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Inhibition of Na^+,K^+-ATPase by the extract of *Stephania cephararantha* HAYATA and bisbenzylisoquinoline alkaloid cycleanine, a major constituent

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Abstract

The *Stephania cephararantha* HAYATA extract, and its constituent bisbenzylisoquinoline alkaloids, such as cycleanine, cepharanthine, isotetrandrine, berbamine, homoaromoline, and cepharanoline were studied for effects on Na^+,K^+-ATPase activity. The *S. cephararantha* HAYATA extract inhibited Na^+,K^+-ATPase activity with an apparent IC_{50} value of 540 μg/mL. Cycleanine markedly inhibited Na^+,K^+-ATPase activity with an IC_{50} value of 6.2×10^{-4} M. It slightly inhibited Mg^{2+}-ATPase, H^+-ATPase, and Ca^{2+}-ATPase. No effects on alkaline and acid phosphatase activities were observed. The inhibition by isotetrandrine, homoaromoline, cepharanthine, and berbamine was less marked, and cepharanoline showed no effect. Five synthetic analogues of cepharanthine slightly inhibited the activity. The mechanism of inhibition by cycleanine on Na^+,K^+-ATPase activity was examined in detail, and the following results were obtained in the overall reaction: (1) the mode of inhibition was noncompetitive with respect to ATP; (2) the degree of inhibition was decreased with a decrease of K^+ concentration; (3) it was not affected by Na^+ concentration; (4) the inhibition mechanism was different from that of ouabain. The activity of K^+-dependent *p*-nitrophenyl phosphatase, a partial reaction of Na^+,K^+-ATPase, did not appear to have been inhibited by cycleanine in the reaction mixture containing 15 mM K^+ (optimum condition). However, cycleanine increased the $K_{0.5}$ value for K^+ and reduced the K_i values for Na^+ and ATP, in K^+-dependent *p*-nitrophenyl phosphatase. Cycleanine might interact with the enzyme in $Na{\cdot}E_1$-P form and prevents the reaction step from $Na{\cdot}E_1$-P to E_2-P.

Keywords: Na^+,K^+-ATPase; Cycleanine; Bisbenzylisoquinoline alkaloid; Cepharanthine; *Stephania cephararantha* HAYATA; K^+-pNPPase

1. Introduction

Bisbenzylisoquinoline alkaloids are widely used as medications. For example, Cepharanthin® drug extracted from *Stephania cephararantha* HAYATA (ScH) has been clinically applied in the treatment of leukopenia [1] and alopecia areata [2]. The ScH extract contains bisbenzylisoquinoline alkaloids, such as cepharanthine, isotetrandrine, cycleanine, and berbamine, each of which occupies approximately 1/4, 1/3, 1/10, and 1/10 of the total alkaloid, respectively. These bisbenzylisoquinoline alkaloids exhibit diverse biological activities. Cycleanine, tetrandrine, and berbamine suppressed hepatic injury and production of tumor necrosis factor in BCG/LPS-treated mice [3,4]. Cepharanthine, cycleanine, and isotetrandrine exhibited suppressive effects on *in vitro* histamine release by rat basophilic leukemia cells (RBL-2H3) [5] and nitric oxide production by LPS-stimulated peritoneal macrophages derived from BCG-treated mice [6]. Furthermore, some of these bisbenzylisoquinoline alkaloids stimulated the proliferate activities on cultured hair cells from murine skin [7]. Cepharanthine was highly potent inhibitor of HIV-replication in chronically infected monocytic cell line [8] and suppressed the production of inflammatory cytokines and neural cell death [9]. Cycleanine had Ca-antagonist property [10]. These alkaloids have

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Abbreviations: ScH, *Stephania cephararantha* HAYATA; K^+-pNPPase, K^+-dependent *p*-nitrophenyl phosphatase; BCG, Bacillus Calmette-Guerin; LPS, lipopolysaccharide; PGG, 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose.

