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ADENOSINE 5′-DIPHOSPHATE ANTAGONISTS AND HUMAN PLATELETS: NO EVIDENCE THAT AGGREGATION AND INHIBITION OF STIMULATED ADENYLATE CYCLASE ARE MEDIATED BY DIFFERENT RECEPTORS

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1 Adenosine 5′-diphosphate (ADP) induces human platelet aggregation and noncompetitively inhibits stimulated human platelet adenylate cyclase; it has been suggested that these two effects are mediated by separate ADP receptors on the platelet surface.

2 Adenosine 5′-triphosphate and seven adenine nucleotide analogues were tested as inhibitors of both effects of ADP on human platelets, and were found to be competitive.

3 pA_2 values were calculated for each antagonist for inhibition of both effects of ADP, and a good correlation (correlation coefficient 0.87; $P < 0.01$) was found between the pA_2 values for inhibition of ADP-induced aggregation and the pA_2 values for inhibition of the effect of ADP on stimulated adenylate cyclase.

4 Such a correlation does not support the suggestion that ADP-induced aggregation and the inhibition by ADP of stimulated adenylate cyclase are mediated by two separate receptors.

Introduction

Adenosine 5′-diphosphate (ADP) is a physiologically important inducer of human platelet aggregation (Born, 1962), and also noncompetitively inhibits stimulated human platelet adenylate cyclase (Haslam, 1973). It has been suggested that these two actions of ADP are mediated by two separate external receptors at the platelet surface (Mills & Macfarlane, 1978), since two ADP receptor agonists, 2-azidoadenosine 5′-diphosphate (2-azido-ADP) and 2-methylthioadenosine 5′-diphosphate (2-methylthio-ADP) are considerably more potent as inhibitors of prostaglandin E_1 (PGE_1)-stimulated adenylate cyclase than they are as aggregating agents (Mills & Macfarlane, 1978; Macfarlane, Srivastava & Mills, 1979). In addition, *p*-chloromercuribenzenesulphonate (PCMBS) inhibits the action of ADP on adenylate cyclase but does not inhibit ADP-induced aggregation (Mills & Macfarlane, 1977), while 5′-*p*-fluorosulphonylbenzoyladenosine (FSBA) inhibits ADP-induced aggregation but not the action of ADP on adenylate cyclase (Mills, Coleman, Figures, Morinelli, Niewiarowski & Colman, 1980).

However, the relationship between receptor occupancy by an agonist and the measured parameter may not be the same for aggregation and for inhibition of adenylate cyclase. Thus the results obtained with 2-azido-ADP and 2-methylthio-ADP could reflect a greater efficacy for inhibition of adenylate cyclase than for induction of aggregation at a single receptor, rather than differing affinities for two separate ADP receptors.

Information about the homogeneity of receptors can be obtained by using reversible, competitive antagonists, since their only action is to occupy the receptors allowing dissociation constants (K_i) to be derived by classical pharmacological methods. This approach has been used on human platelets to establish that shape change and aggregation induced by 5-hydroxytryptamine (5-HT) are mediated by the same receptor, which is different from the receptor responsible for uptake of 5-HT (Born, Juengjaroen & Michal, 1972). However, PCMBS and FSBA would appear to be unsuitable for such a study, since PCMBS is a nonspecific, irreversible thiol reagent, rather than a competitive ADP antagonist (Mills & Macfarlane, 1977), and FSBA is a progressive, irreversible inhibitor of ADP-induced aggregation which has not been shown to be competitive or specific for ADP (Bennett, Colman & Colman, 1978).

Many analogues of adenosine 5′-monophosphate (AMP) and adenosine 5′-triphosphate (ATP) have been tested as inhibitors of ADP-induced aggregation (for a review see Haslam & Cusack, 1981) and of these, ATP (Macfarlane & Mills, 1975), adenylyl (β,γ-methylene)-diphosphonate (AMP-PCP) (Born & Foulks, 1977; Evans, 1979) and some α,ω-

