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Inhibition of H^+,K^+-ATPase by Hinesol, a Major Component of So-jutsu, by Interaction with Enzyme in the E_1 State

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ABSTRACT. Hinesol, a major component of the crude drug "So-jutsu" (Atractylodis Lanceae Rhizoma), strongly inhibited H^+,K^+-ATPase activity with a IC_{50} value of 5.8×10^{-5} M. It also inhibited Na^+,K^+-ATPase, Mg^{2+}-ATPase, Ca^{2+}-ATPase, and H^+-ATPase activities, although the inhibition rate was lower. No effects on alkaline or acid phosphatase activities were observed. The mechanism by which hinesol inhibited H^+,K^+-ATPase activity was studied in detail. The inhibition was uncompetitive with respect to ATP, and it increased as the Mg^{2+} concentration was raised, whereas it was not affected by the K^+ concentration. The activity of K^+-dependent *p*-nitrophenyl phosphatase (K^+-pNPPase), a partial reaction of H^+,K^+-ATPase, was inhibited by hinesol noncompetitively with respect to pNPP (IC_{50} value of 1.6×10^{-4} M), and competitively with respect to K^+, whereas it was not affected by the Mg^{2+} concentration. These results suggest that hinesol is a relatively specific inhibitor of H^+,K^+-ATPase. It appears that hinesol reacts with enzyme in the E_1 state in the presence of ATP and Mg^{2+} and forms the complex hinesol-$H^+ \cdot E_1$-ATP or hinesol $\cdot E_1$-P, blocking the conformational change to the E_2 state. Furthermore, hinesol enhanced the inhibitory effect of omeprazole on H^+,K^+-ATPase, and the inhibitory site of hinesol was different from that of omeprazole. The effect of So-jutsu as an anti-gastric ulcer agent may be ascribed to the inhibitory effect of hinesol on H^+,K^+-ATPase activity. BIOCHEM PHARMACOL **59**;7:881–886, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. H^+,K^+-ATPase; hinesol; inhibitor; omeprazole; So-jutsu; Atractylodis Lanceae Rhizoma; K^+-pNPPase

"So-jutsu" (Atractylodis Lanceae Rhizoma), an important group of Chinese traditional drugs [1], is used to normalize kidney, stomach, and intestinal functions. It contains 5–9% (w/w) sesquiterpenoids such as hinesol (Fig. 1), β-eudesmol, elemol, and β-selinene. It also contains polyacetylene compounds such as atractylodin. A number of the effects of So-jutsu are thought to be attributable to the sesquiterpenoids [2, 3]. A methanol extract of So-jutsu exhibits a stomach antiulcer action, which is attributed to hinesol and β-eudesmol [4–7]. Hinesol inhibits the secretion of gastric juice in rats [7]. It is also used to enhance the circulation in the brain and metabolism [8] and to inhibit the aggregation of platelets [9]. H^+,K^+-ATPase, the proton pump responsible for acid secretion in the stomach, is located in gastric membrane vesicles and catalyzes the electroneutral exchange of intracellular H^+ and extracellular K^+ coupled with the hydrolysis of cytoplasmic ATP [10–12]. Therefore, H^+,K^+-ATPase is a pharmacological target for proton pump inhibitors such as omeprazole [13], SCH28080 [14], and E3810 [15]. We found that H^+,K^+-ATPase activity is inhibited by an ethanol extract of So-jutsu [16], but only weakly inhibited by β-eudesmol [17]. We have now examined the effects of hinesol on H^+,K^+-ATPase. The characteristics of hinesol as an inhibitor of H^+,K^+-ATPase activity are presented.

MATERIALS AND METHODS

Hinesol (98% pure) was purchased from the Kurita Industries Co. Omeprazole was a gift from Yoshitomi Pharm. Industries, LTD.

