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INHIBITION OF Na^+,K^+-ATPase ACTIVITY BY β-EUDESMOL, A MAJOR COMPONENT OF ATRACTYLODIS LANCEAE RHIZOMA, DUE TO THE INTERACTION WITH ENZYME IN THE $Na \cdot E_1$ STATE

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Abstract—β-Eudesmol, a major component of the crude drug "So-jutsu" (Atractylodis Lanceae Rhizoma), inhibited Na^+,K^+-ATPase activity most strongly among the various kinds of phosphatases examined. It also inhibited Ca^{2+}-ATPase and H^+,K^+-ATPase, but to a lesser extent. Its effect on Mg^{2+}-ATPase was minute. No effects on H^+-ATPase or alkaline and acid phosphatase activities were observed. The effects of β-eudesmol on horse kidney Na^+,K^+-ATPase were studied in detail, and the following results were obtained: (1) β-eudesmol inhibited the Na^+,K^+-ATPase activity with an I_{50} value of 1.6×10^{-4} M. The mode of its inhibition was uncompetitive with respect to ATP; (2) it prevented the stimulation of enzyme activity by Na^+. The inhibition gradually increased in accord with the increase of Na^+ concentration, and it was constant when Na^+ was higher than 6.3 mM; (3) it did not alter the K^+ concentration necessary for half-maximal activation ($K_{0.5}$ for K^+); and (4) it inhibited the enzyme activity with a mode of action different from ouabain. Phosphorylation of enzyme with [γ-^{32}P]ATP was inhibited by β-eudesmol with an I_{50} of 1.4×10^{-4} M. The inhibition was greater in 1 M NaCl than in 0.1 M NaCl. It had no effects on dephosphorylation steps, i.e. none of the non-specific, the ADP-sensitive ($Na \cdot E_1$-P → $Na \cdot E_1$) and the K^+-dependent (E_2-P → $K \cdot E_2$) dephosphorylation processes were affected. These results suggest that β-eudesmol, a relatively specific inhibitor of Na^+,K^+-ATPase, interacts with the enzyme in the $Na \cdot E_1$ form and inhibits the reaction step $Na \cdot E_1$ → $Na \cdot E_1$-P.

"Jutsu" is an important group of Chinese drugs, and includes "So-jutsu" (Atractylodis Lanceae Rhizomas) and "Byaku-jutsu" (Atractylodis Rhizoma). Jutsu is used to normalize kidney, stomach and intestine function. It is thought that the versatile effects of Jutsu are due to the major constituents, sesquiterpenoids. So-jutsu contains 5–9% sesquiterpenoids; the major components are β-eudesmol and hinesol, and elemol and β-selinere are also relatively abundant. It also contains polyacetylene compounds such as atractylodin [1]. β-Eudesmol, hinesol and the extract containing both β-eudesmol and hinesol (E–H mixture) produce the effects of sedation and prolongation of hexobarbital-induced sleeping time [2]. All of these compounds are also known to have anticonvulsive effects on mice exposed to electric shocks [2]. However, they do not show a significant effect on stress-induced ulceric gastritis or excess secretion of gastric juice in rats [2]. Alcohol extracts of So-jutsu cause a prolonged fall of blood sugar after oral administration [3]. So-jutsu is used for the treatment of water retention in the body, but its decoction has no diuretic effect in rats administered water *per os* [4]. A recent report has shown that β-eudesmol is a channel blocker for nicotinic acetylcholine receptors in skeletal muscle cells [5].

Na^+,K^+-ATPase is an intrinsic membrane component responsible for the coupled active transport of Na^+ and K^+ across the plasma membrane. In the kidneys, Na^+ and water are absorbed in tubules, especially Henle's loop and distal tubules, where Na^+,K^+-ATPase is abundantly localized. We have reported that the ethanol extract of So-jutsu inhibits the activity of purified horse kidney Na^+,K^+-ATPase [6]. We have now examined the effects of β-eudesmol on Na^+,K^+-ATPase in order to clarify the mechanism of action of So-jutsu at the molecular level. The characteristics of β-eudesmol as a relatively specific inhibitor of Na^+,K^+-ATPase are presented.

