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SHORT COMMUNICATION

Inhibition of Na^+,K^+-ATPase by 1,2,3,4,6-Penta-O-galloyl-β-D-glucose, a Major Constituent of Both Moutan Cortex and Paeoniae Radix

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ABSTRACT. The inhibition of Na^+,K^+-ATPase activity by various constituents of Moutan Cortex and Paeoniae Radix was studied. 1,2,3,4,6-Penta-O-galloyl-β-D-glucose (PGG), a major component of both crude drugs, strongly inhibited Na^+,K^+-ATPase activity ($IC_{50} = 2.5 \times 10^{-6}$ M), whereas galloylpaeoniflorin, benzoic acid, and catechin were weakly inhibitory, and albiflorin, oxypaeoniflorin, paeoniflorin, paeonol, and phenol were ineffective. The inhibition of Na^+,K^+-ATPase activity by PGG was decreased in the presence of BSA or phospholipids. The inhibition mode of PGG was noncompetitive with respect to ATP. The $K_{0.5}$ value for Na^+ was increased by the addition of PGG from 9.1 to 12.3 mM, whereas that for K^+ was not altered. PGG also inhibited K^+-dependent *p*-nitrophenyl phosphatase activity with an IC_{50} value of 5.3×10^{-6} M, and the extent of the inhibition increased at higher concentrations of K^+. The $K_{0.5}$ value for K^+ was decreased by the addition of PGG from 3.3 to 2.0 mM. These results suggested that the inhibition of Na^+,K^+-ATPase activity is caused by interaction of PGG with the enzyme in the E_2 state. The inhibitory effect of Moutan Cortex or Paeoniae Radix is considered to be mainly attributable to PGG. BIOCHEM PHARMACOL 53;4:611–614, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. Na^+,K^+-ATPase; Na^+,K^+-ATPase inhibitor; 1,2,3,4,6-Penta-O-galloyl-β-D-glucose; Moutan Cortex; Paeoniae Radix; K^+-pNPPase

Moutan Cortex, the root cortex of *Paeonia suffruticosa* ANDREWS, and Paeoniae Radix, the root of *Paeonia lactiflora* PALLS var. *trichocarpa* BUNGE, are important Chinese crude drugs, used in many traditional prescriptions. PGG§ (Fig. 1), PN, PF, OPF, GPF, AF, BA and BPF have been isolated from both Moutan Cortex and Paeoniae Radix [1, 2]. Phenol and catechin were also detected in Paeoniae Radix [3, 4]. These constituents have diverse biological activities. For example, PGG has urea-nitrogen reducing activity in rat serum [5], inhibits sialidase activity [6], and shows antimutagenic activity [7]. GPF is a potent oxygen radical scavenger and antioxidant [8]. PN has sedative and anti-inflammatory effects, and inhibits stress-induced gastric erosion and gastric juice secretion [9, 10]. Anti-inflammatory activity of BPF also has been reported [11].

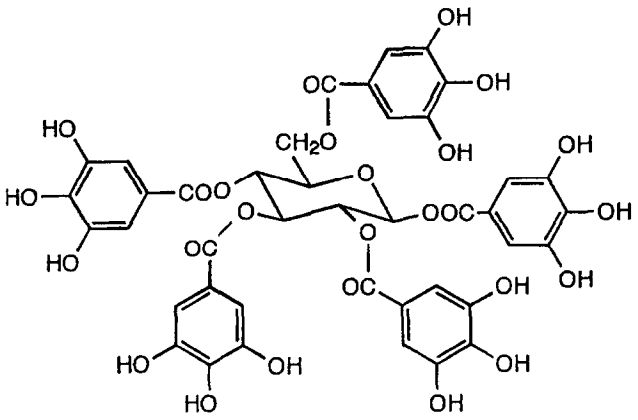

FIG. 1. Structure of PGG.

