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# EFFECTS OF SURAMIN ON INCREASES IN CYTOSOLIC CALCIUM AND ON INHIBITION OF ADENYLATE CYCLASE INDUCED BY ADENOSINE 5′-DIPHOSPHATE IN HUMAN PLATELETS

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**Abstract**—The effects of the $P_2$-purinoceptor antagonist, suramin, on ADP-induced increases in human platelet cytosolic calcium concentration ($[Ca^{2+}]_i$) and inhibition of prostaglandin $E_1$ ($PGE_1$)-stimulated adenylate cyclase activity were investigated. Suramin (50–200 $\mu$M) acted as an antagonist of ADP-induced increases in $[Ca^{2+}]_i$, causing parallel, rightward shifts of the log concentration–response curve to ADP with no apparent depression of the maximal response. However, the slope of the Schild plot was 2.3 ± 0.3, similar to that obtained in previous studies on aggregation, indicating that the antagonism was not simply competitive. The apparent $pA_2$ for suramin, taken from the Schild plot, was 4.63, similar to that for suramin's inhibition of aggregation, which suggests that these two effects are closely related. Suramin was not specific for the ADP receptor, however, as it was also able to inhibit, non-competitively, increases in $[Ca^{2+}]_i$ induced by 5-hydroxytryptamine. Suramin (50–400 $\mu$M) also inhibited the effect of ADP on $PGE_1$-stimulated accumulation of cyclic AMP, causing parallel shifts of the log concentration–response curve to ADP, with a Schild plot slope of 1.00 ± 0.10, suggesting competitive antagonism, and a $pA_2$ value of 5.09. Suramin (400 $\mu$M) did not reduce the inhibition of cyclic AMP accumulation by adrenaline, although it was able to inhibit the accumulation of cyclic AMP caused by $PGE_1$, again showing that suramin has some non-specific effects. These data suggest that suramin is an antagonist at the platelet ADP receptor mediating increases in $[Ca^{2+}]_i$ and inhibition of adenylate cyclase, but that it also shows non-specific effects and can depress platelet responses to other agonists. In addition, the similar $pA_2$ value of suramin for the two effects of ADP does not support suggestion that they are mediated by two different receptors on human platelets.

*Key words:* ADP; aggregation; purinoceptors; cyclic AMP

ADP induces aggregation of human blood platelets via a class of $P_2$-purinoceptor which has been designated $P_{2T}$ [1]. We have recently shown that the trypanocidal drug, suramin, which is an antagonist at $P_{2X}$ and $P_{2Y}$ receptors on vascular and visceral smooth muscle [2–9], is an antagonist of ADP-induced aggregation of washed human platelets with an apparent $pA_2$ value of 4.62 [10]. The effect of suramin on ADP-induced aggregation was not simply competitive, however, as the Schild plot slope obtained was significantly greater than unity, and it was not specific for ADP as suramin was also able to inhibit, non-competitively, platelet aggregation induced by 5-HT†. The apparent $pA_2$ value does, however, correspond well with the published $pA_2$ values for suramin at other $P_2$-purinoceptor subtypes on other tissues, which range between 4.5 and 5.4 [7–9], supporting the classification of the platelet ADP receptor among the $P_2$-purinoceptors.

One possible explanation for the non-selective, non-competitive effects of suramin on human platelet aggregation is that suramin, which is known to bind avidly to plasma proteins [11], may in some way interfere with the interaction of platelets with fibrinogen, a factor which is essential for agonist-induced aggregation [12]. To test this hypothesis it was necessary to study a platelet response which is not dependent on the presence of fibrinogen. The initial biochemical responses of platelets to stimulation with ADP are an increase in $[Ca^{2+}]_i$ [13, 14] and inhibition of stimulated adenylate cyclase activity [15] both of which can be measured in washed platelets and are believed to be independent of fibrinogen. We therefore investigated the effects of suramin on these responses to ADP in the absence of fibrinogen. Some of these results have been published previously in the form of an abstract [16].

## MATERIALS AND METHODS

*Cytosolic calcium measurement.* Venous blood was drawn from healthy human volunteers, who had not taken aspirin for 10 days, into one sixth of the volume of acid–citrate–dextrose anticoagulant (85 mM tri-sodium citrate, 71 mM citric acid, 111 mM glucose) and centrifuged at 260 *g* for 20 min. The PRP was removed and incubated with fura-2-AM

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† Abbreviations: $PGE_1$, prostaglandin $E_1$; $[Ca^{2+}]_i$, cytosolic calcium concentration; 5-HT, 5-hydroxytryptamine; PRP, platelet-rich plasma; IBMX, isobutylmethylxanthine; FSBA, 5′-*p*-fluorosulphonylbenzoyladenosine.