MATERIALS AND METHODS

Horse kidneys were obtained from a slaughterhouse in Tama, Tokyo. [γ-^{32}P]ATP was purchased from New England Nuclear (Boston, MA, U.S.A.). β-Eudesmol (98% pure) was from the Wako Pure Chemical Industries Co. (Osaka, Japan). Other reagents were from the Wako Pure Chemical Industries Co. or the Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Preparation of horse kidney Na^+,K^+-*ATPase.* Crude membrane fraction was prepared from the supernatant obtained from horse kidney outer medulla centrifuged at 4400 *g* for 15 min; the membrane fraction was obtained by centrifuging at 32,000 *g* for 35 min. Purified enzyme (SDS-enzyme)

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was obtained by treatment of the membrane fraction with sodium dodecyl sulfate (SDS‖) according to Jørgensen [7]. The membrane fraction and SDS-enzyme used for the following experiments had specific activities of 0.8 to 1.4 and 28 to 50 μmol P_i released/mg protein/min, respectively. ATPase activity of SDS-enzyme was inhibited almost completely by 1×10^{-4} M ouabain. Protein was determined according to Lowry *et al.* [8] or Bradford [9], using bovine serum albumin as a standard.

Preparation of rat gastric membrane vesicles for the assay of H^+,K^+*-ATPase activity.* Membrane vesicles were prepared from rat (Jcl:SD, Clea, Japan) gastric mucosa essentially according to the report by Maeda *et al.* [10]. The 8000 *g* supernatant of the homogenate was layered over 34% (w/v) sucrose and centrifuged at 100,000 *g* for 50 min. The fraction at the interface between the supernatant and sucrose layer was collected and used for the following experiments. Its specific activity was 0.02 to 0.07 μmol P_i/mg/min.

Assay of ATPase activities. Ouabain-sensitive (Na^+,K^+-) and ouabain-insensitive (Mg^{2+}-) ATPases in horse kidney membrane fraction were determined with and without 0.5 mM ouabain in a total volume of 500 μL for 1–15 min according to a method reported previously [11]. Ouabain was also included at a 0.5 mM concentration in the assay mixture of other ATPase activities. H^+-ATPase [12] was determined by a 30-min incubation of the sonicated membrane fraction, which was prepared from the horse kidney membrane fraction by sonication for 3 min (1 min × 3) with a sonifier (Branson B-12) and used without further purification. Ca^{2+}-ATPase [13] activity was estimated in the horse kidney membrane fraction over a 30-min period. H^+,K^+-ATPase [10] was determined in rat gastric membrane vesicles during a 45-min incubation. After incubation at 37°, the liberated P_i was measured according to Fiske and Subbarow [14]. When the effects of β-eudesmol on the catalytic activity were to be determined, various amounts of β-eudesmol dissolved in 10 μL ethanol-dimethyl sulfoxide (DMSO) (8:2, v/v) were included in the reaction mixture. The concentrations of ethanol and DMSO (1.6 and 0.4%, respectively) in the β-eudesmol solution had no effects on ATPase activities. Each sample containing the solvent at the corresponding concentrations was taken as the control. All assays herein and below were carried out in triplicate in three to nine separate determinations.

Assay of alkaline and acid phosphatase activities. Alkaline and acid phosphatase activities were determined according to Fernley [15] and Hollander [16], respectively, in the horse kidney membrane fraction during a 10-min incubation.

Enzyme phosphorylation and dephosphorylation. Phosphorylation and dephosphorylation of SDS-enzyme were carried out essentially according to a previous report [11]. Briefly, for the phosphorylation experiment, SDS-enzyme (2.5 μg) was incubated at 0° for 10 sec with 20 μL of [γ-^{32}P]ATP (10 μM, 37.0 kBq) in the reaction mixture (180 μL) containing various amounts of β-eudesmol dissolved in 4 μL of ethanol–DMSO (8:2, v/v) [11]. Immediately after the phosphorylation reaction without β-eudesmol, dephosphorylation was initiated by adding ligands such as *trans*-1,2-cyclohexanediamine-*N,N,N',N'*-tetraacetic acid (CDTA), CDTA + ADP or CDTA + KCl in the reaction mixture containing various amounts of β-eudesmol in a total volume of 200 μL, and the phosphoenzyme level was determined after a 5-sec incubation at 0°.

