Binding of 2-Azidoadenosine [β-^{32}P]Diphosphate to the Receptor on Intact Human Blood Platelets Which Inhibits Adenylate Cyclase†

Donald E. Macfarlane,*,‡ David C. B. Mills, and Prem C. Srivastava§

ABSTRACT: [β-^{32}P]-2-Azido-ADP was prepared with a specific activity up to 10000 Ci/mol and used to investigate the ADP receptors on human blood platelets. 2-Azido-ADP was about 5-fold more potent than ADP as an aggregating agent and 10–20-fold more potent as an inhibitor of prostaglandin E_1 stimulated cyclic AMP accumulation in intact platelets. 2-Azido-ADP exists at neutral pH as a mixture of azide and tetrazole forms, the former being favored by low pH: the tetrazole isomer was 2-fold less potent than the azide isomer both as an aggregating agent and as an inhibitor of cyclic AMP accumulation, and the products of photolysis of 2-azido-ADP were also 10–20-fold weaker. Equilibrium binding measured by a centrifugal technique revealed about 500 noninteractive binding sites per platelet with an affinity close to the concentration for half-maximal inhibition of cyclic AMP accumulation. Binding, and dissociation in the presence of excess ADP, were complete in less than 30 s. Both ADP and ATP inhibited the binding of 2-azido-ADP competitively (half-maximal inhibition at 5–10 μM). UDP, GDP, IDP, and AMP were at least 100-fold less effective. Binding was blocked by *p*-mercuribenzenesulfonate, a thiol agent which blocks the action of ADP on the cyclase but does not block the ability of ADP to induce the platelet shape change. When washed platelets were photolyzed with [β-^{32}P]-2-azido-ADP, several macromolecules were covalently labeled, but none of this labeling was suppressed by ADP or ATP. It is concluded that the binding site is the receptor for ADP which regulates the adenylate cyclase, and because this site is blocked by *p*-mercuribenzenesulfonate—an agent which does not inhibit the shape change induced by ADP—it cannot be the receptor responsible for the induction of shape change.

Adenosine diphosphate (ADP) has two distinguishable effects on blood platelets. It induces the shape change leading to aggregation, and it inhibits the adenylate cyclase of intact platelets (Mills & Macfarlane, 1976) and broken cell preparations (Cooper & Rodbell, 1979; Mellwig & Jakobs, 1980). Both of these effects of ADP are antagonized by ATP (Macfarlane & Mills, 1974). We have recently shown that the kinetics of inhibition of the adenylate cyclase of intact platelets suggest a simple receptor mechanism closely coupled to the adenylate cyclase (Macfarlane & Mills, 1981). We also demonstrated that the thiol complexing agent *p*-mercuribenzenesulfonate blocks the inhibition by ADP of the adenylate cyclase but does not affect ADP's ability to induce the shape change, suggesting that two different ADP receptor mechanisms exist on platelets (Mills & Macfarlane, 1977).

The aggregation of platelets is inhibited when they accumulate intracellular cyclic AMP, which is synthesized by the membrane-bound adenylate cyclase. The action of this enzyme is regulated by a number of pharmacological agents, being stimulated by prostacyclin (Tateson et al., 1977), PGD_2 (Mills & Macfarlane, 1976), PGE_1, isoproterenol, and extracellular adenosine (Mills & Smith, 1971). The stimulation of cyclic AMP synthesis is in turn blocked by α-adrenergic agents, ADP (Cole et al., 1971; Mills & Smith, 1972), and high concentrations of adenosine probably acting within the cell (Haslam & Rosson, 1975). As in other cells, agonist regulation is modulated by guanine nucleotides (Krishna et al., 1972), and there is evidence for two guanine nucleotide sensitive sites on the platelet adenylate cyclase complex (Steer & Wood, 1979). Binding sites for prostacyclin (Siegl et al., 1979a), PGD_2 (Siegl et al., 1979b), and α-adrenergic antagonists (Newman et al., 1978; Macfarlane et al., 1981) have been demonstrated, which probably represent the pharmacological receptors.

In this paper we briefly describe the properties of a photoactivatable analogue of ADP, 2-azido-ADP, and its use in demonstrating binding sites on intact platelets with many of the properties of the receptor through which ADP inhibits platelet adenylate cyclase.

