DETERMINATION OF IMMUNOREACTIVE ANTIARRHYTHMIC PEPTIDE (AAP) IN RATS

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A sensitive and specific radioimmunoassay for antiarrhythmic peptide (AAP) has been developed, utilizing 3-(4-hydroxy, 5-[^125I]iodophenyl)propionyl AAP and guinea pig anti-AAP serum. Synthetic AAP was used as a standard and the polyethylene glycol method was employed to separate free AAP from antibody-bound AAP. The minimum detectable dose of AAP was 0.2 pmol. In the assay system, immunoreactivity was shown not to be attributable to fragments derived from AAP, gelatin or collagen peptides, which sequences differ only by one amino acid from those of AAP. Immunoreactive (IR) AAP extracted from rat tissues and serum gave parallel dose-response curves to those of AAP. Thus, the AAP equivalents per g or ml of tissues measured in adult male rats were 203 pmol in heart, 166 pmol in kidney, 4 pmol in serum, and 2 to 6 pmol in the other tissues. IR-AAP was separated into two fractions by Sephadex G-25 chromatography. Fr. I was considered to have a larger molecular mass than AAP, while Fr. II appeared to have a molecular mass equal to AAP. Ratios of Fr. II in total IR-AAP were 50% in heart and 10% in kidney, while serum IR-AAP gave only Fr. II. The IR-AAP level in heart showed a positive correlation increase with age in rats.

Keywords — radioimmunoassay; peptide; heart; arrhythmia; thrombosis; collagen

An antiarrhythmic peptide (AAP), isolated from bovine heart\(^1\) and identified as Gly-Pro-Hyp-Gly-Ala-Gly,\(^2\) has a protective effect against experimental arrhythmias\(^1,3\) and experimental thromboses.\(^4\) In this paper, as a first step towards achieving an understanding of the physiological significance of endogenous AAP, a sensitive and specific radioimmunoassay of AAP has been developed and immunoreactive (IR) AAP has been demonstrated in rats.

MATERIALS AND METHODS

Materials — AAP, its terminal fragments and collagen peptides were synthesized by a solid phase method as described previously.\(^5\) After purification by gel filtration on Sephadex G-10 and by Amberlite CG-120 chromatography, the peptides were shown to possess the appropriate integral molar ratios of their constituent amino acids, as well as the expected NH\(_2\)-terminal amino acids. Bovine serum albumin (BSA, fraction V) and gelatin (type IV from calf skin) were products of Sigma Chemical Co. Bolton-Hunter reagent for protein iodination was a product of Amersham. Ethylenediaminetetraacetic acid disodium salt (EDTA) was from Dojin, bovine \(\gamma\)-globulin from Povite Production and Freund's complete adjuvant from Difco Lab.

Preparation of Antiserum — BSA-AAP conjugate was prepared according to the method of Schick and Singer.\(^6\) To 10 ml of 2% BSA solution in 0.1 M phosphate buffer (pH 7.5), 0.2 ml of toluene-2,4-diisocyanate was added at 0 °C. After vigorous stirring at 0 °C for 25 min, the mixture was centrifuged, and the supernatant fluid was allowed to stand for an additional hour at 0 °C. The solution was then incubated with 5

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ml of a 2.5% AAP solution in 0.2 M borate buffer (pH 9.5) at 37 °C for 1 h, followed by the addition of 1 ml of 1 M ammonium carbonate. The reaction mixture wasfractionated on a Sephadex G-25 column (2.5 × 95 cm) using 20 mM ammonium bicarbonate. Fractions containing the conjugate were lyophilized to give BSA–AAP (yield: 200 mg). The average molar ratio of AAP to BSA in the conjugate was approximately 2.3 as calculated from the hydroxyproline content which was obtained by amino acid analysis of acid hydrolyzate of the conjugate.

The protein conjugate was dissolved in saline (5 mg/ml) and emulsified with an equal volume of Freund’s complete adjuvant. To each 5 Hartley male guinea pigs (300 to 350 g weight), approximately 1 mg of the protein conjugate was administered intradermally by multiple injections. After 12 immunizations at 2- or 3-week intervals, antibody production was detected in 2 animals. Antisera were collected from these animals 1 week after the 13th immunization. The serum showing high antibody titer was stored at -80 °C until use.

