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# STUDIES ON HEART. XXI. AMINO ACID SEQUENCE OF ANTIARRHYTHMIC PEPTIDE (AAP) ISOLATED FROM ATRIA*

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The amino acid sequence of an atrial peptide, AAP, that improves the rhythmicity of cultured myocardial cell clusters, was established as Gly-Pro-4Hyp-Gly-Ala-Gly. This was accomplished by the sequence analyses with dansyl-Edman degradation and carboxypeptidase Y digestion on the isolated native peptide. The hexapeptide and its several constituent peptides were synthesized by using dicyclohexylcarbodiimide as a condensing agent. Synthetic AAP was found to be chemically and biologically indistinguishable from the native one. In a structure-activity correlation of AAP, it was recognized that the whole molecule of this peptide was essential for its antiarrhythmic activity and its promoting effect on spreading phenomenon of myocardial cells in culture.

**Keywords**—antiarrhythmic peptide; myocardial cells; arrhythmia; amino acid sequence; glycine, alanine; proline; hydroxyproline; peptide synthesis

It has been reported that myocardial cells show arrhythmic movements under conditions of low potassium concentration, high calcium concentration, 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.[1] We reported earlier that oxytocin, which suppresses heart arrhythmias, improves the rhythmicity of myocardial cell clusters affected by low potassium condition.[2] These results strongly suggested that severe cardiac arrhythmias such as flutter and fibrillation in adult whole heart originate 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 connection with our studies on the humoral factors affecting heart functions, we reported previously the isolation of an atrial peptide (AAP) which improves the rhythmicity of cultured myocardial cell clusters affected by low potassium, high calcium or addition of ouabain.[3] The analytical data on the isolated peptide showed that it was a hexapeptide with the following amino acid composition: 4Hyp** (1 residue/mol of peptide), Pro (1), Gly (3) and Ala (1).[3a] This paper describes the amino acid sequence determination, synthesis and structure-activity correlation of AAP.

## MATERIALS AND METHODS

*Materials*—AAP was isolated from bovine atria, as described previously.[3a] Carboxypetidase Y was a product of Oriental Yeast Co., Ltd. Z-Cl, Z-Gly-Pro, Gly-Pro, Gly-Gly-Gly, (Pro-Pro-Gly)$_5 \cdot 4H_2O$, Boc-4Hyp (OBzl), Boc-Ala, Boc-Gly, Gly-OBzl · TosOH and dicyclohexylcarbodiimide (DCC) were products of the Protein Research Foundation. All other reagents and sol-

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** Symbols for amino acids, peptides, their derivatives and substituents follow the suggestions of the IUPAC-IUB Commission on Biochemical Nomenclature; *J. Biol. Chem.*, **42**, 977–983 (1972).

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vents used for synthesis of peptides were special-grade products of Nakarai Chemicals Ltd. The reagents in amino acid sequence analysis were Wako sequence-grade reagents. Silica gel was product of Wako Pure Chemical Ltd. Polyamide layer sheets, Sephadex media, chromatography papers and all other reagents were obtained as previously described.[3,4]

*Analytical Procedures* —The procedures for two-dimensional paper chromatography and electrophoresis (peptide-mapping), dansyl-Edman degradation, Sephadex G-15 column chromatography and amino acid analysis were those already described,[3a,4] except the amino acid analyzing condition, that is, single column method, in which the column (Hitachi custom #2611, 0.9×55 cm) was developed with stepwise elution (pH 3.25 buffer to pH 4.25 at 2 h 40 min, then to pH 6.10 at 5 h 40 min) at 30 ml/h at 55°C. According to the method of Hayashi *et al.*,[5] carboxypeptidase Y digestion was performed by incubating a mixture of the peptide and enzyme in 0.05 M phosphate buffer (pH 6.5) at 25°C. Thin layer chromatography (TLC) on silica gel B-5 plate was performed using the solvent systems, A: *n*-butanol/pyridine/acetic acid/water (15 : 10 : 3 : 12), B: ethanol/water (63 : 37), C: chloroform/methanol/17% ammonia water (2 : 2 : 1) and D: chloroform/methanol (9 : 1). The chromatograms were developed with ninhydrin and/or molin reagents. Silica gel C-200 column chromatography was done with stepwise elution using chloroform and chloroform/methanol. The eluate was monitored by TLC.

