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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

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Whereas adenosine itself exerted independent stimulatory and inhibitory effects on the adenylate cyclase activity of a platelet particulate fraction at low and high concentrations respectively, 2-substituted and N^6-monosubstituted adenosines had stimulatory but greatly decreased inhibitory effects. Deoxyadenosines, on the other hand, had enhanced inhibitory but no stimulatory effects. The most potent inhibitors found were, in order of increasing activity, 9-(tetrahydro-2-furyl)adenine (SQ 22536), 2′,5′-dideoxyadenosine and 2′-deoxyadenosine 3′-monophosphate. Kinetic studies on prostaglandin E_1-activated adenylate cyclase showed that the inhibition caused by either 2′,5′-dideoxyadenosine or compound SQ 22536 was non-competitive with MgATP and that the former compound, at least, showed negative co-operativity; 50% inhibition was observed with 4 μM-2′,5′-dideoxyadenosine or 13 μM-SQ 22536. These two compounds also inhibited both the basal and prostaglandin E_1-activated adenylate cyclase activities of intact platelets, when these were measured as the increases in cyclic [^{3}H]AMP in platelets that had been labelled with [^{3}H]adenine and were then incubated briefly with papaverine or papaverine and prostaglandin E_1. Both compounds, but particularly 2′,5′-dideoxyadenosine, markedly decreased the inhibition by prostaglandin E_1 of platelet aggregation induced by ADP or [arginine]vasopressin as well as the associated increases in platelet cyclic AMP, so providing further evidence that the effects of prostaglandin E_1 on platelet aggregation are mediated by cyclic AMP. 2′-Deoxyadenosine 3′-monophosphate did not affect the inhibition of aggregation by prostaglandin E_1, suggesting that the site of action of deoxyadenosine derivatives on adenylate cyclase is intracellular. Neither 2′,5′-dideoxyadenosine nor compound SQ 22536 alone induced platelet aggregation. Moreover, neither compound potentiated platelet aggregation or the platelet release reaction when suboptimal concentrations of ADP, [arginine]vasopressin, collagen or arachidonate were added to heparinized or citrated platelet-rich plasma in the absence of prostaglandin E_1. These results show that cyclic AMP plays no significant role in the responses of platelets to aggregating agents in the absence of compounds that increase the platelet cyclic AMP concentration above the resting value.

Adenosine has been shown to have a biphasic effect on the unstimulated adenylate cyclase activity of platelet particulate fraction, activating the enzyme at low concentrations and inhibiting at higher concentrations (Haslam & Lynham, 1972). In the presence of PGE_1, a potent activator of adenylate cyclase, only the inhibitory activity of adenosine was observed. With intact platelets, the stimulatory activity of adenosine, mediated by externally orientated membrane receptors, predominated, but evidence for an attenuated inhibition of adenylate cyclase by adenosine acting at intracellular sites was also obtained (Haslam & Rosson, 1975). Inhibition by adenosine of adenylate cyclase from other tissues has frequently been observed (Moriwaki & Foa, 1970; Fain *et al.*, 1972; McKenzie & Bär, 1973; Weinryb & Michel, 1974; Zenser, 1976), and various deoxy analogues of adenosine have been found to be even more potent inhibitors, including 2′,5′-dideoxyadenosine with adenylate cyclase from fat-cells (Fain *et al.*, 1972) and epithelial cells (Zenser, 1976) and various 9-substituted adenines, including 9-(tetrahydro-2-furyl)adenine (SQ 22536), with adenylate cyclase from lung (Weinryb & Michel, 1974). We therefore examined the effects of these and other adenosine analogues on platelet adenylate cyclase with a view to finding compounds with inhibitory

* Abbreviations used: PGE_1, prostaglandin E_1; IC_{50}, concentration giving 50% inhibition.

but no stimulatory activity which could potentially be used in intact platelets to investigate the roles of cyclic AMP in platelet function. We were also encouraged to undertake this study by a preliminary report of Harris *et al.* (1975) indicating that some 9-substituted adenines, including compound SQ 22536, blocked the effects of PGE_1 on platelets and by the finding of Zenser (1976) that 2′,5′-dideoxyadenosine suppressed the increases in cyclic AMP in epithelial cells caused by cholera toxin.

Although it has been widely accepted that cyclic AMP mediates the inhibitory effects on platelet function of PGE_1, adenosine and inhibitors of cyclic AMP phosphodiesterase (Salzman, 1972; Haslam, 1973, 1978; Mills & Macfarlane, 1976; Haslam *et al.*, 1978), the question whether or not decreases in platelet cyclic AMP occur during aggregation and may promote the aggregation process, as suggested by Salzman & Levine (1971), has not been resolved. The fact that some aggregation can occur in the presence of increased platelet cyclic AMP concentrations rules out a role for decreases in platelet cyclic AMP as a necessary cause of aggregation (Haslam, 1973, 1975, 1978), but does not eliminate the possibility that aggregation or release of platelet granule constituents could be facilitated by a decrease in the steady-state concentration of cyclic AMP in resting platelets. Measurements of changes in platelet cyclic AMP induced by aggregating agents have given widely divergent results and have not contributed to the solution of this problem (e.g. Salzman, 1972; Haslam, 1975). In the last part of the present study, we have therefore attempted to determine whether or not cyclic AMP exerts a tonic inhibitory effect within the resting platelet by investigation of the effects on platelet function of adenosine analogues that inhibit adenylate cyclase. Some of our results have been reported in a preliminary form (Haslam, 1978; Haslam *et al.*, 1977, 1978).

Experimental

Materials

[2-^{3}H]ATP (27Ci/mmol), [2-^{3}H]adenine (19Ci/mmol), cyclic [adenine-U-^{14}C]AMP (287mCi/mmol) and 5-hydroxy[*side-chain*-2-^{14}C]tryptamine (54mCi/mmol) were obtained from Amersham Corp., Oakville, Ont., Canada. ATP, ADP, cyclic AMP, adenosine, 2′-deoxyadenosine, 3′-deoxyadenosine 2′-deoxy, adenosine 3′-monophosphate, 2-chloroadenosine, N^6-γγ-dimethylallyladenosine, N^6-benzyladenosine, dithiothreitol, EGTA, phosphocreatine, creatine phosphokinase, crystalline bovine serum albumin, protein standard solution *N*-ethylmaleimide, [arginine]vasopressin, bovine tendon collagen, arachidonic acid, heparin and Tris base were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Papaverine was obtained from BDH Chemicals, Toronto, Ont., Canada. Liquid-scintillation 'cocktail' (Quantafluor) was obtained from Mallinckrodt, St. Louis, MO, U.S.A. A number of compounds were gifts from the following sources: PGE_1, Dr. J. Pike, Upjohn Company, Kalamazoo, MI, U.S.A.; 2′,5′-dideoxyadenosine, Dr. H. B. Wood, Drug Synthesis and Chemistry Branch, National Cancer Institute, Bethesda, MD, U.S.A.; 9-(tetrahydro-2-furyl)-adenine (SQ 22536), Dr. D. N. Harris, Squibb Institute for Medical Research, Princeton, NJ, U.S.A.; 5′-deoxyadenosine, Dr. J. W. Daly, National Institute of Arthritis, Metabolism and Digestive Diseases, Bethesda, MD, U.S.A.; N^6-phenyladenosine, N^6-cyclohexyladenosine and 2-benzylthioadenosine, Dr. K. Kikugawa, Kohjin Co., Tokyo, Japan; 2-hydrazinoadenosine and 2-azidoadenosine, Dr. B. E. McCarry, McMaster University, Hamilton, Ont., Canada.

