP (control); lane 2, 0.1 μM GP; lane 3, 0.3 μM; lane 4, 1 μM; lane 5, 3 μM; and lane 6, 10 μM. **B: The inhibitory effects of arecatannin A-1, eugeniin, and GP on the activity of A-kinase *in vitro*.** The inhibitory effects of these three tannins on the activity of A-kinase (heterodimer) were determined using histone H2B as a substrate. After incubation (30 min at 30°C) in the presence of various doses of these compounds, ^{32}P-labeled histone H2B in the reaction mixtures was determined by autoradiography after SDS-PAGE. The autoradiogram was scanned with a spectrophotometer. 100% represents the A-kinase–mediated phosphorylation of histone H2B determined in the absence of these compounds. ■, Arecatannin A-1; ▲, eugeniin; and ●, GP. The inhibitory effect of GP on the activity of A-kinase α (○). Insert: lane 1, absence of GP (control); lane 2, 0.01 μM GP; lane 3, 0.03 μM; lane 4, 0.1 μM; lane 5, 0.3 μM; and lane 6, 1.0 μM.

(α-subunit = 44 kDa and β-subunit = 26 kDa); specific activity: 400 kU/mg protein using a peptide substrate (RRREEETEEE) per minute at 30°C at pH 7.5] was obtained from Biomol Research Laboratories (Plymouth Meeting, PA, USA).

Further Purification of A-Kinase by Mono Q Column HPLC Chromatography—Bovine heart A-kinase (bhA-kinase) was further purified on a Mono Q HPLC column, previously equilibrated with 40 mM Tris-HCl (pH 7.4) containing 0.2 M NaCl, 1 mM DTT and 5% glycerol. Elution was carried out with a linear gradient of 0.2 to 1.0 M NaCl. The bhA-kinase activity was eluted between 0.6 and 0.7 M NaCl as a single active peak, as previously reported (*19*). This purified bhA-kinase was used for most of the present study.

Assay of the Activities of A-Kinase and CK-II—The activities (phosphorylation of histone H2B) of purified bhA-kinase and bhA-kinase α were assayed in reaction mixtures comprising 40 mM Tris-HCl (pH 7.4), purified bhA-kinase (approx. 50 ng) or A-kinase α (approx. 50 ng), 20 μM [γ-^{32}P]ATP (500 cpm/pmol), 10 mM Mg^{2+} and 3 μg histone H2B (substrate). The activity of A-kinase (heterodimer) was measured in the presence of 2 μM cAMP.

The activity (phosphorylation of α-casein) of CK-II was assayed in reaction mixtures comprising 40 mM Tris-HCl (pH 7.4), rhCK-II (approx. 50 ng), 20 μM [γ-^{32}P]ATP (500 cpm/pmol), 3 mM Mn^{2+} and 3 μg α-casein (substrate), as reported previously (*1–3*). After incubation for 30 min at 30°C in the presence of poly-Arg as a CK-II activator, ^{32}P-labeled α-casein or histone H2B in the reaction mixtures was detected directly by autoradiography after SDS-PAGE, as reported previously (*1–3, 19*).

Detection of the Autophosphorylation of A-Kinase β by Autoradiography—Purified bhA-kinase (approx. 50 ng) was directly incubated for 30 min at 30°C in reaction mixtures comprising 40 mM Tris-HCl (pH 7.4), 20 μM [γ-^{32}P]ATP (500 cpm/pmol), and 10 mM Mg^{2+}. The ^{32}P-labeled A-kinase β (p55) in the reaction mixtures was detected by SDS-PAGE followed by autoradiography.

RESULTS

The Inhibitory Effects of Four Tannins and Seven Polyphenol Antioxidant Compounds on the Activities of CK-II and A-Kinase In Vitro—As reported previously (*1, 2*), the inhibitory effects of five tannins (eugeniin, PB-2, AC trimer, and GP) and four polyphenol-containing antioxidant compounds (epicatechin, procyanidin B-3, theaflavin, 3′,4′,7-THI, and 8C-3′,4′,5,7-THI) on the activity of CK-II were examined. Figure 2A shows that (i) GP is the most effective inhibitor (ID_{50} = approx. 0.6 μM) of CK-II among the tested compounds; (ii) quercetin (ID_{50} = approx. 0.8 μM), AC trimer (ID_{50} = approx. 2 μM), and PB-2 (ID_{50} = approx. 2.5 μM) are effective inhibitors of CK-II; and (iii) the inhibitory effects of theaflavin (ID_{50} = approx. 6 μM) and EGCG (ID_{50} = approx. 8 μM) on the activity of CK-II are rather less than those determined for two antioxidant compounds [8C-3′,4′,5,7-THI (ID_{50} = approx. 0.1 μM) and 3′,4′,7-THI (ID_{50} = approx. 0.4 μM)], which effectively inhibit the CK-II–