(4 $\mu$M) at 37° for 45 min. The fura-2-loaded platelets were then harvested by centrifugation at 680 $g$ for 20 min in the presence of prostacyclin (1 $\mu$M) and resuspended at $10^8$ platelets/mL in HEPES–saline (145 mM NaCl, 5 mM KCl, 10 mM HEPES, 10 mM glucose, 2 mg/mL BSA, adjusted to pH 7.4). Aliquots (700 $\mu$L) were incubated in siliconized glass aggregometer tubes at 37° for 3 min in the presence of 1 mM $CaCl_2$ and transferred to a Perkin–Elmer LS50 Luminescence Spectrometer for estimation of the $[Ca^{2+}]_i$. A cyclooxygenase inhibitor was not included because under these conditions ADP is unlikely to induce the release of stimulatory prostaglandins and thromboxane, as this process is known only to occur in the presence of lowered extracellular $Ca^{2+}$ and if the platelets are allowed to aggregate, and addition of a cyclooxygenase inhibitor has been shown not to affect ADP-induced increases in $[Ca^{2+}]_i$ [12, 13, 17]. The stirred suspension was excited at 380 nm and the emitted light intensity was measured at 510 nm. The fluorescence intensity at saturating calcium ($F_{sat}$) and in the absence of calcium ($F_{abs}$) was determined by lysing the platelets with 0.1% Triton X-100 ($F_{sat}$) followed by the sequential addition of 20 mM Tris and 10 mM EGTA. The cytosolic calcium concentration was then calculated using the equation $[Ca^{2+}] = K_d(F - F_{abs})/(F_{sat} - F)$ [18]. Suramin has a broad UV absorption spectrum and absorbs wavelengths up to about 370 nm, and it was therefore not possible to use the more usual dual wavelength calcium measurement with fura-2, as this requires excitation at around 340 nm. Agonists were added in 14 $\mu$L of water and suramin, where used, was added simultaneously with the agonist. Simultaneous addition was employed because our previous studies with suramin on washed platelet aggregation had shown that the inhibitory effects of suramin decrease with incubation time [10]. The response was quantified as the maximal change in $[Ca^{2+}]_i$ following addition of the agonist. Log concentration–response curves were obtained at least three times using blood from different donors and the results were pooled. $EC_{50}$ values were obtained by linear regression of the linear parts of the pooled concentration–response curves and dose ratios for suramin were calculated from these $EC_{50}$ values.

*Measurement of inhibition of $PGE_1$-stimulated adenylate cyclase.* PRP was incubated with 4 $\mu$M [$^{14}$C]adenine for 45 min at 37°. The platelets were then collected by centrifugation at 680 $g$ for 20 min in the presence of prostacyclin (1 $\mu$M) and resuspended at $1.1 \times 10^8$/mL in HEPES–saline. Platelet cyclic AMP levels were determined by a modification of the method of Haslam and Rosson [19]. Aliquots (450 $\mu$L) of platelet suspension, which had been incubated for 3 min at 37° in the presence of 1 mM $CaCl_2$, were treated with solutions (50 $\mu$L) of ADP or adrenaline which contained $PGE_1$ (10 $\mu$M), to stimulate adenylate cyclase, IBMX (1 mM), to inhibit phosphodiesterase, and an appropriate concentration of suramin. After 30 sec the reaction was terminated by addition of 3 M perchloric acid (100 $\mu$L) containing [$^3$H]cyclic AMP (~20,000 dpm) to estimate recovery. After at least 30 min on ice the samples were centrifuged in a microfuge and a 450 $\mu$L aliquot of the supernatant applied to columns of AG50W-X8 [$H^+$] (1.3 mL) to remove the adenine and adenosine. The adenine nucleotides were eluted with 1 mM $KH_2PO_4$ (pH 7.5) and the eluate was twice treated with nascent barium sulphate (by addition of 0.3 mL of 0.25 M zinc sulphate and 0.3 mL of 0.25 M barium hydroxide) and centrifuged to remove adenine nucleotides other than cyclic AMP. An aliquot (5–6 mL) of the supernatant was lyophilized and the $^3$H and $^{14}$C estimated by scintillation counting. $^{14}$C dpm were corrected for recovery of [$^3$H]cyclic AMP, and the baseline level of [$^{14}$C]cyclic AMP (i.e. that in the presence of IBMX alone) was subtracted. Inhibition of adenylate cyclase was expressed as a percentage relative to the level of [$^{14}$C]cyclic AMP in platelets stimulated with $PGE_1$ alone. Log concentration–response curves were obtained in platelets from at least three donors in triplicate and pooled. The $EC_{50}$ values were calculated by linear regression of the linear portion of the pooled concentration–response curves. Dose ratios were calculated from these $EC_{50}$ values.