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been reported to have some effects on cell membranes. Cepharanthine suppressed the release of K^+ from various cells, such as rabbit blood cells, rat mast cells, Ehrlich ascites tumor cells, and AH-130 ascites hepatoma cells, and from liposome caused by treatment with phospholipase A, bilirubin, or lysolecthin [11–15]. Cepharanthine, isotetrandrine, and berbamine showed shape-transforming activity toward invagination and altered resistance against hypotonic hemolysis in erythrocytes [16]. However, the mechanisms of the effects on the cell membrane have not been fully understood. Na^+,K^+-ATPase is an intrinsic membrane component responsible for the coupled active transport of Na^+ and K^+ across the plasma membrane. We have shown that the extracts and constituents of several folk medicines inhibit the activity of Na^+,K^+-ATPase [17–21]. In this article, we report on the inhibitory potential of the ScH extract, its constituent alkaloids, and related compounds on Na^+,K^+-ATPase activity.

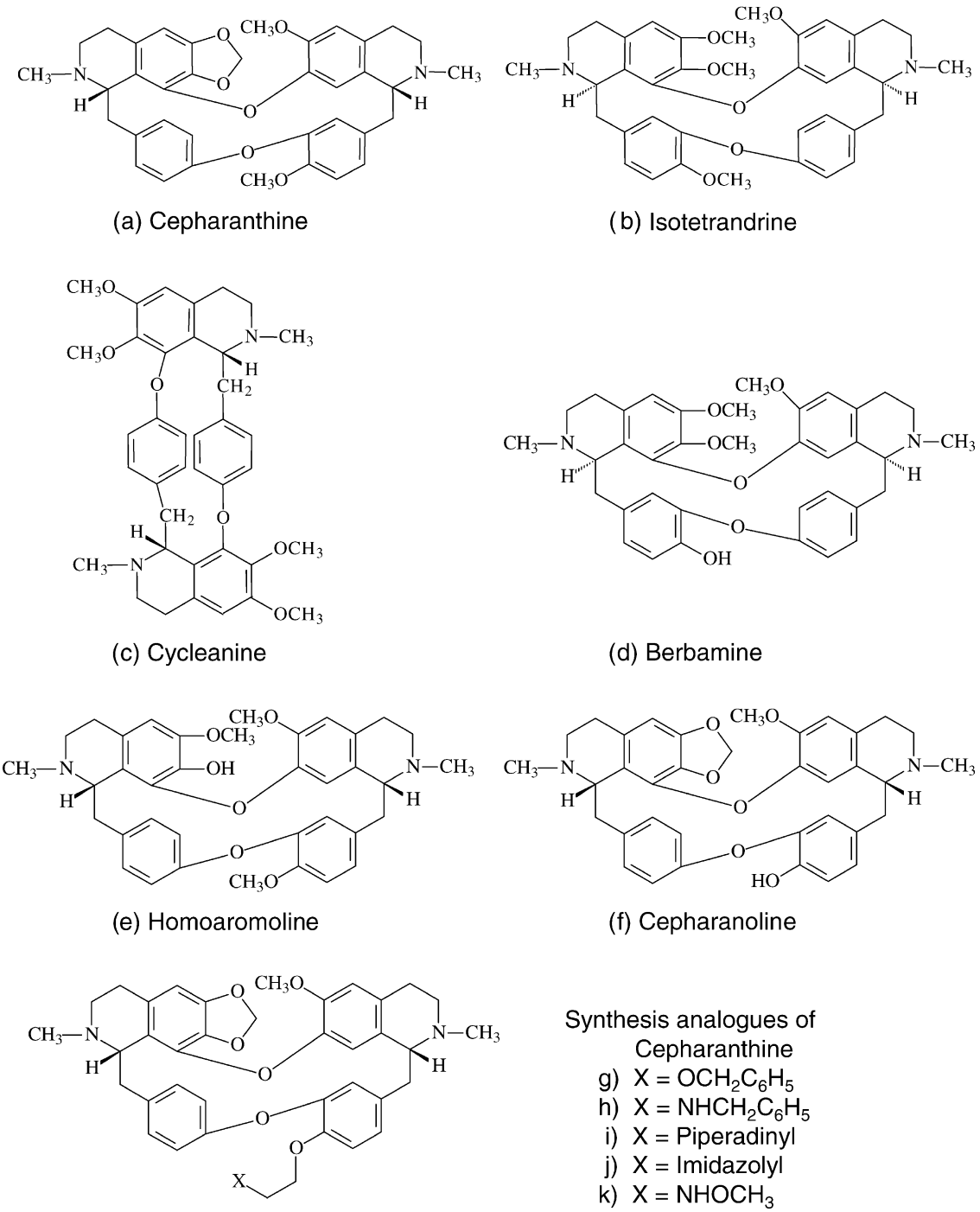

Fig. 1. The chemical structures of bisbenzylisoquinoline alkaloids.

2. Materials and methods

The ScH extract, cepharanthine, isotetrandrine, cycleanine, berbamine, homoaromoline, cepharanoline, and the synthetic analogues of cepharanthine were donated by Kaken Shoyoku Co Ltd. (Fig. 1).

Na^+,K^+-ATPase was prepared from the crude membrane fraction of horse kidney outer medulla. Purified enzyme was obtained by treating the membrane fraction with SDS according to previous reports [17,22,23]. The purified enzyme (SDS-enzyme) used for the following experiments had a specific activity of 32–52 μmol/mg protein/min. The ATPase activity of SDS-enzyme was inhibited almost completely by 1×10^{-5} M ouabain. Other reagents were purchased from the Wako Pure Chemicals Ind. Co or the Sigma Chemical Co.