Recently, we found that the water extracts of Moutan Cortex and Paeoniae Radix strongly inhibit Na^+,K^+-ATPase activity [12]. We have now purified the constituents of Moutan Cortex and Paeoniae Radix, and examined the effect of each component on Na^+,K^+-ATPase in order to identify the compounds responsible for this activity.

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§ *Abbreviations:* PGG, 1,2,3,4,6-penta-O-galloyl-β-D-glucose; GPF, galloylpaeoniflorin; BA, benzoic acid; AF, albiflorin; OPF, oxypaeoniflorin; PF, paeoniflorin; PN, paeonol; BPF, benzoyl-paeoniflorin; K^+-pNPPase, K^+-dependent *p*-nitrophenyl phosphatase; PC, L-α-phosphatidylcholine; and PS, L-α-phosphatidyl-L-serine.

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MATERIALS AND METHODS

PGG was isolated from Moutan Cortex [13, 14]. OPF [14], GPF [8], and AF [15] (98% pure, respectively) were isolated from Paeoniae Radix. PN and PF (98% pure) were purchased from the Yoneyama Yakuhin Ind. Co. (Osaka, Japan). Catechin was purchased from Nakalai Tesque (Kyoto, Japan). BA and phenol were from Wako Pure Chemical Ind. Ltd. (Osaka, Japan). PC and PS were from the Sigma Chemical Co. (St. Louis, MO, U.S.A.), Na^+,K^+-ATPase was prepared from horse kidney outer medulla. The SDS-treated enzyme (purified Na^+,K^+-ATPase) [16] (whose activity was 32–52 μmolP_i/mg protein/min, and was inhibited almost completely by 1.0×10^{-5} M ouabain) was used for the assay of Na^+,K^+-ATPase. Na^+,K^+-ATPase activity was measured principally according to a previous report [16]. PGG and other constituents were each dissolved in DMSO. An aliquot of the DMSO solution (1 μL) was added to the reaction mixture (0.1 mL) containing purified enzyme (1 μg protein/mL), 3 mM ATP, 140 mM NaCl, 14 mM KCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM EGTA, and 50 mM imidazole–HCl buffer (pH 7.2) with or without 0.5 mM ouabain. K^+-pNPPase activity was determined in a reaction mixture (0.1 mL) containing purified enzyme (1 μg protein/mL), 20 mM pNPP, 15 mM KCl, 10 mM $MgCl_2$, 0.1 M Tris–HCl buffer (pH 7.7), and 1 μL of PGG solution at 37° for 10 min [17]. The concentration of DMSO (1%) in the reaction mixture had no effect on the enzyme activities.

RESULTS AND DISCUSSION

Inhibition of Na^+,K^+-ATPase Activity by the Constituents of Moutan Cortex and Paeoniae Radix

The inhibition of Na^+,K^+-ATPase activity by PGG was concentration dependent and was complete at 10^{-4} M (Fig. 2). The apparent IC_{50} value of PGG for Na^+,K^+-ATPase activity is 2.5×10^{-6} M [13]. GPF, BA, and catechin at 10^{-2} M caused only 33, 25, and 16% inhibition, respectively. AF, OPF, PF, PN, and phenol had no inhibitory effect at 10^{-2} M. PGG is thus, by far, the most potent inhibitor of Na^+,K^+-ATPase among these constituents of Moutan Cortex and Paeoniae Radix.

Effects of PGG on Na^+,K^+-ATPase Activity

When Na^+,K^+-ATPase was incubated in reaction mixtures containing PGG at concentrations of 1.0×10^{-4} and 2.5×10^{-6} M, giving 100 and 50% inhibition, respectively, and the enzyme was recovered by centrifugation of the reaction mixtures after dilution with 10 vol. of reaction buffer, the inhibition was not reversed. These results confirmed the irreversibility of inhibition of Na^+,K^+-ATPase activity by PGG.

