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Studies on Heart. XIX.[1] Isolation of an Atrial Peptide that improves the Rhythmicity of Cultured Myocardial Cell Clusters

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A new peptide that improves the rhythmicity of cultured myocardial cell clusters, designated as AAP (antiarrhythmic peptide), has been discovered and isolated in a pure form from bovine atria by extraction with boiling water at pH 4.5, followed by column chromatography procedures, two-dimensional paper chromatography and electrophoresis. The yield was 200 μg per 1.0 kg of wet tissues. The homogeneity of this peptide was confirmed by the chromatographic and/or electrophoretic behavior of the native peptide and its dansyl derivative. It was found that AAP is a hexapeptide composed of hydroxyproline (1), proline (1), glycine (3) and alanine (1), from the results of amino acid analysis and apparent molecular weight determination on a Sephadex G-15 column. The NH_2-terminal amino acid was glycine as determined by dansylation, and the COOH-terminus was also glycine (carboxypeptidase A digestion and dansylation after hydrazinolysis of AAP). This peptide changed the rhythmic beatings with continuous cellular fibrillation of myocardial cell clusters induced by 0.7 mM potassium, 3 mM calcium or 0.05 mM ouabain in modified Eagle's minimum essential medium supplemented with 2.5% bovine serum to normal rhythmic beatings, and changed irregular beatings with cellular fibrillation at 0.5 mM potassium or 5 mM calcium to rhythmic beatings with cellular fibrillation, followed by normal beatings. This peptide also restored the arrhythmic movements of isolated rat atrium induced by low potassium concentration and addition of acetylcholine to a normal rhythm, in the same way as quinidine. The activities of this peptide on myocardial cells and isolated atrium were stronger than those of quinidine. No antiarrhythmic activity was found in amino acids, peptides and proteins chemically related to AAP, such as hydroxyproline, proline, glycine, alanine, Gly–Pro, Gly–Pro–Leu, Gly–Pro–Leu–Gly–Pro, gelatin and heat-treated gelatin.

Keywords——antiarrhythmic peptide; cultured myocardial cell; arrhythmic movements; atrium; hydroxyproline; atrial peptide; collagen; gelatin

Cultured myocardial cells show various functions of differentiation, beating rhythmicity and drug-induced beating properties that are essentially similar to those of an adult intact heart.[3] Culture systems of myocardial cells can be used to study the direct action of materials on heart cells because they are free from nerves and blood vessels. In connection with our studies on the humoral factors affecting heart functions,[4] this paper describes the isolation from bovine atria of a peptide, designated as AAP (antiarrhythmic peptide), that improves the rhythmicity of rat cultured myocardial cells and rat isolated atria.

Experimental

Materials——Bovine atria were freed from fat and other tissues and rinsed with saline to exclude blood within 1 hr after death. The atria, weighing 150—200 g each, were minced and immediately frozen until

1) Part XVIII: S. Aonuma, Y. Kohama, K. Akai, Y. Muramoto, and S. Nakajima, *Chem. Pharm. Bull.*, **26**, 2957 (1978).
2) Location: *133-1, Yamadakami, Suita, Osaka, 565, Japan.*
3) I. Harary and B. Farley, *Exp. Cell Res.*, **29**, 451 (1963); K. Goshima, *ibid.*, **80**, 432 (1973); K. Goshima, *ibid.*, **92**, 339 (1975).
4) *a*) S. Aonuma, Y. Kohama, H. Kawano, M. Wada, E. Ohtsuka, and K. Akai, *Yakugaku Zasshi*, **96**, 1057 (1976); *b*) S. Aonuma, Y. Kohama, K. Akai, and M. Fujioka, *Chem. Pharm. Bull.*, **26**, 709 (1978).

