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INHIBITION OF GASTRIC H^+,K^+-ATPase BY THE ANTI-ULCER AGENT, SOFALCONE

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Abstract—Effects of the anti-ulcer agent, sofalcone, on gastric H^+,K^+-ATPase were studied as well as those of other chalcone derivatives, chalcone and sophoradin. These drugs inhibited pig gastric H^+,K^+-ATPase in a dose-dependent manner. They were 5–10-fold less inhibitory toward Na^+,K^+-ATPase than H^+,K^+-ATPase. The potencies of these drugs on the inhibition of enzymes were as follows: sophoradin > sofalcone > chalcone. Kinetic studies showed that the inhibition of H^+,K^+-ATPase by sofalcone was competitive with respect to ATP and was non-competitive with respect to K^+. Sofalcone also inhibited H^+,K^+-ATPase mediated proton transport and reduced the phosphoenzyme level. These results suggest that sofalcone inhibits gastric H^+,K^+-ATPase competitively with ATP at the ATP site and thereby blocks the phosphorylation of the enzyme. This may be the cause of the anti-secretory activity of sofalcone.

The anti-ulcer agent, sofalcone, is a synthetic derivative of sophoradine, which is an isoprenyl chalcone isolated from the root of the Chinese medicinal plant *Sophora subprostrata* with CHUN et T. CHEN as the active component [1–3] (Fig. 1). Sofalcone is thought to exert its anti-ulcer effect mainly by strengthening gastric defensive factors such as microcirculation [4] and mucus synthesis [5, 6]. These effects are attributed to the inhibition of prostaglandin metabolizing enzyme, 15-hydroxyprostaglandin dehydrogenase, in the gastric mucosa, which leads to an increase in the prostaglandin content [7]. Sofalcone also has a mild anti-secretory effect. Saziki *et al.* [8] reported that sofalcone at 50 mg/kg i.d. significantly reduced the volume and acidity of gastric juice in pylorus-ligated rats. The mechanism, however, by which sofalcone inhibits acid secretion is not known. Gastric H^+,K^+-ATPase plays an important role in acid secretion. This enzyme transports H^+ into the lumen in exchange for K^+ [9, 10]. In the present study, we examined the effects of sofalcone on gastric H^+,K^+-ATPase. The effects of chalcone derivatives, chalcone and sophoradin also were investigated.

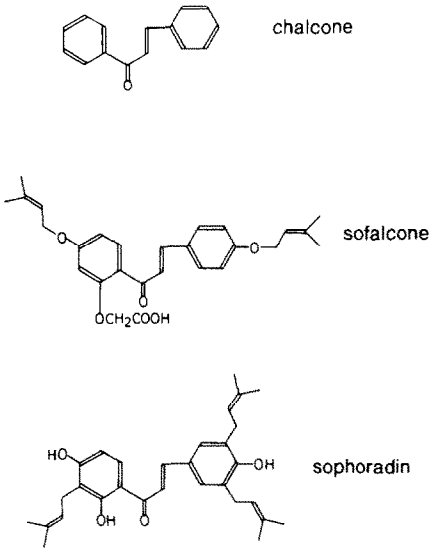

Fig. 1. Chemical structures of chalcone, sofalcone and sophoradin.

MATERIALS AND METHODS

Materials. *p*-Nitrophenyl phosphate (pNPP†), PIPES, acridine orange and valinomycin were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). Chalcone was from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). [γ-^{32}P]ATP (11 Ci/mmol) and Aquasol were from New England Nuclear (Boston, MA, U.S.A.). All other chemicals were of the highest purity commercially available. Sofalcone was synthesized at our laboratory. Sophoradin was extracted from *Sophora subprostrata* CHUN et T. CHEN as described [1]. ATP Tris salt was prepared from ATP Na salt in our laboratory.

