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Antithrombotic effect of β,β′-monochloromethylene diadenosine 5′,5‴-P^1,P^4-tetraphosphate

(antiplatelet aggregation)

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ABSTRACT **The feasibility of using β,β′-monochloromethylene diadenosine 5′,5‴-P^1,P^4-tetraphosphate (AppCHClppA) as an antithrombotic agent was studied in a rabbit intracarotid cannula thrombosis model previously shown to be sensitive to antiplatelet agents. This analogue, having a *P*-C-*P* bridge in place of a *P*-O-*P* internucleoside linkage, has been found resistant to phosphodiesterase activity. Rabbits were infused with the dinucleotide at a dose of 50 mg per kg over a 2-hr period, at a controlled rate by pump. A 1-cm length of polyethylene cannula (1 mm i.d.) was tied into the carotid artery. Animals were stable under general anesthesia during the entire period of the experiment. In the control group, 16 of 20 animals formed clots, an incidence of 80%, whereas in the test animals, 6 of the 20 formed clots (30% incidence, $P < 0.05$). After preincubation of whole blood with 50 μM AppCHClppA at 37°C for up to 3 hr, a consistent suppression of ADP-induced platelet aggregation was observed. The present study suggests that AppCHClppA may be useful as an antithrombotic agent in certain clinical situations, such as hemodialysis, arteriovenous shunts, and introduction of artificial heart valves. It may also possibly prevent extension of recent clots. The toxicity and metabolism of AppCHClppA have, however, yet to be explored.**

Diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A) was found in 1966 in the back reaction of amino acid activation by lysyl-tRNA synthetase, lysine, and ATP (1, 2). It had earlier been discovered as a byproduct of the chemical synthesis of ATP (3). The following information has now been demonstrated: (*i*) Ap_4A is a ubiquitous component of living cells (4); (*ii*) intracellular concentrations of Ap_4A vary, depending on the cell-growth cycle (5); (*iii*) there is a relatively high Ap_4A content in blood platelets, in which Ap_4A is stored exclusively in dense granules (6); (*iv*) Ap_4A is a competitive inhibitor for ADP-induced platelet aggregation (7–9); (*v*) Ap_4A has a short half-life in the blood due to degradation by phosphodiesterase (10); and (*vi*) Ap_4A has antithrombotic effects in a rabbit carotid artery model (10). The background and current status of studies on Ap_4A have recently been reviewed (11).

Phosphonate analogues of Ap_4A are known to be resistant to phosphodiesterase activity (12, 13). We have found that substitution of the oxygen bridge in the β,β′ position of Ap_4A by -CHCl- (β,β′-monochloromethylene diadenosine 5′,5‴-P^1,P^4-tetraphosphate; AppCHClppA) results in a potent inhibitory agent for ADP-induced platelet aggregation, superior to that of Ap_4A. The feasibility of using AppCHClppA as an antithrombotic agent in a rabbit model has now been further studied, in extension of preliminary work (14).

MATERIALS AND METHODS

New Zealand White rabbits (male) weighing 2 kg were anesthetized with ketamine hydrochloride. AppCHClppA or saline was infused continuously for 2 hr via a marginal ear vein. A segment of common carotid artery was isolated and clamped. A 1-cm length of polyethylene tubing (1-mm i.d.; intramedic polyethylene tubing PE-90, Clay Adams) was inserted and secured by silk ligatures; blood flow was then reestablished by removing the clamps. The polyethylene tube was removed at the end of infusion, and the presence or absence of clot inside the cannula aperture was examined as the endpoint.

A Simplate bleeding-time device was used (Organon Teknika) (15). After shaving the dorsal surface of the ear, the site was carefully selected under an illuminator to avoid gross vasculature. Bleeding time was measured under free flow, devoid of pressure. A Chrono-Log whole-blood platelet aggregometer (model 530) equipped with a recorder (Chrono-Log, Havertown, PA) was used for measurement of platelet aggregation. This instrument detects platelet aggregation in whole blood by measuring the electrical impedance between two electrodes (16). Incidence of clot formation in the intracarotid cannula in the two groups was compared by the χ^2 test; the unpaired *t* test was used to compare prolongation of bleeding time in the different groups. AppCHClppA was synthesized as described (12).

RESULTS

Four different concentrations of AppCHClppA were tested for inhibition of ADP-induced platelet aggregation in both human and rabbit blood (Fig. 1). The blood was preincubated in the aggregometer for 1 min with the desired concentration of inhibiting agent, and then 50 μM ADP was added to induce platelet aggregation. As concentration of inhibitor was increased, aggregability was proportionately suppressed, indicating a dose-dependent inhibition. The ID_{50} values (doses that induce 50% of maximal inhibition) for 50 μM ADP in this experiment were 19 μM in human blood and 29 μM in rabbit blood, respectively. Similar results (21 μM and 28 μM in human and rabbit blood, respectively) were observed in another experiment.

