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Adenine nucleotide binding and photoincorporation in Glanzmann's thrombasthenia platelets

Nicholas J. Greco *, Narendra N. Tandon, Barrington Jackson, G.A. Jamieson

American Red Cross, Cell Biology Laboratory, 15601 Crabbs Branch Way, Rockville, MD 20855, USA

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Abstract

Adenosine 5'-(1-thiotriphosphate) (ATPαS) binds to about 25000 high affinity sites in platelets ($K_d \sim 3$ nM), competes fully in inhibiting the binding of ADP and, despite the absence of a specific photoactivatable substituent, is directly photoincorporated into a specific 18 kDa domain beginning at Tyr-198 in the α chain of glycoprotein IIb (GPIIbα) following ultraviolet irradiation of fresh unfixed platelets (Greco et al. (1991) J. Biol. Chem. 266, 13627–13633). 8-azido ATP has now been shown to have similar binding parameters (K_d 8 nM, 20000 sites/platelet) but, in this case, photoincorporation occurred equally in GPIIb and GPIIIa. To determine the possible function of GPIIbα in ADP-induced activation, platelets were isolated from two Glanzmann's thrombasthenia patients whose platelets contain ~6% of normal levels of GPIIb. ADP and ATPαS bound to intact, formaldehyde-fixed Glanzmann's platelets at high affinity sites with dissociation constants of ~30 nM and ~2 nM, respectively. Both nucleotides also bound to low affinity sites with dissociation constants of ~2 μM: these values are similar to those obtained with control platelets. ATPαS antagonized the shape ADP-induced shape change response of Glanzmann's platelets (EC_{50} 5 μM) indicating that it bound to the P_{2T} (ADP) receptor. However, photoincorporation was low (~7% of control) similar to their content of GPIIbα. These results show that ADP binding and photoincorporation are occurring at different sites on the platelet surface but suggest that the ADP binding site may be located in proximity to GPIIbα.

Keywords: ADP; Glanzmann's thrombosthenia; Photoaffinity labeling; Receptor; Binding

1. Introduction

Under appropriate conditions, ADP released from damaged cells and tissues can give rise to a primary wave of platelet activation, inhibition of adenylyl cyclase, and the release of ADP from platelet dense granules. Primary activation and release of ADP can also be induced by other agonists such as thrombin or collagen. In both cases, the released ADP can then react further with platelets to induce a secondary wave of activation. These effects in platelets are thought to be mediated by surface receptors that are designated P_{2T} in the purinergic receptor classification system since they recognize ADP as an agonist and ATP as an antagonist, although ATP acts as an agonist at other types of P_2 receptors [1]. Despite its importance in hemostasis, the platelet ADP receptor has not been characterized and it has not been resolved whether a single type of receptor mediates both activation and inhibition of elevated adenylyl cyclase or whether there are two distinct types of receptors one of which mediates activation and the other the inhibition of elevated adenylyl cyclase (for reviews, see [1–3]).

A difficulty in carrying out equilibrium nucleotide binding studies in fresh platelets is that steady state conditions cannot be achieved because of complications arising from nucleotide metabolism and secretion of ADP from stimulated platelets. To avoid these complications, we have used formaldehyde-fixed platelets as a test system to obtain steady state binding parameters for ADP and other nucleotides and then have compared these binding data with the potency of these compounds as examined in fresh platelets [4–7]. Both high and low affinity binding sites were found for the 20 nucleotides examined in the fixed platelet system, the highest affinity (K_d 3 ± 0.1 nM) being exhibited by adenosine 5'-(1-thiotriphosphate) (ATPαS). Using fresh unfixed platelets, we then showed that ATPαS

Abbreviations: ATPαS, adenosine 5'-(1-thiotriphosphate); 8-azido ATP, 8-azido-5'-adenosine triphosphate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gelelectrophoresis; GPIIb/IIIa, glycoprotein IIb/IIIa.

* Corresponding author. Fax: +1 (301) 7380794.