Experimental Procedures

The preparation of citrated platelet rich plasma, the tubidometric measurement of platelet aggregation and the accumulation of cyclic [^{14}C]AMP in [U-^{14}C]adenine-prelabeled platelets have been described in detail elsewhere (Macfarlane & Mills, 1981). For some experiments, platelets were separated from plasma either by centrifugation at 200*g* for 10 min at 23 °C after being treated with 0.5 μM PGI_2 and resuspension in a buffer containing 108 mM NaCl, 21 mM sodium citrate, 4 mM KCl, 1 mM $MgCl_2$, 2 mM $NaHCO_3$, 10 mM NaH_2PO_4, and 11 mM glucose, pH 7.4. For other experiments platelets were concentrated by two successive centrifugations into an albumin density gradient (Walsh et al., 1977), followed by Sepharose 2B exclusion chromatography.

Binding of [β-^{32}P]-2-azido-ADP was estimated by layering a 0.2–0.5-mL platelet suspension with the isotope and other additions as indicated over a mixture of silicone oils (Dow Corning No. 550 and No. 200; 1 cs) (Feinberg et al., 1974) adjusted to a density between that of platelets and plasma (about 1.028). The platelets were then centrifuged through the oil, and a sample of the supernatant was taken. The tube was then cut through the silicone layer, and the platelet pellet was solubilized in 100 μL of 10 g/100 mL sodium dodecyl sulfate for scintillation counting.

[β-^{32}P]-2-Azido-ADP. 2-Chloroadenosine (Sigma Chemical Co.) was treated with anhydrous hydrazine, and the solid resulting from repeated evaporation with water and 2-propanol was diazotized (Schaeffer & Thomas, 1958). The resulting crystals of 2-azidoadenosine were dried in vacuo at room

† From the Specialized Center of Thrombosis Research, Temple University Hospital, Philadelphia, Pennsylvania 19140. *Received July 8, 1981.* Supported by Grant HL 14217 from the National Institutes of Health.

‡ Correspondence should be addressed to this author at the Department of Medicine, University of Iowa Hospitals, Iowa City, IA 52242.

§ Present address: Cancer Research Center, Department of Chemistry, Brigham Young University, Provo, UT 84602.

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temperature: yield, 88%. Anal. Calcd for $C_{10}H_{12}N_8O_4$: C, 38.39; H, 3.93; N, 36.35. Found: C, 38.69; H, 4.07; N, 36.11; decomposed at 172–173 °C. This material was phosphorylated as described by Sowa & Ouchi (1975) and worked up by applying the reaction mixture, quenched with ice, onto a 4-mL bed column of Dowex 50 H^+. The column was eluted with 4 volumes of 0.1 M acetic acid, followed by 8 volumes of water which were collected and freeze-dried to yield (49%) 2-azido-AMP. The product migrated 22% faster than AMP as a single pale blue fluorescent spot on high-voltage electrophoresis (50 mM sodium citrate, pH 4.05). Anal. Calcd for $C_{10}H_{13}N_8O_7P \cdot 0.5H_2O$: C, 30.24; H, 3.55; N, 28.20; P, 7.80. Found: C, 30.11; H, 3.29; N, 28.12; P, 7.82.

2-Azido-ADP was prepared by the method of Hoard & Ott (1965) using 2-azido-AMP imidazolidate and the tri-*n*-butylammonium salt of orthophosphate or [^{32}P]orthophosphate as appropriate. Finally the reaction mixture was diluted with water or dilute NaOH and purified by electrophoresis. The fluorescent streak migrating 7% faster than ADP was cut out and eluted with 0.1 M acetic acid and freeze-dried. The yield was estimated by UV absorbance (typically 40%) by assuming that a molar extinction coefficient of 2-azido-ADP is the same as that of 2-azido-AMP (15 500 at 271 mM, 0.1 M HCl). The overall yield from 2-chloroadenosine was 17%. Radioactive 2-azido-ADP was typically prepared by using 0.3 μmol of 2-azido-AMP and 10 mCi of ^{32}P and 1 μmol orthophosphate in 7 μL of dimethylformamide. The yield of the product was estimated either by bioassay using platelets and nonradioactive 2-azido-ADP or by UV absorbance.

High-pressure liquid chromatography was performed with a Waters pump and a Whatman Partisil SAX column by using a linear gradient of $NH_3H_2PO_4$ from 10 to 700 mM over 10 min at 3 mL/min. A Shandon L24 apparatus was used for high-voltage electrophoresis at 22 °C on 3 MM paper with 50 mM sodium citrate buffer, pH 4.05. Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN. PGI_2 (prostacyclin) was obtained from Upjohn Co., Kalamazoo, MI. RA233 (2,6-bis(diethanolamino)-4-piperidinopyrimido[5,4-*d*]pyrimidine) was a gift from Boehringer Corp., Isleworth, UK.

