Reversible Inhibitions of Gastric $H^+,K^+$-ATPase by Scopadulcic Acid B and Diacetyl Scopadol
NEW BIOCHEMICAL TOOLS OF $H^+,K^+$-ATPase*

Shinji Asano, Motofumi Mizutani, Toshimitsu Hayashi, Naokata Morita, and Noriaki Takeguchi
From the Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, Sugitani Toyama 930-01, Japan

Scopadulcic acid B (SA-B), a novel diterpenoid, is a main ingredient of the Paraguayan traditional medicinal herb "Typychi kuratii" (Scoparia dulcis L.). SA-B and its debenzoyl derivative, diacetyl scopadol (DAS), specifically inhibit ATP hydrolysis of gastric $H^+,K^+$-ATPase. Both compounds inhibit the $K^+$-dependent dephosphorylation step of the enzyme without any effect on the phosphorylation step. SA-B is a mixed-type inhibitor with respect to the activating cation, $K^+$. SA-B lowers the affinity of $H^+,K^+$-ATPase to $K^+$ and decreases the maximal velocity of ATP hydrolysis, whereas DAS is an uncompetitive inhibitor with respect to $K^+$. Furthermore, the effects of SA-B and DAS on conformational states of the ATPase were studied by measuring the changes in the fluorescence intensity of the fluorescein isothiocyanate-labeled enzyme. The fluorescence study shows that SA-B primarily binds to the $E_2K$ form in the presence of $Mg^{2+}$ and stabilizes the form and that DAS stabilizes the $E_2PK$ form. Therefore, the chemical modification of SA-B, debenzoylation, induced the changes in the pattern of inhibition of $H^+,K^+$-ATPase. Furthermore, the inhibition mechanisms of SA-B and DAS were different from those of omeprazole, which is an irreversible inhibitor, and SCH 28080, which is a reversible, competitive inhibitor with respect to $K^+$. DAS also inhibited the $K^+$-dependent $p$-nitrophenylphosphatase activity, and the inhibition was competitive with respect to $K^+$, indicating that the $K^+$-dependent $p$-nitrophenylphosphatase activity does not represent the partial reaction step of $H^+,K^+$-ATPase.

$H^+,K^+$-ATPase is a proton pump for gastric acid secretion (1-5). Two types of compounds are well known to specifically inhibit $H^+,K^+$-ATPase; one is substituted benzimidazoles such as omeprazole (6), picoprazole (7), and E3810 (8), and the other is substituted imidazolo[1,2-$d$]pyridine such as SCH 28080 (9). Omeprazole is transformed to an active compound in an acidic compartment and modifies the essential cysteine residue of $H^+,K^+$-ATPase (10-13). SCH 28080 is a mixed-type inhibitor with respect to $K^+$. SCH 28080 acts like $Na^+$,$K^+$-ATPase (14, 15). SCH 28080 binds to the dephospho form of $H^+,K^+$-ATPase, but its binding to the phospho form is controversial (16-18). SCH 28080 acts like $K^+$ and forms an enzyme-inhibitor complex, $E_S$ SCH 28080 or $E_D$ (SCH 28080) (17). These $H^+,K^+$-ATPase-specific inhibitors have given important insight into the reaction mechanism of the enzyme. However, how the enzyme actively transports the ions is still unknown. To solve this fundamental problem, functional monoclonal antibodies and other specific inhibitors that have different inhibition mechanisms from those of omeprazole and SCH 28080 are also expected to be useful.

In this report, we showed that scopadulcic acid B (SA-B), one of the major components of Paraguayan traditional medicinal herb "Typychi kuratii" (Scoparia dulcis L.) (19) specifically inhibits $H^+,K^+$-ATPase activity without any effect on $Na^+$,$K^+$-ATPase. The inhibition of the $H^+,K^+$-ATPase activity caused by SA-B was antagonized by $K^+$ on the luminal side. SA-B and SCH 28080 commonly have a benzoyl group. Therefore, to determine the role of the benzoyl group in the inhibition mechanism, we synthesized a debenzoyl derivative of SA-B, diacetyl scopadol (DAS) (Fig. 1). DAS also specifically inhibits $H^+,K^+$-ATPase. Interestingly, the inhibition was stimulated by $K^+$ on the luminal side. In this paper, we studied the inhibition mechanism of these compounds by measuring their effects on partial reactions of $H^+,K^+$-ATPase ($E_1$ $\rightarrow$ $E_2$ $\rightarrow$ $E_2$ $\rightarrow$ $E_3$ $\rightarrow$ $E_4$ $\rightarrow$ $E_5$) and compared the inhibition points in the catalytic reaction of $H^+,K^+$-ATPase. The inhibition mechanism of DAS was the simplest among known proton pump inhibitors such as omeprazole, SCH 28080, SA-B, and DAS.