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diadenosine polyphosphates including P^1,P^5-di(adenosine 5'-)pentaphosphate (Ap_5A) (Harrison, Brossmer & Goody, 1975) have been shown to be competitive. Of the AMP analogues, 2-chloroadenosine 5'-monophosphorothioate (2-chloro-AMPS) and 2-alkylthio derivatives of AMP have been shown specifically to inhibit ADP-induced aggregation (Gough, Nobbs, Middleton, Penglis-Caredes & Maguire, 1978). 2-Methylthioadenosine 5'-monophosphate and 2-(pentan-1-yl)thioadenosine 5'-monophosphate have also been claimed to be competitive inhibitors (Michal, Maguire & Gough, 1969; MacIntyre, Gordon, Drummond, Steer & Salzman, 1977), but a recent study failed to confirm this (Cusack & Hourani, 1982). 2-Chloroadenosine 5'-triphosphate (2-chloro-ATP) (Gough, Maguire & Satchell, 1973), adenosine 5'-O-(3-fluorotriphosphate) (ATP-γ-F) (Haley & Yount, 1972) and the $\mathbf{R_P}$ and $\mathbf{S_P}$ diastereoisomers of adenosine 5'-O-(1-thiotriphosphate) (($\mathbf{R_p}$)-ATP-α-S and ($\mathbf{S_p}$)-ATP-α-S) (Eckstein & Goody, 1976) had not previously been tested on human platelets, but our preliminary studies suggested that they were competitive.

In an attempt to resolve the controversy over whether induction of aggregation and inhibition of stimulated adenylate cyclase were mediated by the same or different ADP receptors, we decided to compare the dissociation constants obtained for inhibition of each effect by these structurally diverse antagonists.

Methods

Aggregation studies

Human platelet-rich plasma (PRP) was obtained by centrifuging citrated venous blood at 260 *g* for 20 min at room temperature and collecting the supernatant. Aggregation was quantified photometrically (Born, 1962; Michal & Born, 1971) with a Born-Michal Mark IV aggregometer as the maximal rate of change in light transmission (arbitrary units/min) through a sample (0.5 ml) of stirred PRP at 37°C on addition of a test solution (15 μl) containing ADP alone or containing ADP and an inhibitor.

Measurement of platelet adenylate cyclase activity

Increases in levels of platelet adenosine 3',5'-cyclic monophosphate (cyclic AMP) were measured in PRP that had been preincubated for 90 min at 37°C with purified [U-^{14}C]-adenine to label platelet adenine nucleotides (Haslam & Rosson, 1975). Aliquots (0.45 ml) at 37°C were treated with solutions (50 μl) of ADP alone, or ADP and an inhibitor, which contained PGE_1 (10 μM) (to stimulate adenylate cyclase) and papaverine hydrochloride (20 mM) (to inhibit phosphodiesterase). After 30 s at 37°C, the incubation was stopped and cyclic AMP extracted by addition of 3 M perchloric acid (0.1 ml) containing [2,8-^{3}H]-cyclic AMP to estimate recovery. The samples were centrifuged and the cyclic AMP in the supernatant purified by chromatography on AG50W-X8 [H^+](1.3 ml), followed by treatment of the cyclic AMP-containing eluate with a suspension of 0.25 M barium sulphate (2 × 0.6 ml) and centrifugation. The supernatant was lyophilized and [^{14}C]-cyclic AMP and [^{3}H]-cyclic AMP estimated by liquid scintillation counting. Measurements of the stimulation of [^{14}C]-cyclic AMP production by PGE_1 were carried out in the presence and absence of the nucleotides, and the % inhibition was calculated from the difference between these values after correction for the baseline effect of papaverine alone.

Drugs

ADP, ATP, 2-chloroadenosine, carbonyl diimidazole and papaverine hydrochloride were obtained from Sigma London. Ap_5A, AMP-PCP and adenosine 5'-monophosphorothioate (AMPS) were obtained from Boehringer Mannheim. [U-^{14}C]-adenine and [2,8-^{3}H]-cyclic AMP were obtained from Amersham International Ltd. AG50W-X8[H^+] was obtained from BioRad Laboratories. Phosphoryl chloride and thiophosphoryl chloride were obtained from Fluka AG. PGE_1 was a generous gift from Dr J. Pike of the Upjohn Company in Kalamazoo, Michigan. Sodium monofluorophosphate of high purity was kindly donated by Mr W. Stoker of Fluorochem Ltd, U.K.