Radioiodination — Radioiodination was carried out according to the method of Bolton and Hunter,7 AAP (20 μg) in 50 μl of 0.1 M borate buffer (pH 8.5) was added to dried iodinated ester (500 μCi), N-succinimidyld 3-(4-hydroxy, 5-[125I]iodophenyl) propionate, and the reaction mixture was agitated for 15 min at 0 °C, followed by the addition of 0.5 ml of 0.2 M glycine. The labelled AAP, 3-(4-hydroxy, 5-[125I]iodophenyl)propionyl AAP (125I-HPP-AAP), was purified by gel filtration on Sephadex G-10.

Radioimmunoassay Procedure — The assay buffer (diluent) was phosphate-buffered saline (pH 7.5)–25 mM EDTA supplemented with 0.5% BSA and 0.01% sodium metehiolate. The 800 μl incubation mixture contained 100 μl of diluted guinea pig anti-AAP serum, 100 μl of 125I-HPP-AAP, 200 μl of sample or standard synthetic AAP solution and 400 μl of the assay buffer. The mixture was incubated at 4 °C for 2 d, and its radioactivity, total (T), was counted with a γ-scintillation counter. The bound type (B) and free type (F) were separated at 4 °C by additions of 0.2 ml of 1% γ-globulin and 1.0 ml of 25% polyethylene glycol 6000 in 50 mM phosphate buffer (pH 7.5). The mixture was allowed to stand at 4 °C for 10 min and centrifuged at 2200 × g at 4 °C for 20 min. The precipitate (B) was counted, and the B/F ratio was calculated. The B/F ratio in the presence of antiserum was corrected for damage and non-specific coprecipitation of 125I-HPP-AAP by subtracting the (B) observed for the appropriate sample in the presence of normal guinea pig serum. The damage and non-specific coprecipitation were found to be less than 2%, suggesting that the tracer is chemically stable. All samples containing standard were assayed in duplicate or triplicate.

Extraction of Endogenous Samples — Animals used were 1-, 4- and 8- to 10-week-old Wistar male rats. Eight to ten-week-old rats were referred to adult rats. Tissues removed immediately after bleeding were washed by cold saline, weighed, minced and extracted. Serum was separated from blood obtained from the abdominal artery. The cold extraction procedure involved homogenizing minced tissues in 3 volumes of 0.05 N acetic acid using a Biomixer. After homogenizing each sample, the homogenizer was washed with one volume of distilled water which was added to the homogenate. After centrifugation at 16000 × g for 10 min, the supernatant fluid was set aside and the precipitate was washed with one volume of distilled water. The combined supernatant was lyophilized. The assay extract was dissolved in 10 mM phosphate buffer (pH 8.0)–2.5 mM EDTA and, if necessary, centrifuged at 2200 × g for 10 min before being subjected to the assay. All the procedures were carried out at 4 °C. The hot and cold extracting procedures were the same except that, in the former, the homogenate was autoclaved for 10 min following the addition of 10 to 20 μl of n-octyl alcohol. The serum assay samples were native serum and extract prepared by the hot procedure, in which an equal volume
RESULTS AND DISCUSSION

Radioimmunoassay Procedure

$^{125}$I-HPP-AAP was purified by gel filtration on Sephadex G-10, as shown in Fig. 1. Bolton and Hunter recommended that Sephadex should be equilibrated and the column eluted with buffer containing gelatin to minimize the loss of labelled peptides by adsorption due to the binding of low molecular weight labelled products to serum protein and Sephadex. However, we did not use any carrier in gel filtration, since the antibody to AAP might cross-react with gelatin because of the resemblance of the chemical structure. Most of $^{125}$I-HPP-AAP, which was recognized by its reactivity to anti-AAP serum, was eluted at a position of approximately 2 column volumes, probably due to its adhesion to the gel. $^{125}$I-HPP adhered to the gel and was not eluted by the position of 3 column volumes in this gel filtration. Fractions of $^{125}$I-HPP-AAP indicated in Fig. 1 were stored at $-20$ °C until radioimmunoassay use.

The final dilution of anti-AAP serum giving a B/F ratio of 1.0 after incubation of the antiseraum (100 µl) with $^{125}$I-HPP-AAP (10000 cpmp) in a total volume of 800 µl for 2 d, had a range of 1/800 to 1/1600.

When $^{125}$I-HPP-AAP (10000 cpmp) was incubated with 100 µl of antiseraum (final dilution 1/800) in a total volume of 800 µl at 4 °C, the equilibrium state of antigen-antibody reaction was sufficiently established on the 2nd day.

Based on the results mentioned above, the standard procedure for AAP radioimmunoassay was established. Figure 2 represents a standard curve for AAP radioimmunoassay. A characteristic curve was obtained when the B/F ratio was plotted versus AAP dose in log scale. The minimum detectable dose in the system was 0.2 pmol of AAP. All unknowns were assayed as much as possible at three dilutions so as to span the sensitive portions (0.6 to 20 pmol) of the standard curve.

