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INHIBITION OF GASTRIC H^+,K^+-ATPase AND ACID SECRETION BY CASSIGAROL A, A POLYPHENOL FROM *CASSIA GARRETTIANA* CRAIB

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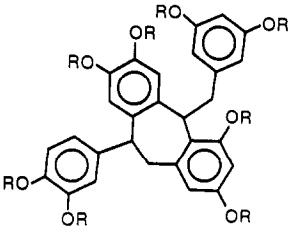
Abstract—The effects of cassigarol A, a naturally occurring polyphenol, on gastric H^+,K^+-ATPase and gastric acid secretion were studied. Cassigarol A inhibited H^+,K^+-ATPase and K-stimulated *p*-nitrophenyl phosphatase from hog gastric mucosa with 50% inhibition of 1.2×10^{-6} and 6.3×10^{-6} M, respectively. The kinetic study showed that the inhibition of H^+,K^+-ATPase by cassigarol A was competitive with respect to ATP and non-competitive with respect to K^+. Cassigarol A inhibited both H^+,K^+-ATPase-mediated proton transport and 2-deoxy-D-glucose-induced acid secretion. On the other hand, cassigarol A acetate, in which phenolic hydroxy groups are acetylated, was not effective in the inhibition of enzyme activity and acid secretion. These results indicate that cassigarol A is a potent inhibitor of gastric H^+,K^+-ATPase, that the anti-secretory activity of cassigarol A is related to the inhibition of H^+,K^+-ATPase and that an important moiety of cassigarol A in the interaction with the enzyme is the phenolic hydroxy groups.

Gastric H^+,K^+-ATPase is a membrane-bound enzyme which catalyses H^+ transport at the expense of ATP hydrolysis [1, 2]. This is an important final step in acid secretion, and the inhibition of the enzyme results in the reduction of gastric acid secretion. A well-known H^+,K^+-ATPase inhibitor, omeprazole, is clinically used in the therapy of peptic ulcers [3, 4].

Cassigarol A (Fig. 1) is a naturally occurring polyphenol isolated from the heartwood of *Cassia garrettiana* Craib which is used as a crude drug in Thailand [5]. In the course of our studies of the biological activity of cassigarol A, an inhibitory effect on gastric H^+,K^+-ATPase was found. The present studies were undertaken to examine in detail the effects of cassigarol A on gastric H^+,K^+-ATPase and acid secretion.



R=H : Cassigarol A

R=Ac : Cassigarol A-Ac

Fig. 1. Chemical structures of cassigarol A and cassigarol A acetate (cassigarol A-Ac).

MATERIALS AND METHODS

Materials. *p*-Nitrophenyl phosphate (pNPP‡), acridine orange, valinomycin, ATP disodium salt and Na^+,K^+-ATPase (dog kidney) were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). 2-Deoxy-D-glucose (2-DG) was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). All other chemicals were of the highest purity commercially available. ATP Tris salt was prepared from ATP disodium salt in our laboratory. Fresh hog stomachs were purchased from the local slaughterhouse. Male Wistar rats were purchased from SLC (Hamamatsu, Japan).

Extraction and isolation of cassigarol A. Cassigarol A was extracted from the heartwood of *Cassia garrettiana* Craib and isolated according to the method described previously [6]. Acetylation of cassigarol A was carried out by the method reported previously [6]. Chemical structure of compounds was determined by means of spectural analysis such as nuclear magnetic resonance and high resolution mass spectrum [6].