Preparation of gastric H^+,K^+*-ATPase.* Gastric microsomal vesicles containing H^+,K^+-ATPase were prepared by density gradient centrifugation from pig fundic mucosa, as described [11]. The purified vesicles were collected and lyophilized to be rendered freely permeable to cations and were stored at −80°. For proton transport experiments, fresh non-lyophilized vesicles were used. Protein was determined by the Lowry method, using bovine serum albumin as a standard [12].

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† Abbreviations: pNPP, *p*-nitrophenyl phosphate; K^+-pNPPase, K^+-stimulated *p*-nitrophenyl phosphatase; PIPES, piperazine-*N*,*N*′-bis-2-ethanesulfonic acid.

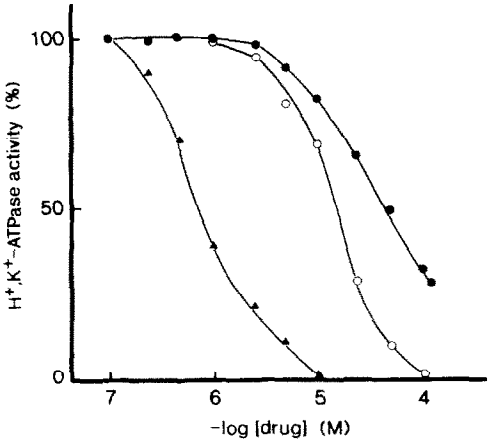

Fig. 2. Effects of chalcone, sofalcone and sophoradin on the H^+,K^+-ATPase from pig gastric mucosa. Microsome membrane (5 μg protein) was incubated in 40 mM Tris–HCl buffer (pH 7.4) containing 2 mM $MgCl_2$, 20 mM KCl and 2.5 mM ATP Tris salt in a total volume of 1 mL for 20 min at 37°. The reaction was terminated by the addition of trichloroacetic acid (final 5%) and liberated inorganic phosphate was determined. Each point represents the mean of two values. Chalcone (●); sofalcone (○); sophoradin (▲).

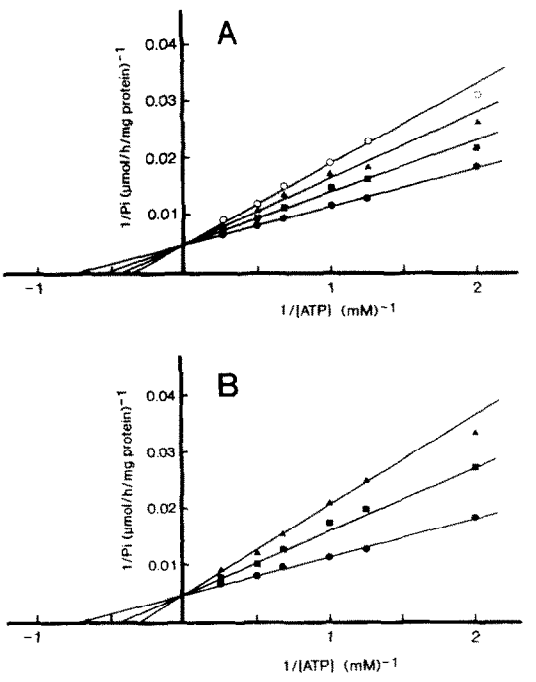

Fig. 3. Double reciprocal plots of the hydrolysis rates of ATP by H^+,K^+-ATPase vs concentrations of ATP in the presence of 0 (●), 30 (■), 60 (▲) and 90 μM (○) chalcone (A), and 0 (●), 10 (■) and 20 μM (▲) sofalcone (B). Each value represents the average of duplicate experiments.