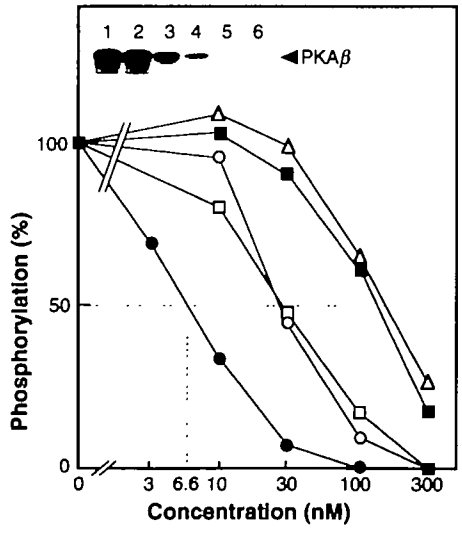

Fig. 3. **The inhibitory kinetics of GP on the autophosphorylation of A-kinase β and CK-IIβ *in vitro*.** The inhibitory effects of GP and its related compounds (eugeniin, sanguiin H-6 and pentagalloylglucose) on the autophosphorylation of A-kinase β. Purified bhA-kinase (approx. 50 ng) was directly incubated for 30 min at 30°C under the standard assay conditions. The inhibitory kinetics of GP on the autophosphorylation of CK-IIβ were also determined in the presence of various doses of GP. After incubation (30 min at 30°C), ^{32}P-labeled p37 (CK-IIβ) in the reaction mixtures was determined by autoradiography after SDS-PAGE. Autoradiograms were scanned with a spectrophotometer. 100% represents the autophosphorylation of A-kinase β or CK-IIβ determined in the absence of these inhibitors. The kinetics of autophosphorylation of bhA-kinase β determined in the presence of GP (●), eugeniin (○), sanguiin H-6 (□), or pentagalloylglucose (△); and GP-induced inhibition of autophosphorylation of CK-IIβ (■). Insert: lane 1, absence of GP (control); lane 2, 1 nM GP; lane 3, 3 nM; lane 4, 10 nM; lane 5, 30 nM; and lane 6, 100 nM.

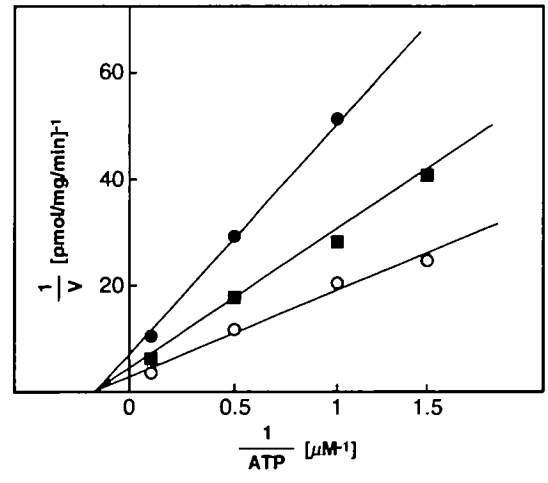

Fig. 4. **Inhibitory kinetics of GP on the autophosphorylation of A-kinase.** The autophosphorylation kinetics of bhA-kinase were determined with various concentrations of ATP in the presence or absence of 10 nM GP. After incubation (20 min at 30°C), the ^{32}P-labeled β-subunit (p55) of bhA-kinase in the reaction mixtures was precipitated with 0.5 ml of 20% trichloroacetic acid (TCA) and 0.5 ml of 0.1 M sodium pyrophosphate containing bovine serum albumin (1 mg/ml) and 10 mM EDTA. The ^{32}P-radioactivity of the TCA-insoluble precipitates on the filter was measured with a liquid scintillation spectrometer, as previously reported (*2, 5*). Lineweaver-Burk plots: (○) in the absence of GP (control); (■) in the presence of 5 nM GP; or (●) 10 nM GP.

mediated phosphorylation of GL-binding functional proteins *in vitro* (*5–7*).

