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EFFECT OF STILBENE DERIVATIVES ON GASTRIC H^+,K^+-ATPase

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Abstract—The effect of naturally occurring hydroxystilbene, 3,3′,4,5-tetrahydroxystilbene (piceatanol), and its derivatives on gastric H^+,K^+-ATPase was studied. Piceatanol inhibited H^+,K^+-ATPase in a dose-dependent manner. The 50% inhibition value was 4.3×10^{-6} M. It was found from the kinetic study that the inhibition of the enzyme by piceatanol was competitive with respect to ATP and was non-competitive with respect to K^+. Piceatanol also effectively inhibited gastric acid secretion. However, methylation of phenolic hydroxy groups of piceatanol resulted in a complete loss of the inhibition of the enzyme and acid secretion, suggesting the role of phenolic hydroxy groups in the inhibition. The study on hydroxystilbene derivatives also showed that phenolic hydroxy groups are important in the interaction with H^+,K^+-ATPase and that stilbenes with neighbouring hydroxy groups are the most effective inhibitors.

Stilbene derivatives have a variety of biological and pharmacological activities [1–3]. In a previous study 3,3′,4,5-tetrahydroxystilbene (piceatanol, Table 1, **15**) [4] from a Thai medicinal plant, *Cassia garrettiana* Craib, was shown to have a broad spectrum of physiological activities including antifungal, phyto-growth inhibition, and an ichthytoxic and coronary artery dilation effect [5, 6]. Nimmanpisut *et al.* [7] and Janthasoot *et al.* [8] reported the effect of a similar hydroxystilbene, 2,4,3′,5′-tetrahydroxystil-bene, on liver mitochondria containing ATPase. Gastric H^+,K^+-ATPase is an ion pump responsible for acid secretion and plays an important role in the terminal step of gastric acid secretion [9, 10]. This enzyme therefore has been regarded as an important target for peptic ulcer therapy. A selective gastric H^+,K^+-ATPase inhibitor, omeprazole, which shows a potent antisecretory activity, is used clinically for the treatment of peptic ulcers [11, 12]. We reported previously that cassigarol A, a polyphenol isolated from the same medicinal plant, inhibits both gastric H^+,K^+-ATPase and gastric acid secretion [13]. Since cassigarol A is thought to be a dimer of piceatanol, we investigated the effects of piceatanol on gastric H^+,K^+-ATPase and acid secretion. We further synthesized hydroxystilbene derivatives and analysed the structure–activity relationships of the compounds in the inhibition of H^+,K^+-ATPase.

MATERIALS AND METHODS

Materials. *p*-Nitrophenyl phosphate, 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 a local slaughterhouse. Male Wistar rats were from SLC, Hamamatsu, Japan.

Isolation of 3,3′,4,5-tetrahydroxystilbene (piceastanol). Piceatanol was extracted from the heartwood of *Cassia garrettiana* Craib and isolated according to the method described previously [4, 14].

Synthesis of hydroxystilbene derivatives. Hydroxy-stilbenes were prepared by applying the procedures appearing in the literature [15–18].

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 towels. 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, 250 mM sucrose and 20 mM Pipes–NaOH pH 7.4, with Physcotron (Nichi-on, Tokyo, Japan). 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*. The pellet was resuspended in homogenization buffer. Gastric microsome vesicles containing H^+,K^+-ATPase were prepared by Ficoll-sucrose discontinuous density gradient centrifugation as described previously [19]. The membrane fraction containing vesicles, which was fractionated above the Ficoll interface, was collected and lyophilized to render the vesicles freely permeable to cations, and stored at −80°. Protein was determined by the Lowry method [20].

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

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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 terminated by the addition of 1 mL ice-cold trichloroacetic acid (10%) and assayed for inorganic phosphate according to the method of Fiske and Subbarow [21]. Drugs were dissolved in dimethylsulfoxide. The concentration of dimethylsulfoxide in the assay solution was below 1.0%, which did not affect the enzyme activity.

