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Adenosine Inhibits the Rise in Intracellular Calcium and Platelet Aggregation Produced by Thrombin: Evidence That Both Effects Are Coupled to Adenylate Cyclase

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SUMMARY

Platelet aggregation and secretion are associated with a rise in intracellular calcium concentration ($[Ca^{2+}]_i$). Adenosine has been postulated as an endogenous inhibitor of platelet aggregation. The antiaggregatory effects of adenosine are related to activation of adenylate cyclase. We studied the effect of adenosine on the rise in $[Ca^{2+}]_i$ and platelet aggregation produced by thrombin. Human platelets were obtained from dextrose/citrate-treated plasma. $[Ca^{2+}]_i$ was determined by fluorescence-dye techniques (fura-2). Adenosine inhibited the slope of the first phase of aggregation and the rise in $[Ca^{2+}]_i$ produced by thrombin, in a dose-dependent manner. The dose that produced 50% inhibition of both aggregation and the rise in $[Ca^{2+}]_i$ was approximately 500 nM. The effects of adenosine on $[Ca^{2+}]_i$ were shared by its stable analogs, 5′-*N*-ethylcarboxamidoadenosine being approximately 10-fold more potent than (−)N^6-phenylisopropyladenosine, suggesting that these effects were mediated through adenosine A2 receptors. Furthermore, caffeine antagonized the inhibitory effects of adenosine on platelet aggregation and $[Ca^{2+}]_i$. The effects of adenosine on $[Ca^{2+}]_i$ appear to be mediated through a rise in intracellular cAMP, because they were prevented by the adenylate cyclase inhibitor 2′,5′-dideoxyadenosine (1 mM) and were potentiated by phosphodiesterase inhibition with papaverine (1 μM). Adenosine also inhibits the rise in $[Ca^{2+}]_i$ produced by thrombin in a calcium-free medium, suggesting that adenosine inhibits both calcium influx and the release of calcium from intracellular stores.

Platelet activation plays a vital role in hemostasis. It is initiated by disruption of vascular endothelium, which results in the generation of thrombin and other mediators. Thrombin is a potent aggregatory agent of human platelets (1), and growth of a thrombus appears to depend largely upon the generation of thrombin at the injury site and around the aggregatory mass.

Adenosine has been postulated as an endogenous inhibitor of platelet aggregation and secretion (2). The putative efficacy of dipyridamole as an antiaggregatory agent could be explained by its ability to block the nucleoside transport system, thus increasing extracellular concentrations of adenosine and potentiating its effect on membrane receptors (3). The antiaggregatory effects of adenosine are mediated through activation of cell surface membrane A2 (Ra) receptors (4) and are associated with an increase in cyclic AMP (5). On the other hand, a rise in the intracellular ionic calcium concentration appears to be essential in the development of platelet aggregation and secretion induced by thrombin (4, 6) and other aggregating agents (7). Although the effects of adenosine on platelet aggregation, secretion, and adenylate cyclase are well established, the effects of adenosine on platelet intracellular calcium and other intracellular signaling mechanisms are not known. The role of adenylate cyclase and/or intracellular calcium as second messengers for the actions of adenosine in other secretory cells is also unclear (8–10). Therefore, we sought to determine whether adenosine would modulate platelet $[Ca^{2+}]_i$. We also wanted to determine whether this action was mediated by activation of cell surface A2 receptors and was coupled to a subsequent rise in cyclic AMP.

Materials and Methods

Preparation of platelets. Blood was obtained from healthy, medication-free volunteers who abstained from methylxanthine-containing beverages for at least 12 hr before the study. After the first milliliter of blood was discarded, blood was drawn into ACD solution (9:1, v/v;

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ABBREVIATIONS: PRP, platelet-rich plasma; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; NECA, *5′-N-ethylcarboxamidoadenosine;* L-PIA, (−)N^6-phenylisopropyladenosine DPSPX, 1,3-dipropyl-8-(*p*-sulfophenyl)xanthine; PPP, platelet-poor plasma; $[Ca^{2+}]_i$, intracellular calcium concentration.

composition, 25 g/liter dextrose, 22 g/liter Na citrate, 8 g/liter citric acid, in water, pH 5) at room temperature and in the presence of 5 μg/ml indomethacin. In preliminary studies, we showed that cyclooxygenase inhibition had no effect on adenosine actions on aggregation or intracellular calcium. PRP was obtained by centrifugation of whole blood at 200 × *g* for 20 min at room temperature. PPP was obtained by spinning the remaining blood at 1000 × *g* for 10 min.