Preparation of hog gastric H^+,K^+*-ATPase.* Stomachs from freshly slaughtered hogs were flushed with tap water and mucus was removed by wiping the tissue with paper towel. The mucosal layer of the fundic region was scraped off from the underlying muscular layer and homogenized in an ice-cold buffer containing 0.2 mM EDTA, 0.25 M sucrose and 20 mM Pipes-NaOH pH 7.4, with a teflon-glass

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‡ Abbreviations: pNPP, *p*-nitrophenyl phosphate; K^+-pNPPase, K-stimulated *p*-nitrophenyl phosphatase; Pipes, piperazine-*N*,*N*′-bis[2-ethanesulfonic acid]; 2-DG, 2-deoxy-D-glucose.

homogenizer. All of the following procedures were carried out at 4°. The homogenate was centrifuged for 30 min at 20,000 *g*. The resulting supernatant was centrifuged for 60 min at 100,000 *g*. Gastric microsome vesicles containing H^+,K^+-ATPase were prepared by Ficoll-sucrose discontinuous density gradient centrifugation as described elsewhere [7]. The crude microsome pellet was resuspended in homogenization buffer and layered over homogenization buffer containing 7% (w/v) Ficoll. Following centrifugation for 60 min at 78,000 *g*, the membrane fraction, which was fractionated above the Ficoll interface, was collected, diluted with the buffer and recovered by centrifugation. In experiments where intact vesicles were used, the osmosity of the enzyme assay medium was maintained at 0.25 M with sucrose. The vesicles were stored at 4° until use on the same day. In cases where the leaky vesicles were required, the vesicle preparation was lyophilized to render them freely permeable to K^+, and stored at −80°. Protein was determined by the Lowry method [8] using bovine serum albumin as the standard.

Assay of H^+,K^+*-ATPase.* The assay medium contained, in a total volume of 1 mL, 2 mM $MgCl_2$, 40 mM Tris–HCl pH 7.4 and 5–10 μg membrane protein with or without 20 mM KCl. The reaction was started with 2 mM ATP Tris salt and incubated for 20 min at 37°. The reaction was stopped by the addition of 1 mL ice-cold trichloroacetic acid (10%) and assayed for inorganic phosphate according to the method of Fiske and Subbarow [9]. Drugs were dissolved in dimethylsulfoxide. The concentration of dimethylsulfoxide in the assay medium was below 1.0%, which did not affect the enzyme activity.

Assay of K^+*-pNPPase.* For K^+-pNPPase, the assay medium contained 5 mM $MgCl_2$, 5 mM pNPP, 40 mM Tris–HCl pH 7.4 and 5–10 μg membrane protein, with or without 20 mM KCl, in a total volume of 1 mL. After 20 min incubation at 37°, the reaction was terminated with 1 mL 1 M NaOH. The absorbance of the reaction mixture was read at 410 nm. Drugs were dissolved in dimethylsulfoxide.

Assay of Na^+,K^+*-ATPase.* The assay medium of Na^+,K^+-ATPase contained 40 mM Tris–HCl buffer pH 7.4, 2 mM $MgCl_2$ and 20 μg enzyme protein, with or without 20 mM KCl and 100 mM NaCl, in a total volume of 1 mL. The reaction was started by ATP Tris salt (final concentration 2.5 mM) and stopped after 20 min of incubation at 37° with 1 mL of 10% trichloroacetic acid. Liberated inorganic phosphate from ATP was measured according to the method of Fiske and Subbarow [9].

Proton transport experiment. Proton transport was measured by a spectrophotometric method, as described previously [10]. The incubation mixture contained 1 mM $MgCl_2$, 150 mM KCl, 0.5 mM EDTA, 20 mM Pipes-NaOH pH 7.4, 300 μg non-lyophilized intact vesicles from hog gastric microsomes and 10 μg acridine orange, with or without drugs, in a final volume of 2 mL. ATP Mg salt 0.48 mM was added before starting the experiment by the addition of 10 μM valinomycin. Incubation was performed at room temperature. Quenching of fluorescence was monitored using a Shimadzu Spectrophotometer UV-240 at 493 nm (excitation) and 530 nm (emission).