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could be photoincorporated into a single component by direct irradiation with ultraviolet light in the absence of any specific photolabile substituents in the nucleotide [7]. The site of incorporation was shown to be in the α-chain of glycoprotein IIb (GPIIb), specifically within an 18 kDa domain beginning at Tyr-198. We have also shown that binding is of relatively low specificity with regard to nucleoside structure and that a range of purine and pyrimidine nucleoside triphosphates yield similar binding parameters and degrees of photoincorporation [6]. An advantage of using the nucleoside triphosphates and thiotriphosphates is that they bind to platelets with high affinity and are antagonists of ADP-induced platelet activation so that interpretation of photoincorporation patterns is not complicated by membrane changes that might be associated with platelet activation.

Although these studies clearly established GPIIbα as the site of nucleotide photoincorporation, it was difficult to reconcile a role for GPIIbα as a possible ADP receptor with the fact that previous reports have indicated that Glanzmann's thrombasthenia platelets, which are markedly deficient in GPIIbα, show normal shape change and elevations in $[Ca^{2+}]_i$ in response to ADP [8,9]. Furthermore, platelet GPIIbα was not labelled by the photoaffinity analogue 2-(*p*-azidophenyl)ethylthioadenosine 5′-phosphate (AzPet-ADP) [10]. On the other hand, incorporation of GPIIb/IIIa into liposomes has suggested a role for this integrin in Ca^{2+} transport [11] and other studies have indicated decreased serotonin release, thromboxane B_2 synthesis and $[Ca^{2+}]_i$ changes in thrombasthenic platelets suggesting defects in ADP-induced responses [12,13].

In order to resolve these discrepancies and to evaluate the possible role of GPIIbα in ADP-induced platelet activation, we have examined ATPαS binding and its photoincorporation using Glanzmann's thrombasthenia platelets. Our results suggest that the ADP receptor is distinct from GPIIbα but may be located in sufficiently close proximity to it that bound ADP can be photoincorporated into the GPIIb/IIIa complex in normal platelets.

2. Methods

The source and purity of reagents used has been previously described [6,7]. Unlabeled 8-azido-5′-adenosine triphosphate (8-azido ATP) was obtained from Sigma and 8-azido-5′-adenosine [γ-^{32}P]triphosphate (5 Ci/mmol) of greater than 95% purity was from ICN Biomedicals (Costa Mesa, CA). All other radiolabeled compounds were from Dupont-New England Nuclear.

Normal human platelets were prepared from volunteer blood donations anticoagulated with citrate-phosphate-dextrose-adenine, as previously described [6]. Citrated whole blood from two patients with Type I Glanzmann's thrombasthenia (patient 1, NL; patient 2, MM) [14] was kindly provided by Dr. Margaret Johnson and Mr. Robert Abel, Christiana Hospital, Wilmington, DE and processed to gel filtered or washed platelets.

2.1. Binding studies

The filtration technique using a Brandel cell harvester for measuring steady state nucleotide binding parameters in fixed platelets has been previously described [4,7]. The ratio of bound/total nucleotide was kept at < 0.1 and binding was determined after 15 min incubation. As previously reported [5–7], platelets were treated with 4% paraformaldehyde (4° C, 48 h). In addition, we have obtained binding data with platelets fixed with 1% paraformaldehyde for 30 min at 4° C which are the conditions routinely used in flow cytometric evaluation of platelet antigens. Binding studies with 8-azido ATP were carried out in semi-darkness.

2.2. Shape change

Shape change of gel-filtered platelets ($1.5 \cdot 10^8$/ml) in the absence of added fibrinogen was evaluated in a total volume of 400 μl in a Payton Dual Channel aggregometer (Payton Associates, Buffalo, NY). Platelet rich plasma (5 ml) was applied to a 50 ml Sepharose 2B column equilibrated in Tyrode's-Hepes. The void volume was collected and the platelets tested for their ability to undergo shape change with ADP. Only isolated platelets that did not aggregate with 10 μM ADP alone (i.e., were free from plasma fibrinogen) were used for shape change experiments.

