

# Adenosine-mediated Inhibition of Platelet Aggregation by Acadesine

## A Novel Antithrombotic Mechanism In Vitro and In Vivo

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### Abstract

Inhibition of platelet aggregation by acadesine was evaluated both in vitro and ex vivo in human whole blood using impedance aggregometry, as well as in vivo in a canine model of platelet-dependent cyclic coronary flow reductions. In vitro, incubation of acadesine in whole blood inhibited ADP-induced platelet aggregation by 50% at  $240 \pm 60 \mu\text{M}$ . Inhibition of platelet aggregation was time dependent and was prevented by the adenosine kinase inhibitor, 5'-deoxy 5-iodotubercidin, which blocked conversion of acadesine to its 5'-monophosphate, ZMP, and by adenosine deaminase. Acadesine elevated platelet cAMP in whole blood, which was also prevented by adenosine deaminase. In contrast, acadesine had no effect on ADP-induced platelet aggregation or platelet cAMP levels in platelet-rich plasma, but inhibition of aggregation was restored when isolated erythrocytes were incubated with acadesine before reconstitution with platelet-rich plasma. Acadesine (100 mg/kg i.v.) administered to human subjects also inhibited platelet aggregation ex vivo in whole blood. In the canine Folts model of platelet thrombosis, acadesine (0.5 mg/kg per min, i.v.) abolished coronary flow reductions, and this activity was prevented by pretreatment with the adenosine receptor antagonist, 8-sulphophenyltheophylline. These results demonstrate that acadesine exhibits antiplatelet activity in vitro, ex vivo, and in vivo through an adenosine-dependent mechanism. Moreover, the in vitro studies indicate that inhibition of platelet aggregation requires the presence of erythrocytes and metabolism of acadesine to acadesine monophosphate (ZMP). (*J. Clin. Invest.* 1994, 94:1524–1532.) **Key words:** ZMP (acadesine monophosphate) • cyclic flow reductions • erythrocyte-platelet interactions • thrombosis • unstable angina

### Introduction

Adenosine inhibits platelet aggregation in vitro via an  $A_2$ -adenosine receptor-mediated mechanism thought to involve increases in cAMP (1). The majority of studies regarding antithrombotic effects of adenosine have been performed in platelet-rich plasma (2–6), however adenosine has been shown to be ineffective as an antiplatelet agent in blood (4, 5) presumably because of its

short half-life of  $< 1$  s in whole blood (7). Consequently, while inhibition of platelet aggregation by adenosine in vitro is well documented, there is a paucity of studies demonstrating antithrombotic actions of adenosine in vivo, in particular those that relate to the importance of adenosine released by blood cells, endothelial cells, or cardiomyocytes as a local modulator of platelet aggregation. In a recent study Kitakaze et al. (8) reported that administration of the adenosine receptor antagonist, 8-phenyltheophylline, resulted in coronary thromboembolization and regional ischemia. They concluded that adenosine, produced locally by the myocardium in response to ischemia, inhibited platelet aggregation to help maintain patency of coronary arteries. Notwithstanding the relative lack of information regarding adenosine's antithrombotic effects, there is a significant body of literature concerning the antithrombotic activity of the adenosine transport inhibitor, dipyridamole in vivo (9). Yet despite the past support for use of dipyridamole and evidence that dipyridamole elevates adenosine concentrations (10, 11), recent studies concur that dipyridamole is a weak inhibitor of aggregation with limited therapeutic benefit (9, 12). Thus, the relative importance of adenosine's antiplatelet activity remains controversial.

The nucleoside analog, acadesine, has received attention recently as a means of protecting the myocardium during ischemia and reperfusion in animal models (13–18) and during coronary artery bypass graft surgery in patients (19, 20). Preclinical data indicate that this action is due to enhanced extracellular adenosine levels during ischemia (13, 21, 22). While the exact mechanism of the adenosine-regulating action is not understood, acadesine is taken up by cells and phosphorylated by adenosine kinase to its monophosphate, ZMP<sup>1</sup> (23, 24, Fig. 1), and both acadesine and ZMP can inhibit enzymes involved in adenosine metabolism and formation, respectively, to augment extracellular adenosine levels during ischemia (18). In view of this adenosine-regulating activity of acadesine, and the proposed antiplatelet activity of adenosine, the present studies were undertaken to (a) evaluate the effects of acadesine on platelet aggregation in vitro, ex vivo, and in vivo; (b) assess the involvement of adenosine in mediating the effects of acadesine on platelet aggregation; and (c) compare the antiaggregatory effects of acadesine to those observed with exogenously administered adenosine and dipyridamole. These studies used impedance aggregometry in whole blood and platelet-rich plasma (PRP) for in vitro and ex vivo assessment of platelet function, and a canine model of platelet-dependent coronary thrombosis for assessment in vivo (25).

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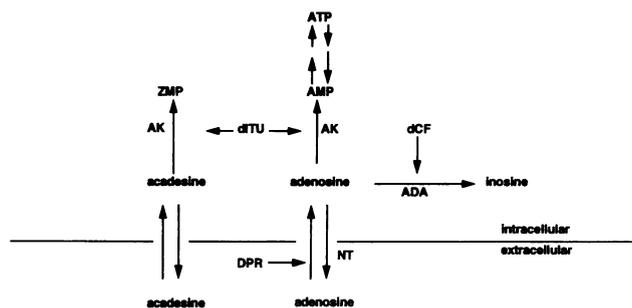
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1. *Abbreviations used in this paper:* ADA, adenosine deaminase; CFR, cyclic coronary flow reduction; dITU, 5'-deoxy 5-iodotubercidin; PPP, platelet-poor plasma; PRP, platelet-rich plasma; RBC, red blood cells; 8-SPT, 8-sulphophenyltheophylline; WBC, white blood cells; ZMP, acadesine monophosphate.



**Figure 1.** Acadesine and adenosine metabolism. Acadesine is taken up into cells and phosphorylated to ZMP in a reaction catalyzed by adenosine kinase (AK). Adenosine is taken up into cells via a nucleoside transporter (NT) where it can be phosphorylated to AMP by AK, deaminated to inosine by ADA or released via the NT. AK is inhibited by *dITU*, ADA by 2'-deoxycoformycin (*dCF*), and NT by dipyrindamole (*DPR*).

## Methods

### *In vitro* studies

**Isolation and fractionation of whole blood.** Whole blood was drawn from healthy donors who had not taken aspirin or aspirin-containing medication for 10 d, alcohol for 24 h, or methylxanthine-containing drinks or food for 12 h before donation. It was collected in plastic tubes containing 129 mM buffered citrate solution (Becton Dickinson Immunocytometry Systems, San Jose, CA) to prevent coagulation. PRP was prepared by centrifuging the citrated blood at 500 g for 15 min at 4°C. White blood cells (WBC) and red blood cells (RBC) were separated by addition of hypaque (1:2 vol/vol) and recentrifugation at 1,400 g for 20 min at 4°C. The upper layer containing WBC and the lower layer containing RBC were resuspended in equal volumes of platelet-poor plasma (PPP). PPP was prepared by centrifuging PRP at 2,000 g for 20 min at 4°C.