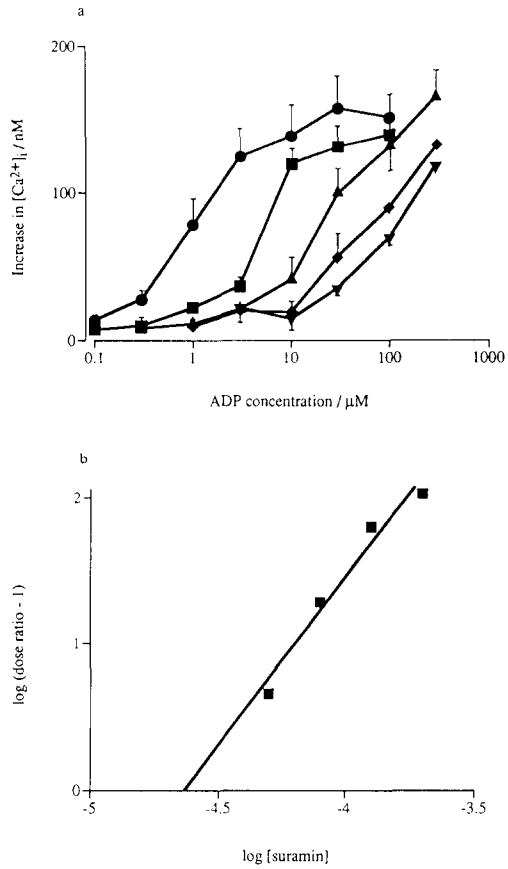


Fig. 1. (a) Increases in platelet $[Ca^{2+}]_i$ induced by ADP in the absence (●) or presence of suramin at 50 (■), 80 (▲), 130 (◆) or 200 (▼) $\mu$M. (b) Schild plot of the data presented in (a). Values are the means of three separate determinations on platelets from different donors and vertical bars show the SEM.

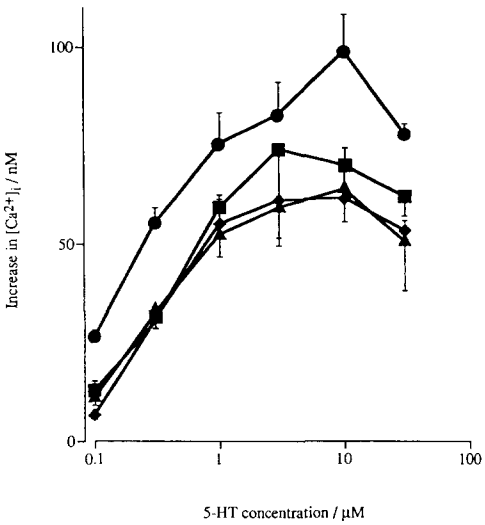


Fig. 2. Increases in $[Ca^{2+}]_i$ induced by 5-HT in the absence (●) or presence of suramin at 100 (■), 200 (▲) or 400 (◆) μM. Values are the means of three determinations on platelets from different donors and vertical bars show the SEM.