‖ Abbreviations: SDS, sodium dodecy sulfate; CDTA, *trans*-1,2-cyclohexanediamine-*N,N,N',N'*-tetraacetic acid; and DMSO, dimethyl sulfoxide.

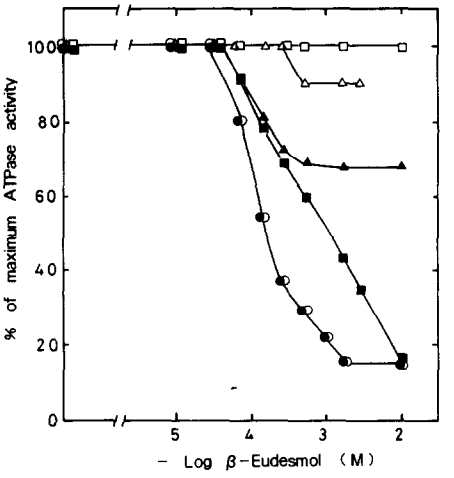


Fig. 1. Effect of β-eudesmol on ATPase activities. The enzyme activities were determined in the presence of various amounts of β-eudesmol. ATPase activities without β-eudesmol were taken as 100%. They were 1.1, 0.2, 0.09, 0.04, 0.04 and 39.6 μmol P_i/mg protein/min for Na^+,K^+-ATPase (●), Mg^{2+}-ATPase (△), Ca^{2+}-ATPase (■) and H^+-ATPase (□) in horse kidney membrane fraction, H^+,K^+-ATPase (▲) in rat gastric membrane vesicles, and Na^+,K^+-ATPase (○) in SDS-enzyme, respectively. SD was < 2.2%.

RESULTS

Effects of β-eudesmol on ATPase and phosphatase activities. The effects of β-eudesmol on Na^+,K^+-ATPase and other ATPase activities were examined (Fig. 1). The activities of Na^+,K^+-ATPase in membrane fraction and SDS-enzyme from horse kidney were inhibited by β-eudesmol. The inhibition curves for Na^+,K^+-ATPase activity with crude and purified enzyme preparations coincided. The inhibition was detected at concentrations as low as 4.7×10^{-5} M and was concentration dependent. β-Eudesmol at 2.7×10^{-3} M caused 85% inhibition of activity; further inhibition was not attained even if the concentration of β-eudesmol was raised. The apparent I_{50} value of β-eudesmol for Na^+,K^+-ATPase was 1.6×10^{-4} M. At this concentration of β-eudesmol, no inhibition of Mg^{2+}-ATPase activity in horse kidney membrane fraction was observed (Fig. 1). The inhibition of Mg^{2+}ATPase activity was only 10% at 5×10^{-4} M and further inhibition was not observed with higher concentrations of β-

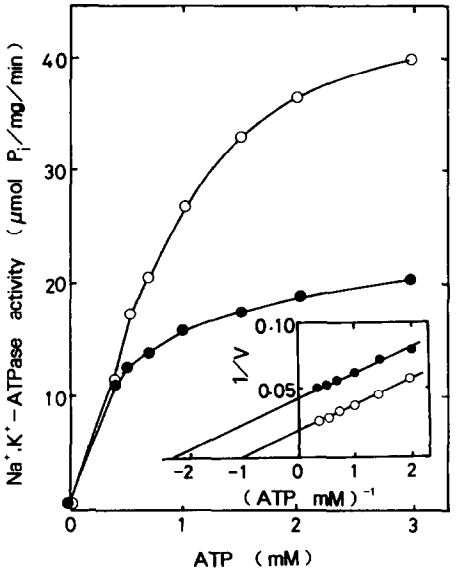


Fig. 2. Effect of ATP concentration on Na^+,K^+-ATPase activity in the presence of β-eudesmol. Na^+,K^+-ATPase activity of SDS-enzyme was determined in various ATP concentrations in the presence (●) or absence (○) of β-eudesmol (1.6×10^{-4} M). A Lineweaver–Burk plot is shown in the inset. SD was < 2.2%.