**Aggregation studies.** Platelet aggregation was elicited by addition of ADP (6–25  $\mu$ M) at the minimum concentration, inducing full aggregation determined for individual donors, and measured by the impedance technique (26) using a whole blood aggregometer (model 500; Chrono-Log Corp., Havertown, PA). The nature of acadesine's antiplatelet actions was investigated using whole blood or fractionated blood (RBC, WBC, PRP) in the following protocols.

In protocol 1, platelet aggregation in whole blood or PRP was determined in the presence or absence of acadesine (0.1–10 mM). Whole blood or PRP was diluted with an equal volume of saline, then incubated with acadesine for 15–120 min at 37°C. In some experiments the relationship between the antiplatelet action of acadesine and its metabolism to ZMP was assessed using the adenosine kinase inhibitor, 5'-deoxy 5-iodotubercidin (*dITU*, 20  $\mu$ M, Fig. 1). In other experiments, adenosine deaminase (ADA, 1.4 U/ml) was added to examine the role of endogenous adenosine in the antiaggregatory effects of acadesine (Fig. 1).

In protocol 2, to assess the contribution of RBC or WBC to the action of acadesine, PRP was reconstituted with either RBC or WBC and incubated in the presence or absence of acadesine (0.1–1 mM) for 60 min at 37°C.

In protocol 3, to evaluate whether pretreatment of RBC with acadesine is required for inhibition of platelet aggregation, isolated RBC were preincubated with acadesine (0.1–10 mM) for 60 min at 37°C before reconstitution with PRP. The effect of pretreatment of PRP with acadesine was assessed in the converse experiment in which PRP was preincubated for 30 min with acadesine before reconstitution with RBC.

In protocol 4, to determine whether intracellular accumulation of ZMP in the RBC contributes to the antiplatelet effect, isolated RBC were incubated with acadesine (0.1–1 mM) for 60 min at 37°C, reisolated, and washed in saline to remove extracellular acadesine. Washed

RBC were then reconstituted with PRP and incubated for 5 min before ADP-induced aggregation.

In all experiments with reconstituted cells (protocols 2–4), volume was adjusted with saline to yield final cell counts of  $1.3 \times 10^6$  platelets/ml and  $1.0 \times 10^9$  RBC/ml.

**Acadesine metabolism in human whole blood.** To evaluate the relationship between acadesine metabolism and its antiplatelet actions, acadesine and ZMP levels were measured in conjunction with adenosine kinase inhibition to inhibit acadesine metabolism (Fig. 1). Whole blood (1 ml) diluted in saline (1:1 vol/vol) was incubated with 1 mM acadesine for 15–120 min at 37°C in the presence or absence of 20  $\mu$ M *dITU*. Plasma was isolated by centrifugation (270 g for 5 min at 23°C) and deproteinized by ultrafiltration (2,000 g for 30 min at 23°C) using Amicon 4104 filters (Amicon, Danvers, MA). Isolated, saline-washed RBC were extracted using 4 M perchloric acid, centrifuged (5,000 g for 10 min at 23°C), and supernatants neutralized with 3 M  $K_2CO_3$ . Plasma acadesine and RBC ZMP levels were determined by HPLC as described by Dixon et al. (27).

**Adenosine metabolism by human whole blood.** The metabolism of isotope-labeled adenosine was measured in the presence and absence of acadesine. The effects of acadesine were compared with inhibitors of adenosine kinase, adenosine deaminase, and adenosine transport (Fig. 1). Whole blood (1 ml) diluted in saline (1:1 vol/vol) was incubated with acadesine (1 mM), *dITU* (10  $\mu$ M), the adenosine deaminase inhibitor 2'-deoxycoformycin (1  $\mu$ M), or the adenosine transport inhibitor dipyrindamole (10  $\mu$ M) for 60 min at 37°C. [ $^{14}C$ ]Adenosine (10  $\mu$ M) was then added and incubated for 0.25, 0.5, 1.2, and 5 min. Blood samples (0.1 ml) were added to ice-cold 3 M perchloric acid, rapidly mixed and centrifuged at 5,000 g for 5 min at 4°C. Acid extracts were neutralized with 1 M  $K_2CO_3$ , recentrifuged, and [ $^{14}C$ ]adenosine in the supernatant was isolated by Kodak-cellulose TLC (Eastman Kodak Co., Rochester, NY) (28) and quantitated by scintillation counting.

**Platelet cAMP measurements in human whole blood or PRP.** To evaluate the effects of acadesine on platelet cAMP levels, platelet nucleotides were labeled by incubating PRP with 2  $\mu$ M [ $2,8-^3H$ ]adenine (23 Ci/mmol; Moravsek, Brea, CA) for 90 min at 37°C (29, 30). Isotope-labeled platelets were applied to a  $1.6 \times 22$  cm Sepharose-2B column (Sigma Chemical Co., St. Louis, MO) and isolated by eluting the column with 10 mM  $K_2HPO_4$ - $KH_2PO_4$ , pH 7.4, containing 0.35% (wt/vol) BSA. Isolated  $^3H$ -labeled platelets (0.4 ml) were added to whole blood (0.5 ml) or PRP (0.5 ml), together with 0.1 ml of acadesine (1 mM), adenosine (10  $\mu$ M), or saline as a control. In some experiments, adenosine deaminase (1.4 U/ml) was added to the incubation mixture. After 60 min at 37°C, reactions were stopped by addition of 0.5 ml ice-cold 2 M perchloric acid, which contained 2,000 cpm [ $8-^{14}C$ ]cAMP (56 mCi/mmol, Moravsek) to correct for recoveries during subsequent isolation of cAMP. cAMP was isolated from whole blood or PRP using alumina and cation exchange columns as described by Haslam and colleagues (29, 30) and counted for  $^3H$  and  $^{14}C$  by scintillation counting. After correcting for the recovery of  $^{14}C$ -labeled cAMP (60–70%), platelet  $^3H$ -labeled cAMP was expressed as a percentage of the total platelet  $^3H$ .

### *Ex vivo* studies

Acadesine (100 mg/kg) was administered by intravenous infusion over 90 min to eight healthy men who had not taken aspirin, aspirin-containing compounds, or non-steroidal antiinflammatory drugs for 7 d, or alcohol and methylxanthine-containing foods or beverages for 24 h before or during the study. Citrated whole blood was collected immediately before infusion and 1, 1.5, 2, 3, 6, and 24 h after infusion. Platelet aggregation induced by ADP (10  $\mu$ M) or collagen (2.5  $\mu$ g/ml) was measured by impedance aggregometry. One subject failed to respond to ADP-induced platelet aggregation and was excluded from the study. All subjects responded to collagen-induced platelet aggregation. The study was approved by the Institutional Review Board of the Clinical Research Centre (New Orleans, LA). Written informed consent was obtained from all patients.