When the reaction mixtures containing 2.5×10^{-6} M PGG (equal to the IC_{50} value) and BSA, PC, or PS were preincubated at 37° for 15 min and then left at 25° for 1 hr

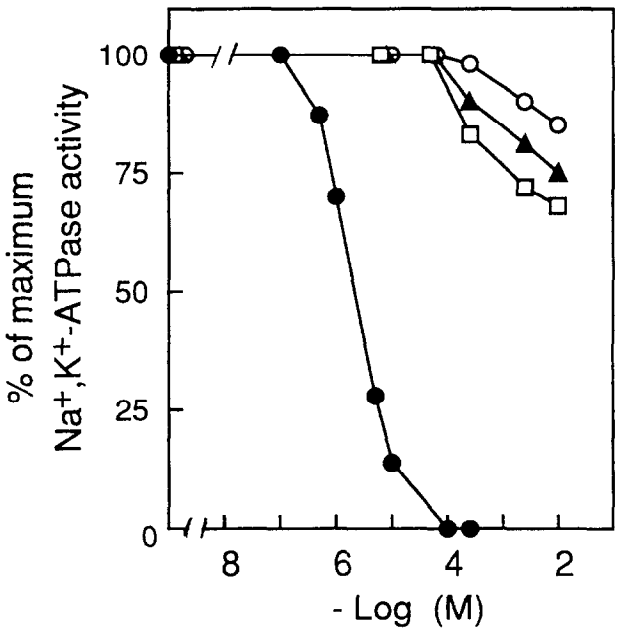

FIG. 2. Effects of various constituents of Moutan Cortex and Paeoniae Radix on the activity of Na^+,K^+-ATPase. The enzyme was incubated in the reaction mixture with various concentrations of PGG (●), GPF (□), BA (▲), or catechin (○). Enzyme activity without PGG was taken as 100% (36.6 μmol P_i/mg protein/min). Values are means ± SD (N = 6).

and the activity was measured, the inhibition of enzyme activity by PGG was decreased from 50 to 26.2, 0, 0, and 36.4% by preincubation with 10 or 50 μg/mL BSA and 30 μg/mL each of PC or PS, respectively. Further, the Na^+,K^+-ATPase activity in a crude preparation (250 μg/mL, sp. act.

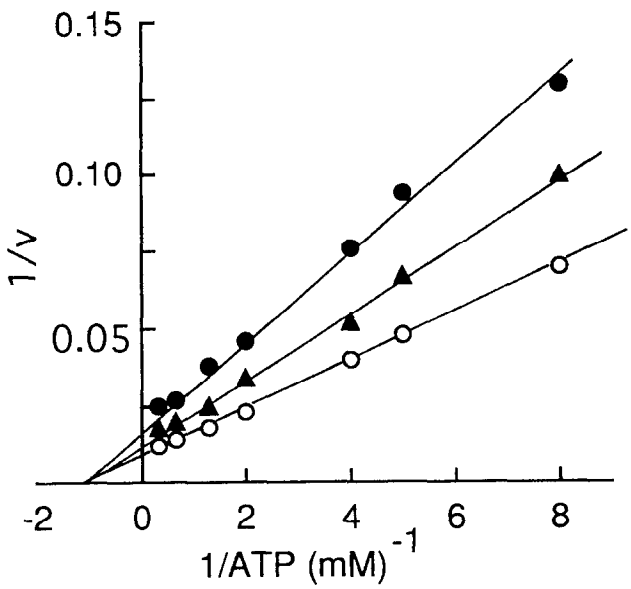

FIG. 3. Double-reciprocal plots of hydrolysis rates of ATP by Na^+,K^+-ATPase. Enzyme activity was determined with PGG at 1.0×10^{-6} (▲) or 2.5×10^{-6} M (●) and without PGG (○). The concentration of ATP was varied. The SD was within 2.3% (N = 6).

