Identification of a receptor for ADP on blood platelets by photoaffinity labelling

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The synthesis of a new analogue of ADP, 2-(*p*-azidophenyl)-ethylthioadenosine 5'-diphosphate (AzPET-ADP), is described. This compound contains a photolabile phenylazide group attached to the ADP molecule by a thioether link at the purine 2 position. It has been prepared in radioactive form with ^{32}P in the β -phosphate at a specific radioactivity of 100 mCi/ μmol . The reagent activated platelets, causing shape change and aggregation, with somewhat lower affinity than ADP. On photolysis the affinity was increased. The reagent also inhibited platelet adenylate cyclase stimulation by prostaglandin E_1 , with considerably higher affinity than ADP. On photolysis the affinity was decreased. AzPET-ADP competitively inhibited the binding of 2-methylthio[β - ^{32}P]ADP, a ligand for the receptor by which ADP causes inhibition of adenylate cyclase. In the dark, AzPET-[β - ^{32}P]ADP bound reversibly and with high affinity to a single population of sites similar in number to the sites that bind

2-methylthio[β - ^{32}P]ADP. Binding was inhibited by ADP and by ATP and by *p*-chloromercuribenzenesulphonic acid (pCMBS). On exposure to u.v. light in the presence of platelets, AzPET-[β - ^{32}P]ADP was incorporated covalently but non-specifically into several platelet proteins, although prominent intracellular proteins were not labelled. Specific labelling was confined to a single region of SDS/polyacrylamide gels, overlying but not comigrating with actin. Incorporation of radioactivity into this region was inhibited by ADP and by ATP as well as by ADP βS , ATP αS and pCMBS, but not by adenosine, GDP or AMP. Inhibition of AzPET-[β - ^{32}P]ADP incorporation was closely correlated with inhibition of equilibrium binding of 2-methylthio[β - ^{32}P]ADP. These results suggest that the labelled protein, which migrates with an apparent molecular mass of 43 kDa in reduced gels, is the receptor through which ADP inhibits adenylate cyclase.

INTRODUCTION

Blood platelets exposed to ADP at micromolar concentrations undergo a change in shape from flat discs to irregular spheres with numerous pseudopodia [1]. In the presence of fibrinogen and Ca^{2+} ions, they also become sticky and, if stirred, they aggregate [2–4]. During the aggregation process, arachidonic acid is released from membrane phospholipids by the action of phospholipases A_2 and C, and is converted into endoperoxides and thromboxane A_2 . ADP and serotonin (5-hydroxytryptamine) are released from the platelet dense storage granules, and adhesive proteins and mitogens are released from the α -granules [5]. These processes are regarded as being important contributors to the participation of platelets in the initial phase of wound healing, through the formation of the haemostatic plug, and also for their involvement in thrombotic processes.

From the time of its discovery as an aggregating agent [2,6], ADP has been assumed to act through a specific receptor mechanism. Highly negatively charged molecules such as nucleotides are unable to penetrate cell membranes without the intervention of specific transporters, and the concentration of ADP present within the cell is much higher than the extracellular concentration required to provoke the response. The platelet responds specifically to ADP; AMP and other natural nucleoside diphosphates are ineffective, and ATP is a specific and reversible competitive inhibitor [7]. Even small modifications of the ADP molecule in general give rise to compounds that have little activity, either as aggregating agents or as inhibitors. Exceptions

are a family of derivatives substituted in the 2- position of the adenine nucleus. 2-ChloroADP [8], 2-azidoADP (2N_3 -ADP) [9,10] and 2-methylthioADP (2MeS-ADP) [11,12] all cause aggregation and shape change with apparent affinities equal to or greater than that of ADP. Like ADP, these analogues also inhibit the response of platelet adenylate cyclase to stimulation by prostaglandins and other agents. This effect is also competitively inhibited by ATP [10,12,13].