Assay of H^+,K^+*-ATPase*. The assay medium consisted of 2 mM $MgCl_2$, 2 mM ATP Tris salt, 40 mM Tris–HCl (pH 7.4) and 5 μg membrane protein, with or without 20 mM KCl in a total volume of 1 mL. The medium was incubated for 20 min at 37°. The reaction was terminated by adding 1 mL cold trichloroacetic acid (10%). The inorganic phosphate derived from the ATP was measured according to Fiske and Subbarow [13]. Drugs were dissolved in dimethylsulfoxide. The concentration of dimethylsulfoxide in the reaction mixture did not exceed 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 μg membrane protein, with or without 20 mM KCl, in a total volume of 1 mL. After 20 min of incubation at 37°, the reaction was terminated with 1 mL 1 N NaOH. The absorbance of the reaction medium was read at 410 nm.

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 [13].

Proton transport experiment. The transport of protons was measured by a spectrophotometric method, as described [14]. The incubation mixture contained 1 mM $MgCl_2$, 150 mM KCl, 0.5 mM EDTA, 20 mM PIPES/NaOH (pH 7.4), 300 μg fresh vesicles from pig 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).

Determination of intermediate phosphoenzymes. Incubation mixture consisted of 2 mM $MgCl_2$, 10 mM Tris/PIPES (pH 7.0), 2.5 μM [γ-^{32}P]ATP and 50 μg lyophilized vesicles, with or without drugs in a final volume of 0.5 mL. After a 15 sec incubation at room temperature, the reaction was quenched with 1 mL ice-cold 10% (w/v) perchloric acid containing 5 mM non-labeled ATP and 40 mM NaH_2PO_4. The precipitated membranes were then collected by filtration on a Whatman GF/B filter. The filter was washed 10 times with 5 mL ice-cold 5% (w/v) perchloric acid containing 10 mM NaH_2PO_4 and was transferred to a liquid scintillation vial and its radioactivity was counted in 10 mL of Aquasol.

RESULTS

Effect on gastric H^+,K^+*-ATPase*

Effects of chalcone, sofalcone and sophoradin on gastric H^+,K^+-ATPase were studied on lyophilized vesicles from pig gastric mucosa. Each drug inhibited the enzyme with 50% inhibition at 4.8×10^{-5}, 1.5×10^{-5} and 7.4×10^{-7} M, respectively (Fig. 2).

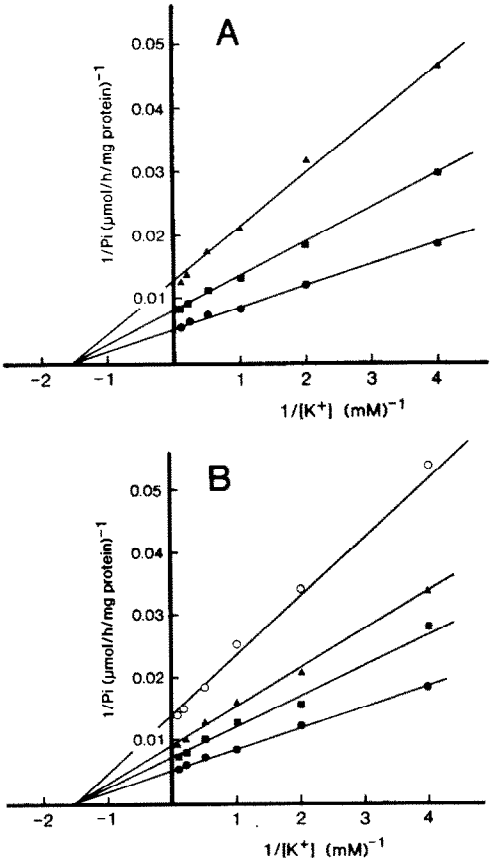

Fig. 4. Double reciprocal plots of the hydrolysis rats of ATP by H^+,K^+-ATPase vs concentrations of KCl in the presence of 0 (●), 30 (■) and 60 μM (▲) chalcone (A), and 0 (●), 5 (■), 10 (▲) and 20 μM (○) sofalcone (B). Each value represents the average of duplicate experiments.