EXPERIMENTAL PROCEDURES

Materials—SA-B was isolated from Scoparia dulcis L. as described elsewhere (19). It is a diterpenoid with a novel skeleton as shown in Fig. 1. DAS (Fig. 1) was prepared by reducing and acetylation of SA-B. For this purpose, 100 mg of LiAlH₄ was added to 10 ml of ether containing 101 mg of SA-B. After stirring for 1 h at room temperature, ethyl acetate was added to the reaction mixture followed by successive washing with 10% aqueous HCl and water. The ethyl acetate layer was concentrated and chromatographed on a silica gel column using CHCl₃ as an eluting solvent to separate reduced SA-B from by-products. The structure of DAS was confirmed by NMR. SA-B and DAS were dissolved in ethanol. The final concentration of ethanol did not exceed 1%.

1 The abbreviations used are: SA-B, scopadulcic acid B; DAS, diacetyl scopadol; FITC, fluorescein isothiocyanate; K"-pNPase, $K^+$-dependent $p$-nitrophenylphosphatase; Pipes, 1,4-piperazine-dithanesulfonic acid.

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Preparations—Gastric vesicles enriched in H⁺,K⁺-ATPase were prepared from mucosa in the fundic region of hog stomachs by differential and density gradient centrifugation as described elsewhere (5). When leaky vesicles were needed, the vesicle suspension was diluted 10 times with distilled water, followed by immediate freezing in liquid N₂. Then the vesicles were lyophilized and resuspended with the original volume of distilled water.

Microsomes of hog kidney abundant in Na⁺,K⁺-ATPase were prepared from the red outer medulla of hog kidney by differential and density gradient centrifugation as described elsewhere (20).

Enzyme Assay—H⁺,K⁺-ATPase activity was measured in 1 ml of the reaction mixture containing gastric vesicles (10 μg of protein), 3 mM MgSO₄, 3 mM ATP, and 40 mM Tris-HCl (pH 7.4) in the presence or absence of 15 mM KCl. When indicated, 10 μg of valinomycin were added to the mixture. The mixture was incubated at 37 °C for 10 min. Inorganic phosphate released was measured by the method of Yoda and Hokin (21). For lyophilized (leaky) vesicles, K⁺-ATPase activity was calculated as the difference between (Mg⁺⁺,K⁺)- and Mg⁺⁺-dependent activities. For nonlyophilized (tight) vesicles, we measured valinomycin-stimulated K⁺-ATPase activity. The valinomycin-stimulated K⁺-ATPase activity was calculated as the difference between (Mg⁺⁺,K⁺,valinomycin)– and (Mg⁺⁺,K⁺)-dependent activities.

Na⁺,K⁺-ATPase activity was measured in 1 ml of the reaction mixture containing enzyme preparation (10 μg of protein), 3 mM MgSO₄, 3 mM ATP, 15 mM KCl, 120 mM NaCl, and 40 mM Tris-HCl (pH 7.4). Na⁺,K⁺-ATPase activity was calculated as the difference between the activities in the absence and presence of 1 mM ouabain.

K⁺-dependent p-nitrophenylphosphatase (K⁺-pNPPase) activity was measured in 1 ml of reaction mixture containing gastric vesicles (10 μg of protein), 6 mM MgSO₄, 6 mM p-nitrophenyl phosphate, 40 mM Tris-HCl (pH 7.4) in the presence or absence of 15 mM KCl. The mixture was incubated at 37 °C for 10 min, and the reaction was stopped by the addition of 0.5 N NaOH. Released p-nitrophenol was measured at 410 nm.