Binding sites for 2-substituted ADP derivatives have been identified by equilibrium binding measurements with 2N_3 -ADP [10] and 2MeS-ADP [12], both compounds being labelled with ^{32}P in the β -phosphate. On average, a single platelet has 534 ± 44 binding sites for 2MeS-ADP [14], with a dissociation constant of 5–10 nM, determined by both equilibrium and kinetic measurements [12]. A similar number of binding sites was identified by equilibrium binding of 2N_3 -ADP, with a dissociation constant of 100 nM. Attempts to label this site covalently by photolysing 2N_3 -ADP in the presence of platelets were unsuccessful [10]. Only non-specific labelling occurred, which was not inhibited by the presence of excess ADP or ATP. We proposed that the analogue, when bound, is oriented in such a way that the nitrene radical generated in the 2- position, where even quite bulky substituents are tolerated, would be unable to contact the binding protein. Photoaffinity labelling of the site might be possible with a reagent in which the nitrene is generated at some distance from the purine ring. We have therefore prepared a reagent of this nature, 2-(*p*-azidophenyl)-ethylthioadenosine 5'-diphosphate (AzPET-ADP) and describe its use for covalent

Abbreviations used: AzPET-ADP, 2-(*p*-azidophenyl)ethylthioadenosine 5'-diphosphate; ADP βS , adenosine 5'-[β -thio]diphosphate; ATP αS , adenosine 5'-[α -thio]triphosphate; 2N_3 -ADP, 2-azidoADP; 2MeS-ADP, 2-methylthioADP; PRP, platelet-rich plasma; PGE $_1$, prostaglandin E_1 ; DMF, dimethylformamide; p-CMBS, *p*-chloromercuribenzenesulphonate.

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labelling of ADP-binding sites on platelets. A preliminary report has been published [15].

MATERIALS AND METHODS

Blood was obtained by venepuncture from normal volunteers of either sex after obtaining informed consent, and was collected into 0.1 vol. of 3.8% trisodium citrate anticoagulant. Platelet-rich plasma (PRP), obtained by centrifugation for 15 min at 950 *g* at room temperature and kept at room temperature, was warmed to 37 °C 5 min before use. Platelet aggregation was measured as the maximum rate of decrease in absorbance of a 0.8 ml sample of PRP in a Payton aggregometer with 6 mm diameter cuvettes at 37 °C and stirring at 1000 rev./min. Shape change was measured as the maximum extent of increase in absorbance of PRP diluted with 4 vol. of Tris-buffered saline (139 mM NaCl, 15 mM Tris/HCl, pH 7.4) containing 5 mM disodium EDTA. Aggregating agents were added in less than 10 μ l by syringe.

Stimulation of adenylate cyclase was measured in washed platelet suspensions previously labelled with tritiated adenine. PRP was incubated at 37 °C for 1 h with 0.2 nM [2,8- 3 H]adenine. The suspension was cooled on ice and 0.1 vol. of 0.1 M disodium EDTA was added. The platelets were sedimented by centrifugation at 4 °C for 10 min at 1350 *g* and resuspended in 0.3 vol. of Tris-buffered saline containing 1 mM EDTA. Samples (0.5 ml) of the labelled suspension were incubated with ADP or other nucleotides for 2 min before the addition of 2 μ M prostaglandin E₁ (PGE₁) and 0.2 mM RA233, a phosphodiesterase inhibitor. After 2 min, the reaction was stopped by the addition of 0.8 ml of cold aq. 15% trichloroacetic acid. After addition of

2500 c.p.m. of [14 C]cyclic AMP, cyclic AMP was isolated by sequential chromatography on Dowex 50 and alumina [16]. [3 H]Cyclic AMP, corrected for recovery of the 14 C standard, was expressed as a percentage of the total intracellular radioactivity.

Binding of labelled ADP analogues to platelets was studied in suspensions of platelets prepared by chilling PRP on ice and adding 10 mM EDTA and centrifuging as described above. Platelets from 60–70 ml of PRP were resuspended in 40 ml of Tris-buffered saline containing 1 mM EDTA and 1 mM KH₂PO₄. The centrifugation was repeated and the pellet suspended in 7–10 ml of the same medium, to give a concentration of 1×10^9 – 1.3×10^9 platelets/ml. This suspension was kept on ice for up to 2 h before use. For equilibrium-binding measurements, 200 μ l of the suspension was added to 50 μ l of Tris-buffered saline containing EDTA and KH₂PO₄ (final concentrations 10 mM and 5 mM respectively) and a range of concentrations of the labelled analogue, with or without competing nucleotides, and incubated at 37 °C for 5–10 min. Duplicate 100 μ l samples were taken into Sarstedt microsediment collection tubes containing a 50 μ l cushion of 20% (w/v) sucrose in Tris-buffered saline containing 10 mM EDTA, a layer of air being left between the cushion and the sample. The tubes were centrifuged in groups of 12 for 2 min at full speed in a Fisher model 59 swing-out microcentrifuge equipped with acrylic microtube holders. The tube contents were frozen with solid CO₂ and the tubes cut 2 mm above the pellet. Both pellet and supernatant were counted by Cerenkov radiation in a Beckman LS1800 scintillation counter. Non-specific binding was determined at two concentrations of the radioligand, in the presence of 0.8 mM ADP.