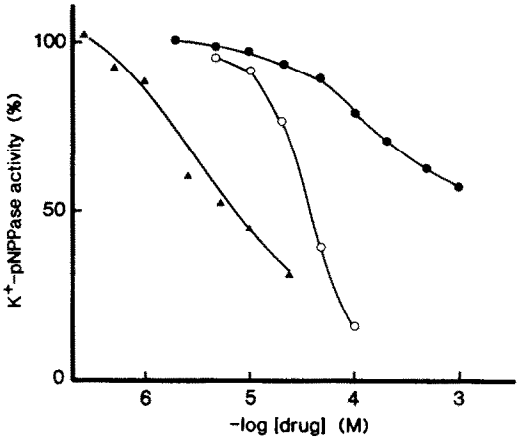

Fig. 5. Effects of chalcone, sofalcone and sophoradin on the K^+-pNPPase from pig gastric mucosa. Microsome membrane (5 μg protein) was incubated in 40 mM Tris–HCl buffer (pH 7.4) containing 5 mM $MgCl_2$, 20 mM KCl and 5 mM pNPP in a total volume of 1 mL for 20 min at 37°. The reaction was terminated by the addition of 1 mL in NaOH and the absorbance was measured at 410 nm. Each value represents the mean of two values. Chalcone (●); sofalcone (○); sophoradin (▲).

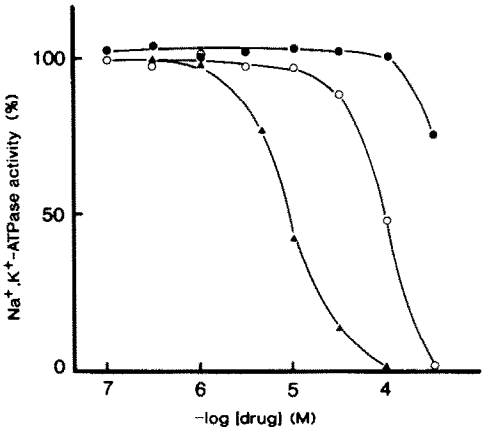

Fig. 6. Effects of chalcone, sofalcone and sophoradin on the Na^+,K^+-ATPase from dog kidney. Microsome membrane (20 μg protein) was incubated in 40 mM Tris–HCl buffer (pH 7.4) containing 2 mM $MgCl_2$, 20 mM KCl, 100 mM NaCl and 2.5 mM ATP Tris salt in a total volume of 1 mL for 20 min at 37°. The reaction was terminated by the addition of trichloroacetic acid (final 5%) and liberated inorganic phosphate was determined. Chalcone (●); sofalcone (○); sophoradin (▲).

Sophoradin was the most potent enzyme inhibitor among these drugs.

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

Kinetic studies were carried out by varying ATP and K^+ concentrations. For ATP, the inhibition of H^+,K^+-ATPase was investigated in the presence of chalcone or sofalcone by changing the ATP concentration from 0.5 to 4 mM. From double reciprocal plots, it was found that chalcone and sofalcone behaved as competitive inhibitors with respect to ATP (Fig. 3A and B). The apparent V_{max} values were increased in the presence of chalcone or sofalcone. The calculated K_i values were 79 and 14 μM with chalcone and sofalcone, respectively. For K^+, its concentration was changed from 0.25 to 10 mM. Double reciprocal plots showed that the inhibitions of H^+,K^+-ATPase by chalcone and sofalcone were non-competitive with respect to K^+ (Fig. 4A and B). The calculated K_i values were 48 and 11 μM with chalcone and sofalcone, respectively.