use. Ouabain octahydrate, carboxypeptidase A (47 U/mg, DFP-treated) and gelatin (Type IV, approx. 60 Bloom from calf skin) were products of Sigma Chemical Co. Heat-treated gelatin were prepared by heating in boiling water at pH 4.5 for 20 min, and separated into 2 fractions (high and low molecular weights) by Sephadex G-25 column chromatography. Gly–Pro, Z–Gly–Pro–Leu, Z–Gly–Pro–Leu–Gly–Pro·H_2O·AcOEt, (Pro–Pro–Gly)$_5$·$4H_2O$ and Des–Arg^9–[Leu^8]-bradykinin were products of the Protein Research Foundation. N-Carbobenzoxy groups were removed with hydrogen in the presence of palladium black. Quinidine sulfate, acetylcholine chloride and fluorescamine (Fluram, Roche) were purchased from Nakarai Chemicals Ltd. The reagents in analytical procedures were special-grade or special biochemical-grade reagents from Wako Pure Chemical Ltd. Sephadex media were obtained from Pharmacia Chemical Co., Dowex 50W-X4 (200—400 mesh) from Muromachi Kagaku Ltd., polyamide layer sheets from Cheng Chin Trading Co. and chromatography papers from Toyo Kagaku Ltd.

Analytical Procedures——The procedures for two-dimensional paper chromatography and electrophoresis (peptide mapping) and dansylation were essentially those already described by the authors.[5]

For amino acid analysis, the peptide was hydrolyzed in 6 N HCl at 110° for 24 hr or 2.5 N NaOH in the presence of starch at 110° for 20 hr under a vacuum. For aminosugar and neutral sugar analyses, the peptide was hydrolyzed in 4 N HCl at 110° for 6 hr and 2 N trifluoroacetic acid at 100° for 2 hr under a vacuum, respectively. The hydrolysates were run on a Hitachi 034 liquid chromatograph.

The COOH-terminal amino acid was determined by 2 different methods, carboxypeptidase digestion and dansylation after hydrazinolysis of the peptide. According to the procedure of Maghuin-Rogister *et al.*,[6] enzyme digestion was performed by incubating a mixture of the peptide and enzyme in 0.2 M N-ethylmorpholine acetate buffer (pH 8.5) at 37°. According to the procedure of Akabori *et al.*,[7] hydrazinolysis was performed by heating the peptide with anhydrous hydrazine at 100° for 6 hr. The reaction mixture was lyophilized and subjected to dansylation. After the removal of dansyl (DNS)-amino acid hydrazides by extraction with ethylacetate at an acidic pH, DNS-amino acid was subjected to polyamide layer chromatography.

A molecular weight determination was carried out by Sephadex G-15 column chromatography.

All the analytical procedures were done in duplicate.

For desalting, a Sephadex G-10 column (2.5 × 90 cm) was used.

Measurements of Antiarrhythmic Activity on Cultured Myocardial Cells and Isolated Atria of Rats——According to the procedure described previously,[4b,8] cultured myocardial cell clusters (2×10^6 cells/dish) of neonatal rats were prepared in a dish containing 2.5 ml of Eagle's minimum essential medium (MEM) supplemented with 10% bovine serum. Arrhythmic movements of cell clusters were induced by low potassium or high calcium, or by addition of ouabain, in Eagle's MEM supplemented with 2.5% serum.[8,9] After the addition of samples into cultures which showed rhythmic beating with continuous cellular fibrillation at 0.7 mM potassium, 3 mM calcium or 0.05 mM ouabain and into cultures which showed irregular beating with fibrillation at 0.5 mM potassium, 5 mM calcium or 0.2 mM ouabain, the types of arrhythmic movements and beating rates were observed by means of an inverted phase-contrast microscope (Nikon MD, magnification; 80 to 160), according to the criterion of Goshima.[9] Various doses of each sample were added to 3 dishes with the same degree of arrhythmia at each concentration of irritant, and 2 regions of each dish, marked before the treatment, were observed. The effects of the sample could easily be judged, and the beating rate of cell clusters was represented as the mean of 6 values obtained from 3 dishes. For screening of fractions during purification, a part of each fraction was desalted, lyophilized and weighed, and its activity at 0.7 mM potassium was checked.

The heart of a male rat (Wistar strain) weighing 250—300 g was removed and placed in a modified Krebs-Henseleit buffer (pH 7.4),[10] equilibrated with O_2: CO_2 (95: 5). The atrium was freed from fat and other tissues, and suspended in a bath (10 ml) of the buffer aerated with O_2: CO_2 (95: 5) at 30—31°. The atrium maintained rhythmical isotonic contraction, and the changes were followed on a kymograph. The resting tension was 0.8 g. Arrhythmic movements were induced by reducing the concentration of KCl to 0.3 mM in the buffer followed by addition of acetylcholine chloride (100 μg/ml final concentration),[11] and of the sample. Each sample was tested in triplicate.