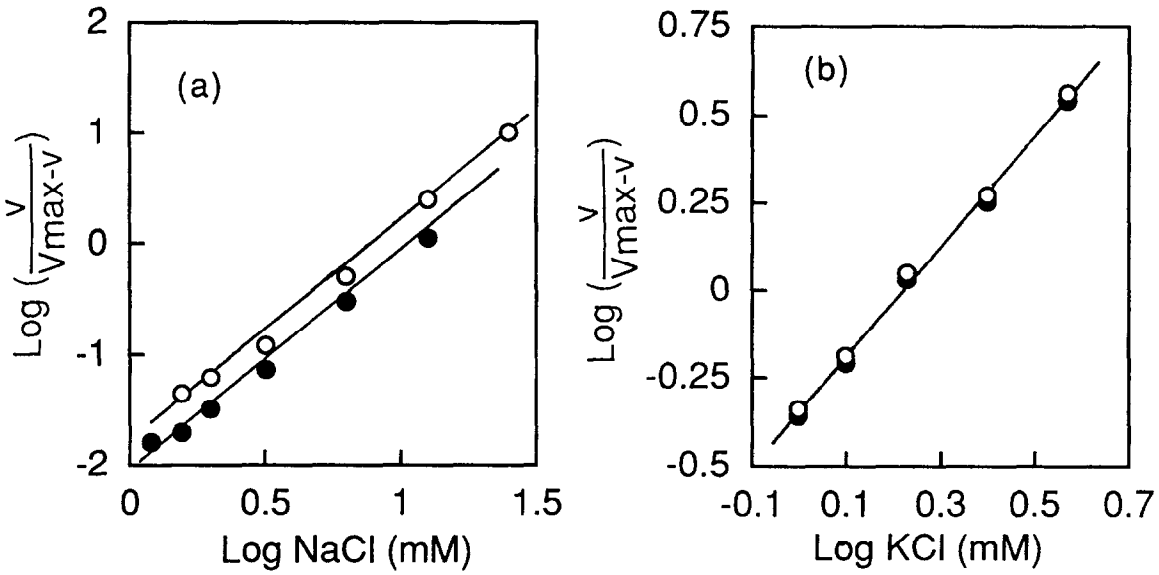

FIG. 4. Hill plots of Na^+,K^+-ATPase activity in the presence and absence of PGG with various concentrations of Na^+ or K^+. Enzyme activity was determined with (●) or without (○) PGG (2.5×10^{-6} M). The concentration of NaCl (a) or KCl (b) was varied. The SD was less than 2.5% (N = 6).

1.1 μmol P_i/mg protein/min) was inhibited by 2.5×10^{-6} M PGG to a smaller extent (about 10%) than the purified enzyme. These results are compatible with the finding by Okuda [18] that PGG binds with proteins or phospholipids. The crude preparation of Na^+,K^+-ATPase contains many components other than the enzyme protein, and the binding of PGG to these components appears to be strong. It is also possible that PGG interacts with other compounds in the prescription of the crude drugs.

Kinetic studies were carried out by varying the concentrations of ATP, Na^+, and K^+. The inhibition of Na^+,K^+-ATPase activity by PGG was examined in the presence of various concentrations of ATP, and the double-reciprocal plots are shown in Fig. 3. The apparent V_{max} value was decreased by raising the concentration of PGG, but the K_m value was not altered. This result indicates that the mode of inhibition was noncompetitive with respect to ATP. Na^+,K^+-ATPase activity was assayed in the presence of various concentrations of Na^+ or K^+ with or without 2.5×10^{-6} M PGG, and the Hill plots were obtained (Fig. 4). The $K_{0.5}$ values for Na^+ were 9.1 and 12.3 mM in the absence and presence of PGG, respectively, and the Hill coefficient was not affected by PGG (Fig. 4a). On the other hand, the $K_{0.5}$ values for K^+ (1.7 mM) and the Hill coefficients were