Assay of adenylate cyclase in platelet particulate fraction

Human platelets were isolated and washed as described previously (Haslam & Lynham, 1972), except that they were finally resuspended in 150mM-Tris/HCl, pH7.4. This suspension was rapidly frozen by using a solid Co_2/acetone mixture and then shaken in a water bath at 37°C until just completely thawed, after which the lysate was centrifuged at 48000*g* for 20min at 0°C. The pellet was resuspended at approx. 0.5mg of platelet protein/ml in a solution at 0°C containing all the usual components of the adenylate cyclase assay mixture (including unlabelled ATP but excluding [^{3}H]ATP and the compounds under investigation; see below). Platelet protein was determined by the method of Lowry *et al.* (1951), with correction for the protein present in the suspending medium. Within 10min, 150μl of resuspended enzyme was added to 50–80μl of other additions, the mixture was incubated for 1min at 30°C to permit temperature equilibration and the assay was then started by addition of 20–50μl of [^{3}H]ATP (2μCi). Unless otherwise indicated, the final assay mixtures (250μl) contained 75mM-Tris/HCl, pH7.4, 5mM-$MgCl_2$, 1mM-cyclic AMP, 1mM-papaverine, 0.4mM-[^{3}H]ATP, 4mM-phosphocreatine, 8 units (μmol/min) of creatine phosphokinase/ml, 1mg of bovine serum albumin/ml, 0.4mM-dithiothreitol and 0.4mM-Mg–EGTA and were incubated for 10min at 30°C. Assays were stopped by addition of 100μl of solution containing 1μmol of unlabelled ATP and 0.2μmol of cyclic [^{14}C]AMP (1000d.p.m.), followed by heating for 3min at 100°C. Cyclic AMP was isolated by a modification (Haslam & Rosson, 1975) of the method of Krishna *et al.* (1968) and was counted for ^{3}H and ^{14}C by liquid-scintillation spectrometry. The

^3H found in zero-time controls was subtracted from the cyclic [^3H]AMP found in actual assays, and corrections were made for the recovery of cyclic [^{14}C]AMP (approx. 50%). Assays were carried out in triplicate in each experiment unless stated otherwise; standard errors were on average less than 5% of the mean, except when adenylate cyclase activities were inhibited by more than 80%.

Measurement of changes in platelet cyclic AMP

These were determined by extraction of labelled cyclic AMP from human heparinized platelet-rich plasma that had been preincubated for 90min with labelled adenine. The experimental procedures were as described previously (Haslam & Rosson, 1975), except that the platelets were labelled by incubation with 2μM-[^3H]adenine (1Ci/mmol) instead of with 2μM-[^{14}C]adenine (287mCi/mmol), and added cyclic [^{14}C]AMP (0.4nCi) rather than cyclic [^3H]AMP (0.01μCi) was used to monitor and correct for variations in the recovery of labelled cyclic AMP, which averaged 50% in the present study. Labelled platelet cyclic AMP was extracted from incubation mixtures by method 2 of Haslam & Rosson (1975) and was expressed as a percentage of the total radioactivity incorporated into the platelets. As the standard errors of replicate measurements by this technique were on average only about 3% of the mean values (see e.g. Table 3), single assays were performed in large experiments, which were repeated with minor variations with platelet-rich plasma from at least three separate donors.

Studies on platelet aggregation and the release reaction

These were carried out with either human citrated platelet-rich plasma [prepared from blood anticoagulated with 0.1vol. of 3.8% (w/v) trisodium citrate] or human heparinized platelet-rich plasma, in both of which the platelet count was, if necessary, adjusted by dilution with platelet-free plasma to be within the range 350000–470000/mm^3. Further details of the preparation of platelet-rich plasma have been given elsewhere (Haslam & Rosson, 1972). For measurements of platelet aggregation, incubation mixtures comprising 0.85ml of platelet-rich plasma and a final total of 0.15ml of additions in 0.154M-NaCl were stirred in an aggregometer (Payton Associates, Scarborough, Ont., Canada) linked to a potentiometric recorder (Photovolt Corp., New York, NY, U.S.A.), which was operated in a logarithmic mode to record changes in absorbance (with a 606 filter) with respect to a blank containing platelet-free plasma. In experiments in which the platelet release reaction was studied, platelet-rich plasma was first incubated with 0.2μM-5-hydroxy-[^{14}C]tryptamine for 1h before experiments were begun; over 90% of the ^{14}C was taken up by the platelets. Platelet aggregation was then recorded for 2min after addition of the aggregating agent, at which time the release of 5-hydroxy[^{14}C]tryptamine was stopped by addition of 0.2ml of 9% (w/v) paraformaldehyde; the mixture was then cooled to 0°C and centrifuged to determine the percentage of platelet ^{14}C released into the supernatant by the aggregating agent (Costa & Murphy, 1976). The values obtained were corrected for any ^{14}C found in supernatants from platelet-rich plasma to which only 0.154M-NaCl was added. Collagen and arachidonate were prepared for use as aggregating agents as described elsewhere (Haslam *et al.*, 1975; Davies *et al.*, 1976).

Liquid-scintillation counting

Sample to be counted for radioactivity were freeze-dried to remove traces of ^3H_2O, redissolved in 1ml of water mixed with 7.5ml of Quantafluor and counted for ^3H and ^{14}C in a Beckman LS 230 scintillation counter. Counting efficiencies were approx. 16 and 50% for ^3H and ^{14}C respectively, under dual-isotope counting conditions. Results were corrected for background radioactivity, channel cross-over and variations in quenching.

Results

Effects of adenosine analogues on the adenylate cyclase activity of platelet particulate fraction

We have previously reported that adenosine activates platelet adenylate cyclase at low concentrations (2–25μM) and inhibits the enzyme at concentrations above about 100μM (Haslam & Lynham, 1972). Two concentrations, 5 and 400μM, which clearly show these two effects of adenosine, were therefore chosen for comparison of a wide range of adenosine analogues (Table 1). Modification of different parts of the adenosine molecule had radically different effects. Substitution in the 2-position decreased (2-benzylthioadenosine and 2-chloroadenosine) or abolished (2-hydrazinoadenosine and 2-azidoadenosine) the inhibitory activity observed with 400μM-adenosine, while some or all of the stimulatory activity observed with 5μM-adenosine was retained. Of these four compounds, only 2-azidoadenosine was at least as effective an activator of adenylate cyclase as adenosine at 5μM and also showed marked stimulatory activity at 400μM. All compounds tested with single substituents in the N^6-position were stimulatory rather than inhibitory at 400μM, but showed less stimulatory activity at 5μM than did adenosine. In contrast with the above compounds, analogues of adenosine in which the ribosyl moiety was modified by removal of one or more oxygen atoms had no stimulatory

Table 1. *Effects of adenosine analogues on the adenylate cyclase activity of platelet particulate fraction*
In this experiment the activity of platelet adenylate cyclase in the absence of adenosine or analogues was 0.59± 0.01 nmol of cyclic AMP formed/10 min per mg of protein (means±S.E.M. from five determinations on the same enzyme preparation). Assays in the presence of adenosine were carried out in quintuplicate and with other compounds in triplicate. The changes in mean adenylate cyclase activities ± the S.E. of the differences are expressed as percentages of the mean control value. The significance of changes (Student's *t* test) are indicated as follows: *$2P < 0.05$; **$2P < 0.001$.