2.3. Photoaffinity labeling

In brief, unfixed washed platelets ($1 \cdot 10^8$) in the presence of 1 μg/ml PGE_1 and in the absence of bovine serum albumin were incubated for up to 5 min at 0° C with 85 nM [^{35}S]ATPαS [6,7], an amount equal to 30-times the K_d of high affinity ATPαS binding to fixed platelets [7], and were then irradiated at 360 μW/cm^2 (254 nm) for an additional 5 min at 0° C. Platelets were recovered by centrifugation ($12\,000 \times g$, 2 min) and solubilized in 1% Triton X-100 in a buffer of 50 mM Tris, pH 7.4 containing as protease inhibitors leupeptin (50 μg/ml), phenylmethylsulfonyl fluoride (2 mM) and benzamidine (2 mM). Insoluble material was removed by centrifugation ($217\,000 \times g$, 30 min) and the supernatant and insoluble (cytoskeleton-associated) fractions were separately analyzed by 7% SDS-PAGE and subsequent autoradiography.

A similar procedure was used to measure the photoincorporation of 8-azido [^{32}P]ATP (25–800 nM) into washed unfixed control platelets.

2.4. Protein blotting

Proteins were separated by 7% SDS-PAGE and electrophoretically transferred to 0.45 μm nitrocellulose in a

semidry Western blot apparatus (E and K Scientific Products, Saratoga, CA) (2.5 mA/cm^2 for 1 h) or in a Bio-Rad Trans Blot Apparatus (50 mA/13–16 h then 100 mA for 1–2 h). Unoccupied binding sites on the nitrocellulose were blocked (2 h, 22° C) with 2% bovine serum albumin in Tris-buffered saline (10 mM Tris, 150 mM NaCl, pH 7.5) before incubation (2 h, 22° C) with a polyclonal anti GPIIb/IIIa antiserum (1:1000 dilution) kindly provided by Dr. Leo Lin (Cetus Corporation). After extensive washing of the nitrocellulose membranes, ^{125}I-protein A (0.95 μCi) was added and the membranes incubated (1 h, 22° C) to visualize GPIIb. Protein A (Sigma) was iodinated (Iodobeads, Pierce, Rockford, IL) in phosphate-buffered saline (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.35) to a specific activity of 2 μCi/μg. Autoradiographs of photoaffinity labeling and Western blotting experiments were scanned using an LKB soft-laser densitometer.

3. Results

3.1. Effect of ATPαS on shape change

As previously reported [9], Glanzmann's thrombasthenia platelets showed a normal shape change response to 2.5 μM ADP. We showed that this response was antagonized by ATPαS (Fig. 1) with an IC_{50} of ~ 5 μM similar to that previously seen with normal platelets suggesting that ATPαS could be used for direct photoincorporation studies with Glanzmann's platelets [6,7]. We have confirmed the previous report [8] that Glanzmann's platelets show negligible serotonin secretion but a normal rate and extent of $[Ca^{2+}]_i$ increase in response to ADP as measured by fura-2 (data not shown).

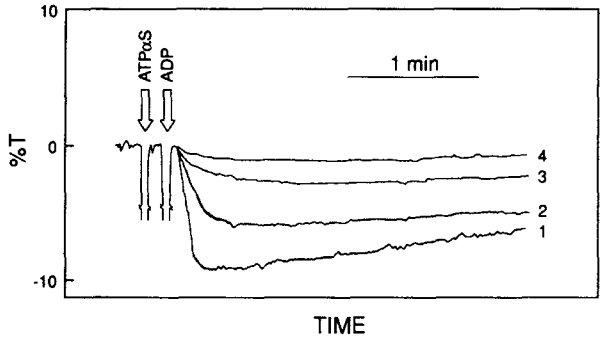

Fig. 1. Shape change in gel-filtered Glanzmann's platelets. Shape change response induced by 2.5 μM ADP (curve 1) was inhibited by increasing concentrations of ATPαS (curve 2, 2.5 μM; curve 3, 5 μM; and curve 4, 10 μM). These results were identical to results obtained using gel-filtered normal platelets in the absence of exogenous fibrinogen.