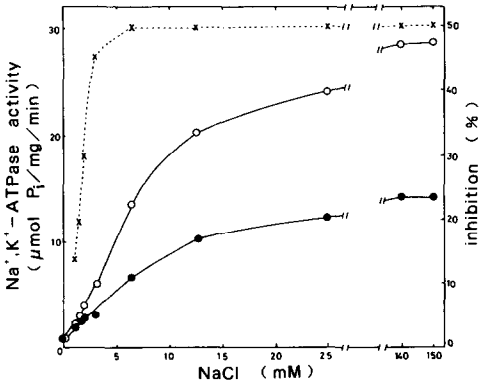


Fig. 3. Effect of NaCl concentration on Na^+,K^+-ATPase activity in the presence of β-eudesmol. The SDS-enzyme was incubated with (●) or without (○) β-eudesmol (1.6×10^{-4} M). The concentration of NaCl was varied from 0.7 to 150 mM. The percent inhibition caused by β-eudesmol at each point is indicated (×). SD was < 2.1%.

eudesmol. The Ca^{2+}-ATPase activity in horse kidney membrane fraction was inhibited by β-eudesmol with an I_{50} value of 1.1×10^{-3} M, which was much higher than that for Na^+,K^+-ATPase. The inhibition of Ca^{2+}-ATPase by 1.6×10^{-4} M β-eudesmol (I_{50} for Na^+,K^+-ATPase) was about 20%. Not more than 30% of H^+,K^+-ATPase activity in rat gastric membrane vesicles was inhibited by β-eudesmol in a range higher than 5.4×10^{-4} M. β-Eudesmol had no effect on the H^+-ATPase activity of sonicated membrane fraction in horse kidney (Fig. 1) or on that in rat heart (data not shown). Furthermore, β-eudesmol had no effect on alkaline or acid phosphatase activities (data not shown) in horse kidney membrane fraction. These results demonstrate that β-eudesmol inhibited Na^+,K^+-ATPase activity most prominently among the phosphatases examined. Purified preparation (SDS-enzyme) was used to investigate in detail the inhibition of Na^+,K^+-ATPase activity by β-eudesmol.

The inhibition of Na^+,K^+-ATPase activity by β-eudesmol was completely reversible, i.e. the activity returned to the control value when β-eudesmol was removed by centrifugation following dilution of the mixture.

The effects of β-eudesmol at 1.6×10^{-4} M (I_{50}) on Na^+,K^+-ATPase activity were examined with various concentrations of ATP (Fig. 2). The Na^+,K^+-ATPase activity was dependent on the concentration of ATP in the assay medium (Fig. 2). The V_{max} obtained from Lineweaver–Burk plots was decreased by the presence of β-eudesmol from 52.1 to 23.8 μmol P_i/mg protein/min (Fig. 2, inset). The two lines of the Lineweaver–Burk plot (with and without β-eudesmol) were parallel. The mode of inhibition appeared to be uncompetitive with respect to ATP.

Na^+,K^+-ATPase activity was stimulated by an increase of Na^+ concentration in the assay medium, and the $K_{0.5}$ for Na^+ of the SDS-enzyme obtained from a Hill plot was 6.3 mM. β-Eudesmol at 1.6×10^{-4} M inhibited the Na^+,K^+-ATPase activity by about 15% in 1.2 mM NaCl, and the inhibition increased gradually depending on the concentration of Na^+ (Fig. 3). When the concentration of Na^+ was higher than 6.3 mM, the extent of inhibition of Na^+,K^+-ATPase activity by 1.6×10^{-4} M β-eudesmol was almost constant (50%).

Na^+,K^+-ATPase activity was stimulated by raising the concentration of K^+ (Fig. 4). The inhibition

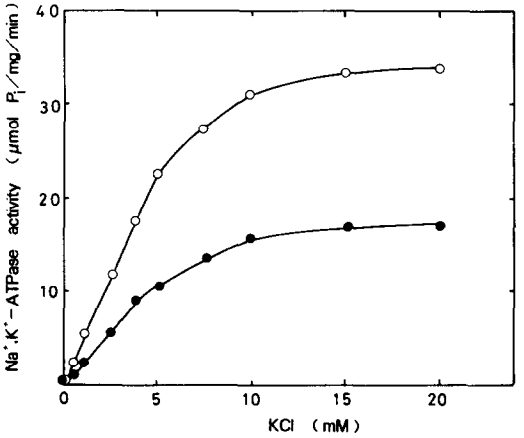


Fig. 4. Effect of KCl concentration on Na^+,K^+-ATPase activity in the presence of β-eudesmol. The reaction was performed essentially in the same way as in Fig. 3, but the concentration of KCl was varied from 0.625 to 20 mM. SD was < 1.9%. Key: (●) with β-eudesmol; and (○) without β-eudesmol.