By primary screening of polyphenol-containing antioxidant compounds and tannins, three tannins [arecatannin A-1, eugeniin and GP (Fig. 1)] were selected as potent CK-II inhibitors, and their inhibitory effects on the activities of bhA-kinase (heterodimer) and A-kinase α were examined *in vitro*. Figure 2B shows that (i) arecatannin A-1 inhibits the activity of A-kinase in a dose-dependent manner (ID_{50} = approx. 0.2 μM); and (ii) GP (ID_{50} = approx. 50 nM) as well as eugeniin (ID_{50} = approx. 80 nM) inhibit it effectively. In contrast, the ID_{50} of GP on the activity of A-kinase α was approx. 0.3 μM (Fig. 2B).

Characterization of GP as a Potent Inhibitor of the Autophosphorylation of A-Kinase β In Vitro—Since different GP sensitivities were observed between A-kinase (heterodimer) and A-kinase α (Fig. 2B), the direct effects of eugeniin and GP on the autophosphorylation of A-kinase β (p55) were examined, and the results were compared with their effects on the autophosphorylation of CK-IIβ. As expected, GP inhibits the autophosphorylation of A-kinase β (p55) in a dose-dependent manner with an ID_{50} of approx. 6.6 nM (Fig. 3), which is about one-thirtieth the ID_{50} of GP required to inhibit the activity of A-kinase (Fig. 2B). GP also inhibits the autophosphorylation of CK-IIβ (p37) in a dose-dependent manner with the ID_{50} of approx. 0.2 μM (Fig. 3). Under the same experimental conditions, the inhibitory kinetics of sanguiin H-6 (MW = 1,870), which is composed of two molecules of GP (*17*), on the autophosphorylation of A-kinase β are similar to those observed for eugeniin (Fig. 3). Eugeniin (ID_{50} = approx. 30 nM) and pentagalloylglucose (ID_{50} = approx. 0.2 μM) also inhibit the autophosphorylation of A-kinase β (Fig. 3), but require doses about 4.5- and 30-fold higher, respectively, to induce the same inhibition observed with GP (Fig. 3). The GP-induced inhibition of the autophosphorylation of A-kinase β from bovine heart was reproduced exactly with A-kinases purified from porcine heart and liver (data not shown).

The inhibitory kinetics of GP on the autophosphorylation of A-kinase β *in vitro* were measured using purified bhA-kinase. The V_{max} for ATP of bhA-kinase shifted from 0.29 to 0.12 (pmol/mg/min) when incubated with 10 nM GP. However, no change in the apparent K_m (4.28 × 10 μM) for ATP of bhA-kinase was detected by incubation with 10 nM GP under the given experimental conditions (Fig. 4). These results indicate that GP inhibits the autophosphorylation of bhA-kinase in a noncompetitive manner with ATP.

Inhibitory Effect of GP on the Suppressive Effect of A-Kinase β on the Activity of A-Kinase α In Vitro—It is well known that (i) the β-subunit of A-kinase directly suppresses the phosphorylating activity of the α-subunit; and (ii) the specific binding of cAMP to the β-subunit stimulates the activity of the α-subunit *in vitro* (*20*). Therefore, it was confirmed that the β-subunit reduces the activity of the α-subunit in a dose-dependent manner in the absence of cAMP (Fig. 5). Preincubation of the β-subunit with GP reduced the suppressive effect on the activity of the α-subunit and significantly stimulated phosphorylation of histone H2B by the α-subunit (Fig. 6). The GP-induced stimulation of the activity of the α-subunit is about half that observed with 0.3 μM cAMP (Fig. 6). These results show that the