Na^+,K^+-ATPase activity was determined according to a previous report [18]. Various amounts of extract or alkaloids dissolved in 1 μL DMSO was added to the reaction mixture (0.1 mL) containing purified enzyme (1 μg protein/mL), 3 mM ATP, 140 mM NaCl, 14 mM KCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM EGTA, and 50 mM imidazole–HCl buffer (pH 7.2). These reaction mixtures were incubated at 37° for 15 min.

K^+-dependent *p*-nitrophenyl phosphatase (K^+-pNPPase) activity was determined at 37° for 10 min in a reaction mixture (0.1 mL) containing purified enzyme (1 μg protein/mL), 20 mM pNPP, 15 mM KCl, 10 mM $MgCl_2$, 0.1 M Tris–HCl buffer (pH 7.7), and 1 μL of the alkaloid solution [19].

The activities of Mg^{2+}-ATPase, H^+-ATPase, Ca^{2+}-ATPase, and alkaline and acid phosphatases were also determined [18,20].

The concentration of DMSO (1%) in the reaction mixture had no effect on the enzyme activities.

3. Results

3.1. Effects of the ScH extract, alkaloid constituents, and the synthetic analogues of cepharanthine on ATPases and phosphatase activity

Na^+,K^+-ATPase activity was inhibited by the ScH extract in a concentration-dependent manner, and maximal inhibition (29%) was attained with 57 μg/mL. No further increase in inhibition was observed at higher concentrations. The apparent IC_{50} value of the extract for Na^+,K^+-ATPase activity is 540 μg/mL (Fig. 2).

Some alkaloid components of ScH were examined. The inhibition of Na^+,K^+-ATPase activity by cycleanine was relatively strong among these alkaloids. The inhibition was concentration dependent, but it was not complete at 10^{-3} M (about 68% inhibition). The apparent IC_{50} value of cycleanine for enzyme activity is 6.2×10^{-4} M (Fig. 3). Cepharanoline, isotetrandrine, homoaromoline, cepharanthine, and

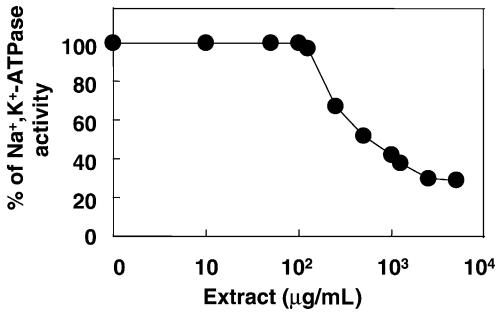

Fig. 2. Effect of *Stephania cephararantha* Hayata extract on Na^+,K^+-ATPase activity. The Na^+,K^+-ATPase activity was determined in the presence of various amounts of ScH extract. Na^+,K^+-ATPase activity without the extract was taken as 100% (42.5 μmol P_i/mg protein/min). The SD was less than 2.4% (N = 3).