Results

2-Azidopurines undergo rearrangement to tetrazole forms (Temple et al., 1966), and certain inconsistencies in our initial results led us to investigate the azide–tetrazole equilibrium with 2-azido-AMP in aqueous solutions. Figure 1b shows the UV spectra of 2-azido-AMP obtained when the compound was kept at 4 °C in 0.1 N HCl or 0.1 N NaOH for 18 h and then neutralized. A slow transition occurred after neutralization to an intermediate spectrum (imbedded dashed line). The half-time for this transition was about 20 min at 23 °C. The equilibrated spectrum of 2-azido-AMP was relatively unaffected by pH between 3 and 11, and reversible transitions occur with p*K*'s of approximately 2 and 12. These data were interpreted as showing that at neutral pH, nucleotides of 2-azidoadenosine exist as approximately equal parts of azide and tetrazole forms. These forms were separated by high-pressure liquid chromatography (performed with 2-azido-ADP) which showed that equilibration in alkali favored an earlier eluting peak and equilibration in acid favored a later peak. The form favored by acid was photolabile (quantum yield about 1 over the range 270–320 nm; the products were not identified) but not fluorescent, whereas the form favored by alkali was photostable and exhibited a weak fluorescence (λ_{max} 460 emission; λ_{max} 312 excitation; quantum yield about 2×10^{-3}). We assume that the acid-favored form is the azide, and the alkali

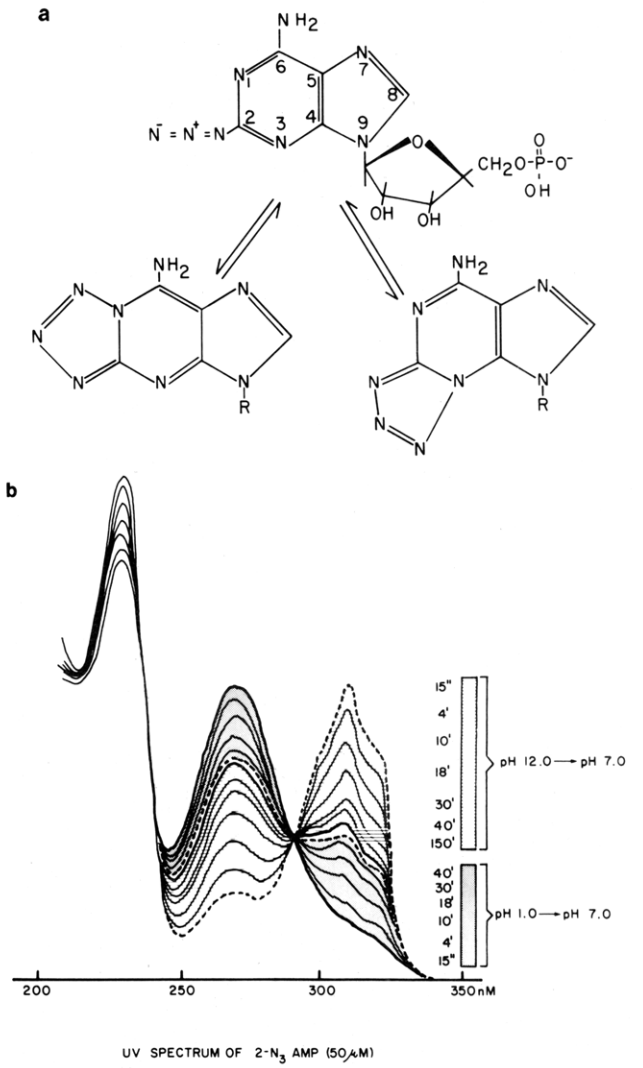

FIGURE 1: Azido–tetrazole transitions of 2-azido-AMP. (a) Structures of the two possible tetrazoles. (b) Changes in UV spectra after neutralization. Solutions of 2-azido-AMP at 1 mM were prepared in 0.1 N NaOH or 0.1 N HCl and equilibrated for 18 h. The samples were then neutralized by dilution to 50 μM in 0.1 M phosphate buffer, pH 7, and repeated spectra were obtained, beginning at the times indicated. Each spectrum took 75 s. Equilibration with acid is shown by stippling and with alkali by hatching.

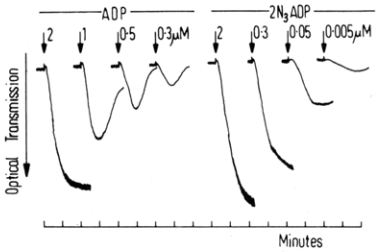

FIGURE 2: Aggregation of platelets by ADP and 2-azido-ADP. Samples of 0.8 mL of platelet-rich plasma in aggregometer cuvettes were warmed to 37 °C for 3 min before adding the nucleotides in 1–10 μL of Tris-buffered saline, pH 7.4. The final concentration of each agent (μM) is given above each trace.

form is one or a mixture of the two possible tetrazoles (Figure 1a). The acid form was also favored by increase in temperature (ΔS = 10.6 cal mol^{-1} deg^{-1}; ΔH = 2830 cal mol^{-1}).