Photoaffinity labelling with 32 P-labelled AzPET-ADP was performed with platelets isolated as for equilibrium binding. Usually 10–20 samples of 0.8×10^8 – 1.2×10^8 platelets in 100–150 μ l were irradiated simultaneously in adjacent wells of a flat-bottomed plastic 96-well microtiter plate, cooled on ice. Unfiltered light from an American Optical high-pressure mercury lamp was directed down on the plate from a distance of 50 cm by reflection from a sheet of aluminium foil. After irradiation, the samples were transferred to microcentrifuge tubes with a 0.2 ml wash of cold Tris-buffered saline containing 10 mM EDTA, diluted with 1.0 ml of the same solution and centrifuged at 15000 *g* for 2 min in the cold-room. The pellets were suspended in 50 μ l of Tris-buffered saline containing 1 mM EDTA, and dissolved by addition of 50 μ l of 0.5 M Tris/HCl, pH 8.3, containing 4% (w/v) SDS, 12% (v/v) 2-mercaptoethanol and 20% (w/v) glycerol. The samples were loaded on a 2 mm-thick 15% Laemmli polyacrylamide slab gel [17] and subjected to electrophoresis for 16 h at 10 mA/gel and 5 h at 15 mA. Standard proteins for molecular mass determination were run at the same time. Gels were fixed in aq. 40% methanol/10% acetic acid, stained with Coomassie Brilliant Blue in the same solvent, destained and dried in air between cellophane sheets. Molecular masses were determined from a second-order polynomial fit to the relation between the log of the molecular mass of the standards and their mobility.

Autoradiographs were made on Dupont Chronex film at –70 °C in a cassette equipped with intensifying screens. After exposure for 2–10 days, relevant regions of the gel were cut out with scissors after first softening the brittle gel by exposing it to water vapour while clamped between supporting plastic mesh screens. Radioactivity was quantified by Cerenkov counting.

Synthesis of AzPET-ADP (5) (Scheme 1)

AMP was converted into the N¹-oxide with *m*-chloroperoxybenzoic acid. Ring cleavage with NaOH followed by con-

Scheme 1 Synthesis of AzPET-ADP

densation with carbon disulphide gave 2-thioAMP (3) [18,19]. AzPET-ADP was prepared by the condensation of (3) with 1-(2-bromoethyl)-4-azidobenzene (2) followed by phosphorylation of the monophosphate.

2-(4-Nitrophenyl)ethyl bromide was catalytically reduced to the corresponding amine (1) [20] which was diazotized and converted into the azido derivative (2) with NaN_3 . Condensation of (2) with 2-thioAMP gave the monophosphate (4), the structure of which was confirmed by microanalysis and u.v. and n.m.r. spectra. The monophosphate was converted into the imidazolate and allowed to react with tri-*n*-butylammonium phosphate [21]. The diphosphate (5) was purified by chromatography on DEAE-Sephadex. For the radioactive compound, ^{32}P was used in the final stage giving a compound labelled in the β position with an initial specific radioactivity of 100 mCi/ μmol .

To 1 g (5 mmol) of 1-(2-bromoethyl)-4-aminobenzene (1) in 10 ml of 10 M HCl stirred at 4 °C was added dropwise a solution of 400 mg of NaNO_2 in 1 ml of ice-cold water. The mixture was stirred for 20 min at 4 °C and an ice-cold solution of 360 mg of NaN_3 in 1 ml of water was added slowly and the mixture allowed to stand for 2 h at 4 °C. The precipitate was removed by filtration and the filtrate was extracted three times with 100 ml of chloroform. The combined extracts were washed with water, dried with Na_2SO_4 and evaporated *in vacuo*. The residue was dissolved in chloroform and applied to a silica gel column. Elution with *n*-hexane and evaporation *in vacuo* gave 0.6 g of compound 2 (53 %) as a chromatographically pure oil. I.r. 2096 cm^{-1} (N_3); ^1H n.m.r. (^{2}H)dimethyl sulphoxide) δ 31.1 (t, 2H, CH_2 -1), 3.71 (t, 2H, CH_2 -2), 7.06 (d, 2H, H-2 and H-6 aromatic), 7.32 (d, 2H, H-3 and H-5 aromatic). (Found: C, 42.6; H, 3.7; N, 18.2. Calc. for $\text{C}_8\text{H}_8\text{BrN}_3$: C, 42.5; H, 3.6; N, 18.6).