Proton Transport into Gastric Vesicles—Proton transport into gastric vesicles was monitored by measuring the quench of acidine orange fluorescence (22). The vesicles were incubated in either of the following two ways. 1) Twenty micrograms of gastric vesicles were incubated in 1 ml of solution containing SA-B, 150 mM KCl, 2.5 mM MgCl₂, and 1 mM Pipes-NaOH (pH 7.4) at 25 °C for 10 min, and then incubated with 5 μM acidine orange and 10 μg of valinomycin at 25 °C for 1 min. 2) Twenty micrograms of gastric vesicles were incubated in 1 ml of solution containing 150 mM KCl, 2.5 mM MgCl₂, and 1 mM Pipes-NaOH (pH 7.4) at 4 °C for 12 h and at 25 °C for 2 h. The mixture was incubated with SA-B at 25 °C for 10 min and then with 5 μM acidine orange at 25 °C for 1 min. Proton uptake was started by the addition of 0.3 mM Mg-ATP. Fluorescence of acidine orange was measured with an excitation wavelength of 493 nm and an emission wavelength of 530 nm.

Enzyme Phosphorylation and Dephosphorylation—Enzyme phosphorylation was measured as described by Maeda et al. (23). Fifty micrograms of lyophilized gastric vesicles were incubated in a solution containing inhibitor (SA-B or DAS) at the indicated concentration, 2 mM MgSO₄, and 40 mM Tris-HCl (pH 7.4) at 25 °C for 20 min. Then, the mixture was incubated with [γ-³²P]ATP (1 × 10⁵ cpm) and 5 μM ATP at 25 °C for 10 s. The reaction was stopped by the addition of 1 ml of ice-cold stop solution containing 10% trichloroacetic acid and 10 mM inorganic phosphate. The precipitated enzyme was collected on a Millipore filter (HAWP 0.45 μm) and washed thoroughly with the ice-cold stop solution, and the radioactivity of the filter was counted.

To study the effect of SA-B or DAS on the dephosphorylation step, H⁺,K⁺-ATPase in lyophilized gastric vesicles was phosphorylated in the presence or absence of SA-B or DAS as described above, and then the mixture was incubated with various concentrations of KCl at 25 °C for 10 s. The K⁺-dependent decrease in the ³²P-phosphoenzyme level was regarded as the parameter of dephosphorylation on the basis of the present result that neither SA-B nor DAS affected the phosphorylation step of the enzyme.

FITC Labeling of the Enzyme—Lyophilized gastric vesicles (750 μg/ml) were incubated in 1 ml of solution containing 2 mM EDTA, 100 mM Tris-HCl (pH 9.2), and 5 μM FITC for 30 min at 25 °C (24). Then the solution was applied to a Sephadex G-50 column equilibrated with 40 mM Tris-HCl (pH 7.4) to remove unbound FITC.

Fluorescence Measurements—Fluorescence intensity of FITC was measured at 517 nm (excited at 495 nm). The slit width was 2 nm for excitation and 10 nm for emission. The maximal volume of ligands added was 0.5% of the total volume in order to avoid dilution effects on the fluorescence intensity.

RESULTS

Specificity of SA-B and DAS to H⁺,K⁺-ATPase—Fig. 2 shows the effects of SA-B (A) and DAS (B) on activities of hog gastric H⁺,K⁺-ATPase and hog kidney Na⁺,K⁺-ATPase. Both compounds dose dependently inhibited the H⁺,K⁺-ATPase activity without any effect on the activity of Na⁺,K⁺-ATPase, which is a cation-transporting ATPase related to H⁺,K⁺-ATPase. The relative extent of inhibition of H⁺,K⁺-ATPase caused by high concentrations of SA-B was greater in tight vesicles than in leaky vesicles. The half-maximum inhibitory concentration (IC₅₀) of SA-B was 25 μM for tight vesicles and 54 μM for leaky vesicles. On the other hand, DAS was more potent in leaky vesicles than in tight vesicles. The half-maximum inhibitory concentration of DAS in lyophilized vesicles was 17 μM, i.e., DAS is 3-fold more potent than SA-B. In tight vesicles, DAS even at 50 μM inhibited the activity by 40% (Fig. 2B), and we could not measure the effect of DAS at 100 μM, because DAS at concentrations higher than 50 μM was insoluble in the assay medium. The H⁺ concentration in tight vesicles is greater than in leaky vesicles. Since SA-B and DAS are stable compounds, neither protonation nor acid activation of these compounds is considered for the cause of difference in their potency between the two kinds of vesicles.

