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NOVEL PEPTIDE INHIBITOR (SPAI) OF Na^+, K^+-ATPASE FROM PORCINE INTESTINE

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Three unique inhibitors(SPAI-1, -2, and -3) were first purified from porcine duodenal extract based on the Na^+, K^+-ATPase inhibitory activity. These peptide inhibitors had four disulfide bridges in common. The sequencing results of their S-carboxymethyl derivatives, lysilendopeptidase fragments, and chymotryptic peptides disclosed their entire primary structures. Both SPAI-2 and -3 consisted of 61 amino acids, respectively, and had almost the same sequences except for two amino acid substitutions, while SPAI-1 was found to lack the N-terminal twelve amino acid sequence of SPAI-2. The kinetics study revealed that SPAIs inhibited Na^+, K^+-ATPase by the competitive mode against Na^+ and were uncompetitive with K^+. © 1989 Academic Press, Inc.

It has been well known that diarrhea is sometimes a gastrointestinal adverse effect of digitalis in its clinical use (1). This fact prompted us to investigate whether the intestine had not only receptors for digitalis but also endogenous digitalis-like factors. Cardiac steroids have been thought to exert their cardiotonic activity by Na^+, K^+-ATPase inhibition, and many reports have claimed the presence of similar endogenous inhibitory factors in blood, urine, and some organs, as well (2). No endogenous factor, however, has been precisely identified so far. We report herein the isolation procedures of three unique peptides from porcine duodena and the structures of these three novel peptides SPAI-1, -2, and -3, (an acronym of sodium potassium ATPase inhibitors) which have Na^+, K^+-ATPase inhibitory activity. These peptides inhibited Na^+, K^+-ATPase by the competitive mode against Na^+ while ouabain, a cardiac steroid, was competitive with K^+, as is well known. Although the structure homology search revealed the significant similarity to a recently isolated human mucous proteinase inhibitor (3), these peptides did not show any proteinase inhibitory activities.

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MATERIALS AND METHODS

Purification procedures: We used as starting material the vasoactive intestinal peptide (VIP) fraction obtained from 25,000 porcine duodena (4). The crude material (2 g) was first adsorbed onto a CM-Sephadex column (8 x 400 mm) and the column was washed with a linear gradient of ammonium formate pH 6.5 (100 mM, 600 ml to 600 mM, 600 ml). Active material was then eluted with 2 M ammonium formate pH 8 and lyophilized. Preparative HPLC on a YMC-GEL C18 column (10 x 300 mm, Yamamura, Kyoto) was used for the next purification step with a linear gradient of acetonitrile from 20 to 40 % containing 0.1 % trifluoroacetic acid (TFA) for 30 min with a flow rate of 2.5 ml/min. Further purifications were achieved by two consecutive reverse phase HPLCs first using YMC-GEL CN (6 x 300 mm) and then C18 (4.6 x 250 mm) columns. A linear gradient of acetonitorile from 20 to 37 % containing 0.1 % TFA for 30 min was used for the CN column with a flow rate of 1.2 ml/min. By the final isocratic elution of 26 % acetonitrile containing 0.1 % TFA, three active peaks emerged as SPAI-1, -2, and -3, respectively.

Enzyme assay: Inhibitory activity of Na^+, K^+-ATPase was determined by the modified method of Kelly et al. (5) using dog kidney Na^+, K^+-ATPase (Sigma). Trypsin, Chymotrypsin, and leukocyte elastase were purchased from Sigma and human cathepsin G was from Protogen, Switzerland.

Amino acid analyses: Each peptide was hydrolyzed with 6 N hydrochloric acid containing 0.1 % phenol and then the amino acid composition was analyzed by either the Pico-Tag method (Waters) (6) or by the Hitachi Amino Acid Analyser Model 835.