Effect on K^+-pNPPase

Synthetic substrate pNPP is also known to be hydrolysed by the H^+,K^+-ATPase system. Effects of chalcone, sofalcone and sophoradin on K^+-pNPPase in pig gastric membrane were tested using the same preparation as for H^+,K^+-ATPase. These drugs inhibited K^+-pNPPase in a dose-dependent manner (Fig. 5). The values for 50% inhibition were as follows: chalcone $> 10^{-3}$, sofalcone 3.7×10^{-5}, sophoradin 6.0×10^{-6} M. The inhibitory effects of these drugs on K^+-pNPPase were weaker than those

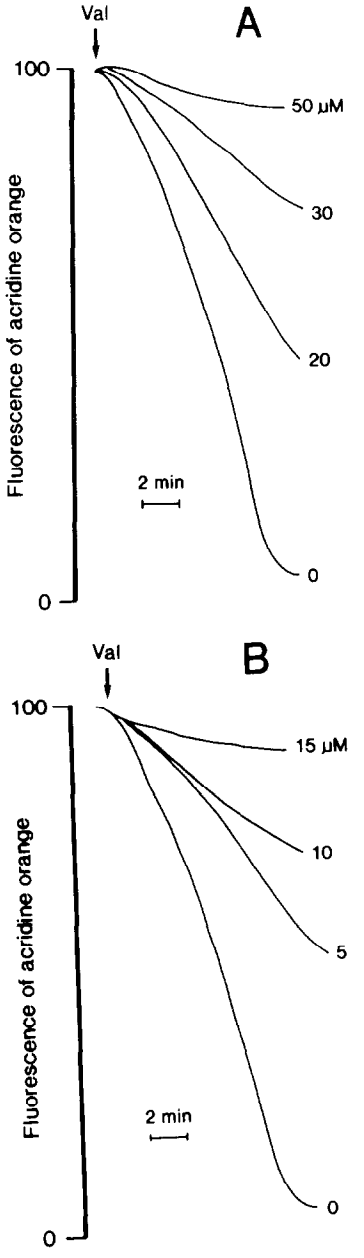

Fig. 7. Effects of chalcone (A) and sofalcone (B) on H^+,K^+-ATPase mediated proton transport. The incubation mixture contained 1 mM $MgCl_2$, 150 mM KCl, 0.5 mM EDTA, 300 μg fresh vesicles and 10 μM acridine orange with or without the drugs. ATP Mg salt was added before the reaction was started by the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

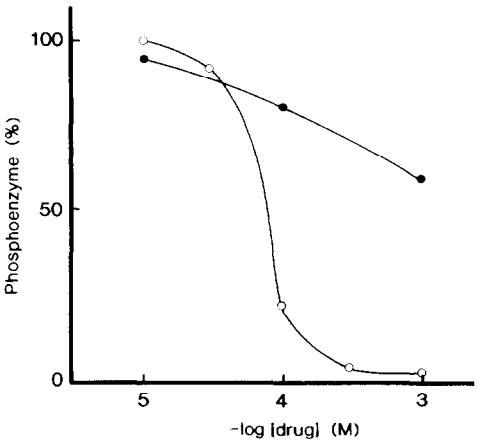

Fig. 8. Effects of chalcone and sofalcone on phosphoenzyme level. Lyophilized vesicles (50 μg protein) were incubated in 10 mM Tris–PIPES buffer (pH 7.0) containing 2 mM $MgCl_2$ and 2.5 μM [γ-^{32}P]ATP with or without the drugs in a final volume of 0.5 mL for 15 sec at room temperature. The reaction was quenched and the radioactivity of collected vesicles was counted. Chalcone (●); sofalcone (○).

on H^+,K^+-ATPase. However, sophoradin was the most potent inhibitor as seen with H^+,K^+-ATPase.

Effect on Na^+,K^+-*ATPase*

Effects of chalcones on Na^+,K^+-ATPase were studied on the enzyme from dog kidney. Sofalcone and sophoradin inhibited the enzyme with 50% inhibition at 9.5×10^{-5} and 8.9×10^{-6} M, respectively (Fig. 6). Inhibition by chalcone was below 30% even at 3×10^{-4} M. The potency of these chalcones in the inhibition of Na^+,K^+-ATPase was 5–10-fold weaker than that of H^+,K^+-ATPase.