Like ADP, 2-azido-ADP-induced platelet aggregation was preceded by a shape change. Figure 2 shows typical aggregation traces, and it can be seen that 2-azido-ADP is some 2–5-fold more potent than ADP. The traces differ in form

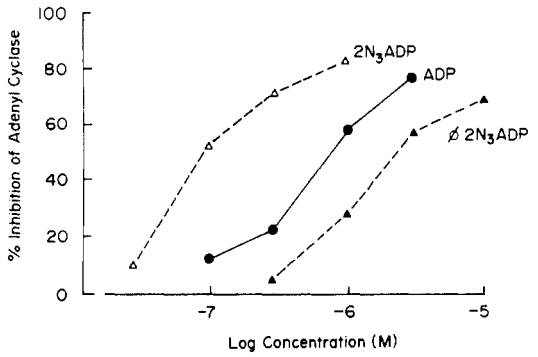

FIGURE 3: Inhibition of PGE_1-stimulated cyclic AMP accumulation by ADP, 2-azido-ADP (△), and the photolytic products of 2-azido-ADP (▲). Nucleotides were added in buffered saline, pH 7.4, simultaneously with PGE_1 (2 μM) and the phosphodiesterase inhibitor RA233 (0.2 mM) to platelet-rich plasma prelabeled by incubation for 1 h at 37 °C with 1.5 μM [^{14}C]adenine. After 1 min at 37 °C, perchloric acid containing a standard amount of cyclic [^{3}H]AMP was added to stop the reaction, and the content of cyclic [^{14}C]AMP determined. Results are expressed as the degree of inhibition of increase in cyclic AMP due to PGE_1 plus RA233 (1.20% of total intracellular radioactivity).

and show that aggregation induced by 2-azido-ADP is more delayed and has less tendency to reverse than aggregation induced by ADP. The products of photolysis of 2-azido-ADP were some 10-fold less potent than ADP. The acid-equilibrated (azide) form was 2-fold more active than the alkali-equilibrated (tetrazole) form (data not shown).

Also like ADP, 2-azido-ADP inhibited the stimulation of cyclic AMP accumulation in intact platelets exposed to PGE_1. Figure 3 shows a typical experiment, in which 2-azido-ADP was 8.4-fold more active than ADP, having half-maximal inhibition at about 100 nM. The products of photolysis were 3-fold less active than ADP.

The higher apparent affinity of platelets for 2-azido-ADP than for ADP enabled us to use [β-^{32}P]-2-azido-ADP as a radioligand to determine the number of binding sites on the platelet surface. Platelets were incubated with the ligand and then centrifuged through a layer of silicone oil. The radioactivity of the pellet and supernatant were then determined. Figure 4 gives the raw data of such an experiment, in which a concentration range of [β-^{32}P]-2-azido-ADP of 7.5–1500 nM was used. The shape of the curve is consistent with a simple saturable process superimposed on a nonsaturable one. The nonsaturable component was estimated from the slope of the upper portion of the curve and corresponds to the inclusion in the pellet of a volume of supernatant amounting to 2.3 fL/platelet. This component was subtracted from the raw data which were then calculated as molecules bound per platelet and are shown in the Scatchard plot (insert). This yields a line corresponding to 530 sites per platelet with a dissociation constant of 134 nM. The other insert shows a Hill plot of the data, with a slope of approximately unity, showing no cooperative interaction between sites. The curve drawn to the points of the raw data is computed as the sum of the saturable and nonsaturable processes. In other experiments we estimated the nonsaturable process by using excess ADP to block access to the receptor. When ATP was used, slightly lower values were found, and [^{14}C]insulin, [^{14}C]sucrose, and radioiodinated albumin all gave slightly different values for the trapped volume.