The inhibition of the H⁺,K⁺-ATPase activity by SA-B or DAS was restored by dilution (data not shown), indicating that the inhibition by SA-B or DAS is reversible as in the case of SCH 28080 (14).

Effects of K⁺ on the Inhibition of H⁺,K⁺-ATPase by SA-B or DAS—K⁺ is transported actively from the luminal (intravesicular) side to the cytosolic (external) side of gastric vesicles in exchange for H⁺, i.e., K⁺ acts as a substrate of the ATPase. We studied the effect of the K⁺ concentration in the medium on the inhibition caused by SA-B or DAS of the K⁺-ATPase activity in lyophilized vesicles. The inhibition by SA-B was reduced as the K⁺ concentration increased. In contrast, the inhibition caused by DAS was enhanced as the K⁺ concentration in the medium increased. Fig. 3, A and B, shows the double-reciprocal (Lineweaver-Burk) plots between the K⁺-ATPase activity and the K⁺ concentration. The plots in
was more sensitive to DAS in lyophilized vesicles than in tight vesicles (Fig. 2A). The Na+,K+-ATPase activity in the absence of inhibitors was 11.5 ± 0.7 μmol/mg/h, and the valinomycin-stimulated K+-ATPase activity of nonlyophilized vesicles was 24.9 ± 1.9 μmol/mg/h. The Na+,K+-ATPase activity in the absence of inhibitors was 11.5 ± 0.7 μmol/mg/h. Data shown are averages ± S.E. for three observations.

Fig. 3A show that the inhibition of H+,K+-ATPase by SA-B is a mixed type and that SA-B lowers not only the affinity of H+,K+-ATPase to K+ but also the maximal velocity (Vmax) of the H+,K+-ATPase reaction. Thus, the fact that the H+,K+-ATPase activity of tight vesicles was more sensitive to SA-B than that of lyophilized vesicles (Fig. 2A) can be explained by this protective effect of K+. In lyophilized vesicles, the intravesicular K+ concentration can be assumed to be close to that of the reaction medium. In tight vesicles, the former is lower than the latter, since the KCl conductance across the vesicle membrane even in the presence of valinomycin is limited (25). The plots in Fig. 3B show that DAS is an uncompetitive inhibitor with respect to K+, indicating that DAS binds to the enzyme-substrate complex (26), that is DAS binds to the K+-bound form of H+,K+-ATPase (E2K or E2PK) and forms a stable inhibitory complex. Generally, in the case of uncompetitive inhibition, the degree of inhibition increases when the substrate concentration increases (28). This finding explains the above finding that H+,K+-ATPase was more sensitive to DAS in lyophilized vesicles than in tight vesicles (Fig. 2B).

Fig. 4. Effects of SA-B on proton uptake into gastric vesicles. A, 20 μg of gastric vesicles were incubated in 1 ml of solution containing SA-B at the indicated concentration, 150 mM KCl, 2.5 mM MgCl₂, 1 mM Pipes-NaOH, pH 7.4, at 25°C for 10 min and then incubated with 5 μM acridine orange and 10 μg of valinomycin at 25°C for 1 min. Proton uptake was initiated by the addition of 0.3 mM MgATP and monitored by measuring the fluorescence quench of acridine orange. The x-axis shows the time after the addition of ATP, and the y-axis shows the relative change in the fluorescence. Concentrations (μM) of SA-B were as follows: a, 0; b, 5; c, 10; d, 20; e, 50; f, 80; g, 100; h, 500. B, 20 μg of gastric vesicles were preincubated in a solution containing 150 mM KCl, 2.5 mM MgCl₂, and 1 mM Pipes-NaOH, pH 7.4, at 4°C for 12 h and then at 25°C for 2 h. The mixture was incubated with SA-B at 25°C for 10 min and then with 5 μM acridine orange at 25°C for 1 min. Proton uptake was initiated as in panel A.