Preparation of Porcine Gastric Membrane Vesicles

Porcine gastric membrane vesicles containing H^+,K^+-ATPase were prepared as described [18, 19]. Briefly, mucosal scrapings from porcine stomachs obtained at a slaughterhouse were homogenized in 0.25 M sucrose buffered with 5 mM Tris–HCl (pH 6.8, buffer A). The homogenate was centrifuged at 8000 *g* for 20 min. The supernatant was layered over 34% (w/v) sucrose and centrifuged at 100,000 *g* for 50 min. The interface fraction was collected and centrifuged at 100,000 *g* for 15 min. The precipitate was resuspended in buffer A and was layered over a step

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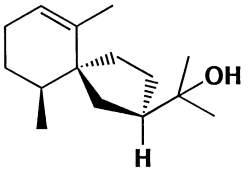

FIG. 1. Structure of hinesol.

gradient consisting of 10% sucrose (w/v), 7% Ficoll (Pharmacia) + 10% sucrose (w/v), 45% sucrose (w/v), and 70% sucrose (w/v) in buffer A, and centrifuged at 27,000 *g* for 16 hr. The interface between the 7% Ficoll + 10% sucrose and 45% sucrose layers was collected and centrifuged at 100,000 *g* for 15 min. The resulting pellet was suspended in buffer A and stored at −80° until needed for experiments. The specific activity of H^+,K^+-ATPase was 18–60 μmol P_i/mg protein/hr. All steps of the preparation were carried out at 0–4°.

Assays of ATPase and Phosphatase Activities

The activity of H^+,K^+-ATPase was determined as described [16, 20]. Various concentrations of hinesol and/or omeprazole dissolved in DMSO (1 μL) were added to the reaction mixture (0.1 mL) containing porcine gastric membrane vesicles (50 μg protein/mL), 1 mM ATP, 2 mM $MgCl_2$, and 20 mM Tris–HCl buffer (pH 6.8) with or without 10 mM KCl, and the whole mixture was incubated at 37° for 15 min. K^+-pNPPase* activity was determined in a mixture (0.1 mL) containing porcine gastric membrane vesicles (50 μg/mL), 5 mM *p*-nitrophenyl phosphate, 5 mM $MgCl_2$, and 20 mM Tris–HCl buffer (pH 6.8) with or without 10 mM KCl and 1 μL of a solution of hinesol and/or omeprazole at 37° for 10 min [20].

The activities of Na^+,K^+-ATPase [21], Mg^{2+}-ATPase [17], H^+-ATPase [17], Ca^{2+}-ATPase [17], and alkaline and acid phosphatase [20] were also determined. The 2% concentrations of DMSO in the reaction mixture had no effect on enzyme activities.

RESULTS

Effects of Hinesol on ATPase and Phosphatase Activities

The inhibition of H^+,K^+-ATPase activity by hinesol was detected at a concentration as low as 1.0×10^{-5} M and was concentration dependent (Fig. 2). Hinesol at 4.0×10^{-4} M completely inhibited H^+,K^+-ATPase activity. The IC_{50} value of hinesol for H^+,K^+-ATPase was 5.8×10^{-5} M. Hinesol also inhibited Na^+,K^+-ATPase, Ca^{2+}-ATPase, Mg^{2+}-ATPase, and H^+-ATPase activity, and these apparent IC_{50} values were 1.7×10^{-4}, 2.2×10^{-4}, 4.0×10^{-4}, and 5.0×10^{-4} M, respectively (Fig. 2). Furthermore, hinesol had no effect on alkaline or acid phosphatase activities. These results demonstrated that hinesol inhibited H^+,K^+-ATPase most prominently among the phosphatases examined.

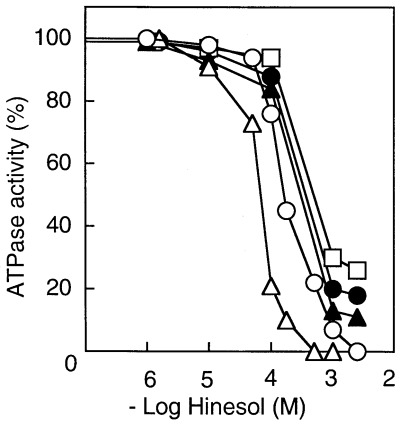

FIG. 2. Effect of hinesol on ATPase activities. The enzyme activities were determined in the presence of various amounts of hinesol. ATPase activities in the absence of hinesol were taken as 100%. They were 18.6, 2420, 13.8, 2.4, and 6.6 mol P_i/mg protein/hr for (△) H^+, K^+-ATPase activity in porcine gastric membrane vesicles, (○) Na^+, K^+-ATPase in purified horse kidney enzyme, and (●) Mg^{2+}-ATPase, (□) H^+-ATPase, and (▲) Ca^{2+}-ATPase in horse kidney crude membrane fractions, respectively. The SD was less than 2.4% (N = 8).