Synthesis of 2-thioAMP (3)

1- β -D-Ribofuranosyl-5-aminoimidazole-4-carboxamidoxime 5'-monophosphate was prepared by base-catalysed hydrolysis of AMP N^1 -oxide, and purified by chromatography on a column (1.5 cm $\times$ 25 cm) of Dowex AG50Wx4 (200 mesh, H^+ form), elution with water and evaporation to dryness *in vacuo*. Then 0.75 g (2.1 mmol) of this material was incubated with 5 ml of carbon disulphide in a sealed glass tube at 120 °C for 24 h. The reaction mixture was applied to a column (1.5 cm $\times$ 25 cm) of Dowex 1x2 (200 mesh, HCO_3^- form), washed with 100 ml of water and eluted with a 2-litre linear gradient of 0–1 M NH_4HCO_3 at 1.7 ml/min. The material eluted between 450 and 1050 ml was concentrated *in vacuo* and precipitated by addition of methanol, yielding 0.4 g (50 %) of 2-thioAMP (3) as a white solid. ^1H n.m.r. (^{2}H)dimethyl sulphoxide) δ 3.81 (m, 2H, CH_2 -5'), 4.01 (m, 1H, H-4'), 4.18 (m, 1H, H-3'), 4.51 (m, 1H, H-2'), 5.80 (d, J 6.4 Hz, 1H, H-1'), 8.35 (s, 1H, H-8); u.v._{max} nm ($\times 10^{-3}$) pH 1.5, 235 (13.4), 288 (17.0); pH 11.5, 241 (19.6), 282 (14.3).

Synthesis of 2-{[2-(4-azidophenyl)ethyl]thio}adenosine 5'-monophosphate (AzPET-AMP) (4)

To 0.34 g (0.9 mmol) of 3 in 27 ml (2.7 mmol) of aq. 0.1 M NaOH was added 0.75 g (3.3 mmol) of 1-(2-bromoethyl)-4-azidobenzene (2) in 30 ml of ethanol. The solution was allowed to stand at room temperature overnight, neutralized and evaporated *in vacuo*. The residue was dissolved in water and extracted twice with chloroform. The aqueous layer was evaporated to a small volume and 2 M HCl was added dropwise. The white precipitate was collected by filtration, washed with

cold water and dried *in vacuo* to give 0.24 g (50 %) of AzPET-AMP (4) as a chromatographically pure solid: m.p. 202–204 °C (decomp.); i.r. 2080 cm^{-1} (N_3); ^1H n.m.r. (^{2}H)dimethyl sulphoxide) δ 2.97 (t, 2H, CH_2 -2), 3.28 (t, 2H, CH_2 -1), 3.97 (m, 2H, CH_2 -5'), 4.10 (m, 1H, H-4'), 4.18 (m, 1H, H-3'), 4.60 (m, 1H, H-2'), 5.94 (d, J 6.4 Hz, 1H, H-1'), 7.07 (d, 2H, H-2 and H-6 aromatic), 7.40 (d, 2H, H-3 and H-5 aromatic), 8.26 (s, 1H, H-8); u.v._{max} nm ($\times 10^{-3}$) pH 1.5, 258 (13.5); pH 8.5, 238 (15.3) 252sh (14.1) 274sh (10.8); pH 11.5, 239 (16.2) 276sh (10.8). (Found: C, 39.5; H, 4.1; N, 20.3. Calc. for $\text{C}_{18}\text{H}_{21}\text{N}_8\text{O}_7\text{PS}$, H_2O : C, 39.8; H, 4.3; N, 20.6).