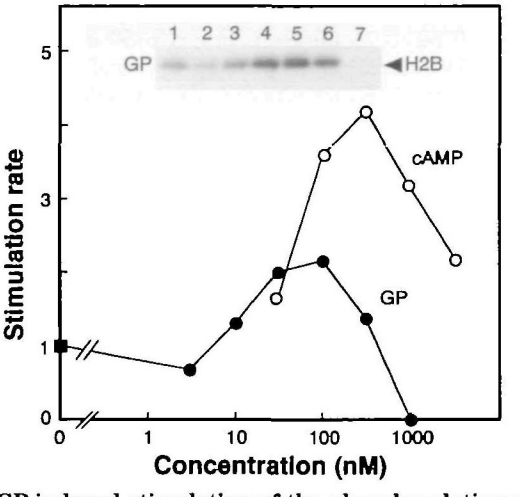

Fig. 6. **GP-induced stimulation of the phosphorylating activity of the α-subunit.** The stimulatory effect of GP on the phosphorylation of histone H2B by the α-subunit was determined after preincubation of purified β-subunit (approx. 0.1 unit) for 20 min at 30°C with various doses of GP in the absence of cAMP (●). In a parallel experiment, the stimulatory effect of cAMP (○) on the β-subunit was determined. The autoradiogram (insert) was scanned with a spectrophotometer. Stimulation rate 1 (■) represents phosphorylation of histone H2B by the α-subunit in the absence of cAMP. Lane 1, absence of GP (control); lane 2, 3 nM GP; lane 3, 10 nM; lane 4, 30 nM; lane 5, 100 nM; lane 6, 300 nM; and lane 7, 1 μM.

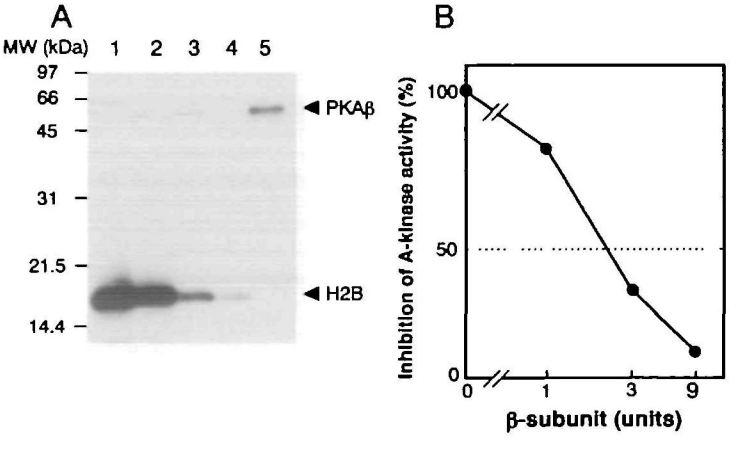

Fig. 5. **GP-induced reduction of the suppressive effect of A-kinase β on the phosphorylating activity of the α-subunit *in vitro*.** A: Phosphorylation of histone H2B by the α-subunit was determined after incubation of purified α-subunit (approx. 0.1 unit) for 20 min at 30°C with various doses of the β-subunit in the absence of cAMP (assay conditions: c.f. those described in the legend to Fig. 4). Lane 1, absence of the β-subunit (control); lane 2, α-subunit incubated with the β-subunit [1 unit (lane 2), 3 units (lane 3), and 9 units (lane 4)]; and lane 5, β-subunit incubated without histone H2B. B: The autoradiogram was scanned with a spectrophotometer. 100% represents phosphorylation of histone H2B by the α-subunit in the absence of cAMP.

direct binding of GP to the β-subunit may prevent the physiological interaction between the α- and β-subunits of A-kinase *in vitro*.

Selective Precipitation of A-Kinase with GP—The above results (Figs. 3 and 5) suggest that GP may bind directly to the β-subunit (p55) of A-kinase. In addition, it has been reported that GP can precipitate glamisigin S (*10*) as well as proline-rich proteins (*11*) under low pH conditions. Since the A-kinase β contains a proline-rich domain (*13*, *14*), the direct binding of GP to the β-subunit was tested. After preincubation of purified bhA-kinase with 20 μM [γ-^{32}P]ATP (500 cpm/pmol) for 20 min at 30°C, GP (final 0.1 mM or 1 mM) was added to reaction mixtures containing ^{32}P-labeled bhA-kinase β (p55) in 0.1 M acetate buffer (pH 5.0). Autoradiography detected p55 in the precipitates as a phosphorylated polypeptide, and the amount increased significantly with increasing concentrations of GP (Fig. 7A, lanes 2 and 3). Under the same experimental conditions, a similar precipitation of A-kinase was also observed with eugeniin. These results show that the direct binding of GP or eugeniin to the A-kinase β results in the selective precipitation of A-kinase at pH 5.0.