For intracellular calcium determinations, PRP was mixed with ACD (9:1, v/v), placed over 50% albumin, and centrifuged at 1000 × *g* for 10 min (11). The platelet layer was resuspended in a buffer containing 150 mM NaCl, 2.7 mM KCl, 0.37 mM NaH_2PO_4, 1 mM $MgSO_4$, 5 mM D-glucose, 10 mM HEPES-NaOH, 50 units/ml heparin, 0.35% bovine serum albumin, 4 mM phosphocreatinine, and 8 units/ml creatinine phosphokinase, pH 6.55. Platelets were loaded with fura-2 by incubation of this platelet suspension for 30 min at 37° with 1 μM fura-2/acetoxymethyl ester. Cells were then centrifuged over 50% albumin at 1000 × *g* for 10 min. The platelet layer was resuspended in the same buffer, except that the pH was 7.4 and the buffer contained 2 mM $CaCl_2$ and no heparin. Some experiments were done in calcium-free buffer, as indicated.

Platelet aggregation. Aggregation was assessed by the principle of light transmission (660 Platelet Calcium Ionized Aggregometer; Chronolog Co., Havertown, PA). Drugs (5 μl) were added to 300 μl of PRP, which was continuously stirred at 37°. PPP was used as blank. The slope of the primary phase of aggregation was taken as the response parameter for agonist-stimulated platelet aggregation and was expressed in arbitrary units.

Cyclic AMP measurements. cAMP levels were determined in PRP by radioimmunoassay (12). Samples of 0.3 ml of PRP were incubated at 37° for 1 min in the aggregometer. Then, 5 μl of either buffer or drug as indicated were added to the PRP. After addition of 0.3 unit/ml thrombin, PRP was incubated for 1 min and then the reaction was stopped by addition of trichloracetic acid (final volume, 6%). The mixtures were centrifuged for 10 min at 12,000 × *g*, at 4°. The supernatant was extracted five times with 4 volumes of water-saturated ether, and the aqueous phase was lyophilized and solubilized in 500 μl of water. Fifty microliters were used for cAMP determination after acetylation. Cyclic AMP levels were expressed in fmol/10^6 platelets. Cells were counted in an automated cell counter (Coulter Counter Model ZM; Coulter Electronics, Inc., Hialeah, FL).

Intracellular calcium measurements. Cytosolic free calcium concentrations were determined by the fura-2 technique. Fluorescence was measured in 1-cm plastic cuvettes containing 1800 μl of buffer and 200 μl of platelet suspension, at room temperature. The emission wavelength was 510 nm and the excitation wavelengths were 340 and 380 nm. Maximal fluorescence was determined at the end of each incubation by addition of 40 μl of 2% Triton X-100. Minimal fluorescence was then determined by addition of 40 μl of 1 mM EGTA. Intracellular calcium was calculated using previously described formulas, using a K_d of 135 nM and an S_{f2}/S_{b2} of 15.3 (13). Fluorescence was measured either in a SPF-500 spectrofluorometer (American Instrument Co., Silver Spring, MD) or in a CM1T11I cation measurement system (Spex Industries Inc., Edison, NJ).

Drugs. The stable adenosine analogs L-PIA and NECA were purchased from Research Biochemicals, Inc. (Natick, MA). The effects of these analogs on platelet function and cAMP levels have been reported previously (14). 2′,5′-Dideoxyadenosine was a generous gift from Dr. Roger Johnson, Department of Biochemistry, Stony Brook University, NY. DPSPX was synthesized using a variation (15) of a previously described method (16). All other drugs were purchased from Sigma Chemical Company (St. Louis, MO).

Calculation of EC_{50} values from dose-response curves was done with a microcomputer, using ALLFIT software. Results are presented as a mean ± standard error.

Results

Effects of adenosine on platelet aggregation and intracellular calcium. Thrombin produced platelet aggregation in association with a rise in intracellular calcium. The rise in intracellular calcium reached a peak at approximately 30 sec and was stable for at least 2 min. Calcium measurements were done at baseline (227 ± 66 nM) and at peak response to thrombin (1920 ± 273 nM; 12 experiments). Adenosine, added immediately before thrombin, inhibited platelet aggregation and the rise in intracellular calcium induced by thrombin. The inhibitory actions of adenosine were dose dependent (Fig. 1). The dose of adenosine that produced 50% inhibition (EC_{50} was virtually identical for inhibition of aggregation (510 ± 43 nM) and for inhibition of the rise in intracellular calcium (490 ± 49 nM). The inhibitory effects of adenosine were maximal at 100 μM. At this dose, adenosine produced virtually complete inhibition of platelet aggregation (94%). In contrast, inhibition of the rise in intracellular calcium was partial (55%).

Effects of stable adenosine analogs on intracellular calcium. the stable adenosine analogs NECA and L-PIA inhibited the rise in intracellular calcium produced by thrombin. The effects were also dose dependent. NECA was approximately 10-fold more potent than L-PIA in inhibiting $[Ca^{2+}]_i$ (Fig. 2). However, EC_{50} values were not calculated, because solubility problems with higher concentrations precluded us achieving maximal response.

