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COMPETITIVE INHIBITION BY ADENOSINE 5′-TRIPHOSPHATE OF THE ACTIONS ON HUMAN PLATELETS OF 2-CHLOROADENOSINE 5′-DIPHOSPHATE, 2-AZIDOADENOSINE 5′-DIPHOSPHATE AND 2-METHYLTHIOADENOSINE 5′-DIPHOSPHATE

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1 Adenosine 5′-diphosphate (ADP) induces human platelet aggregation and noncompetitively inhibits stimulated human platelet adenylate cyclase; these two effects are mediated by the same ADP receptor, at which adenosine 5′-triphosphate (ATP) is a competitive antagonist.

2 Two ADP analogues, 2-azidoadenosine 5′-diphosphate (2-azido-ADP) and 2-methylthioadenosine 5′-diphosphate (2-methylthio-ADP) have been reported to be more potent as inhibitors of adenylate cyclase than they are as aggregating agents, but no evidence has been presented that these actions are mediated solely by the ADP receptor.

3 We therefore tested the ability of ATP to inhibit the actions of these compounds and of another ADP analogue, 2-chloroadenosine 5′-diphosphate (2-chloro-ADP).

4 2-Chloro-ADP, 2-azido-ADP and 2-methylthio-ADP each induced aggregation and inhibited stimulated adenylate cyclase. Both of these actions were competitively inhibited by ATP (50 μM) with pA_2 values similar to those previously found for inhibition by ATP of these effects of ADP.

5 The reported greater potency of 2-azido-ADP and of 2-methylthio-ADP as inhibitors of adenylate cyclase than as aggregating agents is therefore due only to their greater efficacy for this effect, not to some extra actions elsewhere.

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; Haslam & Rosson, 1975). Some 2-substituted analogues of ADP, in particular 2-chloroadenosine 5′-diphosphate (2-chloro-ADP), 2-azidoadenosine 5′-diphosphate (2-azido-ADP) and 2-methylthioadenosine 5′-diphosphate (2-methylthio-ADP) are more potent than ADP as aggregating agents (Gough, Maguire & Michal, 1969; Gough, Maguire & Penglis, 1972; Cusack & Born, 1977). Two of these analogues, 2-azido-ADP and 2-methylthio-ADP, are considerably more potent as inhibitors of stimulated adenylate cyclase than they are as aggregating agents, and this was interpreted as suggesting that these two actions of ADP are mediated by two different ADP receptors at the platelet surface (Mills & Macfarlane, 1978; Macfarlane, Srivastava & Mills, 1979). However, using a range of structurally diverse competitive ADP antagonists, we have found a good correlation between the pA_2 values (the negative logarithms of the dissociation constants) for inhibition of ADP-induced aggregation and the pA_2 values for the inhibition of the effect of ADP on stimulated adenylate cyclase. This is strong evidence that these two actions of ADP on platelets are mediated by the same receptor (Cusack & Hourani, 1982b).

The greater potency of the two ADP analogues as inhibitors of stimulated adenylate cyclase than as aggregating agents is therefore likely to be due to a greater efficacy for inhibition of adenylate cyclase, rather than to a greater affinity for a separate 'adenylate cyclase receptor'. However, 2-azido-ADP is a photolysable analogue of ADP (Cusack & Born, 1977), and is therefore potentially capable of interacting irreversibly with the ADP receptor or with other membrane components and so its action might not be entirely straightforward. 2-Methylthio-ADP would not be expected to have any irreversible effect, but the action of the related compound 2-methylthioadenosine 5′-triphosphate (2-methylthio-ATP) is anomalous. Although adenosine 5′-

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triphosphate (ATP) is a competitive ADP antagonist (Macfarlane & Mills, 1975; Cusack & Hourani, 1982b), 2-methylthio-ATP is a specific but noncompetitive inhibitor of ADP-induced platelet aggregation (Cusack & Hourani, 1982a).

The possibility remains therefore that the greater potency of 2-azido-ADP and 2-methylthio-ADP as inhibitors of stimulated adenylate cyclase than as aggregating agents could be due to some extra effects, not simply to a reversible interaction with the platelet ADP receptor. We used ATP, a known competitive antagonist of both effects of ADP (Macfarlane & Mills, 1975; Cusack & Hourani, 1982b), to test whether these two ADP analogues were indeed acting solely at the ADP receptor. 2-Chloro-ADP was also included in this study, since its effects on adenylate cyclase have not been reported. In addition, 2-chloroadenosine 5'-triphosphate (2-chloro-ATP), like ATP, is a competitive inhibitor of both actions of ADP on human platelets (Cusack & Hourani, 1982b), and so the 2-chloro substituent appears not to introduce anomalous behaviour.

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) 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 (10 μl) containing an ADP analogue alone or an ADP analogue plus ATP (final ATP concentration 50 μM).