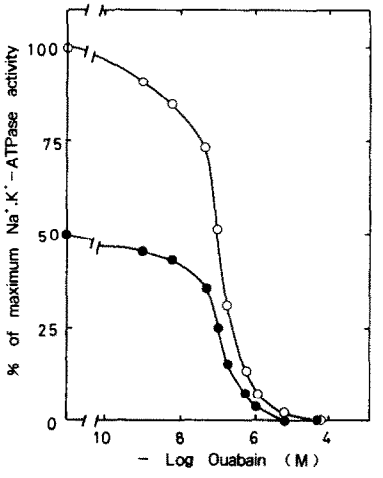


Fig. 5. Effect of ouabain on Na^+,K^+-ATPase activity in the presence of β-eudesmol. The enzyme activity was determined in the presence of various concentrations of ouabain with (●) or without (○) β-eudesmol (1.6×10^{-4} M). The Na^+,K^+-ATPase activity of SDS-enzyme without β-eudesmol and ouabain was taken as 100% (28.0 μmol P_i/mg protein/min). SD was < 1.9%.

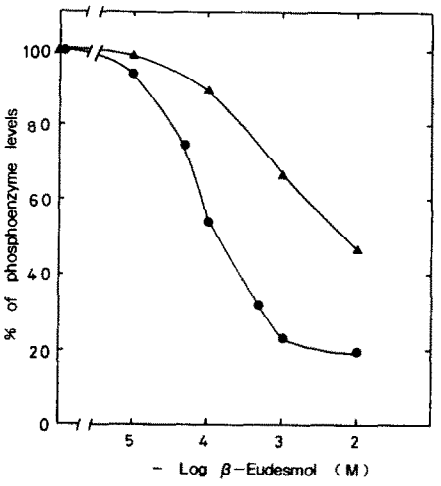


Fig. 6. Effect of β-eudesmol on the phosphorylation of Na^+,K^+-ATPase. The SDS-enzyme (sp. act. 48 μmol P_i/mg protein/min) was phosphorylated with 10 μM [γ-^{32}P] ATP for 10 sec at 0° in a solution of 2 mM $MgCl_2$, 20 mM histidine-HCl buffer, pH 7.5, and 1 M or 0.1 M NaCl in the presence of various concentrations of β-eudesmol. The phosphoenzyme levels without β-eudesmol in 1 M and 0.1 M NaCl were each taken as 100% [(●) 13.4 nmol/mg, and (▲) 12.6 nmol/mg, respectively]. SD was < 5.0%.

caused by β-eudesmol was constant regardless of the K^+ concentration. The $K_{0.5}$ value for K^+ obtained from the Hill plots was 3.8 mM both in the presence and in the absence of β-eudesmol.

The inhibition of Na^+,K^+-ATPase activity by a specific inhibitor, ouabain, was examined in the presence of β-eudesmol (Fig. 5). The shapes of the inhibition curves by ouabain in the presence and absence of β-eudesmol were similar but the activity levels were different. The I_{50} value of ouabain for Na^+,K^+-ATPase activity was approximately 1.4×10^{-7} M in both the presence and the absence of β-eudesmol (Fig. 5).

Effect of β-eudesmol on phosphorylation and dephosphorylation of the enzyme. The effect of β-eudesmol on phosphoenzyme formation, which is dependent on ATP, Na^+ and Mg^{2+}, was examined at steady state (Fig. 6). The decrease of the phosphoenzyme formation was dependent on the concentration of β-eudesmol in the assay medium. Inhibition of phosphorylation by β-eudesmol was greater in 1 M NaCl than in 0.1 M NaCl. The I_{50} value of β-eudesmol for phosphorylation in 1 M NaCl was 1.4×10^{-4} M, which was similar to that for Na^+,K^+-ATPase activity. With the same concentration of β-eudesmol (1.4×10^{-4} M), the inhibition of phosphorylation in 0.1 M NaCl was only 10%.