Effects of adenosine antagonists. Caffeine antagonized

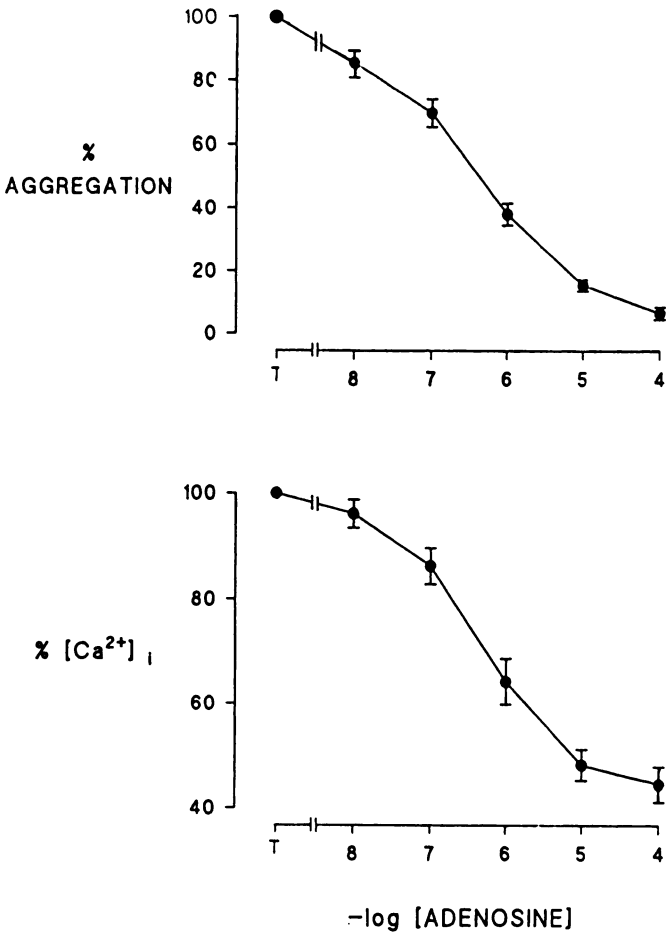

Fig. 1. Effect of increasing concentrations of adenosine on thrombin-induced platelet aggregation (*upper*) and peak increase in free cytosolic calcium concentration (*lower*) (12 experiments). In both cases, the response to 0.3 units/ml thrombin (*T*) was taken as 100%.

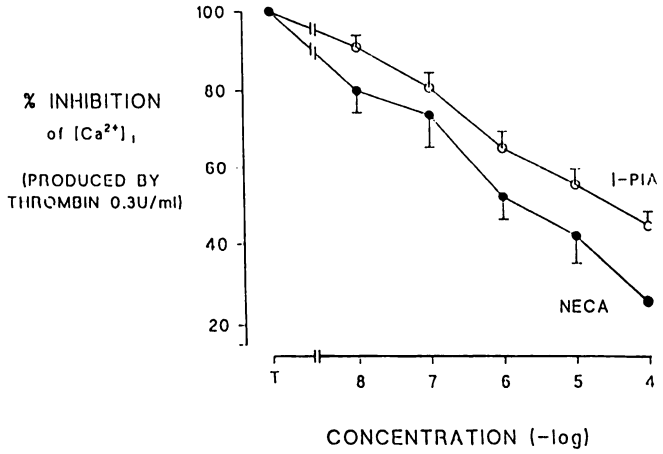

Fig. 2. Effect of increasing concentration of NECA or L-PIA on thrombin-induced increases in free cytosolic calcium concentration. The response to 0.3 units/ml thrombin (*T*) was taken as 100%. Each point represents the mean ± standard error of three experiments.