Procedures for the Isolation of AAP——The tissues, usually 1.0 kg, were homogenized to a uniform

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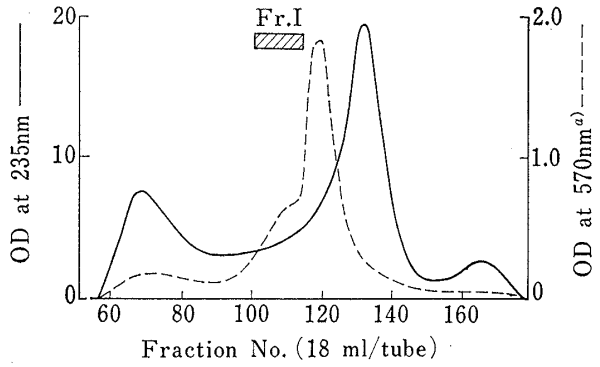

Fig. 1. Gel Filtration of Atrial Extract on a Sephadex G-25 Column

Column size, 5.0×127 cm; solvent, distilled water.
a) A 10 μl aliquot was taken for ninhydrin reaction.

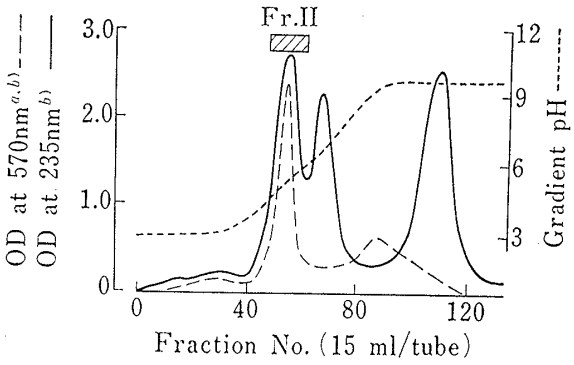

Fig. 2. Chromatography of Fr. I on a Dowex 50W Column

Column size, 2.0×120 cm. The column (Na form) was developed with 0.1 M sodium citrate buffer (pH 3.0), followed by a linear gradient with 0.3 M Tris-HCl buffer (pH 9.5).
a) A 50 μl aliquot was taken for ninhydrin reaction.
b) Corrected for Tris or citrate.

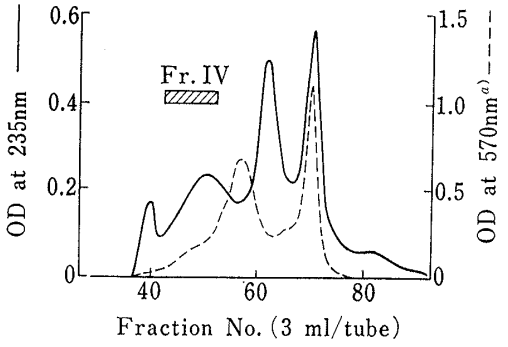

Fig. 3. Gel Filtration of Fr. III on a Sephadex G-10 Column

Column size, 1.8×118 cm; solvent, 0.1 N acetic acid.
a) A 100 μl aliquot was taken for ninhydrin reaction.

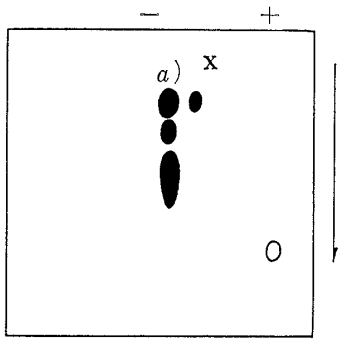

Fig. 4. Two-dimensional Chromatography and Electrophoresis of Fr. IV

One mg of Fr. IV was applied at the origin (×) with phenol red as a marker on Toyo Filter Paper No. 50 (42×45 cm), and subjected to descending chromatography with *n*-butanol/pyridine/acetic acid/water (15: 10: 3: 12) for 18 hr in the direction of the arrow, then to electrophoresis with pyridine/acetic acid/water (10: 0.4: 90, pH 6.5) at 33 V/cm for 1 hr in a perpendicular direction (− +). Solid circles show Fluram-staining materials, and the open circle shows phenol red.
a) Designated AAP.