Washed platelets displayed a higher affinity for 2-azido-ADP. A typical result is shown in Figure 5. The raw data are shown (Figure 5a), together with a Scatchard plot of the data corrected for trapped volume which shows 450 sites per

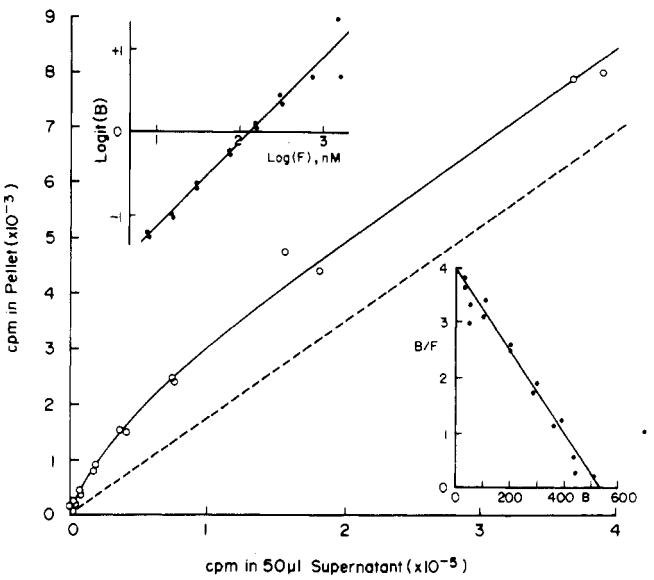

FIGURE 4: Binding of 2-azido-ADP to platelets in platelet-rich plasma. One milliliter of plasma containing 6.8×10^8 platelets was mixed with 50 μL of 0.1 M Na_2EDTA, pH 7.4, and an appropriate concentration of [β-^{32}P]-2-azido-ADP (2.5 Ci/mmol) between 7.5 and 1500 nM and layered over silicone oil (0.20 mL) in an Eppendorf tube and centrifuged. Counts in the pellets are plotted (ordinate) against counts in 50 μL, of supernatant (abscissa) without correction. The dashed line, drawn parallel to upper segment of the binding curve and through the origin, corresponds to nonsaturable binding equivalent to a trapped plasma volume of 2.3 fL/platelet. The lower insert shows a Scatchard plot of the data corrected for trapped plasma. B (abscissa) is the number of molecules of 2-azido-ADP bound per platelet and F is the free concentration of 2-azido-ADP (nM). The line drawn by eye corresponds to 530 molecules bound per platelet with a dissociation constant of 134 nM. The upper insert shows a Hill plot of the same data where logit B = log $[B/(B - 530)]$. The line is drawn with slope = 1.

platelet with a dissociated constant of 17.5 nM. Also shown on this figure is the effect of 100 μM p-mercuribenzenesulfonate, which was as effective in blocking specific binding as was 1 mM ADP.

ADP and ATP competed with 2-azido-ADP for binding with half-maximal displacement at about 8.3 μM for ADP (Figure 6a) and 10 μM ATP (Figure 6b), respectively. Other naturally occurring nucleotides including IDP, UDP, GDP, and AMP were at least 100-fold less effective in competing for binding (Figure 6b).

We attempted to determine the time course of binding of 2-azido-ADP to the platelet receptor and the rate of dissociation of the complex, but the earliest observation we could make was at about 30 s, and by this time dissociation in the presence of excess ADP and association were substantially complete.

We attempted to label the ADP receptor on washed platelets covalently with [β-^{32}P]-2-azido-ADP (25–500 nM) with or without an excess of ADP or ATP to block access to the receptor. So that the optical properties of the suspension could be equalized, adenine or adenosine was added in place of ADP or ATP in some experiments. The mixture was then irradiated with UV light from a Bausch & Lomb SP200 mercury source by using a 10 mM solution of AMP (20-mm thickness) to filter out wavelengths below 295 nm.

Irradiation was continued for periods of 30–120 s, until $^1/_3$–$^2/_3$ of the [β-^{32}P]-2-azido-ADP was photolyzed as revealed by high-voltage electrosphoresis. The platelets were sedimented through a layer of 10% metrizamide. The pellet was washed 3 times by resuspension, lysed with urea and 2% sodium dodecyl sulfate, and electrophoresed on a 3%–7.5% po-