### In vivo studies

These studies examined (a) the antithrombotic dose response to acadesine, (b) the effect of adenosine receptor blockade on acadesine's action, and (c) the antithrombotic effects of exogenously infused adenosine and dipyridamole. All experiments were conducted according to the National Institutes of Health Guide for the Care and Use of Animals, and protocols were approved by the Institutional Committee for Animal Use and Care at Gensia Inc. (San Diego, CA).

**Experimental preparation.** 23 random source mongrel dogs weighing between 18 and 28 kg were used in these studies. Dogs were fasted overnight, sedated with acepromazine (1 mg/kg, i.m.), and anesthetized with pentobarbital sodium (30 mg/kg, i.v.). Powdered pentobarbital sodium was dissolved in 0.9% NaCl to avoid the antiplatelet effects of the vehicle used in commercially available liquid formulations. Blood platelet counts were determined in venous blood using a Cell-Dyn 900 hematology analyzer (Sequoia-Turner Corp., Mountain View, CA). Left circumflex coronary artery platelet thrombi and cyclic coronary flow reductions (CFRs) were initiated using the method described by Folts (25). CFRs were quantified by two criteria: frequency (no. of CFRs/30 min) and nadir of flow (ml/min). Thus, decreased frequency and increased nadir of flow indicated inhibition of platelet aggregation, epitomized by the abolition of CFRs when coronary flow was stable with no cyclical changes present.

**Experimental protocols.** In all studies, baseline hemodynamic data were obtained before establishing CFRs. CFRs were initiated and considered stable only when frequency and nadir of flow were stable for 30 min. Four groups of dogs were studied.

Group I ( $n = 7$ ) examined the antithrombotic dose-response to acadesine. After stabilization of CFRs, acadesine was infused intravenously at doses of 0.1, 0.25, and 0.5 mg/kg per min sequentially for 30 min each. CFRs were monitored for a further 30 min after termination of infusion.

Groups II ( $n = 7$ ) and III ( $n = 6$ ) tested the antithrombotic effect of acadesine in the absence (Group II) and presence (Group III) of adenosine receptor blockade with 8-sulphophenyltheophylline (8-SPT), respectively. In Group II, CFRs were established and an intracoronary infusion of saline was initiated. After 30 min, acadesine (0.5 mg/kg per min, i.v.) was infused for a period of 60 min, at which time the infusion was discontinued. CFRs were monitored for an additional 60 min after termination of the acadesine infusion. In Group III, CFRs were established and a continuous intracoronary infusion of 8-SPT (10 mg/kg per h) was initiated. After 30 min, acadesine (0.5 mg/kg per min, i.v.) infusion was initiated and continued for 60 min. After termination of acadesine infusion, CFRs were monitored for an additional 30 min. To verify that 8-SPT specifically inhibited the action of acadesine, the effects of aspirin were then tested (5 mg/kg, i.v. bolus) after termination of acadesine infusion, but during the continued infusion of 8-SPT.

Group IV ( $n = 4$ ) tested the antithrombotic effects of adenosine and dipyridamole. After baseline hemodynamic data were established, two doses of adenosine (250 and 500  $\mu$ g/kg per min, i.v.) were infused for 30 min each, followed by 60 min of restabilization, and two doses of dipyridamole (10 and 25  $\mu$ g/kg per min, i.v.) for 30 min each. Dipyridamole vehicle (4 mM tartaric acid) was infused intravenously for 30 min before dipyridamole infusion as a control.

### Materials

Acadesine (5-aminoimidazole-4-carboxamide-1- $\beta$ -D-ribofuranoside; Pro-tara<sup>®</sup>) was manufactured by Gensia Inc. ZMP, adenosine deaminase (Type IV from calf intestinal mucosa), and Hypaque were purchased from Sigma Chemical Co. (St. Louis, MO). ADP and collagen were obtained from Chrono-Log Corp. 8-SPT was purchased from Research Biochemicals Inc. (Natick, MA). dITU was synthesized by the Department of Chemistry at Gensia Inc.

### Statistical analysis

In the in vitro studies, statistical comparison between groups was performed by one-way ANOVA, and subsequent comparison of means

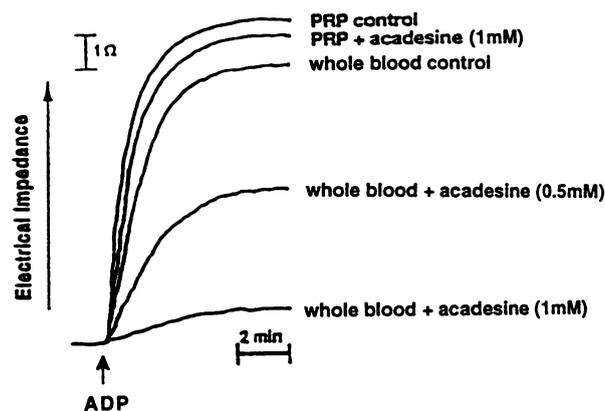


Figure 2. Effect of acadesine on ADP-induced platelet aggregation in whole blood and PRP. Representative traces are superimposed showing aggregation elicited by ADP in whole blood and PRP measured by impedance aggregometry. Acadesine (0.5 or 1 mM) inhibits aggregation in whole blood, but not PRP.

using Scheffe's  $F$  test;  $P < .05$  was accepted as statistically significant. In the ex vivo study, differences from preinfusion values were determined for each subject at each time point. These differences were subjected to ANOVA;  $P < .05$  was taken as statistically significant. In all studies performed in vivo, each dog served as its own control, with the effects of drug at each 30-min time point compared with baseline. Thus, data were compared using paired  $t$  tests with Bonferroni correction for four comparisons (31). Statistical significance was accepted if  $P < 0.0125$ . All data are presented as mean  $\pm$  SEM.

## Results

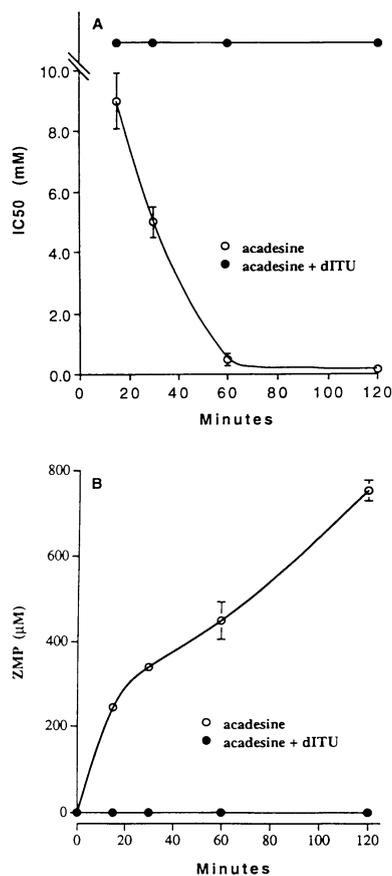
### In vitro studies

#### PROTOCOL 1

**Effect of acadesine on platelet aggregation in whole blood and PRP.** Incubation of 1 mM acadesine in human whole blood for 60 min at 37°C resulted in a  $> 90\%$  inhibition of platelet aggregation elicited by ADP (Fig. 2). In contrast, incubation of acadesine with human PRP at concentrations up to 10 mM had no effect on ADP-induced platelet aggregation. Inhibition of platelet aggregation by acadesine in whole blood was concentration-dependent and increased with incubation time such that the  $IC_{50}$  for acadesine was  $9 \pm 2$  mM after 5 min and  $0.24 \pm 0.06$  mM after 120 min (Fig. 3). Extended incubations with acadesine were not evaluated because of loss of control aggregation responsiveness in untreated whole blood incubated for longer than 120 min.

