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Na^+,K^+-ATPase inhibition by an endogenous peptide, SPAI-1, isolated from porcine duodenum

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SPAI-1, a peptide isolated from porcine duodenum, has been shown to inhibit Na^+,K^+-ATPase *in vitro* (Araki et al. (1989) Biochem. Biophys. Res. Commun. 164, 496–502). The characteristics of ATPase inhibition by this novel peptide were examined. SPAI-1 inhibited Na^+,K^+-ATPase preparations isolated from various organs of dog or rat or from sheep kidney with similar potency. Three isoforms of rat Na^+,K^+-ATPase had similar sensitivity to inhibition by SPAI-1 although these isoforms had remarkable differences in their sensitivity to the inhibitory effect of ouabain. Ca^{2+}-ATPase isolated from the sarcoplasmic reticulum of rabbit skeletal muscle was insensitive to inhibition by SPAI-1. Ouabain-insensitive Mg^{2+}-ATPase activity was unaffected by low concentrations of SPAI-1, but was stimulated at high concentrations. SPAI-1 inhibited H^+,K^+-ATPase from hog stomach in concentrations similar to that required for Na^+,K^+-ATPase inhibition. These results indicate that SPAI-1 is a specific inhibitor for monovalent cation transporting ATPases.

Introduction

The cardiac glycosides, such as ouabain, are selective inhibitors of Na^+,K^+-ATPase and hence the active transport of Na^+ and K^+ [1]. There are remarkable differences in ouabain sensitivity of this enzyme dependent on species and organs, or the isoforms of the enzyme [2]. The pattern of isoenzyme-dependent differences in glycoside-sensitivity is unique to the glycoside and related compounds. Another inhibitor of Na^+,K^+-ATPase, prednisolone-bisguanylhydrazone (PBGH), shows species and organ-dependent differences in inhibitory potency; however, the spectrum of isoenzyme sensitivities to PBGH is different from that observed with the cardiac glycoside [3]. Inhibition of Na^+,K^+-ATPase by either ouabain or PBGH is enhanced by high concentrations of Na^+ and reduced by K^+ in the presence of Mg^{2+} and ATP [1,3,4], presumably because binding sites for these inhibitors are more easily accessible when the enzyme protein is in the Na^+-induced conformation.

SPAI (an acronym of sodium potassium ATPase inhibitors) family of endogenous peptides are isolated from porcine duodenum, and include three closely related peptides, SPAI-1, SPAI-2 and SPAI-3 [5]. Their structures have been elucidated; each of the peptides has four disulfide bridges within its molecule [6]. These proline-rich peptides have strong basic characteristics. Kinetic analysis of the inhibitory effect of SPAI on Na^+,K^+-ATPase indicates that inhibition is competitive with respect to Na^+ and uncompetitive with respect to K^+ [5]. Thus, the effects of monovalent cations on the interaction between SPAI and Na^+,K^+-ATPase are quite different from the effects of cations on interaction of either cardiac glycosides or PBGH with

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Na^+,K^+-ATPase. SPAI seems to be one of the most potent inhibitors among known endogenous inhibitors of $Na^+.K^+$-ATPase.

In the present study, the effects of SPAI-1, consisting of 49 amino acids, were examined using partially purified Na^+,K^+-ATPase preparations obtained from dogs, rats or sheep by sodium dodecyl sulfate (SDS) or NaI treatment of crude cell membrane fractions. Effects on Na^+,K^+-ATPase were compared with those on other ATPases to determine the selectivity of SPAI action.

Methods

Enzyme preparations

Crude cell membrane fractions were obtained using the method described by Nakao et al. [7] with minor modifications. Animals were anesthetized by either nembutal or diethyl ether. After exsanguination, organs were dissected, minced with a pair of scissors, and homogenized in a 0.32 M sucrose solution containing 2 mM EDTA and 5 mM Tris-HCl buffer (pH 7.5). A 10% homogenate was prepared using a Polytron homogenizer in an ice bath. Centrifugation and resuspension to obtain crude membrane fractions were carried out as described by Nakao et al. [7].

Various organs and tissues of rats have been shown to contain distinct combinations of the Na^+,K^+-ATPase isoforms [8]. The predominant form in the kidney is the α_1 isoform. The skeletal muscle contains the α_1 and α_2 but not the α_3 isoform. The axolemma has been reported to contain α_2 and α_3 isoforms. Taking advantage of the above differential distribution, enzyme preparations enriched with particular isoform(s) were obtained.

Enzyme preparations enriched with the α_1 isoform were obtained from rat kidney following the method described by Jørgensen [9]. Crude cell membrane fractions (2 mg protein/ml) were mixed with 2 mM Na_2ATP, 2 mM EDTA and SDS (0.71 mg/ml) in a 25 mM imidazole-HCl buffer (pH 7.5) at 20°C. After an incubation for 30 min, the mixture was layered over discontinuous density gradients of 10%, 15%, 30% and 38.5% sucrose containing 25 mM imidazole-HCl (pH 7.5) and 1 mM EDTA, and centrifuged at $100000 \times g$ for 3 h at 4°C. The membrane fraction was collected from the interface between 30% and 38.5% sucrose layers.