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

Effects of Hinesol on H^+,K^+-ATPase Activity in the Presence of Various Ligands

The effects of hinesol at 4.0×10^{-5} and 5.8×10^{-5} M on H^+,K^+-ATPase activity were examined with various concentrations of ATP (Fig. 3). The V_{max} obtained from double-reciprocal plots was decreased from 167 to 100 and 45 μmol P_i/mg protein/hr in the presence of 4.0×10^{-5} and 5.8×10^{-5} M hinesol, respectively (Fig. 3 inset). The three lines of the double-reciprocal plots were parallel. The mode of inhibition was uncompetitive with respect to ATP.

H^+,K^+-ATPase activity was assayed at various concentrations of Mg^{2+} with or without 5.8×10^{-5} M hinesol (Fig. 4a). Hinesol had no effect on H^+,K^+-ATPase activity at low concentrations of Mg^{2+} ($<1.0 \times 10^{-5}$ M). However, raising the Mg^{2+} concentration gradually increased the extent of inhibition, with a maximum at 1.0×10^{-3} M Mg^{2+}. When the concentration of Mg^{2+} was 1.0×10^{-4}, 2.0×10^{-3}, 2.0×10^{-2}, and 1.0×10^{-1} M, the extent of inhibition was 23, 50, 71, and 100%, respectively.

H^+,K^+-ATPase activity was assayed in the presence of various concentrations of K^+ with or without 5.8×10^{-5} M hinesol (Fig. 4b). Raising the concentration of K^+ stimulated H^+,K^+-ATPase activity, and the extent of inhibition caused by hinesol was constant regardless of the K^+ concentration. The concentration of K^+ giving half-maximal velocity was 2.3 mM in both the absence and the presence of hinesol.

* *Abbreviation*: K^+-pNPPase, K^+-dependent *p*-nitrophenyl phosphatase.

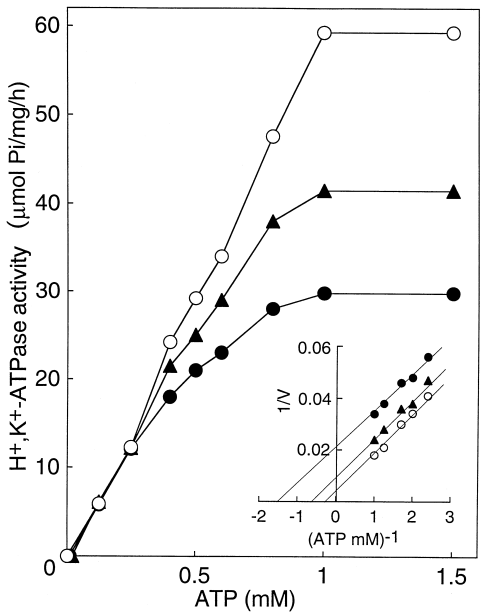

FIG. 3. Effect of ATP concentration on H^+,K^+-ATPase activity in the presence of hinesol. H^+, K^+-ATPase activity was determined with various ATP concentrations in the presence (▲, 4.0×10^{-5} M; ●, 5.8×10^{-5} M) or absence (○) of hinesol. A double-reciprocal plot is shown in the inset. The SD was less than 2.3% (N = 5).

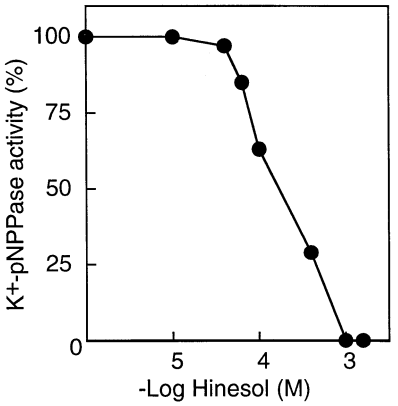

FIG. 5. Inhibition of K^+-pNPPase activity by hinesol. The enzyme activity was determined in the presence of various amounts of hinesol. The activity in the absence of hinesol was taken as 100% (20.4 μmol/mg protein/hr). The SD was less than 1.9% (N = 7).

Inhibition of K^+-pNPPase Activity by Hinesol

K^+-pNPPase activity was inhibited by hinesol in a concentration-dependent manner, and the inhibition was almost complete at 1.0×10^{-3} M (Fig. 5). The IC_{50} value of hinesol for K^+-pNPPase activity was 1.6×10^{-4} M from the Hill plot.

