A potent platelet antiaggregant action of an antiarrhythmic peptide (AAP) was demonstrated to be a cause of the antithrombotic effect. AAP (10, 20 or 40 mg/kg, i.v.) inhibited ex vivo platelet aggregation induced by collagen in a dose-dependent manner. AAP also inhibited the platelet aggregation of platelet-rich plasma (PRP) induced by collagen, Ca-ionophore A-23187, adenosine diphosphate (ADP), thrombin or arachidonic acid in vitro. The IC$_{50}$ was 2.5 mM for collagen, 1.7 mM for A-23187, 5 mM for ADP, 0.4 mM for thrombin and 0.15 mM for arachidonic acid. The aggregation inhibitory activity of the peptide on washed platelet (WP), in a Ca$^{2+}$-free medium, was stronger than on PRP. The IC$_{50}$ was 1 mM for collagen and 20 μM for A-23187. No significant difference was found between antiaggregant activities of platelet-free plasma (PPP) from AAP-treated rats and PPP from normal rats supplemented with AAP. The direct action of AAP on platelets was also supported by the incorporation of AAP into platelet cytoplasm which caused a decrease of Ca$^{2+}$-dependent 3',5'-cyclic nucleotide phosphodiesterase (Ca-PDE) activity. It was considered that AAP showed its platelet aggregation inhibitory activity by decreasing intracellular Ca$^{2+}$ concentration through the inhibition of Ca-PDE activity.

**Keywords** — peptide; arrhythmia; thrombosis; platelet; calcium ion; phosphodiesterase; calcium-ionophore

A hexapeptide (AAP), isolated from bovine atria, and identified as Gly-Pro-Hyp-Gly-Ala-Gly, has a protective effect against experimental drug-induced heart arrhythmias via the improvement of permeability of ions through the myocardial cell membrane. AAP was also effective in various thrombosis models including white thrombus formation in the extracorporeal shunt, coronary thromboembolism, pulmonary thrombosis and peripheral arterial thrombosis, in which platelets were mainly involved. In the present study, it was demonstrated that the potent platelet antiaggregant action of AAP was a cause of the antithrombotic effect.

**MATERIALS AND METHODS**

**Materials** — Synthetic AAP prepared in our laboratory was used in this study. AAP dissolved in saline and adjusted to pH 7.3 was used throughout the experiment except for the determination of Ca$^{2+}$-dependent 3',5'-cyclic nucleotide phosphodiesterase (Ca-PDE). Collagen (type I from bovine achilles tendon), bovine albumin (BA, Fr.V), arachidonic acid sodium salt (approx. 99%, from porcine liver) and adenosine 3',5'-cyclic monophosphate (cyclic AMP) were products of Sigma Chemical Co. Adenosine 5'-diphosphate disodium salt (ADP, Kohjin Co., Ltd.), Ca-ionophore, A-23187 (Calbiochem-Behring Co.), thrombin (Mochida Seiyaku Ltd.), diethylaminoethyl (DEAE)-cellulose (DEAE-SH, Serva), Sepharose 4B (Pharmacia) and glutaraldehyde (EMS-51) were obtained from Nakarai Chemicals Ltd. HEPES (Dojin), Somnopentyl (sodium pentobarbital, Pitmann-Moore Inc.), ethylenediamine tetracetic acid disodium salt (EDTA, Dojin) and heparin sodium salt (165 units/mg) were obtained from Wako Pure Chemical Ind., Ltd.

**Platelet Preparation** — Blood was collected from the abdominal artery of healthy Wistar male rats weighing 250 to 350 g under pentobarbital anesthesia (30 mg/kg, i.p.) into a plastic syringe containing 3.008% sodium citrate (1/10 volume of the blood). The citrate blood was centrifuged at 270 g for 10 min at room temperature to obtain platelet-rich plasma (PRP). The residual blood cell precipitate was further centrifuged at 2000 g for 15 min at 4 °C to obtain platelet-poor plasma (PPP). The PPP was centrifuged at 20000 g for 45 min at 4 °C to obtain platelet-free plasma (PPP). Using a platelet counter (Sysmex PL-100), the platelet concentration of PRP was adjusted to approximately 10$^9$ platelets/ml by adding PPP. For arachidonic acid-induced platelet aggregation, blood was...
Anti-platelet Action of AAP

prevented from coagulation by the addition of a heparin solution (30 units/ml, 1/10 volume of the blood) instead of the citrate solution.