Compound tested	Changes in adenylate cyclase activity caused by adenosine and its analogues (%)	
	5 μM	400 μM
Adenosine	+30 ± 2**	−44 ± 3**
2-Benzylthioadenosine	+ 6 ± 2*	−10 ± 2*
2-Chloroadenosine	+25 ± 2**	−14 ± 5*
2-Hydrazinoadenosine	+15 ± 2**	+29 ± 2**
2-Azidoadenosine	+34 ± 4**	+41 ± 5**
N^6-$\gamma\gamma$-Dimethylallyladenosine	+12 ± 2**	+29 ± 2**
N^6-Benzyladenosine	+11 ± 2*	+50 ± 4**
N^6-Phenyladenosine	+24 ± 2**	+44 ± 4**
N^6-Cyclohexyladenosine	+19 ± 2**	+37 ± 4**
2′-Deoxyadenosine	−13 ± 5	−71 ± 3**
3′-Deoxyadenosine	−11 ± 4*	−79 ± 2**
5′-Deoxyadenosine	−5 ± 6	−72 ± 2**
2′,5′-Dideoxyadenosine	−46 ± 2**	−86 ± 2**
2′-Deoxyadenosine 3′-monophosphate	−55 ± 3**	−92 ± 2**
Compound SQ 22536	−20 ± 7*	−81 ± 2**

activity at either concentration tested and were, in several instances, potent inhibitors of adenylate cyclase at a concentration of 5 μM as well as 400 μM. In order of increasing inhibitory activity, the most potent compounds found were SQ 22536, 2′,5′-dideoxyadenosine and 2′-deoxyadenosine 3′-phosphate (Table 1). It is of interest that modification of 2′-deoxyadenosine, either by removal of the 5′-oxygen atom or by addition of a 3′-phosphate residue, greatly enhanced its inhibitory activity.

Compound SQ 22536 and 2′,5′-dideoxyadenosine were the only inhibitors of platelet adenylate cyclase listed in Table 1 that were found to have marked effects in intact platelets, so further studies on particulate-fraction adenylate cyclase were carried out with these two compounds. Previous observations had indicated that, when platelet adenylate cyclase was activated by PGE_1, adenosine lacked stimulatory activity and appeared to be a more effective inhibitor of the enzyme than in the absence of PGE_1 (Haslam & Lynham, 1972). The effects of 2′,5′-dideoxyadenosine and of compound SQ 22536 were therefore compared in the presence and absence of 1 μM-PGE_1. The percentage changes in adenylate cyclase activities in three such experiments, which were much less variable than the specific activities of the different enzyme preparations, are shown in Table 2 as mean values ±S.E.M. With 20–400 μM-2′,5′-dideoxyadenosine, more potent inhibitions of platelet adenylate cyclase were observed in the presence than in the absence of PGE_1 (77–94% and 66–85% respectively), though the differences were much smaller than those seen with 400 μM-adenosine. However, with 20–400 μM-compound SQ 22536 there was no significant effect of PGE_1 on the inhibitions observed (57–84%). As a result, the difference between the inhibitory activities of 2′,5′-dideoxyadenosine and compound SQ 22536 was only statistically significant at lower concentrations ($\leq$20 μM) in the absence of PGE_1, but was significant at all concentrations up to 400 μM in the presence of PGE_1 ($2P < 0.05$, Student's *t* test). At the highest concentration tested (2 mM; not shown in Table 2), 2′,5′-dideoxyadenosine and compound SQ 22536 inhibited PGE_1-activated adenylate cyclase activity by 96 and 90% respectively.

The kinetics of the inhibition of platelet adenylate cyclase by 2′,5′-dideoxyadenosine and compound SQ 22536 were studied in the presence of PGE_1. Adenylate cyclase activity was linear for at least 10 min at all MgATP concentrations from 0.04 mM to 1.0 mM, both in the presence and absence of these inhibitors. Results from three experiments, one of which is shown in Fig. 1, indicated that the inhibitions were non-competitive and that the K_m value for MgATP was about 60 μM. As the double-reciprocal plots intersected on or close to the abscissa the non-competitive inhibition approximates to the simple type. Studies with a range of inhibitor concentrations indicated that the IC_{50} values were 4 μM for 2′,5′-dideoxyadenosine and 13 μM for compound SQ 22536 and showed that the

Table 2. *Effects of adenosine, 2′,5′-dideoxyadenosine and compound SQ 22536 on the basal and PGE_1-stimulated adenylate cyclase activities of platelet particulate fraction*

In each of three experiments, the changes in adenylate cyclase activities observed in the presence of adenosine or analogues were calculated as percentages of the corresponding control activity in the presence or absence of 1 μM-PGE_1. Mean values ±S.E.M. for the percentage changes in activity in the three experiments are given. The significance of changes (Student's *t* test) are indicated as follows: *$2P < 0.05$; **$2P < 0.01$. PGE_1 increased adenylate cyclase activity from 0.43±0.13nmol of cyclic AMP formed/10min per mg of protein to 7.2±2.2nmol of cyclic AMP formed/10min per mg of protein in these three experiments (mean values ±S.E.M.).

Addition	Concn. (μM)	Changes in adenylate cyclase activity caused by adenosine and its analogues (%)	
		−PGE_1	+PGE_1
Adenosine	20	+20 ± 4*	−23 ± 2**
	100	−14 ± 5	−53 ± 3**
	400	−47 ± 3**	−73 ± 2**
2′,5′-Dideoxyadenosine	20	−66 ± 2**	−77 ± 2**
	100	−80 ± 2**	−89 ± 0**
	400	−85 ± 2**	−94 ± 1**
Compound SQ 22536	20	−57 ± 2**	−58 ± 3**
	100	−77 ± 0**	−81 ± 2**
	400	−84 ± 1**	−88 ± 1**

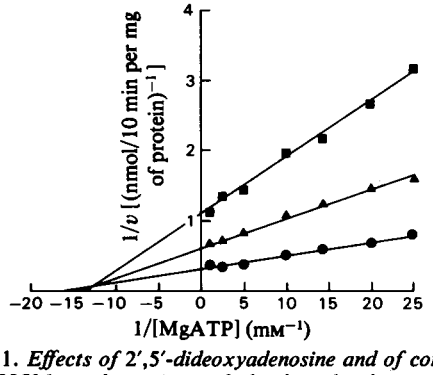

Fig. 1. *Effects of 2′,5′-dideoxyadenosine and of compound SQ 22536 on the activity of platelet adenylate cyclase in the presence of PGE_1 and different substrate concentrations*

Double-reciprocal plots of the rate of cyclic [^{3}H]AMP formation (v) against MgATP concentration (0.04–1.0mM) are shown in the absence of inhibitors (●), in the presence of 20μM-2′,5′-dideoxyadenosine (■) and in the presence of 20μM-compound SQ 22536 (▲). Individual points are means from four determinations of enzyme activity (two 5min incubations + two 10min incubations); average S.E.M. values (as percentages of individual means) were ±2.3% without inhibitors, ±2.8% with 2′,5′-dideoxyadenosine and ±2.2% with SQ 22536. PGE_1 (1μM) was present in all incubation mixtures; an excess of 4.6mM-$MgCl_2$ was present at all MgATP concentrations. This experiment gave a K_m value for MgATP of 60μM.

same inhibitions were observed whether the substrate concentration was 60 or 400μM (Fig. 2*a*). The non-linearity of plots of v_0/v_i against [I] indicated an apparent negative co-operativity in the actions of both inhibitors (Fig. 2*a*); Hill plots of the same results gave interaction coefficients (h) of 0.66 for 2′,5′-dideoxyadenosine (Fig. 2*b*) and of 0.75 for compound SQ 22536. These estimates are dependent on the assumption that saturation of the inhibitor sites results in 100% inhibition of enzyme activity. Linear extrapolation of the available results (plotted as $1/(v_0-v_i)$ against 1/[I]) gives minimum values for the maximum possible inhibition of PGE_1-activated adenylate cyclase by 2′,5′-dideoxyadenosine and compound SQ 22536 of 97 and 91% respectively. For 2′,5′-dideoxyadenosine, this is sufficiently close to 100% to sustain the view that there is negative co-operativity in the action of the compound (e.g. if the maximum inhibition were 97%, $h = 0.74$), but in the case of compound SQ 22536 this question remains open.