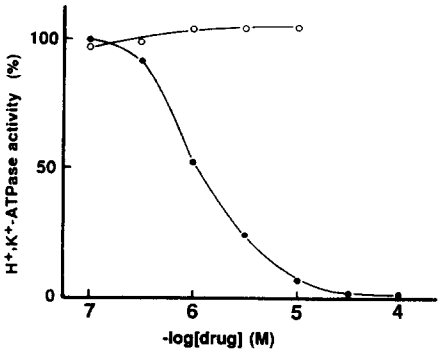

Fig. 2. Effects of cassigarol A and cassigarol A acetate on H^+,K^+-ATPase from hog gastric mucosa. Microsome membrane (5 μg protein) was incubated in 40 mM Tris–HCl buffer pH 7.4 containing 2 mM $MgCl_2$, 20 mM KCl and 2.5 mM Tris–ATP for 20 min at 37°. The reaction was terminated by the addition of trichloroacetic acid (final concentration, 5%) and liberated inorganic phosphate was determined. Results are expressed as per cent of control, which was 124 μmol P_i/hr/mg protein. Each point represents the mean of four values. Cassigarol A (●), cassigarol A acetate (○).

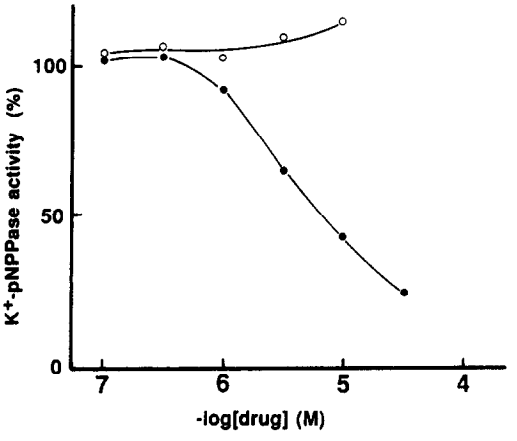

Fig. 3. Effects of cassigarol A and cassigarol A acetate on K^+-pNPPase from hog gastric mucosa. Microsome membrane (5 μg protein) was incubated in 40 mM Tris–HCl buffer pH 7.4 containing 5 mM $MgCl_2$, 20 mM KCl and 5 mM pNPP for 20 min at 37°. The reaction was terminated by the addition of 1 mL of 1 M NaOH and the absorbance was measured at 410 nm. Results are expressed as per cent of control, which was 57 μmol P_i/hr/mg protein. Each point represents the mean of four values. Cassigarol A (●); cassigarol A acetate (○).

Antisecretory study. Measurement of the gastric acid secretion was performed by the method by Maeda-Hagiwara and Watanabe [11] with slight modification. The trachea was exposed and cannulated. A dual polyethylene gastric cannula was inserted into the gastric lumen after ligation of the pylorus. The inlet and outlet tubes of the dual

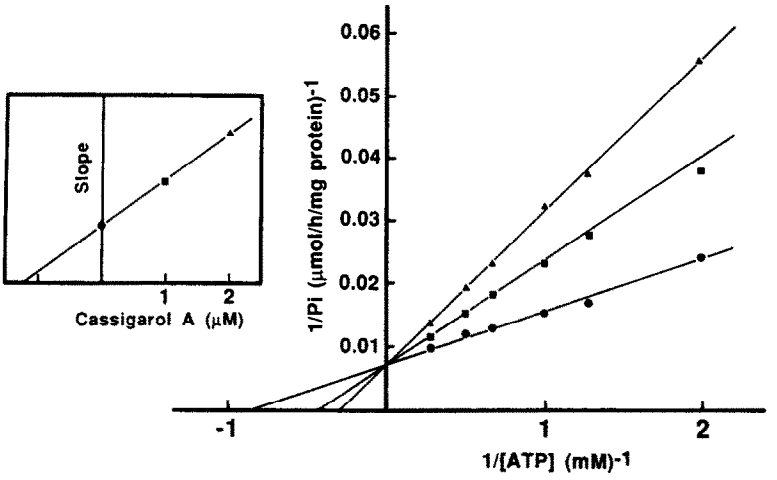

Fig. 4. Double reciprocal plots of the hydrolysis rates of ATP by H^+,K^+-ATPase vs concentrations of ATP in the presence of 0 (●), 1.0 (■), 2.0 μM (▲) cassigarol A. Each value represents the average of duplicate experiments (N = 4).