In one experiment, the *in vitro* preincubation of rabbit blood with 100 μM AppCHClppA at 37°C was extended to 3 hr. Complete suppression of aggregation after a 2-hr preincubation and 95% suppression at 3 hr was seen (Fig. 2*A*). In another experiment, 100 mg of AppCHClppA dissolved in 10 ml saline was slowly infused at a controlled rate by pump throughout a 2-hr period in a 2-kg rabbit; blood samples were then obtained. Blood platelets of this postinfusion sample

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Abbreviations: Ap_4A, diadenosine 5′,5‴-P^1,P^4-tetraphosphate; AppCHClppA, β,β′-monochloromethylene diadenosine 5′,5‴-P^1,P^4-tetraphosphate.

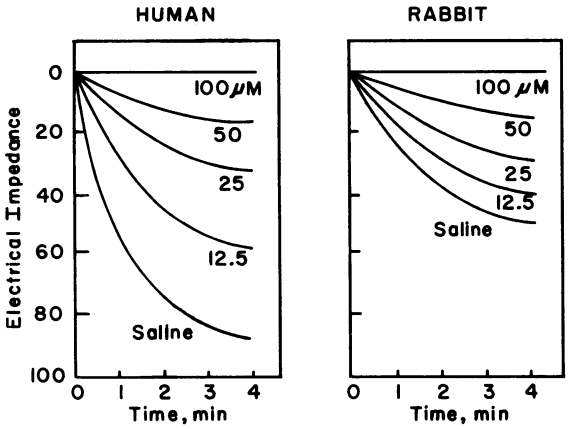

FIG. 1. Inhibitory effects of AppCHClppA on ADP-induced whole-blood platelet aggregation. Fresh normal blood anticoagulated with 0.38% sodium citrate was examined for inhibition of ADP-induced aggregation by AppCHClppA. An aggregometer designed to detect platelet aggregation in whole blood by continuously recording the electrical impedance between two electrodes (15) was used. The concentration of ADP to induce aggregation was 50 μM. Four different concentrations of AppCHClppA were examined for their inhibitory effects in both human and rabbit blood.

also did not respond to 50 μM ADP stimulation. A control blood sample after saline infusion showed a good platelet-aggregation response to the same amount of ADP (Fig. 2*B*).

The antithrombotic effect of AppCHClppA was determined in a rabbit model with the intracarotid cannula technique (10). Twenty rabbits were infused at a constant rate with 100 mg of the dinucleotide in 10 ml of saline over a 2-hr period. The presence or absence of clot formation in the cannula at the end of infusion was examined. The incidence of thrombosis in the AppCHClppA-treated group was 30% (6 out of 20 rabbits). Sixteen rabbits were infused with a single bolus dose of 30 mg of AppCHClppA in 5 ml of saline and were evaluated for clot formation inside the intracarotid cannula, in the same way as in the previous group. Incidence of clot formation in the latter group was 38% (6 out of 16 rabbits). The incidence of thrombosis in both groups treated with AppCHClppA was significantly lower than that with saline infusion in the control group (80% incidence, 16 out of 20 rabbits) (Table 1).

A series of microvascular bleeding times was measured before treatment, during infusion, and at the end of infusion

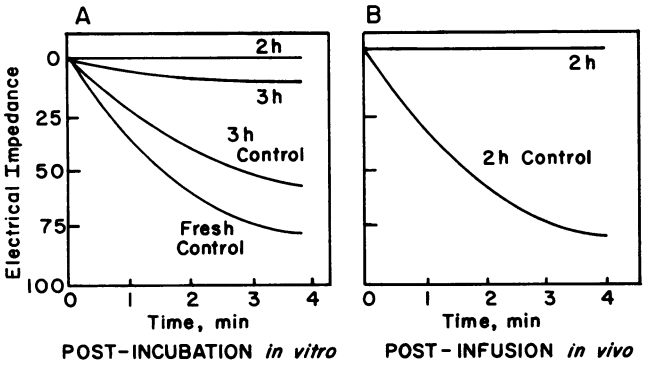

FIG. 2. Inhibition of ADP-induced whole-blood platelet aggregation. (*A*) Normal fresh rabbit blood was anticoagulated with 0.38% sodium citrate and incubated with 100 μM AppCHClppA or saline (control) at 37°C for 3 hr (h) of incubation, and ADP-induced (50 μM) platelet aggregation was measured in a whole-blood aggregometer. (*B*) Aggregation curves of blood samples taken at the end of a continuous 2-hr infusion of either saline control or inhibiting agent (50 mg per kg) in rabbits.

