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Short communication

Ptilomycalin A, a novel Na^+,K^+- or Ca^{2+}-ATPase inhibitor, competitively interacts with ATP at its binding site

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Abstract

Ptilomycalin A inhibited the brain Na^+,K^+-ATPase and Ca^{2+}-ATPase from skeletal sarcoplasmic reticulum with an IC_{50} value of 2 μM and 10 μM, respectively. Kinetic analysis of the inhibitory effects of ptilomycalin A suggests that the inhibition of Na^+,K^+-ATPase is a competitive-, an uncompetitive- and an anticompetitive-type with respect to ATP, Na^+ and K^+, respectively. The inhibition of Ca^{2+}-ATPase by ptilomycalin A is a competitive- or an uncompetitive-type with respect to ATP or Ca^{2+}, respectively. These results suggest that ptilomycalin A interacts with ATP at the ATP binding site of Na^+,K^+-ATPase or Ca^{2+}-ATPase. Ptilomycalin A has become a useful biochemical tool for clarifying the ATP binding site in both enzymes.

Keywords: Ptilomycalin A; Na^+,K^+-ATPase; Ca^{2+}-ATPase; ATPase inhibitor

1. Introduction

Plasma membrane generates ionic gradients which are essential for excitable function. The transport of Na^+ or K^+ and Ca^{2+} is mediated by the Na^+,K^+-pump and Ca^{2+}-pump, also referred to as Na^+,K^+-ATPase and Ca^{2+}-ATPase, respectively. Na^+,K^+-ATPase accomplishes ATP-coupled efflux of 3 Na^+ and influx of 2 K^+ across the cell membrane (Skou, 1990). Ca^{2+}-ATPase accomplishes ATP-coupled transport of $2Ca^{2+}$ from the cytoplasm to the extracellular space (Forge et al., 1993). The sequences of several isoforms of Na^+,K^+-ATPase and Ca^{2+}-ATPase have been determined (Shull et al., 1985; Campbell et al., 1991).

Recently, we have isolated several ATPase modulators such as purealin (Takito et al., 1986), [8]-gingerol (Kobayashi et al., 1988) and xestoquinone (Kobayashi et al., 1991) from plants and marine organisms and revealed their pharmacological and biochemical properties. In our further and continuing search for physiologically active substances we found that ptilomycalin A (Fig. 1A) isolated from the marine sponge *Ptilocaulis spiculifer* (Kashman et al., 1989) showed a marked inhibitory effect on Ca^{2+}-ATPase and Na^+,K^+-ATPase. Competitive ATPase inhibitors with respect to ATP have not been reported except the ATP analogue (Stewart and Grisham, 1988) and furylacryloylphosphate (Inesi et al., 1980). Here, we present the first report indicating that ptilomycalin A interacts competitively with ATP at its binding site on both enzymes.

2. Materials and methods

2.1. Materials

ATP, creatine phosphate and creatine kinase were supplied by Oriental Yeast (Japan). Phosphodiesterase was supplied by Sigma. All other chemicals were obtained from Wako Pure Chemical Industries (Japan).

2.2. Methods

Ptilomycalin A was isolated from the marine sponge *P. spiculifer* as previously reported (Kashman et al., 1989). Briefly, the freeze-dried *P. spiculifer* was extracted with

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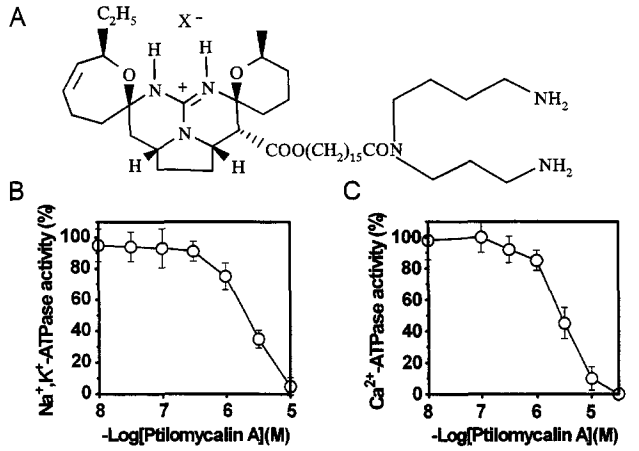