**Importance of acadesine metabolism for inhibition of ADP-induced platelet aggregation in whole blood.** Acadesine rapidly enters cells and is metabolized by the intracellular enzyme adenosine kinase to its 5'-monophosphate, ZMP (23, 24). As shown in Fig. 3, when acadesine was incubated in whole blood, ZMP rapidly accumulated in the red blood cells. Addition of the adenosine kinase inhibitor, dITU (32) completely inhibited the conversion of acadesine to ZMP, and abolished the inhibition of platelet aggregation (Fig. 3). ZMP itself, at concentrations up to 1 mM, had no effect on ADP-induced platelet aggregation in whole blood.

**Evidence that endogenous adenosine mediates inhibition of ADP-induced platelet aggregation by acadesine in whole blood.** Adenosine deaminase, which degrades adenosine to inosine,



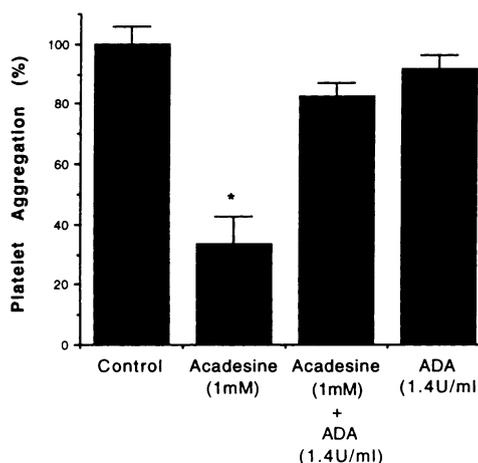
**Figure 3.** Time and metabolism-dependent inhibition of platelet aggregation by acadesine. *A* shows IC<sub>50</sub>s determined for inhibition of ADP-induced platelet aggregation by acadesine in whole blood treated for 15–120 min in the absence (○) or presence (●) of 20 μM dITU. Values are mean±SEM of separate determinations from at least three blood donors. *B* shows ZMP levels in RBC isolated from whole blood treated with 1mM acadesine for 15–120 min in the absence (○) or presence (●) of 20 μM dITU. Values are mean±SEM for three determinations at each incubation time.

was used to examine whether inhibition of platelet aggregation by acadesine is mediated by adenosine. An adenosine deaminase concentration of 1.4 U/ml was chosen based on preliminary experiments in which inhibition of platelet aggregation in PRP by 10 μM adenosine (25±3% of control, *n*=3) was completely blocked (100±1%, *n*=3) by this concentration of adenosine deaminase. As shown in Fig. 4, the addition of adenosine deaminase substantially (80±9%) reversed the inhibition of platelet aggregation in whole blood by acadesine (1 mM).

#### PROTOCOLS 2–4

**Importance of red blood cells for inhibition of ADP-induced platelet aggregation by acadesine.** An important role for the red cells in the inhibition of platelet aggregation by acadesine is suggested by the inability of acadesine to inhibit platelet aggregation in PRP and the temporal association between acadesine metabolism to ZMP in red cells and inhibition of platelet aggregation. This was investigated further in a series of experiments using fractionated human whole blood.

Inhibition of platelet aggregation by acadesine was first evaluated in PRP to which either isolated RBC or WBC were added. When acadesine was incubated in PRP containing RBC, acadesine inhibited platelet aggregation with an IC<sub>50</sub> of 0.6±0.2 mM (*n*=3). In contrast, acadesine (1 mM) had no effect on platelet aggregation in PRP to which WBC had been added. These results suggest that the presence of RBC, but not WBC, are important for inhibition of platelet aggregation by acadesine. However, these experiments do not address whether pretreatment of RBC alone is sufficient for inhibition of aggregation. Therefore, RBC were incubated with acadesine before the addi-



**Figure 4.** Effect of adenosine deaminase on inhibition of platelet aggregation by acadesine. Inhibition of ADP-induced platelet aggregation by 1 mM acadesine is prevented by 1.4 U/ml ADA. ADA was added at the same time as acadesine and incubated for 60 min prior to ADP-induced aggregation. Control response (100% aggregation) is 7.3±2.6 Ω. Values are mean±SEM for four determinations. \**P* < 0.05 vs. control, acadesine + ADA, and ADA.

tion of platelets and compared with platelets treated with acadesine prior to the addition of RBC. Acadesine-treated RBC inhibited ADP-induced platelet aggregation with an IC<sub>50</sub> of 0.8±0.2 mM (*n*=3), whereas no inhibition was observed when RBC was added to acadesine (1 mM)-treated platelets. An identical IC<sub>50</sub> of 0.8±0.1 mM (*n*=3) was determined in parallel experiments for inhibition of ADP-induced platelet aggregation by acadesine in unfractionated whole blood, under the same conditions.

To evaluate whether acadesine-treated RBC alone were sufficient to inhibit platelet aggregation, additional experiments were carried out in which the acadesine-treated RBC were isolated and washed to remove extracellular acadesine prior to addition to platelets. ADP-induced platelet aggregation was inhibited by washed, acadesine-treated RBC in these experiments with an IC<sub>50</sub> of 0.8±0.1 mM (*n*=3).

These experiments were further elaborated to assess the importance of acadesine metabolism by RBC per se and the role of adenosine in the inhibition of platelet aggregation by acadesine-treated RBC. Inhibition of platelet aggregation by 1 mM acadesine (33±4% of control, *n*=6) was reduced when RBC were incubated with acadesine in the presence of dITU (71±6%, *n*=3), whereas dITU had no effect (36±4%, *n*=3) if added at the end of the incubation of RBC with acadesine. Inhibition of platelet aggregation by acadesine was also attenuated if adenosine deaminase was added during the incubation (78±6%, *n*=4) or at the end of the incubation (73±6%, *n*=3). These results therefore provide further evidence that acadesine metabolism by RBC is important for inhibition of ADP-induced platelet aggregation in whole blood. Moreover, these data suggest that addition of acadesine-treated RBC to platelets promotes inhibition of aggregation via an adenosine-mediated action.