3.2. Binding studies

Homologous and heterologous 18 duplicate point binding studies with ADP and ATPαS using intact, fixed Glanzmann's platelets gave binding isotherms (not shown) and binding constants (Table 1) that were essentially identical with those found in the present work with two normal controls as well as with the numerous normal controls previously reported [6]. The high affinity binding constants for the two patients calculated from homologous binding isotherms using ADP were 35 nM and 24400 sites per platelet for patient 1 and 31 nM and 9300 sites per platelet for patient 2. Further studies with ATPαS using platelets from patient 2 gave 19 000–33 000 high affinity sites/platelet in both homologous and heterologous competition studies, similar to the values found in control platelets [6,7]. Although our mean value for the number of

Table 1
Homologous and heterologous binding parameters for Glanzmann's thrombasthenia platelets

		$K_{d,1}$	$K_{d,2}$	$K_{i,1}$	$K_{i,2}$	S1 (sites/plt)	S2 (sites/plt)
[^{3}H]ADP vs. ADP	control 1	20	380	–	–	27205	47037
	control 2	39	1900	–	–	7698	111478
	control [a]	29	5600	–	–	11202	132755
	patient 1	35	1800	–	–	24400	182300
	patient 2	31	360	–	–	9300	55400
[^{35}S]ATPαS vs. ATPαS	control 2	2.0	5500	–	–	25880	741552
	control 2	1.2	2400	–	–	22811	430223
	patient 2	1.4	1100	–	–	26500	297000
	patient 2	3.3	5100	–	–	33000	865000
[^{35}S]ATPαS vs. ADP	control 2	–	–	53	1300	28186	159826
	control 2	–	–	69	1800	32105	155863
	patient 2	–	–	30	646	19100	131800
	patient 2	–	–	100	5300	32300	65700
[^{3}H]ADP vs. 8-azido ATP (n = 3)	control	–	–	48 ± 22	757 ± 227	21000 ± 3600	113000 ± 35000
8-azido AT^{32}P vs. 8-azido ATP (n = 3)	control	8 ± 2	770 ± 390	–	–	21000 ± 5300	41000 ± 10500

Binding isotherms with eighteen duplicate points were carried out at B/T ratios < 0.1 and subjected to Scatchard analysis using the LIGAND program. To obtain this ratio the platelet concentration was 1.5 · 10^8/0.5 ml in experiments with [^{3}H]ADP and 3 · 10^7/0.5 ml in experiments with [^{35}S]ATPαS. Tracer concentrations were 10 nM for [^{3}H]ADP and 8-azido ATP and 2.5 nM for [^{35}S]ATPαS with increasing concentrations of the competing nucleotide. Values for K_d and K_i are given in nM.

[a] Platelets were fixed with 1% paraformaldehyde for 30 min at 4° C prior binding studies (n = 2).

ADP binding sites on control platelets is ~ 25 000 ($n = 14$) [6,7], the low value (9500) obtained with patient 2 is within biological variability and it may be noted that a similar value was found with control 2, although values of ~ 25 000 were found for ATPαS binding in both cases.

Because of concerns that the conditions of fixation might have deleterious effects on the platelet membrane and its nucleotide binding sites, we also carried out binding studies on platelets fixed under the conditions used for flow cytometric evaluation which are generally considered to preserve platelet membrane antigens; namely, 1% paraformaldehyde/4° C/30 min. Binding parameters obtained under these milder conditions were similar to those obtained with more prolonged fixation (Table 1).