Effect on proton transport

Proton transport was measured by observing the quenching of acridine orange fluorescence, *in vitro*. Chalcone and sofalcone showed dose-dependent inhibition of proton transport (Fig. 7A and B). Chalcone at 50 μM and sofalcone at 15 μM almost completely inhibited proton transport.

Effect on phosphoenzyme level

Sofalcone reduced the level of phosphoenzyme intermediates with a 50% inhibition value at 5.9×10^{-5} M (Fig. 8). Although chalcone also reduces the phosphoenzyme level in a dose-dependent manner, the inhibition was about 40% at 10^{-3} M.

DISCUSSION

We previously showed that naturally occurring chalcone derivatives, xanthoangelol and 4-hydroxyderricin from the root of *Angelica keiskei* KOIDZUMI, are potent inhibitors of gastric H^+,K^+-ATPase [15]. The anti-ulcer agent, sofalcone, which is a chalcone derivative, was developed as a stable synthetic compound from sophoradin, because of difficulties in the stability and synthesis of sophoradin. It has also been reported that sofalcone has mild anti-secretory activity [8]. In the present study, therefore, we examined whether sofalcone and sophoradin, including chalcone, have inhibitory effects on gastric H^+,K^+-ATPase. Our results demonstrated that chalcone, sofalcone and sophoradin are inhibitors of gastric H^+,K^+-ATPase. The order of potency in inhibiting the enzyme was as

follows: sophoradin > sofalcone > chalcone. This is compatible with the anti-secretory activity shown in pylorus-ligated rats *in vivo*, in which sophoradin was more effective than sofalcone in reducing the acid output and chalcone had little effect on acid output [2, 8]. These compounds were also shown to be effective in the *in vitro* proton transport experiment which was mediated by H^+,K^+-ATPase. Taking these observations into consideration, it is responsible for the anti-secretory effect of these chalcone derivatives.

It has been reported that the ATP hydrolytic sites for H^+,K^+-ATPase are located at cytosolic sites and the high affinity K^+ sites are on the luminal face across the membrane [16]. The enzyme is phosphorylated at cytosolic sites by ATP in the presence of Mg^{2+}. Then the enzyme–phosphate complex is dephosphorylated by luminal K^+. This kinetic study demonstrated that the inhibition of gastric H^+,K^+-ATPase by sofalcone and chalcone was competitive with respect to ATP, and non-competitive with respect to K^+. Sofalcone also inhibited the phosphoenzyme level. These results suggest that sofalcone and chalcone may bind to ATP sites competitively with ATP and inhibit the formation of intermediate phosphoenzymes, thereby inhibiting H^+,K^+-ATPase activity.

Chalcone derivatives tested here also showed inhibitory effect on Na^+,K^+-ATPase. Since recent reports have shown that the primary structure of H^+,K^+-ATPase is highly homologous to that of Na^+,K^+-ATPase [17], this may be related to the fact that these chalcones have interactions with enzymes at the ATP sites. However, the inhibition of Na^+,K^+-ATPase by chalcone derivatives was 5–10-fold weaker than that of H^+,K^+-ATPase. This suggests that these chalcones could recognize the delicate differences of the structure at the ATP site, and inhibit H^+,K^+-ATPase selectively to a certain extent. A previous report [15] showed that the chalcone derivatives, xanthoangelol and 4-hydroxyderricin, inhibited H^+,K^+-ATPase by the same pattern as observed here. This indicates that the key moiety in the interation between the enzyme is the chalcone structure itself and that the substituents on the phenyl rings are not responsible for the mode of action. However, it is possible to enhance the potency by varying the substituents as observed in chalcone and sophoradin.

Though it is not clear to what extent the anti-secretory activity of sofalcone contributes to its anti-ulcer effects, the present study suggests that the anti-secretory activity of sofalcone is partly due to the inhibition of H^+,K^+-ATPase.

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