berbamine at 10^{-3} M caused only 0, 5, 11, 14, and 25% inhibition, respectively. The synthetic analogues of cepharanthine (Fig. 1g–k) were examined, but these chemicals at 10^{-3} M caused inhibition less than 12%, and the inhibitory effects were not remarkable (data not shown).

Cycleanine slightly inhibited Mg^{2+}-ATPase, H^+-ATPase, and Ca^{2+}-ATPase, and the extents of inhibition at 10^{-3} M were 5, 3, and 6%, respectively (data not shown). No effects on alkaline and acid phosphatase activities were observed.

The inhibition of Na^+,K^+-ATPase activity by cycleanine was completely reversible, i.e. the activity returned to control values when cycleanine was removed by centrifugation following dilution of reaction mixture (data not shown).

3.2. Effects of cycleanine on Na^+,K^+-ATPase activity in the presence of various ligands

The effects of cycleanine at 2.0×10^{-4} M on Na^+,K^+-ATPase activity were examined at various concentrations of ATP. The activity was increased, depending on an increase in the concentration of ATP in the absence and presence of cycleanine (Fig. 4). The maximum activities (V_{max}) obtained from Lineweaver–Burk plots were 107.6

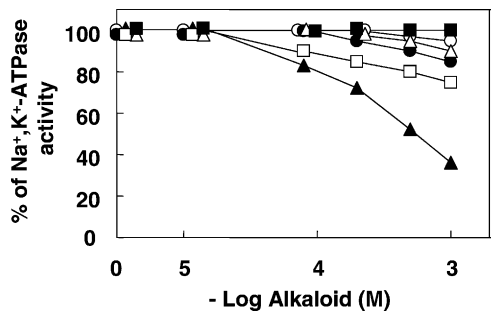

Fig. 3. Effects of bisbenzylisoquinoline alkaloids on Na^+,K^+-ATPase activity. The Na^+,K^+-ATPase activity was determined in the presence of various amounts of bisbenzylisoquinoline alkaloids. Na^+,K^+-ATPase activity without the alkaloids was taken as 100% (52.1 μmol P_i/mg protein/min). (■) Cepharanoline, (○) isotetrandrine, (△) homoaromoline, (●) cepharanthine, (□) berbamine, (▲) cycleanine. The SD was less than 1.9% (N = 5).

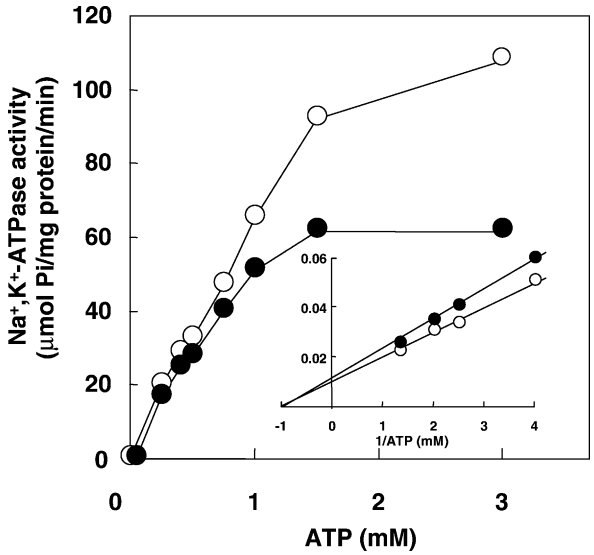

Fig. 4. Effects of ATP concentration on Na^+,K^+-ATPase activity in the presence of cycleanine. The Na^+,K^+-ATPase was determined in the presence (●) or absence (○) of cycleanine (2.0×10^{-4} M). Lineweaver–Burk plot is shown in the inset. *V* means Na^+,K^+-ATPase activity. The SD was less than 2.2% (N = 5).

and 61.5 μmol P_i/mg protein/min in the absence and presence of cycleanine, respectively, though the K_m values (0.9 mM) for ATP were the same (Fig. 4, inset). These results indicated that the mode of inhibition appeared to be noncompetitive.