The preparation enriched with the α_2 isoenzyme was obtained from the gastrocnemius muscle of rats by the method previously described [10]. The muscle was minced and then homogenized in 0.25 M sucrose solution containing 2 mM EDTA and 30 mM histidine buffer (pH 7.2) at 0°C using a Polytron homogenizer. To solubilize myosin and to suppress Mg^{2+}-ATPase activity, the homogenate was treated with KSCN by adding KSCN powder to the final concentration of 1 M. After stirring for 120 min at 24°C, the suspension was centrifuged at $100000 \times g$ for 30 min at 2°C. The pellet was washed twice by resuspending to its original volume and centrifuging again. The final suspension was treated with SDS as above.

Preparations enriched with the α_3 isoform of Na^+,K^+-ATPase were obtained from rat brainstem axolemma by a selective SDS extraction as described by Sweadner [11]. Subsequently, preparations were treated with trypsin [12] as follow. The preparation (320 μg protein/ml) in a solution containing 0.3 M sucrose, 1 mM EDTA, 10 mM Tris-HCl buffer (pH 7.2) and 15 mM KCl was incubated with 2 μg of trypsin-TPCK (268 μg/ml, Worthington) at 37°C for 9 min. Trypsin digestion was stopped by the soybean trypsin inhibitor (Sigma Chemical Company, St. Louis, MO). The suspension was centrifuged at $100000 \times g$ for 30 min using a Beckman TL-100 ultracentrifuge. The resulting pellet was suspended in a 0.3 M sucrose solution containing 1 mM EDTA and 10 mM Tris-HCl buffer (pH 7.2).

Canine small intestinal Na^+,K^+-ATPase preparations were obtained as described by Nakao et al. [7] by NaI treatment because SDS treatment failed to yield satisfactory enzyme preparations from this tissue. Crude membrane fractions (final protein concentration 2 mg/ml) were treated with a mixture of 2 M NaI, 1 mM EDTA, 1 mM dithiothreitol and 24 mM Tris-HCl buffer (pH 8.0) at 20°C for 30 min under gentle stirring. The suspension was diluted to 0.5 M NaI, and centrifuged at $100000 \times g$ for 70 min. The pellet was washed three times with 5 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA by resuspension and centrifugation.

Other canine Na^+,K^+-ATPase preparations and sheep kidney enzyme were obtained by the Jørgensen's method [9] by SDS treatment of 'microsomal' fractions.

Ca^{2+}-ATPase preparations were obtained from rabbit skeletal muscle by the method described by Nunogaki and Kasai [13]. The white skeletal muscle was minced in a juicer/mixer and homogenized in a solution containing 100 mM NaCl, 5 mM Tris-maleate buffer (pH 7.0) at 0°C using a Polytron homogenizer. After centrifuging at $4000 \times g$ for 30 min, the supernatant solution was filtered through a nylon mesh and centrifuged at $9500 \times g$ for 30 min. The sediment was rehomogenized in a solution containing 0.6 M KCl and 5 mM Tris-maleate buffer (pH 6.5). The homogenate was centrifuged at $53000 \times g$ for 60 min at 4°C. The resulting pellet was washed twice by resuspending and centrifuging as above. The final suspension (sarcoplasmic reticulum fraction, protein concentration 15 mg/ml) was purified by mixing with a 10% glycerol solution containing 5 mM β-mercaptoethanol and 50 mM Tris-HCl buffer (pH 8.5). The mixture was incu-

bated and then Triton X-100 was added to the final concentration of 20 mg/ml. After an additional 5-min incubation, $CaCl_2$ (final concentration, 5 mM) was added and incubated for 5 h. Subsequently, the mixture was centrifuged at $87000 \times g$ for 30 min. The supernatant solution containing purified sarcoplasmic reticulum was washed twice by centrifuging as above and resuspending in a 0.15 M sucrose solution containing 20 mM KCl, 20 μM $CaCl_2$ and 10 mM Hepes-Tris buffer (pH 8.5). The final sediments was resuspended in a solution containing 20 mM KCl, 10 mM Hepes-Tris buffer (pH 7.0), 20 μM $CaCl_2$ and 0.15 M sucrose.

H^+,K^+-ATPase preparations were obtained from hog stomach by the method of Maeda et al. [14]. After immersing in 3 M NaCl, gastric mucosa was dissected out from the smooth muscle and minced in a solution containing 0.25 M sucrose and 5 mM Tris-HCl buffer (pH 7.4), and then homogenized using a Polytron homogenizer. After centrifugation at $2000 \times g$ for 5 min, the supernatant solution was centrifuged at $100000 \times g$ for 60 min. The pellet was resuspended and centrifuged again. The final sediment was resuspended and layered over a discontinuous gradient consisting of 15 and 30% sucrose solutions containing 5 mM Tris-HCl (pH 7.4), and centrifuged at $100000 \times g$ for 120 min at 4°C. The enzyme preparation obtained at the interface between two sucrose layers was suspended in a 0.25 M sucrose buffer solution.