Present findings also suggest that the reactive sites of SA-B and DAS are on the luminal side. The present inhibition mechanisms of SA-B (a mixed type) and DAS (an uncompetitive type with respect to K+) are different from a competitive type found for SCH 28080 (14, 15).

Effect of SA-B on Proton Uptake into Gastric Vesicles—We studied the effect of SA-B on proton uptake into tight gastric vesicles. Fig. 4A shows the effect of SA-B on proton uptake into gastric vesicles in the presence of 150 mM KCl and 10 μg of valinomycin. The quenching of acridine orange fluoresent...
cience reflects proton uptake into vesicles (3, 22). SA-B dose dependently inhibited the rate of proton transport, its IC₅₀ being 20 μM as determined from the initial slopes. SA-B at 100 μM inhibited the rate of proton uptake by 94%. Fig. 4B shows the effect of SA-B on proton uptake into gastric vesicles preincubated sufficiently in a solution containing 150 mM KCl in the absence of valinomycin for 12 h at 4 °C and then for 2 h at 25 °C. In this case, the proton uptake reached the maximal value rapidly compared with the case shown in Fig. 4A, and the effects of SA-B on the rate of proton uptake were less effective. SA-B at 100 μM inhibited the rate of proton uptake by about 55%. This fact indicates that the K⁺ concentration in the vesicles preincubated for 1 min in the presence of 10 μg of valinomycin plus 150 mM K⁺ (Fig. 4A) was much smaller than that in the vesicles incubated sufficiently in the 150 mM KCl solution (Fig. 4B).

Effects of SA-B and DAS on Phosphorylation of H⁺,K⁺-ATPase—Hereafter, we studied the effects of SA-B or DAS on partial reactions of H⁺,K⁺-ATPase to know the inhibition mechanism of these inhibitors. Here we studied the effects of SA-B or DAS on the formation of the phosphorylated intermediate of H⁺,K⁺-ATPase in lyophilized vesicles. When the enzyme was phosphorylated from 5 μM ATP in the absence of K⁺ for 10 s, the phosphoenzyme level was 1140 pmol of EP/mg of protein, indicating that 22% of the catalytic subunit was phosphorylated under the assumptions that H⁺,K⁺-ATPase constituted about 60% of the vesicle protein (27) and that this ATPase was composed of equivalent 114-kDa subunits (28, 29). Neither SA-B even at 100 μM nor DAS even at 50 μM affected the steady state level of phosphoenzyme in the absence of K⁺ (data not shown). Therefore, these compounds did not affect the phosphorylation step of H⁺,K⁺-ATPase (E₁ → E₂P plus E₃P).

Effects of SA-B and DAS on the Dephosphorylation Step of H⁺,K⁺-ATPase—The phosphorylated intermediate of H⁺,K⁺-ATPase is rapidly degraded in the presence of K⁺ (23, 30, 31). After phosphorylating H⁺,K⁺-ATPase in the K⁺-free solution, we added K⁺ into the solution to start the partial reaction of the K⁺-dependent dephosphorylation. In the absence of the inhibitor (SA-B or DAS), the level of phosphorylated enzyme 10 s after the addition of KCl (2, 15, or 150 mM) decreased to about 20% of the original level, irrespective of the K⁺ concentrations (2–150 mM). Fig. 6A shows that SA-B dose dependently inhibits the K⁺-sensitive dephosphorylation. The extent of the inhibition by SA-B depended on the K⁺ concentration in the medium, that is the higher the K⁺ concentration, the smaller the inhibition. The IC₅₀ of SA-B was 47 μM at 2 mM K⁺. SA-B (100 μM) inhibited only 20% of the dephosphorylation level at 15 mM K⁺. The dephosphorylation was hardly inhibited by SA-B at 150 mM K⁺. The K⁺ antagonistic effect on the dephosphorylation step is similar at least qualitatively to that on the H⁺,K⁺-ATPase activity. This result indicates that SA-B binds to the phospho form of the enzyme.

Fig. 5B shows that DAS dose dependently inhibits the K⁺-sensitive dephosphorylation of H⁺,K⁺-ATPase. The extent of inhibition by DAS increased as the K⁺ concentration in the medium increased, in contrast to the case of SA-B. The half-maximum inhibitory concentration (IC₅₀) of DAS was 16 μM at 150 mM K⁺.