Specificity

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**FIG. 1. Separation of $^{125}$I-HPP-AAP on Sephadex G-10**

The iodination mixture was applied on a column (1.0 × 51 cm), which was eluted with 50 mM phosphate buffer (pH 7.5). Fractions were collected in tubes containing 0.2 ml of 10% BSA in buffer.

a) One hundred µl of antiseraum (final dilution 1/800) was incubated with a 5000 cpmp aliquot of the described fractions in a total volume of 0.8 ml for 2 h.

**FIG. 2. Standard Curve for AAP Radioimmunoassay**
The amino acid sequence of AAP resembles partial sequences of the collagen molecule and collagen-like protein. Since collagen is constructed as a structural and fibrous protein, its soluble degradation products may cause difficulty in cross-reactivity to AAP rather than collagen itself in the determination of endogenous samples. Nine kinds of collagen peptides, whose sequences differ only by one amino acid from those of AAP in the primary structures of collagens α1 chains of rat (residues 1–402) and bovine (residues 403–1011) skins, were synthesized and assayed in AAP radioimmunoassay. Their biological activities were also examined. As shown in Table I, immunoreactivities of all collagen peptides were less than 0.03% of AAP, and there were no peptides which possessed both antiarrhythmic and antithrombotic activities like AAP. Gelatin showed only 0.003% immunoreactivity to AAP. Thus, peptides derived from the collagen molecule were biologically and immunologically different from AAP.

While NH₂-terminal partial sequences, Gly-Pro-Hyp-Gly-Ala and Gly-Pro-Hyp-Gly, which were included in collagen peptides, were devoid of biological and immunological activities, COOH-terminal tetrapeptide possessed 0.31% immunoreactivity and no biological activity. It is well known that the peptide bonds in tetrapeptide, Gly-Pro-Hyp-Gly, have much resistance to peptidases and proteases including collagenase and proline-specific peptidase in animals. These findings were strongly suggestive that most of AAP-related peptides do not compete with ¹²⁵I-HPP-AAP in binding to anti-AAP serum in the system.

**Determination of IR-AAP in Rats**

Validation of extracting procedures for IR-AAP was investigated by measuring recovery of added synthetic AAP from rat heart and serum. As shown in Table II, recovery of added AAP after the hot procedure using 0.05 N acetic acid was more than 90% in heart, while that

**TABLE I. Comparative Biological Activity and Immunoreactivity of AAP and Its Related Peptides**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Antithrombotic activity</th>
<th>Antithrombotic activity</th>
<th>% immunoreactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (saline)</td>
<td>86 ± 4</td>
<td>38.6 ± 4.0</td>
<td></td>
</tr>
<tr>
<td>AAP (Gly-Pro-Hyp-Gly-Ala-Gly)</td>
<td>103 ± 4</td>
<td>20.7 ± 3.1</td>
<td>100</td>
</tr>
<tr>
<td>Gly-Pro-Hyp-Gly-Ser-Ala-Gly</td>
<td>83 ± 7</td>
<td>44.4 ± 0.9</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>Gly-Pro-Hyp-Gly-Glu-Ala-Gly</td>
<td>83 ± 7</td>
<td>26.4 ± 3.5</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>Gly-Pro-Hyp-Gly-Pro-Ala-Gly</td>
<td>84 ± 9</td>
<td>49.6 ± 8.7</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>Gly-Pro-Hyp-Gly-Leu-Ala-Gly</td>
<td>80 ± 4</td>
<td>34.3 ± 4.9</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>Gly-Pro-Hyp-Gly-Ala-Hyp-Gly</td>
<td>81 ± 6</td>
<td>47.0 ± 3.8</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>Gly-Pro-Hyp-Gly-Ala-Asp-Gly</td>
<td>86 ± 8</td>
<td>40.2 ± 3.8</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>Gly-Pro-Hyp-Gly-Ala-Thr-Gly</td>
<td>84 ± 5</td>
<td>43.6 ± 6.7</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>Gly-Pro-Hyp-Gly-Ala-Val-Gly</td>
<td>86 ± 6</td>
<td>30.2 ± 3.8</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>Gly-Pro-Hyp-Gly-Ala-Arg-Gly</td>
<td>85 ± 10</td>
<td>46.1 ± 3.5</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>Hyp-Gly-Ala-Gly</td>
<td>94 ± 11</td>
<td>31.1 ± 2.6</td>
<td>0.31</td>
</tr>
<tr>
<td>Gly-Ala-Gly</td>
<td>88 ± 6</td>
<td>32.6 ± 4.7</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>Gelatin</td>
<td>N.D.</td>
<td>N.D.</td>
<td>0.003</td>
</tr>
</tbody>
</table>