The phosphoenzyme was chased by CDTA (non-specific dephosphorylation), CDTA + ADP (ADP-sensitive dephosphorylation) or CDTA + KCl (K^+-dependent dephosphorylation) at 0° for 5 sec (Fig. 7). The phosphoenzyme formed in 0.1 M NaCl was decreased to an undetectable level after the chase with 1 mM KCl, but that formed in 1 M NaCl retained the level of 73% after the chase (data not shown). Therefore, for the estimation of K^+-dependent dephosphorylation in 0.1 M NaCl, a low concentration of KCl (0.1 mM), which slows the

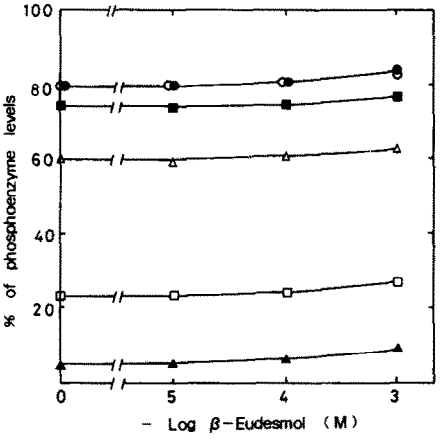
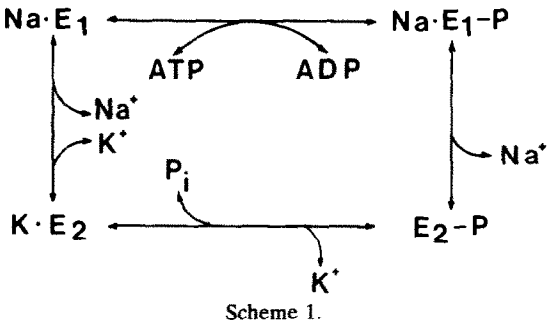


Fig. 7. Effect of β-eudesmol on the dephosphorylation of Na^+,K^+-ATPase. The dephosphorylation reaction of phosphoenzyme, formed without β-eudesmol (Fig. 6), was performed for 5 sec at 0° in the presence of 20 mM CDTA (●, ○), 20 mM CDTA + 5 mM ADP (▲, △) or 20 mM CDTA + 0.1 mM KCl (■, □) with various concentrations of β-eudesmol. Closed and open symbols denote the phosphorylation condition of 1 M and 0.1 M NaCl, respectively. These values are given as percentages relative to each initial phosphoenzyme level (100%) formed without β-eudesmol as described in the legend to Fig. 6. SD was < 4.8%.



Scheme 1.

reaction enough to give an intermediary value after a 5-sec chase, was adopted. When β-eudesmol was not included, phosphoenzyme levels after a 5-sec chase with 20 mM CDTA, 20 mM CDTA + 5 mM ADP and 0.1 mM KCl were 80, 6 and 74% of the initial phosphoenzyme formed in 1 M NaCl, respectively, and the corresponding values of phosphoenzyme formed in 0.1 M NaCl were 80, 60 and 23%, respectively. Phosphoenzyme levels remaining after the chase in the presence of β-eudesmol were not notably altered, even when the concentration of β-eudesmol was raised (Fig. 7). β-Eudesmol had no effect on dephosphorylation processes. However, a very slight increase of the phosphoenzyme levels with 10^{-5} M β-eudesmol was discernible under all the chasing conditions including the CDTA ligand. These phenomena were thought to be insignificant. There is a possibility that the radioactivity was present in the precipitate of phosphoenzyme with β-eudesmol, which was insoluble because the critical micellar concentration was exceeded.