consistency with 2 volumes of distilled water. The homogenate was adjusted to pH 4.5, boiled for 20 min, cooled to room temperature and then readjusted to pH 4.5. The resulting precipitates were removed by centrifugation at 12000 ***g*** for 5 min, and the supernatant was adjusted to pH 7.0 and centrifuged. The supernatant was concentrated to 1/20 of the initial volume at 45—50° under a vacuum. Fractionation of the concentrated fluid, atrial extract, was first performed on a column of Sephadex G-25. As shown in Fig. 1, 50 ml of the extract was applied to the column and eluted with distilled water. The eluates were monitored in terms of the absorptions at 235 nm and 570 nm upon ninhydrin staining. Fr. I, which was eluted in fractions 101—114, was adjusted to pH 3.0 and then applied to a Dowex 50W column (Na form). As shown in Fig. 2, 300 ml of 0.1 M sodium citrate buffer (pH 3.0) was passed through the column, then a linear gradient was established with 0.3 M Tris-HCl buffer (pH 9.5). A ninhydrin-positive peak, Fr. II, which was eluted at about pH 4.5, was diluted with an equal volume of distilled water and then applied to another Dowex 50W column (2.0×80 cm) (H form). The column was developed with 1.0 l each of distilled water, 0.5 N HCl and finally 4 N HCl. Fr. III, which was eluted with 4 N HCl and showed absorption peaks at 235 nm and 570 nm, was evaporated to dryness under a vacuum below 40°, then after addition of a little distilled water, was again evaporated to dryness to remove HCl. The residue was dissolved in 1 ml of distilled water and applied to a Sephadex G-10 column. As shown in Fig. 3, Fr. IV (fractions 43—53) was collected and lyophilized. The peptide mapping procedure was used as a final preparative step. As shown in Fig. 4, the

active Fr. IV (usually 1 mg per one run) was dissolved in 10 μl of distilled water and applied to the paper for chromatography and then electrophoresis. An active principle, AAP, was extracted with distilled water from the appropriate region of the paper and lyophilized.

Results

Yield and Homogeneity

Table I summarizes a typical purification of AAP from 1.0 kg of bovine atria. The yield was 200 μg, which corresponded to about 0.4 μ mol of peptide. By this procedure, AAP was purified approximately 20000-fold (based on the minimum effective concentration) from Fr. I, which was the active fraction in the first fractionation with Sephadex G-25. The activity of the extract could not be measured because it contained large amounts of salts and impurities, and damaged the cultured cells.

Table I. Summary of the Purification of AAP from Bovine Atria

Fraction	Activity[a]		
	Minimum effective concentration (μg/ml)	Relative activity (% of AAP)	Yield (mg) from atria 1.0 kg
Fr. I	2000[b]	0.005	1478
Fr. II	200	0.05	399
Fr. III	20	0.5	95
Fr. IV	1	10	6
AAP	0.1	100	0.2

a) The arrhythmic movements of myocardial cell clusters at 0.7 mM potassium were used for assay.
b) The arrhythmia was incompletely suppressed, and concentrations higher than 2 mg/ml made some of the cells shrink.

Several lines of evidence indicate that the isolated peptide is homogeneous. In the final preparative step on paper (Fig. 4), AAP ran as a single peptide spot with *Rf* 0.21; the migration rate from the origin was 6 cm/hr. When AAP was treated with DNS-chloride and chromatographed on polyamide layer sheet, only one fluorescent peptide spot other than DNS–NH_2 and DNS–OH was visible in 5 different solvent systems, as shown in Fig. 5. The *Rf* values were 0.68 in solvent I, 0 in II, 1.0 in III, 0.79 in IV and 0.61 in V. Further, this peptide was eluted as a symmetrical peak, which stained with ninhydrin reagent, at a position of K_{av} 0.28 on a Sephadex G-15 column equilibrated with pH 4.5 buffer.

