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Effects of the P_2-purinoceptor antagonist, suramin, on human platelet aggregation induced by adenosine 5'-diphosphate

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1 The effects of suramin, a trypanocidal drug which has been reported to be a P_2-purinoceptor antagonist on smooth muscle, were investigated in human platelets, where adenosine 5'-diphosphate (ADP) induces aggregation by acting on a subtype of purinoceptors which has been called P_{2T}.

2 Suramin (100 μM) had no inhibitory effect on ADP-induced platelet aggregation in plasma, even after 40 min incubation in the presence of bacitracin, a peptidase inhibitor, and did not affect the ability of adenosine 5'-triphosphate (ATP) (40 μM) to inhibit competitively ADP-induced aggregation. This lack of effect of suramin on platelets in plasma is probably due to its extensive binding to plasma proteins.

3 In washed platelets, suramin (50–400 μM) acted as an apparently competitive antagonist, causing parallel shifts to the right of the log concentration-response curve to ADP. No depression of the maximal response to ADP was observed at concentrations of suramin (50–150 μM) for which full log concentration-response curves to ADP could be obtained, but the slope of the Schild plot was around 2, indicating that this antagonism was not simply competitive. The apparent pA_2 value for suramin, taken from this Schild plot, was 4.6.

4 Suramin (200–400 μM) also noncompetitively inhibited aggregation induced by U46619 (a thromboxane receptor agonist) or by 5-hydroxytryptamine in the presence of adrenaline (100 μM), and caused a depression of the maximal response to these agonists. This nonspecific effect of suramin may explain the high Schild plot slope obtained against ADP.

5 These results provide evidence that the ADP receptor on human platelets is indeed similar to the P_2-purinoceptors responding to adenine nucleotides on smooth muscle and other tissues, and show that suramin cannot distinguish between the proposed subtypes of the P_2-purinoceptors.

Keywords: Human platelets; purinoceptors; adenine nucleotides; suramin

Introduction

The adenine nucleotides adenosine 5'-diphosphate (ADP) and adenosine 5'-triphosphate (ATP) have potent extracellular effects on many tissues which are mediated by specific receptors known as P_2-purinoceptors. Two subclasses of these, P_{2X} and P_{2Y}, have been proposed to exist on smooth muscle causing contraction and relaxation respectively, with ADP and ATP being equipotent as agonists on these subclasses (Burnstock & Kennedy, 1985). Two other subclasses, P_{2Z} and P_{2T}, have been identified on immune cells and on platelets respectively, and differ from the P_{2X} and P_{2Y} subclasses in that on P_{2Z}-purinoceptors the agonist is ATP^{4-} and ADP is inactive, whereas on P_{2T}-purinoceptors ADP is the agonist and ATP is a competitive antagonist (Gordon, 1986). By use of synthetic analogues of adenine nucleotides, different structure-activity relationships have also been found for these four subclasses of P_2-purinoceptor, which supports the proposed subdivision. The structure-activity relationships for the P_{2T} subtype are more similar to those for the P_{2Y} subtype than to those for the P_{2X}, as 2-substituted analogues of ADP and ATP are more potent than the parent nucleotides, whereas methylene phosphonate analogues are less potent (Cusack & Hourani, 1990). Until recently no selective, reversible, competitive P_2 antagonists have been found, so this subclassification can only be provisional. Although ATP and its analogues are antagonists at P_{2T}-purinoceptors (Cusack & Hourani, 1982), they are agonists on the other subclasses and are therefore not useful for receptor classification.