**Effect of acadesine on adenosine utilization in human whole blood: comparison with inhibitors of adenosine kinase, adenosine deaminase and adenosine transport.** Adenosine was rapidly

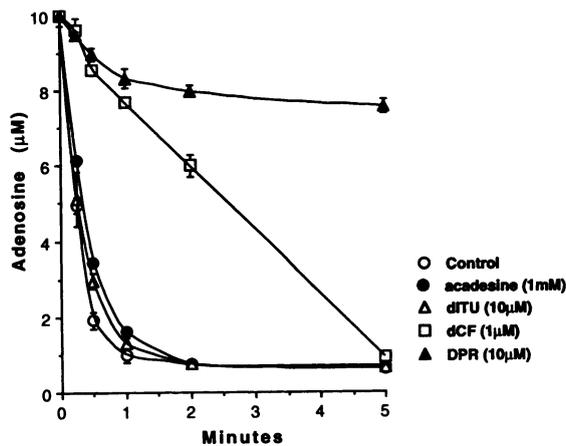


Figure 5. Effect of acadesine, dITU, dCF and dipyridamole on adenosine utilization in whole blood. Adenosine is rapidly utilized in acadesine and dITU-treated whole blood but utilization is inhibited in 2'-deoxycoformycin (dCF) and dipyridamole (DPR)-treated blood. Values are mean  $\pm$  SEM for three determinations of blood adenosine levels at 0.25–5 min after addition of 10  $\mu$ M adenosine to whole blood.

degraded in human whole blood (> 90% in 1 min, Fig. 5). Incubation of acadesine (1 mM) with whole blood for 60 min at 37°C had no effect on adenosine utilization (Fig. 5). Similar results were obtained when adenosine was added to blood incubated with the adenosine kinase inhibitor, dITU (10  $\mu$ M). In contrast, adenosine was metabolized much more slowly (< 25% after 1 min) in blood treated with either 2'-deoxycoformycin (1  $\mu$ M) to inhibit adenosine deaminase or with dipyridamole (10  $\mu$ M) to block adenosine transport.

**Effect of acadesine on platelet cAMP levels in human whole blood.** To provide insight into the mechanism by which acadesine inhibits platelet aggregation, platelet cAMP levels were measured in whole blood. When 1 mM acadesine was incubated in whole blood, platelet cAMP levels were increased by 43  $\pm$  10% (0.022  $\pm$  0.002% of total platelet  $^3$ H,  $n = 3$ , vs. 0.015  $\pm$  0.002%,  $n = 3$ , in untreated blood,  $P < .05$ ). Moreover, acadesine-induced elevation of platelet cAMP was prevented by 1.4 U/ml adenosine deaminase (0.014  $\pm$  0.001% of total platelet  $^3$ H,  $n = 3$ ,  $P = \text{NS}$  vs. untreated blood). To confirm the erythrocyte dependence of acadesine's antiplatelet activity, similar experiments were performed in PRP. In these studies 10  $\mu$ M adenosine increased platelet cAMP levels by 71  $\pm$  9% (0.044  $\pm$  0.002% of total platelet  $^3$ H,  $n = 3$ , vs. 0.026  $\pm$  0.002%,  $n = 3$ , in untreated PRP,  $P < .05$ ). In contrast, 1 mM acadesine had no effect on platelet cAMP levels in PRP (0.022  $\pm$  0.001%,  $n = 3$ ,  $P = \text{NS}$  vs. untreated PRP), consistent with its lack of an antiaggregating effect in this setting.

#### Ex vivo studies

In healthy human volunteers, before acadesine infusion, ADP-induced platelet aggregation measured by the impedance method was 7.7  $\pm$  1.0  $\Omega$  ( $n = 6$ ) and collagen-induced platelet aggregation was 18.2  $\pm$  1.2  $\Omega$  ( $n = 8$ ). Administration of acadesine (100 mg/kg, i.v.) resulted in significant inhibition of ex vivo platelet aggregation elicited by ADP or collagen (Fig. 6). Inhibition was time dependent and maximal after 2 h; i.e., 30 min after termination of the 90-min infusion of acadesine. At 2

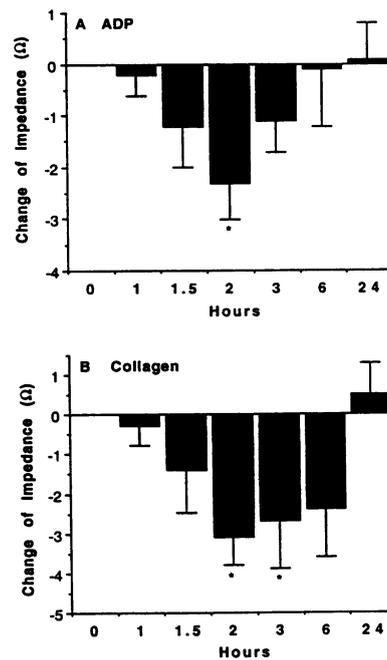


Figure 6. Effect of acadesine on platelet aggregation measured ex vivo in whole blood. Acadesine (100 mg/kg, i.v.) administered over a period of 1.5 h inhibited platelet aggregation elicited by 10  $\mu$ M ADP (A) and 2.5  $\mu$ g/ml collagen (B). Impedances prior to acadesine infusion were 7.7  $\pm$  1.0  $\Omega$  and 18.2  $\pm$  1.2  $\Omega$  for ADP and collagen, respectively. Values are mean  $\pm$  SEM for four averaged determinations from six (ADP) or eight (collagen) subjects. \* $P < 0.05$  vs. preinfusion aggregatory response.

h, ADP-induced platelet aggregation was decreased by 32  $\pm$  10% ( $P < .05$ ) and collagen-induced platelet aggregation by 18  $\pm$  3% ( $P < .05$ ). Platelet aggregation was attenuated after 3 h, but after 6 h was not significantly different from preinfusion levels when either ADP or collagen was used to induce aggregation.

#### In vivo studies

Platelet counts were not different in the four groups of dogs, averaging 325  $\pm$  27, 329  $\pm$  25, 315  $\pm$  39, and 282  $\pm$  31  $\times 10^3$  platelets/ $\mu$ l in Groups I-IV, respectively.

Fig. 7 illustrates the dose-response effects of acadesine on CFRs. During the control period, there were an average of 5.0  $\pm$  0.6 CFRs in 30 min, with a nadir of 5.5  $\pm$  1.6 ml/min.

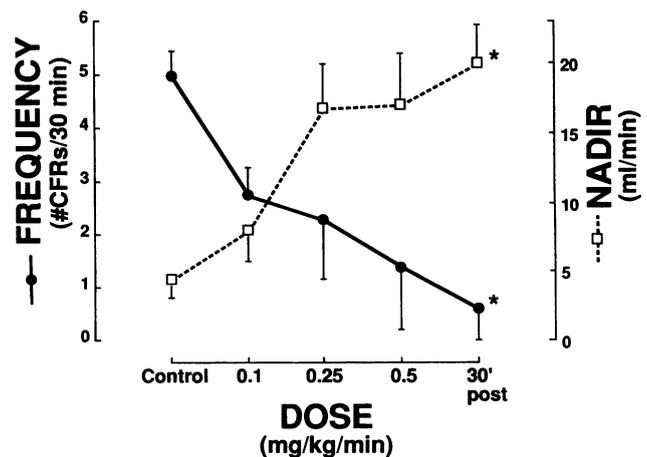


Figure 7. Dose response to acadesine on the frequency of CFRs (●) and the nadir of coronary blood flow (□) in anesthetized dogs. Each dose of acadesine was infused for 30 min in a stepwise manner. Acadesine dose-dependently reduced the frequency and increased the nadir of flow. \* $P < 0.0125$  vs. control.