Synthesis of 2-{[2-(4-azidophenyl)ethyl]thio}adenosine 5'-diphosphate (AzPET-ADP) (5)

To 5.25 mg (10 μmol) of 4 in 50 μl of dry dimethylformamide (DMF) was added 2.4 μl (10 μmol) of tri-*n*-butylamine. The solvent was removed under a stream of nitrogen. *NN'*-Carbonyldi-imidazole [8 mg (50 μmol)] in 50 μl of dry DMF was added and the mixture left in the desiccator for 1 h. Methanol [3 μl (75 μmol)] was added and left for 30 min. Then 0.2 ml of 0.5 M tri-*n*-butylammonium phosphate in dry DMF was added and left for 3 h at room temperature. NaOH (10 ml; 1 M) was added and a white precipitate was removed by centrifugation. Solvent was removed by evaporation and the residue dissolved in 0.1 M NH_4HCO_3 (pH 7.8) and applied to a column (1 cm $\times$ 4 cm) of Sephadex A50 (HCO_3^- form). The column was washed with 30 ml of 0.1 M NH_4HCO_3 and eluted with a 200 ml linear gradient of 0.1–1 M NH_4HCO_3 at 15 ml/h. Fractions containing AzPET-ADP were pooled and lyophilized, yielding 3.3 μmol (33 %) estimated by the u.v. absorbance, assuming the same absorption coefficient as AzPET-AMP. (Found: C, 34.4; H, 3.7; N, 17.7. Calc. for $\text{C}_{18}\text{H}_{22}\text{N}_8\text{O}_{10}\text{P}_2\text{S}$, H_2O : C, 36.7; H, 3.9; N, 18.0).

For the preparation of AzPET-ADP labelled with ^{32}P , the scale of the synthesis was reduced. Typically, 5 μl of 10 mM tri-*n*-butylammonium phosphate (0.05 μmol) was added to 5 mCi of carrier-free $\text{H}_3^{32}\text{PO}_4$ in 5 μl of water in a plastic tube and dried under N_2 and then left in an evacuated desiccator over P_2O_5 at room temperature overnight. To this was added 5 μl of DMF containing 0.04 μmol of the imidazolate of AzPET-AMP prepared as described above. The reaction was allowed to proceed overnight at room temperature over P_2O_5 . The mixture was taken up in 10 μl of 0.1 M H_3PO_4 and applied to a 2.5 cm strip of Whatman no. 3MM paper and subjected to electrophoresis for 35 min at 56 V/cm in 50 mM sodium citrate buffer, pH 4.05, with cooling. A companion strip was run at the same time with markers of AMP, ADP and ATP, and a small sample of the reaction mixture obtained by rinsing out the reaction tube. After electrophoresis, the preparative strip was placed between acrylic plates and transferred to the deep-freeze. The companion strip was dried and autoradiographed to locate the product, and examined under u.v. light to locate the markers. The appropriate section of the preparative strip was cut out and eluted with 5 ml of water overnight at 4 °C. The specific radioactivity of the product was assumed to be the same as that of the phosphate from which it was prepared. Purity of the product was determined by h.p.l.c. on a Whatman Partisil SAX column with gradient elution in 10 mM–1 M KH_2PO_4 .

Reagents

[2,8- ^3H]Adenine (1.06 TBq/mmol), [8- ^{14}C]cyclic AMP and $\text{H}_3^{32}\text{PO}_4$ (carrier-free) were from ICN, Costa Mesa, CA, U.S.A. Dowex AG50, urea and acrylamide were from Bio-Rad,

Figure 1 U.v. spectrum of AzPET-ADP and its product after 2 h irradiation at 254 nm in the fluorimeter (pH 8.5)

Richmond, CA, U.S.A. PGE₁ was a gift from Dr. J. E. Pike, Upjohn Ltd., Kalamazoo, MI, U.S.A. The phosphodiesterase inhibitor RA233 {2,6-bis-(diethanolamino)-4-piperidinopyrimido[5,4-d]pyrimidine} was a gift from Dr. J. W. Bell, Boehringer Corp., Isleworth, Middx., U.K. Sp-ATP α S was from Boehringer, Mannheim, Germany. Other nucleotides and biochemicals were from Sigma, St. Louis, MO, U.S.A. Inorganic

chemicals were of analytical grade. Protein molecular-mass standards were from Gibco BRL, Gaithersburg, MD, U.S.A.

RESULTS

As expected, AzPET-ADP proved to be unstable when exposed to u.v. light at 254 nm. The shoulder at 252 nm in the absorption spectrum disappeared after exposure, and was replaced with a trough (Figure 1). There was no change in the spectrum on standing for 2 h at room temperature under normal (fluorescent) laboratory lighting. From the rate of disappearance of absorbance at 254 nm, the half-time for photolytic decomposition was estimated at pH 6.4, pH 7.4 and pH 8.4 to be 8.7, 11 and 12.3 s respectively.