Assay of Na^+,K^+*-ATPase.* For Na^+,K^+-ATPase, the assay medium contained 2 mM $MgCl_2$, 40 mM Tris–HCl, pH 7.4, and 10 μg enzyme protein, with or without 20 mM KCl and 100 mM NaCl, in a total volume of 1 mL. The reaction was started with 2.5 mM ATP Tris salt 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 [21]. Drugs were dissolved in dimethylsulfoxide. The concentration of dimethylsulfoxide in the assay solution was below 1.0%, which did not affect the enzyme activity.

Antisecretory study. Measurement of the gastric acid secretion was carried out by the method of Maeda-Hagiwara and Watanabe [22] with a 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 cannula were connected to a saline reservoir and the stomach was continuously perfused at a 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, Tokyo, Japan) with a personal computer (PC-9800, NEC, Tokyo, Japan). Acid output was recorded every 5 min for 6 hr. Gastric acid secretion was induced by the subcutaneous administration of 300 mg/kg 2-DG.

RESULTS

Effect of piceatanol on H^+,K^+*-ATPase and* Na^+,K^+*-ATPase*

Piceatanol inhibited gastric H^+,K^+-ATPase from hog gastric mucosa in a dose-dependent manner with a 50% inhibition value of 4.3×10^{-6} M (Fig. 1). The effect of methylpiceatanol, whose hydroxy groups are protected by methyl groups, was also examined. This compound was not effective in the inhibition of gastric H^+,K^+-ATPase. For Na^+,K^+-ATPase, inhibition by piceatanol was only 35% even at 10^{-4} M (data not shown).

Kinetics of H^+,K^+*-ATPase inhibition*

A kinetic study was carried out by varying ATP and K^+ concentrations to investigate the mechanism by which piceatanol inhibits H^+,K^+-ATPase. When the ATP concentration was varied at fixed K^+, a competitive interaction between ATP and piceatanol was observed (Fig. 2). The apparent K_m values were increased from 1.3 to 1.9 and 2.8 mM in the presence

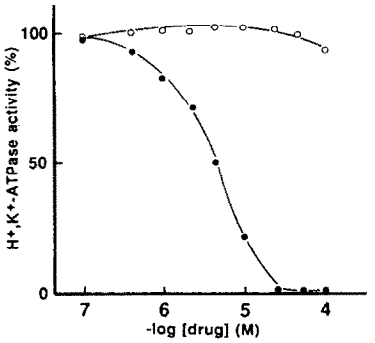


Fig. 1. Effects of piceatanol and methylpiceatanol on the H^+,K^+-ATPase from gastric mucosa. Microsomal membrane (5–10 μg) was incubated in 40 mM Tris–HCl buffer pH 7.4 containing 2 mM $MgCl_2$, 20 mM KCl and 2.5 mM ATP Tris salt for 20 min at 37°. The reaction was terminated by the addition of trichloroacetic acid (final concentration 5%) and liberated inorganic phosphate was determined. Results are expressed as per cent of control, which was 126 μmol P_i/hr/mg protein. Each point represents the mean of four values of two separate experiments. Piceatanol (●), methylpiceatanol (○).

of 2.5 and 5 μM inhibitor, respectively, without changing the apparent V_{max} value. The calculated K_i value was 4.2 μM. When the inhibition of H^+,K^+-ATPase activity by piceatanol was measured as a function of the K^+ concentration, a non-competitive interaction was observed (Fig. 3). The apparent V_{max} value was changed from 113 to 69 and 52 μmol/hr/mg protein in the presence of 2.5 and 5 μM piceatanol, respectively, while the apparent K_m value remained unchanged.

Effect of piceatanol on gastric acid secretion

Gastric acid secretion was induced by 2-DG, and continued for 6 hr (Fig. 4). The stimulated acid secretion was markedly inhibited by the intraperitoneal administration of piceatanol (50 mg/kg) up to 6 hr. On the other hand, methylpiceatanol (50 mg/kg) had no effect on the stimulated acid secretion.