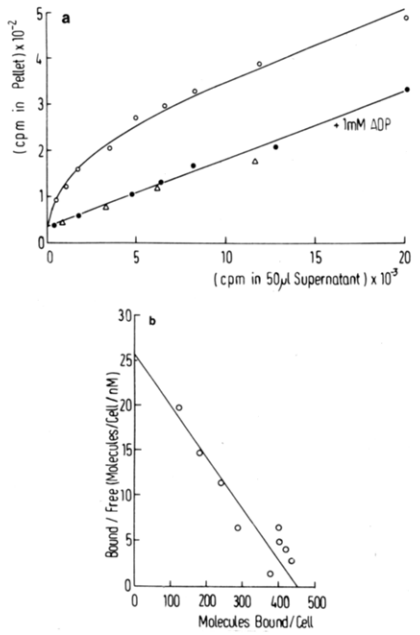

FIGURE 5: (a) Binding of [β-^{32}P]-2-azido-ADP to isolated platelets. Samples (0.5 mL) of the platelet suspension (7.78×10^8/mL) were added to appropriate concentrations of [β-^{32}P]-2-azido-ADP (0.7 Ci/mmol) equilibrated in 0.1 M HCl. After the mixture was mixed, 0.2 mL aliquots were layered on silicone in microsediment collection tubes and centrifuged. The figure shows uncorrected pellet radioactivity (ordinate) plotted against radioactivity in 50 μL of supernatant (O). In the presence of 1 mM ADP (●), the straight line was obtained passing through the counting background (35 cpm) corresponding to a trapped volume of 4.8 fL/platelet. The curve is calculated from the sum of this straight line and the counts expected from saturable binding to 450 sites per platelet with a dissociation constant of 17.5 nM (from b). Binding was also measured (△) to the platelet incubated for 3 min with 10^{-4} M *p*-mercuribenzenesulfonate. (b) Scatchard plot of data after correction for trapped volume and counter background.

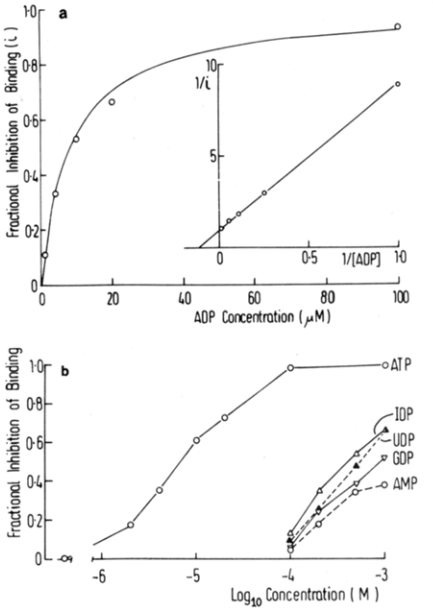

FIGURE 6: (a) Inhibition of binding of [β-^{32}P]-2-azido-ADP to platelets by ADP. Binding was measured in a suspension containing 1.01×10^9 isolated platelets per mL and with EDTA added to 4 mM immediately before centrifugation, using the technique described for Figure 5. The concentration of [β-^{32}P]-2-azido-ADP was 5 nM (acidified) and ADP was added to the range 1–1000 μM. The fractional inhibition of binding (O) is plotted (ordinate) against the ADP concentration, assuming that 1 mM ADP causes complete inhibition of binding. The curve is calculated from the concentration of ADP (8.3 μM) for half-maximal inhibition of binding, obtained from the intercept of the double-reciprocal plot (insert), and assuming that ADP inhibits binding with simple competitive kinetics. (b) Inhibition of binding of [β-^{32}P]-2-azido-ADP to platelets by other naturally occurring nucleotides.

lyacrylamide slab gel (Neville, 1971).

No radioactivity from [β-^{32}P]-2-azido-ADP was covalently bound to platelets unless the mixture was irradiated, nor was there incorporation when platelets were incubated with previously photolyzed [β-^{32}P]-2-azido-ADP. When the reagent was photolyzed with platelets, radioactivity was incorporated into at least four distinct fractions, two of which corresponded to PAS positive material (Figure 7). No protection from labeling any of these bands was seen when irradiation was carried out in the presence of an excess of either ADP or ATP (Figure 7), nor was there any evidence that the intensity of the labeling was saturable over the range of concentrations used (25–500 nM; data not shown).

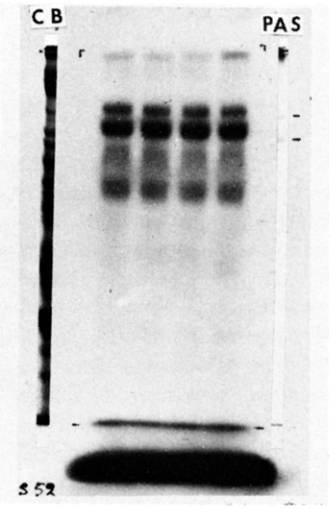

FIGURE 7: Autoradiograph of slab gel electrophoretogram of platelets irradiated with UV light in the presence of [β-^{32}P]-2-azido-ADP (50 nM). Strips of the same gel were stained with Coomassie blue (left) and with periodic acid–Schiff stain (right). The four channels of the autoradiograph correspond to samples photolyzed in the presence of the following additions (1 mM), from the left: adenine, ADP, adenosine, and ATP.