Recently it has been reported that the trypanocidal drug suramin is a selective, competitive antagonist at P_{2X} and P_{2Y} receptors on vascular and visceral smooth muscle preparations, although it does not distinguish between these two receptor subtypes, having a pA_2 value of around 5 in each case (Dunn & Blakeley, 1988; Den Hertog *et al.*, 1989a, b; Hoyle *et al.*, 1990; Leff *et al.*, 1990; Von Kugelgen *et al.*, 1990). Suramin also selectively and competitively inhibits the effects of ATP on PC12 phaeochromocytoma cells with similar potency, the reported pA_2 value being 4.52 (Nakazawa *et al.*, 1990; Inoue *et al.*, 1991). As well as antagonizing P_2-purinoceptors, suramin is also known to inhibit a number of other proteins with nucleotide binding sites, for example yeast hexokinase (Wills & Wormall, 1950), erythrocyte membrane Na^+/K^+-ATPase (Fortes *et al.*, 1973), firefly luciferase (Fortes *et al.*, 1973), vacuole-type H^+-ATPases (Moriyama & Nelson, 1988; Calcaterra *et al.*, 1988), smooth muscle ecto-nucleotidases (Hourani & Chown, 1989), protein kinase C (Mahoney *et al.*, 1990), mitochondrial adenine nucleotide exchanger (Calcaterra *et al.*, 1988), the GTPase activity associated with G_i (Butler *et al.*, 1988) and various polynucleotide synthesizing enzymes (Broder *et al.*, 1985; Ono *et al.*, 1988; Offensperger *et al.*, 1988).

In this study we report the effects of suramin on the P_{2T}-purinoceptors on human platelets, in which ADP induces a change in shape, aggregation and the release of mediators from storage granules (Born, 1962).

Methods

Platelet aggregation

Venous blood was drawn from healthy human volunteers into one sixth of its volume of Acid-Citrate-Dextrose anticoagulant (trisodium citrate dihydrate 25 $g\,l^{-1}$, citric acid monohydrate 15 $g\,l^{-1}$, glucose 20 $g\,l^{-1}$), and centrifuged at 290 g for 20 min. Volunteers denied taking aspirin for 10 days before the experiment. The platelet-rich plasma (PRP) was removed and the platelets isolated by centrifugation at 680 g for 20 min in the presence of prostacyclin (1 μM). The supernatant was discarded and the platelets resuspended at a density of $10^8\,ml^{-1}$ in

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HEPES-saline buffer of the following composition (mM): NaCl 145, KCl 5, $MgCl_2$ 1, HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid) 10, glucose 10 and bovine serum albumin (BSA) 2 mg ml^{-1}, adjusted to pH 7.4 with 1 M NaOH. Aggregation and shape change were followed photometrically (Born, 1962) with a Chrono-log Lumi-Aggrometer, and aggregation was quantified as the maximal rate of change in light transmission (expressed as arbitrary units min^{-1}) through a stirred sample (500 μl) at 37°C on addition of agonist. Human fibrinogen (0.3 mg ml^{-1}) was added to all samples 20 s before addition of agonist, and calcium (1 mM, as calcium chloride) was added at the same time as suramin or at least 3 min before addition of agonist. Suramin was added either simultaneously with the agonist or preincubated with the platelet suspension for various times at 37°C. The agonists used were ADP (0.1–300 μM), U46619 (11α,9α-epoxymethanoprostaglandin H_2) (0.3–30 μM) and 5-hydroxytryptamine (5-HT) (0.1–100 μM). In the case of 5-HT, adrenaline (100 μM) was added simultaneously to potentiate its effects and result in a measurable aggregation response.

In studies on platelets in plasma, blood was drawn into one ninth of its volume of trisodium citrate (38 g l^{-1}) and centrifuged at 290 g for 20 min, and the PRP was removed. Aggregation was quantified as above in 500 μl samples of PRP, without addition of calcium or fibrinogen.

EC_{50} values were obtained by regression analysis of the linear portion of the log concentration-response curve to ADP.

Drugs

ADP, ATP, prostacyclin, U46619, adrenaline, 5-HT, fibrinogen (fraction 1 from human plasma, essentially plasminogen-free) and bacitracin were obtained from Sigma Chemical Co., Poole. Suramin was a generous gift from Bayer, UK, and all other chemicals were AnalaR Grade from BDH, Poole. Prostacyclin was dissolved at 100 μg ml^{-1} in 10 mM NaOH and U46619 was dissolved initially at 30 mM in absolute ethanol then diluted to 10 mM with distilled water and both drugs were stored frozen. All other drugs were dissolved in distilled water and the bacitracin, suramin, fibrinogen, 5-HT and adrenaline were made up freshly each day while the nucleotides were stored frozen.