Molecular Weight, Composition and Terminal Amino Acids

Fig. 6 shows the elution position of AAP from the column of Sephadex G-15 calibrated with peptides of known molecular weight, chosen as having some chemical similarity to AAP. The calibration curve was based on the elution volumes of Gly–Pro–Leu, Gly–Pro–Leu–Gly–Pro and $(Pro–Pro–Gly)_5$, but Des–Arg^9–[Leu^8]–bradykinin was unduly retarded probably because of its phenylalaninyl residue. AAP was eluted just before Gly–Pro–Leu–Gly–Pro, and its apparent molecular weight obtained from the calibration curve was 450. The elution positions of the standard peptides and AAP at 45° were all the same as at 20°. Table II shows the results of amino acid analysis of the pure peptide. Amino acid analysis of the hydrolysate with 6 N HCl gave hydroxyproline, proline, glycine and alanine without any other amino acids or unknown peaks. Analysis of the hydrolysate with 2.5 N NaOH showed the absence of tryptophan, and the sugar analyses showed no detectable sugar. The following amino acid composition was obtained: hydroxyproline (1), proline (1), glycine (3) and alanine (1). The molecular weight of this peptide calculated from this amino acid composition was 470.

Two-dimensional polyamide layer chromatography of a hydrolysate of DNS–AAP gave only DNS–Gly as a DNS–amino acid derivative in solvent systems I and II. Treatment of

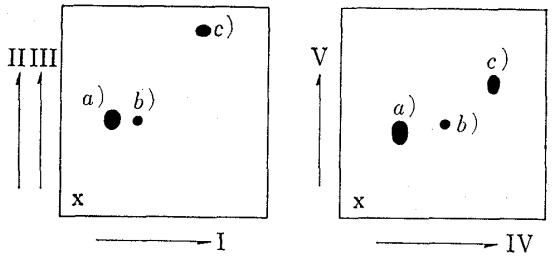

Fig. 5. Two-dimensional Chromatography of DNS–AAP on Polyamide Layer Sheets

The DNS-peptide was applied at the origin (×) and chromatographed in the direction of the arrows with the following solvents: I, 1.5% formic acid; II, benzene/acetic acid (9: 1); III, 28% ammonia water/water (1: 100); IV, *n*-butanol/acetic acid/water (3: 1: 1); V, *n*-butanol/pyridine/water (1: 1: 1). Solid circles show fluorescent spot : *a*) DNS–OH, *b*) DNS–NH_2 and *c*) DNS–AAP.

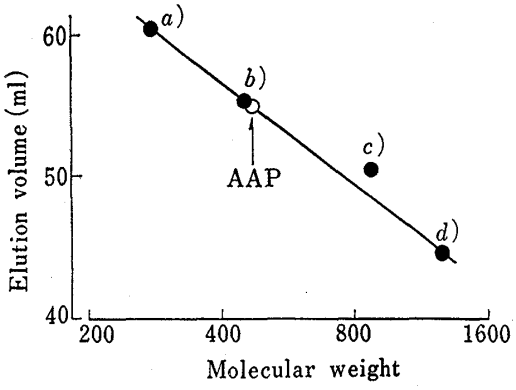

Fig. 6. Molecular Weight Determination of AAP on a Sephadex G-15 Column

Two hundred μl of AAP solution was applied to the column (0.9×156 cm), and the column was developed with pH 4.25 buffer (containing $Na_3C_6H_5O_7 \cdot 2H_2O$ (19.7 g) and HCl (8.4 ml) in a volume of 1.0 l; 0.2 M in Na) at 20 ml/hr at 20°. The eluates were automatically monitored with ninhydrin reagent at a flow rate of 10 ml/hr. The column was calibrated with *a*) Gly–Pro–Leu (molecular weight; 285), *b*) Gly–Pro–Leu–Gly–Pro (439), *c*) Arg–Pro–Pro–Gly–Phe–Ser–Pro–Leu (870) and *d*) (Pro–Pro–Gly)$_5$ (1273). The void volume estimated with blue dextran was 38.2 ml. The elution position of AAP is shown by the arrow.

Table II. Amino Acid Composition of AAP

Amino acid	Residues/450 g[a] of AAP	Nearest integer/450 g of AAP	Integer × molecular weight of residues
Hyp	1.06	1	113
Pro	1.03	1	97
Gly	2.88	3	171
Ala	0.91	1	71
Total		6	470

a) The molecular weight of 450 obtained by gel filtration was used in the calculation. The values are the means of 2 experiments.
b) 18 added for 1 H_2O.