*Measurements of Antiarrhythmic and Spreading Activities* —The procedures for the measurements of activities on cultured myocardial cells were essentially those as described previously.[2,3] Briefly, for the antiarrhythmic activity, cultured myocardial cell clusters ($2\times10^6$ 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 in modified Eagle's MEM supplemented with 2.5% serum. After the addition of sample into culture which showed rhythmic beating with continuous cellular fibrillation at 0.7 mM potassium and into cultures which showed irregular beating with fibrillation at 0.5 mM potassium, the types of arrhythmic movements and beating rate were observed by means of an inverted phase-contrast microscope (Nikon MD). For spreading assay in serum-free culture, myocardial single cells ($10^5$ cells/dish) were cultivated with the test sample in Eagle's MEM containing 0.5% bovine serum albumin for 2 d in $CO_2$-incubator. Attached cells and spreading cells in 6 $mm^2$ on the bottom surface of the dish were counted, and the ratio of spreading cells to attached cells (spreading %) was calculated.

*Synthetic Procedures of Peptides* —Peptides were synthesized by using DCC as a codensing agent.[6] The purity of each product was established by TLC. All melting points are uncorrected. Rotations were determined with an Atago polax machine. The retention time of final product on ion-exchange chromatography under the condition for standard amino acid analysis was estimated. The final products were confirmed for their amino acid compositions in acid hydrolysates (6N HCl, 24 h) and their amino acid sequences by the dansyl-Edman degradation.

1) Gly-Pro-4Hyp-Gly-Ala-Gly: i) Boc-Ala-Gly-OBzl: Boc-Ala (7.3 g) and DCC (7.8 g) were dissolved in methylene chloride (58.0 ml) at 0°C. After stirring for 30 min at 0°C, a solution of Gly-OBzl · TosOH (12.9 g) in a mixture of methylene chloride (22.0 ml) and triethylamine (3.6 ml) was added. The reaction was allowed to proceed for 3 h at 0°C, then overnight at room temperature. DCurea was removed by filtration and the filtrate was concentrated *in vacuo*. The residue was dissolved in ethyl acetate, and the solution was washed successively with distilled water, 4% sodium bicarbonate and 10% citric acid. The organic layer was then dried with anhydrous sodium sulfate, and ethyl acetate was removed by evaporation. Boc-Ala-Gly-OBzl was crystallized by addition of petroleum ether, (yield: 68%, mp: 76°C, *Rf*: 0.96 solvent A and 0.93 in B).

ii) Ala-Gly-OBzl · HCOOH: Boc-Ala-Gly-

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OBzl (8.7 g) was dissolved in 98% formic acid (435 ml) and the solution was kept at room temperature for 30 min.[7] The solvent was then removed *in vacuo* under 30°C, and the residue was triturated with anhydrous ether (109 ml). The crude solid was recrystallized from ethanol-petroleum ether to give Ala-Gly-OBzl · HCOOH, (yield: 95%, mp: 72—74°C, *Rf*: 0.75 in solvent A and 0.10 in D).

iii) Ala-Gly-OBzl: Ala-Gly-OBzl · HCOOH (6.94 g) was dissolved in minimum amount of distilled water. Sodium bicarbonate was added to the residual aqueous solution to bring about alkaline, and the isolated oil was extracted with ethyl acetate several times. The organic layer was removed by evaporation to give Ala-Gly-OBzl, (yield: 58%, mp: 170—172°C, *Rf*: 0.67 in solvent A and 0.63 in B).

iv) Boc-Gly-Ala-Gly-OBzl: This compound was prepared from Boc-Gly (2.46 g) and Ala-Gly-OBzl (3.73 g) as described in i). Boc-Gly-Ala-Gly-OBzl was purified by silica gel column chromatography. A yellow oil obtained was chromatographically pure, but could not be crystallized, (yield: 40%, *Rf*: 0.73 in solvent A and 0.44 in D).