Effects of Hinesol on K^+-pNPPase Activity in the Presence of Various Ligands

K^+-pNPPase activity was determined at various concentrations of pNPP, Mg^{2+}, or K^+ with or without 1.6×10^{-4} M hinesol, and its effects were analyzed by double-reciprocal plots. In the study with various concentrations of substrate (Fig. 6a) and Mg^{2+} (Fig. 6b), the V_{max} values were both decreased by hinesol, and the K_m for pNPP and the $K_{0.5}$ value for Mg^{2+} were the same regardless of whether hinesol was present or not. These results indicated that the mode of inhibition appeared to be noncompetitive with respect to pNPP and Mg^{2+}. The effect of K^+ concentration on K^+-pNPPase activity was expressed in the same manner. The $K_{0.5}$ value was increased from 12.5 to 31.3 mM in the presence of hinesol, whereas the V_{max} value was not altered (Fig. 6c). The hinesol-induced inhibition of the K^+-pNPPase activity was competitive with respect to K^+.

Effects of Hinesol on the Inhibition of H^+,K^+-ATPase or K^+-pNPPase Activities by Omeprazole

The inhibition of H^+,K^+-ATPase or K^+-pNPPase activity by hinesol was examined in the presence of omeprazole, a specific inhibitor of H^+,K^+-ATPase. The K_i value of

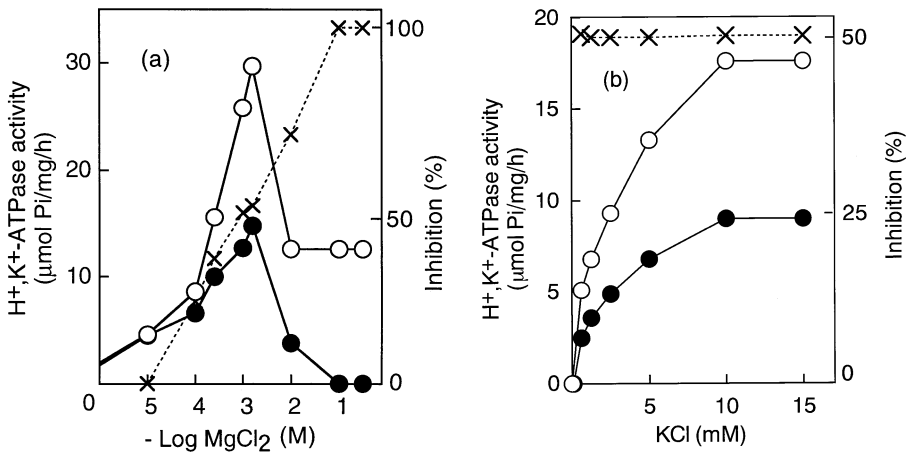

FIG. 4. Effects of Mg^{2+} and K^+ concentrations on the inhibition of H^+, K^+-ATPase activity by hinesol. The enzyme was incubated with (●) or without (○) 5.8×10^{-5} M hinesol. (a) The concentration of $MgCl_2$ was varied. (b) The concentration of KCl was varied. The percent inhibition caused by hinesol at each point is indicated (x). The SD was less than 3.1% (N = 6).