Washed platelets (WP) were prepared by two methods, one of which was that of Timmons et al. Briefly, PRP was chromatographed on a Sepharose 4B column using HEPES buffer (NaCl 8 g, KCl 0.2 g, MgCl₂ 6H₂O 0.2 g, glucose 1 g, BA 3.5 g, Na₂HPO₄ 0.45 g and HEPES 0.9 g/l, pH 7.4). Fractions in the void volume were centrifuged at 1500 g for 5 min at 4 °C. The platelet precipitate was resuspended in HEPES buffer, HEPES buffer containing 2 mM Ca²⁺ or PFP (10⁹ platelets/ml) and was referred to as gel-filtered platelets (WP-GF). The second procedure to obtain WP preparation was as follows: PRP was added to an equal volume of 25 mM tris-HCl buffer (pH 7.4)-130 mM NaCl (TBS) containing 1.5 mM EDTA and centrifuged at 1500 g for 10 min at 4 °C. The platelet was washed again with TBS containing 1.5 mM EDTA and then with TBS containing 0.5 mM EDTA (1500 g for 5 min at 4 °C). Finally, the platelet precipitate was resuspended in various media (10⁹ platelets/ml) and referred to as the centrifuged platelet (WP-C).

Platelet Aggregation — Platelet aggregation was measured photometrically by a Payton aggregation module at 37 °C while stirring at 1100 rpm. Aggregation was initiated by adding less than 10 µl of an aggregation agent to the mixture of 10 µl of sample and 250 µl of PRP or WP suspension. The agents included collagen (stock solution: 27 mg/ml), A-23187 (1 mM), ADP (87 µM), thrombin (26 NIH units/ml) and arachidonic acid (26 mM). TBS was used as a solvent or diluent of the agent, except for A-23187 which was dissolved in methanol.

Incorporation of AAP into Platelet — WP-C (10⁹ platelets/ml) resuspended in HEPES buffer was incubated with AAP (1 mM) at 37 °C for a prescribed time. The reaction mixture was chilled and then centrifuged at 1500 g for 10 min at 4 °C, after the addition of two volumes of cold TBS containing 0.3 mM EDTA. The precipitate was washed twice with the same buffer. One half of the platelets were subjected to an AAP radioimmunoassay. The residual half were resuspended in 25 mM potassium phosphate buffer (pH 7.4) and glutaraldehyde was introduced (final concentration 0.05%), followed by incubation for 30 min at 4 °C with gentle agitation. The procedure fixed unwashed free AAP onto the platelet cell membrane. The platelet suspension was centrifuged at 1500 g for 10 min at 4 °C, the platelets resuspended in phosphate buffer, and sonicated for 20 min/ml at 4 °C. The cytoplasm, recovered in the supernatant fluid of the sonicated platelet after centrifugation at 105000 g for 45 min at 4 °C, was subjected to a radioimmunoassay. The radioimmunoassay of AAP was performed as described in our previous paper. The assay sample was prepared by the hot extracting procedure using 0.05 N acetic acid.

Determination of Ca-PDE Activity — Ca-PDE was extracted by the method of Hidaka et al. WP-C was homogenized in 50 mM tris-HCl buffer (pH 7.4) containing 1 mM MgCl₂ using a teflon homogenizer and was sonicated (30 s/ml) followed by centrifugation at 105000 g for 60 min at 4 °C. The supernatant fluid was applied to a DEAE-cellulose column (1.5 x 20 cm) equilibrated with 50 mM tris-acetate buffer (pH 6.0) containing 3.75 mM 2-mercaptoethanol. The column was developed with several column volumes of the buffer containing 2-mercaptoethanol, followed by a linear gradient elution from 0 to 1 M sodium acetate in a total volume of 300 ml at a flow rate of 18 ml/h at 4°C. The Ca-PDE fraction was monitored by absorbance at 280 nm and by measuring Ca-PDE activity using a calmodulin preparation obtained from rat brain. An appropriate dilution of Ca-PDE was incubated in 50 mM tris-HCl buffer (pH 8.0) containing 5 mM MgCl₂, 50 µg of BA, 10 or 100 µM cyclic AMP and sample in total volume of 0.5 ml. After 20 min at 30 °C, the reaction was terminated by boiling the mixture for 5 min. After centrifugation at 1500 g for 10 min at 4 °C, the remaining cyclic AMP in the supernatant fluid was assayed using a cyclic AMP assay kit to estimate Ca-PDE activity.