AzPET-ADP caused shape change and aggregation when added to human platelets in diluted PRP and in PRP respectively (Figure 2). Dose-response relationships obtained with AzPET-ADP and AzPET-ADP after extensive exposure to u.v. light were parallel to those obtained with ADP, indicating that all three compounds have similar intrinsic activities for both effects. The azide was somewhat less potent than ADP (EC_{50} for ADP/ EC_{50} for AzPET-ADP = 0.1 for shape change and 0.3 for aggregation). The potency after photolysis was increased in each case to a value closer to that of ADP.

AzPET-ADP and its photolytic decomposition product both inhibited the stimulation of platelet adenylate cyclase activity by PGE₁, with dose-response curves that were also parallel to that for ADP, and with apparently similar intrinsic activities, when measured in a washed platelet suspension in the presence of EDTA. The azide proved 120-fold more potent in this system than ADP, and the effect of photolysis was to reduce its potency (Figure 3).

We measured the binding of AzPET-[β -³²P]ADP to washed human platelets by means of a modification of a previously described technique, replacing the silicone oil barrier with a 20 % sucrose cushion, with a reduction in non-displaceable binding due to trapping of extracellular fluid and an improved recovery of platelets in the pellet. AzPET-ADP bound reversibly to 437

Figure 2 Tracings of absorbance recordings of (a) shape change in platelets in PRP diluted with 4 vol. of Tris-buffered saline containing 5 mM EDTA, and (b) aggregation of human platelets in PRP, made with a Payton aggregometer at 37 °C and stirring at 1000 rev./min

ADP or AzPET-ADP were added at the arrows to give the final concentrations shown beside each trace.

Figure 3 Dose-response curves for ADP (●) and for AzPET-ADP (▲) and its photolytic product (△) for inhibition of PGE_1 -stimulated adenylate cyclase

...., Basal; —, no addition

Figure 4 Equilibrium binding of AzPET-ADP to washed platelets resuspended in Tris-buffered saline containing 1 mM EDTA and 1 mM KH_2PO_4

The Scatchard plot (inset) was used to calculate values for affinity ($K_D = 10.9$ nM) and for maximum binding ($B_{\text{max}} = 412$ molecules per platelet). These values were used with the non-specific binding (0.30%) to calculate the line drawn through the experimental points.

sites on each platelet (Figure 4). At half-maximal specific binding, which occurred at 10.9 nM ligand, the ratio of specific to non-specific binding measured in the presence of 0.8 mM ADP was 10:1. There was no evidence for more than one class of sites. The same sample of platelet suspension revealed 412 binding sites for 2MeS- $[\beta\text{-}^{32}\text{P}]\text{ADP}$, with an affinity of 13.8 nM. Non-radioactive AzPET-ADP inhibited the equilibrium binding of 2MeS- $[\beta\text{-}^{32}\text{P}]\text{ADP}$ to platelets in a dose-dependent manner.

When AzPET- $[\beta\text{-}^{32}\text{P}]\text{ADP}$ was exposed to u.v. light in the presence of washed human platelets, radioactivity was incorporated into several protein bands identified by autoradiography

Figure 5 SDS/PAGE (15% gels)

On the right is a representative lane stained for protein with Coomassie Brilliant Blue, with the positions and molecular masses in kDa of the protein standards indicated. On the left is an autoradiograph of eight lanes of the same gel showing patterns of incorporation of AzPET- $[\beta\text{-}^{32}\text{P}]\text{ADP}$ in the presence of: lane 1, ATP; lane 2, ADP; lane 3, AMP; lane 4, adenosine; lane 5, saline; lane 6, pCMBS; lane 7, GDP; lane 8, 8-bromo-ADP. Platelets (10^8) were incubated with 3×10^5 c.p.m. of AzPET- $[\beta\text{-}^{32}\text{P}]\text{ADP}$ at 83 nM in 120 μl , and photolysed for 3 min. Competing compounds were present at 0.8 mM.

of dried polyacrylamide slab gels, after electrophoresis in the Laemmli buffer system (Figure 5). The most prominently labelled band ran as an apparent doublet in the 41–45 kDa area, with an appearance reminiscent of a hamburger bun, with the beef corresponding to a lighter zone on the autoradiographs, overlying the prominent Coomassie Blue staining band due to platelet actin. This suggests that the labelled protein is a single band with the same mobility as actin, but not comigrating with it. We therefore refer to this band as a 43 kDa protein. Another prominently labelled band was seen at 68 kDa, probably due to albumin present as a contaminant of the platelet suspension. Labelling of this material was not affected by the presence of competing nucleotides.

