Inhibition of ADP-induced Platelet Responses by Covalent Modification of Aggregin, a Putative ADP Receptor, by 8-(4-Bromo-2,3-dioxobutylthio)ADP*

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ADP is an important platelet agonist which initiates platelet shape change, aggregation, exposure of fibrinogen receptors, and calcium mobilization. Because of the limitations of previously used affinity analogs and photo-labelling studies as well as controversies surrounding the identity of an ADP receptor on platelets, we have used an affinity label capable of alkylating a putative exofacial receptor on platelets. We now report that 8-(4-bromo-2,3-dioxobutylthio)adenosine-5′-diphosphate (8-BDB-TADP), which is an analog of the natural ligand ADP, blocked ADP-induced platelet shape change, aggregation, exposure of fibrinogen-binding sites, secretion, and calcium mobilization. Following modification by 8-BDB-TADP, the rates of aggregation of platelets induced by thrombin, a calcium ionophore (A23187) or a stimulator of protein kinase C (phorbol myristate acetate) were minimally affected. However, the 8-BDB-TADP-modified platelets exhibited decreased rates of aggregation in response to ADP, as well as collagen and a thromboxane mimetic (U46619), both of which partially require ADP. Autoradiograms of the gels obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of solubilized platelets modified by either [β-35S]8-BDB-TADP, or 8-BDB-TADP and NaB[3H]4 showed the presence of a single radiolabeled protein band at 100 kDa. The intensity of this band was reduced when platelets were preincubated with ADP, ATP, and 8-BDB-TADP prior to labeling by the radioactive 8-BDB-TADP. The results show that 8-BDB-TADP selectively and covalently labeled aggregin (100 kDa), a putative ADP receptor, resulting in a loss of ADP-induced platelet responses.

Although ADP (Fig. 1A) is one of the earliest known agonists for platelet activation (1, 2), the identity of the receptor is still uncertain. ADP receptors on platelets and magakaryocytes are unique (3); they constitute a subtype P2T of a class of P2 puri-
nergic receptors where ADP is the strongest agonist and ATP is an antagonist (4). The presence of a P2T receptor on human erythroleukemia cells has recently been demonstrated (5). Previous work from our laboratory demonstrated that 5′-p-fluorosulfonfylbenzoyladenosine (FSBA)1 (Fig. 1B), an ADP affinity label, blocked ADP-induced platelet shape change (6), aggregation, and exposure of fibrinogen-binding sites (7) with concomitant covalent modification of a single surface protein, aggregin (100 kDa), an ADP receptor on platelet surface (8, 9). Covalent modification of platelets by FSBA was shown to block platelet aggregation induced by U46619 (a thromboxane mimetic) (10) and collagen (11), suggesting that aggregation induced by these agonists, in part, depends on interaction of ADP with aggregin. Other investigators have proposed different candidates for a putative ADP receptor on platelet surface. Greco et al. (12) suggested that there is an ADP-binding site on platelet glyco-
protein IIb (GP IIb) based on results obtained by photolabeling of platelets by [35S]adenosine-5′-(1-thiotriphosphate), ATP-
α-S. The same group has recently proposed that an ADP-bind-
ing site does not reside on GP IIb but is in close proximity to it (13). However, only ATP (but not ADP analogs) inhibits binding of fibrinogen to its receptor, the GP IIb-IIIa complex (12, 14). Patients with thromboasplenia, lacking this receptor, exhibit normal ADP-induced platelet shape change and mobilization of intracellular Ca2+ (15). Although 2-azido-ADP (2-N3-ADP) was shown to block ADP-induced aggregation of platelets (16), no covalently labeled ADP-binding protein was detected by gel electrophoresis. Photolabeling by 2-(p-azidophenyl)-ethylthio-
adenosine-5′-diphosphate (AzPET-ADP) was shown to label several proteins on the platelet surface (17). The fact that labeling of one of these proteins (43 kDa) was reduced in the presence of ADP led the authors to conclude that the 43-kDa protein might be an ADP receptor on platelet surface. Although photoaffinity labeling has provided useful information concerning the structure-function relationship of both pur-
urified enzymes (18) and functional proteins in intact cellular systems (14, 19), it suffers from a number of drawbacks. The nature of the chemical reaction and the product(s) formed during “photoaffinity” labeling (13, 17) remain uncertain. Pho-
toaffinity labeling often yields multiple labeling patterns, thus complicating the process of identification of labeled proteins (13, 17). UV radiation can mimic the action of small ligands in activating signal transduction pathways that activate and regu-
late mammalian cell functions (20–22).