Fig. 1. Chemical structure of ptilomycalin A (A) and the concentration-response curves for ptilomycalin A on the activities of Na^+,K^+-ATPase from pig brain (B) and Ca^{2+}-ATPase from skeletal muscle (C). Specific activities of Na^+,K^+-ATPase and Ca^{2+}-ATPase were 22.5 and 10.3 μM/mg/min. Each value is expressed as mean ± S.E.M. ($n = 3$). X, unspecified.

$CHCl_3$-MeOH (9:1) to give a brown oil, which was separated by chromatography (3 ×) on an NS-gel column (Nippon Seimitsu Kagaku) eluted with MeOH-H_2O, affording ptilomycalin A.

Na^+,K^+-ATPase was prepared from pig cerebral cortex according to Nakao et al. (1973).

The light fraction of fragmented sarcoplasmic reticulum was prepared from rabbit skeletal muscle by the method of Mori et al. (1986). Ca^{2+}-ATPase of the light fraction of fragmented sarcoplasmic reticulum was purified with deoxycholate according to Meissner et al. (1973).

The heavy fraction of fragmented sarcoplasmic reticulum was prepared by the method of Nakamura et al. (1986).

The measurements of Na^+,K^+-ATPase and Ca^{2+}-ATPase activities were carried out as previously described (Nakamura et al., 1986). The enzyme reaction mixture (final volume, 0.5 ml) was as follows: 50 mM Tris (Tris(hydroxymethyl)aminomethane)-HCl buffer (pH 7.4), 100 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$, 0.01 mg/ml NaI-treated enzyme, 0.3 mM ATP and ptilomycalin A for Na^+,K^+-ATPase; 50 mM MOPS (3-(*N*-morpholino)propanesulfonic acid)-KOH buffer (pH 7.0), 90 mM KCl, 5 mM $MgCl_2$, 0.75 mM $CaCl_2$, 1 mM EGTA, 0.0065 mg/ml light fraction of fragmented sarcoplasmic reticulum, 0.3 mM ATP and ptilomycalin A for Ca^{2+}-ATPase.

The extravesicular Ca^{2+} concentration of the heavy fraction of fragmented sarcoplasmic reticulum suspension was measured at 30°C with a Ca^{2+} electrode prepared as described previously (Nakamura et al., 1986). The Ca^{2+} electrode showed Nernstein response (slope 27–29 mV/pCa unit) in the calibration solutions containing Ca^{2+}-EGTA between pCa 3 and 6.5.

3. Results

Fig. 1B shows the concentration-response curve for ptilomycalin A on the activity of the brain Na^+,K^+-ATPase. The Na^+,K^+-ATPase activity decreased by ptilomycalin A in a concentration-dependent manner with an IC_{50} value of 2 μM. Fig. 1C shows the concentration-response curve for ptilomycalin A on the activity of Ca^{2+}-ATPase from skeletal muscle. The Ca^{2+}-ATPase activity decreased by ptilomycalin A in a concentration-dependent manner with an IC_{50} value of 10 μM. Ptilomycalin A also caused concentration-dependent inhibition of bovine cardiac phosphodiesterase (Sigma) with an IC_{50} value of 100 μM.