Na^+,K^+-ATPase activity was assayed in a reaction mixture containing various concentrations of K^+ with or without 2.0×10^{-4} M cycleanine. Activity was observed at concentrations as low as 1.3 mM K^+ in the absence of cycleanine, but activity was not detected in the presence of cycleanine. The extent of inhibition by cycleanine was 90, 47, 25, and 0% at the K^+ concentration of 3.8, 14, 45, and 360 mM, respectively. The inhibition rate gradually decreased with an increase in K^+ concentration. The $K_{0.5}$ values for K^+ were 3.3 and 10.5 mM in the absence and presence of cycleanine, respectively (Fig. 5a).

Furthermore, Na^+,K^+-ATPase activity was determined in a reaction mixture containing various concentrations of Na^+ with or without 2.0×10^{-4} M cycleanine. The extent of inhibition by cycleanine was constant at 50% regardless of the Na^+ concentration, and the $K_{0.5}$ value (6.9 mM) for Na^+ was not altered (Fig. 5b).

The inhibition of Na^+,K^+-ATPase activity by a specific inhibitor ouabain was examined in the absence and presence of 2.0×10^{-4} M cycleanine (Fig. 6). The activities were completely inhibited by 1.0×10^{-5} M ouabain. The shapes of the inhibition curves for ouabain in the absence and presence of cycleanine were similar, but the activity levels were different. The K_i value (1.5×10^{-7} M) for ouabain determined from the Hill plot was the same both in the absence and presence of cycleanine (Fig. 6, inset). The K_i value of cycleanine was not altered by ouabain at 1.5×10^{-7} M.

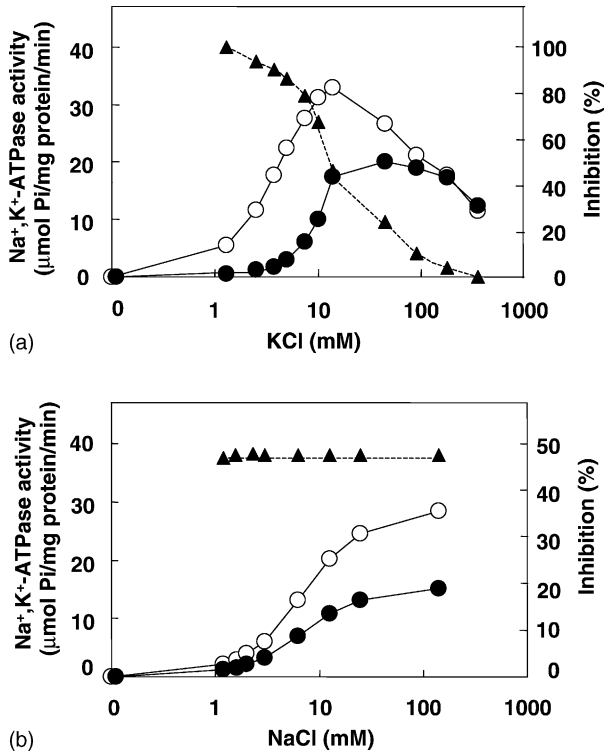

Fig. 5. Effects of KCl or NaCl concentration on the inhibition of Na^+,K^+-ATPase activity by cycleanine. The Na^+,K^+-ATPase was incubated with (●) or without (○) 2.0×10^{-4} M cycleanine. (a) The concentration of KCl was varied and that of NaCl was fixed at 140 mM. (b) The concentration of NaCl was varied and that of KCl was fixed at 14 mM. The percent inhibition caused by cycleanine at each point is indicated (▲). The SD was less than 3.1% (N = 6).