Effects of 2′,5′-dideoxyadenosine and of compound SQ 22536 on adenylate cyclase in intact platelets

It was repeatedly observed that neither 100μM-2′,5′-dideoxyadenosine nor 100μM-compound SQ 22536 alone had any detectable effect on the amount of cyclic [^{3}H]AMP in platelets labelled with [^{3}H]-adenine, but that both markedly suppressed the increases in cyclic [^{3}H]AMP observed in the presence of PGE_1, papaverine or PGE_1 and papaverine. When the effects of a range of concentrations of these two adenosine analogues were compared, differences in their effects were observed, depending on the agent used to increase platelet cyclic [^{3}H]AMP (Fig. 3). With PGE_1 alone, 2′,5′-dideoxyadenosine was substantially more effective than compound SQ

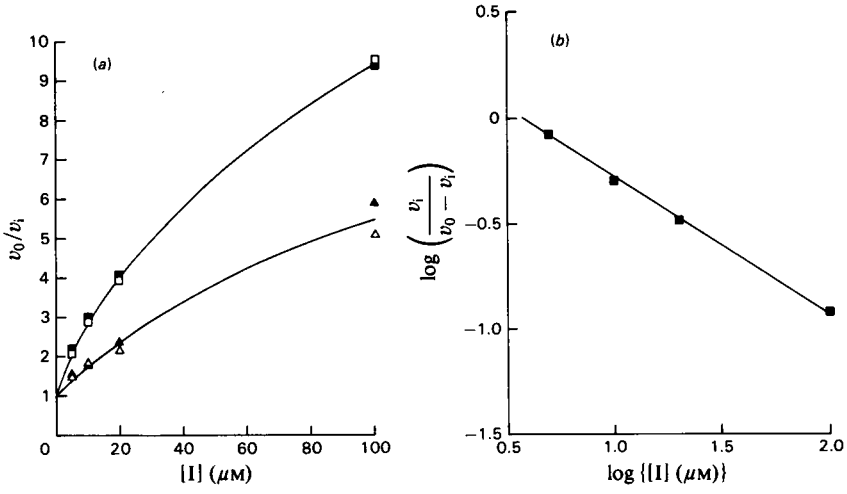

Fig. 2. *Effects of different concentrations of 2′,5′-dideoxyadenosine and of compound SQ 22536 on platelet adenylate cyclase activity in the presence of PGE_1*

(*a*) Plots of the ratio (v_0/v_i) of enzyme activities in the absence (v_0) and presence (v_i) of inhibitor against inhibitor concentration ([I]). PGE_1 (1 μM) was present in all assay mixtures. Concentrations of 2′,5′-dideoxyadenosine (□, ■) and of compound SQ 22536 (△, ▲) ranged from 5 to 100 μM. Two MgATP concentrations were used: 0.06 mM (open symbols) and 0.4 mM (closed symbols). IC_{50} values of 4 μM and 13 μM were obtained with 2′,5′-dideoxyadenosine and compound SQ 22536 respectively. (*b*) Hill plot of the PGE_1-activated adenylate cyclase activities observed with 0.4 mM-MgATP and 5–100 μM-2′,5′-dideoxyadenosine (from *a*). The interaction coefficient (*h*) was 0.66.

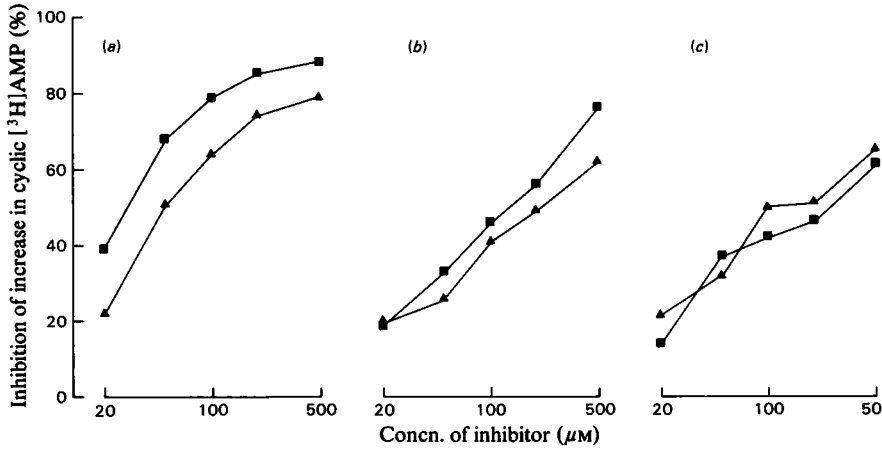

Fig. 3. *Inhibition by 2′,5′-dideoxyadenosine and by compound SQ 22536 of the increases in cyclic [^{3}H]AMP caused by PGE_1 and papaverine in intact platelets*

Incubation mixtures contained 0.83 ml of platelet-rich plasma labelled with [^{3}H]adenine and 0.17 ml of 0.154 M-NaCl containing 2′,5′-dideoxyadenosine (■) or compound SQ 22536 (▲), and other additions as follows (final concentrations given): (*a*) 0.4 μM-PGE_1; (*b*) 0.4 μM-PGE_1 + 2 mM-papaverine; (*c*) 2 mM-papaverine. Incubation times were 20s (*a* and *b*) or 60s (*c*). The effects of 2′,5′-dideoxyadenosine and of compound SQ 22536 are expressed as the percentage inhibitions of the increases in platelet cyclic [^{3}H]AMP observed in controls without inhibitors. These control increases in cyclic [^{3}H]AMP (means of duplicate determinations expressed as percentages of total platelet ^{3}H) were: (*a*) 0.233%; (*b*) 0.341%; (*c*) 0.039%; the average resting concentration of platelet cyclic [^{3}H]AMP was 0.026%. The effect of each concentration of 2′,5′-dideoxyadenosine or compound SQ 22536 studied was determined in a single assay.

Table 3. *Effects of N-ethylmaleimide on the inhibitions by 2',5'-dideoxyadenosine and by ADP of the increases in platelet cyclic [^{3}H]AMP concentration caused by PGE_1*

Incubation mixtures contained 0.85ml of platelet-rich plasma labelled with [^{3}H]adenine and a final total of 0.15ml of additions in 0.154M-NaCl. Samples of labelled platelet-rich plasma were incubated for 5min with or without 0.4mM-*N*-ethylmaleimide before other additions were made and the incubations were then continued for a further 0.5min. Concentrations of other additions were: PGE_1, 0.4μM; 2',5'-dideoxyadenosine, 100μM; ADP, 2μM. The concentration of *N*-ethylmaleimide used was that found in other experiments to be just sufficient to block the decrease in platelet cyclic AMP caused by ADP. Values given for cyclic [^{3}H]AMP are means ± S.E.M. from four determinations in the same experiment.