The kinetics of inhibition by ptilomycalin A (2 μM and 10 μM) were examined with respect to ATP, Na^+ and K^+ for Na^+,K^+-ATPase. Fig. 2A shows the double-reciprocal plot for the ATP concentration in Na^+,K^+-ATPase. The slope and intercept were increased by ptilomycalin A and all of the lines crossed at a point on the vertical axis. The K_m value increased by ptilomycalin A from 0.157 to 0.641 μM without changing the V_{max} value (25.06 μmol/min/mg). This indicates that ptilomycalin A shows a competitive inhibition pattern with respect to ATP in Na^+,K^+-ATPase. Fig. 2B shows the double-reciprocal plot for the Na^+ concentration in Na^+,K^+-ATPase. The slopes and intercept were increased by ptilomycalin A. The V_{max}

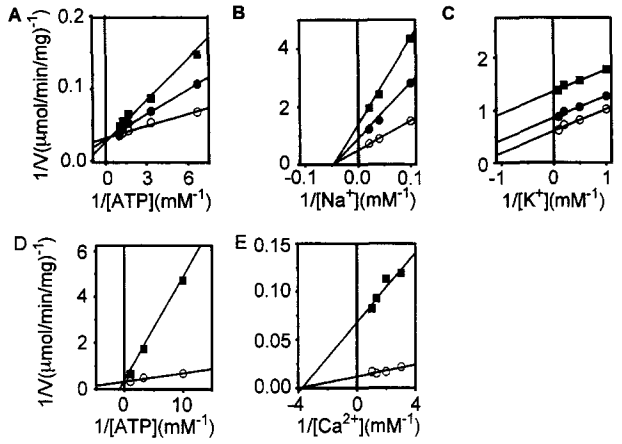

Fig. 2. The double-reciprocal plot for ATP (A), Na^+ (B) or K^+ (C) concentration in Na^+,K^+-ATPase inhibition by ptilomycalin A and for ATP (D) or Ca^{2+} (E) concentration in Ca^{2+}-ATPase inhibition by ptilomycalin A. Control (○); 2×10^{-6} M ptilomycalin A (●); 10^{-5} M ptilomycalin A (■). Each enzyme reaction mixture (final volume, 0.5 ml) was as follows: 50 mM Tris (Tris(hydroxymethyl)aminomethane)-HCl buffer (pH 7.4), 100 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$, 0.01 mg/ml NaI-treated enzyme, 0.3 mM ATP and ptilomycalin A for Na^+,K^+-ATPase; 50 mM MOPS (3-(*N*-morpholino)propanesulfonic acid)-KOH buffer (pH 7.0), 90 mM KCl, 5 mM $MgCl_2$, 0.75 mM $CaCl_2$, 1 mM EGTA, 0.0065 mg/ml light fraction of fragmented sarcoplasmic reticulum, 0.3 mM ATP and ptilomycalin A for Ca^{2+}-ATPase. The concentration range of ATP (A), NaCl (B), KCl (C), ATP (D) and $CaCl_2$ (E) was 0.15–1.00 mM, 10–100 mM, 1–20 mM, 0.1–10 mM and 0.3–1 mM, respectively.

value decreased from 416.7 to 73.53 μmol/min/mg, while the K_m value of 0.00154 μM was constant. In a reciprocal plot for the K^+ concentration, of the intercepts were increased by ptilomycalin A, while the slopes remained nearly constant and thus the lines were almost parallel to one another (Fig. 2C).

The kinetics of inhibition by ptilomycalin A (10 μM) were examined with respect to ATP and Ca^{2+} for Ca^{2+}-ATPase. Fig. 2D shows the double-reciprocal plot for the ATP concentration in Ca^{2+}-ATPase. The slope and intercept were increased by ptilomycalin A and all of the lines crossed at a point on the vertical axis. The K_m value increased by ptilomycalin A from 0.0966 to 1.26 mM, while the V_{max} value (6.4 μmol/min/mg) was not changed. This indicates that ptilomycalin A shows a competitive inhibition pattern with respect to ATP in Ca^{2+}-ATPase. In a reciprocal plot for the Ca^{2+} concentration, the slope and intercept increased by ptilomycalin A (Fig. 2E). The V_{max} value decreased from 416.7 to 73.53 μmol/min/mg, while the K_m value of 1.54 μM was constant.