3.3. Effects of cycleanine on K^+-pNPPase activity in the presence of various ligands

K^+-pNPPase activity, which reflects reaction in the E_2 state of Na^+,K^+-ATPase, was not inhibited by 1.0×10^{-3} M cycleanine in the reaction mixture containing

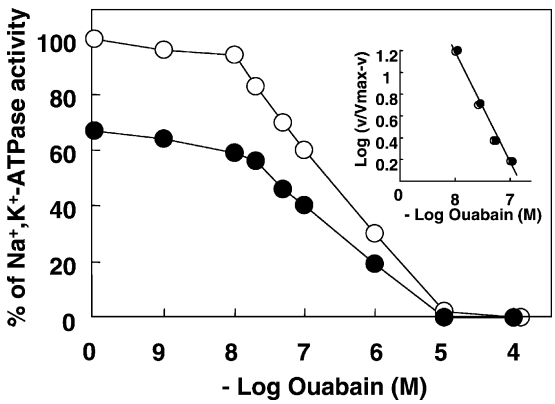

Fig. 6. Effects of ouabain on Na^+,K^+-ATPase activity in the presence of cycleanine. The Na^+,K^+-ATPase activity was determined in the presence of various concentrations of ouabain with (●) or without (○) 2.0×10^{-4} M cycleanine. Na^+,K^+-ATPase activity without ouabain and cycleanine was taken as 100% (42.5 μmol P_i/mg protein/min). Hill plot is shown in the inset. The SD was less than 2.1% (N = 6).

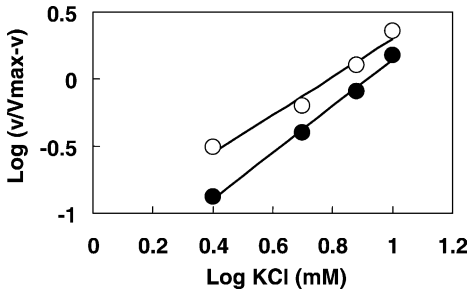

Fig. 7. Hill plots of K^+-pNPPase activity in the presence and absence of cycleanine with various concentrations of KCl. K^+-pNPPase activity was determined with (●) or without (○) 1.0×10^{-3} M cycleanine, and KCl was varied. The SD was less than 2.4% (N = 6).

15 mM K^+ (optimal condition), but inhibition gradually appeared as the K^+ concentration decreased. The $K_{0.5}$ values for K^+ determined from the Hill plots were 6.2 and 8.2 mM in the absence and presence cycleanine, respectively (Fig. 7).

K^+-pNPPase activity was examined in the reaction mixture containing various concentrations of Na^+ (6.3–200 mM) with or without 1.0×10^{-3} M cycleanine. K^+-pNPPase activity was decreased as the Na^+ concentration increased in the absence or presence of cycleanine, though lower activity was observed in the presence of cycleanine. The extent of inhibition by cycleanine was 0, 34, 64, and 100% at the Na^+ concentration of 0, 12.5, 50, and 150 mM, respectively (Fig. 8a). The K_i value for Na^+ obtained from