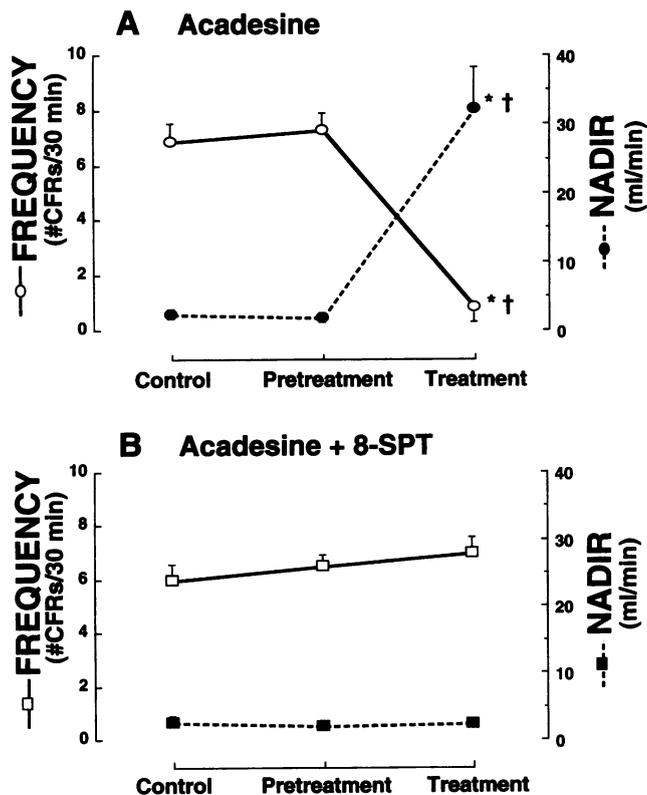


Figure 8. Effects of acadesine (0.5 mg/kg per min, i.v.) (A) and acadesine + 8-SPT (10 mg/kg per hr, i.c.) (B) on frequency of CFRs (solid line) and nadir of coronary blood flow (broken line). Control = 30 min of no treatment; pretreatment = 30 min of saline or 8-SPT, i.c.; treatment = 60 min of acadesine or acadesine + 8-SPT. Acadesine significantly reduced the frequency and increased the nadir of CFRs. Intracoronary 8-SPT did not affect CFRs, but prevented the antiplatelet action of acadesine. \* $P < 0.025$  vs. control; † $P < 0.01$  vs. acadesine + 8-SPT.

Acadesine dose-dependently reduced the frequency and increased the nadir of coronary blood flow, such that frequency and nadir of coronary blood flow at termination of infusion averaged  $1.4 \pm 1.2$  cycles/30 min and  $17.1 \pm 3.3$  ml/min, respectively. CFRs were abolished in five of six animals at termination of infusion, and did not return within 30 min after infusion. Left ventricular and arterial pressures, first derivative of left ventricular pressure, and heart rate were unchanged at any time during the infusion of acadesine. From these preliminary experiments, a dose of acadesine of 0.5 mg/kg per min was chosen for use in subsequent studies. Plasma concentrations of acadesine at termination of 0.5 mg/kg per min infusion were  $40 \pm 3$   $\mu$ M.

Groups II and III tested the effects of 8-SPT on acadesine's antithrombotic effect. In these studies, blockade of adenosine receptors with 8-SPT was confirmed by the abolition of coronary vasodilation to adenosine. In the absence of 8-SPT, adenosine (200 mg/kg, i.v.) increased coronary flow by  $44 \pm 7$  ml/min from a baseline of  $31 \pm 2$  ml/min. In the presence of 8-SPT (10 mg/kg per h), coronary flow increased only by  $5 \pm 1$  ml/min ( $P < 0.05$ ). Fig. 8 illustrates the effects of acadesine infusion (0.5 mg/kg per min) on CFRs in the absence (Group II) and presence (Group III) of an intracoronary infusion of 8-SPT. Frequency of CFRs and nadir of flow did not differ between the two groups during the 30-min control period, and the CFRs

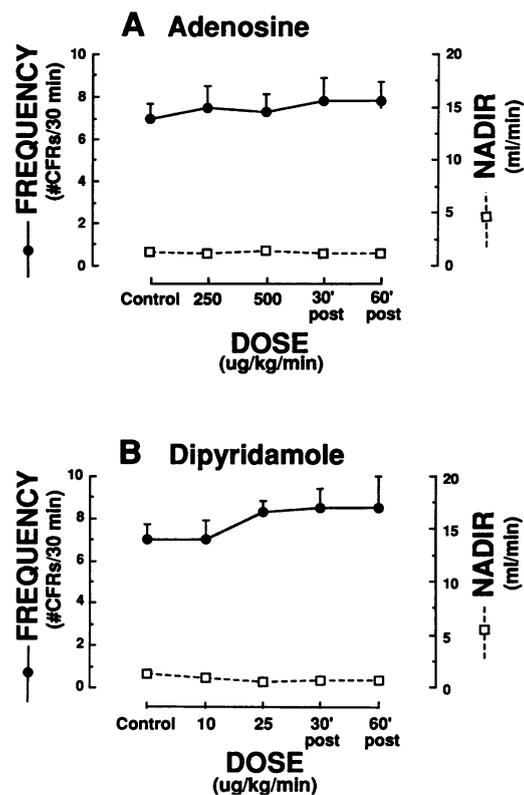


Figure 9. Effect of adenosine (A) and dipyridamole (B) on frequency of CFRs (●) and nadir or coronary blood flow (□). Neither agent affected CFRs during infusion or up to 60 min after infusion.

were unchanged by intracoronary infusion of saline in the control group or 8-SPT in the treated group. Infusion of acadesine alone significantly reduced the frequency of CFRs and increased the nadir of coronary flow, abolishing CFRs in six of seven dogs within  $19 \pm 3$  min. In contrast, infusion of acadesine in dogs pretreated with 8-SPT failed to alter either the frequency of CFRs or the nadir of coronary flow. Hemodynamic parameters did not differ in the two groups of animals at any time point, and were unchanged by infusion of acadesine (data not shown).

To verify that 8-SPT selectively inhibited the action of acadesine, the effects of aspirin (5 mg/kg, i.v.) were tested in each of the dogs pretreated with 8-SPT. Aspirin abolished CFRs in six of six dogs within  $7 \pm 4$  min, resulting in a CFR frequency of 0 and a stable coronary blood flow of  $31 \pm 1$  ml/min, similar to that observed in dogs treated with acadesine alone.