Steady state binding parameters for the interaction of 8-azido ATP with platelets have not been previously reported. Using increasing concentrations of 8-azido ATP to compete in the binding of both [^{3}H]ADP and 8-azido [^{32}P]ATP to fixed normal platelets, the best fit line to the data points, as calculated by the LIGAND program [15], corresponded in each case to a two site model of high and low affinity sites with low non-specific binding (Fig. 2). Calculation of the binding parameters showed that 8-azido ATP bound to the same number of high affinity sites (20 000) and low affinity sites (114 000) as did ADP. Homologous binding isotherms (8-azido [^{32}P]ATP vs. 8-azido ATP) gave dissociation constants of 8 ± 2 nM at the high affinity site and 770 ± 390 nM at the low affinity site while heterologous binding isotherms ([^{3}H]ADP vs. 8-azido ATP) gave values of 48 ± 22 nM and 757 ± 227 nM, respectively, at those sites: these values are similar to those obtained with ADP itself (Table 1).

3.3. Photoaffinity labeling

With normal platelets, ATPαS was photoincorporated mainly into GPIIbα with much less incorporation into GPIIIa in both the Triton soluble (Fig. 3, lane 1) and Triton-insoluble (cytoskeleton-associated) fractions (Fig. 3, lane 2) but labeling was greatly reduced if the platelets were preincubated with EDTA (10 mM, 10 min, 37° C) to dissociate the GPIIb/IIIa complex (Fig. 3, lane 3). Following autoradiography for one month to obtain visible bands, Glanzmann's platelets gave a pattern of radiolabeling that would be expected in the absence of the specific labeling of GPIIb/IIIa and, as in control platelets labeling was much more evident in the Triton-soluble than in the Triton-insoluble fractions (Fig. 3, lanes 4 and 5). ATPαS was also photoincorporated into a band of molecular weight (125 kDa) slightly higher than that of GPIIbα (120 kDa) in platelets from both Glanzmann's patients (Fig. 3, lane 4) but was not observable in control platelets (Fig. 3, lane 1 or 2). We have confirmed the recent report [16] that 8-azido ADP is photoincorporated into GPIIb/IIIa in control platelets (Fig. 3, lane 6) and that ADP is fully competitive for its photoincorporation (data not shown).

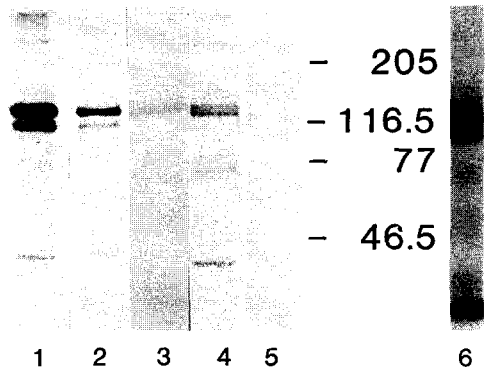

Fig. 3. Photoaffinity labeling: platelets ($1 \cdot 10^8$) were labeled with 85 nM [^{35}S]ATPαS and analyzed by 7% SDS-PAGE followed by autoradiography. Lane 1, control Triton-soluble fraction; lane 2, control Triton-insoluble (cytoskeleton-associated) fraction; lane 3, photoincorporation into control platelets previously incubated at 37° C, 10 min with EDTA (10 mM); lane 4, Glanzmann's Triton-soluble fraction; lane 5, Glanzmann's Triton-insoluble fraction; lane 6, control Triton-soluble fraction of normal platelets photoaffinity labeled with 300 nM 8-azido [^{32}P]ATP. Note the position in lane 4 of the higher molecular weight component that is photoaffinity labeled in Glanzmann's platelets but is not detectable in control platelets. Autoradiography was carried out for about one month to allow band detection in Glanzmann's samples although normally the labeled GPIIbα band in control platelets is visible within three days of exposure. Identical labeling patterns were observed using 140 nM [^{35}S]GTPαS.