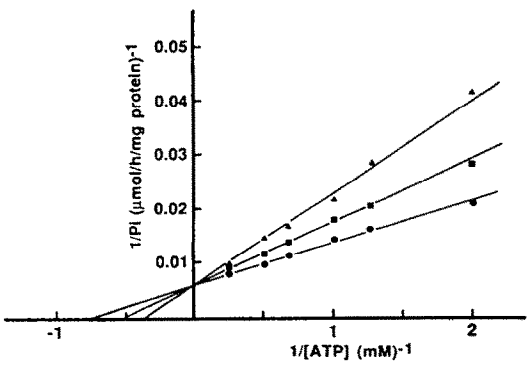


Fig. 2. Double reciprocal plots of the hydrolysis rates of ATP by H^+,K^+-ATPase vs concentration of ATP in the presence of 0 (●), 2.5 (■) and 5 μM (▲) piceatanol. Each value represents the mean of four values of two separate experiments.

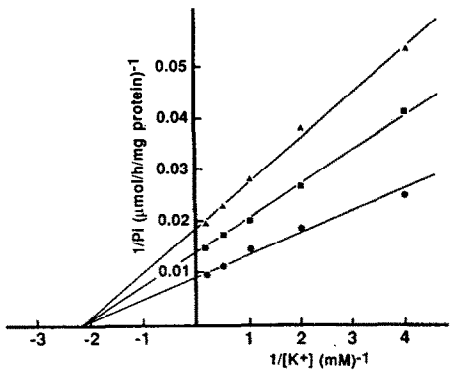


Fig. 3. Double reciprocal plots of the hydrolysis rates of ATP by H^+,K^+-ATPase versus concentration of K^+ in the presence of 0 (●), 2.5 (■) and 5 μM (▲) piceatanol. Each value represents the mean of four values of two separate experiments.

Structure–activity relationships of stilbene derivatives in the inhibition of H^+,K^+-*ATPase*

trans-Stilbene derivatives with hydroxy groups on phenyl rings were synthesized and the inhibitory activity on H^+,K^+-ATPase was assayed (Table 1). Non-substituted parent compound, *trans*-stilbene (**1**), had little inhibitory effect even at 300 μM. For the stilbene derivatives with one hydroxy group on the A ring (**2–4**), 50% inhibition (IC_{50}) of H^+,K^+-ATPase was 40–130 μM. Among these three derivatives, the *meta*-substituted derivative (**3**) was the most potent, while the *ortho*-substituted derivative (**4**) was a poor inhibitor. The inhibition was markedly potentiated by introducing another hydroxy group to the A ring (**5–7**). In particular, compounds with neighbouring hydroxy groups (catechol-type) showed potent inhibition (**5**, **6**).On the other hand, the introduction of another hydroxy group to the B ring led to a reduction in the inhibition (**8–10**). This may be due to the reduced hydrophobicity of the molecule caused by introducing hydroxy groups. For the compounds with two hydroxy groups on the A ring and one hydroxy group on the B ring (**11–13**), the compounds with neighbouring hydroxy groups (**11** and **12**) showed potent inhibition, as observed for derivatives with two hydroxy groups substituted (**5**, **6**), while compound **13**, which is a non-catechol type, was less inhibitory. Compounds with two hydroxy groups on each phenyl ring, including piceatanol (**15**), were potent inhibitors of H^+,K^+-ATPase. All these derivatives have neighbouring hydroxy groups in the molecule (catechol-type). However, the inhibition was not potentiated by introducing another pair of neighbouring hydroxy groups (**5** vs **14**). In order to investigate the presence of the olefin structure, derivatives with the double bond reduced to a single bond were synthesized and assayed for inhibition of H^+,K^+-ATPase activity (**17–19**). The inhibition by **18** and **19**, which corresponds to **12** and **15**, respectively, was not decreased, suggesting that the *trans*-olefin structure (double bond) is not essential for the inhibition of the enzyme. Compound **17** also inhibited similarly to the corresponding compound **10**. Compound **16**, which has neighbouring hydroxy groups at the positions of *ortho* and *meta*, also showed similar potency to the *meta* and *para*-substituted derivatives.