ATPase assay

Na^+,K^+-ATPase activity was estimated from the amount of inorganic phosphate released from ATP during a 10 or 20 min incubation at 37°C. The standard incubation mixture contained, in the final volume of 0.1 ml, 3 mM Na_2ATP, 4 mM $MgCl_2$, 0.1 mM EDTA, 40 mM Hepes-imidazole buffer (pH 7.4), 114 mM NaCl and 20 mM KCl unless otherwise noted. ATPase reaction was started by the addition of enzyme protein (final concentration, 10 or 20 μg/ml). The reaction was stopped by cooling the mixture in an ice bath. The amount of inorganic phosphate was estimated by the method of Fiske and SubbaRow [15]. Ouabain-insensitive Mg^{2+}-ATPase activity was estimated under the similar condition as Na^+,K^+-ATPase assay except that the incubation medium contained 1 mM ouabain, 10 mM NaCl, 4 mM KCl, 3 mM $MgCl_2$ and 25 mM histidine-HCl buffer (pH 7.0).

Ca^{2+}-ATPase activity was estimated in a solution containing 0.2 mg of enzyme protein per ml, 5 or 50 μM $CaCl_2$, 0.1 mM EGTA, 100 mM KCl, 4 mM $MgCl_2$, 0.01% Triton X-100 and 20 mM Hepes buffer (pH 7.0). H^+,K^+-ATPase activity was estimated in a solution containing 0.2 mg enzyme protein per ml, 2 mM $MgCl_2$, 25 mM Tris-HCl buffer (pH 7.4) with or without 5 mM KCl and 2 mM ouabain.

Miscellaneous

Protein concentrations were estimated by the method of Lowry et al. [16] using bovine serum albumin as the standard. SDS gel electrophoresis was performed with Laemli system using a 5% homogenous SDS-polyacrylamide gel [17]. Immunoblotting method was performed to identify isoform-rich preparations using the isoenzyme-specific antibodies McB2 against the α_2-isoenzyme. Rabbit antiserum, raised against the pig kidney Na^+,K^+-ATPase, was also used. These results indicate that kidney preparations contain α_1- but not α_2-isoenzyme, muscle preparations contain both α_1- and α_2-isoenzymes, and the trypsin-treated axolemmal preparations contain only α_3-isoenzyme although crude axolemmal preparations contain both α_2- and α_3-isoforms.

SPAI-1 was kindly supplied by Dr. Shinro Tachibana of Eizai Company or purchased from Peptide Institute (Osaka, Japan). Ouabain was obtained from Boehringer Mannheim and oxidized glutathione was from Sigma. Heat-stable enterotoxin from *Escherichia coli* (STh) was generously supplied by Dr. Tae Takeda, Department of Infectious Diseases, National Children's Medical Research Center, Tokyo.

Results

Tissue dependency

Tissue and organ specificity of the inhibitory effect of SPAI-1 on Na^+,K^+-ATPase was examined using enzyme preparations obtained from canine brain, kidney, heart or intestine. In all preparations, SPAI-1 inhibited Na^+,K^+-ATPase activities in a concentration dependent manner (Fig. 1B). Significant inhibition was observed at 3 to 10 μM SPAI-1 with Na^+,K^+-ATPase preparations obtained from brain, kidney or heart. Enzyme preparations obtained from intestine appear to be slightly less sensitive: 30 μM SPAI-1 caused approx. 30% inhibition. With either brain, kidney or heart enzyme, the above concentration of SPAI-1 caused more than 70% inhibition of enzyme activity. With enzyme preparations obtained from dog, concentration-inhibition curves for ouabain were nearly superimposable (Fig. 1A). Concentrations of ouabain needed to cause a 50% inhibition of enzyme preparations obtained from either brain, kidney, heart or intestine were more than an order of magnitude lower than those of SPAI-1. The slopes of concentration-inhibition curves for SPAI-1 seem to be steeper than those for ouabain. These results indicate that inhibitory effects of SPAI-1 are relatively unaffected by the source of the enzyme, and inhibition curves for SPAI-1 appear steeper than those for the cardiac glycoside.