Structure analyses: SPAI (10 nmol) was dissolved in 150 µl of 1.75 M Tris-HCl buffer pH 8.6 containing 7.3 M guanidine and 0.5 % EDTA and was reduced by adding 20 µl of 0.6 M dithiothreitol (DTT). The reaction mixture was incubated under nitrogen gas at 37 C for 2.5 hr and then 30 ul of 0.6 M carboxymethyl iodide (Wako Chemicals , Osaka) and 30 µl of 0.5 N sodium hydroxide were added. S-carboxymethyl derivatives were purified by the HPLC on a C18 column (4.6 x 250 mm) with a linear gradient of acetonitrile for 30 min, 24 to 38 %, 0.1 % TFA. S- carboxymethyl SPAI-2 (1 nmol) was applied to a gas phase semiautomated sequencer (Applied Biosystems). S-carboxymethyl peptide (2 nmol) was dissolved in 100 µl of 50 mM Tris-HCl buffer pH 9 and digested with lysilendopeptidase[1] (0.2 µg, E.C. 3.4.21.50) at 37 °C for 6 hr. The digest was directly loaded to a C18 column and fragments were separated by a linear gradient of acetonitrile from 1 to 36 % containing 0.1 % TFA for 60 min with a flow rate of 1 ml/min.

S-carboxymethyl SPAI-2 (2 nmol) in 100 µl of 100 mM Tris-HCl buffer pH 8 was also digested with TLCK-treated α-chymotrypsin (1 µg, Sigma) and chymotryptic peptides were separated by the same HPLC condition as that used in lysilendopeptidase fragments. The carboxymethyl SPAI-2 (360 pmol) dissolved in 300 µl of 300 mM ammonium formate pH 6.5 was digested with carboxypeptidase (CPase) Y (0.6 µg, Sigma) at 25° C and the amino acids thus released were analyzed after 10, 30 min, 2, 4, and 12 hr incubation period. S-carboxymethyl derivatives of SPAI-1 and -3 were similarly obtained and treated with enzymes.

RESULTS

Purification procedures and the yield of each peptide are shown in Fig. 1. Unfortunately, we did not notice the presence of the third active peak at first so we missed collecting SPAI-3. That is the reason why SPAI-3 resulted in a low yield. The structure analyses were mainly performed with SPAI-2 due to its high yield.

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[1] A generous gift from Dr. T. Masaki (7).

	Protein
VIP fraction	110 g
↓	
CM-Sephadex C-25 column chromatography	21.5 g
↓	
Sephadex G-25 column chromatography	5.9 g
↓	
Preparative HPLC using YMC-GEL C_{18} (10x300 mm)	250 mg
↓	
HPLC using YMC-GEL CN (6x300 mm)	-
↓	
HPLC using YMC-GEL C_{18}(4.6x250 mm) isocratic elution	
SPAI-1	0.8 mg
SPAI-2	1.2 mg
SPAI-3	0.2 mg

Fig. 1. Purification procedures and yields of SPAIs.

The three peptides showed rather similar amino acid compositions and each peptide had four disulfide bridges. SPAI-2 consisted of 61 amino acids : Asx 5, Glx 2, Ser 3, Gly 5, His 1, Arg 5, Ala 2, Pro 8, Tyr 2, Val 3, CysH 8, Ile 1, Leu 5, Phe 3, Lys 7 and Trp 1. Tryptophan was found by the sequencing of S-carboxymethyl SPAI-2 at a later time. The direct sequencing of S-carboxymethyl SPAI-2 disclosed indisputably N-terminal 27 amino acid residues. In order to obtain the remaining sequence, S-carboxymethyl SPAI-2 was digested with lysilendopeptidase. Seven fragments (L-1 to L-7) were obtained by HPLC separation of the digest. About one tenth of each fragment was spent for the amino acid analysis and the residual material was sequenced respectively. Whole sequences of all the fragments were elucidated, which coincided with the results of their amino acid analyses. Three fragments, L-1, -2, and -6, out of the seven peptides, were found to reside in the already disclosed N-terminal part.

To estimate the alignment of the remaining four fragments, S-carboxymethyl SPAI-2 was then digested with chymotrypsin, followed by HPLC separation to obtain eight chymotryptic peptides (C-1 to C-8). Similarly about one tenth of each chymotryptic fragment was analyzed for its amino acid composition and the residue was applied for sequencing. The sequencing results of C-3 and C-6 as well as the enzyme specificity allowed us to estimate the alignment of the remaining four lysilendopeptidase fragments (L-3, -4, -5, and -7).