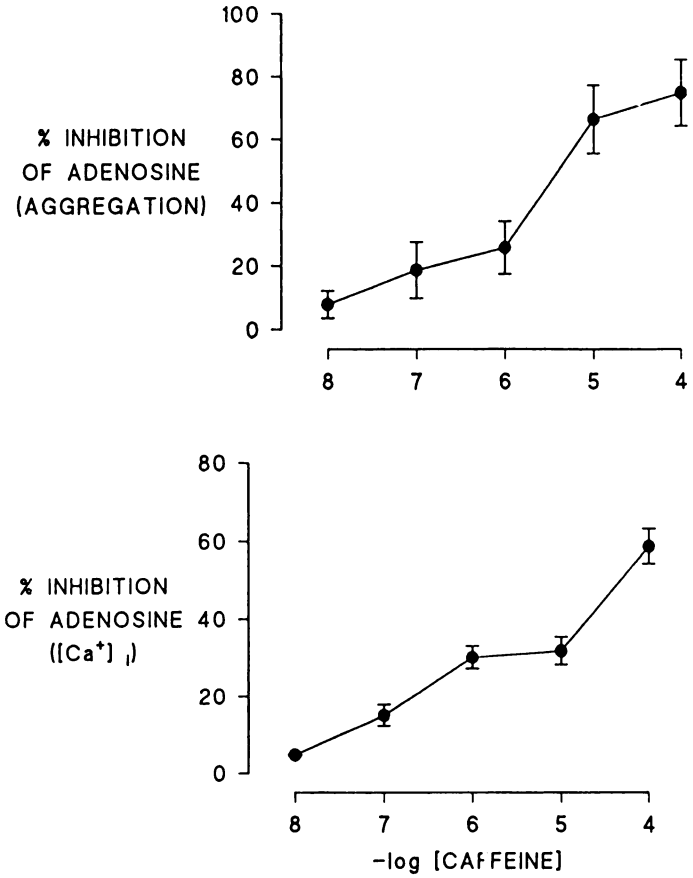

Fig. 3. Effect of increasing concentrations of caffeine on the inhibitory effects of 1 μM adenosine on the platelet aggregation (*upper*) and the increase in cytosolic free calcium concentration (*lower*) produced by 0.3 units/ml thrombin. Each *point* represents the mean ± standard error of three experiments

the actions of adenosine on platelet aggregation (Fig. 3, *upper*) and intracellular calcium (Fig. 3, *lower*), in a dose-dependent manner. At the concentrations used, caffeine had no effect on baseline intracellular calcium. At concentrations above 1 mM, caffeine reportedly releases calcium from the sarcoplasmic reticulum of skeletal, myocardial, and vascular smooth muscle cells (17). Caffeine is also a relatively weak phosphodiesterase inhibitor. However, even if this effect were operative, caffeine would potentiate rather than inhibit adenosine. Because caffeine may have other actions unrelated to adenosine receptor antagonism, the effect of a specific adenosine receptor antagonist, DPSPX, was also determined (16). DPSPX (100 μM) antagonized the effect of adenosine on platelet aggregation (Fig. 4) and increased the EC_{50} of adenosine from 438 to 4807 nM. The effects of DPSPX on intracellular calcium could not be determined, because DPSPX emitted fluorescence that interfered with $[Ca^{2+}]_i$ measurements.

Role of cyclic AMP in the actions of adenosine. The role of cAMP in the actions of adenosine on intracellular calcium was evaluated either by preventing the rise in cAMP produced by adenosine with 2′,5′-dideoxyadenosine, an inhibitor of the catalytic subunit of adenylate cyclase, or by potentiating the rise in cAMP through inhibition of phosphodiesterase with papaverine.

As shown in Fig. 5, 1 μM adenosine inhibited thrombin-induced aggregation by 73% and the rise in intracellular calcium by 43%. Cyclic AMP levels found in thrombin-stimulated PRP were 95 ± 25 fmol/10^6 cells. Adenosine increased cAMP by 72% above levels found in the presence of thrombin alone, to 162 ± 38 fmol/10^6 cells. However, in the presence of 2′,5′-dideoxyadenosine, the adenosine-induced increase in cAMP was prevented (119 ± 92 fmol/10^6 cells, or 25% above thrombin alone). The inhibitory effects of adenosine on aggregation and intracellular calcium were also reversed by the prior addition of 1 mM 2′,5′-dideoxyadenosine (Fig. 5), which acts on the intracellular inhibitory "P site" of the catalytic subunit of adenylate cyclase (18). This concentration of 2′,5′-dideoxyadenosine had no effect on baseline or thrombin-stimulated intracellular calcium concentration or aggregation.

We chose papaverine as a phosphodiesterase inhibitor over methylxanthines because of the potential for methylxanthines to antagonize adenosine receptors. NECA, a stable adenosine analog, was used instead of adenosine because papaverine can also block adenosine uptake into cells, thus potentiating its action on extracellular receptors. NECA (0.1 μM) inhibited thrombin-induced aggregation by 22 ± 6% and inhibited the rise in calcium produced by thrombin by 18 ± 3% in the presence of adenosine deaminase (2 units/ml). Phosphodiesterase inhibition with 1 μM papaverine resulted in potentiation of

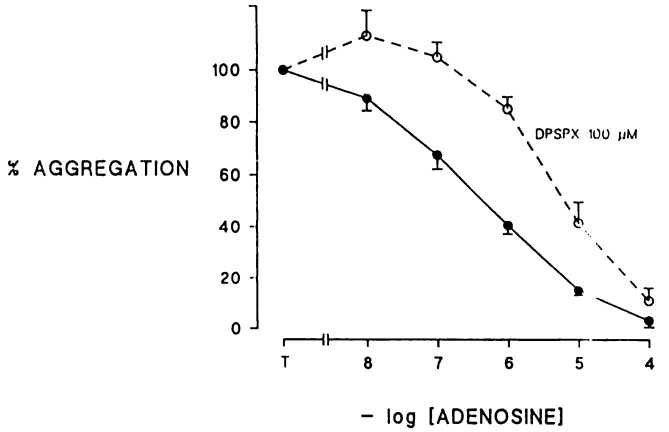

Fig. 4. Effect of adenosine on thrombin-induced platelet aggregation in the absence (——) or in the presence (– – –) of 100 μM DPSPX. Each *point* represents the mean ± standard error of three experiments.