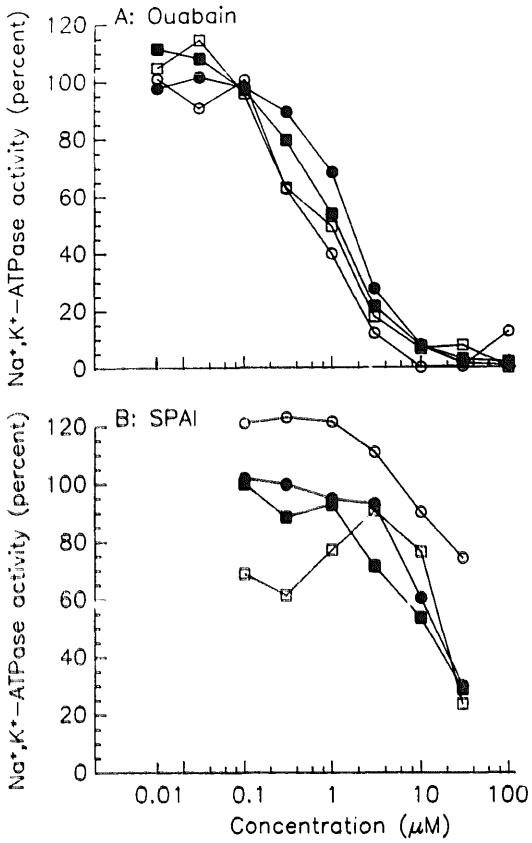

Fig. 1. Ouabain (A) or SPAI-1 (B) sensitivity of Na^+,K^+-ATPase obtained from various organs of dogs. Na^+,K^+-ATPase activity was assayed in the presence of 114 mM NaCl, 20 mM KCl, 4 mM $MgCl_2$, 3 mM Na_2ATP and 40 mM Hepes-imidazole buffer (pH 7.4) at 37°C. Mg^{2+}-ATPase activity assayed in the presence of 2 mM ouabain was subtracted. Values are expressed as percent of control activity. Control enzyme activities in μmol P_i/mg protein per h (set at 100%) were: brain, 86.0 (filled squares); kidney, 87.6 (filled circles); heart, 37.9 (open squares) and intestine, 23.4 (open circles). Each point represents the mean of duplicate determinations representative of three experiments.

Isoenzyme dependency

Isoenzymes of Na^+,K^+-ATPase obtained from rats have been shown to have marked differences in their affinities for ouabain. Present enzyme preparations obtained from various organs of the rat had marked differences in ouabain sensitivity (Fig. 2A) confirming earlier reports [18,19]. Enzyme preparations obtained from axolemma with or without trypsin treatment had a high sensitivity for ouabain whereas those obtained from the kidney required more than two orders of magnitudes higher concentrations of ouabain for comparable inhibition (Fig. 2A). Inhibition of Na^+,K^+-ATPase by ouabain showed two phases with the enzyme preparation obtained from the skeletal muscle, apparently indicating the presence of two isoforms having high and low affinity for the glycoside, respectively.

Na^+,K^+-ATPase preparations obtained from axolemma, skeletal muscle or kidney of rats were inhibited by SPAI-1 (Fig. 2B). Concentrations of SPAI-1 required to cause a 50% inhibition of Na^+,K^+-ATPase (IC_{50} values) were 8.7 to 24.6 μM depending upon enzyme preparations; however, marked differences in sensitivity comparable to those for ouabain were not observed. Specific activity of the trypsin-resistant α_3 isoform of Na^+,K^+-ATPase obtained from the axolemma was 138.6 μmol P_i/mg protein per h. This value was 56% of the untreated axolemmal enzyme preparations. The IC_{50} value for SPAI-1 was slightly decreased (the affinity was slightly increased) by the trypsin treatment (Fig. 2B) indicating that the trypsin-resistant α_3 isoform of Na^+,K^+-ATPase may have a slightly higher affinity for SPAI-1. The slopes of concentrations-inhibition curves for SPAI-1 were similar with four enzyme preparations and were steeper than those for ouabain.

Kinetic analyses

The effects of Na^+ on the inhibitory action of SPAI-1 were examined using enzyme preparations obtained from sheep kidney. As the concentration of Na^+

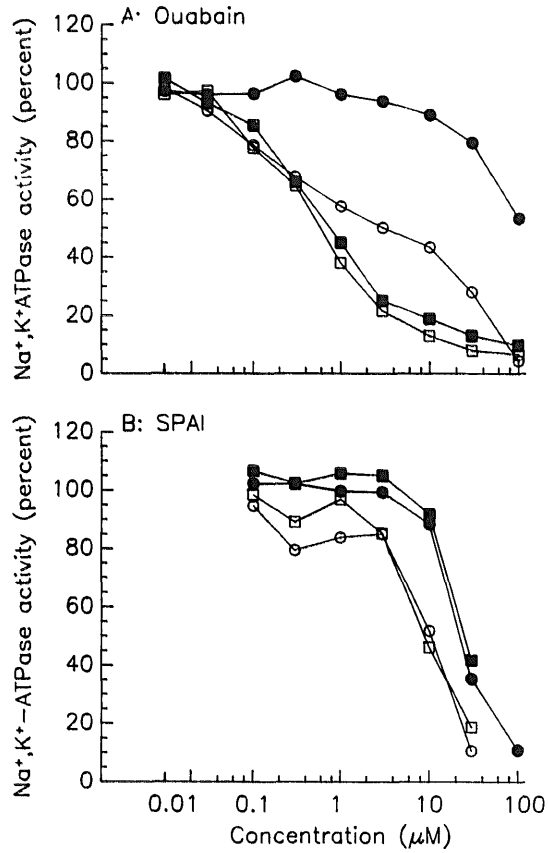

Fig. 2. Ouabain (A) or SPAI-1 (B) sensitivity of isoforms of Na^+,K^+-ATPase obtained from rats. See legend to Fig. 1. Control enzyme activities in μmol P_i/mg protein per h (set at 100%) were; brainstem axolemma, 245.9 (open squares), trypsin-treated axolemmal fraction, 138.6 (filled squares), kidney, 211.9 (filled circles) and skeletal muscle, 20.7 (open circles). Each point represents the mean of duplicate determinations representative of three experiments.