ATP-γ-F was synthesized from ADP and monofluorophosphate as described by Haley & Yount (1972). 2–Chloroadenosine 5'-monophosphate (2-chloro-AMP) was synthesized by phosphorylation of 2-chloroadenosine with phosphoryl chloride (Gough, Maguire & Michal, 1969) and converted to 2-chloro-ATP by pyrophosphorylation with pyrophosphate and carbonyl diimidazole (Gough *et al.*, 1973). 2-Chloro-AMPS was synthesized by thiophosphorylation of 2-chloroadenosine with thiophosphoryl chloride (Gough *et al.*, 1978).

ATP-α-S was synthesized by pyrophosphorylation of AMPS (Eckstein & Goody, 1976), and the $\mathbf{R_P}$ and $\mathbf{S_P}$ diastereoisomers obtained were separated by isocratic (0.01 M KH_2PO_4, 2 ml/min) high performance liquid chromatography (h.p.l.c.) on a reverse phase column (μ Bondapak C18, Waters Associates). The $\mathbf{S_P}$ configuration of the first eluted diastereoisomer (retention time 5 min) and the $\mathbf{R_P}$ configuration of the second eluted diastereoisomer (retention time 7 min) were established by comparison with the pro-

ducts of enzymatic phosphorylation of the S_P and R_P diastereoisomers respectively of adenosine 5'-O-(1-thiodiphosphate) (ADP-α-S) (Eckstein & Goody, 1976; Burgers & Eckstein, 1978; Cusack & Hourani, 1981).

All nucleotides were purified by ion exchange chromatography and examined by h.p.l.c. immediately before use, and stock solutions were assayed by ultraviolet spectroscopy.

Calculation of pA_2 values

Log dose-response curves were constructed to ADP alone and in the presence of various concentrations of the inhibitors. Lines were constructed through each log dose-response curve and the weighted mean of these slopes was used to redraw parallel lines through each log does-response curve. The dose-ratio (DR) was calculated from the shift in the redrawn parallel lines, and log (DR−1) was plotted against the log of the inhibitor concentration (I) according to Arunlakshana & Schild (1959). A line was drawn through this Schild plot and the intercept on the log (I) axis was taken as the negative log of the dissociation constant of the inhibitor (pA^2). All lines were drawn by least squares linear regression analysis and all calculations were performed by computer, uusing the method described by Tallarida & Jacob (1979).

Results

ATP, AMP-PCP, Ap_5A, 2-chloro-AMPS, 2-chloro-ATP, ATP-γ-F, (R_P)-ATP-α-S and (S_P)-ATP-α-S all caused parallel shifts to the right of the log dose-response curves to ADP, both for aggregation (Figure 1) and for inhibition of PGE_1-stimulated adenylate cyclase (Figure 2). Several concentrations of the inhibitors were used to construct Schild plots (inhibition by only one concentration is shown in Figures 1 and 2 for clarity). The slopes of the Schild plots and the K_i and pA_2 values derived from these Schild plots are shown in Table 1. A plot of pA_2 values for inhibition of aggregation against pA_2 values for inhibition of the effect of ADP on PGE_1-stimulated adenylate cyclase is shown in Figure 3. Linear regression analysis of this gave a line with a slope of 0.89 ± 0.21, an intercept on the ordinate of 0.90 ± 0.97 and a correlation coefficient of 0.87 ($P < 0.01$).

Discussion

These results show that ATP, AMP-PCP, Ap_5A, 2-chloro-AMPS, 2-chloro-ATP, ATP-γ-F, (R_P)-ATP-α-S and (S_P)-ATP-α-S all inhibited both ADP-induced human platelet aggregation and the effect of ADP on PGE_1-stimulated adenylate cyclase. In each case the inhibition was competitive, and the Schild slopes were close to unity (Table 1). The K_i for inhibition of ADP-induced aggregation by ATP (23 μM) is in good agreement with the published values of 20 μM (Macfarlane & Mills, 1975) and 25 μM (Cusack & Hourani. 1981). Our K_i values for the inhibition of ADP-induced aggregation by Ap_5A (35 μM) and AMP-PCP (84 μM) are not in agreement with the published values of < 0.7 μM (Harrison *et al.*, 1975) and133 μM (Lips, Sixma & Schiphorst, 1980) respectively, but in those studies washed platelets, preincubated with the inhibitors, were used. Washing platelets always results in some degree of cell damage as well as the loss of plasma cofactors, and consequently their pharmacological behaviour is different from that of platelets in their native plasma (Akkerman, Doucet-de Bruïne, Gorter, de Graaf, Holme, Lips, Nijmeijer, Over, Starkenburg, Tries-