RESULTS

Effect of AAP on Platelet Aggregation

PRP was prepared from rats given AAP intravenously at a dose of 10, 20 or 40 mg/kg 5 min before the blood collection, and the platelets were subjected to collagen-induced platelet aggregation. AAP inhibited the extent, the rate and the onset of platelet aggregation in a dose dependent manner (Fig. 1). To clarify that AAP
interacts with a receptor or with other components of the effector mechanism to inhibit the action of collagen, we plotted the log dose-effect curves for collagen in ex vivo experiments. AAP caused a decrease in the maximum height of the extent of platelet aggregation caused by collagen, suggesting that the effect was not a competitive inhibition (Fig. 2). In the case of collagen (10 µg/ml)-induced platelet aggregation, the ID$_{50}$ was in the range of 20 to 40 mg/kg.

**FIG. 1. Effect of AAP on Tracing Profiles of Collagen-Induced Platelet Aggregation of PRP ex Vivo**
Collagen, 10 µg/ml; A, Saline; B-1, AAP (10 mg/kg, i.v.); B-2, AAP (20 mg/kg, i.v.); B-3, AAP (40 mg/kg, i.v.).

**FIG. 2. Effect of AAP on Collagen-Induced Platelet Aggregation of PRP ex Vivo**
AAP was given i.v. 5 min before blood collection. Each point shows the mean ± S.E. of 12 experiments. ( ), Saline; ( ), AAP (10 mg/kg); ( ), AAP (20 mg/kg); ( ), AAP (40 mg/kg).

Normal rat PRP was preincubated with AAP in vitro at 37 °C for 3 min and the AAP-treated PRP was subjected to various drug-induced platelet aggregations. The concentration of each drug utilized was the dose which caused 50—60% of platelet aggregation. The peptide inhibited the extent, the rate and the onset of

**FIG. 3. Effect of AAP on Tracing Profiles of Platelet Aggregation of PRP in Vitro**
Collagen, 10 µg/ml; A-23187, 2 µM; ADP, 3.5 mM; thrombin, 0.8 U/ml; arachidonic acid, 1 mM. A, saline; B-1, AAP (0.2 mM); B-2, AAP (1 mM); B-3, AAP (2 mM).
platelet aggregation induced by collagen or arachidonic acid, and the extent and the rate of the platelet aggregation induced by A-23187, thrombin or ADP (Fig. 3). In the thrombin-induced platelet aggregation, AAP did not affect the coagulation (Fig. 3). As shown in Fig. 4, the inhibition caused by AAP was characteristic of the drugs tested for platelet aggregation. The IC$_{50}$ of AAP in the platelet aggregation of PRP caused by collagen, A-23187, ADP, thrombin and arachidonic acid in vitro was 2.5, 1.7, 5, 0.4 and 0.15 mM, respectively.

In the next experiment, WP-GF obtained from normal rats was preincubated with AAP in vitro at 37°C for 3 min in HEPES buffer and was subjected to collagen- and A-23187-induced platelet aggregation tests. The peptide also showed inhibitory activity on the extent and the rate of platelet aggregations in these models (Fig. 5). The inhibitory actions observed were stronger in these models than in those models used in the platelet aggregation of PRP. The effect of AAP on A-23187-induced platelet aggregation in HEPES buffer containing 2 mM Ca$^{2+}$ and in PFP was the same. The IC$_{50}$ in HEPES buffer was 1 mM for collagen and 20 μM for A-23187 (Fig. 6).

In order to clarify whether AAP acts directly on platelets only, PFP obtained from AAP-treated rats and PFP obtained from normal rats and treated with AAP in vitro were compared for its effect on collagen-induced platelet aggregation. To obtain an equal concentration of AAP level in both of the PFP preparations, we calculated the concentration of AAP using the results of the pharmacodynamics of (1-14C) acetylamidino AAP (14C-AAP). $^{51}$ 14C-AAP given i.v. to mice was distributed in the body according to a one compartment model and its half life in blood was 10 min. $^{51}$ The incubation of platelets with 1.1 mM (0.1 mM) AAP for 3 min in vitro was calculated to correspond to the incubation with PFP collected 5 (120) min after AAP administration in a dose of 20 (40) mg/kg, i.v. using the area under the blood concentration versus time curve (AUC). The concentration of AAP in PFP was almost 0.87 mM 5 min after AAP (20 mg/kg, i.v.) was administered. WP-GF (10$^9$ platelets/ml) was preincubated with either PFP from AAP-treated rats, PFP from normal rats and treated with AAP or HEPES buffer containing AAP at 37°C for 3 min and the effects of the incubation on collagen-induced platelet aggregation were examined. The PFP from rats which received AAP (20 mg/kg, i.v.) 5 min before the PFP from normal rats and treated with AAP (1.1 or 0.87 mM) showed almost the same inhibitory effect (ca. 17%) on the platelet aggregation.