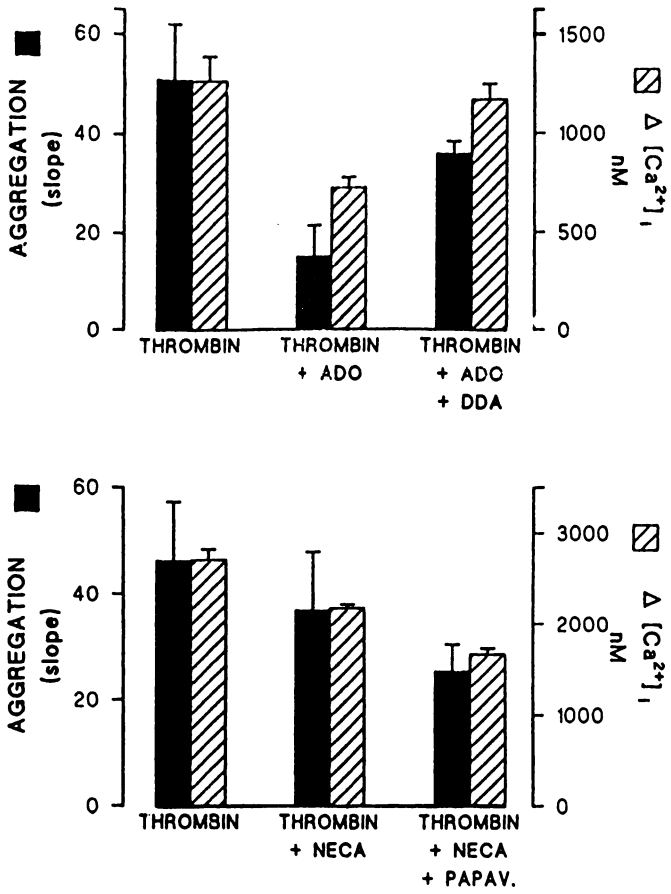

Fig. 5. Effect of 0.3 units/ml thrombin on platelet aggregation (■), expressed as the slope of the primary phase of aggregation in arbitrary units (*left axis*), and free cytosolic calcium concentration (▨) (*right axis*). *Upper*, effects of thrombin alone (*left bars*) and in the presence of 1 μM adenosine (*ADO*) (*middle bars*) and the effects of 1 mM 2′,5′-dideoxyadenosine (*DDA*) on adenosine actions (*right bars*). *Lower*, effects of thrombin alone (*left bars*) and in the presence of 100 nM NECA (*middle bars*) and the effects of 1 μM papaverine (*PAPAV*) on adenosine actions (*right bars*). Each *bar* represents the mean ± standard error of three experiments.

the inhibitory effects of NECA on both aggregation (to 46 ± 6%) and intracellular calcium (to 37 ± 4%) (Fig. 5). Papaverine also shifted the dose-response curve of NECA for platelet aggregation to the left (Table 1). We chose this concentration of papaverine because in ancillary studies it was found to have little if any effect on basal intracellular calcium or on the effects of thrombin on intracellular calcium or aggregation. Even at higher concentrations (0.1–0.4 mM), papaverine reportedly does not increase baseline cAMP significantly (19).

Effects of adenosine on calcium influx and intracellular calcium mobilization. To determine whether the effects of adenosine on $[Ca^{2+}]_i$ were due to inhibition of calcium influx and/or inhibition of calcium mobilization from intracellular stores, the rise in intracellular calcium produced by thrombin was determined in parallel studies either in the presence of 2 mM calcium or in a calcium-free buffer containing 1 mM EGTA. The increase in the fura-2 signal produced by thrombin in the presence of extracellular calcium reflects both calcium entry and intracellular calcium mobilization. On the other hand, the increase in fura-2 signal produced by thrombin in the absence of extracellular calcium reflects only release of calcium from intracellular stores (20). Finally, the mathematical difference between these two signals can be used as an indirect measurement of calcium influx. In three parallel experiments, thrombin increased intracellular calcium from 56 ± 18 nM to 1958 ± 261 nM in the presence of extracellular calcium and from 51 ± 24 nM to 893 ± 236 nM in the absence of extracellular calcium. As can be seen in Fig. 6 and in Table 2, adenosine produced a dose-dependent inhibition of the rise in intracellular calcium produced by thrombin, both in the presence and in the absence of extracellular calcium. The EC_{50} values were 151 ± 39 nM and 135 ± 31 nM, respectively. When the difference between the calcium signal produced by thrombin in the presence and in the absence of extracellular calcium was used to determine calcium influx, adenosine also inhibited calculated calcium influx, with an EC_{50} of 178 ± 52 nM (Table 2).

Discussion

Adenosine has been shown to inhibit activation of many secretory cells in addition to platelets. Adenosine appears to act on A2 receptors in leukocytes, as determined by the relative potency of stable adenosine analogs (NECA > L-PIA) (21). However, it is unclear whether adenosine receptors on these secretory cells are linked to adenylate cyclase and whether their activation modulates intracellular calcium concentrations (8, 9). Adenosine also inhibits neurotransmitter release from nerve terminals (22). This effect appears to be associated with an inhibition of intracellular calcium. However, the characteristics of the receptor that mediates this action, and the second messengers involved, remain unclear. The existence of a third adenosine receptor that modulates intracellular calcium but is not linked to adenylate cyclase has been proposed (10).