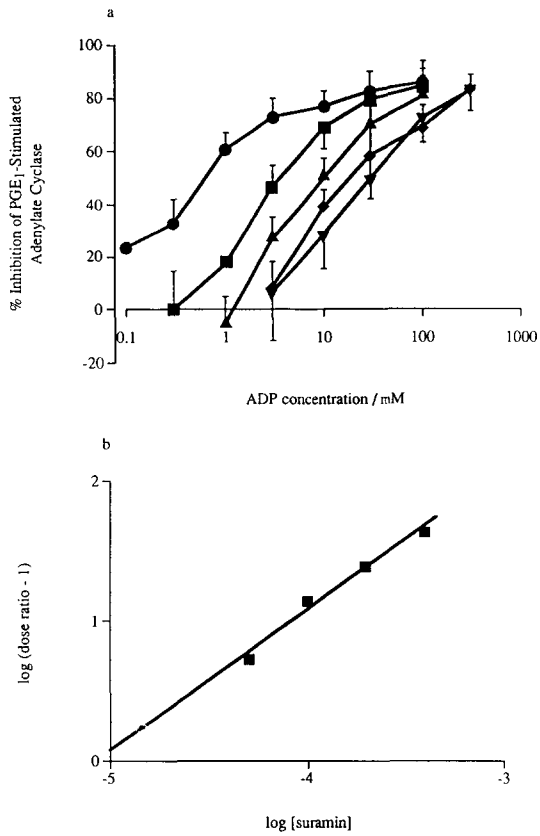


Fig. 3. (a) Inhibition of $PGE_1$-stimulated cyclic AMP accumulation by ADP in the absence (●) or presence of suramin at 50 (■), 100 (▲), 200 (◆) or 400 (▼) μM. (b) Schild plot of the data presented in (a). Values are the means of at least three determinations on platelets from different donors and vertical bars show the SEM.

Concentration–response curves to $PGE_1$ were performed in a similar manner to above, but in the absence of ADP or adrenaline, and the results were expressed in dpm as [$^{14}$C]cyclic AMP present in the platelet suspension, corrected for the baseline effects of IBMX. These experiments were performed in the presence of a constant concentration of ethanol corresponding to that present at the highest concentration of $PGE_1$ assayed.

*Materials.* ADP, ATP, adrenaline, BSA, fura-2-AM, 5-HT, IBMX, prostacyclin, $PGE_1$ and Triton X-100 were obtained from the Sigma Chemical Co. (Poole, U.K.). AG50W-X8 [$H^+$] was obtained from BioRad. [2,8-$^3$H]Cyclic AMP, ammonium salt (41.5 Ci mmol$^{-1}$) in 50% aqueous ethanol and [U-$^{14}$C]adenine (270 mCi mmol$^{-1}$) in 2% aqueous ethanol were from Amersham International (Amersham, U.K.). Suramin was a generous gift from Bayer (U.K.) and all other chemicals were AnalaR grade from BDH (Poole, U.K.). Prostacyclin was dissolved at 100 μg/mL in 10 mM NaOH, $PGE_1$ was dissolved at 1 mM in 50% aqueous ethanol and fura-2-AM was dissolved at 5 mM in DMSO, and these solutions were stored at −20°. IBMX was dissolved in HEPES–saline and all other drugs were dissolved in water. Adrenaline, IBMX, 5-HT and suramin were made up freshly each day while the nucleotides were stored frozen.

## RESULTS

The basal $[Ca^{2+}]_i$ in the platelets under the conditions of this study was 35.1 ± 3.4 nM (N = 21). ADP induced concentration-dependent increases in $[Ca^{2+}]_i$ with an $EC_{50}$ value of 1.0 μM. This effect of ADP was inhibited by suramin which caused dose-dependent, rightward shifts of the log concentration–response curve to ADP (Fig. 1a). Schild analysis of these data gave a slope of 2.3 ± 0.3, which was significantly greater than unity ($P < 0.05$, Student's *t*-test) and an apparent $pA_2$ value (negative log of the concentration causing a dose ratio of 2) of 4.63 (Fig. 1b). Suramin (100–400 μM) was also able to inhibit increases in platelet $[Ca^{2+}]_i$ induced by 5-HT, although this inhibition was not competitive as suramin caused a decrease in the maximal response to this agonist and the effect was limited, 200 and 400 μM suramin causing the same degree of inhibition (Fig. 2). Suramin alone did not affect the fluorescence of the platelet suspension and therefore had no effect on the basal platelet $[Ca^{2+}]_i$ at any concentration tested (data not shown).