N.D.: Not determined.  

a) Dose: 10 mg/kg, i.v. for AAP and its terminal sequence, 25 mg/kg, i.v. for collagen peptide. Data are means ± S.E. of onset time (s) of arrhythmia after start of aconitate infusion (1.9 μg/0.25 ml/25 g b.w./min) in mice. n=32 for control, n=6 to 12 for peptide.  

b) Dose: 10 mg/kg, i.v. Data are means ± S.E. of thrombus formation (mg) in the extracorporeal shunt of rats. n=7 for control, n=3 to 7 for peptide.  

c) Determined by radioimmunoassay.  

d) p < 0.05, e) p < 0.01: versus control.
TABLE II.  Recovery of Added AAP from Rat Heart and Serum

<table>
<thead>
<tr>
<th>Sample</th>
<th>Added AAP (per g heart or per ml serum)</th>
<th>Extracting procedure $^a$)</th>
<th>% recovery (mean $\pm$ S.E., $n = 3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>10 $\mu$g</td>
<td>Cold 0.05 N acetic acid</td>
<td>33.1 $\pm$ 6.3 $^b$)</td>
</tr>
<tr>
<td></td>
<td>10 $\mu$g</td>
<td>Hot 0.05 N acetic acid</td>
<td>94.0 $\pm$ 5.6 $^b$)</td>
</tr>
<tr>
<td></td>
<td>10 ng</td>
<td>Hot 0.05 N acetic acid</td>
<td>98.5 $\pm$ 8.4 $^c$)</td>
</tr>
<tr>
<td>Serum</td>
<td>10 $\mu$g</td>
<td>None</td>
<td>10.1 $\pm$ 7.2 $^b$)</td>
</tr>
<tr>
<td></td>
<td>10 $\mu$g</td>
<td>Hot 0.05 N acetic acid</td>
<td>90.4 $\pm$ 3.8 $^b$)</td>
</tr>
<tr>
<td></td>
<td>10 ng</td>
<td>Hot 0.05 N acetic acid</td>
<td>92.2 $\pm$ 9.8 $^c$)</td>
</tr>
</tbody>
</table>

$a$) See details in the method.  $b$) The amount of endogenous IR-AAP was neglected.  $c$) The amount of endogenous IR-AAP was subtracted from the determined value.

TABLE III.  IR-AAP Levels in Rats

<table>
<thead>
<tr>
<th>Sample $^a$)</th>
<th>IR-AAP (pmol/g or/ml)</th>
<th>Sample $^a$)</th>
<th>IR-AAP (pmol/g or/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>203.4</td>
<td>Blood vessel</td>
<td>3.6</td>
</tr>
<tr>
<td>Lung</td>
<td>5.5</td>
<td>Fat tissue</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Spleen</td>
<td>5.1</td>
<td>Serum</td>
<td>4.0</td>
</tr>
<tr>
<td>Kidney</td>
<td>165.1</td>
<td>Blood</td>
<td>3.8</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>2.6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$a$) The parts pooled from 10 adult rats were extracted by the hot procedure.

After the cold procedure was one-third of the hot procedure. In serum, more than 90% of added AAP was also recovered after the hot procedure, but only 10% was recovered from native serum. As shown in Fig. 3, the amounts of IR-AAP extracted by the hot procedure from rat heart and serum were linearly related to the amounts of tissue and serum, and their dose-response curves paralleled those of AAP. The B/F ratio was calculated after the 2 d incubation of hot extracts with antibody and $^{125}$I-HPP-AAP. The preincubation of the antibody with the hot extracts for 2 d prior to the 2 d incubation with the $^{125}$I-HPP-AAP did not affect the B/F ratio. The result suggested that there was no interfering substance (i.e. degrading enzymes) of the antibody action. The AAP equivalents extracted from heart by the cold procedure were, however, much lower than those extracted by the hot procedure and were not related to the amounts of tissue, especially in a high concentration of extract. The B/F ratio obtained was not in the range of 0 to 1.2 (which was recorded in the standard assay), when native serum was subjected to the assay. Poor recovery of added AAP from heart by the cold procedure and from native serum may be a result of high concentration of large molecular size substances interrupting the antigen-antibody reaction and/or the separation of B and F by the polyethylene glycol method. Therefore, all endogenous samples for the assay were prepared by the hot extracting procedure.