Additions other than *N*-ethylmaleimide	Amount of cyclic [^{3}H]AMP present (% of total platelet radioactivity)	
	N-Ethylmaleimide absent	*N*-Ethylmaleimide present
None	0.039 ± 0.001	0.039 ± 0.003
PGE_1	0.418 ± 0.006	0.646 ± 0.018
PGE_1 + ADP	0.066 ± 0.001	0.710 ± 0.010
PGE_1 + 2',5'-dideoxyadenosine	0.080 ± 0.002	0.101 ± 0.003

22536, particularly at concentrations below 50μM (Fig. 3*a*). More than 80% inhibition of the increase in cyclic [^{3}H]AMP in response to PGE_1 alone was observed with concentrations of 2',5'-dideoxyadenosine over 100μM. However, in the presence of 2mM-papaverine, the inhibitory effects of both 2',5'-dideoxyadenosine and compound SQ 22536 were less powerful (Figs. 3*b* and 3*c*). Moreover, the former compound appeared to be only slightly more effective than the latter in the presence of both PGE_1 and papaverine, and there was little difference between their activities in the presence of papaverine alone. Approx. 200μM-2',5'-dideoxyadenosine or compound SQ 22536 was required to inhibit the accumulation of cyclic [^{3}H]AMP caused by 2mM-papaverine with or without PGE_1 by 50%.

Because of an apparent similarity between the actions of the inhibitory nucleosides and of ADP on adenylate cyclase in intact platelets, we studied the effect on the action of 2',5'-dideoxyadenosine of *N*-ethylmaleimide, which blocks the inhibition of adenylate cyclase by ADP (Mills, 1974). Table 3 shows that 0.4mM-*N*-ethylmaleimide caused a moderate increase in the accumulation of cyclic [^{3}H]AMP in platelets in response to PGE_1 alone and completely prevented the inhibition of the effect of PGE_1 by ADP. However, 0.4mM-*N*-ethylmaleimide had no effect on the inhibition of cyclic [^{3}H]AMP accumulation by 2',5'-dideoxyadenosine (Table 3).

Effects of 2',5'-dideoxyadenosine and of compound SQ 22536 on the inhibition of platelet aggregation by PGE_1

The inhibition of ADP-induced platelet aggregation by prior addition of PGE_1 was markedly diminished by 100μM-2',5'-dideoxyadenosine added with the PGE_1, as also was the increase in platelet cyclic [^{3}H]AMP that preceded the addition of ADP (Fig. 4). Compound SQ 22536 at 100μM was slightly less potent than 2',5'-dideoxyadenosine in both respects. Essentially the same effects of these adenosine analogues on both the inhibition of platelet aggregation by PGE_1 and the associated increase in platelet cyclic [^{3}H]AMP were observed in two further experiments with ADP as the aggregating agent and in three experiments with [arginine]-vasopressin as the aggregating agent (e.g. Fig. 5). However, as reported previously (Haslam & Rosson, 1972; Haslam, 1975), PGE_1 was a much more potent inhibitor of platelet aggregation induced by [arginine]vasopressin than of that caused by ADP (compare Figs. 4 and 5). It is therefore noteworthy that the inhibition of vasopressin-induced aggregation was blocked particularly effectively by these compounds, though 2',5'-dideoxyadenosine was again more potent than compound SQ 22536 (Figs. 5 and 6). Additional experiments confirmed that, when the PGE_1 concentrations were adjusted to give roughly equally inhibited responses to ADP and vasopressin, both 2',5'-dideoxyadenosine and compound SQ 22536 were more effective in preventing inhibition of aggregation induced by the latter agent. This can be attributed to the fact that vasopressin, unlike ADP, does not itself inhibit the accumulation of cyclic [^{3}H]AMP (Haslam, 1975). In each experiment, the inhibition of aggregation was related to the concentration of platelet cyclic [^{3}H]AMP resulting from the combined actions of PGE_1, adenosine analogue and aggregating agent (Figs. 4 and 5). However, the extent to which 100μM-2',5'-dideoxyadenosine proved more effective than 100μM-compound SQ 22536 in reversing the inhibition of platelet aggregation by PGE_1 varied widely from experiment to experiment (e.g. Figs. 4–6). This may be explained by the observation that a relatively small decrease in the elevation of platelet cyclic [^{3}H]AMP concentration by PGE_1 was often associated with a transition from

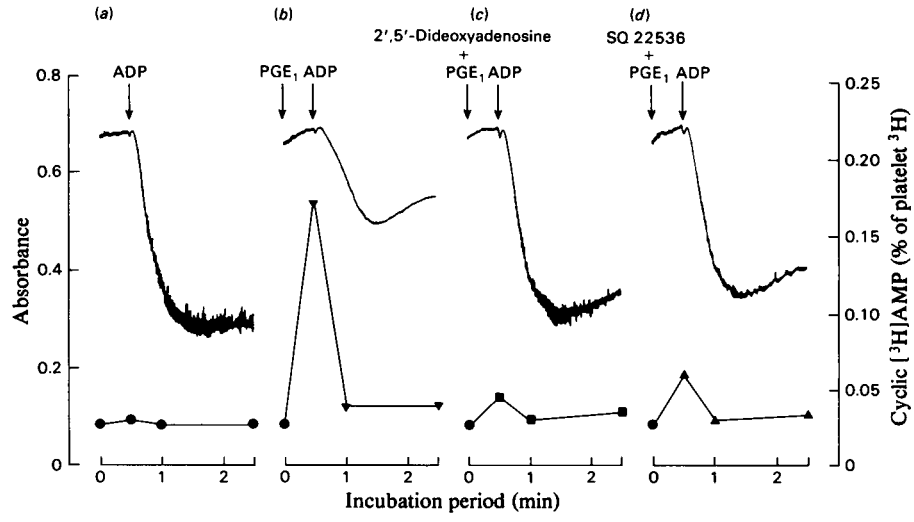

Fig. 4. *Effects of 2',5'-dideoxyadenosine and of compound SQ 22536 on the inhibition by PGE_1 of platelet aggregation induced by ADP and on the associated increase in platelet cyclic AMP*

Incubation mixtures consisting of 0.85ml of labelled platelet-rich plasma and a final total of 0.15ml of additions in 0.154M-NaCl were stirred continuously in an aggregometer. Additions (final concentrations) were as follows: (*a*) 1μM-ADP after 0.5min; (*b*) 0.5μM-PGE_1 at 0min and 1μM-ADP after 0.5min; (*c*) 100μM-2',5'-dideoxyadenosine and 0.5μM-PGE_1 at 0min and 1μM-ADP after 0.5min; (*d*) 100μM-compound SQ 22536 and 0.5μM-PGE_1 at 0min and 1μM-ADP after 0.5min. For the measurements of platelet cyclic [^{3}H]AMP single separate incubations were terminated after 0, 0.5, 1 and 2.5min to give the values shown (●, PGE_1 absent; ▼, PGE_1 but no adenosine analogue present; ■, 2',5'-dideoxyadenosine and PGE_1 present; ▲, compound SQ 22536 and PGE_1 present; ADP was added only to the 1 min and 2.5 min incubations). The aggregation recordings are from the 2.5 min incubations.