**FIG. 4. Effect of AAP on Platelet Aggregation of PRP in Vitro**
Each point shows the mean ± S.E. of 3 experiments. (○), Collagen (10 μg/ml); (●), A-23187 (2 μM); (△), ADP (3.5 mM); (▲), thrombin (0.8 U/ml); (□), arachidonic acid (1 mM).

**FIG. 5. Effect of AAP on Tracing Profiles of WP-GF Aggregation**
Collagen, 10 μg/ml; A-23187, 4 μM. A, Saline; B-1, AAP (0.1 mM); B-2, AAP (1 mM).
TABLE I. Effect of PFP from AAP-Treated Rats on WP-GF Aggregation in Rats

<table>
<thead>
<tr>
<th>Mediuma)</th>
<th>Inhibition (%) of extent of aggregation (mean ± S.E. of 6 experiments)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PFP from AAP-treated rats (20 mg/kg, i.v.) 5 min before blood collection</td>
<td>17.8 ± 3.0</td>
</tr>
<tr>
<td>Normal rat PFP added to AAP (1.1 mM)</td>
<td>17.6 ± 1.8</td>
</tr>
<tr>
<td>Normal rat PFP added to AAP (0.87 mM)</td>
<td>16.5 ± 1.4</td>
</tr>
<tr>
<td>HEPES buffer added to AAP (1.1 mM)</td>
<td>40.5 ± 2.4</td>
</tr>
<tr>
<td>PFP from AAP-treated rats (40 mg/kg, i.v.) 120 min before blood collection</td>
<td>0.0 ± 2.8</td>
</tr>
<tr>
<td>Normal rat PFP added to AAP (0.1 mM)</td>
<td>1.1 ± 1.8</td>
</tr>
<tr>
<td>HEPES buffer added to AAP (0.1 mM)</td>
<td>3.1 ± 1.2</td>
</tr>
</tbody>
</table>

a) Normal rat WP-GF was suspended in each medium and incubated at 37°C for 3 min, followed by a collagen (10 μg/ml)-induced platelet aggregation test.

while HEPES buffer added AAP (1.1 mM) showed a stronger inhibitory effect (ca. 41%)(Table I). However, PFP from AAP administered (40 mg/kg, i.v.) rats 120 min prior to the blood collection showed no inhibitory effect on the platelet aggregation as well as normal rat PFP and HEPES buffer added with AAP (0.1 mM) (Table I).

**Incorporation of AAP into Platelet**

The incorporation of AAP into platelet was examined by incubating AAP (1 mM) with WP-C at 37 °C in vitro. The incorporation of AAP into platelet cytoplasm reached equilibrium state within 1 min (Fig. 7). The amount of AAP incorporated into platelet plasma was not different from that incorporated into whole platelet at 3 min (Fig. 7).

**Effect of AAP on Ca-PDE Activity**

The effect of AAP on hydrolysis of cyclic AMP by Ca-PDE was examined using WP-C prepared from normal rats. AAP inhibited the

---

**FIG. 6. Effect of AAP on WP-GF Aggregation**

Each point shows the mean ± S.E. of 3 experiments. (○), Collagen in HEPES buffer (10 μg/ml); (●), A-23187 in HEPES buffer (4 μM); (△), A-23187 in HEPES buffer containing 2 mM CaCl₂ (2 μM); (▲), A-23187 in PFP (2 μM).

**FIG. 7. Incorporation of AAP into Platelet in Vitro**

Each point shows the mean ± S.E. of 3 experiments. (○), Whole platelet; (●), 105000 g supernatant fluid.

**FIG. 8. Effect of AAP on Ca-PDE Activity**

Each point shows the mean ± S.E. of 4 experiments.
Ca-PDE activity in a dose dependent manner and the ED$_{50}$ was 40 $\mu$M. AAP at a dose of 100 $\mu$M almost completely inhibited the Ca-PDE activity (Fig. 8). A Dixon-plot analysis of the result of this experiment revealed that AAP inhibited the Ca-PDE activity in a noncompetitive and an uncompetitive manner (Fig. 9).