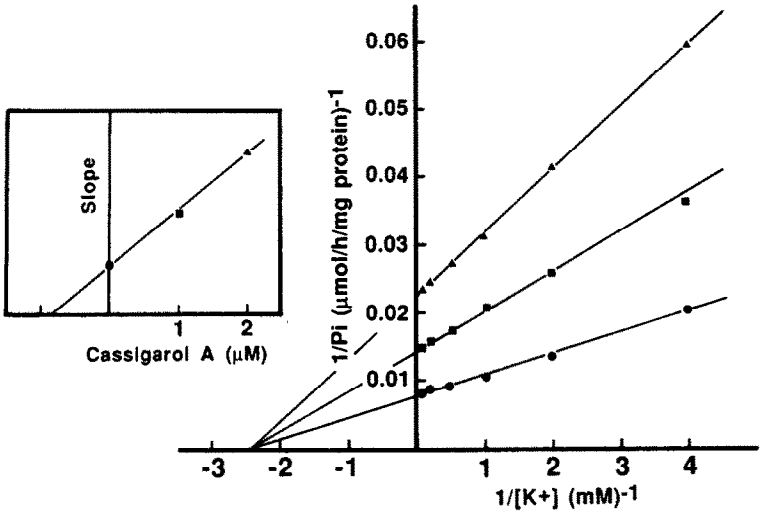

Fig. 5. Double reciprocal plots of the hydrolysis rates of ATP by H^+,K^+-ATPase vs concentrations of KCl in the presence of 0 (●), 1.0 (■) and 2.0 μM (▲) cassigarol A. Each value represents the average of duplicate experiments (N = 4).

cannula were connected to a saline reservoir and the stomach was perfused continuously at the rate of 10 mL/min with saline solution (adjusted to pH 7.0 with NaOH) through the gastric cannula using a perfusion pump. The perfusate was titrated in the reservoir with 0.01 N NaOH at pH 7.0 using an automatic titrator (GT-05, Mitsubishi Kasei) with a personal computer (PC-9800, NEC). The acid output during the 5-min period in the perfusate was recorded continuously for 6 hr. Gastric acid secretion was induced by 2-DG (300 mg/kg, s.c.).

RESULTS AND DISCUSSION

Cassigarol A inhibited gastric H^+,K^+-ATPase from gastric mucosa with a 50% inhibition of 1.2×10^{-6} M (Fig. 2). The synthetic substrate pNNP is also hydrolysed by the H^+,K^+-ATPase system (K^+-pNPPase) [12]. Cassigarol A dose-dependently inhibited the K^+-pNPPase activity with a 50% inhibition of 6.4×10^{-6} M (Fig. 3). These results show that cassigarol A is a potent inhibitor of gastric H^+,K^+-ATPase. On the other hand, cassigarol A acetate, in which the hydroxy groups are protected by acetate, had no inhibitory effects on either H^+,K^+-ATPase or K^+-pNPPase (Figs 2 and 3). We showed previously that acetylation of phenolic hydroxy groups results in the complete loss of inhibition of gastric H^+,K^+-ATPase by the naturally occurring depside, salvianolic acid A [13].