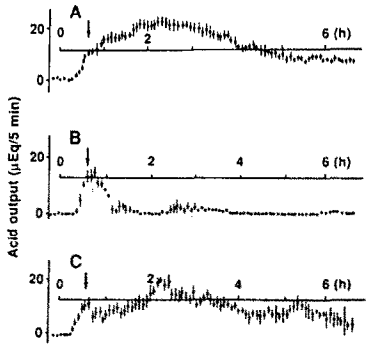


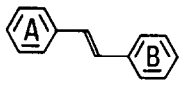
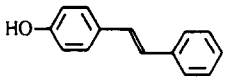
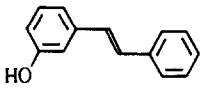
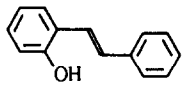
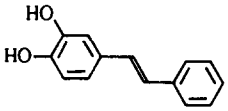
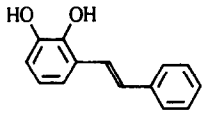
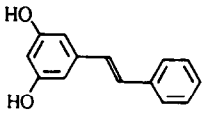
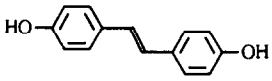
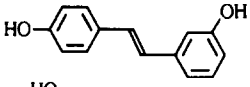
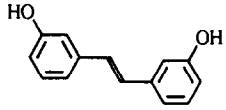
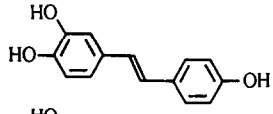
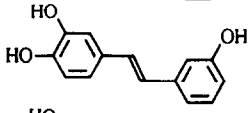
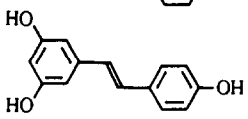
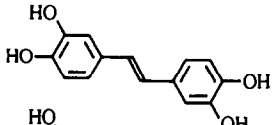
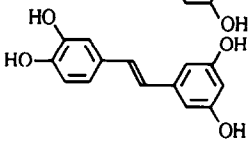
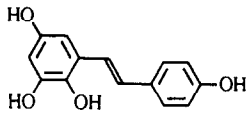
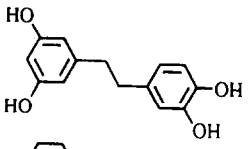
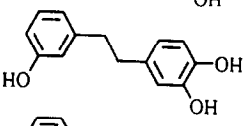
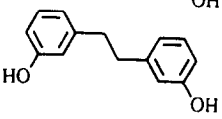
Fig. 4. Effects of piceatanol and methylpiceatanol on 2-DG-induced gastric acid secretion in rats. 2-DG was dissolved in saline solution and given subcutaneously (300 mg/kg) at time 0. Drugs were suspended in 5% gum arabic solution and given intraperitoneally (50 mg/kg) (indicated by arrow). Ordinates 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) piceatanol + 2-DG; (C) methylpiceatanol + 2-DG. Each value represents the mean ± SE for three to six animals.

DISCUSSION

3,3′,4,5′-Tetrahydroxystilbene, piceatanol, is a naturally occurring hydroxystilbene derivative which has been reported to have a variety of physiological and biological activities [5, 6]. The present results show that hydroxystilbene derivatives are potent inhibitors of gastric H^+,K^+-ATPase. We have shown previously that naturally occurring polyphenol, cassigarol A, isolated from the same medicinal plant as piceatanol, is a potent inhibitor of gastric H^+,K^+-ATPase [13]. The inhibitory effect on the H^+,K^+-ATPase by cassigarol A was thought to be responsible for its antisecretory activity. Since cassigarol A, which is a dimer of piceatanol, is thought to be synthesized from piceatanol in the plant, we investigated in the present study the effect of piceatanol on gastric H^+,K^+-ATPase and acid secretion. We found that piceatanol is also a potent inhibitor of gastric H^+,K^+-ATPase similarly to cassigarol A. On the other hand, piceatanol is more than 20-fold less inhibitory toward Na^+,K^+-ATPase than H^+,K^+-ATPase, suggesting that the inhibition of ATPases by piceatanol is selective to H^+,K^+-ATPase to a certain extent.