The effects of adenosine and dipyridamole on CFRs are illustrated in Fig. 9. Infusion of adenosine (250 and 500  $\mu$ g/kg per min) failed to alter either frequency or the nadir of coronary blood flow, despite significant reductions in mean arterial pressure (peak change =  $-38 \pm 5$  mm Hg). Similarly, infusion of dipyridamole vehicle (4 mM tartaric acid) or dipyridamole (10 and 25  $\mu$ g/kg per min) had no effect on frequency of CFRs or nadir of coronary flow, yet dipyridamole elicited significant reduction in mean arterial pressure (peak change =  $-18 \pm 5$  mm Hg). Coronary conductance in the normal (nonstenotic) left anterior descending coronary artery increased with infusion of adenosine ( $0.38 \pm 0.06$  to  $0.94 \pm 0.17$  ml/min per mm Hg) and dipyridamole ( $0.30 \pm 0.04$  to  $0.62 \pm 0.04$  ml/min per

mm Hg), indicating that concentrations of these agents in the coronary vasculature were sufficient to cause significant vasodilation.

## Discussion

The present studies addressed whether regulation of endogenous adenosine levels by acadesine could elicit inhibition of platelet aggregation *in vitro* and *in vivo*. The important results of this investigation are: (a) acadesine inhibits platelet aggregation in whole blood *in vitro*; (b) the intravenous administration of acadesine to healthy subjects results in inhibition of platelet aggregation as assessed *ex vivo* in whole blood; (c) acadesine inhibits intracoronary thrombosis in a canine model of unstable angina *in vivo*; (d) the antiplatelet actions of acadesine require the presence of RBCs; and (e) acadesine-induced antiaggregatory effects are mediated by endogenous adenosine both *in vitro* and *in vivo*. Thus, these results confirm the adenosine-regulating activity of acadesine previously associated with its cardioprotective antiischemic actions, and extend those activities to include an antithrombotic effect.

Adenosine inhibits platelet aggregation in PRP via an adenosine A<sub>2</sub>-receptor-mediated stimulatory guanyl nucleotide protein-linked activation of adenylate cyclase that increases platelet cAMP (1). However, in whole blood the activity of adenosine is limited by its rapid uptake and metabolism by RBCs. Indeed, while the IC<sub>50</sub> of adenosine in PRP is approximately 1 μM, in whole blood the IC<sub>50</sub> is greater than 1 mM (33). The antiplatelet effect of adenosine in blood can be unmasked by prevention of adenosine removal with nucleoside transport inhibitors (4, 5) or inhibition of adenosine metabolism with a combination of 2'-deoxycoformycin and 5-iodotubercidin to inhibit adenosine deaminase and adenosine kinase, respectively (4) (Fig. 1). Under these conditions, the IC<sub>50</sub> for adenosine as an inhibitor of platelet aggregation in whole blood is decreased to 1.5 μM (4), similar to the value obtained in PRP. Thus, under normal circumstances, adenosine levels are tightly regulated and adenosine is unable to exert an antiplatelet effect. However, if adenosine levels are augmented, e.g., by inhibition of adenosine metabolism, this platelet inhibitory activity can be expressed.

Whole blood impedance aggregometry developed by Cardinal and Flower in 1980 offers the advantage of studying platelet behavior in the presence of other blood cells. This technique has been critically evaluated (34–36) and offers distinct advantages over the turbidimetric device using PRP developed by Born (37), particularly when studying interactions between blood elements in the process of aggregation. As originally noted by Cardinal and Flower (26), RBCs and leukocytes are frequently observed trapped within the platelet aggregate when performing aggregation studies in blood, thereby providing the opportunity for dynamic cell-platelet interactions to influence the aggregatory response. Indeed the ability of erythrocytes to enhance platelet aggregation (38), independent of their ability to act as a source of ADP, has become recognized. The present study extends this concept to demonstrate that the erythrocytes can be targeted by acadesine to bestow an antithrombotic activity upon the platelets. Thus, preincubation of isolated RBCs with acadesine, followed by reconstitution in PRP, confers an inhibitory effect against platelet aggregation, whereas the converse experiment in which platelet-rich plasma is preincubated

with acadesine before the addition of isolated RBCs bestows no antiplatelet effect.

The present studies provide insight into the mechanism by which acadesine inhibits platelet aggregation. It is an indirect effect involving endogenous adenosine because the antiplatelet activity and associated increased platelet cAMP levels are blocked if adenosine is removed using adenosine deaminase *in vitro* or the effects of adenosine are prevented with the adenosine receptor antagonist 8-SPT *in vivo*. Acadesine itself is not a ligand for adenosine A<sub>1</sub> or A<sub>2</sub> receptors or the S(4-nitrobenzyl)-6-thioinosine-sensitive adenosine transporter at concentrations up to 1 mM (39), nor a substrate for adenosine deaminase  $V_{max} < 10^{-4}V_{max}$  for adenosine (Jim Appleman, personal communication). Moreover, the antiplatelet activity of acadesine is dependent upon its phosphorylation by adenosine kinase to the monophosphate, ZMP, because inhibition of platelet aggregation is temporally related to ZMP formation in the red blood cells, and prevented by the adenosine kinase inhibitor, dITU. If adenosine kinase is a major enzyme which regulated adenosine levels, it could be argued that competition for this enzyme between acadesine and adenosine could result in elevated adenosine levels. However, in whole blood inhibition of adenosine kinase alone does not alter the removal of radiolabeled adenosine (40 and Fig. 5, present study) indicating that adenosine kinase inhibition is not sufficient to augment adenosine levels significantly. Furthermore, dITU itself was ineffective at preventing platelet aggregation despite inhibition of adenosine kinase (Fig. 3). Acadesine, but not ZMP, is also a competitive inhibitor of adenosine deaminase ( $K_i = 362 \mu\text{M}$  in calf intestinal mucosa, reference 41), another enzyme which regulates adenosine removal. However, inhibition of adenosine deaminase does not appear to account for the antithrombotic activity because it is independent of ZMP, and a potent adenosine deaminase inhibitor, 2'-deoxycoformycin was ineffective as an inhibitor of platelet aggregation *in vitro* (40). Thus, inhibition of either adenosine kinase or adenosine deaminase does not account for the erythrocyte ZMP-dependent, adenosine-mediated antiplatelet activity of acadesine, and the actual mechanism requires further investigation.

Inhibition of platelet aggregation *in vitro* by acadesine is analogous to that described for dipyridamole, an inhibitor of adenosine transport. In both cases the response is observed in whole blood but not PRP, and is inhibited by adenosine receptor antagonists or adenosine deaminase (33). Indeed, Gressele et al. (33) proposed that erythrocytes release precursors of adenosine during "microtrauma" suggested to occur during aggregation, although no evidence for erythrocyte injury during aggregation was found by these investigators or others. However, other features of the antiplatelet activity of acadesine distinguish it from adenosine transport inhibitors like dipyridamole. Dipyridamole delayed the removal of radiolabeled adenosine from blood, in contrast to acadesine (Fig. 5). Furthermore, the inhibition of aggregation with dipyridamole *in vitro* is partial (33), whereas complete inhibition can be achieved with acadesine. In addition, *ex vivo* studies of platelet inhibition in subjects receiving dipyridamole show very little antiaggregatory response (42, 43). In contrast, human subjects that received 100 mg/kg acadesine over 90 min showed significant reductions in *ex vivo* platelet aggregation elicited by both ADP and collagen (Fig. 6). Finally, acadesine effectively suppressed platelet-dependent cyclic coronary flow reductions in the open-chest dog, a model in which dipyridamole is ineffective (reference 10, and

present results). Thus, acadesine is a far more effective inhibitor of platelet aggregation *in vitro*, *ex vivo*, and *in vivo* than dipyridamole, and evidence suggests that the two agents operate via different mechanisms. In fact, recent clinical studies concur that dipyridamole is a weak inhibitor of aggregation with limited therapeutic benefit (9, 44). In contrast, the present studies suggest that acadesine has a more pronounced activity that is evident even after administration to human subjects using an *ex vivo* assay, and where adenosine has a half-life in human blood of  $< 1$  s (7). The purpose of the present clinical study was to confirm an antiplatelet effect in man. Additional studies are required to define other issues such as the dose-related activity, the role of adenosine, and the clinical significance of the inhibitory effect.