Table 1 Data derived from Schild plot analysis of inhibition of the effects of ADP on human platelets

	Aggregation				*Inhibition of PGE_1-stimulated adenylate cyclase*			
Inhibitor	*Range (and number) of dose-ratios*	*Schild slope*	*pA_2*	*K_i(μM)*	*Range (and number) of dose-ratios*	*Schild slope*	*pA_2*	*K_i(μM)*
ATP	1.8– 8.4 (4)	0.92	4.64	23	3.9–29.1 (4)	0.98	5.21	6.2
AMP-PCP	1.5– 3.3 (3)	0.96	4.08	84	1.8– 4.6 (3)	0.91	4.22	60
Ap_5A	1.5– 8.2 (4)	1.13	4.45	35	2.3– 3.7 (2*)	0.86	4.79	16
2-Chloro-AMPS	1.5– 3.8 (3)	1.05	4.13	74	2.3– 8.3 (3)	1.07	4.54	29
2-Chloro-ATP	3.9–26.3 (4)	0.93	5.18	6.6	2.8–25.2 (4)	1.13	5.95	1.1
ATP-γ-F	1.8– 3.6 (3)	1.07	3.91	122	4.3–10.5 (3)	0.97	4.57	27
(R_P)-ATP-α-S	2.0– 9.6 (4)	0.89	4.74	18	4.3–28.0 (4)	0.94	5.26	5.4
(S_P)-ATP-α-S	2.1–10.2 (4)	0.93	5.44	4.0	1.9– 9.4 (4)	0.98	5.34	4.6

*Only two concentrations could be used because high concentrations precipitated with papaverine.