Effect of DAS on K⁺-pNPPase Activity—K⁺-pNPPase activity has been regarded as the dephosphorylation step of H⁺,K⁺-ATPase (32). It is reported that K⁺-occluded conformation of Na⁺,K⁺-ATPase, E₂(K), has the phosphatase activity (33, 34). Fig. 6 shows the effect of DAS on the K⁺-pNPPase activity in gastric vesicles. In this experiment, we used lyophilized vesicles as an enzyme preparation to regulate the intravesicular K⁺ concentration. DAS dose dependently inhibited the K⁺-pNPPase activity. Its IC₅₀ values were 8.9, 30, and 56 μM in the presence of 2, 15, and 150 mM K⁺, respectively. In contrast with the results of the H⁺,K⁺-ATPase activity and its dephosphorylation step, the DAS-induced inhibition of the K⁺-pNPPase activity was antagonized by K⁺. Fig. 7 shows Lineweaver-Burk plots between the K⁺-pNPPase activity and the KCl concentration. The DAS-induced inhibition of the K⁺-pNPPase activity was competitive with respect to K⁺. Because we used gastric vesicles as the enzyme preparation of H⁺,K⁺-ATPase, we cannot rule out the possibility that this preparation includes K⁺-dependent phosphatase other than H⁺,K⁺-ATPase. We measured K⁺-pNPPase activity in the presence of 10 mM NiCl₂ to remove a possible contribution from 5'-nucleotidase activity (35). Even in the presence of NiCl₂, the inhibition by DAS was also competitive with respect to K⁺ (data not shown).

Effects of SA-B and DAS on Change in FITC Fluorescence
Reversible Inhibition of Gastric H⁺,K⁺-ATPase

FIG. 6. Effect of DAS on K⁺-pNPPase activity in lyophilized leaky vesicles. The vesicles were preincubated in a solution containing various concentrations of DAS for 10 min at 37 °C. The K⁺-pNPPase activity was measured in the presence of 2 ( ), 15 ( ), and 150 (Δ) mM KCl. The K⁺-pNPPase activity is expressed as a percentage of control in the absence of DAS. The K⁺-pNPPase activities in the absence of DAS were 10.2 ± 0.1 (for 2 mM K⁺), 25.2 ± 0.7 (for 15 mM K⁺), and 23.7 ± 0.9 μmol/mg/h (for 150 mM K⁺), respectively. Data shown are average ± S.E. for three experiments.

FIG. 7. Lineweaver-Burk plots between the K⁺-pNPPase activity of lyophilized vesicles and the KCl concentration of the medium (0.5–10 mM) in the presence of DAS at 0 ( ), 10 (Δ), and 50 ( ) μM. Typical results in one of three experiments are shown.

Intensity of H⁺,K⁺-ATPase—Here, we studied the effects of SA-B and DAS on the conformation of the ATPase by using a fluorescence probe, FITC. FITC has been used for Na⁺,K⁺-ATPase and Ca²⁺-ATPase, because its fluorescence level changes in response to ligand-induced conformational changes of these ATPases (36-40). FITC covalently binds to the lysine residue (Lys-518 in hog gastric H⁺,K⁺-ATPase) in or near the ATP binding site of the enzyme (24, 29). The addition of K⁺ (1, 5, or 15 mM) to a solution containing the FITC-labeled H⁺,K⁺-ATPase in the presence of 5 mM Mg²⁺ dose dependently quenched the fluorescence intensity (data not shown). Fifteen mM K⁺ decreased the fluorescence by 4–5% of the total fluorescence (Figs. 8 and 9). The decrease reflects the conformational change from the E₁ form to the E₂K form.

FIG. 8. Effects of successive additions of Mg²⁺, K⁺, and SA-B (A) or DAS (B) on the fluorescence intensity of FITC-labeled H⁺,K⁺-ATPase. Twenty micrograms of the FITC-labeled enzyme were suspended in 1 ml of 40 mM Tris-HCl buffer, pH 7.4, at 25 °C. The fluorescence was excited at 495 nm and emitted at 517 nm. Five microliters of indicated ligands were added at the arrows indicated. Final concentrations of SA-B (A) or DAS (B) are shown in the figure. The vertical scale shows 1% change of the fluorescence intensity of the FITC-labeled enzyme, taking the initial fluorescence intensity as 100%.