Since 1963, it has been known that adenosine inhibits platelet aggregation (23). It was initially thought that the effects of adenosine on blood platelets could be explained by antagonism of ADP receptors. It is now apparent that adenosine activates A2 receptors located in the platelet cell surface, leading to an increase in intracellular cAMP (3, 5). Although the role of endogenous adenosine in platelet homeostasis has not been fully elucidated, it has been postulated that the actions of dipyridamole are at least partially mediated through potentiation of the actions of endogenous adenosine (3). Also, the

TABLE 1

Effect of increasing concentrations of NECA on thrombin-induced platelet aggregation in the absence or presence of papaverine (1 μM)

Results are shown as mean ± standard error of three experiments. The response to thrombin was taken as 100%.

	Platelet aggregation (%)				
	Thrombin	Thrombin + NECA			
		10 nM	50 nM	100 nM	250 nM
Control	100	95 ± 2	78 ± 7	58 ± 7	12 ± 0.3
Papaverine	97	84 ± 2	68 ± 5	47 ± 6	8 ± 0.9

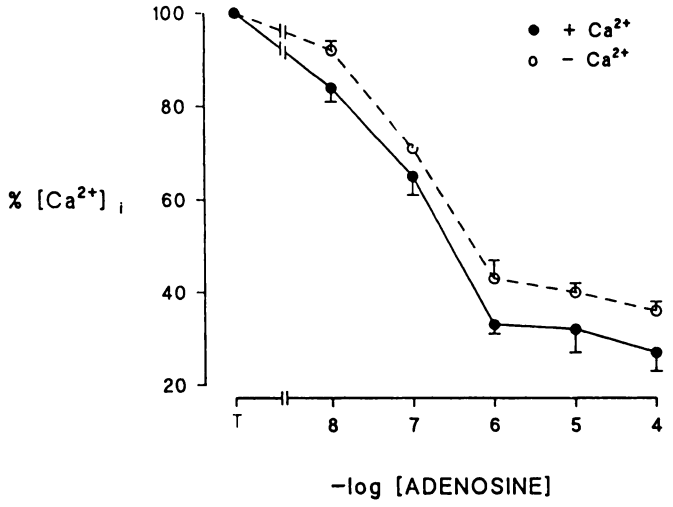

Fig. 6. Effects of adenosine on thrombin-induced increases in free cytosolic calcium concentration in the presence of 2 mM calcium in the incubation buffer (——) or in the absence of extracellular calcium (in the presence of 1 mM EGTA) (– – –). Each *point* represents the mean ± standard error of three experiments.

intracellular processes that mediate adenosine-induced inhibition of platelet aggregation following the rise in cAMP have not been determined. Because a rise in intracellular calcium is essential for many secretory processes, including platelet aggregation and secretion (7), we studied the effect of adenosine on intracellular calcium. Furthermore, other agents, such as prostaglandins and forskolin, that are known to raise cAMP reportedly lead to a decrease in intracellular calcium (24–26).

Adenosine inhibited thrombin-induced platelet aggregation, as previously reported (12). The dose that produced 50% inhibition of platelet aggregation was approximately 500 nM, a concentration relatively close to that found in plasma of normal subjects (161 ± 75 nM) (27). It must be noted that, although the EC_{50} values of adenosine for platelet aggregation and inhibition of intracellular calcium were virtually identical, the maximal effect of adenosine produced a 94% inhibition of platelet aggregation while producing only a 55% inhibition of the rise in intracellular calcium (Fig. 1). This can be interpreted as meaning that adenosine inhibits calcium mobilization from a discrete pool that is critical for the development of platelet aggregation but this is not reflected as a complete reduction in total cytosolic free calcium. Alternatively, adenosine may be acting on other intracellular processes responsible for inhibition of platelet aggregation, in addition to intracellular calcium. For example, it has been suggested that activation of adenylate cyclase results in inhibition of protein kinase C (28).

The observed effects of adenosine on intracellular calcium appear to be mediated through cell surface adenosine A2 receptors. The greater potency of NECA over L-PIA and the antagonism of caffeine and DPSPX support this conclusion. Because activation of adenosine A2 receptors on platelets results in activation of adenylate cyclase with cAMP accumulation (4), it seemed likely that the increase in cAMP was responsible for adenosine-induced inhibition of intracellular calcium. Our results suggest that this is indeed the case. Adenylate cyclase inhibition with 2′,5′-dideoxyadenosine (an inhibitor of the catalytic subunit of the enzyme, acting at the P site) prevented the actions of adenosine on intracellular calcium. Conversely, prevention of the removal of cAMP by phosphodiesterases with papaverine resulted in potentiation of the effects of adenosine receptor activation on intracellular calcium. We believe this approach provides further proof of a link between adenylate cyclase and the calcium signaling mechanism, as reported previously (24–26).