**DISCUSSION**

The present study revealed that AAP has a potent antiaggregant activity against platelet aggregation induced by various agents. Since collagen at a dose of 10 $\mu$g/ml induced about 80% of maximum aggregation (Fig. 2), the effect of AAP in the collagen-induced platelet aggregation was determined at this dose. AAP inhibited ex vivo collagen-induced platelet aggregation in a dose-dependent manner, and the IC$_{50}$ on collagen (10 $\mu$g/ml)-induced platelet aggregation was found to be in the range of 20 to 40 mg/kg, i.v. In our previous paper, we reported that intravenously administered AAP significantly inhibited thrombus formation in the extracorporeal shunt model in rats. The ID$_{50}$ value reported for thrombus formation was 30 mg/kg which is very similar to that of the ex vivo platelet aggregation. In this model, white thrombus was formed by platelet adhesion to silken threads and subsequent aggregation under inhibition of blood coagulation by continuous injection of heparin. This evidence strongly suggested that AAP showed antithrombotic action via its platelet antiaggregant effect in vivo. In in vitro experiments, the inhibition of platelet aggregation of PRP was most clearly observed when arachidonic acid was used and the inhibition was weaker in the order of use of thrombin, A-23187, collagen and ADP. Recently, a mechanism of platelet activation has been proposed to involve factors such as receptor binding, phosphatidylinositol (PI) response, arachidonic acid cascade, intracellular Ca$^{2+}$ mobilization, platelet cytoskeleton system and their mutual couplings. It is known that arachidonic acid activates platelet via its direct participation in arachidonic acid cascade coupled with PI response, thrombin via intracellular Ca$^{2+}$ mobilization and PI response through receptor binding. A-23187 via intracellular Ca$^{2+}$ mobilization and extracellular Ca$^{2+}$ influx, collagen via PI response through receptor binding and ADP via phospholipase (PLase) C activation through receptor binding and its direct participation in arachidonic acid cascade. Considering these factors, it seemed that AAP interacted with a substance existing inside the platelet rather than on the cell membrane. AAP showed a strong platelet aggregation inhibitory activity when WP suspended in Ca$^{2+}$ free media was used. In particular, the inhibitory activity of AAP on the A-23187-induced platelet aggregation model was 85 times stronger than that shown in the platelet aggregation of PRP in the presence of extracellular Ca$^{2+}$. The PI response enhanced by thrombin, collagen and ADP is closely associated with the increase of cytosolic free Ca$^{2+}$. On the other hand, A-23187 can activate platelet without the presence of extracellular Ca$^{2+}$ and does not couple with the rapid PI response. Moreover, A-23187 can directly release Ca$^{2+}$ from a dense tubular system (DTS). Therefore, the present results strongly suggest that AAP interacts with intracellular Ca$^{2+}$ mobilization.

PFP obtained from AAP-treated rats was used to investigate whether the activity of AAP in vivo derived only from its direct action on platelet or involved a second effect in the body. As shown in the results, there was no significant difference between antiaggregant activities of PFP from AAP-treated rats and PFP from normal rats and treated with AAP. HEPES buffer containing AAP showed stronger antiaggregant activity than did AAP added to PFP. This difference can be explained by the fact that the action of AAP was emphasized in the Ca$^{2+}$-free medium.

Supported by the results that AAP was incorporated into platelet plasma and could directly decrease the Ca-PDE activity, AAP was regarded to have a direct effect on platelets, interacting
with intracellular Ca\(^{2+}\) mobilization. The A-23187-induced platelet activation is inhibited only by cyclic AMP.\(^{12b,15b}\) It is a well-known fact that cyclic AMP is biologically synthesized by adenylyl cyclase and is hydrolyzed by phosphodiesterase.\(^{11}\) Adenylyl cyclase is activated by an increase of intracellular Ca\(^{2+}\) concentration, while phosphodiesterase activity is regulated by both intracellular Ca\(^{2+}\) and calmodulin.\(^{11}\) In the cyclic AMP metabolism, AAP inhibited the Ca-PDE activity via its direct action on the site of Ca-PDE, which differed from both calmodulin and cyclic AMP binding sites (Fig. 9). It is highly probable that AAP must decrease Ca\(^{2+}\) concentration via cyclic AMP uptake in platelets. Although it is difficult to directly compare the dose efficiency of AAP on the platelet aggregation and Ca-PDE activity (since both experimental conditions are different), it seemed that the minimum concentration which caused the inhibition of Ca-PDE activity was higher than that of the platelet aggregation. Therefore, it was assumed that AAP may also relate to different kinds of Ca\(^{2+}\)-dependent enzymes or Ca\(^{2+}\)-binding proteins other than Ca-PDE.

Acknowledgement We are grateful to Miss Kaori Ikeda for her technical assistance.

REFERENCES