Measurement of platelet adenylate cyclase activity

Increases in levels of platelet adenosine 3',5'-cyclic monophosphate (cyclic AMP) were measured in PRP which 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 an ADP analogue alone or an ADP analogue plus ATP (final ATP concentration 50 μM), which contained prostaglandin E_1 (PGE_1) (final concentration 1 μM) (to stimulate adenylate cyclase) and papaverine hydrochloride (final concentration 2 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 sample was centrifuged and the cyclic AMP in the supernatant was purified by chromatography on AG50W-X8[H^+] ion exchange resin (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 lyophilised and [^{14}C]-cyclic AMP and [^{3}H]-cyclic AMP estimated by liquid scintillation counting.

Measurements of the stimulation of [^{14}C]-cyclic AMP formation 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

2-Chloroadenosine, ATP and papaverine hydrochloride were obtained from Sigma London. [U-^{14}C]-adenine and [2,8-^{3}H]-cyclic AMP were obtained from Amersham International. AG50W-X8[H^+] was obtained from BioRad Laboratories. PGE_1 was a generous gift from Dr J.E. Pike of the Upjohn Company in Kalamazoo, Michigan.

2-Chloroadenosine was converted to 2-azidoadenosine by treatment with hydrazine followed by nitrous acid (Schaeffer & Thomas, 1958), and to 2-methylthioadenosine by treatment with methanethiol (Maguire, Nobbs, Einstein & Middleton, 1971). 2-Chloro-ADP, 2-azido-ADP and 2-methylthio-ADP were synthesized by successive phosphorylation of 2-chloroadenosine (Gough *et al.*, 1969), 2-azidoadenosine (Cusack & Born, 1977) and 2-methylthioadenosine (Gough *et al.*, 1972) respectively.

ATP and the ADP analogues were purified immediately before use by ion exchange chromatography, and stock solutions were assayed before use by ultraviolet spectroscopy.

Calculation of pA_2 values

Log dose-response curves were constructed to each ADP analogue alone and in the presence of ATP, and a line was drawn through the linear region of each log dose-response curve. The statistical significance of the difference between the slopes of the lines drawn through each pair of log dose-response curves was tested using the method described by Tallarida & Murray (1981). For each ADP analogue the weighted mean of the slopes of the pair of lines was used to redraw a pair of parallel lines through the log dose-response curves, and the dose-ratio (DR) was calculated from the shift in these redrawn parallel lines. The pA_2 value was calculated from the relationship

$$pA_2 = -\log(\text{ATP concentration}/(DR - 1))$$

All lines were drawn by least squares linear regression analysis, and the calculations were performed by computer according to the method described by Tallarida & Jacob (1979).

Results

2-Chloro-ADP, 2-azido-ADP and 2-methylthio-ADP each induced human platelet aggregation (Figure 1) and inhibited PGE_1-stimulated adenylate cyclase (Figure 2). Both of these effects were competitively inhibited by ATP (50 μM) added simultaneously, which caused a parallel shift to the right of the log dose-response curves (Figures 1 and 2). In each case the slopes of the lines drawn through the linear regions of the log dose-response curves in the absence and presence of ATP were not significantly different ($P>0.05$). The pA_2 values of ATP for inhibition of aggregation induced by 2-chloro-ADP, 2-azido-ADP and 2-methylthio-ADP were 4.79,

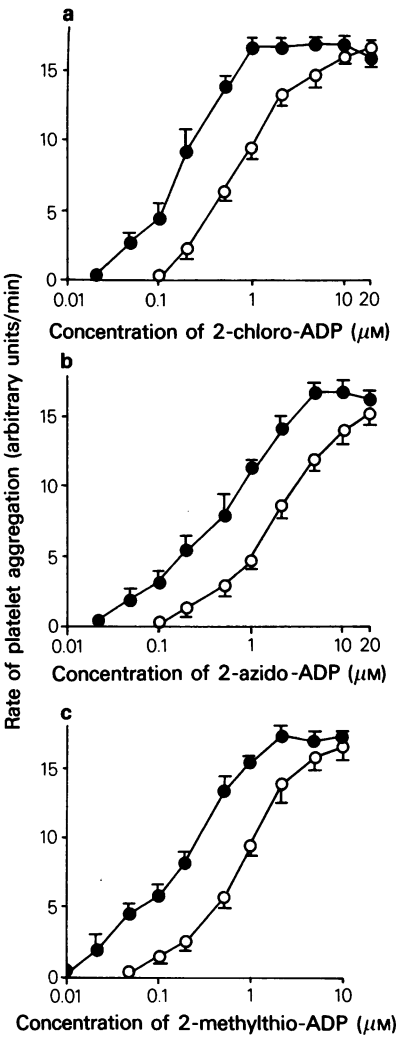

Figure 1 Human platelet aggregation induced by three ADP analogues alone (●) or in the presence (○) of ATP (50 μM). (a) 2-Chloro-ADP; (b) 2-azido-ADP; (c) 2-methylthio-ADP. Each point is the mean of at least three determinations. Vertical bars show the standard deviations.