ADP induced a concentration-dependent inhibition of $PGE_1$-stimulated cyclic AMP accumulation with an $EC_{50}$ value of 0.47 μM. Suramin inhibited this effect of ADP in a concentration-dependent manner (Fig. 3a) and Schild analysis of this inhibition resulted in a slope of 1.00 ± 0.10 and a $pA_2$ value of 5.09 (Fig. 3b). Suramin (400 μM) had no effect on the inhibition of stimulated adenylate cyclase by

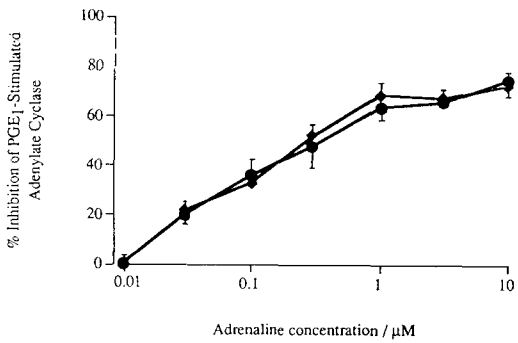


Fig. 4. Inhibition of $PGE_1$-stimulated cyclic AMP accumulation by adrenaline in the absence (●) or presence (◆) of suramin (400 μM). Values are the means of at least three determinations on platelets from different donors and vertical bars show the SEM.

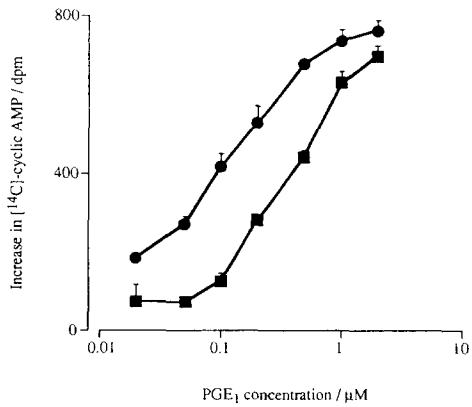


Fig. 5. A representative graph showing the accumulation of cyclic AMP in human platelets stimulated with $PGE_1$ in the absence (●) or presence (■) of suramin (400 μM). Values are the means of triplicate determinations and vertical bars shown the SEM.

adrenaline (Fig. 4), but was able to inhibit accumulation of cyclic AMP induced by sub-maximal concentrations of $PGE_1$, causing a 4-fold shift to the right of the log concentration–response curve (Fig. 5). Suramin alone (400 μM) had no significant effect either on the basal level of cyclic AMP in platelets [289 ± 34 dpm (N = 7) in the absence of suramin, 292 ± 41 dpm (N = 3) in its presence] or on the effect of $PGE_1$ (1 μM) in the experiments measuring inhibition by ADP or adrenaline [933 ± 128 dpm (N = 7) in the absence of suramin, 897 ± 117 dpm (N = 7) in its presence].

## DISCUSSION

These results show that suramin caused concentration-dependent inhibition of ADP-induced increases in $[Ca^{2+}]_i$ with a Schild plot slope of 2.3 ± 0.3 and an apparent $pA_2$ value of 4.63. These results are in good agreement with those obtained in our previous studies on the inhibition by suramin of ADP-induced aggregation of washed platelets [10], which showed a Schild plot slope of 1.82 ± 0.21 and an apparent $pA_2$ value of 4.62. Our previous studies on a number of adenine nucleotide analogues as agonists and antagonists of ADP receptor-mediated increases in platelet $[Ca^{2+}]_i$ [14] showed a good correlation between the effects of these compounds on $[Ca^{2+}]_i$ and their reported effects on ADP receptor-mediated platelet aggregation [20–25]. These results along with our present findings with suramin suggest a close relationship between these two measures of platelet activation.

A possible reason for the non-unit Schild plot slope obtained for suramin against aggregation was that suramin, because of its tendency to bind to plasma proteins [11], was interfering in some way with the ability of fibrinogen to take part in aggregation. This could also have explained the ability of suramin to inhibit, non-competitively, aggregation induced by 5-HT [10]. Fibrinogen is an essential factor for aggregation caused by all but the strongest aggregating agents [12]; thus, any agent which could block its interaction with platelets would cause a general non-selective, non-competitive inhibition of the response of platelets to all such aggregating agents. In the present study we have shown that suramin is able to inhibit ADP-induced increases in $[Ca^{2+}]_i$ in a manner almost identical to its inhibition of aggregation and that it is also able to inhibit, non-competitively, increases in $[Ca^{2+}]_i$ induced by 5-HT. In the case of 5-HT the effect of suramin on increases in $[Ca^{2+}]_i$ was less pronounced than its effect on aggregation and was limited, as 200 and 400 μM suramin caused almost identical levels of inhibition. Overall, these results suggest that any interaction of suramin with fibrinogen is not responsible for the non-selective inhibitory effects of suramin on platelet aggregation.