To confirm the selective precipitation of A-kinase in the partially purified A-kinase fraction (DEAE-cellulose fraction) prepared from porcine liver, the A-kinase fraction (approx. 20 μg) was preincubated for 30 min at 30°C with 20 μM [γ-^{32}P]ATP. When GP (1 mM) was added to the reaction mixtures at pH 5.0, at least four ^{32}P-labeled polypeptides (p29, p27, p18, and p16) and p55 (A-kinase β) were detected in the precipitated fraction (Fig. 7B, lane 2). These results show that A-kinase and its associated polypeptides (p29, p27, p18, and p16) in the DEAE-cellulose fraction from porcine liver are coprecipitated with GP at pH 5.0.

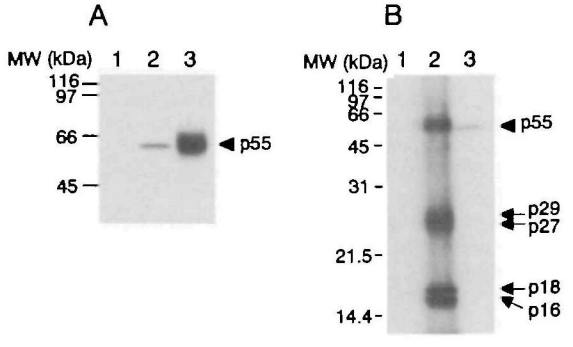

Fig. 7. **A: Precipitation of purified A-kinase with GP *in vitro*.** To determine the direct binding of GP to the A-kinase β, purified bhA-kinase (approx. 10 μg) was preincubated for 30 min at 30°C under the A-kinase assay conditions. The ^{32}P-labeled β-subunit (p55) of bhA-kinase was directly incubated with GP (final 0.1 mM or 1 mM) in 0.1 M acetate buffer (pH 5.0). The resulting precipitates were washed four times with 0.1 M acetate buffer (pH 4.5). After centrifugation for 20 min at 14,000 ×*g* at 0°C, the obtained precipitates containing ^{32}P-labeled A-kinase β (p55) were dissolved in 40 mM Tris-HCl (pH 7.6) containing 0.1 M NaCl and 2 mM DTT. ^{32}P-labeled p55 (A-kinase β) in the supernatant and precipitated fractions were determined by autoradiography after SDS-PAGE. Lane 1, supernatant separated from the polypeptides precipitated with 1 mM GP; lane 2, bhA-kinase precipitated with 0.1 mM GP; and lane 3, bhA-kinase precipitated with 1 mM GP. **B: Precipitation of A-kinase and its associated polypeptides from the partially purified A-kinase fraction with GP.** The partially purified A-kinase fraction (DEAE-cellulose fraction, approx. 20 μg) was incubated for 30 min at 30°C in A-kinase assay mixtures containing 2 μM cAMP, 10 mM Mg^{2+}, and 20 μM [γ-^{32}P]ATP (500 cpm/pmol). After that, GP (1 mM) was added to the mixtures at pH 5.0. The precipitates formed were removed by centrifugation (for 20 min at 14,000 ×*g* at 0°C). ^{32}P-labeled p55 (A-kinase β) and other phosphorylated polypeptides in the supernatant and precipitated fractions were determined by autoradiography after SDS-PAGE. ^{32}P-labeled polypeptides, including p55, in the reaction mixtures were determined by autoradiography after SDS-PAGE. Lane 1, supernatant removed from polypeptides precipitated with 1 mM GP; lane 2, polypeptides, including p55, in the fraction precipitated with 1 mM GP; and lane 3, purified bhA-kinase incubated directly with [γ-^{32}P]ATP (control).

DISCUSSION

In the present study, we have confirmed that GP and eugeniin weakly inhibit the activity of A-kinases from bovine and porcine heart *in vitro* (Fig. 2), as has been reported by Kashiwada *et al.* (*8*). However, we found that (i) at least four tannins (AC trimer, eugeniin, GP, and PB-2) among those tested compounds effectively inhibit CK-II activity, and that GP is the most effective against CK-II (Fig. 2A); (ii) GP inhibits the autophosphorylation of A-kinase β as well as CK-IIβ (Fig. 3) in a dose-dependent manner, and GP (ID_{50} = approx. 6.6 nM) is about 7.6-fold more effective in autophosphorylating A-kinase β than in inhibiting A-kinase activity (ID_{50} = approx. 50 nM) (Figs. 2B and 3); (iii) the inhibition of A-kinase β autophosphorylation by GP is about 4.5-fold more effective rather than the inhibition by eugeniin or sanguiin H-6 (Fig. 3); and (iv) GP prevents the suppressive effect of the β-subunit on the phosphorylating activity of the α-catalytic subunit (Fig. 6). Taken together, these results suggest that (i) GP is a selective inhibitor of the autophosphorylation of A-kinase β; and (ii) the GP-induced inhibition of A-kinase activity is due to the direct binding of GP to the β-subunit of A-kinase *in vitro*.