The canine model of CFRs described by Folts (25) is associated with the formation of platelet-rich thrombi. Acadesine effectively abolished CFRs in 12 of 14 dogs studied, and significantly reduced CFRs in the remaining two dogs. These antiplatelet effects of acadesine were prevented by pretreatment with the adenosine receptor antagonist, 8-SPT, at a dose that abolished the coronary vasodilator response to adenosine but did not block the antiplatelet activity of aspirin. In the absence of evidence for direct binding of acadesine to adenosine receptors (39), these data support the *in vitro* results demonstrating an adenosine-mediated action of acadesine.

While the present studies demonstrate antiplatelet activity *in vitro*, *ex vivo*, and *in vivo*, comparison between the antithrombotic efficacy in these systems is difficult because the pharmacokinetics of acadesine and its conversion to ZMP need to be taken into consideration. *In vitro*, 1 mM acadesine provides almost complete inhibition of aggregation and this is associated with erythrocyte ZMP levels of  $448 \pm 42$   $\mu\text{M}$  (Fig. 3). In human subjects, acadesine exhibited maximal effects 30 min after the infusion was terminated, when plasma acadesine levels were  $50 \pm 3$   $\mu\text{M}$ . In a separate study, an identical dose of acadesine yielded ZMP levels of 750  $\mu\text{M}$  ( $n = 2$ ) which peaked 30 min after the infusion was terminated (Ross Dixon, personal communication). The plasma level of acadesine ( $40 \pm 3$   $\mu\text{M}$ ) which abolished CFRs in dogs is similar to that observed in humans ( $50 \pm 3$   $\mu\text{M}$ ). We have not measured ZMP levels in dog platelets after acadesine infusion, but *in vitro* comparisons of acadesine-induced inhibition of platelet aggregation in dog vs. human blood reveal no substantial differences ( $\text{IC}_{50}$ s of  $240 \pm 60$   $\mu\text{M}$  and  $220 \pm 40$   $\mu\text{M}$  for dog and human blood, respectively, after 120 min of incubation). Thus the data available from dog and human in different assays are internally consistent with respect to plasma acadesine concentrations and ZMP levels in RBCs. It is clear, however, that acadesine is more potent *in vivo* vs. *in vitro* ( $40$ – $50$   $\mu\text{M}$  vs. 1 mM). One potential explanation resides in the presence of additional cell types *in vivo*, such as endothelial cells or cardiomyocytes (45, 46), which might also contribute some adenosine; or that there are additional factors which contribute to the antithrombotic effect *in vivo*, such as adenosine-mediated inhibition of platelet adherence to the injured vessel wall. It should be noted that the half-life of ZMP in human RBC is  $\sim 24$ – $36$  h, suggesting that ZMP levels achieved in all the current studies should be relatively constant for the duration of the experiments (47). We have not examined the pharmacokinetics of ZMP in other cell types.

In contrast to the antithrombotic effects of acadesine *in vivo*, an infusion of exogenous adenosine or dipyridamole had no effect on CFRs. This is consistent with a previous study by

Folts and Rowe (10), which demonstrated that dipyridamole, either alone or in combination with aspirin, had no effect on CFRs in the dog. While the failure of adenosine itself to prevent CFRs despite exerting hemodynamic effects may appear inconsistent with the proposed mechanism of action for acadesine, there are a number of potential explanations for this apparent conundrum. One clear difference in activity between adenosine and acadesine is the accompanying hemodynamic changes. Acadesine is hemodynamically "silent" because it does not increase adenosine levels in the systemic circulation but, rather, augments local adenosine levels in situations where adenosine production is increased, e.g., during myocardial ischemia (13). The decrease in coronary perfusion pressure secondary to hypotension elicited by adenosine and dipyridamole could act to reduce the shear stress in the stenotic vessel, which normally helps to dislodge the thrombus and thus offsets any antithrombotic activity. In addition, adenosine and dipyridamole are potent coronary vasodilators, in contrast to acadesine (13, 18). The former two agents are used to provoke coronary steal to aid in the diagnosis of ischemic heart disease using thallium scintigraphy (48). The diversion of coronary flow away from the stenosed vessel might also serve to reduce shear stress.

Another potential explanation for the failure of hemodynamically active levels of adenosine to influence CFRs is the relative potency of adenosine for its different pharmacological actions. Bellardinelli and Shyrook (49) highlighted the different concentrations of adenosine required to induce coronary vasodilation, bradycardia, negative dromotropic actions, and effects on contractile function, indicating that a wide range of adenosine concentrations are required to elicit the complete repertoire of responses. It is clear that adenosine is much less potent as an inhibitor of platelet aggregation (1) when compared with its effects on coronary vasodilation (49). Consequently, doses of adenosine which induce hemodynamic changes might be insufficient for antithrombotic activity. The pronounced cardiovascular effects at dose levels of adenosine required for platelet inhibition could limit this utility of adenosine in the clinic.

Despite the recognition of the antiplatelet activity of adenosine, and speculation that this activity might contribute to the cardioprotective effects of the purine, there is a paucity of *in vivo* studies using adenosine to inhibit platelet aggregation. The only study addressing the antithrombotic role of local endogenous adenosine, of which we are aware, was recently published by Kitakaze et al. (8). In this study, blockade of adenosine receptors in the presence of a coronary stenosis precipitated declines in coronary blood flow and coronary venous platelet counts, attributed to thromboembolism in the small coronary arteries. It was concluded that endogenous adenosine, formed in the ischemic myocardium, exerted an antiplatelet effect. The results of the present studies are consistent with the observations of Kitakaze et al. (8), and support the hypothesis that local augmentation of adenosine exhibits antithrombotic properties.

In conclusion, recent clinical studies with acadesine in the setting of coronary artery bypass graft surgery demonstrate reductions in perioperative myocardial infarction, stroke, and mortality (19, 20). Because of the proposed link between intracoronary thrombosis and acute myocardial infarction, it could be speculated that the antiplatelet activity of acadesine described in this study contributes to the benefits seen in the clinic. Certainly, this newly recognized antiplatelet activity extends the cardioprotective profile of acadesine, previously demonstrated to alleviate ischemia reperfusion injury.

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