v) Gly-Ala-Gly-OBzl (I): This compound was prepared from Boc-Gly-Ala-Gly-OBzl (2.24 g) as described in ii) and iii), (yield: 69%, *Rf*: 0.65 in solvent A and 0.80 in B).

vi) Boc-4Hyp(OBzl)-Gly-Ala-Gly-OBzl: Preparation was carried out as described in i) from Boc-4Hyp(OBzl) (1.26 g) and Gly-Ala-Gly-OBzl (1.15 g), except that dimethylformamide instead of methylene chloride was used as the solvent. An oil which was apparently pure on TLC, was obtained, (yield: 28%, *Rf*: 0.69 in solvent A and 0.56 in D).

vii) 4Hyp(OBzl)-Gly-Ala-Gly-OBzl · HCOOH: This was prepared from Boc-4Hyp (OBzl)-Gly-Ala-Gly-OBzl (0.655 g) as in ii), (yield: 98%, *Rf*: 0.75 in solvent A and 0.81 in B).

viii) 4Hyp(OBzl)-Gly-Ala-Gly-OBzl: 4Hyp(OBzl)-Gly-Ala-Gly-OBzl · HCOOH (0.584 g) was dissolved in chloroform (27.0 ml) and washed with 4% sodium bicarbonate and saturated sodium chloride solution. The organic layer was dried with anhydrous sodium sulfate and removed by evaporation to give an oil, (yield: 51%, *Rf*: 0.68 in solvent A and 0.43 in D).

ix) Z-Gly-Pro-4Hyp(OBzl)-Gly-Ala-Gly-OBzl: Preparation was carried out from Z-Gly-Pro (0.173 g) and 4Hyp(OBzl)-Gly-Ala-Gly-OBzl (0.273 g) as in vi). An oil was obtained, (yield: 59%, *Rf*: 0.71% in solvent A and 0.59 in D).

x) Gly-Pro-4Hyp-Gly-Ala-Gly: Z-Gly-Pro-4Hyp(OBzl)-Gly-Ala-Gly-OBzl (0.262 g) dissolved in *tert*-butanol (3.9 ml) and distilled water (0.7 ml) was hydrogenated over palladium black (0.05 g) for 5 h. After filtration, the filtrate was evaporated to dryness to give a free hexapeptide, (yield: 81%, *Rf*: 0.51 in solvent A and 0.45 in B, retention time: 2 h 45 min, amino acid composition: 4Hyp (1.01), Pro (0.94), Gly (3.00) and Ala (1.05), amino acid sequence: $\overrightarrow{\text{Gly-Pro-4Hyp-Gly-Ala-Gly}}$, $[\alpha]_D^{20}$ : −133°C ($c$ =1, 0.5 N HCl)).

2) Gly-Pro-4Hyp: i) 4Hyp-OBzl · HCl: 4Hyp (10.0 g) was esterified with dry HCl in benzyl alcohol (125 ml), followed by removal of HCl and water *in vacuo* at 85°C.[8] The insoluble residue was filtered off and the ester hydrochloride was crystallized from the mother liquor by adding anhydrous ether, (yield: 16%, mp: 146—148°C, *Rf*: 0.75 in solvent A).

ii) Z-Gly-Pro-4Hyp-OBzl: Preparation was carried out from Z-Gly-Pro (3.7 g) and 4Hyp-OBzl · HCl (3.1 g) as in 1)-vi). An oil was obtained, (yield: 27%, *Rf*: 0.30 in solvent A and 0.53 ini D).

iii) Gly-Pro-4Hyp: This was prepared from Z-Gly-Pro-4Hyp-OBzl (1.67 g) as described in 1)-x), (yield: 90%, *Rf*: 0.41 in solvent A and 0.61 in B, retention time: 4 h 43 min, amino acid composition: 4Hyp (1.03), Pro (1.11) and Gly (1.00), amino acid sequence: $\overrightarrow{\text{Gly-Pro-4Hyp}}$, $[\alpha]_D^{20}$: −121°C ($c$ =1, 0.5 N HCl)).

