Effects of photolysable 2-azido analogues of adenosine, AMP and ADP on human platelets

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The aggregating effect of ADP on human platelets and the inhibiting effect of adenosine are apparently mediated by different receptors on the cells' outer membranes. Photolysable azido-analogues of adenosine, AMP and ADP have been prepared so that the receptors for them can be labelled. 2-Azidoadenosine inhibited platelet aggregation by ADP more than adenosine itself and increased platelet cAMP as effectively as did adenosine or 2-chloroadenosine. 2-Azido-AMP inhibited aggregation much more effectively than AMP itself or than 2-chloro-AMP. 2-Azido-ADP was about five times more potent than ADP in causing aggregation. All these 2-azido derivatives were photolysable by irradiation at 365 nm which does not affect platelet functions.

INTRODUCTION

Human blood platelets are activated to change shape and subsequently to aggregate by various agents including ADP (see CIBA Foundation Symposium 1976) and these effects are inhibited by adenosine (Born & Cross 1963). There is indirect evidence that activation by ADP and inhibition by adenosine are mediated by specific receptors on the platelet membranes (Born 1965) and that the receptor for ADP is different from that for adenosine (Haslam & Rosson 1975).

Adenosine causes inhibition by activating adenylate cyclase in the platelet membrane and increasing the cAMP concentration in the cell (Mills & Smith 1971). Therefore, a receptor for adenosine appears to be linked to the adenylate cyclase complex. There is also at least one uptake system for adenosine, the properties of which suggest the presence of a high affinity receptor for adenosine (Sixma, Lips, Trieschnigg & Holmsen 1976). So far there is no evidence as to whether these receptors for adenosine are identical or different; no receptor has been isolated and characterized.

One way to begin with the isolation of adenosine and ADP receptors is to label them in situ with affinity analogues. Such analogues must, on the one hand, produce the same physiological responses and, on the other, be capable of binding irreversibly to the platelets under conditions in which they are not damaged.

In a previous paper (Cusack & Born 1976) we described 2-azidoadenosine, a photolysable analogue of adenosine, and showed that it inhibited the enzyme...
adenosine aminohydrolase (E.C. 3.5.4.4) which has a specific reaction site for adenosine. The inhibition was competitive before, and non-competitive after, the mixture of enzyme and inhibitor had been irradiated with u.v. light at a wave length of 254 or 365 nm.

We also indicated briefly that 2-azidoadenosine inhibited platelet aggregation like adenosine but more strongly. This paper describes the experiments which have established this, as well as others showing that the 2-azido derivative of AMP is also inhibitory whereas that of ADP is a more potent aggregating agent than ADP itself.

**Methods**

Adenosine, 2-chloroadenosine, adenosine 5'-monophosphate (AMP), adenosine 5'-diphosphate (ADP) and papaverine were obtained from Sigma Chemical Co., London. 2-Azidoadenosine was prepared from 2-chloroadenosine (Schaeffer & Thomas 1958). [U-14C]-adenine (286 mCi/mM) was obtained from the Radiochemical Centre, Amersham and purified by two-dimensional thin-layer chromatography (t.l.c.) on cellulose (Haslam & Rosson 1975).

2-Chloro-AMP was prepared by phosphorylation of 2-chloroadenosine with phosphoryl chloride (Gough, Maguire & Michal 1969); and 2-chloro-ADP by further phosphorylation with tri-n-butylamine orthophosphate and 1,1'-carbonyldiimidazole (Gough, Maguire & Penglis 1972). Similarly, crystalline 2-azido-AMP (m.p. 158–160 °C dec., \( \lambda_{\text{max}}^{\text{PH}} = 271, 309 \) nm, \(-N_3\) \( \nu_{\text{max}}^{\text{DMSO}} = 2155 \) cm\(^{-1}\), purine: total P: acid labile P: inorganic P, 1:1:0:0) was synthesized by phosphorylation of 2-azidoadenosine with phosphoryl chloride in trimethyl orthophosphate at 0°. Further phosphorylation with 1,1'-carbonyldiimidazole and tri-n-butylamine orthophosphate in dimethylformamide, followed by column chromatography on DEAE-cellulose [\( \text{HCO}_3^- \)] gave the ammonium salt of 2-azido-ADP (\( \lambda_{\text{max}}^{\text{PH}} = 271, 309 \) nm, \(-N_3\) \( \nu_{\text{max}}^{\text{DMSO}} = 2155 \) cm\(^{-1}\), purine: total P: acid labile P: inorganic P, 1:2:1:0).

2-Azido-AMP and 2-azido-ADP had identical chromatographic and electrophoretic migrations on cellulose and PEI-cellulose as AMP and ADP respectively.

Photolysis of the azido analogues was demonstrated by irradiation at 254 or 365 nm of the nucleotides (0.1 \( \mu \)l) together with AMP, ADP, 2-chloro-AMP and 2-chloro-ADP controls spotted on to cellulose or PEI-cellulose followed by ascending chromatography (2-propanol, 0.25 \( \text{m} \) ammonium hydrogen carbonate 2:1; \( n \)-butyric acid, \( \text{m} \) ammonium hydroxide, 7:3, 0.5; \( \text{m} \) lithium chloride), and also by following changes in their u.v. spectra (Cusack & Born 1976).