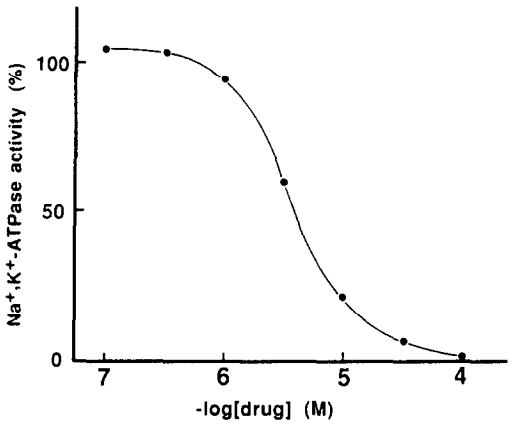

Fig. 6. Effect of cassigarol A on the Na^+,K^+-ATPase from dog kidney. Microsome membrane (20 μg protein) was incubated in 40 mM Tris–HCl buffer pH 7.4 containing 2 mM $MgCl_2$, 20 mM KCl, 100 mM NaCl and 2.5 mM Tris–ATP in a total volume of 1 mL for 20 min at 37°. The reaction was terminated by the addition of trichloroacetic acid (final concentration, 5%) and liberated inorganic phosphate was determined. Results are expressed as per cent of control, which was 152 μmol P_i/hr/mg protein. Each point represents the mean of four values.

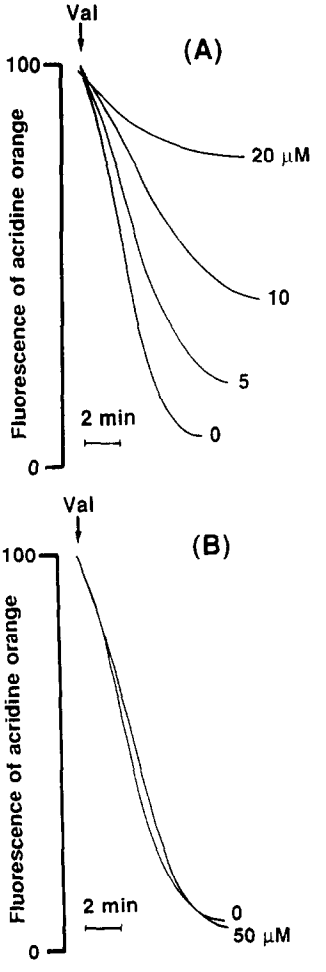

Fig. 7. Effects of cassigarol A (A) and cassigarol A acetate (B) on H^+,K^+-ATPase-mediated proton transport. The incubation mixture contained 1 mM $MgCl_2$, 150 mM KCl, 0.5 mM EDTA, 300 μg fresh vesicles and 10 μM acridine orange, with or without the drugs. Mg-ATP was added before the reaction was started by the addition of 10 μM valinomycin (Val). Quenching of fluorescence was monitored.

To elucidate the inhibition patterns by cassigarol A, the effects of ATP and K^+ concentration on the H^+,K^+-ATPase activity were studied in the presence of cassigarol A. When the inhibition of H^+,K^+-ATPase activity was measured as a function of the concentration of ATP, a competitive interaction between ATP and the inhibitor was found from double reciprocal plot analysis (Fig. 4). The apparent K_m values were increased from 1.2 to 2.3 and 3.3 mM in the presence of 1.0 and 2.0 μM cassigarol A, respectively, a change in the V_{max} value of 143 μmol P_i/hr/mg protein. The calculated K_i value was 1.2 μM. Determination of the effect of cassigarol A on H^+,K^+-ATPase activity in the presence of various K^+ concentrations showed a non-competitive interaction between the inhibitor and K^+ (Fig. 5). The apparent V_{max} values changed from 141 to 68 and 44 μmol P_i/hr/mg protein in the presence of 1.0 and 2.0 μM cassigarol A, respectively. The calculated K_i value was 0.89 μM. In the H^+,K^+-ATPase system, the enzyme is phosphorylated on the cytosolic side in the presence of Mg^{2+} and ATP, which leads to phosphoenzyme intermediates. It is then dephosphorylated by luminal K^+ [14]. From the kinetic study showing that the inhibition of gastric H^+,K^+-ATPase by cassigarol A is competitive with respect to ATP and non-competitive with respect to K^+, it is suggested that cassigarol A may compete with ATP presumably at the ATP site, thereby inhibiting phosphorylation of the enzyme. We reported previously that naturally occurring polyphenol, ellagic acid [15] and the depside, salvianolic acid A [13], are inhibitors of gastric H^+,K^+-ATPase. All these compounds have phenolic hydroxy groups which are thought to be important in the interaction with H^+,K^+-ATPase and these inhibitors show the same inhibition patterns as cassigarol A. These findings indicate an important role of phenolic hydroxy groups in inhibiting gastric H^+,K^+-ATPase.