3) 4Hyp-Gly and Gly-Pro-4Hyp-Gly: i) Z-4Hyp: This was synthesized by the method of Patchett and Witkop[8] (yield: 73%, *Rf*: 0.54 in solvent A and 0.44 in D).

ii) Z-4Hyp-Gly-OBzl: Preparation was ac-

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cording to 1)-i) from Z-4Hyp (3.19 g) and Gly-OBzl · TosOH (4.02 g), (yield: 28%, mp: 147—150°C, *Rf*: 0.88 in solvent A and 0.61 in D).

iii) 4Hyp-Gly: This was prepared from Z-4Hyp-Gly-OBzl (1.36 g) as in 1)-x), (yield: 89%, *Rf*: 0.31 in solvent A and 0.75 in D, amino acid composition: 4Hyp (1.01) and Gly (1.00), amino acid sequence: $\overrightarrow{\text{4Hyp-Gly}}$).

iv) 4Hyp-Gly-OBzl · TosOH: This was prepared from 4Hyp-Gly (0.47 g) as described by Greenstein and Winitz,[9] (yield: 80%, *Rf*: 0.78 in solvent A and 0.77 in B).

v) Z-Gly-Pro-4Hyp-Gly-OBzl: Preparation was carried out from Z-Gly-Pro (0.61 g) and 4Hyp-Gly-OBzl · TosOH (0.91 g) as in 1)-i). An oil was obtained, (yield: 34%, *Rf*: 0.76 in solvent A and 0.51 in D).

vi) Gly-Pro-4Hyp-Gly: This was prepared from Z-Gly-Pro-4Hyp-Gly-OBzl (0.38 g) as in 1)-x), (yield: 82%, *Rf*: 0.17 in solvent A and 0.42 in B, retention time: 4 h 10 min, amino acid composition: 4Hyp (0.93), Pro (0.96) and Gly (2.00), amino acid sequence: $\overrightarrow{\text{Gly-Pro-4Hyp-Gly}}$, $[\alpha]_D^{20}$: −121°C ($c$ =1, 0.5 N HCl)).

4) Gly-Ala-Gly: This was prepared by catalytic reduction of compound (I), as in 1)-x), (yield: 67%, *Rf*: 0.44 in solvent A and 0.73 in C, retention time: 5 h 18 min, amino acid composition: Gly (2.00) and Ala (1.02), amino acid sequence: $\overrightarrow{\text{Gly-Ala-Gly}}$, $[\alpha]_D^{20}$: −74°C ($c$ =1, 0.5 N HCl)).

## RESULTS AND DISCUSSION

### *Amino Acid Sequence of Native AAP*

Previously, we reported that AAP was a hexapeptide composed of 4Hyp (1), Pro (1), Gly (3) and Ala (1) from the results of amino acid analysis and apparent molecular weight determination on a Sephadex G-15 column, and that the $NH_2$-ter-

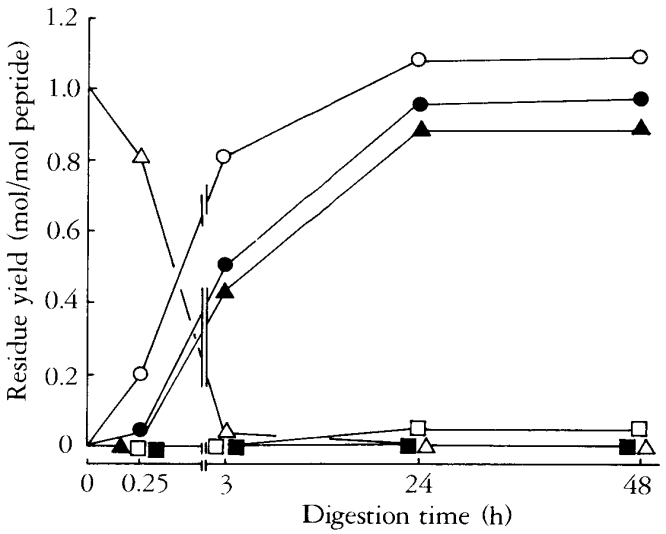


FIG. 1. *Kinetics of the Digestion of Native AAP with Carboxypeptidase Y*

*One hundred nmol of pure peptide was digested with 15 μg of carboxypeptidase Y. Aliquots were withdrawn for amino acid and peptide analyses at the times indicated. Gly (○), Ala (●), Gly-Pro-4Hyp-Gly-Ala-Gly (△), Gly-Pro-4Hyp-Gly (▲), Gly-Pro-4Hyp (□), Pro, 4Hyp, and Gly-Pro (■).*