AAP with carboxypeptidase A at 37° released only glycine in amounts of 0 for 5 min, 0.011 mol per mol of peptide for 30 min, 0.035 for 120 min and 0.069 for 360 min, but gave no hydroxyproline, proline or alanine. Polyamide layer chromatography of the DNS-amino acid obtained after hydrazinolysis of the peptide yielded only DNS–Gly as a fluorescent spot. It was thus concluded that the NH_2-terminal amino acid of AAP is glycine and that its COOH-terminus is also glycine.

Activity on Arrhythmic Movements

The effects of AAP on 2 types of arrhythmic movements of myocardial cell clusters are shown in Figs. 7, 8 and 9. When the potassium concentration of the culture medium was 0.7 mM, the cells showed rhythmic beating with continuous cellular fibrillation. This arrhythmia was improved to normal beating by 0.1 μg/ml of AAP (Fig. 7). Similarly, rhythmic beating with cellular fibrillation at 3 mM calcium or 0.05 mM ouabain was improved by concentrations of 1 or 10 μg/ml, respectively (Figs. 8 and 9). Irregular beating with cellular fibrillation at 0.5 mM potassium was improved to incompletely rhythmic beating with cellular fibrillation and then to normal beating by concentrations of 1—20 μg/ml (Fig. 7). The severe

arrhythmic movements at 5 mM calcium were also improved by 10 μg/ml of the peptide (Fig. 8), but the similar arrhythmic movement at 0.2 mM ouabain was not influenced by this peptide (Fig. 9). In all the cultures used for assays of AAP, it was found that this peptide tended to decrease the beating rate of myocardial cell clusters.

The effect of AAP on the arrhythmic movements of isolated atria of rats is illustrated in Fig. 10. When the concentration of KCl in the buffer was reduced to 0.3 mM, followed by

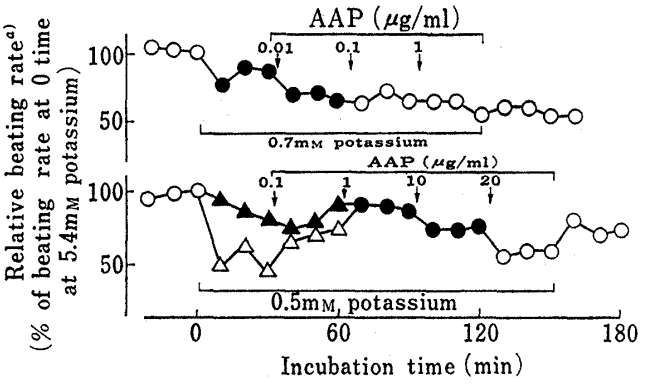

Fig. 7. Effect of AAP on the Arrhythmic Movement of Myocardial Cell Clusters induced by Low Potassium

a) The mean of 3 dishes (6 regions of cell clusters).
(○) Rhythmic beating, (●) rhythmic beating with continuous cellular fibrillation, (△,▲) irregular beating with fibrillation; (▲) strong and weak beating counted, (△) only strong beating counted.

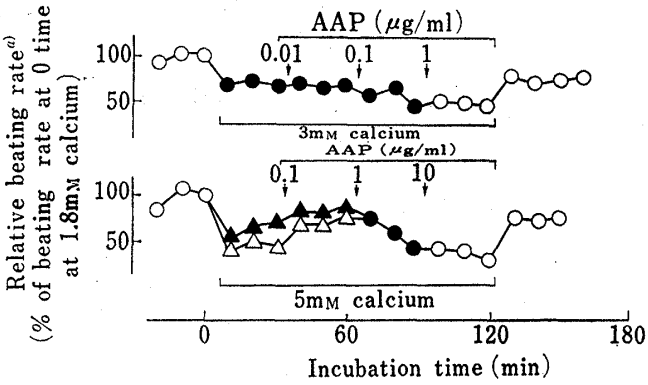

Fig. 8. Effect of AAP on the Arrhythmic Movements of Myocardial Cell Clusters induced by High Calcium

a) The mean of 3 dishes (6 regions of cell clusters).
(○) Rhythmic beating, (●) rhythmic beating with continuous celluar fibrillation, (△,▲) irregular beating with fibrillation; (▲) strong and weak beating counted, (△) only strong beating counted.