Table 1. Antithrombotic effects of AppCHClppA in rabbit intracarotid-cannula model

	Rabbits tested, no.	Rabbits with thrombi, no.	Incidence of thrombosis, %
AppCHClppA			
100 mg (2 hr)	20	6	30*
30 mg (1 min)	16	6	38*
Saline control	20	16	80

*$P < 0.05$.

of AppCHClppA in 10 rabbits and also in 5 saline-control rabbits (Table 2). Normal bleeding time (preinfusion) of the rabbits under anesthesia was 104 ± 23 sec (15 determinations). The rabbits receiving AppCHClppA infusion were divided into two subgroups: (A) four animals that exhibited intracannular thrombosis and (B) six animals that did not exhibit intracannular thrombosis. The latter group showed a moderate prolongation of bleeding time during infusion and also at the end of infusion. The former group showed a mild prolongation of bleeding time during infusion, but bleeding time returned to normal by the end of infusion. There was no change in bleeding time of the control group given saline infusion. The *t* test revealed significant prolongation ($P < 0.05$ level) of the bleeding time in both groups during infusion and in the group without thrombosis at the end of infusion, compared to the respective bleeding time seen in the saline control group (Table 2).

DISCUSSION

We have demonstrated a potent antithrombotic effect of AppCHClppA in a rabbit intracarotid-cannula thrombosis model. This animal model has been reported (17) to be sensitive to antiplatelet agents. The reduction in thrombosis (from 80% in controls to 30% in test groups) by AppCHClppA indicates a superior effectiveness over that observed with the native molecule, Ap_4A (from 84% in controls to 56% in test groups) (10). Prolonged bleeding time after infusion of the agent was significant both at the midpoint and at the endpoint in the group without thrombosis.

The more potent antithrombotic effectiveness of AppCHClppA may partly be due to its stability in blood (resistance to hydrolytic enzymes) (13). Moreover, AppCHClppA was designed so that it cannot be hydrolyzed to ADP by any of the known Ap_4A pyrophosphohydrolases and can only slowly be hydrolyzed to AMP (18). The configuration of the molecule (two ADP moieties linked by a carbon bridge) may also play a role in its increased effectiveness over Ap_4A. A signal-producing conformational change of the platelet membrane occurs upon stimulation with ADP and is not as yet clearly understood, but AppCHClppA may compete with ADP at the receptor sites. Our inhibition kinetic study of ADP-induced platelet aggregation showed the inhibition to be competitive in nature and the K_i value for AppCHClppA to be low. This result suggests a high affinity for the platelet ADP receptor

Table 2. Effects of AppCHClppA on microvascular bleeding time in rabbits

	Bleeding time, % preinfusion control	
Group (*n*)	During infusion (60 min)	Infusion end
Control (5)	104 ± 12	109 ± 18
A (4)	142 ± 32*	110 ± 25
B (6)	183 ± 31*	157 ± 21*

Normal preinfusion bleeding time was 104 ± 23 sec (100%) in 15 determinations. Ten animals were infused with inhibitor, and the control group was infused with saline. Group A (four animals) had clot formation, and group B (six animals) had no clot formation.
*$P < 0.05$.

(19). We also found a potent suppression of the ADP-induced platelet-release reaction and of ADP-enhanced platelet factor 3 activity (19).

We have not as yet determined the AppCHClppA level in the rabbit blood. However, blood samples obtained at the end of the *in vivo* rabbit experiment still showed that ADP-induced platelet aggregation was completely inhibited. This result indicates that the dose of the infused agent was sufficient to suppress thrombosis throughout the 2-hr experimental period. The toxicity and pharmacokinetics of this analogue have yet to be examined.

CONCLUSION

Our results suggest that AppCHClppA might prove to be a clinical antiplatelet, antithrombotic agent, provided that future toxicity and pharmacokinetic studies are favorable. Its use might, for example, be considered in instances such as hemodialysis, arteriovenous shunts, heart-valve replacements, and cardiopulmonary bypass procedures. Its site of action, at activation of the platelets resulting in thrombosis, suggests possible therapeutic benefit from the use of Ap_4A or certain of its analogues to prevent incipient thrombosis. These compounds also might be used concurrently with other antithrombotic agents acting later in the sequence of events leading to thrombosis to help prevent extension of an existing fresh thrombus.

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