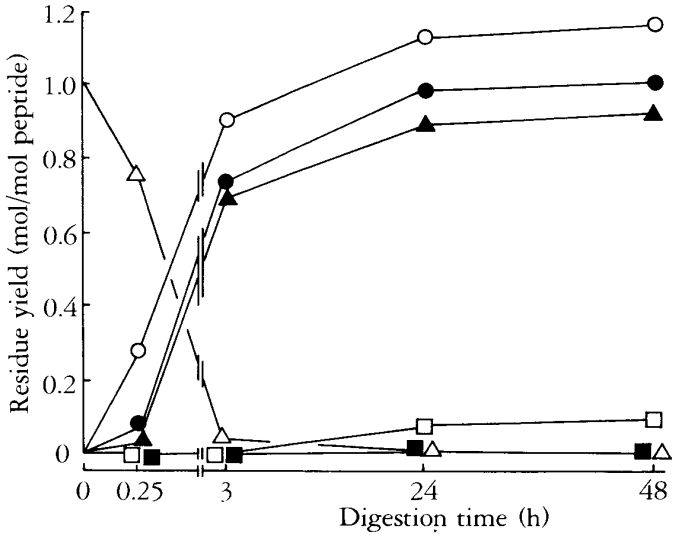


FIG. 2. *Kinetics of the Digestion of Synthetic AAP with Carboxypeptidase Y*

*Four hundred nmol of synthetic peptide was digested with 60 μg of carboxypeptidase Y. Aliquots were withdrawn for amino acid and peptide analyses at the times indicated. Gly (○), Ala (●), Gly-Pro-4Hyp-Gly-Ala-Gly (△), Gly-Pro-4Hyp-Gly (▲), Gly-Pro-4Hyp (□), Pro, 4Hyp and Gly-Pro (■).*

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minal amino acid was Gly as determined by dansylation and the COOH-terminus was also Gly as determined by carboxypetidase A digestion and dansylation after hydrazinolysis of the peptide.[3a] In this experiment, subsequent application of dansyl-Edman degradation on native AAP gave dansyl-Gly, dansyl-Pro, dansyl-4Hyp, dansyl-Gly, dansyl-Ala and dansyl-Gly, successively from the $NH_2$-terminus to residue No. 6, but no longer detectable dansyl-amino acid derivatives after residue No. 6. The amino acid composition of the peptide obtained by the dansyl procedure agreed entirely with that by amino acid analysis after acid-hydrolysis and apparent molecular weight determination. It seems that AAP offers much resistance against sequential hydrolysis by aminopeptidases[10] and carboxypeptidases[11] because that this peptide consisted of 4Hyp, Pro, Gly and Ala, and its both termini were Gly. In fact, the treatment of AAP with carboxypeptidase A at 37°C released only Gly in the amount of 0.07 mol/mol peptide even for 6 h.[3a] Then, the COOH-terminal sequence analysis was attemped by using carboxypeptidase Y which possesses comparatively wide susceptibility to COOH-terminal amino acids containing Pro and Gly.[5] After incubation of the native peptide with carboxypeptidase Y, the digest was subjected to amino acid analysis, in addition to analysis of peptides using synthetic Gly-Pro-4Hyp-Gly-Ala-Gly, Gly-Pro-4Hyp-Gly, Gly-Pro-4Hyp, and Gly-Pro (retention time: 5 h 26 min) with an amino acid analyzer, as shown in Fig. 1. Fifteen min after the start of digestion, 0.2 mol of Gly and 0.05 mol of Ala were released per mol of peptide as free amino acids, and their releases increased with the digestion time up to 24 h. At 24 and 48 h, 1.1 mol of Gly and 1.0 mol of Ala were released, but any detectable 4Hyp and Pro were not released. On the other hand, non-amino acid components in the digest were qualitatively identified by comparing with each retention time of synthetic peptide as standard, and their amounts were determined by calculating each area of elu-