Results

In citrated human plasma, ADP induced platelet aggregation with an EC_{50} value of 1.1 μM, and this aggregation was competitively inhibited by simultaneous addition of ATP (40 μM), with a K_B value of 10 μM derived from the shift in the ADP concentration-response curve. Suramin (100 μM) added simultaneously had no effect on the ADP-induced aggregation or on the inhibition of this by ATP (Figure 1a). Preincubation with suramin (100 μM) for 40 min at 37°C did not inhibit ADP-induced aggregation (Figure 1b), even in the presence of the peptidase inhibitor bacitracin (2 units ml^{-1}) (Figure 1c). Suramin alone did not induce aggregation or shape change at concentrations up to 1 mM (results not shown).

In washed platelets, ADP induced aggregation with an EC_{50} value of 4.3 μM, and suramin added simultaneously with ADP caused a dose-dependent parallel shift to the right of the log concentration-response curve (Figure 2a). Schild analysis of these data gave a slope of 1.82 ± 0.21, which was significantly greater than unity ($P < 0.005$, Student's t test), and an apparent pA_2 value (the negative log of the concentration causing a dose-ratio of 2) of 4.62 (Figure 2b). Under these conditions ATP also caused dose-related parallel rightward shifts of the log concentration-response curve for ADP, but gave Schild plots with slopes close to unity and pA_2 values around 5 (results not shown). Incubation of washed platelets with suramin for 10 or 40 min at 37°C before addition of ADP also yielded Schild plots with slopes significantly greater than unity (2.01 ± 0.16 and 2.06 ± 0.01 respectively), and with increasing time of incubation there was a reduction in the potency of suramin (apparent pA_2 values of 4.43 and 4.05 after 10 and 40 min respectively) (Figure 2c).

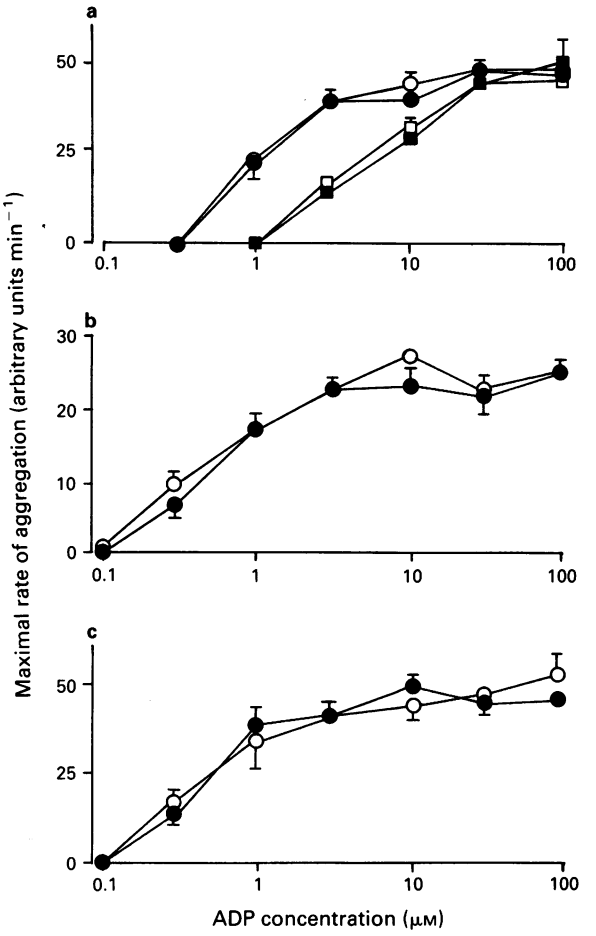

Figure 1 Effects of suramin on ADP-induced human platelet aggregation in citrated plasma. (a) Aggregation induced by ADP alone (○) or in the presence of suramin (100 μM) (●), ATP (40 μM) (□) or both suramin (100 μM) and ATP (40 μM) (■), added simultaneously with ADP. (b) Aggregation induced by ADP after preincubation of platelet-rich plasma for 40 min at 37°C with distilled water (○) or suramin (100 μM) (●). (c) Aggregation induced by ADP after preincubation of platelet-rich plasma for 40 min at 37°C with bacitracin (2 units ml^{-1}) (○) or with bacitracin (2 units ml^{-1}) and suramin (100 μM) (●). Each point is the mean of 3 determinations, and vertical bars show the s.e.mean.