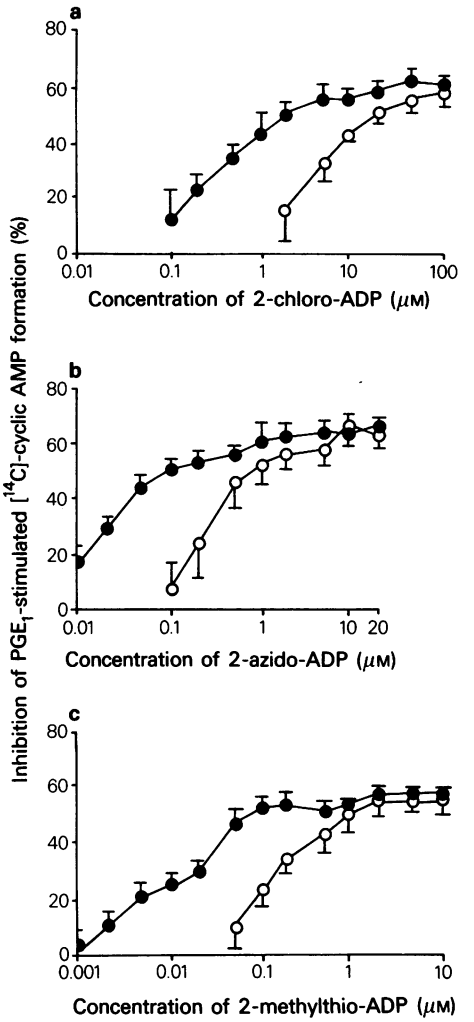

Figure 2 Inhibition of prostaglandin E_1 (1 μM)-stimulated formation of [^{14}C]-cyclic AMP in human platelets by three ADP analogues alone (●) or in the presence (○) of ATP (50 μM). (a) 2-Chloro-ADP; (b) 2-azido-ADP; (c) 2-methylthio-ADP. All samples contained papaverine (2 mM). Each point is the mean of at least three determinations. Vertical bars show the standard deviations.

4.87 and 4.83 respectively, and for inhibition of the effects of these analogues on PGE_1-stimulated adenylate cyclase were 5.35, 5.30 and 5.42 respectively.

Discussion

These results show that ATP competitively inhibited the effects of 2-chloro-ADP, 2-azido-ADP and 2-methylthio-ADP both as aggregating agents and as inhibitors of PGE_1-stimulated adenylate cyclase. The pA_2 values of ATP for inhibition of the effects of these analogues were very similar to the pA_2 values of ATP obtained for inhibition of ADP-induced aggregation (4.64) and for inhibition of the effect of ADP on PGE_1-stimulated adenylate cyclase (5.21) (Cusack & Hourani, 1982b). This suggests that the actions of these ADP analogues are mediated solely by a reversible interaction with the platelet ADP receptor, and that the reported greater potency of 2-azido-ADP and 2-methylthio-ADP as inhibitors of adenylate cyclase than as aggregating agents (Mills & Macfarlane, 1978; Macfarlane *et al.*, 1979) is due only to a greater efficacy for this action, rather than to some additional effect elsewhere.

Inhibition by ATP of aggregation induced by ADP is immediate in onset and appears to be independent of the time of preincubation (Macfarlane & Mills, 1975). In our present study, as in our previous study with competitive antagonists of ADP (Cusack & Hourani, 1982b), ATP was not preincubated with PRP to avoid any complications which could arise from its breakdown to ADP and adenosine.

The pA_2 values of ATP for inhibition of the three ADP analogues are somewhat lower for aggregation than they are for their effect on stimulated adenylate cyclase. This trend was also observed for inhibition of the actions of ADP by ATP (see above) and by the other ADP antagonist used in our prevous study, but the good correlation found there between the pA_2 values for inhibition of aggregation and inhibition of the effect of ADP on adenylate cyclase (Cusack & Hourani, 1982b) strongly suggests that this is due to the very different experimental techniques used to study aggregation and the inhibition of PGE_1-stimulated adenylate cyclase. In particular, in the aggregation studies the platelets are stirred and aggregate, whereas in the studies on adenylate cyclase activity the platelets are not stirred and therefore do not aggregate, and the experiments are performed in the presence of PGE_1 and a high concentration (2 mM) of papaverine.

The results presented here, taken together with the results from our previous study (Cusack & Hourani, 1982b) show that ADP, 2-chloro-ADP, 2-azido-ADP and 2-methylthio-ADP all induce human platelet aggregation and all inhibit stimulated human platelet adenylate cyclase by interacting reversibly with the same ADP receptor, at which ATP is a competitive antagonist.

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