TABLE I. *Comparison of Chromatographic and Electrophoretic Behavior of Native and Synthetic AAP*

| System | Property | Value[a] | |
|---|---|---|---|
| | | Native | Synthetic |
| Gel filtration on Sephadex G-15 with 0.2 M citrate buffer (pH 4.25) | $K_{av}$[b] | 0.28 | 0.28 |
| Ion-exchange chromatography on Hitachi custom #2611 resin | Retention time | 2 h 45 min | 2 h 45 min |
| Paper electrophoresis with pyridine/acetic acid/water (10:0.4:90, pH 6.5) at 33 V/cm | $M_{pr}$[c] | −0.76 | −0.81 |
| Paper chromatography with *n*-butanol/pyridine/acetic acid/water (15:10:3:12) | *Rf* | 0.21 | 0.20 |
| Polyamide layer chromatography of dansyl-AAP with; | | | |
| 1.5% formic acid | *Rf* | 0.68 | 0.73 |
| Benzene/acetic acid (9:1) | *Rf* | 0 | 0 |
| 0.3% ammonia water | *Rf* | 1.00 | 0.95 |
| *n*-Butanol/acetic acid/water (3:1:1) | *Rf* | 0.79 | 0.78 |
| *n*-Butanol/pyridine/water (1:1:1) | *Rf* | 0.63 | 0.68 |

*a) Mean of 2 experiments. b) $K_{av}$ = ratio of (elution volume − void volume) versus (column volume − void volume). c) $M_{pr}$ = relative mobility versus phenol red.*

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tion band in comparison with that of standard under a given set of operating condition. The hexapeptide which existed at 15 min, almost disappeared at 3 h. Gly-Pro-4Hyp-Gly which first appeared at 3 h, increased slightly up to 24 h, but an additional increase was not found at 48 h. A little amounts of Gly-Pro-4Hyp were detected only 24 h later, but Gly-Pro was not detected even at 48 h. Independently, when synthetic Gly-Pro-4Hyp-Gly and Gly-Pro-4Hyp were digested with this enzyme for 24 h, only 0.1 mol of Gly was released from the former peptide, but no free amino acid was released from the later. The result that Gly of residue No. 4 and its following $NH_2$-terminal amino acids offered much resistance to carboxypetidase Y, is in accord with the report that the Gly residue in sequence of insulin and

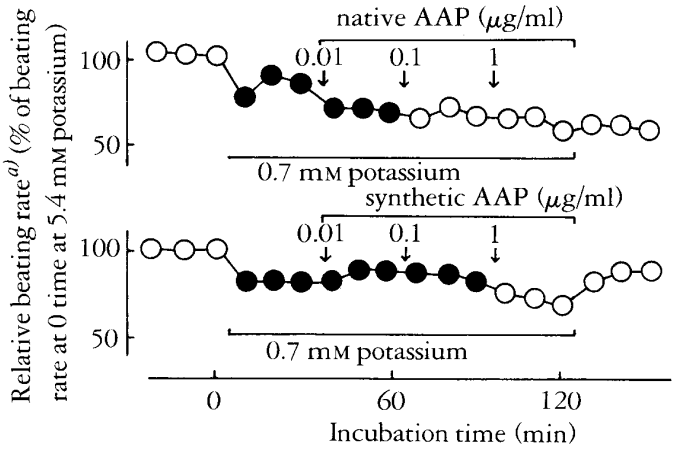


FIG. 3. *Comparison of Effect of Native and Synthetic AAP on the Continuous Cellular Fibrillation of Myocardial Cell Clusters Induced by Low Potassium Condition*

*a) The mean of 3 dishes (6 regions of cell clusters). Rhythmic beating (○), Rhythmic beating with continuous cellular fibrillation (●).*