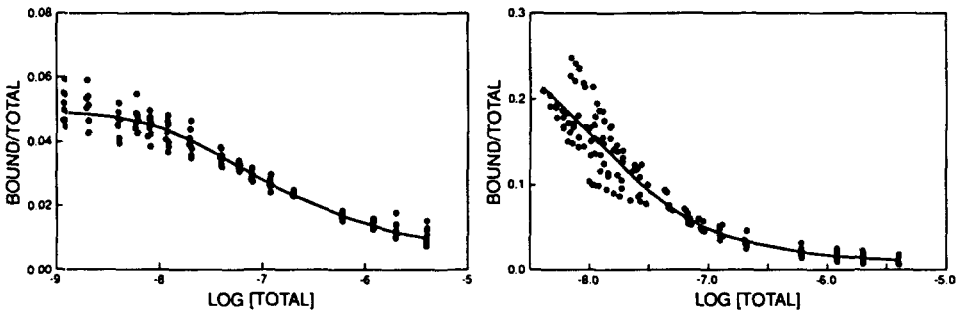

Fig. 2. Binding studies of 8-azido ATP with fixed normal platelets. These studies determined 18 nucleotide concentrations in triplicate and the data was subjected to Scatchard analysis using the LIGAND program. The platelet concentration was $1.5 \cdot 10^8$/0.5 ml for heterologous experiments of [^{3}H]ADP vs. 8-azido ATP (Fig. 2, left) and $3 \cdot 10^7$/0.5 ml for homologous experiments of 8-azido [^{32}P]ATP vs. 8-azido ATP (Fig. 2, right). Tracer concentrations were 10 nM for [^{3}H]ADP and 2.5 nM for 8-azido [^{32}P]ATP with increasing concentrations of competing 8-azido ATP.

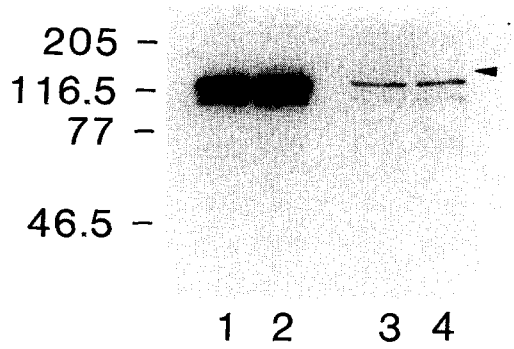

Fig. 4. Protein blots. The Triton X-100 soluble fraction and insoluble residue of platelets ($5 \cdot 10^7$) was subjected to SDS-PAGE and probed with polyclonal anti-GPIIb and ^{125}I-protein A. Lane 1, control Triton-soluble fraction; lane 2, control Triton-insoluble fraction; lane 3, Glanzmann's Triton-soluble fraction; lane 4, Glanzmann's Triton-insoluble fraction. The arrowhead marks the expected position of the 125 kDa component labeled with [^{35}S]ATPαS. Autoradiography was carried out for 2 days for controls and 1 month for Glanzmann's platelets.

3.4. Protein blotting

A higher molecular weight variant of GPIIb (122/128 kDa; red/unred) has been reported in a case of Glanzmann's thrombasthenia [17]. To determine whether this GPIIb variant corresponded to the higher molecular weight band detected in the photoincorporation studies with Glanzmann's platelets, protein blotting was carried out using a polyclonal anti GPIIb/IIIa antibody (Fig. 4). This clearly showed labeling of GPIIbα and GPIIIa in the Triton-soluble and Triton-insoluble fractions from both control (Fig. 4, lanes 1 and 2) and Glanzmann's platelets (Fig. 4, lanes 3 and 4) but there was not a αGPIIb/IIIa immunoreactive band at the position expected for the 125 kDa component (arrow).

4. Discussion

Attempts to affinity label the platelet ADP receptor have been largely unsuccessful. Multiple bands were labeled with 2-azidoadenosine [β-^{32}P]diphosphate but the labeling was not suppressed by ADP nor by ATP indicating the observed labeling was non-specific [18]. A 100 kDa component has been labeled with the adenosine analogue 5′-*p*-fluorosulfonyl adenosine [2] but evidence that this is a P_{2T} receptor is inconclusive. More recently, a 43 kDa component of the platelet membrane has been shown to be labeled with the new photoaffinity analogue AzPet-ADP [10]. This observation is of particular interest since ADP has been shown to activate a G protein-coupled receptor [19] and this is the molecular weight range expected for such receptors. P_{2x} receptors which react with ATP but which are pharmacologically distinct from P_{2T} receptors have also been described although not fully characterized [20].