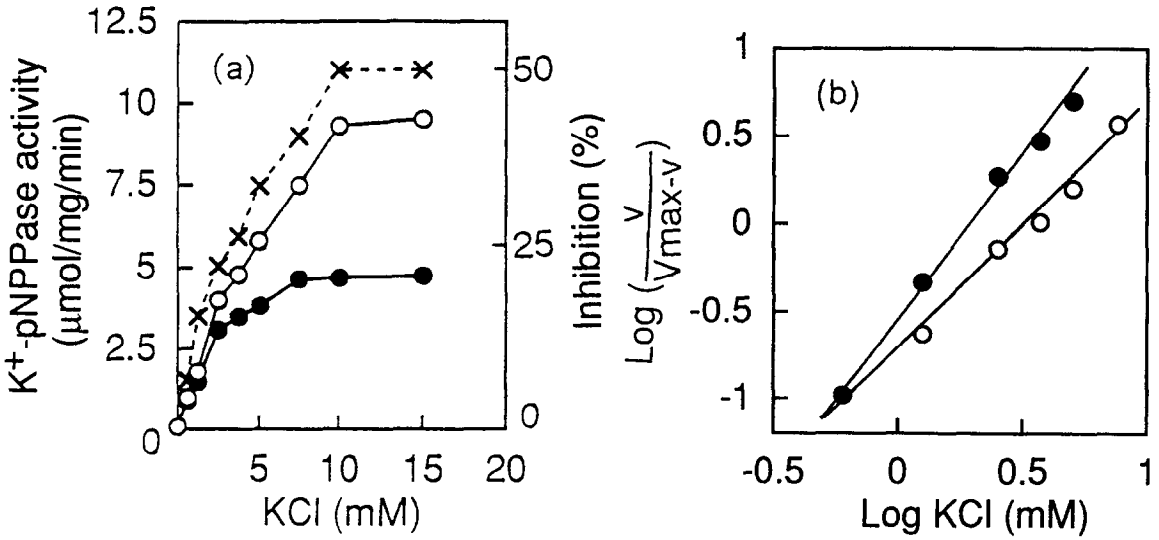

FIG. 5. Effect of PGG on K^+-pNPPase activity in the presence of various concentrations of K^+. (a) Enzyme activity (μmol/mg protein/min) was determined with (●) or without (○) PGG (5.3×10^{-6} M). The percent inhibition by PGG is indicated (x). (b) Hill plot of the data in (a). The SD was less than 2.1% (N = 6).

the same, regardless of the presence or absence of PGG (Fig. 4b).

Effects of PGG on K^+-pNPPase Activity

In the overall reaction of Na^+,K^+-ATPase, the conformation of the enzyme changes according to the ligand conditions. When the Na^+ concentration reaches a certain level, $K \cdot E_2$ is converted to $Na \cdot E_1$, and $Na \cdot E_1$ is phosphorylated by ATP to $Na \cdot E_1$-P; this is converted to E_2-P, which is dephosphorylated to $K \cdot E_2$ when the K^+ concentration is raised [19, 20]. K^+-pNPPase activity, which reflects the partial reaction in the E_2 state [21], was inhibited by PGG in a concentration-dependent manner. Complete inhibition by PGG was observed at 3.3×10^{-4} M and the apparent IC_{50} value was 5.3×10^{-6} M. In the presence of 5.3×10^{-6} M PGG, weak inhibition was observed when the concentration of K^+ was low; for example, at 0.6 mM K^+ the inhibition was only 7% (Fig. 5a). The extent of the inhibition was increased by raising the K^+ concentration, and became almost constant (50%) when the concentration of K^+ was higher than 10 mM (Fig. 5a). The $K_{0.5}$ values for K^+ were 3.3 and 2.0 mM, and the Hill coefficients (n) were 2.5 and 2.9 in the absence and presence of PGG, respectively (Fig. 5b).

The effects of PGG on the overall and partial reactions of Na^+,K^+-ATPase support the idea that PGG interacts with the enzyme in the E_2 state and inhibits the reaction step from $K \cdot E_2$ to $Na \cdot E_1$. PGG appears to be mainly responsible for the inhibitory effects of Moutan Cortex and Paeoniae Radix.

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