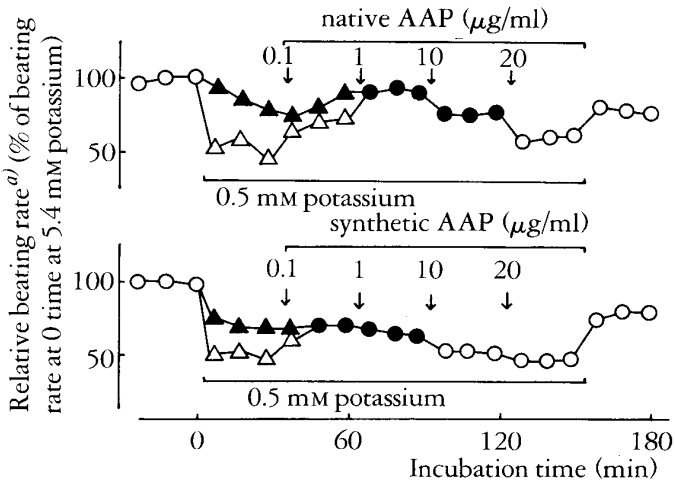


FIG. 4. *Comparison of Effect of Native and Synthetic AAP on the Irregular Beating with Continuous Cellular Fibrillation of Myocardial Cell Clusters Induced by Low Potassium Condition*

*a) The mean of 3 dishes (6 regions of cell clusters). Rhythmic beating (○), Rhythmic beating with continous cellular fibrillation (●), Irregular beating with fibrillation (△, ▲); Strong and weak beating counted (▲), Only strong beating counted (△).*

TABLE II. *Comparison of Effect of Native and Synthetic AAP on the Spreading of Myocardial Single Cells in Serum-free Culture*

| Sample | No. of experiments | Final concentration (ng/ml) | Spreading %[a] |
|---|---|---|---|
| Solvent | 5 | 0 | 17.7±0.9 |
| Native AAP | 5 | 10 | 26.8±1.2[b] |
| Native AAP | 5 | 100 | 31.6±1.9[b] |
| Synthetic AAP | 5 | 10 | 28.4±2.5[b] |
| Synthetic AAP | 5 | 100 | 33.7±2.6[b] |

*a) Mean ± s.e. b) $p < 0.01$, versus solvent.*

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RNase were only slowly released by this enzyme digestion,[5] and suggests that the 4Hyp residue can not be hydrolyzed by this enzyme. Assuming that the amino acid sequence of AAP is Gly-Pro-4Hyp-Gly-Ala-Gly, there was clearly seen a quite reasonable relationship between releases of free amino acids and appearances of residual peptide fragments.

*Identity of Synthetic AAP (Gly-Pro-4Hyp-Gly-Ala-Gly) to Isolated Native AAP*

Identity was established by comparisons of chemical and biological properties of the two peptides. Amino acid compositions of acid-hydrolysates of the two peptides were the same. Sequence analyses by the dansyl-Edman degradation on the two peptides gave comparable results. As shown

TABLE III. *Effects of Synthetic Peptides Related to AAP on Arrhythmic Movements of Rat Cultured Myocardial Cell Clusters induced by Low Potassium Condition*

| Peptide | Antiarrhythmic activity (μg/ml)[a] | |
|---|---|---|
| | Regular beating with cellular fibrillation at 0.7 mM potassium | Irregular beating with cellular fibrillation at 0.5 mM potassium |
| Gly-Pro- 4Hyp- Gly-Ala-Gly | + (1) | + (10) |
| Gly-Pro- | − (500) | − (500) |
| Gly-Pro- 4Hyp | ± (500) | − (500) |
| Gly-Pro- Leu | − (500) | − (500) |
| Gly-Pro- 4Hyp- Gly | − (500) | − (500) |
| Gly-Pro- Leu-Gly-Pro | − (500) | − (500) |
| (Pro- Pro-Gly)$_5$ | − (500) | − (500) |
| 4Hyp- Gly | − (500) | − (500) |
| Gly-Ala-Gly | − (500) | − (500) |
| Gly-Gly-Gly | − (500) | − (500) |

*a) The samples 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. See Figs. 3 and 4 for AAP. The results are indicated as follows: +; improvement (minimum effective concentration), −; no effect (maximum concentration tested), ±; incomplete improvement to partial fibrillation (effective concentration).*