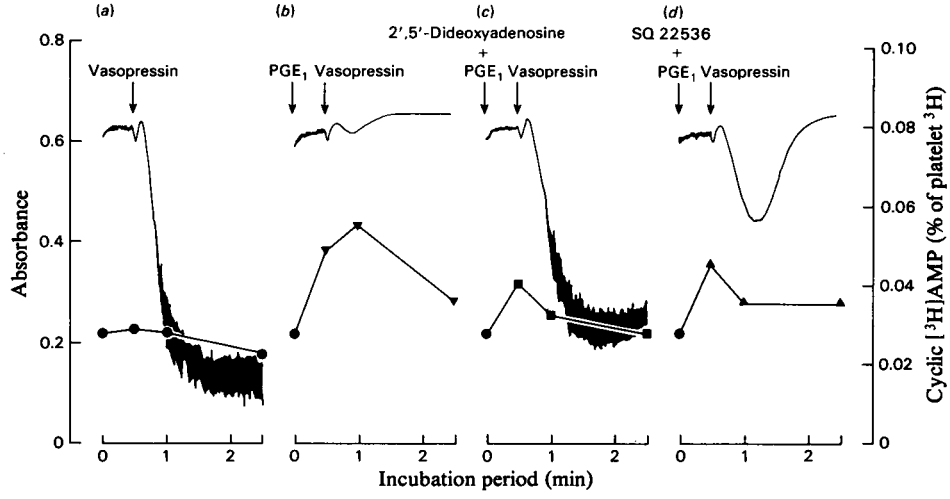

Fig. 5. *Effects of 2',5'-dideoxyadenosine and of compound SQ 22536 on the inhibition by PGE_1 of platelet aggregation induced by [arginine]vasopressin and on the associated increase in platelet cyclic AMP*

The experimental procedure was as for Fig. 4, but the following additions were made (final concentrations given): (*a*) 50munits of [arginine]vasopressin/ml after 0.5min; (*b*) 0.05μM-PGE_1 at 0min and 50munits of [arginine]-vasopressin/ml after 0.5min; (*c*) 100μM-2',5'-dideoxyadenosine and 0.05μM-PGE_1 at 0min and 50munits of [arginine]-vasopressin/ml after 0.5min; (*d*) 100μM-compound SQ 22536 and 0.05μM-PGE_1 at 0min and 50munits of [arginine]-vasopressin/ml after 0.5min. The symbols used for cyclic [^{3}H] AMP values (●, ▼, ■ and ▲) have the same signficance as in Fig. 4, except that vasopressin rather than ADP was added to the 1 min and 2.5 min incubations.

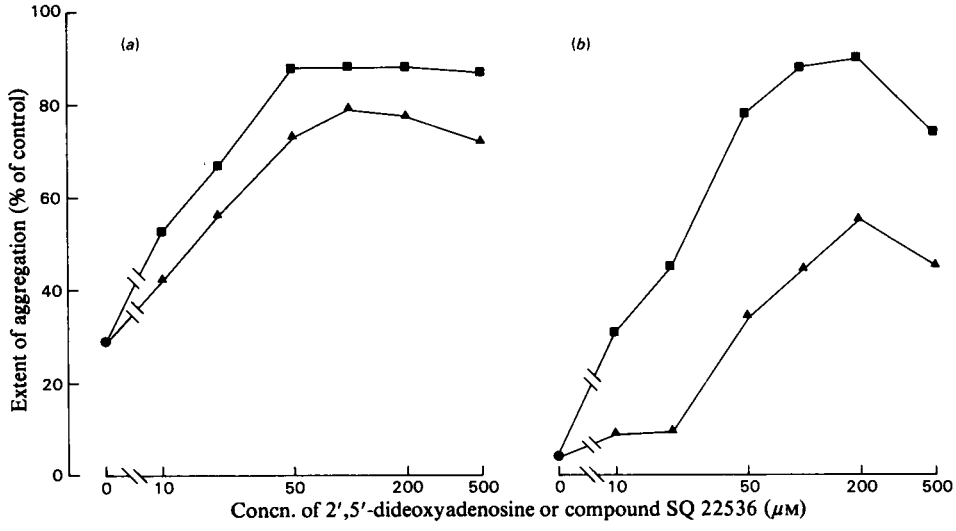

Fig. 6. *Effectiveness of different concentrations of 2',5'-dideoxyadenosine and of compound SQ 22536 in preventing the inhibition by PGE_1 of platelet aggregation induced by [arginine]vasopressin*

Incubation mixtures consisting of 0.85ml of heparinized platelet-rich plasma and a final total of 0.15ml of additions in 0.154M-NaCl were stirred in an aggregometer. [Arginine]vasopressin (final concentration 50munits/ml) was added 0.5min after the following additions: ●, PGE_1; ■, PGE_1 + 2',5'-dideoxyadenosine; ▲, PGE_1 + compound SQ 22536. Experiments were carried out with (*a*) 0.05μM-PGE_1 and (*b*) 0.1μM-PGE_1. The extent of platelet aggregation was measured as the decrease in absorbance of the stirred suspension 0.5min after addition of vasopressin and was expressed as a percentage of the average value from four controls with vasopressin alone (0.330).

a primary reversible aggregation to a secondary irreversible aggregation (compare Figs. 5*c* and 5*d*). Thus the difference between the effects of 2',5'-dideoxyadenosine and compound SQ 22536 was much smaller when the PGE_1 concentration was sufficiently low for both compounds to transform a primary aggregation response into an irreversible aggregation (Fig. 6*a*) than when, with twice the PGE_1 concentration, only 2',5'-dideoxyadenosine was able to do so (Fig. 6*b*). With both [arginine]-vasopressin (Fig. 6) and ADP (not shown), concentrations of 2',5'-dideoxyadenosine or compound SQ 22536 in the range 100–200μM were optimal in preventing the inhibitory effects of PGE_1 on platelet aggregation. Higher concentrations were less effective.

Although 2'-deoxyadenosine 3'-monophosphate was the most potent inhibitor of particulate-fraction adenylate cyclase tested, concentrations in the range 20–500μM had no detectable effect on the inhibition of ADP-induced platelet aggregation by PGE_1.

Effects of 2',5'-dideoxyadenosine and of compound SQ 22536 on platelet aggregation and on release of platelet 5-hydroxy[^{14}C]tryptamine in the absence of exogenous inhibitors of these processes

Neither 2',5'-dideoxyadenosine nor compound SQ 22536 in the concentration range 10–500μM caused aggregation of human platelets in heparinized or citrated platelet-rich plasma. Moreover, in an exhaustive series of 16 separate experiments with platelet-rich plasma from 15 different donors, no potentiation of the aggregation or the release of 5-hydroxy[^{14}C]tryptamine induced by a variety of aggregating agents was observed in the presence of 100μM-2',5'-dideoxyadenosine or 100μM-compound SQ 22536. The aggregating agents used (usually two were studied in a single experiment) were ADP, collagen, [arginine]vasopressin and arachidonate in heparinized platelet-rich plasma, and ADP, collagen and arachidonate in citrated platelet-rich plasma. In most experiments, replicate aggregation tests with each of several concentrations of each aggregating agent were carried out in a random order, and the results were analysed statistically (see e.g. Table 4). One concentration of each aggregating agent was carefully selected to give a small release of 5-hydroxy[^{14}C]tryptamine (or biphasic aggregation with ADP) in the absence of the adenosine analogues, so as to maximize the chances of detecting potentiation in the presence of these compounds. However, the only significant changes were slight inhibitions of the release of 5-hydroxy[^{14}C]tryptamine induced by collagen that were occasionally observed with 2',5'-dideoxyadenosine (Table 4).