Discussion

Several groups of workers have determined the potency of analogues of ADP as aggregating agents. Changes in the pyrophophate bond, the ribose hydroxyls, substitution into the 8 position, replacement of the 6-amino group as in IDP, and attachment of oxygen to the 1 nitrogen all result in compounds having less than 1% of the activity of ADP (Mills & Macfarlane, 1976). Analogues with substituent in the 2 position such as 2-chloro-ADP, 2-thio-ADP, and 2-methylthio-ADP are powerful aggregating agents (Gough et al., 1972). For this reason we undertook the synthesis of radioactive 2-azido-ADP as a photoaffinity label. We were encouraged by the demonstration of Cusack and Born that 2-azidoadenosine, like aromatic azides, is photolabile and can photolabel the active site of adenosine deaminase (Cusack & Born, 1976) and that 2-azido-ADP induces platelet aggregation and inhibits the adenylate cyclase (Cusack & Born, 1977).

While the synthesis of 2-azido-ADP involves no novel chemistry, the propensity for azidopurines to rearrange to tetrazoles poses some problems. 2-Azido-AMP, which we used as a model compound, slowly equilibrates between the azide and tetrazole forms, the former being favored by a strongly acid environment and the latter by alkali. Equilibration at neutral pH results in a mixture of about equal parts of the two

forms, which makes purification and experimental design difficult. The tetrazole form is fluorescent, as might be expected for a three-ring heterocycle, and the azide form is highly photolabile at wavelengths up to 320 nm.

We confirmed that 2-azido-ADP aggregates platelets, being some 2–5-fold more powerful than ADP, and also that it is about 10-fold more potent than ADP as an inhibitor of the platelet adenylate cyclase.

We have previously attempted to demonstrate binding of ADP to intact platelets, but the number of bound counts was small compared with the number of counts passively trapped in the platelet pellet. The increased affinity of platelets for 2-azido-ADP improves this ratio, and data we present demonstrate about 500 noninteractive binding sites per platelet, with a dissociation constant close to the concentration for half-maximal inhibition of cyclic AMP accumulation.

This estimate of the number of binding sites is much lower than those obtained by other workers. Born & Feinberg (1975) used radioactive ADP in studies similar to ours. They obtained a curved Scatchard plot which they interpreted as showing 88 000 binding sites, and similar results have been found in Lips et al. (1980). Nachman & Ferris (1974) demonstrated slow divalent cation dependent binding of ADP to platelet membranes equivalent to about 100 000 sites per platelet. Subsequently it was shown that the binding activity was solubilized by freezing and thawing and was a protein of 160 000 moleuclar weight (Nachman, 1975). Adler & Handin (1979), using similar methods, found an ADP binding protein of 61 000 molecular weight which was distinguishable from nucleoside diphosphate kinase.

Two groups of workers have used affinity labeling techniques to characterize platelet ADP receptors, but neither the 2′,3′-dialdehyde derivative of ADP (Pearce & Scrutton, 1980) nor 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine (Bennett et al., 1978) have high affinity for the receptor that mediates the regulation of adenylate cyclase. An attempt to use a spin-label analogue of ADP was frustrated by rapid chemical reduction of the label (Robey et al., 1979).

The data we have presented make a persuasive case that the binding we observe is to the receptor responsible for the inhibition of the adenylate cyclase. First, half-maximal binding occurred at a concentration close to that causing half-maximal inhibition of the adenylate cyclase. Second, ADP and ATP inhibited binding in a simple competitive fashion. IDP, UDP, GDP, and AMP were much less effective in this regard, and the rank order of their effects was the same as that for their action on the adenylate cyclase (Macfarlane & Mills, 1981). Third, binding to and dissociation from the site was rapid, as is the action of ADP on the adenylate cyclase. Last, binding occurred in the absence of divalent cation and was blocked by prior incubation of platelets with the nonpenetrating sulfydryl reagent *p*-mercuribenzenesulfonate. We have previously shown that this reagent blocks the inhibitory effect of ADP on adenylate cyclase, without influencing the ability of ADP to induce shape change. This differential effect led us to postulate that the platelet has two different receptor mechanisms for detecting extracellular ADP (Mills & Macfarlane, 1977). We observed no binding of 2-azido-ADP to platelets treated with *p*-mercuribenzenesulfonate, suggesting that if the receptor mechanism for the induction of shape change requires the binding of ADP, the amount bound at equilibrium is too small to be detected by our methods.

We have shown that the inhibition of the adenylate cyclase by ADP is rapid and conforms to a simple saturable process (Macfarlane & Mills, 1981). On this basis we suggested that the ADP receptor and the enzyme are closely coupled, i.e., that there is relatively direct communication from receptor to enzyme. This in turn suggests the possibility of a one to one stoichiometry of receptors to adenylate cyclase units. Our figure of 500 or so ADP sites can be compared with 100 or so prostacyclin sites (Siegl et al., 1979a) and 200 α-adrenergic sites (Newman et al., 1978). Each of these numbers is subject to a variety of errors; in our case the accuracy depends on the radiochemical purity and on our estimation of specific activity. Further study is required before it can be concluded that platelets do not present the same number of ADP, adrenergic, and prostacyclin receptors.