Washed platelet aggregation induced by U46619 or by 5-HT (in the presence of 100 μM adrenaline) was also inhibited by suramin at concentrations of 200 or 400 μM, but this inhibition was not competitive and suramin caused a marked reduction in the maximal response to these agonists. ATP (100 μM) also reduced the maximal response to U46619, the effect being similar to that of 200 μM suramin, but did not affect aggregation induced by 5-HT (Figure 3).

Discussion

In this study we have shown that suramin acts as an antagonist of ADP-induced aggregation of human platelets in buffer but has no effect on platelets in plasma. The lack of effect of suramin in plasma was unlikely to be due to degradation of the antagonist by plasma enzymes, as varying the incubation time between 0 and 40 min even in the presence of the peptide inhibitor bacitracin did not affect the results. Indeed, in pharmacokinetic studies *in vivo* suramin has been shown not to be significantly metabolised but does bind non-specifically and

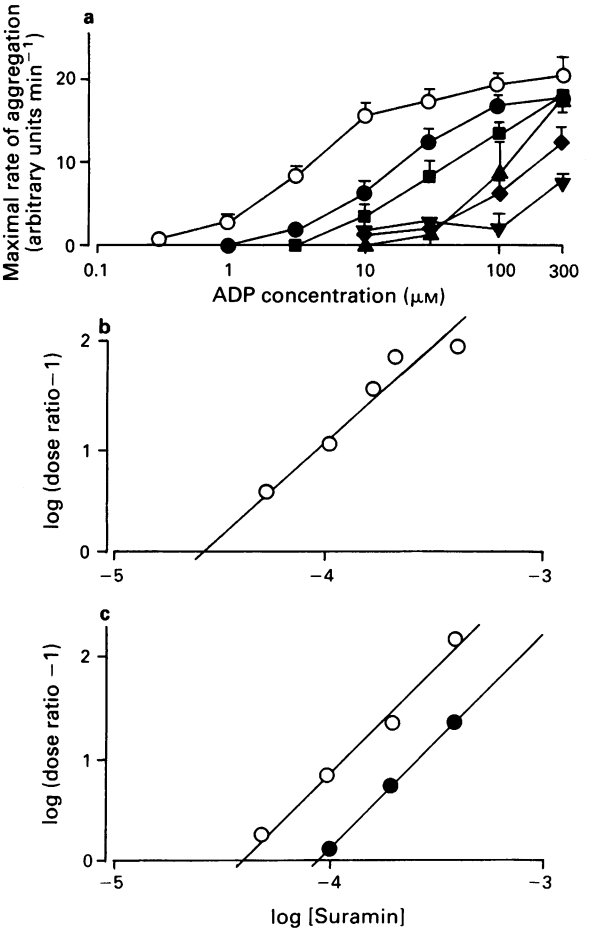

Figure 2 Effects of suramin on ADP-induced washed human platelet aggregation. (a) Aggregation induced by ADP alone (○) or in the presence of suramin at 50 μM (●), 100 μM (■), 150 μM (▲), 200 μM (◆) or 400 μM (▼). These results are the pooled data derived from a number of separate experiments on blood from several donors. Each point is the mean of at least 3 determinations, and the vertical bars show s.e.mean. (b) Schild plot of the data presented in (a). (c) Corresponding Schild plots obtained when platelets were preincubated with suramin for 10 (○) or 40 (●) min before addition of ADP.