Table 4. *Effects of 2',5'-dideoxyadenosine and of compound SQ 22536 on the platelet aggregation and release of 5-hydroxy-[^{14}C]tryptamine from platelets induced by collagen*

Incubation mixtures consisting of 0.85ml of platelet-rich plasma labelled with 5-hydroxy-[^{14}C]tryptamine and a final total of 0.15ml of additions in 0.154M-NaCl were stirred in an aggregometer. 2',5'-Dideoxyadenosine or compound SQ 22536 (final concentrations 100μM) or an equivalent volume of 0.154M-NaCl was added 30s before collagen (final concentrations indicated). Aggregation was recorded for 2min before addition of 0.2ml of 9% (w/v) paraformaldehyde and measurement of the release of platelet 5-hydroxy[^{14}C]tryptamine. For each concentration of collagen tested three aggregation and release measurements were made both with and without added compound. The sequence of these tests was randomized. The significance of any differences observed was evaluated by unpaired Student's *t* tests (NS indicates $2P > 0.05$). s.c., An increase in absorbance occurred, indicating a platelet shape change without aggregation.

				Aggregation			Release of 5-hydroxy-[^{14}C]tryptamine		
Expt. no.	Anti-coagulant	Compound investigated	Collagen concn. (μg/ml)	Without compound (ΔA after 2min)	With compound (ΔA after 2min)	$2P$	Without compound (%)	With compound (%)	$2P$
1	Citrate	2',5'-Dideoxy-adenosine	2.0	s.c.	s.c.	NS	2 ± 1	2 ± 1	NS
			6.0	0.42 ± 0.00	0.42 ± 0.00	NS	62 ± 0	58 ± 1	<0.05
2	Citrate	SQ 22536	3.0	s.c.	s.c.	NS	2 ± 1	0 ± 0	NS
			6.0	0.22 ± 0.05	0.18 ± 0.01	NS	14 ± 4	10 ± 1	NS
			10.0	0.44 ± 0.00	0.47 ± 0.00	NS	53 ± 1	51 ± 1	NS
3	Heparin	2',5'-Dideoxy-adenosine	0.5	0.22 ± 0.05	0.08 ± 0.03	NS	0 ± 1	1 ± 0	NS
			10.0	0.47 ± 0.00	0.46 ± 0.00	NS	65 ± 1	61 ± 0	<0.05
4	Heparin	SQ 22536	1.5	0.30 ± 0.01	0.28 ± 0.01	NS	3 ± 2	1 ± 0	NS
			3.0	0.35 ± 0.01	0.35 ± 0.01	NS	18 ± 2	23 ± 1	NS
			6.0	0.37 ± 0.01	0.37 ± 0.00	NS	37 ± 1	36 ± 1	NS

Discussion

Structure–activity relationships for the activation and inhibition of platelet adenylate cyclase by adenosine analogues

In general, 2-substituted and N^6-monosubstituted adenosines were found to be activators of platelet particulate-fraction adenylate cyclase, whereas deoxyadenosines had no stimulatory activity. The most potent stimulatory adenosine analogue that also lacked inhibitory activity against adenylate cyclase at high concentrations was 2-azidoadenosine. Those analogues previously found to be inhibitors of human platelet aggregation (Born *et al.*, 1965; Kikugawa *et al.*, 1973*a*,*b*; Agarwal & Parks, 1975; Cusack & Born, 1977) were also found to activate platelet adenylate cyclase, providing further evidence that the effects of these compounds on platelet aggregation are mediated by increases in platelet cyclic AMP. Similar structure–activity relationships have been found for the ability of adenosine analogues to increase cyclic AMP concentrations in brain tissue (Huang *et al.*, 1972) and myocardium (Huang & Drummond, 1976).

The inhibitory activity of adenosine on platelet adenylate cyclase was, in contrast with its stimulatory activity, markedly decreased or abolished by 2-substituents or N^6-monosubstitution and was enhanced by removal of oxygen atoms from the ribosyl moiety. These results are consistent with those obtained in a study of the inhibitory effects of adenosine analogues on hepatic adenylate cyclase (Londos & Preston, 1977). The most potent inhibitor of platelet adenylate cyclase that we found was 2'-deoxyadenosine 3'-monophosphate, reported by Sahyoun *et al.* (1976*a*) to be a naturally occurring inhibitor of adenylate cyclase; 2',5'-dideoxyadenosine was slightly less active and compound SQ 22536 was less active again. Relative to these three compounds, the 2'-deoxy-, 3'-deoxy- and 5'-deoxyadenosines were much weaker inhibitors. Adenosine analogues could, in principle, act as antagonists of the stimulatory action of adenosine, as well as being inhibitors in their own right, and 2'-deoxyadenosine (Shimizu *et al.*, 1975) and 5'-deoxyadenosine (Huang & Drummond, 1976) have been reported to show such antagonist activities in brain and heart preparations respectively. However, as less than one-third of the basal adenylate cyclase activity of platelet particulate fraction can be attributed to the stimulatory effect of traces of adenosine in the assay (unpublished observations on the effects of adenosine deaminase; R. J. Haslam, M. M. L. Davidson & J. V. Desjardins) and the inhibitions by all the deoxyadenosines exceeded 70% at 400μM, it is clear that most of their effect on basal adenylate cyclase activity is not due to competition with adenosine. In the presence of PGE_1, when no further stimulation

of adenylate cyclase by adenosine could be demonstrated, the whole of the inhibitory effect of the deoxyadenosines must be exerted at sites distinct from the stimulatory receptor.

The structure–activity relationships for activation and inhibition of adenylate cyclase by adenosine analogues reported here and in studies with other tissues indicate that the optimal molecular configurations required for the two effects are quite different, and strongly support our previous evidence that adenosine exerts independent stimulatory and inhibitory effects on the enzyme at different binding sites (Haslam & Lynham, 1972; Haslam & Rosson, 1975).* Conformational studies on adenosine and its analogues provide further support for this conclusion (Miles *et al.*, 1977).

Mechanism of inhibition of platelet adenylate cyclase by adenosine and its analogues

A previous study with intact platelets in which the uptake of adenosine was blocked with *p*-nitrobenzylthioguanosine indicated that the stimulatory action of adenosine on adenylate cyclase was mediated by externally oriented membrane receptors, and that the inhibitory action of the compound which was much diminished in intact platelets was exerted intracellularly, presumably at a site on or close to the catalytic subunit of the enzyme (Haslam & Rosson, 1975). The latter conclusion is supported by the present finding that 2′,5′-dideoxyadenosine and compound SQ 22536 inhibited adenylate cyclase in intact platelets, whereas 2′-deoxyadenosine 3′-monophosphate, an even more potent inhibitor of the particulate-fraction adenylate cyclase, did not appear to do so. Presumably, 2′-deoxyadenosine 3′-monophosphate, like other nucleotides, is unable to penetrate the platelet plasma membrane.

Our observations that 2′,5′-dideoxyadenosine and compound SQ 22536 inhibited platelet adenylate cyclase non-competitively with respect to MgATP and that the former and possibly the latter compound showed negative co-operativity are consistent with similar reports for the inhibition of lung adenylate cyclase by compound SQ 22536 (Weinryb & Michel, 1974) and of adipocyte adenylate cyclase by 2′-deoxyadenosine 3′-monophosphate (Sahyoun *et al.*, 1976*b*). 2′,5′-Dideoxyadenosine has also been shown to inhibit fat-cell (Fain *et al.*, 1972), epithelial-cell (Zenser, 1976) and liver (Londos & Preston, 1977) adenylate cyclases, but we are not aware of any kinetic study with this compound. The inhibitory action of adenosine has been reported to have similar kinetic properties to those we observed for the more potent deoxy analogues (McKenzie & Bär, 1973; Londos & Preston, 1977). With liver membranes, adenosine has also been shown to act at sites distinct from those through which glucagon, Mg^{2+} ions and GTP promote adenylate cyclase activity (Londos & Preston, 1977).