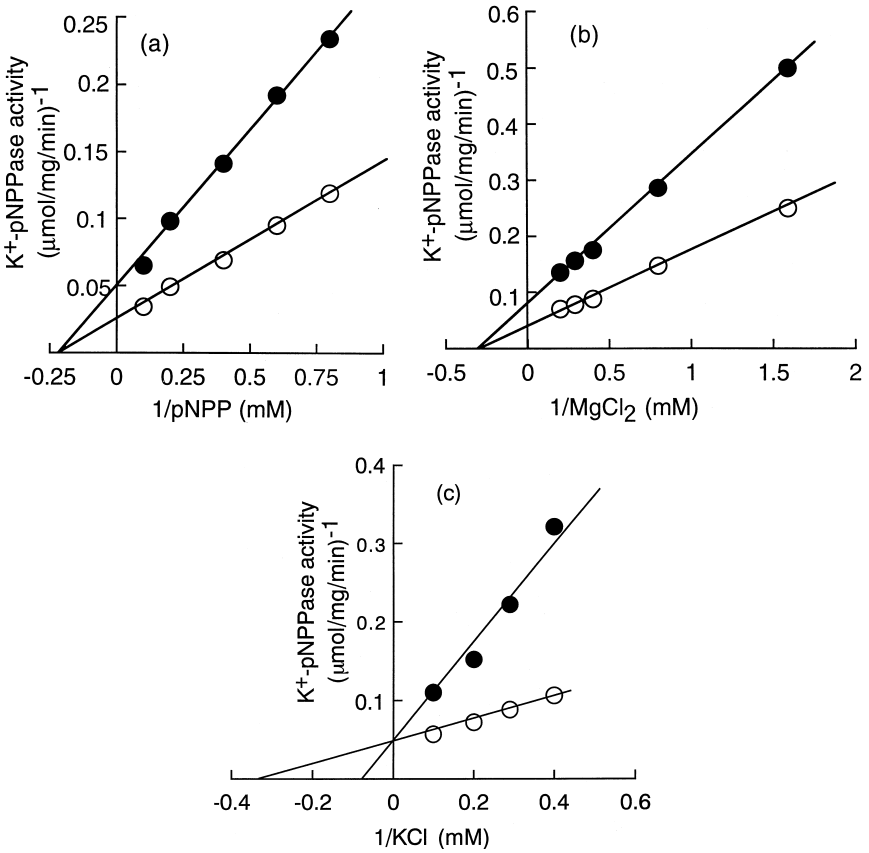

FIG. 6. Double-reciprocal plots of K^+-pNPPase activity and the concentration of pNPP, Mg^{2+}, or K^+ in the presence and absence of hinesol. K^+-pNPPase was incubated with (●) or without (○) 1.6×10^{-4} M hinesol. (a) The concentration of pNPP was varied. (b) The concentration of $MgCl_2$ was varied. (c) The concentration of KCl was varied. The SD was less than 2.3% (N = 6).

hinesol for H^+,K^+-ATPase in the presence of omeprazole was decreased from 5.8×10^{-5} to 1.5×10^{-5} M (Fig. 7a), as determined from Hill plots. Those for K^+-pNPPase were both 1.6×10^{-4} M (Fig. 7b), and this indicated that the presence of omeprazole did not alter the effect of hinesol on K^+-pNPPase.

DISCUSSION

Hinesol, a major component of So-jutsu, was found to inhibit H^+,K^+-ATPase (IC_{50} value of $5.8 \times 10^{\times 5}$ M) most prominently among the phosphatases examined (Na^+,K^+-ATPase, Mg^{2+}-ATPase, Ca^{2+}-ATPase, H^+-ATPase, and alkaline and acid phosphatase). H^+,K^+-ATPase has a catalytic center including a phosphorylation site and an ATP binding site [12]. In the reaction of H^+,K^+-ATPase, the conformation of the enzyme changes from $K^+ \cdot E_2$ to $H^+ \cdot E_1$ when K^+ is released, and $H^+ \cdot E_1$ is phosphorylated by ATP to form the phosphoenzyme $H^+ \cdot E_1$-P in the presence of a certain level of Mg^{2+} concentration. $H^+ \cdot E_1$-P converts to E_2-P, which dephosphorylates to $K^+ \cdot E_2$ when the K^+ concentration reaches a certain level in the presence of Mg^{2+} (Fig. 8) [10, 11, 22]. The study of the effect of hinesol on H^+,K^+-ATPase revealed that hinesol inhibited both H^+,K^+-ATPase and K^+-pNPPase activity. As the concentration of Mg^{2+} was raised, the extent of inhibition of H^+,K^+-ATPase by hinesol was increased, but it was not affected by K^+. With respect to ATP, hinesol uncompetitively inhibited H^+,K^+-ATPase. K^+-pNPPase, which reflects the E_2 state of H^+,K^+-ATPase, was inhibited by hinesol noncompetitively with respect to pNPP and competitively with respect to K^+. Therefore, hinesol may interact with the enzyme in the E_1 state rather than the E_2 state. Inhibition of K^+-pNPPase activity might be caused by hindrance of the conformational change from the E_1 to the E_2 state by hinesol. These results suggest that hinesol reacts with enzyme in the E_1 state and forms the hinesol-$H^+ \cdot E_1$-ATP $\cdot$ Mg^{2+} or hinesol $\cdot$ E_1-P complex, which blocks the transition to the E_2 form.