The kinetic study revealed that piceatanol inhibits H^+,K^+-ATPase following the same pattern as cassigarol A, and is competitive with respect to ATP and non-competitive with respect to K^+. In the H^+,K^+-ATPase system, the enzyme is phosphorylated at cytosolic sites by ATP in the presence of Mg^{2+} to form the phosphoenzyme. The enzyme–phosphate complex is then dephosphorylated by

Table 1. Inhibition by hydroxystilbene derivatives of H^+,K^+-ATPase from hog gastric mucosa

Compound	Structure	IC_{50} (μM)
1		>300
2		74
3		40
4		130
5		2.6
6		2.4
7		50
8		170
9		120
10		140
11		2.8
12		5.5
13		84
14		3.5
15		4.3
16		3.4
17		210
18		2.3
19		4.6

The IC_{50} was determined under the assay conditions as described in Materials and Methods, and was expressed as the mean of triplicate experiments.

luminal K^+ [23]. The result of the kinetic study suggests that piceatanol competes with ATP and thereby inhibits the formation of phosphoenzyme. Hydroxy groups are important in the inhibition of H^+,K^+-ATPase by piceatanol, because methylation of hydroxy groups led to a complete loss of inhibition of the enzyme. We have obtained the same result by using naturally occurring phenolic compounds, salvianolic acid A [24] and ellagic acid [25], in which hydroxy groups are essential to the inhibition of H^+,K^+-ATPase. Also, from the *in vivo* study, methylation of hydroxy groups of piceatanol led to a loss of the inhibition of acid secretion. These results suggest that H^+,K^+-ATPase inhibition is responsible for the antisecretory activity of piceatanol.

Since the present study with the hydroxy stilbene, piceatanol, revealed the important role of phenolic hydroxy groups in the interaction with the enzyme, we synthesized a variety of hydroxystilbene derivatives and screened the inhibition of H^+,K^+-ATPase. Structure–activity relationships of hydroxystilbene indicated that the introduction of hydroxy groups to the phenyl ring of the parent stilbene skeleton, in particular, introduction of neighbouring hydroxy groups (catechol-type) could potentiate the inhibitory effect on H^+,K^+-ATPase, and that these catechol-bearing stilbenes, including piceatanol, were the most effective inhibitors among the compounds tested. As observed for compound **14**, introduction of other neighbouring hydroxy groups to the phenyl ring failed to potentiate the inhibitory activity. The reason for this is not clear from the present study. However, it may be related to the fact that though the introduction of hydroxy groups to the phenyl ring leads to an increase in the moiety interacting with the enzyme, it causes decreased hydrophobicity of the molecule itself at the same time. The decreased hydrophobicity may impair the interaction of the inhibitor with the enzyme.

For the inhibition of gastric H^+,K^+-ATPase by stilbene derivatives, the presence of phenolic hydroxy groups is essential, while the *trans*-olefin structure is not essential, since when the double bond of the hydroxystilbene was reduced the inhibition potency was retained. Inamori *et al.* [5] reported that the *trans*-olefin structure and phenolic hydroxy groups are necessary for coronary vasodilator activity, and that only phenolic hydroxy groups are necessary for ichthytoxic and antimicrobial activities. The result obtained for gastric H^+,K^+-ATPase inhibition is the same as for the latter case. The phenolic hydroxy groups are also essential for the inhibition of mitochondrial ATPase by 2,4,3′,5-tetrahydroxystilbene [8].

In conclusion, our studies show that hydroxystilbenes are inhibitors of gastric H^+,K^+-ATPase, though the parent non-substituted stilbene has no inhibitory effect. A typical potent inhibitor, piceatanol, competed with ATP, which may be the mechanism of inhibition of the enzyme by hydroxystilbenes. It was found from analysis of the structure–activity relationships that the presence of phenolic hydroxy groups is essential for the inhibition of the enzyme and that, among the hydroxystilbenes, catechol-bearing stilbenes are the most effective inhibitors.

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