As noted before (Haslam & Lynham, 1972), adenosine appeared to be a much more potent inhibitor of platelet adenylate cyclase in the presence than in the absence of PGE_1. This difference was much less marked with 2′,5′-dideoxyadenosine and was absent with compound SQ 22536. Measurement of the inhibitory action of adenosine in the absence of PGE_1 is complicated by the simultaneous and independent stimulation of the enzyme by the compound, so it is difficult to determine with the platelet enzyme whether PGE_1 has a real effect on the inhibitory component of the action of adenosine. This, however, may be the case, as adenosine has been shown to inhibit adenylate cyclase more powerfully in the presence of stimulatory hormones in other tissues in which the enzyme was not activated by adenosine (McKenzie & Bär, 1973; Londos & Preston, 1977).

Inhibition of platelet adenylate cyclase by adenosine analogues in intact platelets

Both 2′,5′-dideoxyadenosine and compound SQ 22536 were effective inhibitors of the basal and PGE_1-stimulated adenylate cyclase activities of intact platelets, when these were measured as the increases in labelled cyclic AMP in platelets that had been preincubated with labelled adenine and then incubated briefly with papaverine or papaverine and PGE_1. Papaverine was added to block cyclic AMP phosphodiesterase, but, as the adenosine analogues studied suppressed the increases in labelled cyclic AMP caused by PGE_1 more effectively in the absence than in the presence of papaverine, it is possible that papaverine also decreased their rate of entry into the platelets. Moreover, the greater effectiveness of 2′,5′-dideoxyadenosine relative to compound SQ 22536 in the absence of papaverine might reflect a differential effect of papaverine on the entry of the two compounds. Papaverine is known to inhibit one of the mechanisms responsible for the transport of adenosine into platelets (Haslam & Rosson, 1975; Sixma *et al.*, 1976). The alternative explanation of these observations, that these adenosine analogues potentiate cyclic AMP phosphodiesterase as well as inhibiting adenylate cyclase, seems less probable, but has not been eliminated. The inhibition of platelet adenylate cyclase by deoxyadenosine compounds differed in two respects from the inhibitory action on the enzyme of the aggregating agent ADP; thus the effect of the latter

* Shortly after submission of this paper for publication, a report (Londos & Wolff, 1977) appeared which described the effects of adenosine analogues on adenylate cyclases from several tissues, including platelets, from which essentially the same conclusion was drawn.

is observed in intact but not broken platelets (Salzman & Levine, 1971; Haslam, 1973) and is blocked by *N*-ethylmaleimide (Mills, 1974).

Our observations, that 2′,5′-dideoxyadenosine and compound SQ 22536 markedly decrease both the inhibition of platelet aggregation and the associated increase in platelet cyclic AMP caused by PGE_1, confirm and extend the preliminary report by Harris *et al.* (1975) on the effects of compound SQ 22536. These results are in accord with a large body of evidence indicating that the effect of PGE_1 on platelet aggregation is mediated by cyclic AMP formed as a result of the activation of adenylate cyclase (for reviews see Haslam, 1975, 1978; Haslam *et al.*, 1978). Moreover, our experiments suggest that the criteria of Sutherland *et al.* (1968) for identification of cyclic AMP as the second messenger of hormone or drug action in other biological systems could be increased to include the results of studies with 2′,5′-dideoxyadenosine or related compounds as potential inhibitors of adenylate cyclase. In the only previous attempts to use 2′,5′-dideoxyadenosine in this manner known to us, it was found that, in contrast with adenosine itself, which appears to act by a different mechanism, 2′,5′-dideoxyadenosine blocked the hormone-stimulated accumulation of cyclic AMP in fat-cells and liver cells, but did not inhibit lipolysis or glycogenolysis (Fain *et al.*, 1972; Fain & Shepherd, 1977). These observations were interpreted as indicating that factors in addition to cyclic AMP are involved in the regulation of these processes (Fain & Shepherd, 1977; Moreno & Fain, 1978). Another material known to inhibit adenylate cyclase from many tissues is the adenylate cyclase inhibitor obtained from rat liver perfusate (Levey *et al.*, 1975), which, however, also affects guanylate cyclase and cyclic GMP phosphodiesterase (Lehotay *et al.*, 1977). The specificity of 2′,5′-dideoxyadenosine and related compounds remains to be established.

Lack of effect of inhibition of adenylate cyclase on platelet function in the absence of drugs that increase platelet cyclic AMP

As both 2′,5′-dideoxyadenosine and compound SQ 22536 inhibited the basal adenylate cyclase activity in intact platelets, these compounds should, in principle, decrease the steady-state concentration of cyclic AMP in resting platelets. Although no decrease in the cyclic [^{3}H]AMP extracted from labelled platelets was detected, it may be very difficult to measure decreases in platelet cyclic AMP because of some form of compartmentation of the compound (Haslam, 1975). Whether a decrease in platelet cyclic AMP will affect platelet function will presumably depend on whether or not the steady-state concentration in resting platelets is sufficient to activate to some extent the cyclic AMP-dependent protein kinases involved in the effects of increased cyclic AMP concentrations. This cannot be predicted without investigation and it is, therefore, of great interest that neither 2′,5′-dideoxyadenosine nor compound SQ 22536 caused aggregation or potentiated aggregation or release of 5-hydroxy[^{14}C]-tryptamine induced by any of the aggregating agents studied. These results are consistent with previous evidence (Haslam, 1975, 1978) that a decrease in platelet cyclic AMP is not required for aggregation to occur, and show more convincingly than has previously been possible that the basal concentration of cyclic AMP in platelets is too low to have any significant functional role. Thus our results indicate that cyclic AMP is an essentially unidirectional regulator of platelet function.

These results and conclusions are in conflict with a preliminary report from another laboratory (Salzman *et al.*, 1977), in which compound SQ 22536 was found to potentiate the effects of critical concentrations of certain aggregating agents, including ADP, collagen and arachidonate, but not vasopressin. However, despite reinvestigation with freshly synthesized compound SQ 22536, we were not able to detect any potentiating effects in the absence of PGE_1. In addition, the observation by Salzman *et al.* (1977) that compound SQ 22536 could potentiate the action of ADP, which itself inhibits adenylate cyclase, but not that of vasopressin, which does not, is in apparent conflict with the relative effects of compound SQ 22536 on the inhibitions of ADP- and vasopressin-induced aggregation by PGE_1. Salzman (1977) has also reported that compound SQ 22536 potentiates aggregation induced by prostaglandin G_2. This could be due to inhibition by compound SQ 22536 of cyclic AMP formation induced by prostaglandin D_2, which is generated from prostaglandin G_2 in platelet-rich plasma (Smith *et al.*, 1976) and is a known activator of platelet adenylate cyclase (Mills & Macfarlane, 1974). However, E. W. Salzman (personal communication) has recently observed that a new batch of compound SQ 22536, which inhibited platelet adenylate cyclase, did not potentiate aggregation induced by ADP or adrenaline, suggesting that the material used in his earlier studies may have contained a contaminant that potentiated aggregation. We therefore conclude that cyclic AMP plays no significant role in the regulation of platelet function in the absence of compounds that increase platelet cyclic AMP above the concentration normally found in preparations of platelet-rich plasma.

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