The results we obtained on photolyzing 2-azido-ADP with platelets show that a stable covalent reaction did occur with several classes of proteins, two of which comigrated with major glycoproteins. We can be confident that this labeling was to macromolecules on the platelet surface, since the membrane is essentially impermeable to nucleotide diphosphates. The blocking studies do demonstrate that none of the observed bands represent the high-affinity binding site for 2-azido-ADP, and we therefore suppose that the incorporated radioactivity is due to a chance interaction of the generated nitrene with the major proteins presented on the platelet surface.

Specific labeling would occur only if photolysis generates a nitrene close enough to the receptor protein for interaction to occur; the orientation of 2-azido-ADP within the receptor pocket may be such as to favor a reaction between the nitrene and water in the surrounding medium. This could possibly be overcome by the use of a spacer arm between the photolabile group and the purine nucleus. The fact that a stable covalent bond is not formed with the high-affinity sites does not detract from our equilibrium binding studies.

Acknowledgments

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Binding of Calcium and Magnesium to Myosin in Skeletal Muscle Myofibrils†

Julian Borejdo*,‡ and Moshe M. Werber

ABSTRACT: Binding profiles for divalent cation to myosin have been obtained in myofibrils where myosin is assembled in arrays typical of the in vivo organization. Protection by Ca^{2+} and Mg^{2+} ions of the regions of myosin susceptible to chymotryptic attack provided the means to monitor metal ion binding. The effect of various concentrations of divalent cations on the chymotryptic digestion patterns was assessed by densitometry of Coomassie Blue stained gels obtained by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and by the rate of myofibrillar solubilization. The results indicate the presence of two classes of binding sites differing in affinity by 4 orders of magnitude. The fractional saturation of the high-affinity site associated with the 5,5′-dithiobis(2-nitrobenzoic acid)-dissociable light chains of myosin regulated the production of subfragment 1 of myosin. From the digestion profiles as a function of metal ion concentration, binding constants for Mg^{2+} and Ca^{2+} were obtained. The value for Mg^{2+} was 5.7×10^6 M^{-1}, which is about 1 order of magnitude higher than the most recently determined values for free myosin in solution; the value for Ca^{2+} was 6.3×10^6 M^{-1}. Binding to the low-affinity site regulated the production of the heavy meromyosin fragment and yielded association constants for Ca^{2+} and Mg^{2+} of 0.9×10^3 and 0.7×10^3 M^{-1}, respectively.

It is well-known that skeletal myosin has two protease-sensitive regions. Proteolysis of the first region leads to the formation of subfragment 1 (S-1)[1] of myosin and can be regulated in vitro by binding of divalent cations to the metal binding site associated with the Nbs_2-dissociable light chains (Nbs_2 light chains; Bálint et al., 1971). Saturation of these sites (two per mole of myosin) with either Ca^{2+} or Mg^{2+} inhibits formation of S-1 (Weeds & Pope, 1977). Proteolysis of the second protease-sensitive region of myosin leads to the formation of HMM. Until now, there were no reports suggesting that the rate of proteolysis at this site can be regulated by binding of divalent cations.

The protease-sensitive regions may play an essential part in the mechanism of muscle contraction (Huxley, 1969; Highsmith et al., 1977). However, it is not at all clear whether binding of divalent cations to these sites has any physiological relevance. In particular, it has been shown that binding of divalent cations to the Nbs_2 light chains does not affect the disposition or rotational mobility of myosin heads with respect to the thick filament backbone (Mendelson & Cheung, 1976; Sutoh & Harrington, 1977).

† From the Polymer Research Department, Weizmann Institute of Science, Rehovot 76 100, Israel (J.B.), and Medical Laboratories, Meir Hospital, Kfar Sava 44 281, Israel (M.M.W.). *Received July 6, 1981.* This work was supported by a grant from the U.S.-Israel Binational Science Foundation.
‡ Established Investigator of the American Heart Association.

[1] Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; HMM, heavy meromyosin; S-1, heavy meromyosin subfragment 1; S-2, heavy meromyosin subfragment 2; Nbs_2 light chain, 19 000 molecular weight subunit of myosin dissociated by treatment with 5,5′-dithiobis(2-nitrobenzoic acid); LMM, light meromyosin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; K_a, association constant; rms, root mean square; K_d, dissociation constant.

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