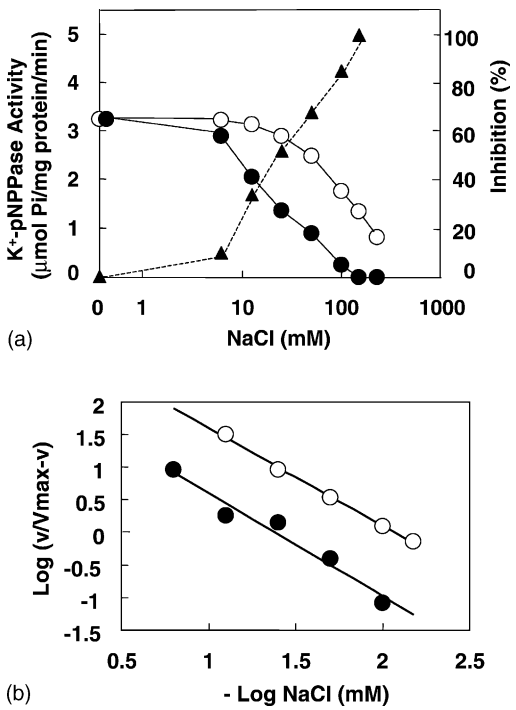

Fig. 8. Effects of NaCl concentration on the inhibition of K^+-pNPPase activity by cycleanine. (a) The K^+-pNPPase was incubated with (●) or without (○) 1.0×10^{-3} M cycleanine. The concentration of NaCl was varied and that of KCl was fixed at 15 mM. The percent inhibition caused by cycleanine at each point is indicated (▲). (b) Hill plots of the data in panel a. The SD was less than 3.2% (N = 6).

Table 1
The K_i values for ATP on K^+-pNPPase activity in the presence and absence of cycleanine and/or NaCl

Cycleanine (1.0×10^{-3} M)	NaCl (25 mM)	K_i value for ATP[a] (mM)
−	−	0.75
−	+	0.75
+	−	0.75
+	+	0.61

The K^+-pNPPase activity was determined in the presence of various concentrations of ATP (from 0.1 to 1.0 mM) with or without 1.0×10^{-3} M cycleanine and/or 25 mM NaCl at 15 mM KCl.

[a] The K_i values were obtained from the Hill plot. The SD was less than 3.2% (N = 6).

the Hill plots was 117 and 24 mM in the absence and presence of cycleanine, respectively (Fig. 8b).

The effect of 1.0×10^{-3} M cycleanine on K^+-pNPPase activity was determined in the reaction mixture containing various concentrations of ATP (0.1–1.0 mM) with or without 25 mM Na^+ (Table 1). ATP decreased K^+-pNPPase activity, and the K_i values for ATP were 0.75 mM both in the absence and presence of Na^+. The K_i value for ATP was not altered by cycleanine in the absence of Na^+, but was decreased from 0.76 to 0.61 mM in the presence of Na^+.

4. Discussion

The ScH extract containing several bisbenzylisoquinoline alkaloids inhibited the Na^+,K^+-ATPase activity. Cycleanine, one of the major components of ScH, is the most potent inhibitor of Na^+,K^+-ATPase ($IC_{50} = 6.2 \times 10^{-4}$ M) among the examined six alkaloids and related compounds. Cycleanine has a tubocuraran structure, two isoquinoline rings are combined with two 4-hydroxybenzyl-type bridges between C1–C8′ and C8–C1′ (Fig. 1c). However, the other bisbenzylisoquinoline alkaloids, such as isotetrandrine, berbamine, homoaromoline, and cepharanoline, have oxyacanthan structures, two isoquinoline rings are combined with an ether bond between C8–C7′ (Fig. 1a–k, except c). The structure of cycleanine may be necessary for the inhibition of Na^+,K^+-ATPase activity. These results suggest that the inhibitory effect of ScH on Na^+,K^+-ATPase is attributable mainly to cycleanine, and not to cepharanthine, isotetrandrine, berbamine, homoaromoline, or cepharanoline.

In the reaction of Na^+,K^+-ATPase, the conformation of the enzyme changes from $K{\cdot}E_2$ to $Na{\cdot}E_1$ when the Na^+ concentration reaches a certain level, and $Na{\cdot}E_1$ is phosphorylated by ATP to $Na{\cdot}E_1$-P. The $Na{\cdot}E_1$-P is converted into E_2-P. E_2-P is dephosphorylated to $K{\cdot}E_2$ when the K^+ concentration reaches a certain level (Fig. 9) [24,25]. We studied the effects of cycleanine on the overall reaction of Na^+,K^+-ATPase and on the partial reaction, K^+-pNPPase.