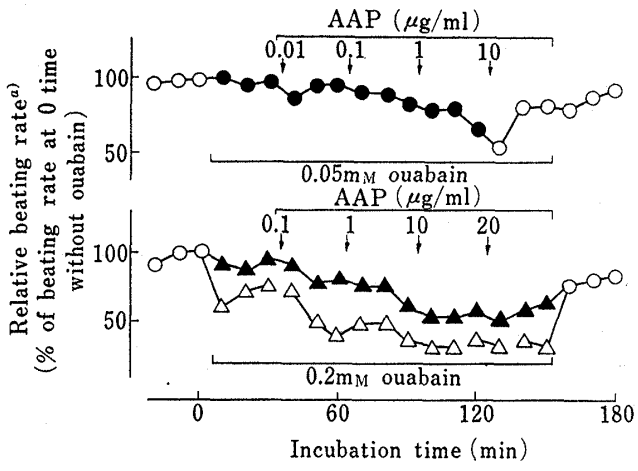

Fig. 9. Effect of AAP on the Arrhythmic Movements of Myocardial Cell Clusters induced by Ouabain

a) The mean of 3 dishes (6 regions of cell clusters).
(○) Rhythimc beating, (●) rhythmic beating with continuous cellular fibrillation, (△,▲) irregular beating with fibrillarion; (▲) strong and weak beating counted, (△) only strong beating counted.

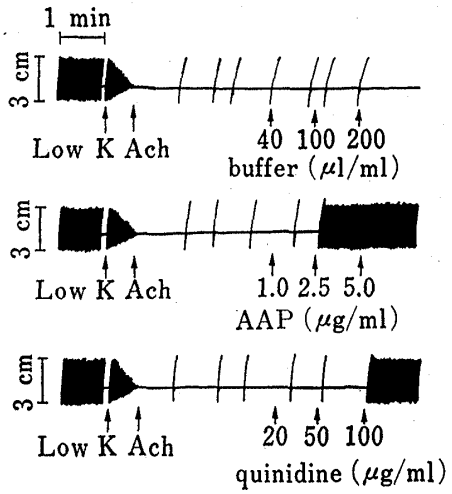

Fig. 10. Typical Effect of AAP on the Arrhythmic Movements of Rat Isolated Atria induced by Low Potassium and Addition of Acetylcholine

The isolated atria were suspended in a 10 ml bath containing modified Krebs-Henseleit buffer (pH 7.4) equilibrated with O_2: CO_2 (95: 5) at 30—31°. Low K: KCl concentration was reduced to 0.3 mM in the buffer. After the addition of Ach (acetylcholine chloride, 100 μg/ml final concentration), portions of the sample dissolved in the buffer (up to 200 μl in total) were added stepwise, as shown by arrows.

Table III. Effects of Amino Acids, Peptides and Proteins chemically related to AAP on the Arrhythmic Movements of Myocardial Cell Clusters and Isolated Atria

Sample	Activities on arrhythmic movements[a] (μg/ml)	
	Myocardial cell clusters[b]	Isolated atria
AAP	+(0.1)[c]	+(2.5)[d]
Mixture of Hyp (1 in molar ratio), Pro (1), Gly (3) and Ala (1)	−(100)	−(1000)
Gly–Pro	−(500)	−(1000)
Gly–Pro–Leu	−(500)	−(1000)
Gly–Pro–Leu–Gly–Pro	−(500)	−(1000)
$(Pro–Pro–Gly)_5$	−(500)	−(1000)
Gelatin	−(1000)	−(2000)
High molecular weight fraction of heat-treated gelatin	−(1000)	−(2000)
Low molecular weight fraction of heat-treated gelatin	−(1000)	−(2000)
Quinidine sulfate	+(1)	+(100)[d]

a) The activities were determined on the arrhythmic movements induced at 0.7 mM potassium in myocardial cell clusters and 0.3 mM KCl and 0.5 mM acetylcholine for isolated atria. The results are indicated as follows: +; improvement (minimum effective concentration), −; no effect (maximum concentration tested).
b) The sample other than AAP were firstly tested at concentrations increasing by 10-fold from 0.1 to 100 μg/ml (final concentrations), and then at 500 μg/ml of peptides and 500 and 1000 μg/ml of proteins for cells, and 500 and 1000 μg/ml of amino acids and peptides and 500, 1000 and 2000 μg/ml of proteins for atria.
c) See Fig. 7.
d) See Fig. 10.

addition of acetylcholine, the atrium developed arrhythmic movements. This arrhythmia was restored to a normal rhythm by the addition of AAP 2.5—5.0 μg/ml (final concentration), as was the case with 100 μg/ml of quinidine sulfate. Experiments on 3 different atria gave the same results. Neither AAP nor quinidine sulfate affected the arrhythmia at lower doses.