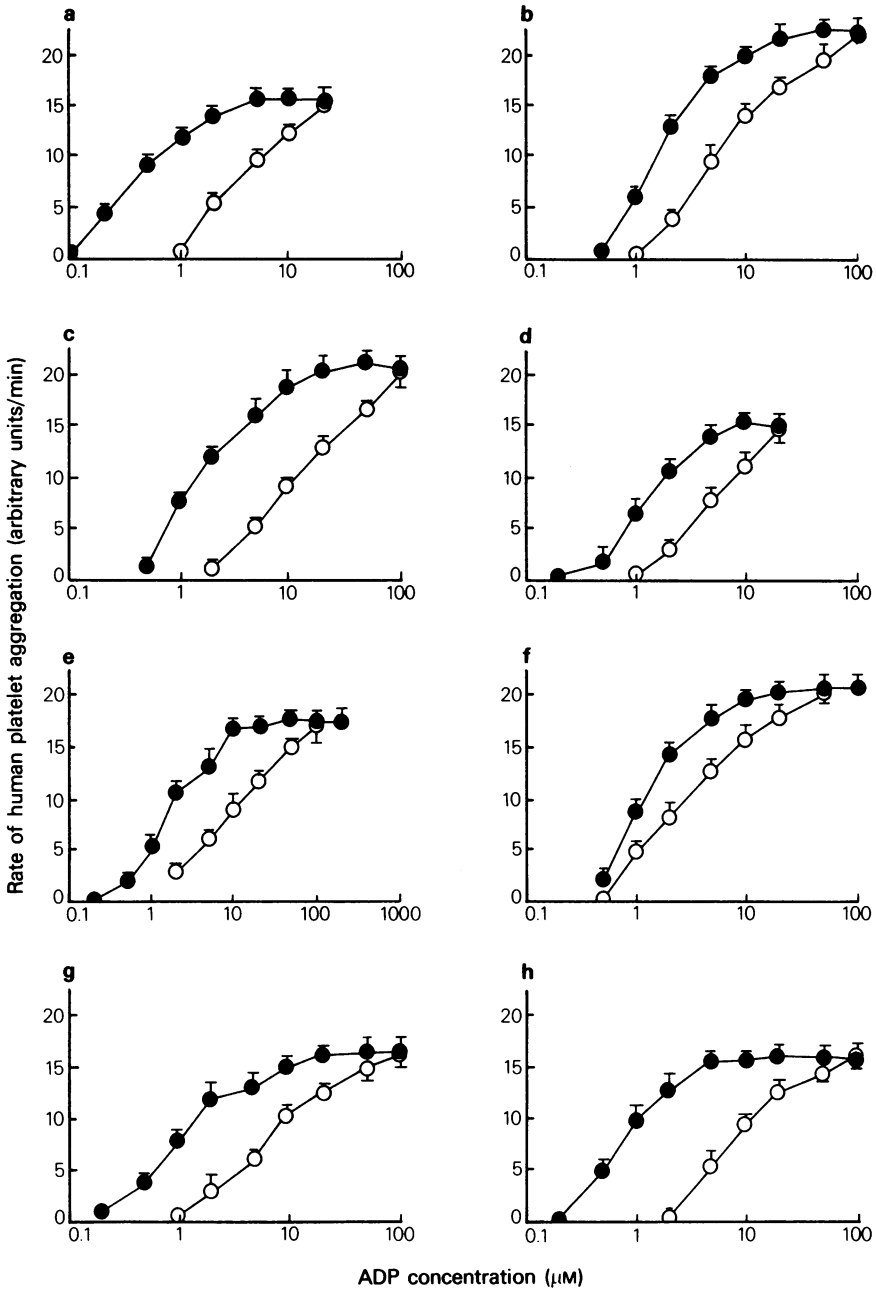

Figure 1 Human platelet aggregation induced by ADP alone (●) or in the presence (○) of an inhibitor: (a) ATP (200 μM); (b) AMP-PCP (200 μM); (c) Ap_5A (200 μM); (d) 2-chloro-AMPS (200 μM); (e) 2-chloro-ATP (40 μM); (f) ATP-γ-F (180 μM); (g) (R_P)-ATP-α-S (100 μM); (h) (S_P)-ATP-α-S (40 μM). Each point is the mean of at least three determinations. Vertical bars show the standard deviations.