Inhibition by cycleanine appeared to be noncompetitive with respect to ATP. The binding site of cycleanine might

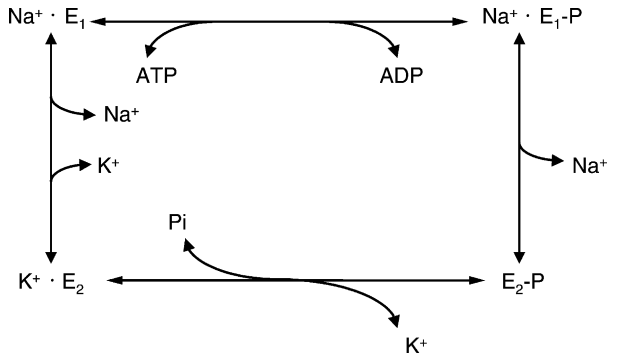

Fig. 9. Reaction mechanism of Na^+,K^+-ATPase. The scheme by Post *et al.* [22] and Taniguchi and Post [25] was modified slightly.

be unrelated to the ATP-binding site in the enzyme. The inhibition rate of Na^+,K^+-ATPase activity by cycleanine was decreased with an increase in the K^+ concentration. K^+ takes parts in the dephosphorylation of E_2-P. Therefore, cycleanine may interact with the enzyme at the high energy E_1 state and inhibit Na^+,K^+-ATPase activity. However, cycleanine probably does not interact with $Na{\cdot}E_1$ because the inhibition rate was not affected by the Na^+ concentration.

In the activity of K^+-pNPPase, that is, the reaction at the low energy E_2 state of Na^+,K^+-ATPase [26], cycleanine decreased the affinity for K^+, but increased the affinities for Na^+ and ATP. The underlying mechanism is that Na^+ favors transition of enzyme from $K{\cdot}E_2$ to $Na{\cdot}E_1$, whereas ATP favors the overall reaction leading to the E_2 state *via* $Na{\cdot}E_1$-P. The studies of K^+-pNPPase activity might indicate that cycleanine did not bind to the E_2 state of Na^+,K^+-ATPase and interact with the E_1 state.

β-Eudesmol, a specific inhibitor of Na^+,K^+-ATPase, which is a major component of So-jutsu, binds to $Na{\cdot}E_1$ and blocks the reaction step from $Na{\cdot}E_1$ to $Na{\cdot}E_1$-P [18,19]. The inhibitory potential of β-eudesmol increases as the Na^+ concentration increases, but was not affected by the K^+ concentration. Furthermore, the inhibition of K^+-pNPPase activity by β-eudesmol was reduced as the K^+ concentration was increasing, whereas a greater inhibition was observed with high concentration of either Na^+ or ATP. In comparison with the inhibition mechanisms of cycleanine and β-eudesmol, we suggest that cycleanine binds to the high energy E_1-P, and blocks the reaction step from $Na{\cdot}E_1$-P to E_2-P.

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 [22]. Cycleanine exerts its inhibitory effect by a mode of action different from ouabain, since the K_i value for ouabain was not altered by cycleanine.

In addition to β-eudesmol, we have found several Na^+,K^+-ATPase inhibitors in the components of crude drugs, e.g. atractylon, a major component of Byaku-jutsu [20], and PGG [21], a major component of Moutan Cortex. Each compound was suggested to interact with a different site on the E_2 state of Na^+,K^+-ATPase.

The inhibition mechanism of cycleanine was different from that of β-eudesmol, atractylon, or PGG on Na^+,K^+-ATPase activity. Cycleanine should be a useful inhibitor probe. It should be interesting to identify the binding sites at the molecular level. Na^+,K^+-ATPase has been shown to be involved in the clinical manifestations of the inflammatory or septic conditions, such as electrolyte disturbance, lung edema, and cholestasis, which are mediated by cytokines and endotoxins [27–31]. On the other hand, it has been known that ScH bears various kinds of anti-inflammatory effects *in vivo* and *in vitro* [5,6,8,13]. As cycleanine is one of the major components of ScH and inhibits Na^+,K^+-ATPase activity as shown by us, it may be possible that cycleanine interferes with these cytokines and endotoxins for Na^+,K^+-ATPase. The ScH extract may work to maintain the adequate concentrations of Na^+ and K^+ ion by suppressing Na^+,K^+-ATPase activity.

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