FIG. 9. Effects of successive additions of SA-B, Mg²⁺, and K⁺ on the fluorescence intensity of FITC-labeled H⁺,K⁺-ATPase.
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After forming the E,K conformation of the H+,K+-ATPase in the presence of 5 mM Mg" and 15 mM K+, we added SA-B (Fig. 8A) or DAS (Fig. 5B) to the vesicle solution. SA-B dose dependently increased the fluorescence level of the enzyme (Fig. 8A), suggesting that binding of SA-B to the E,K conformation forms an inhibitory conformation different from E,K. On the other hand, DAS did not reverse the quenching (Fig. 8B), indicating that DAS does not react with the E,K conformation. When the SA-B-treated enzyme was reacted with 5 mM Mg" and 15 mM K+ successively, the fluorescence change induced by K" was dose dependently inhibited by SA-B (Fig. 9). The final fluorescence level at each SA-B concentration was almost the same as the corresponding fluorescence level observed in Fig. 8A. That is, the final inhibitory products seem to be the same irrespective of whether SA-B is added before or after the formation of E,K.

We name this inhibitory product E,2KI ("I" means SA-B). When the FITC-labeled enzyme preincubated in a solution containing 15 mM K" was reacted with SA-B in the absence of Mg" (data not shown), SA-B did not affect the fluorescence level of E,2K. SA-B did not bind to the E,K form in the absence of Mg". In contrast, successive additions of DAS, Mg"+, and K+ formed the E,K conformation (data not shown), indicating that DAS did not bind to the E,K form of the enzyme.

**DISCUSSION**

Several drugs have been reported to specifically inhibit gastric H+,K+-ATPase. For example, omeprazole (6), E3810 (8), and SCH 28080 (9) are very potent inhibitors of H+,K+-ATPase. In this study, we have shown that a natural compound, SA-B, and its debenzoyl derivative, DAS, have inhibitory effects on the H+,K+-ATPase. We will discuss their inhibitory mechanisms by comparing them with each other and with those of omeprazole and SCH 28080.

Omeprazole was reported to be transformed into an active compound in acidic compartment (10-13). Complete inhibition of the enzyme activity was caused when about 2 mol of omeprazole/mol of phosphoenzyme bound to the H+,K+-ATPase (41-43). Recently Morii et al. (13) reported that omeprazole specifically modified Cys-322 of hog gastric H+,K+-ATPase. The inhibitory mechanism of omeprazole seems not to be specific to a particular reaction step, since omeprazole inhibits the H+,K+-ATPase activity, formation of phosphorylated intermediates, and the K+-dependent p-nitrophenylphosphatase activity (11).

The inhibition of H+,K+-ATPase by SCH 28080 is kinetically competitive with respect to the activating cation, K+ (14, 15). Studies of fluorescence quenching of FITC-labeled H+,K+-ATPase have shown that SCH 28080 binds to the dephospho form of the enzyme and forms an inhibitory conformation of H+,K+-ATPase, E,K-SCH 28080 (17, 18), which prevents rephosphorylation from ATP. SCH 28080 as a true K" site inhibitor would be expected to block reaction steps in the catalytic cycle of H+,K+-ATPase, especially the K"-stimulated dephosphorylation of the E,P form of the enzyme. Wallmark et al. (17) reported that SCH 28080 inhibited the steady state level of phosphoenzyme intermediate in the absence of K", but not observed rate constant of phosphorylation. However, in their rapid reaction experiments 10 μM SCH 28080 did not affect the K"-stimulated dephosphorylation of the enzyme (17). On the other hand, Keeling et al. (16) reported that SCH 28080 binds to both phospho and dephospho forms of H+,K+-ATPase. They proposed that this discrepancy was due to slow binding of SCH 28080 to H+,K+-ATPase (especially at low temperature) (18).

SA-B and DAS also specifically inhibit the H+,K+-ATPase activity. The kinetic pattern of inhibition of SA-B is a mixed type with respect to K" (Fig. 3A); it inhibits the ATPase activity by lowering the affinity of the enzyme to K" and also the Vmax value of the enzyme. The pattern of inhibition of DAS is an uncompetitive type with respect to K" (Fig. 3B). The extent of inhibition increases as the K" concentration increases, indicating that DAS reacts with the K"-bound enzyme (E,K or E,PFK form).