Figure 4 shows inhibition curves produced by extracts of rat endogenous samples, and Table III shows IR-AAP levels in rats, which were calculated from their inhibition curves in Figs. 3 and 4. The antigen-antibody interaction was not
affected by the extracts of tissues and blood (used in Table III), like hot extracts from heart and serum. The highest level of IR-AAP was found in heart (203 pmol/g), followed by kidney IR-AAP contents were assayed. In regard to the immunoreactivity, 90 to 100% of applied extract was recovered after the gel filtration. As shown in Fig. 5, immunoreactivities in extracts of heart and kidney were separated into Fr. I, which was eluted at the position of void volume and Fr. II which was eluted at the same position as that of AAP. Immunoreactivity of heart Fr. II reached 50% of IR-AAP in extract, and only 10% in kidney. In serum all were derived from Fr. II. Figure 6 shows inhibition curves produced by Sephadex G-25 fractions of extracts of rat heart, kidney and serum. All fractions gave dose-response curves paralleled to those of AAP. Considering the fact that AAP-related peptides showed little immunoreactivity in the assay system as shown in Table I, it was highly probable that Fr. II contained AAP itself. Although the chemical properties of Fr. I were not clear, our interpretation of the data was that a large molecular size substance such as an AAP precursor or an AAP-binding protein was eluted in the position of Fr. I. In any event IR-AAP was present in the highest concentration in heart, serum were fractionated with Sephadex G-25 column chromatography. This was the first step used in the purification of AAP from bovine atria.\(^1\)\(^2\) Extracts were applied on the same column, which was calibrated with synthetic AAP. The eluates were lyophilized and their G-25 fractions of extracts of rat heart, kidney and serum. All fractions gave dose-response curves paralleled to those of AAP. Considering the fact that AAP-related peptides showed little immunoreactivity in the assay system as shown in Table I, it was highly probable that Fr. II contained AAP itself. Although the chemical properties of Fr. I were not clear, our interpretation of the data was that a large molecular size substance such as an AAP precursor or an AAP-binding protein was eluted in the position of Fr. I. In any event IR-AAP was present in the highest

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**FIG. 3. Inhibition Curves Produced by Extracts of Rat Heart and Serum**

The solid line is the standard curve. Tissues were pooled from 10 adult rats.

(○), heart extract by the hot procedure; (●), heart extract by the cold procedure; (△), serum extract by the hot procedure; (▲), native serum.

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**FIG. 4. Inhibition Curves Produced by Rat Endogenous Samples**

The solid line is the standard curve. Tissues pooled from 10 adult rats were extracted by the hot procedure.

(○), kidney; (●), lung; (△), spleen; (▲), blood; (□), skeletal muscle; (■), fat tissue.
FIG. 5. Gel Filtration of Extracts of Rat Heart, Kidney and Serum on Sephadex G-25 Column
Tissues pooled from 7 adult rats extracted by the hot procedure. A three ml aliquot of extracts (1 g tissue/ml, 2 ml serum/ml) was applied on the column (1.2 × 160 cm), which was eluted with distilled water.
a) Elution area of synthetic AAP. 
(○), heart extract; (△), kidney extract; (□), serum extract.

FIG. 6. Inhibition Curves Produced by Sephadex G-25 Fracions of Extracts of Rat Heart, Kidney and Serum
The solid line is the standard curve. Fractions were obtained from the gel filtration in Fig. 5.
(○), heart Fr. I; (●), heart Fr. II; (△), kidney Fr. I; (▲), kidney Fr. II; (■), serum Fr. II.

FIG. 7. IR-AAP Levels in Hearts of 1-, 4- and 10-Week-Old Rats
Hearts were extracted by the hot procedure. Each point shows the level determined for extract of hearts, which were pooled from 5 to 30 rats in every group.
(○), per g heart; (●), per heart.

concentration in heart.

The IR-AAP level (203 pmol/g) found in rat heart was about one half of the amount of that in bovine atrial level (420 pmol/g) as estimated from the purification yield. The higher level of IR-AAP seen in kidney coincided with the fact that 14C-AAP injected i.v. in mice mainly distributed in kidney. Not merely excreted in urine, the kidney AAP might have some physiological roles different from heart AAP.

Figure 7 shows IR-AAP levels in hearts of 1-, 4- and 10-week-old rats. Total IR-AAP in heart increased with age. The concentrations of IR-AAP in heart increased greatly from 1- to 4-week-old rats, and then slightly to 10-week-old rats.

proved to be sensitive and specific for AAP. Practical utilization of the system in determination of AAP in various body fluids and tissues may provide important information about its physiological significance.

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