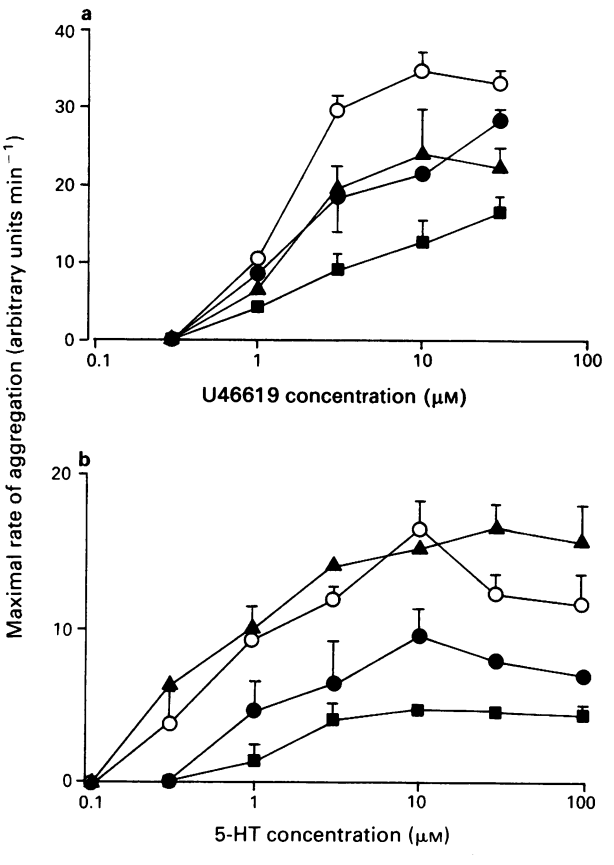

Figure 3 Effects of ATP and suramin on washed human platelet aggregation induced by (a) U46619 or (b) 5-hydroxytryptamine (5-HT) in the presence of adrenaline (100 μM), alone (○) or in the presence of suramin (200 μM (●) or 400 μM (■)) or ATP (100 μM) (▲). Each point is the mean of 3 determinations and the vertical bars show s.e.mean.

with high capacity to plasma proteins, greater than 99% of the drug being protein-bound (Collins *et al.*, 1986). It is likely therefore that the lack of effect of suramin on platelets in plasma is due to this extensive binding to plasma proteins.

In washed platelets, suramin antagonized the aggregation induced by ADP in an apparently competitive manner, causing parallel, dose-dependent shifts to the right of the log concentration-response curve to ADP. However, Schild analysis (Arunlakshana & Schild, 1959) of these data revealed that suramin was not acting as a pure competitive antagonist as the slope of the Schild plot was around 2. In a detailed study of the antagonism by suramin of the effect of ATP on P_{2X}-purinoceptors in the rabbit ear artery, Leff *et al.* (1990) also reported a Schild slope significantly greater than unity, and attributed this to slow equilibration of suramin with the receptors, as increasing the incubation times for low concentrations of suramin reduced the Schild slope to unity. From an analysis of the time-dependence of the inhibitory effect of suramin, Leff *et al.* (1990) concluded that very long incubation times, up to 220 min, were necessary to reach equilibrium, but in our study incubation times longer than 40 min could not be used because responses to ADP could not be reliably obtained. However, in platelets slow equilibration with the receptors is unlikely to be the explanation for the steep Schild plot, as increasing the incubation time with suramin from 0 to 40 min did not affect the slope of the Schild plot but merely reduced the potency of suramin, possibly due to slow binding of suramin to the BSA which was routinely present in the assay buffer. In addition, in a stirred suspension of platelets access of suramin to the receptors is likely to be more rapid and complete than in smooth muscle preparations, and long incubation times are not necessary to achieve Schild slopes of unity with other ADP antagonists in platelet-rich plasma (Cusack & Hourani, 1982).

Other possible explanations for a Schild slope greater than unity are that more than one molecule of the antagonist is binding to the receptor, that the antagonist is acting on a heterogeneous population of receptors, that it is acting non-competitively or that it is having multiple effects on the response (Kenakin, 1987). A Schild slope near 2 could imply that on average 2 molecules of suramin must bind to the receptor, but although from our results it is not possible to draw firm conclusions about the stoichiometry of the interaction, there is no evidence that any other ADP antagonist exhibits multiple binding interactions (Cusack & Hourani, 1982). The dimeric structure of suramin may, however, confer different binding characteristics from those of previously tested ADP antagonists, which are analogues of AMP or of ATP. The second possibility, that there is a heterogeneous population of ADP receptors on platelets, has been suggested by Colman *et al.* (1980; see also Colman 1990), although this suggestion is not consistent with results from antagonist studies (Cusack & Hourani, 1982) or from radioligand binding studies, in which only one site was detected (Macfarlane *et al.*, 1982; 1983; for discussion see Macfarlane, 1987; Hourani & Cusack, 1991). In any case, of the two receptor types proposed by Colman *et al.* (1980), only one was thought to mediate aggregation, the other being proposed to mediate the inhibition by ADP of adenylate cyclase, and as in our study we have investigated only aggregation this would not explain the steep Schild slope obtained for suramin. Although the third