The effects of amino acids, peptides and proteins chemically related to AAP on the arrhythmic movements of myocardial cell cluster and isolated atria are summarized in Table III. The mixture of amino acids contained in AAP was inactive at final concentrations of 100 μg/ml for cells and 1000 μg/ml for atria. The peptides were also inactive even at 500 μg/ml for cells and 1000 μg/ml for atria, which were much higher than the effective concentrations of AAP. Gelatin and heat-treated gelatin were inactive. The minimum effective doses of quinidine sulfate required to improve the rhythmicity of myocardial cells and isolated atria were clearly higher than those of AAP.

Discussion

It has been reported that myocardial cells show arrhythmic movements under conditions of low potassium, high calcium, addition of ouabain or addition of digitoxin, which are known to induce arrhythmias in the whole heart, and that these arrhythmias can be improved by additions of antiarrhythmic drugs such as quinidine and procainamide.[9,12] Previously, we reported that oxytocin, which suppresses heart arrhythmias, improves the rhythmicity of myocardial cell clusters affected by low potassium conditions.[8] These results strongly suggest that severe cardiac arrhythmias such as flutter and fibrillation in adult whole heart originate

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at least in part from cellular arrhythmias of the myocardium, and that the antiarrhythmic effects of drugs are due to the improvement of arrhythmias at the cellular level.

In this work, a new antiarrhythmic peptide, designated as AAP, was isolated from bovine atria as an active principle that improves the rhythmicity of myocardial cell clusters, and was found to be a hexapeptide composed of hydroxyproline (1), proline (1), glycine (3) and alanine (1). The apparent molecular weight of the purified peptide determined by gel filtrarion was 450, which is in good agreement with the minimum molecular weight of the peptide as calculated from its amino acid composition. The NH_2– and COOH-terminal amino acids were both glycine. These results indicate that AAP may be chemically related to a structural and fibrous protein, collagen, which is composed of about 3000 amino acid residues of which one-third is glycine and one-quarter is proline or hydroxyproline.[13] However, there were many differences between AAP and collagen or gelatin (which is denatured collagen). Since the molecular size of AAP evidently differs from that of collagen, the amino acid composition of AAP was compared with those of partial sequences of the collagen molecule[13] and of peptides from enzymatic digests and partial acidic or basic hydrolysates of collagen or gelatin.[14] However, no product with the same composition was seen in these proteins and peptides. Collagen and polytripeptides related to collagen form the triple-helical conformation.[15] Even (Pro–Pro–Gly)$_{10}$ and (Pro–Hyp–Gly)$_5$ exist as trimers at high temperature in aqueous solution.[16] However, it is probable that AAP exists as a monomer, because its size was clearly less than 1000 in the molecular weight determination, in which Gly–Pro–Leu and (Pro–Pro–Gly)$_5$ moved as monomers,[15,16a] and this was also the case in the fractionation of Fr. III on Sephadex G-10 (Fig. 3). Collagen was insoluble in the assay medium, but gelatin and AAP-related compounds were tested for antiarrhrthmic activity. However, gelatin and heat-treated gelatin prepared by the same procedure as was used in the isolation of AAP were biologically inactive towards both myocardial cell clusters and isolated atria, even at much higher concentrations than that of AAP. Gly–Pro–Leu, Gly–Pro–Leu–Gly–Pro and a mixture of the amino acids which composed AAP were also inactive. Collagen fibers are major protein components of skin, tendon, bone, tooth, cartilage, connective tissues and even vessels and connective tissues of vascular systems associated with atria. The collagen from the vascular systems has a composition showing some similarities to that of AAP, but it is still unclear whether AAP exists as a native hexapeptide or as part of a larger molecule such as collagen in atria.

It is also unclear whether the antiarrhythmic effect of AAP on isolated atria is due to its anticholinergic effect.

The mode of action of AAP on myocardial cells will be described in the following paper, and the primary structure of AAP will be presented shortly.

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