The rise in intracellular calcium produced by thrombin is the result of both an increase in calcium influx from the extracellular medium and mobilization of calcium from intracellular stores (20). Adenosine inhibited the rise in intracellular calcium produced by thrombin, both in the presence of calcium (reflecting both pathways) and in a calcium-free medium (reflecting only the mobilization of intracellular stores). Our results suggest that adenosine inhibits both the influx of calcium and the mobilization of internal stores produced by thrombin.

In summary, adenosine receptor activation in human platelets results in inhibition of thrombin-induced aggregation. The antiaggregatory actions of adenosine can be explained at least partially by inhibition of both calcium influx and mobilization of internal stores. The effects of adenosine on platelet aggregation and intracellular calcium are coupled to adenylate cyclase activation.

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References

1. Rittenhouse-Simmons, S. Differential activation of platelet phospholipase by thrombin and ionophore A23187. *J. Biol. Chem.* **256**:4153–4155 (1981).

TABLE 2

Effect of increasing concentrations of adenosine on the rise in intracellular calcium produced by thrombin (0.3 units/ml) in human platelets

Platelets were studied in the presence of 2 mM calcium (the increase in $[Ca^{2+}]_i$ produced by thrombin results from both calcium influx and release of internal stores) and in a calcium-free medium containing 1 mM EGTA (the increase in $[Ca^{2+}]_i$ produced by thrombin results from release of intracellular stores). The mathematical difference between these two is shown, to estimate calcium influx. Adenosine produced a dose-dependent inhibition of all three estimates of intracellular calcium. Results are expressed as mean ± standard error of three experiments. Absolute values of intracellular calcium concentration are given.

	$[Ca^{2+}]_i$						
	Basal	After thrombin (0.3 units/ml)	Thrombin + adenosine				
			10^{-8} M	10^{-7} M	10^{-6} M	10^{-5} M	10^{-4} M
			nM				
Presence of extracellular calcium (2 mM)	56 ± 18	1958 ± 261	1685 ± 262	1271 ± 237	684 ± 147	650 ± 173	613 ± 182
Absence of extracellular calcium	51 ± 24	893 ± 236	811 ± 202	637 ± 171	361 ± 121	347 ± 78	323 ± 68
Difference		1065 ± 170	874 ± 215	634 ± 211	823 ± 127	302 ± 178	290 ± 178