TABLE I

Concentrations of SPAI-1 or ouabain to cause half-maximal inhibition of Na^+,K^+-ATPase (IC_{50} values) and Hill coefficients (n)

Hill coefficients were graphically obtained from data lying between 10 and 90% of control Na^+,K^+-ATPase activity. Values are the mean of duplicate determinations representative of three experiments.

Enzyme source	SPAI-1		Ouabain	
	IC_{50} value (μM)	n	IC_{50} value (μM)	n
Rat				
Axolemma	24.6	2.43	0.71	0.82
(Trypsin)	8.7	1.44	0.57	0.84
Kidney	21.8	1.90	121.0	1.07
Skeletal muscle	10.3	1.67	[a]	[a]
Dog				
Brain	11.6	1.08	1.15	1.19
Kidney	14.7	2.46	1.62	1.46
Heart	17.5	2.09	0.85	1.14
Intestine	-	-	0.55	1.87

[a] Values could not be determined because inhibition curves had two phases apparently corresponding to two isoforms of Na^+,K^+-ATPase having different affinities for ouabain.

was increased, percent inhibition of enzyme activity caused by 10 μM SPAI-1 decreased reaching a plateau at about 100 mM NaCl (data not shown). These results confirm those of an earlier study [5] that Na^+,K^+-ATPase inhibition by SPAI-1 is competitive with respect to Na^+.

Because slopes of the SPAI-1 inhibition curves for Na^+,K^+-ATPase preparations obtained from the dog and rat were steeper than those for ouabain, the positive cooperativity and reversibility of the SPAI-1 action were examined. The results of Hill plot analyses of Na^+,K^+-ATPase inhibition by either SPAI-1 or ouabain in rat or dog enzyme preparations are shown in Table I. The IC_{50} values were not remarkably different with SPAI-1 in rat enzyme preparations obtained from axolemma with or without trypsin treatment, or from the kidney or skeletal muscle. The IC_{50} values for ouabain, however, were approximately 200 times different between trypsin-treated axolemmal enzyme (predominantly α_3 isoform) and kidney enzyme (predominantly α_1 isoform). With enzyme preparations obtained from various organs of dog, no remarkable differences in IC_{50} value for either SPAI-1 or ouabain dependent on the source of the enzyme were observed (Table I).

The Hill plots of the data obtained with rat or dog enzyme preparations yielded nearly straight lines when data representing less than 10% or more than 90% inhibition were excluded. The Hill coefficients were not markedly different from the unity for ouabain (Table I). The values for SPAI-1 were generally higher than unity; however, whether these values are significantly different from one could not be ascertained from the present study. These results failed to clearly establish the presence of positive cooperativity for SPAI-1-induced inhibition of Na^+,K^+-ATPase.

SPAI-1 induced inhibition being irreversible is an alternative explanation for the steep concentration-inhibition curves. Several agents have been shown to irreversibly inhibit Na^+,K^+-ATPase [20–22]. To test the reversibility of SPAI-1 inhibition, enzyme preparations obtained from rat axolemma were preincubated for 30 min with 20 μM SPAI-1 in the presence of Na^+, Mg^{2+} and ATP at 37°C. This concentration of SPAI-1 should cause approximately 50% inhibition of Na^+,K^+-ATPase (Fig. 2). The mixture was diluted with Tris-HCl buffer (pH 7.4) and centrifuged at 200 000 × g for 20 min. The resultant sediment was washed by resuspension and centrifugation as above, and assayed for Na^+,K^+-ATPase activity. The enzyme activity observed at the end of SPAI-1 treatment was 53.4% of the corresponding control value, whereas the activity observed after dilution, centrifugation and resuspension was 74.0% of the corresponding control value after one wash and 81.3% of the activity of similarly treated control preparations after two washes. These results indicate that inhibition of the rat axolemmal Na^+,K^+-ATPase by SPAI-1 is apparently reversible.