The C-terminal part of SPAI-2 was also confirmed by CPase Y digestion. CPase Y first released Lys, followed by Pro, Tyr, Leu and CM-CysH in this order. From these results, we could determine the entire primary amino acid sequence of SPAI-2. The whole structure and the strategies employed for strutural analyses are summarized in Fig. 2.

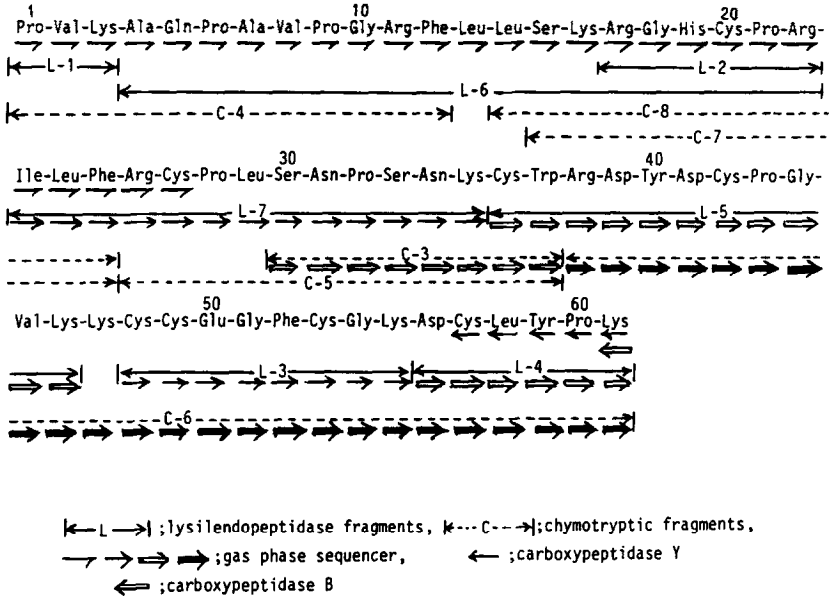

Fig. 2. Amino acid sequence of SPAI-2 and schematic outline of the strategies employed for structure determination.

The structures of SPAI-1 and -3 were determined by comparison of their lysilendopeptidase fragments with those of SPAI-2. The amino acid sequence of SPAI-1 was found to lack the N-terminal twelve amino acid sequence of SPAI-2 that corresponded to the fragment from Leu^{13} to Lys^{61} of SPAI-2. On the other hand, the amino acid analysis of SPAI-3 suggested that it consisted of two more Gly, one less Arg and Ser than SPAI-2. From the HPLC separation of the lysilendopeptidase fragments of SPAI-3, a new peak emerged which was not seen in the case of SPAI-2. About one tenth of the fragment contained in this peak was spent for the amino acid composition and the residual material was sequenced. These results suggested that the positions of Arg^{22} and Ser^{30} in SPAI-2 were substituted for Gly in SPAI-3. The primary structures of these newly isolated three peptides were disclosed as shown in Fig. 3.

A comparison between the effects of SPAI and ouabain on Na^+, K^+-ATPase was studied by using dose-response curves and double-reciprocal plots of

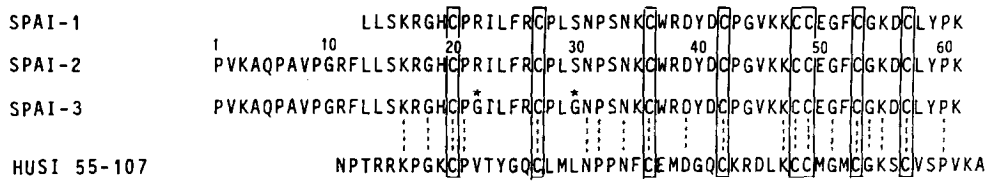

Fig. 3. Sequence homologies between SPAIs and the second domain of HUSI. Dotted lines indicate sequence identities and cysteine residues are boxed. *At positions 22 and 30 of SPAI-3 amino acids are different from those of SPAI-1 ,and -2.