Several compounds were tested for the ability to inhibit the incorporation of AzPET-ADP into the 43 kDa protein, and the results were compared with those obtained, under nearly identical conditions, with equilibrium binding of 2MeS-ADP. The specific activities of both ligands, which were prepared from the same batch of ^{32}P , were identical (17.5 c.p.m./fmol on the day of counting). The concentration of AzPET-ADP was 0.25 μM and the concentration of 2MeS-ADP was 0.32 μM . Competing compounds were included at 0.8 mM. Labelling of the 43 kDa material was blocked by ATP, ADP and *p*-chloromercuribenzenesulphonate (pCMBS). AMP, adenosine and GDP did not prevent labelling, and 8-bromo-ADP was partly effective. Similar effects of these compounds were seen when equilibrium binding of 2MeS-ADP was measured (Table 1).

The four nucleotides that were most effective in blocking incorporation of AzPET-ADP into 43 kDa protein were examined over a range of concentrations from 1 mM to 1 μM for the ability to interfere with equilibrium binding of 2MeS-ADP and covalent incorporation of AzPET-ADP, under closely similar conditions, and with samples of the same platelet suspension. AMP, which had little or no effect, was included as a control.

Table 1 Comparison between equilibrium binding of MeS- $[\beta\text{-}^{32}\text{P}]\text{ADP}$ and covalent incorporation of AzPET- $[\beta\text{-}^{32}\text{P}]\text{ADP}$ into 43 kDa protein in washed platelets from the same donor, in the presence of different additional compounds

All of the additions were at a final concentration of 0.8 mM.

Addition	2MeS-ADP binding (mol/platelet)	Incorporation of AzPET-ADP (c.p.m.)
Saline	793	924
Adenosine	855	770
ADP	132	108
AMP	775	639
ATP	118	98
pCMBS	134	217
GDP	717	663
8-Bromo-ADP	617	406

Figure 6 Inhibition by compounds related to ADP of (a) equilibrium binding of 2MeS- $[\beta\text{-}^{32}\text{P}]\text{ADP}$ and (b) incorporation of AzPET- $[\beta\text{-}^{32}\text{P}]\text{ADP}$ into the 43 kDa protein in washed platelets from the same donor

There were small differences between the potencies of these compounds as inhibitors of binding of the two ligands (Figure 6), but in general a strong correlation was seen between the two

effects. In contrast with the ability of the active compounds to block incorporation of radioactivity into the 43 kDa band, incorporation into the band at 64 kDa was not greatly affected except at the highest concentrations, where some effect of u.v. absorbance by the nucleotides on the efficiency of photolysis might be expected.

DISCUSSION

Our first attempt to identify a receptor for ADP on blood platelets by photoaffinity labelling using $2\text{N}_3\text{-ADP}$ [10] was unsuccessful. From the fact that small changes in the ADP molecule at any of several positions can cause a marked loss of affinity, judged by the potency of the analogue as either an agonist or an antagonist [22], it can be argued that the molecule is held rigidly in its binding site and makes multiple contacts with it. However, at the 2 position, quite bulky substituents, including a spin-labelled radical [18], can be attached without loss of potency as aggregating agents. It is therefore likely that the nitrene generated by the photolysis of $2\text{N}_3\text{-ADP}$ is unable to interact with the binding protein. We therefore constructed AzPET-ADP, in which the nitrene is produced at some distance from the purine ring. AzPET-ADP proved to have the characteristics expected of a useful probe. It is active as an aggregating agent and causes shape change, although with lower potency than ADP. It also inhibits the stimulation of adenylate cyclase by prostaglandins, and in this it is substantially more potent than ADP. Photolysis in water apparently leads to the formation of a single compound, with potency intermediate between those of ADP and AzPET-ADP, that is, more active than the azide as an aggregating agent and less active as an inhibitor of adenylate cyclase. It binds reversibly in the dark with high affinity to an equivalent number of binding sites to those that bind 2MeS-ADP, and compounds that block 2MeS-ADP binding also block the binding of AzPET-ADP.