Ouabain-insensitive ATPases

During the above studies, stimulation of Mg^{2+}-ATPase activity by high concentrations of SPAI-1 was noted. Therefore, the effect of SPAI-1 on ouabain-in-

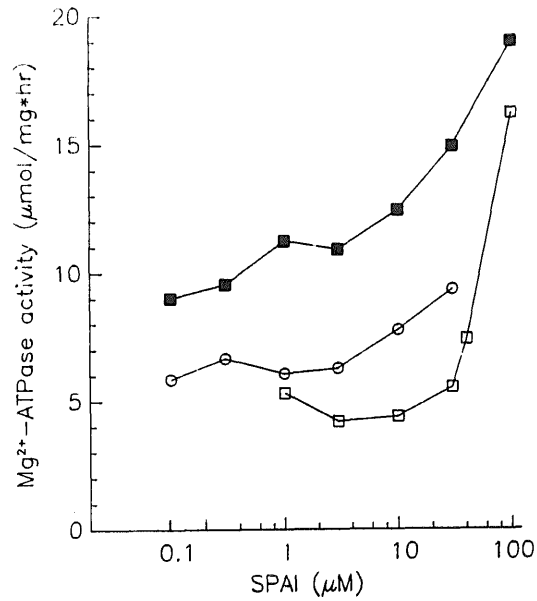

Fig. 3. Stimulation of ouabain-insensitive Mg^{2+}-ATPase by SPAI-1. Mg^{2+}-ATPase activity was assayed in the presence of 1 mM ouabain, 10 mM NaCl, 4 mM KCl, 3 mM $MgCl_2$ and 25 mM histidine-HCl buffer (pH 7.0) at 37°C. Enzyme preparations were obtained from dog brain (filled squares), dog heart (open squares) or rat skeletal muscle (open circles). Each point represents the mean of duplicate determinations representative of two experiments.

sensitive ATPases was examined using a medium containing 1 mM ouabain, 10 mM NaCl, 4 mM KCl, 3 mM $MgCl_2$ and 25 mM histidine-HCl buffer (pH 7.0). In low concentrations, SPAI-1 failed to affect ATPase activities in enzyme preparations obtained from dog brain, dog heart or rat skeletal muscle (Fig. 3). In high concentrations, however, SPAI-1 stimulated activities of all three enzyme preparations.

SPAI-1 has four disulfide bonds within the molecule. Therefore, the hypothesis that high concentrations of disulfide-containing compounds activate ATPase was tested. The effect of glutathione on Mg^{2+}-ATPase was examined using enzyme preparations obtained from dog heart. This compound containing two sulfhydryl groups failed to activate or inhibit ouabain-insensitive ATPase activity in concentrations ranging from 30 μM to 12 mM (data not shown). The effect of STh, the heat stable enterotoxin produced by toxic *E. coli* and contains three disulfide bonds within its molecule [23], was examined using enzyme preparations obtained from sheep kidney. STh failed to activate or inhibit the ouabain-insensitive ATPase in concentrations ranging from 2 to 40 μM (data not shown). These results indicate that inhibition of Na^+,K^+-ATPase or stimulation of ouabain-insensitive Mg^{2+}-ATPase by SPAI-1 is not shared by other compounds containing intracellular disulfide bonds.

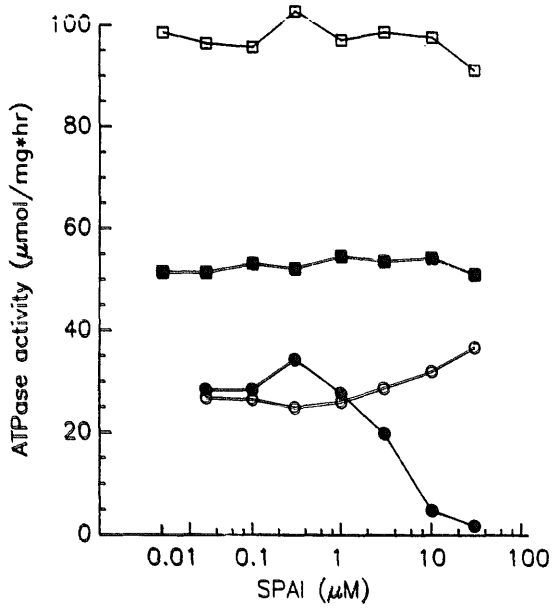

Fig. 4. Effects of SPAI-1 on Ca^{2+}-ATPase and H^+,K^+-ATPase activity. Ca^{2+}-ATPase preparations were obtained from rabbit skeletal muscle. Ca^{2+}-ATPase activity was assayed in the presence of 0.1 mM EGTA, 100 mM KCl, 4 mM $MgCl_2$, 0.01% Triton X-100, 20 mM Hepes-imidazole buffer (pH 7.4) and either 5 μM $CaCl_2$ (filled squares) or 50 μM $CaCl_2$ (open squares) at 37°C. Background ATPase activity assayed in a Ca^{2+}-free EGTA buffer was subtracted to calculate the Ca^{2+}-ATPase activity. H^+,K^+-ATPase preparations were obtained from hog stomach. H^+,K^+-ATPase activity was assayed in the presence of 2 mM $MgCl_2$, 2 mM Tris-ATP, 2 mM Tris-HCl buffer (pH 7.4) with or without 5 mM KCl and 2 mM ouabain at 37°C. K^+-insensitive ATPase activity (open circles) assayed in the absence of KCl was subtracted from the total activity to calculate H^+,K^+-ATPase activity (filled circles). Each point represents the mean of duplicate determinations representative of two experiments.