It has been reported that, *in vitro*, *N*-[2-(methylamino)ethyl]-5-isoquinoline-sulfonamide (H-8) (*20*), *N*-(2-bromocyanylamino)-5-isoquinoline-sulfonamide (H-89), and *N*-(2-cinnamylaminoethyl)-5-isoquinorine-sulfonamide (H-88) (*21*) are potent inhibitors of A-kinase (ID_{50} of H-8 = approx. 1.2 μM; ID_{50} of H-89 = approx. 48 nM; and ID_{50} of H-88 = approx. 0.36 μM). The ID_{50} of GP (ID_{50} = approx. 6.6 nM) on the autophosphorylation of A-kinase β *in vitro* is about one-seventh the ID_{50} of H-89 on A-kinase activity. Since the GP-induced inhibition of autophosphorylation of A-kinase β is typically noncompetitive with ATP (Fig. 4), its actions differ clearly from the inhibitory actions of H-88 and H-89, which inhibit A-kinase activity competitively with ATP (*20*). Although there are no chemical similarities between GP and staurosporine, the inhibitory effect (ID_{50} = approx. 6.6 nM) of GP on autophosphorylation of A-kinase β *in vitro* is similar to that (ID_{50} = approx. 8.2 nM) determined for staurosporine on A-kinase activity (*22*).

Since it has been reported that polyphenolic tannins preferentially interact with proline-rich proteins and can precipitate them under acidic conditions (*11*, *12*), similar experiments were carried out using purified and partially purified A-kinase fractions, because A-kinase β includes a proline-rich domain (*13*, *14*). As expected, ^{32}P-labeled p55 of purified bhA-kinase (Fig. 7A) and A-kinase in the DEAE-cellulose fractions prepared from porcine liver (Fig. 7B) could be precipitated by excess GP (about 1 mM) at pH 5.0. Three observations suggest that GP binds selectively to the A-kinase β *in vitro*: (i) GP selectively inhibits the autophos-

phorylation of A-kinase β (Fig. 3); (ii) direct binding of GP to the β-subunit reduces its suppressive effect on the activity of the α-subunit (Fig. 6); and (iii) GP can selectively precipitate purified A-kinase (Fig. 7A) and also coprecipitate A-kinase and its associated polypeptides (p29, p27, p18, and p16) in the DEAE-cellulose fraction from porcine liver (Fig. 7B).

The GP-induced inhibition of the phosphorylating activity of A-kinase (heterodimer) may be due to the direct binding of GP to the β-subunit of A-kinase, thus preventing the interaction between the β- and α-subunits of A-kinase *in vitro* (Fig. 6). This conclusion is supported by evidence that (i) the β-subunit of A-kinase includes a proline-rich domain for interaction with tannins (*14*, *15*); and (ii) preincubation of the β-subunit with GP significantly stimulates the activity of the α-subunit (Fig. 5). Estimations from kinetic studies of the GP-induced inhibition of the β-subunit and comparison with the stimulatory effect of cAMP on the activity of the α-subunit (Figs. 5 and 6) suggest that approx. one molecule of GP is bound directly to a β-subunit molecule of A-kinase under the present assay conditions (pH 7.8), whereas the binding of plural GP molecules to the β-subunit under acidic pH conditions may precipitate A-kinase.

However, for a clear understanding of the inhibitory action of GP on the physiological activity of A-kinase β and the A-kinase β–mediated regulation of functional cellular proteins involved in signal transduction at the cellular level, further analytical studies will be required (i) to determine the specific binding sites for GP on the A-kinase β; (ii) to identify other GP-binding, proline-rich proteins involved in physiological regulation during cell differentiation and proliferation; and (iii) to determine the GP-induced selective inhibition of the phosphorylation of acidic proline-rich salivary protein 1 (PRP1) by CK-II (*23*) and CR16 (proline-rich protein) by mitogen-activated protein kinase (MAP-kinase) in rat brain neurons (*24*) *in vitro*.

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