The effect of ptilomycalin A on the Ca^{2+}-pumping activity of the heavy fraction of fragmented sarcoplasmic reticulum was examined using a Ca^{2+} electrode system. After pretreatment with ptilomycalin A, the slope of the time course curve of Ca^{2+} uptake was much more gentle than that of the control. The Ca^{2+}-pumping activity decreased by ptilomycalin A (1–30 μM) in a concentration-dependent manner with an IC_{50} value of approximately 8 μM, in close agreement with concentrations that inhibit the Ca^{2+}-ATPase activities. The activity of the heavy fraction of fragmented sarcoplasmic reticulum Ca^{2+} release channels can clearly be visualized by monitoring directly the extravesicular Ca^{2+} concentration of the heavy fraction of fragmented sarcoplasmic reticulum with a Ca^{2+} electrode. Ptilomycalin A (100 μM) did not induce Ca^{2+} release and gave no effect on caffeine (1 mM)-induced Ca^{2+} release from the heavy fraction of fragmented sarcoplasmic reticulum.

4. Discussion

The activities of the brain Na^+,K^+-ATPase and the purified Ca^{2+}-ATPase from skeletal sarcoplasmic reticulum decreased by ptilomycalin A in a concentration-dependent manner with IC_{50} values of 2 μM and 10 μM, respectively. Ptilomycalin A also caused a concentration-dependent inhibitory effect at substantially higher concentrations (IC_{50} = 100 μM). Ca^{2+}-pumping activity of the heavy fraction of fragmented sarcoplasmic reticulum was inhibited by ptilomycalin A (IC_{50} = approximately 8 μM), whereas Ca^{2+} release channel activity was not affected by ptilomycalin A even at a concentration of 100 μM. The Ca^{2+}-ATPase activity decreased by ptilomycalin A with an IC_{50} value approximately equal to that which blocks Ca^{2+}-pumping activity, suggesting a correlation in these inhibitory effects. These results indicate that there is some specificity in the inhibitory effect of ptilomycalin A.

The kinetics of inhibition by ptilomycalin A were examined with respect to ATP for Na^+,K^+-ATPase and Ca^{2+}-ATPase. In a reciprocal plot for the ATP concentration in Na^+,K^+-ATPase and Ca^{2+}-ATPase, each K_m value was increased by ptilomycalin A without changing the value of V_{max}. These results suggest that ptilomycalin A shows a competitive inhibition pattern with respect to ATP in Na^+,K^+-ATPase and Ca^{2+}-ATPase. The kinetics of inhibition by ptilomycalin A were examined with respect to Na^+ and K^+ for Na^+,K^+-ATPase and Ca^{2+} for Ca^{2+}-ATPase. In a reciprocal plot for the Na^+ or Ca^{2+} concentrations, each V_{max} value decreased, while that of K_m was constant, indicating an uncompetitive inhibition by ptilomycalin A. In a reciprocal plot for the K^+ concentration, all of the intercepts increased by ptilomycalin A, while the slopes remained nearly constant and thus lines were almost parallel to one another. This indicates that ptilomycalin A causes an anticompetitive inhibition pattern with respect to K^+.

The ATP analogues are known to bind to the ATP binding site of ATPase. Previous studies have shown that $Co(NH_3)_4ATP$ is a competitive inhibitor with respect to MnATP for Na^+,K^+-ATPase (Stewart and Grisham, 1988). The structure of ptilomycalin A is quite different from that of the ATP analogues (Stewart and Grisham, 1988) and furylacryloylphosphate (Inesi et al., 1980). Therefore, except for the ATP analogues and furylacryloylphosphate, ptilomycalin A is the first substance that competitively interacts with ATP at its binding site on Na^+,K^+-ATPase and Ca^{2+}-ATPase. Thus, ptilomycalin A is an essential chemical probe for clarifying molecular properties of the ATP binding site for both the enzymes.

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