Ca^{2+}-ATPase and H^+,K^+-ATPase

Effects of SPAI-1 on Ca^{2+}-ATPase activity was examined using enzyme preparations obtained from rabbit skeletal muscle. Enzyme activity was assayed in the presence of either 50 μM $CaCl_2$ which maximally activates the Ca^{2+}-ATPase, or 5 μM $CaCl_2$ which causes a half-maximal activation. SPAI-1 failed to neither inhibit nor activate Ca^{2+}-ATPase activity in concentrations up to 30 μM in the presence of either half-maximally or maximally activating concentrations of Ca^{2+} (Fig. 4).

Effects of SPAI-1 on H^+,K^+-ATPase was examined using enzyme preparations obtained from hog stomach. SPAI-1 inhibited H^+,K^+-ATPase activity at 1–30 μM, and stimulated K^+-insensitive Mg^{2+}-ATPase activity (Fig. 4). The concentration of SPAI-1 required to cause a 50% inhibition of H^+,K^+-ATPase activity was 5 μM. These results indicate that SPAI-1 is capable of inhibiting H^+,K^+-ATPase but not Ca^{2+}-ATPase.

Discussion

Present results indicate that SPAI-1, a novel peptide extracted from porcine duodenum, inhibits Na^+,K^+-ATPase. Moreover, the results confirm an earlier report by Araki et al. [5] that Na^+,K^+-ATPase inhibition by SPAI-1 is apparently antagonized by Na^+. Potencies for inhibition were relatively unaffected by the source or isoforms of Na^+,K^+-ATPase, unlike those for the cardiac glycoside (e.g., ouabain) or PBGH. A slight difference in IC_{50} values for SPAI-1 was noted. For example, the trypsin-resistant isoform of Na^+,K^+-ATPase obtained from rat brainstem axolemma had a slightly higher sensitivity to the inhibitory effect of SPAI-1. This finding would indicate that the α_3 isoform of Na^+,K^+-ATPase which is resistant to trypsin digestion [12] may have a higher affinity for SPAI-1. Statistical significance of these relatively minor differences in IC_{50} values, however, could not be established. Small differences in affinity of Na^+,K^+-ATPase preparations for SPAI-1 may result from differences in membrane lipids that may indirectly modify the apparent affinity.

It should be noted that SPAI-1 has been extracted from the duodenum. The distribution of SPAI-1 seems to be uneven among organs. Immunohistochemical studies have shown that SPAI's are found in relatively high concentrations in intestinal mucosa and kidney, but not in the brain (personal communication from Dr. Tachibana). The Na^+,K^+-ATPase preparations ob-

tained from dog intestine or kidney do not have particularly high sensitivities to SPAI-1 inhibition.

The physiological significance of stimulation of ouabain-insensitive Mg^{2+}-ATPase activity observed with high concentrations of SPAI-1 is unclear. Three preparations with which the effect of SPAI-1 on Mg^{2+}-ATPase activity was examined, namely dog brain, dog heart and rat skeletal muscle enzyme preparations, are likely to contain mixtures of Mg^{2+}-ATPases representing different biochemical entities. With all these enzyme preparations, relatively high concentrations of SPAI-1 caused a marked stimulation of ouabain-insensitive Mg^{2+}-ATPase activities. Dithiothreitol is used in the procedures for obtaining some of partially purified enzyme preparations to protect Na^+,K^+-ATPase from inactivation. In high concentrations, however, dithiothreitol inhibits Na^+,K^+-ATPase. This inhibition might be reversed by reagents containing disulfide bonds. The present findings that SPAI-1 stimulated Mg^{2+}-ATPase, however, is not the artifact resulting from re-activation by disulfide bonds of SPAI-1. This is because SPAI-1 stimulated Mg^{2+}-ATPase activity but not Na^+,K^+-ATPase activity. Moreover, the disulfide-containing peptide, glutathione, or the disulfide bond rich endogenous peptide enterotoxin, STh, failed to stimulate the ouabain-insensitive Mg^{2+}-ATPase activity. These results indicate that stimulation of Mg^{2+}-ATPase activity observed with high concentrations of SPAI-1 is not an artifact, but is a specific property of SPAI-1. The concentrations of SPAI-1 to inhibit Na^+,K^+-ATPase (3–100 μM) were similar to SPAI-1 concentrations needed to stimulate Mg^{2+}-ATPase (4–200 μM). Mg^{2+}-ATPase activities are approximately 10% of Na^+,K^+-ATPase in these preparations. Therefore, there is a possibility that a fraction of SPAI-1-inhibited Na^+,K^+-ATPase loses its sensitivity to Na^+ or K^+ and ouabain, and shows activities of Mg^{2+}-ATPases.