In the experiments to clarify the inhibitory reaction step(s) of SA-B and DAS, we found that they specifically inhibit the K"-dependent dephosphorylation step of H+,K+-ATPase without any effect on the steady state level of phosphoenzyme formation (E1 → E,P plus E2P) (Fig. 5, A and B). The inhibition by SA-B is dose dependently antagonized by K", but that by DAS is enhanced by K".

The fluorescence level of FITC-labeled H+,K+-ATPase decreased when E,2K was formed from E,1 in the presence of K+ and Mg" (24). We studied the effects of SA-B and DAS on this fluorescence change of FITC. SA-B bound to the E,K conformation of H+,K+-ATPase and formed an inhibitory complex E,2KI, the fluorescence level of which was different from that of E,K (Fig. 8A). This inhibitory complex was not formed in the absence of Mg"+. The present results suggest that SA-B forms a stable inhibitory complex E,2KI in the presence of Mg"+. On the other hand, DAS does not bind to the E,K form (Fig. 8B).

Present results show that SA-B can bind to both the phospho and dephospho form of gastric H+,K+-ATPase. The binding of SA-B to the phospho form of the enzyme inhibits H+,K+-ATPase by inhibiting its dephosphorylation step (Fig. 5A), lowering the affinity of the ATPase to K". The binding of SA-B to the dephospho form inhibits H+,K+-ATPase by stabilizing the nonphosphorylated, K" high affinity form (E,KI).

In Na+,K+-ATPase, ouabain can bind to both the phospho and dephospho form of Na+,K+-ATPase (44). The binding of ouabain to the dephospho form of the enzyme results in inhibition of phosphorylation, whereas binding of ouabain to the phospho form blocks the K"-stimulated dephosphorylation by stabilizing the phosphorylated, K" high affinity form (E,P). Oubain does not affect the gastric H+,K+-ATPase. Therefore, SA-B is a counterpart of ouabain for H+,K+-ATPase.

Taking all above results on DAS into consideration (Figs. 3B, 5B, and 8B), we suggest that DAS inhibits H+,K+-ATPase by binding to the E,P form of the enzyme and forming an inhibitory complex of E,PK.I. This is different from the case of ouabain, which binds to the E,P form of Na+,K+-ATPase as stated above.

The K"-pNPPase activity has been regarded as a partial reaction step (dephosphorylation step) of H+,K+-ATPase (32), because the K"-pNPPase and H+,K+-ATPase activities are copurified in the same fraction (45, 49), and because both

![Fig. 10. Proposed inhibition points of SA-B (*) or DAS (**) in the reaction cycles of H+,K+-ATPase. I, SA-B or DAS. Proton was omitted in the reaction cycle for simplicity.](image-url)
enzymes have the same sensitivity to cations and some inhibitors. In fact, SCH 28098, which binds to the K+ high affinity site of H+,K+-ATPase inhibits both enzyme activities. However, Ray and Nandi reported that Na+ inhibits the K+-pNPPase activity without affecting the H+,K+-ATPase activity (50), and reported that spermine inhibits the K+-pNPPase activity by competing with K+ without affecting the H+,K+-ATPase activity (51). From these and other related findings, they proposed the presence of three distinct K+ sites on H+,K+-ATPase; one for the H+ and K+-transporting ATPase reaction and two for the K+-pNPPase reaction (51), and that K+-pNPPase does not represent a partial reaction step of H+,K+-ATPase activity by competing with K+ without affecting the H+,K+-ATPase activity.

The inhibitory mechanisms of SA-B and DAS are summarized in the reaction scheme of H+,K+-ATPase in Fig. 10. SA-B binds to both the phospho and dephospho form (E2PK) and DAS specifically binds only to the phospho form (EPKI).

DAS is a debenzoyl derivative of SA-B. The present study shows that the diterpenoid structure in both compounds is essential for inhibition of the H+,K+-ATPase activity, although the benzoyl group of SA-B partially engages in recognition of the K+ high affinity site.

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