As noted in the Introduction, there is evidence both for and against a role for GPIIb/IIIa in ADP-mediated platelet activation. In order to obtain further information on this point we have compared control and Glanzmann's thrombasthenia platelets with regard to adenine nucleotide binding and direct photoincorporation.

Previous studies have reported that isolated membranes from Glanzmann's thrombasthenia platelets bind ADP similarly to membranes from normal controls with either one or two binding sites and dissociation constants in the micromolar range [21,22]. Our present results, using intact fixed platelets, also demonstrate similarity of binding between Glanzmann's thrombasthenia platelets and normal platelets but in this case we find two sites: a low affinity site with a dissociation constant (~ 2 μM) similar to that previously reported [21,22] and, in addition, another site with an affinity (35 nM) about two orders of magnitude greater and similar to that which we found in our other studies on nucleotide binding [6,7]. These differences could arise if formaldehyde fixation enhances the affinity for ADP at the binding site which would appear to be unlikely or, alternatively, if membrane isolation results in the loss of the high affinity binding component. This question cannot be resolved by use of intact unfixed platelets since we have shown that it is not possible to obtain steady state binding conditions in that case due to ligand degradation [4]. Moreover, we have found that changing the conditions of fixation to those known to retain immunologic function in flow cytometric experiments does not affect the nucleotide binding parameters.

About 90% of the ADP bound to fixed platelets would be present at the high affinity binding site [4]. This binding site has several similarities to what would be expected for an ADP receptor in fresh, unfixed platelets: namely, (i) the dissociation constant at the high affinity binding site (35 nM) is in the concentration range (~ 50 nM) at which ADP-induced shape change is observed; (ii) nucleotides known to be antagonists for ADP-induced activation compete in ADP binding; (iii) *p*-chloromercuribenzoate and 5′-*p*-fluorosulfonylbenzoyl adenosine were shown to inhibit the binding of ADP to fixed platelets and are known to inhibit ADP-induced responses of fresh platelets; (iv) 8-bromo ADP was shown to inhibit ADP binding to fixed platelets and was then shown also to inhibit ADP-induced activation of fresh platelets [4].

ADP analogues have strict structural requirements in order to act as platelet agonists and only substitution in the C2 position is tolerated. However, previous studies from this laboratory have shown that a wide variety of purine and pyrimidine analogues of ADP can act as antagonists of ADP-induced activation and can bind to intact fixed platelets with affinities similar to those of ADP itself [4,6,7]. Several of these nucleoside triphosphates have been directly photoincorporated into unfixed platelets following ultraviolet irradiation, specifically into a domain in GPIIbα beginning at Tyr-198 and with little incorporation into GPIIIa [6,7].

We have shown that ATPαS competes in the ADP-induced shape change of Glanzmann's thrombasthenia and is thus a valid probe for photoincorporation studies. Although there is the possibility of nucleotide metabolism when using fresh platelets, and this is the rationalization for using fixed platelets in the binding studies, possible metabolic effects have been minimized by carrying out photoincorporation studies at ice-bath temperatures. Moreover, ADPαS formed as the metabolic product of ATPαS would also bind to the ADP binding site, albeit with lower affinity and could, therefore, also be photoincorporated into GPIIbα. In the absence of GPIIb/IIIa, only one additional photolabeled bands is observed at 125 kDa. We do not know the relevance of this photoaffinity labeled band but it does not react with anti GPIIb/IIIa antibodies. With Glanzmann's platelets the amount of direct photoincorporation of [^{35}S]ATPαS into GPIIbα was about 7% of that in normal controls, which is in agreement with the amount of GPIIb/IIIa detected in these two patients [14]. In contrast, the number of high affinity ADP binding sites found in Glanzmann's thrombasthenia platelets ($\sim$ 25 000) are quantitatively identical to those found in normal platelets (22 300 $\pm$ 4100). These numbers agree reasonably well with the number of GPIIb/IIIa complexes found from the binding of fibrinogen to stimulated platelets (14 000–38 000) [23–25] although lower than the approximately 40 000 sites reported for the binding of anti-GPIIb monoclonal antibodies [26,27]. These numbers may suggest a stoichiometric ratio between the number of high affinity ADP binding sites and the number of GPIIb/IIIa complexes on the surface of normal platelets.