The concentration-inhibition curves for SPAI-1 were steeper than those for ouabain. The steep concentration-inhibition curves have been frequently observed with putative 'endogenous inhibitors' of Na^+,K^+-ATPase. Most of these inhibitors have subsequently been identified to cause a non-specific destruction (irreversible inhibition) of Na^+,K^+-ATPase [20–22]. Inhibition of Na^+,K^+-ATPase by SPAI-1, however, was reversible upon dilution, centrifugation and resuspension. Steep slopes of the concentration-inhibition curves are apparently related to seemingly higher Hill coefficient; however, positive cooperativity for interaction of SPAI-1 with Na^+,K^+-ATPase could not be established.

Inhibition of Na^+,K^+-ATPase by SPAI-1 was observed in concentrations ranging from 3 to 100 μM. These concentrations were generally higher than those of ouabain required to cause similar degrees of enzyme inhibition. Inhibitory effect of ouabain, however, was markedly affected by isoforms of Na^+,K^+-ATPase. For example, the IC_{50} values for ouabain were about 200 times different between trypsin-treated axolemmal Na^+,K^+-ATPase (presumably the α_3 isoform) and kidney enzyme (the α_1 isoform) obtained from rats. For the rat kidney enzyme, therefore, SPAI-1 is significantly more potent (the IC_{50} value = 21.8 μM) than ouabain (the IC_{50} value = 121 μM).

The Na^+,K^+-ATPase preparations obtained from rat skeletal muscle seem to contain two isoforms of Na^+,K^+-ATPase. The gastrocnemius muscle has been shown to contain both type I and type II fibers [24]; however, the predominant form of the Na^+,K^+-ATPase expressed in the skeletal muscle has been reported to be the α_2 isoform [25,26]. The present results, however, indicate that our preparations contain two isoforms having a high and a low affinity for ouabain; the concentration-inhibition curves for ouabain clearly had two phases. Low concentrations of ouabain (0.1 to 3 μM) caused approximately 50% inhibition, and remaining activity was inhibited by concentrations of ouabain ranging from 10 to 300 μM. These concentrations of ouabain correspond to those required for inhibiting the high-affinity form (e.g., axolemmal enzymes with or without trypsin treatment) and the low-affinity form (e.g., kidney enzyme), respectively, shown in Fig. 2. With SPAI-1, two-phase inhibition curves were not observed consistent with the finding that various isoforms of Na^+,K^+-ATPase have similar sensitivity to this inhibitor.

Three isoforms of Na^+,K^+-ATPase have high structural homology; Shull and Lingrel [27], therefore, deduced that relatively limited sites in which three isoforms have structural variations must be the glycoside binding sites. These sites are facing external surface of the cell membrane. The three isoforms of Na^+,K^+-ATPase apparently have similar structures at the site that recognizes SPAI-1. Moreover, SPAI-1 inhibits H^+,K^+-ATPase which is not inhibited by ouabain. The present results, therefore, indicate that binding sites for SPAI-1 are separate from the glycoside binding site. This conclusion is consistent with the finding that the accessibility of ouabain to its binding sites is enhanced by Na^+ and reduced by K^+ [1], whereas accessibility of SPAI-1 to its binding sites is reduced by both Na^+ and K^+ [5]. Because SPAI-1 inhibits Na^+,K^+-ATPase and H^+,K^+-ATPase, but not Ca^{2+}-ATPase, the active center on the enzyme (ATP binding sites) which is common to all three enzymes [28] are not the inhibitory site for SPAI-1.

The sequence homology data show that the H^+,K^+-ATPase is closely related to the Na^+,K^+-ATPase. For example, the homology between Na^+,K^+-ATPase and H^+,K^+-ATPase is 62% [29] whereas the homology is approximately 30% between Na^+,K^+-ATPase and Ca^{2+}-ATPase [30] and 24% between Ca^{2+}-ATPase and

H^+,K^+-ATPase [31]. Moreover, the H_4 region, the most conserved of the hydrophobic domains of H^+,K^+-ATPase has a 72% homology with Na^+,K^+-ATPase [31]. These differences in the structural homology could explain the selectivity of SPAI-1 for Na^+,K^+-ATPase and H^+,K^+-ATPase over Ca^{2+}-ATPase.

In summary, SPAI-1 inhibits Na^+,K^+-ATPase and H^+,K^+-ATPase in similar concentrations, but fails to inhibit Mg^{2+}-ATPase or Ca^{2+}-ATPase. Although SPAI family of the endogenous peptides were isolated from hog duodenum, intestinal enzyme did not have a specifically high affinity for SPAI-1. In high concentrations, SPAI-1 stimulates ouabain-insensitive Mg^{2+}-ATPase. The site on Na^+,K^+-ATPase that recognizes SPAI-1 is unrelated to the site that recognizes the cardiac glycoside, and seems to be shared by three isoforms of Na^+,K^+-ATPase and the H^+,K^+-ATPase.

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