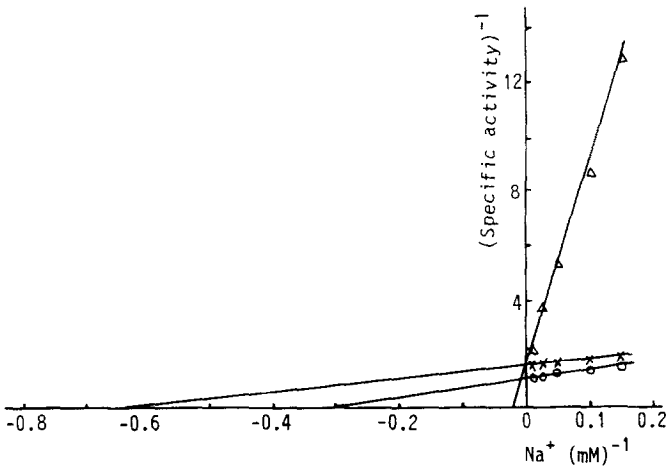

Fig. 4. Double-reciprocal plot of Na^+, K^+-ATPase acitivity against Na^+ of dog kidney. Specific activity is expressed by umoles inorganic phosphate / mg protein/ min. Standard errors (n=3) were so small that they resided within the symbol marks.
○——○; control, X——X ; 20 nM ouabain,
△——△; 10 μM SPAI-2.

Na^+, K^+-ATPase activity against Na^+ and K^+. The inhibitory activity of SPAI was competitive with Na^+ and uncompetitive with K^+ (Fig. 4) while ouabain, as is well known, inhibited the enzyme competitively with K^+ and uncompetitively with Na^+. When the inhibitory potency of SPAI was compared with that of ouabain in terms of IC_{50}, the concentration required for 50 % inhibition of Na^+, K^+-ATPase, the potency of SPAI (IC_{50} = 1.2 x 10^{-5} M) was about one thirtieth of that of ouabain (IC_{50} = 4.2 x 10^{-7} M).

DISCUSSION

In addition to VIP we have already purified some bioactive peptides from this VIP fraction (8), (9). SPAI seems to belong to a new family of peptides which have characteristics of four disulfide bridges, a strong basic and proline rich nature. The Arg^{22}-Ile^{23} bond in SPAI-2 was unusually split by lysilendopeptidase as reported in the case of the Arg-Ala bond (10). The reason could be ascribed to the digestion conditions because this fission never happened when the amount of enzyme was decreased and the incubation time was shortened. The sequence homology search using computer data bases revealed the significant homology to the two domains of human mucous proteinase inhibitor (HUSI) (3), rat whey phosphoprotein (RWP) (11), and the second domain of the basic protease inhibitor of the red sea turtle (RTPI) (12). The highest sequence homology to SPAI was seen in the second domain of HUSI which was compared with the whole sequences of three SPAIs (Fig. 3). HUSI belongs to acid-stable low molecular weight proteinase inhibitors and is, therefore, known to show inhibitory activities

against trpsin, chymotrypsin, leukocyte elastase and cathepsin G. On the contrary, SPAIs showed no inhibitory activities against these enzymes although the twenty postitions were common in these four peptides. However, we have not yet obtained enough evidence to estimate the secondary structure of SPAI. The differences of disulfide connectivities between SPAI and HUSI may explain the biological diversity of the four disulfide core proteins because the differences of disulfide connectivities have been found among the three homologous proteins, HUSI, RWP, and RTPI (13).

The kinetics studies show that the inhibitory potency of SPAI cannot simply be compaired with that of ouabain due to the differences of the inhibitory mechanism between both inhibitors. However, the relative potency of SPAI to ouabain suggests that SPAI is the most potent Na^+, K^+-ATPase inhibitor among the putative endogenous digitalis-like factors so far isolated (14) (15) (16). Although the data are not shown, SPAI could not displace radiolabeled ouabain in the binding study using dog kidney Na^+, K^+-ATPase as the receptors. This fact also supports the differences of the inhibitory mechanism between SPAI and ouabain.

In addition to cardiac steroids, melittin and cardiotoxins which are polypeptide toxins isolated from been venom and snake venom, respectively, have been known to inhibit Na^+, K^+-ATPase in a similar manner to ouabain (17). However, no homology among SPAIs, merittin and cardiotoxines was observed. It is the first time that peptide inhibitors against Na^+, K^+-ATPase were isolated from mammalian organs. The physiological and pathological roles of SPAI are not known at present but these peptides may offer new clues to study heart diseases as well as essential hypertension.

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