TABLE IV. *Effects of AAP Constituent Peptides Synthesized on Spreading of Myocardial Single Cells in Serum-free Culture*

| Sample | No. of experiments | Final concentration (ng/ml) | Spreading %[a] |
|---|---|---|---|
| Solvent | 5 | 0 | 18.4±1.2 |
| Gly-Pro-4Hyp | 5 | 10 | 22.3±1.8 |
| Gly-Pro-4Hyp | 5 | 100 | 25.3±1.8[b] |
| Gly-Pro-4Hyp-Gly | 5 | 10 | 18.3±2.7 |
| Gly-Pro-4Hyp-Gly | 5 | 100 | 16.3±2.3 |
| Gly-Ala-Gly | 5 | 10 | 21.6±2.7 |
| Gly-Ala-Gly | 5 | 100 | 21.9±1.4 |

*a) Mean ± s.e. b) $p < 0.05$, versus solvent.*

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in Figs. 1 and 2, kinetic data of digestion of the two peptides with carboxypeptidase Y were indistinguishable. In addition, chromatographic and electrophoretic behavior of two peptides was the same, as demonstrated with gel filtration on a Sephadex G-15 column, ion-exchange chromatography on amino acide analyzing resin, peptide-mapping and polyamide layer chromatography of their dansyl-derivatives, as shown in Table I. As to the biological aspects, native and synthetic peptides were found to be almost equipotent in regard to their antiarrhythmic activities on the continuous cellular fibrillation (Fig. 3) and on the irregular beating with fibrillation (Fig. 4) of myocardial cell clusters induced by low potassium condition, and their promoting effects on spreading phenomenon (Table II) of myocardial single cells in serum-free culture, which were typical effects of the isolated native peptide on cultured myocardial cells.[3)].

As mentioned above, synthetic hexapeptide was found to be chemically and biologically indistinguishable from isolated native AAP. Consequently, it was determined that the amino acid sequence of AAP is Gly-Pro-4Hyp-Gly-Ala-Gly.

The amino acid sequence of AAP was compared with those of several other peptides, especially involved in the regulation of heart rhythmicity, but no similarities were found in regard to the presence of 4Hyp, sequence or size with calcitonin,[12)] insulin,[2)] oxytocin,[2)] glucagon,[13)] some endogenous polypeptides[14)] and synthetic peptides.[15)] The sequence differed also from collagen[16)] and peptides related to collagen.[17)] So far as we know, the amino acid sequence determined AAP is unique and is not contained within any other peptides and proteins.

*Structure-activity Correlation of AAP*

In order to aid in elucidating structure-activity correlation of AAP, effects of several sythetic peptides related to AAP on the arrhythmic movements of myocardial cell clusters induced by low potassium condition and on the spreading phenomenon of myocardial single cells in serum-free culture were assayed. As shown in Table III, only the $NH_2$-terminal tripeptide, Gly-Pro-4Hpy, among the peptides tested, improved incompletely the continuous cellular fibrillation to partial fibrillation at only 500-fold higher concentration than that of AAP, but dit not improve the irregular beating with fibrillation. Gly-Pro, 4Hyp-Gly, Gly-Pro-4Hyp-Gly and Gly-Ala-Gly which are constituents of AAP, never show the activities. Also, Gly-Pro-Leu, Gly-Pro-Leu-Gly-Pro, Gly-Gly-Gly and $(Pro\text{-}Pro\text{-}Gly)_5$ were inactive. On the spreading phenomenon, only Gly-Pro-4Hyp showed also the promoting effect at a 100 ng/ml concentration which is higher than the effective concentration of AAP, but all other AAP constituent peptides tested did not show any activities, as shown in Table IV.

Judging from these results, we consider that the whole molecule of AAP is really essential for the AAP activity on the myocardial cell functions.

Although it is still unclear whether AAP exists as a native hexapeptide or as part of a larger molecule such as collagen in heart, it seems very interesting to ressolve what physiological significances AAP may possess *in vivo*, because this peptide was isolated from atria, improved the rhythmicity of myocardial cells, prolonged the survival and spontaneous activity of myocardial cells in continuous culture with 1% serum,[3b)] contained unique amino acid of 4Hyp which lacks of a corresponding codon and offered much resistance against proteases.

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