Omeprazole, which is used for the treatment of gastric ulcers, is known as an irreversible inhibitor of H^+,K^+-ATPase activity, and it inhibits the secretion of HCl in the stomach [23, 24]. It is reported that omeprazole inhibits H^+,K^+-ATPase activity, formation of phosphorylated intermediates, and K^+-pNPPase activity [25]. In another report, omeprazole was found to inhibit the conformational change from E_2 to E_1, and omeprazole-bound enzyme was in the E_2 form, as observed by measuring the fluorescence of fluorescein isothiocyanate-labeled enzyme [26]. Studies on H^+,K^+-ATPase and K^+-pNPPase activities in the presence of hinesol and omeprazole suggested that the inhibitory site of hinesol was different from that of omeprazole, and that

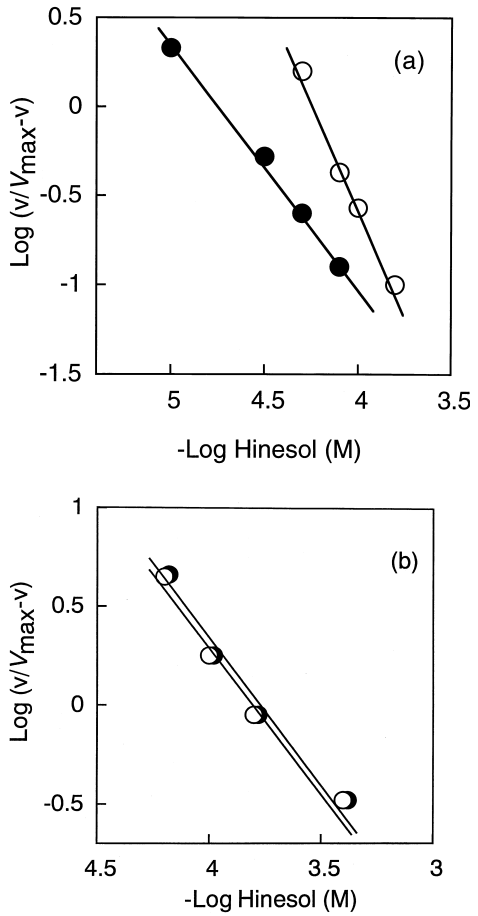

FIG. 7. Effects of hinesol on H^+, K^+-ATPase and K^+-pNPPase activity in the presence of omeprazole. The enzyme activity was determined in the presence of various concentrations of hinesol with (●) or without (○) omeprazole. (a) H^+,K^+-ATPase activity. The concentration of omeprazole was 1.7×10^{-5} M. (b) K^+-pNPPase activity. The concentration of omeprazole was 7.0×10^{-5} M. The H^+, K^+-ATPase and K^+-pNPPase activities in the absence of hinesol and omeprazole were taken as 100% (20.8 and 22.0 μmol/mg protein/hr, respectively). The SD was less than 1.8% (N = 5).

hinesol enhances the inhibitory effect of omeprazole on H^+,K^+-ATPase. It is postulated that the suppression of gastric HCl secretion by hinesol [7] is caused by the inhibition of H^+,K^+-ATPase in gastric membrane vesicles. Hinesol is at least one major component of So-jutsu that exerts an effect on gastric ulcers. Since hinesol and omeprazole have different modes of action, the administration of both compounds at the same time might produce an enhanced anti-ulcer effect.

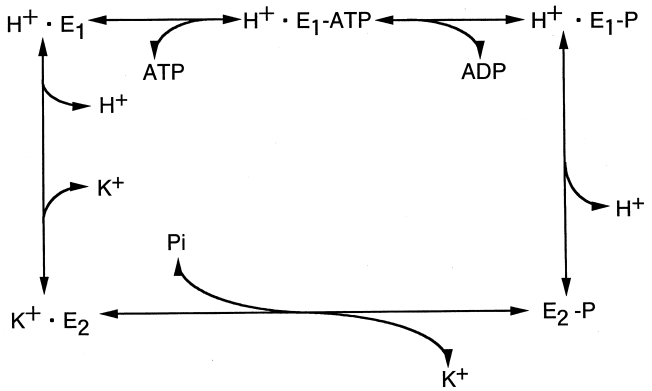

FIG. 8. Reaction mechanism of H^+, K^+-ATPase. The scheme by Faller *et al.* [22] was modified slightly.

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