Suramin also acted as an antagonist of the inhibition by ADP of $PGE_1$-stimulated platelet adenylate cyclase activity. In this case, Schild analysis resulted in a slope of 1.00 ± 0.10 indicating that the effect was competitive, the $pA_2$ value being 5.09 (corresponding to a dissociation constant of 8.1 μM). Suramin did not reduce the inhibition of stimulated adenylate cyclase activity by adrenaline, which was used here as a control agonist in preference to 5-HT which has little effect on platelet adenylate cyclase activity. However, suramin did inhibit the accumulation of cyclic AMP induced by $PGE_1$, although at the concentration of $PGE_1$ used in the inhibition assays (1 μM, which is close to being maximally effective), the effect of 400 μM suramin was insignificant. The inhibition of the effect of $PGE_1$ by suramin appears to be competitive, in that the log concentration–response curves are parallel and the maximal response does not appear to be depressed. However, suramin was somewhat less potent at inhibiting this response than at inhibiting responses to ADP, 400 μM suramin causing only a 4-fold shift in the log concentration–response curve to $PGE_1$. This concentration of suramin caused an approximately 100-fold shift of the log concentration–response for ADP in inducing aggregation [10]

and an approximately 40-fold shift in its log concentration–response curve for inhibition of adenylate cyclase. Interestingly, both ADP and $PGE_1$ are negatively charged at physiological pH. In early studies with suramin as an enzyme inhibitor [26] it was suggested that this compound has its effects by binding to positively charged regions of proteins. Thus, these data suggest that suramin acts as an antagonist at the platelet $P_{2T}$ receptor, as it does at smooth muscle $P_{2X}$ and $P_{2Y}$ receptors, possibly due to its ability to bind to positive charges in the receptor binding site, but that it also has various non-specific inhibitory effects possibly due to its ability to bind positively charged regions of other proteins on the platelet surface.

It has been shown previously that there is little correlation between the potencies of ADP receptor agonists as aggregating agents and as inhibitors of stimulated adenylate cyclase (see Ref. 27 for review), and it has been suggested that this may indicate the presence of two types of ADP receptor on platelets mediating these two responses [28, 29] (see Refs 27, 30 for discussion). The main support for this hypothesis has been claimed from the observation that the photoaffinity reagent FSBA is able to inhibit ADP-induced shape change and aggregation but does not inhibit the effects of ADP on stimulated adenylate cyclase, and a 100-kDa protein labelled by FSBA has been claimed to be the ADP receptor mediating aggregation (see Ref. 31 for review). However, the specificity of FSBA for the ADP receptor is in serious doubt as it is also able to inhibit aggregation induced by a number of other aggregating agents, although this has been taken to reflect a central role for ADP in mediating platelet aggregation [32]. It is also unable to inhibit platelet calcium mobilization induced by ADP [33] a response which is accepted as one of the initial second messenger responses induced by ADP [13] (see Ref. 12 for review), and for which the structure–activity relationships of agonists and antagonists are identical to those for aggregation [14]. Thus, as FSBA is unable to inhibit either of the second messenger responses known to be induced by ADP, it is unlikely that it is acting at the ADP receptor but probably acts at a site distal to it in the aggregation pathway, which casts doubt on the suggestion that it can distinguish between the two putative ADP receptors. Also in a study of a number of analogues of ATP and AMP, which were shown by Schild analysis to be competitive antagonists at the ADP receptor [23], there was a good correlation between the $pA_2$ values of these compounds for inhibition of ADP-induced aggregation and the effect of ADP on adenylate cyclase, which is good evidence that these two responses are in fact mediated by a single class of receptor. A criticism which could be made of this study is that all the compounds used were adenine nucleotide analogues and were therefore structurally very similar. However, we have now shown that suramin, a compound which is structurally unrelated to ADP, also inhibits ADP-induced aggregation [10] and inhibition of stimulated adenylate cyclase with a similar $pA_2$ value for both responses. In spite of the rather non-specific nature of the inhibition by suramin, this strengthens the conclusions of the previous study and provides further support for the suggestion that a single class of ADP receptor mediates aggregation, increases in $[Ca^{2+}]_i$ and inhibition of adenylate cyclase in human platelets.

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