DISCUSSION

β-Eudesmol is a major component of the crude drug "So-jutsu", Atractylodis Lanceae Rhizoma. When the effects of β-eudesmol on a group of phosphatase activities were examined, neither H^+-ATPase nor alkaline and acid phosphatases were affected. Mg^{2+}-ATPase activity was inhibited very slightly by a high concentration of β-eudesmol. Ca^{2+}-ATPase and H^+,K^+-ATPase were inhibited, although to a lesser extent than Na^+,K^+-ATPase. Both Ca^{2+}-ATPase and H^+,K^+-ATPase form covalent phosphoenzyme intermediates (E-P form) during conformational transition as in the case of Na^+,K^+-ATPase, but H^+-ATPase does not [17]. It could be assumed that the inhibition of enzyme activities by β-eudesmol is attributable to the interaction with the enzyme protein at a site relevant to the phosphoenzyme formation. However, the experimental results indicate that it is a relatively specific inhibitor of Na^+,K^+-ATPase with an I_{50} value of 1.6×10^{-4} M.

The mode of inhibition of Na^+,K^+-ATPase by β-eudesmol is uncompetitive with respect to ATP. The stimulation of enzyme activity by Na^+ was inhibited by β-eudesmol. However, the inhibition was less when the concentration of Na^+ was low, although it was constant at high Na^+. The conformation of the enzyme changes from $K \cdot E_2$ to $Na \cdot E_1$ (Scheme 1) [18, 19], when the Na^+ concentration reaches a certain level [20–22]. The results indicate that β-eudesmol interacts with Na^+,K^+-ATPase when it is in the E_1 state in the presence of Na^+. Furthermore, it is speculated that β-eudesmol had no effects on the conversion process from E_1-P to $K \cdot E_2$, since β-eudesmol did not change the $K_{0.5}$ value for K^+, which is a stimulator of this conversion process [20–22].

Ouabain inhibits Na^+,K^+-ATPase activity by binding to the outside of the cell membrane, when the enzyme is in the E_2-P form [18]. β-Eudesmol, which is also an inhibitor of the enzyme activity, exerts its inhibitory effect by a mode of action different from ouabain, since the I_{50} for ouabain was not altered by β-eudesmol.

β-Eudesmol inhibited the phosphorylation reaction and the inhibition was greater in 1 M Na^+ than in 0.1 M Na^+. In contrast, none of the dephosphorylation steps was inhibited. It is known that, when the phosphorylation reaction is carried out in 0.1 M NaCl, the phosphoenzyme $Na \cdot E_1$-P is further converted to E_2-P for the most part [23]. On the other hand, phosphoenzyme formed in 1 M NaCl is mainly $Na \cdot E_1$-P [23]. ADP, produced from ATP by a coupled reaction in the process of $Na \cdot E_1 \rightarrow Na \cdot E_1$-P conversion, stimulates the reverse reaction, leading to the formation of $Na \cdot E_1$ in 1 M NaCl [20]. The enzyme is likely to stay in equilibrium between the two forms, $Na \cdot E_1$ and $Na \cdot E_1$-P, in 1 M NaCl in the presence of ATP [19]. It is possible that β-eudesmol inhibited the phosphorylation reaction ($Na \cdot E_1 \rightarrow Na \cdot E_1$-P). Furthermore, the reaction of β-eudesmol with the $Na \cdot E_1$ form was more likely, since it had no effect on the reverse reaction (the ADP-sensitive dephosphorylation step $Na \cdot E_1$-P $\rightarrow$ $Na \cdot E_1$). Overall, the data on Na^+,K^+-ATPase activity, and on phosphorylation and dephosphorylation experiments support the idea that β-eudesmol interacts with enzyme in the $Na \cdot E_1$ form and inhibits the reaction of the $Na \cdot E_1 \rightarrow Na \cdot E_1$-P conversion. Another methodological approach such as a study of the binding of β-eudesmol to the enzyme would confirm their interaction.

Chinese drugs are usually administered in a mixed form, and the effects of drugs as a medicine are complex, i.e. sometimes additive or cooperative effects of single components are found. Furthermore, concomitant ingredients of crude drugs such as carbohydrate, lipid, protein and others would enhance the cooperativity. However, the diuretic effect of Atractylodis Lanceae Rhizoma may at least be explained by the notion that β-eudesmol inhibits the reabsorption of water due to the inhibition of Na^+,K^+-ATPase activity.

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