Venous blood from apparently healthy human volunteer donors was collected into plastic centrifuge tubes containing 0.1 vol. 3.8% sodium citrate as anticoagulant (see for example, Born & Hume 1967). The blood was centrifuged at 260 g for 20 min at room temperature and platelet-rich plasma was carefully separated. For quantifying aggregation photometrically (Michal & Born 1971), 1 ml samples of platelet-rich plasma were incubated at 37 °C for 3 min before addition of ADP or its analogues. Maximum velocities of aggregation during the
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first 30 s were plotted against log [aggregating agent] to give dose–response curves.

To quantify inhibitory activity, substances were incubated at 37 °C in 1 ml of platelet-rich plasma for 3 min before addition of ADP to initiate aggregation and its maximum velocity was measured as before. All determinations were done in triplicate.

Activation of adenylate cyclase was quantified in platelets which had been incubated with purified [14C]-adenine for 90 min by determining the intracellular adenosine-3',5'-cyclic monophosphate (cAMP) concentration in the presence of papaverine (2 mM) to inhibit phosphodiesterase (Haslam & Rosson 1975).

**Results**

When adenine nucleotides and their azido analogues were spotted on thin layer plates and irradiated at 254 nm for 5 min or at 365 nm for 60 min after chromatographic development only the azido analogues remained at the origin indicating that, like 2-azidoadenosine (Cusack & Born 1976), they had become covalently attached to the cellulose. The spectral changes produced in the azido nucleotides during u.v. irradiation at 365 nm were similar to the change found with 2-azidoadenosine (Cusack & Born 1976).

The aggregating potency of 2-azido-ADP was much greater than that of ADP and almost as great as that of 2-chloro-ADP (figure 1).

Inhibition of platelet aggregation by 2-azidoadenosine at 10^{-5} M was as great as with adenosine or with 2-chloroadenosine at the same concentration. At 10^{-4} M 2-azidoadenosine was more inhibitory than adenosine though less so than 2-chloroadenosine at the same concentration (figure 2.) The inhibitory effect of 2-azidoadenosine persisted for at least 60 min.

The concentration of cAMP in platelets was increased by 10^{-5} M 2-azidoadenosine after 2 min to about the same extent as by adenosine or 2-chloroadenosine at the same concentration (figure 3).

Platelet aggregation was inhibited by 2-azido-AMP at 10^{-5} M as effectively as by adenosine at that concentration, unlike AMP itself or 2-chloro-AMP which are only weak inhibitors.

**Discussion**

The results show that 2-azido-ADP caused human platelets to aggregate almost as effectively as 2-chloro-ADP which is about ten times more potent than ADP itself. This suggests that substitutions in the 2 position, increase the affinity of these derivatives for the ADP receptor; and as 2-azido-ADP is readily photolysable it has properties appropriate to an affinity label for that receptor.

The inhibitory effect of adenosine on platelet aggregation is preserved in those of its analogues which are substituted either in the N^6 position, as in N^6-phenyl-adenosine (Kikugawa, Iizuka & Ichino 1973) or in the C-2 position as in...
Figure 1. Aggregation of human platelets by ADP (○—○), 2-chloro-ADP (□—□), or 2-azido-ADP (△—△) under the conditions described in the text.

Figure 2. Inhibition by adenosine (△—△), 2-chloroadenosine (●—●), or 2-azidoadenosine (□—□) at 10 μM concentration of human platelets by ADP; control (saline only) (○—○).
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2-chloroadenosine (Born 1964). Like adenosine, 2-chloroadenosine raises the concentration of cAMP in platelets (Mills & Smith 1971).

The 2-azido analogue of adenosine could be expected to behave as a photolysable affinity label for adenosine-reactive sites and we showed earlier that this was so with the enzyme adenosine deaminase (Cusack & Born 1976). We have now confirmed that 2-azidoadenosine acts like adenosine in inhibiting the aggregation of human platelets and in increasing their concentration of cAMP. These similarities make it reasonable to conclude that 2-azidoadenosine acts on the same platelet membrane receptors as adenosine. Furthermore, it can be expected that irradiation of platelets at 365 nm, which appears not to damage them (Doery, Dickson & Hirsh 1973), in the presence of 2-azidoadenosine should cause it to be covalently bound to adenosine receptors.

Unlike AMP and 2-chloro-AMP (Born & Cross 1963; Gough et al. 1969), 2-azido-AMP inhibited platelet aggregation at $10^{-5}$ M about as effectively as adenosine. Inhibition by 2-azido-AMP could result either from competition at the ADP receptor or from an action like that of adenosine and its 2-substituted inhibitory analogues on an adenylate cyclase receptor to bring about a rise in cAMP; which of these effects accounts for the inhibition has still to be established.

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We have since found that $10^{-5}$ m AMP, 2-chloro-AMP and 2-azido-AMP increase platelet cAMP by similar amounts which are small compared to the increase caused by adenosine at the same concentration. This suggests that the inhibitory potency of 2-azido-AMP is due in part to competition with ADP for its receptor site.

References


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