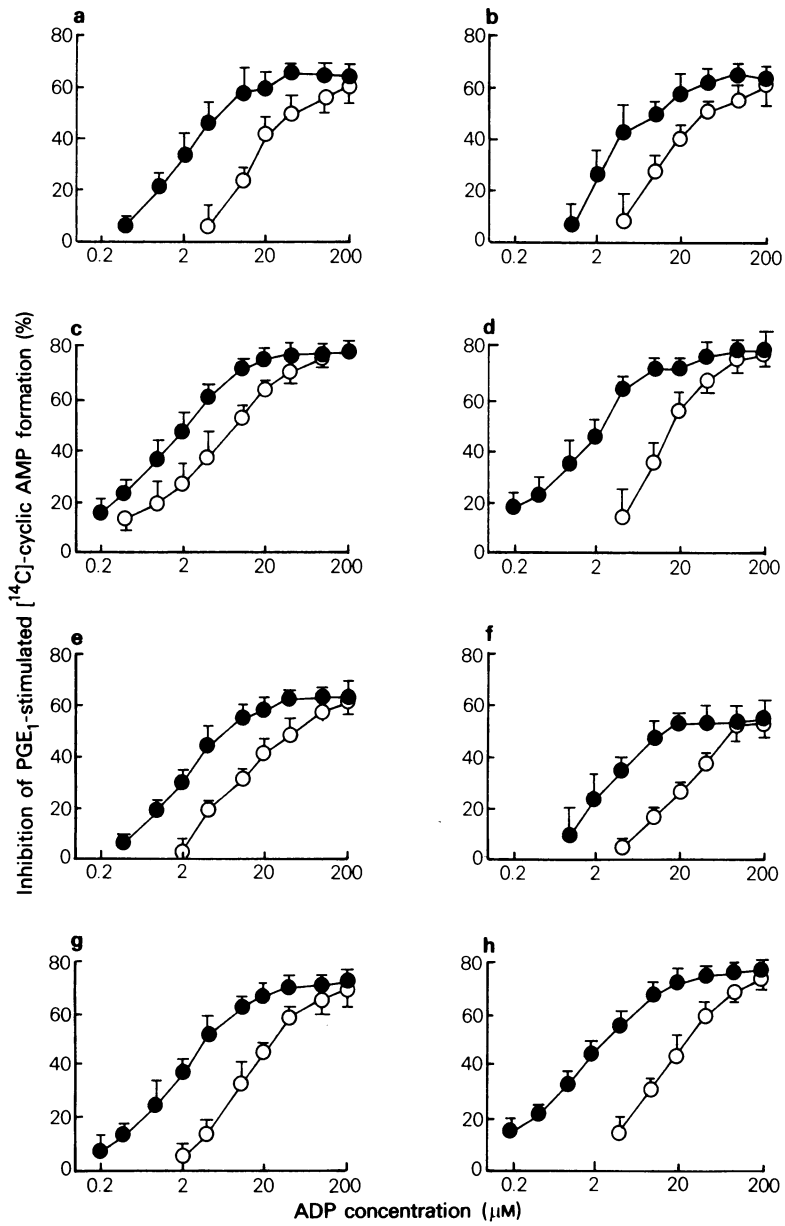

Figure 2 Inhibition of prostaglandin E_1 (PGE_1)(1 μM)-stimulated formation of [^{14}C]-cyclic AMP in human platelets by ADP alone (●) or in the presence (○) of an inhibitor: (a) ATP (40 μM); (b) AMP-PCP (200 μM); (c) Ap_5A (40 μM); (d) 2-chloro-AMPS (200 μM); (e) 2-chloro-ATP (4 μM); (f) ATP-γ-F (180 μM); (g) (R_P)-ATP-α-S (40 μM); (h) (S_P)-ATP-α-S (40 μM). All samples contained papaverine (2 mM). Each point is the mean of at least three determinations. Vertical bars show standard deviations.

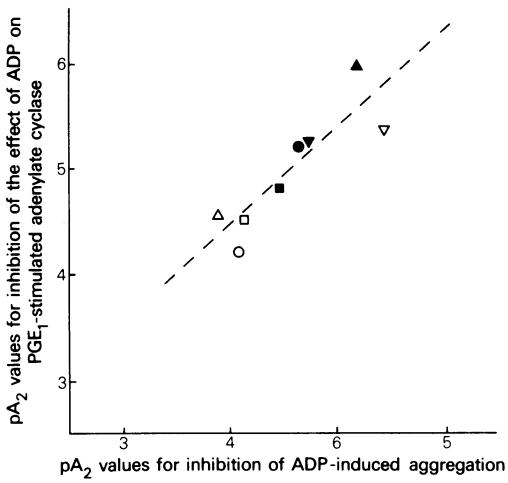

Figure 3 Relationship between the pA_2 values for inhibition of ADP-induced aggregation and the pA_2 values for the inhibition of the effect of ADP on prostaglandin E_1 (PGE_1)-stimulated adenylate cyclase, using the data presented in Table 1. (●) ATP; (○) AMP-PCP; (■) Ap_5A; (□) 2-chloro-AMPS; (▲) 2-chloro-ATP; (△) ATP-γ-F; (▼) (R_P)-ATP-α-S; (▽) (S_P)-ATP-α-S. The broken line was fitted by computer using least squares linear regression analysis.

chnigg, Veen, Vlooswijk, Wester & Sixma, 1978). In addition, preincubation of platelets with adenine nucleotides is undesirable since although intact nucleotides are not removed by uptake, they can be enzymatically dephosphorylated to pharmacologically active products such as ADP and adenosine (Chambers, Salzman & Neri, 1968). ADP, as well as being an aggregating agent, can cause desensitization during preincubation (Holme & Holmsen, 1975), while adenosine inhibits aggregation noncompetively by stimulating adenylate cyclase (Haslam & Rosson, 1975). Preincubation of inhibitors may therefore result in an inhibition which is no longer purely competitive, but represents the nett effect of an undefined mixture of these actions, leading to a different apparent K_i. In view of these considerations and the lack of any obvious barrier in this cell suspension to access of the nucleotides to the ADP receptor, it seemed preferable to avoid preincubation of these inhibitors before addition of ADP.

If ADP-induced aggregation and the inhibition by ADP of stimulated adenylate cyclase were mediated by two separate receptors, no correlation would be expected between the pA_2 values for inhibition of each effect by the eight structurally diverse ADP antagonists used in this study. However, despite the necessarily different experimental procedures used to study aggregation and the inhibition of stimulated adenylate cyclase, and the fact that each pA_2 value was derived from an experiment using blood from a different donor, a good correlation (correlation coefficient $0.87: P < 0.01$) was obtained (Figure 3). Such a correlation does not support the suggestion (Mills & Macfarlane, 1978) that there are two distinct ADP receptors on human platelets for aggregation and for inhibition of stimulated adenylate cyclase.

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