When photolysed in the presence of platelets, radioactive AzPET-ADP was specifically incorporated into a single predominant band, of apparent molecular mass in the Laemmli system of 43 kDa, overlying, but not comigrating with, platelet actin. Photolytic incorporation was inhibited in dose-dependent fashion by ADP and ATP, and by their thio analogues, ADP β S and ATP α S. ADP β S and ATP α S are, respectively, a partial agonist and a competitive antagonist of the effects of ADP on platelets [23,24]. The potency of each of these nucleotides was essentially the same as its potency as an inhibitor of 2MeS-ADP binding. AMP and adenosine, which do not compete with the actions of ADP at its receptor on human platelets and do not inhibit 2MeS-ADP binding, were without effect on the incorporation of AzPET-ADP into the 43 kDa platelet protein. 8-Bromo-ADP is a rather weak inhibitor of aggregation induced by ADP [25] and partially blocked photolabelling by AzPET-ADP.

The photolytic incorporation of AzPET-ADP was surprisingly efficient. Although only 0.17% of the isotope was covalently bound, this represents an efficiency of 59% on the basis of the number of binding sites in the same platelet preparation identified by equilibrium binding of 2MeS-ADP. This suggests the presence of a nucleophilic group in the receptor within the range of the flexible side chain of AzPET-ADP. The presence of a reactive exofacial thiol group closely associated with the ADP-binding site was suggested by Robey et al. [18] who found that their nitroxyl spin-labelled ADP analogue was rapidly reduced when bound to platelets, and is supported by the effects of pCMBS. This non-penetrating mercurial thiol reagent inhibits the effect of ADP on adenylate cyclase, and blocks the binding of $2\text{N}_3\text{-ADP}$

[10] and 2MeS-ADP [12], although it does not inhibit ADP-induced shape change. This reagent also blocks the equilibrium binding of AzPET-ADP, as well as the covalent incorporation of AzPET-ADP on photolysis (Figure 5).

Arguing from the selective effect of pCMBS, we have suggested that the platelet may have two distinct receptors for ADP, one associated with shape change and aggregation and the other with inhibition of adenylate cyclase [12]. However, evidence from the comparison of several competitive inhibitors of both effects of ADP suggests that they are mediated by a single receptor [24]. The possibility that aggregation occurs as a consequence of adenylate cyclase inhibition has been advanced [26] but rejected. There is a wide discrepancy between the potencies of different ADP analogues as aggregating agents and inhibitors of adenylate cyclase [12], and adenosine analogues that inhibit adenylate cyclase by an effect on the intracellular P site, do not induce aggregation or potentiate other aggregating agents [27]. It is possible that a single ADP receptor may cause different actions by interaction with different guanine-nucleotide-binding proteins, as has recently been proposed for the platelet receptor for thrombin [28].

The binding site associated with the 43 kDa protein is clearly different from the nucleotide-binding sites identified by other investigators. The nucleotide affinity reagent *p*-fluoro-sulphonylbenzoyl adenosine, which non-competitively inhibits aggregation induced by ADP, labels a 100 kDa protein on the platelet membrane [29]. This reagent does not inhibit the effect of ADP on adenylate cyclase, nor does it block the binding of 2MeS-ADP [30]. However, unlike ATP [7], it also inhibits aggregation induced by adrenaline and by low concentrations of thrombin, suggesting that its effect is not restricted to interaction with the ADP-binding site. Photoinduced incorporation of ATP α S labels a site on glycoprotein IIb α (120 kDa, reduced) [31]. The relevance of this site to the ADP receptor is questioned by the observation that, in Glanzmann's thrombasthenia, platelet shape change and Ca²⁺ influx induced by ADP are normal, despite a profound defect in glycoproteins IIb and IIIa [32].

The procedure used in these experiments for the isolation of platelets, i.e. centrifugation in the cold in the presence of EDTA, leads to a considerable loss in their responsiveness to shape change and aggregation induced by ADP, but to full retention of their sensitivity to the inhibitory effect of ADP on adenylate cyclase. We therefore propose that the protein identified by photoaffinity labelling with AzPET-ADP under these conditions represents the ADP receptor through which ADP inhibits adenylate cyclase.

The discovery of an efficient mechanism for covalently labelling the platelet receptor identified by 2MeS-ADP binding offers an approach to its purification and characterization. The relevance

of this receptor to the physiological function of platelets is supported by the observation that in volunteers given the experimental antithrombotic drug, clopidogrel, which causes marked prolongation of the cutaneous bleeding time and specific inhibition of platelet aggregation by ADP, 2MeS-ADP binding is greatly diminished [14].

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