possibility, that suramin could be acting non-competitively, cannot be ruled out, the log concentration-response curves to ADP were shifted to the right with no apparent depression of the maximal response, at least at concentrations of suramin (50–150 μM) for which full log concentration-response curves to ADP could be obtained. Suramin, however, clearly had non-specific effects in addition to its antagonism of ADP, as it non-competitively inhibited the aggregation induced by U46619 or by 5-HT, which act at thromboxane ('TP') and 5-HT$_2$ receptors respectively on platelets (Hourani & Cusack, 1991). Given its avid binding to plasma proteins, it is possible that at high concentrations, suramin may bind to fibrinogen and make it unavailable for aggregation, resulting in non-competitive inhibition. The fourth explanation for the steep Schild slope, that suramin is having multiple effects, is therefore the most likely. Although ATP at high concentrations also non-competitively inhibited aggregation induced by U46619, this is probably due to its inhibition of the effect of released ADP, as ATP did not inhibit aggregation induced by 5-HT, which is a weaker platelet aggregating agent than U46619 and does not induce release of stored nucleotides from platelets. Indeed, to achieve aggregations to 5-HT comparable with those induced by ADP and U46619, it was necessary to potentiate the effects of 5-HT with a fixed concentration of adrenaline, as either agonist alone did not induce reliable aggregations.

Although the pA$_2$ values for suramin we obtained in this study have to be treated with caution because of the steep slope of the Schild plot and consequent uncertainty as to the mechanism of action of suramin, they do correspond closely to those obtained in other studies. As an inhibitor of ADP-induced aggregation suramin had an apparent pA$_2$ value between 4.62 and 4.05 depending on the incubation time, which is close to the values ranging from 4.5 to 5.4 which have been reported for the inhibition of the effects of ATP on smooth muscle preparations and phaeochromocytoma cells (Hoyle *et al.*, 1990; Leff *et al.*, 1990; Von Kugelgen *et al.*, 1990; Inoue *et al.*, 1991). In those cases in which no pA$_2$ value has been reported, the concentrations which have shown an inhibitory effect against ATP are generally in the micromolar range, and are also therefore consistent with these values (Den Hertog *et al.*, 1989a, b; Hoiting *et al.*, 1990). The similarity of the apparent pA$_2$ values for suramin found for the inhibition of ADP-induced aggregation to that found for suramin for antagonism of ATP on other tissues, suggests that the platelet ADP receptor is indeed similar to P$_2$-purinoceptors elsewhere, and that suramin is unable to distinguish between the subtypes of P$_2$-purinoceptors. The concentrations of suramin required for antagonism at P$_2$-purinoceptors are also similar to the IC$_{50}$ or K_i values reported for inhibition by suramin of the various purine-binding enzymes, which are generally in the micromolar range (Wills & Wormall, 1950; Fortes *et al.*, 1973; Butler *et al.*, 1988; Calcaterra *et al.*, 1988; Moriyama & Nelson, 1988; Ono *et al.*, 1988; Mahoney *et al.*, 1990), suggesting that these binding sites may be structurally related.

In conclusion, our results have shown that suramin is an antagonist of the ADP receptor mediating aggregation of human platelets, although it is not effective in plasma, it is not specific for ADP and its antagonism is not simply competitive. The pA$_2$ values are consistent with values found for P$_2$-purinoceptors elsewhere, showing that although it is unique in that ATP is an antagonist rather than an agonist, the platelet ADP receptor is indeed a type of P$_2$-purinoceptor.

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