Cassigarol A inhibited also Na^+,K^+-ATPase with a 50% inhibition of 4.5×10^{-6} M (Fig. 6). However, the inhibition potency towards Na^+,K^+-ATPase was about four times lower than that towards H^+,K^+-ATPase. Recent studies on the structure of a series of ion-transporting ATPases including H^+,K^+-ATPase, Na^+,K^+-ATPase and Ca^{2+}-ATPase have revealed that the primary amino acid sequence of gastric H^+,K^+-ATPase is highly homologous to that of Na^+,K^+-ATPase, in particular at the ATP hydrolytic site. On the other hand, the homology of the cation-binding site is rather low [16]. The synthetic compound, SCH 28080, which is a highly potent, K^+ competitive inhibitor of the H^+,K^+-ATPase, inhibits H^+,K^+-ATPase selectively [17–20]. Thus, the inhibition of both H^+,K^+-ATPase and Na^+,K^+-ATPase by cassigarol A, shown by the

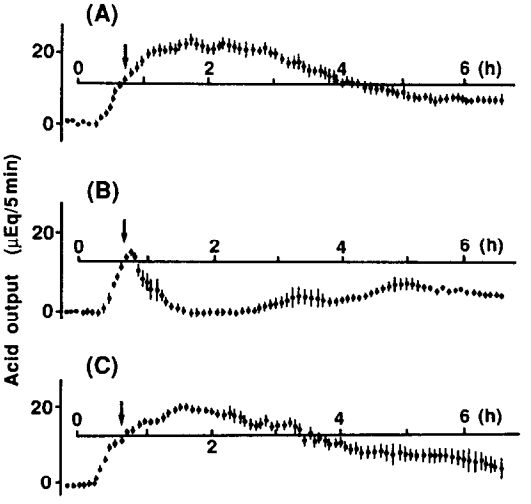

Fig. 8. Effects of cassigarol A and cassigarol A acetate on 2-DG-induced gastric acid secretion in rats. 2-DG was dissolved in saline solution and given s.c. (300 mg/kg) at time 0. Cassigarol A and cassigarol A acetate were suspended in 5% gum arabic solution and given i.p. (50 mg/kg) (indicated by arrow). Ordinates show acid output calculated and recorded by personal computer every 5 min. Abscissas show the time after 2-DG administration (hr). (A) Control, 5% gum arabic solution + 2-DG; (B) cassigarol A + 2-DG; (C) cassigarol A acetate + 2-DG. Each value represents mean ± SE for 3–6 animals.

present data, is related to its competition with ATP at the ATP site of the enzyme.

The effect of cassigarol A on proton transport was measured by observing the accumulation of the dye, acridine orange. A dose-dependent inhibition of acridine orange fluorescence quenching was seen, suggesting that cassigarol A inhibits H^+,K^+-ATPase-mediated proton transport (Fig. 7). On the other hand, cassigarol A acetate did not inhibit proton transport even at 50 μM. The effect of cassigarol A on gastric acid secretion was investigated in stomach-perfused rats. Intraperitoneal administration of cassigarol A (50 mg/kg) showed a potent inhibitory effect on 2-DG induced acid secretion at least up to 6 hr after the stimulation (Fig. 8). Cassigarol A acetate, however, had no effect on acid secretion. Since cassigarol A inhibits H^+,K^+-ATPase activity and proton transport *in vitro*, long term reduction of acid secretion by cassigarol A may be due to the inhibitory effect on H^+,K^+-ATPase. Moreover, these inhibitory effects were completely lost in cassigarol A acetate-treated rats. When considered with these results, it is suggested that the antisecretory effect of cassigarol A is related closely to the inhibition of gastric H^+,K^+-ATPase and that the hydroxy groups of cassigarol A are important in the interaction with the enzyme.

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