Based on the inhibitory effects of 8-bromo ADP on platelet activation [4], the photoactivatable analogue 8-azido ATP has recently been shown to be photoincorporated almost equally into GPIIb and GPIIIa and the specificity of labeling was examined by irradiation in the presence of added 1 mM ATP [16]. As we have previously emphasized [6,7], careful controls are required to ensure that any observed decrease in photoincorporation is due to competition and not to a reduced degree of activation due to absorption of ultraviolet light by the increased concentration of total nucleotide: in general, this means that the incident ultraviolet light irradiating the control must be passed through a filter comprising a petri dish containing the competing nucleotide in the same concentration as that present in the experimental sample.

In the present studies, we have established that 8-azido ATP has similar binding parameters to ADP at the high and low affinity sites in control platelets, we have confirmed that it is incorporated approximately equally into GPIIb and GPIIIa and we have shown that this incorporation is inhibited by ADP when precautions are taken to ensure equal irradiation as outlined above.

Direct photoincorporation of purine nucleotides probably arises from photoactivation of the C8 position and, with ATPαS, appears to be selective for photolabeling GPIIbα [7]. However, when an azido group is present in the C8 position to act as a linker, photolabeling is essentially equal for GPIIbα and GPIIIa. These studies suggest that precise configurational relationships between GPIIb, GPIIIa and the platelet nucleotide binding site are required for photoincorporation and are consistent with our observations that dissociation of the GPIIb/IIIa complex with EDTA results in decreased photoincorporation into both components.

With platelets, the ribose phosphate side chain appears to be an important determinant of binding affinity since ATP binds equally with ADP while ATPαS binds with about 10-fold greater affinity. The requirements for the structure of the nucleoside base appear, however, to be relatively permissive and the 5′-(1-thiotriphosphates) of guanosine (GTPαS), adenosine (ATPαS), cytidine (CTPαS) and uridine (UTPαS) all show comparable binding parameters in fixed platelets and comparable ability to inhibit ADP-induced shape change and to be photoincorporated into GPIIbα in fresh unfixed platelets [6]. In the present experiments, we have found similar patterns of photoincorporation with Glanzmann's platelets using either ATPαS or GTPαS. These results suggest that ADP, or other nucleotides, may bind in platelets through their ribose phosphate moieties at an as-yet-unidentified binding site. Since ADP would be in its extended *anti* configuration, the domain most distal to the ribose phosphate binding moiety would be the C8 position which is thought to be the position that is activated when nucleotides are irradiated with ultraviolet light [28]. Under normal conditions, GPIIbα may be in sufficient proximity to this extended nucleotide configuration that it could become covalently labeled through the C8 position following photoactivation of the nucleotide. Similar crosslinking of approximated domains in skeletal myosin fragment 1 with a bifunctional ATP analogue has been reported [29].

These experiments support the hypothesis that the ADP binding site of platelets is distinct from GPIIbα but suggest that the two may be in sufficiently close proximity that ADP could interact with both under conditions of photoaffinity labeling with ADP or its analogues. This is consistent with the report that Ca^{2+} influx in response to ADP occurs predominantly via a channel closely adjacent to the GPIIb/IIIa complex [8]. The nature of this important receptor remains to be elucidated.

Acknowledgements

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