2. Gerlach, E., B. F. Becker, and S. Nees. Formation of adenosine by vascular endothelium: a homeostatic and antithrombogenic mechanism? in *Topics and Perspectives in Adenosine Research* (E. Gerlach and B. F. Becker, eds.), Springer-Verlag, Berlin, 309–320 (1987).
3. Dawicki, D. D., K. C. Agarwal, and R. E. Parks, Jr. Potentiation of the antiplatelet action of adenosine in whole blood by dipyridamole or dilazep and the cAMP phopshodiesterase inhibitor, RA 233. *Thromb. Res.* **43**:161–175 (1986).
4. Martison, E. A., R. A. Johnson, and J. N. Wells. Potent adenosine receptor antagonists that are selective for the A_1 receptor subtype. *Mol. Pharmacol.* **31**:247–252 (1987).
5. Haslam, R. J., and G. M. Rosson. Effects of adenosine on levels of adenosine cyclic 3′,5′-monophosphate in human blood platelets in relation to adenosine incorporation and platelet aggregation. *Mol. Pharmacol.* **11**:528–544 (1975).
6. Rao, G. H. R., J. D. Peller, and J. G. White. Measurement of ionized calcium in blood platelets with a new generation calcium indicator. *Biochem. Biophys. Res. Commun.* **132**:652–657 (1985).
7. Salzman, E. W., and J. A. Ware. Ionized calcium as an intracellular messenger in blood platelets. *Prog. Thromb. Haem.* 177–202 (1989).
8. Cronstein, B. N., S. B. Kramer, E. D. Rosenstein, H. M. Korchak, G. Weissmann, and R. Hirschhorn. Occupancy of adenosine receptors raises cyclic AMP alone and in synergy with occupancy of chemoattractant receptors and inhibits membrane depolarization. *Biochem. J.* **252**:709–715 (1988).
9. Nielson, C., and R. E. Vestal. Effects of adenosine on polymorphonuclear leucocyte function, cyclic 3′,5′-adenosine monophosphate, and intracellular calcium. *Br. J. Pharmacol.* **97**:882–888 (1989).
10. Ribeiro, J. A., and A. M. Sebastiao. Adenosine receptors and calcium: basis for proposing a third (A_3) adenosine receptor. *Prog. Neurobiol.* **26**:179–209 (1986).
11. Hollister, A. S., J. H. J. Nadeau, G. A. FitzGerald, and D. Robertson. Acute reduction in human platelet α_2-adrenoreceptor affinity for agonist by endogenous and exogenous catecholamines. *J. Clin. Invest.* **72**:1498–1505 (1983).
12. Cusack, N. J., and S. M. O. Hourani. 5′-*N*-Ethylcarboxamidoadenosine: a potent inhibitor of human platelet aggregation. *Br. J. Pharmacol.* **72**:443–447 (1981).
13. Grynkiewicz, G., M. Poenie, and R. Y. Taien. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260**:3440–3450 (1985).
14. Huttermann, E., D. Ukena, V. Lenschow, and U. Schwabe. Ra adenosine receptors in human platelets. Characterization by 5′-*N*-ethylcarboxamido-[^{3}H]adenosine binding in relation to adenylate cyclase activity. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **325**:226–233 (1984).
15. Biaggioni, I., L. S. King, N. Enayat, D. Robertson, and J. Newman. Adenosine produces pulmonary vasoconstriction in sheep. Evidence for thromboxane A_2 prostaglandin endoperoxide receptor activation. *Circ. Res.* **65**:1516–1525 (1989).
16. Daly, J. W., W. Padgett, M. T. Shamin, P. Burrs-Lamb, and J. Waters. 1,3-Dialkyl-8-(*p*-sulfophenyl)xanthines: potent water-soluble antagonists for A_1- and A_2-adenosine receptors. *J. Med. Chem.* **4**:487–492 (1985).
17. Kobayashi, S., H. Kanaide, and M. Nakamura. Complete overlap of caffeine- and K^+ depolarization-sensitive intracellular calcium storage site in cultured rat arterial smooth muscle cells. *J. Biol. Chem.* **261**:15709–15713 (1986).
18. Haslam, R. J., M. M. L. Davidson, and J. V. DesJardins. Inhibition of adenylate cyclase by adenosine analogues in preparations of broken and intact human platelets. Evidence for the unidirectional control of platelet function by cyclic AMP. *Biochem. J.* **176**:83–95 (1978).
19. Zahavi, M., J. Zahavi, and V. V. Kakkar. Effect of adenylcyclase activators, phosphodiesterase inhibitors and pyridoxal-5-phosphate on platelet aggregation and adenosine-3′-5′-cyclic monophosphate accumulation. *Thromb Haemostas.* **52**:205–209 (1984).
20. Pollock, W. K., and T. J. Rink. Thrombin and ionomycin can raise platelet cytosolic Ca^{2+} to micromolar levels by discharge of internal Ca^{2+} stores: studies using FURA-2. *Biochem. Biophys. Res. Commun.* **139**:308–314 (1986).
21. Cronstein, B. N., R. I. Levin, J. Belarder, G. Weissman, and R. Hirschhorn. Adenosine: an endogenous inhibitor of neutrophil-mediated injury to endothelial cells. *J. Clin. Invest.* **78**:76–77 (1986).
22. Kuan, C. J., and E. K. Jackson. Role of adenosine in noradrenergic neurotransmission. *Am. J. Physiol.* **255**:H386–H393 (1988).
23. Born, G. V. R., A. J. Honour, and J. R. A. Mitchell. Inhibition by adenosine and by 2-chloroadenosine of the formation and embolization of platelet thrombi. *Nature Lond.* **202**:761–765 (1964).
24. Zavoico, G. B., and M. B. Feinstein. Cytoplasmic calcium in platelets is controlled by cyclic AMP: antagonism between stimulators and inhibitors of adenylate cyclase. *Biochem. Biophys. Res. Commun.* **120**:579–585 (1984).
25. Feinstein, M. B., J. J. Egan, R. I. Sha'afi, and J. White. The cytoplasmic concentration of free calcium in platelets is controlled by stimulators of cyclic AMP production (PGD_2, PGE_1, forskolin). *Biochim. Biophys. Acta* **113**:598–604 (1983).
26. Sage, S. O., and T. J. Rink. Inhibition by forskolin of cytosolic calcium rise, shape change and aggregation in quin-2 loaded human platelets. *FEBS Lett.* **188**:135–140 (1985).
27. Biaggioni, I., J. Onrot, A. S. Hollister, and D. Robertson. Cardiovascular effects of adenosine infusion in man and their modulation by dipyridamole. *Life Sci.* **39**:2229–2236 (1986).
28. Imai, A., H. Hattori, M. Takahashi, and Y. Nozawa. Evidence that cyclic AMP may regulate calcium mobilization in phospholipase in thrombin-stimulated human platelets. *Biochem. Biophys. Res. Commun.* **112**:693–700 (1983).
29. Paul, S., A. S. Hollister, D. Robertson, and I. Biaggioni. Adenosine inhibits the rise in intracellular calcium and platelet aggregation produced by thrombin. *Clin. Res.* **37**:341A (1989).
30. Feoktistov, I. A., S. Paul, A. S. Hollister, D. Robertson, and I. Biaggioni. Mechanism of adenosine inhibition of intracellular calcium rise in human platelets. *Circulation* **80**:II-126 (1989).

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