FSBA previously used to probe the ADP receptor also has cer-
tain limitations. Although FSBA contains a carbonyl group in a position sterically equivalent to the α-phosphoryl group of

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§The abbreviations used are: FSBA, 5′-p-fluorosulfonfylbenzoyladenosine; 8-BDB-TADP, 8-(4-bromo-2,3-dioxobutylthio)adenosine-5′-diphosphate; 2-N3-ADP, 2-azidoadenosine-5′-diphosphate; AzPET-ADP, 2-(p-azidophenyl)ethylthioadenosine-5′-diphosphate; ATPα-S, adenosine-5′-(1-thiotriphosphate); A23187, a calcium ionophore; U46619, 9,11-dideoxy-9α,11α-methanoepoxy prostaglandin F2; PMA, phorbol-12-myristate-13-acetate; p-APMSF, p-aminophenylmethylsulfonyl fluoride; GPIIb, glycoprotein IIb; GPIIIa, glycoprotein IIIa; PAGE, polyacrylamide gel electrophoresis; MES, 4-morpholineethanesulfonic acid.
Covalent Labeling of a Platelet P2T Receptor by an ADP Analog

ADP, it has a benzoyl group and thus lacks the hydrophilicity of ADP. Since the reagent includes an ester bond (Fig. 1B), hydrolysis of FSBA yields adenosine, which is an inhibitor of platelet functions and thus adenosine deaminase has to be included in the incubation mixtures (23). Furthermore, the available method of synthesis of [3H]FSBA yields a product of relatively low specific radioactivity. These properties have frustrated attempts to purify the ADP receptor using [3H]FSBA as a covalent label.

In light of the limitations on the use of FSBA and the controversy surrounding the identity of an ADP receptor on the platelet surface, we decided to investigate the effect of other nucleotide-based affinity labels with the potential for alkylation of a putative receptor. 8-(4-Bromo-2,3-dioxobutythio)ADP (8-BDB-TADP) (Fig. 1C) has been previously used as an ADP affinity label to probe the structure-function relationship of ADP-requiring enzymes such as pyruvate kinase (24) and glutamate dehydrogenase (25, 26). 8-BDB-TADP has the characteristic diphosphate of the natural ligand ADP, and is both hydrophilic and negatively charged at neutral pH. Furthermore, it can be synthesized as the 32P-labeled reagent with high specific radioactivity. In order to further investigate and test our hypothesis that aggregin is a putative ADP receptor, we chose 8-BDB-TADP as a true ADP affinity ligand to label the ADP receptor and ascertain its effect on ADP-induced platelet responses. In this case the nature of the chemical reaction is well understood and chemically defined products have been isolated (26, 27). This report describes in detail the effect of 8-BDB-TADP on ADP-induced platelet responses and the protein(s) modified on the surface of human platelets. A preliminary account of the work has previously appeared (28).

**Experimental Procedures**

Materials—AMP, ADP, ATP, 8-bromo-ADP, p-chloromercuri benzilate, benzamidine hydrochloride, FSBA, leupeptin, PMA (phorbol-12-myristate-13-acetate), apyrase (from potato), adenosine deaminase (from calf intestinal mucosa, Type VII), bovine serum albumin, and luciferase-luciferin reagent (in glycine buffer) were obtained from Sigma. U46619 (9,11-dideoxy-9a,11a-methanoepoxy prostaglandin F2α) was from Cayman Chemicals (Ann Arbor, MI). A23187 (a calcium ionophore), collagen and p-APMSF (p-aminomethylbenzenesulfonyl fluoride) were from Calbiochem (San Diego, CA). Adenosine, GDP, and ATP–s were obtained from Boehringer Mannheim. 2-Methylthio-ADP was supplied by Research Biochemicals, Inc. (Natick, MA). Prestained molecular weight standards used as markers to estimate molecular weight of proteins by gel electrophoresis were from Life Technologies, Inc., and Bio-Rad. Quin-2/AM (AM = acetoxyethyl ester) and Fura-2/AM were obtained from Molecular Probes (Eugene, OR). ACP-binding protein assay kit was purchased from Diagnostic Corp. (Los Angeles, CA). NaB[3H]4 was supplied by DuPont NEN. [1H]FSBA was prepared as described previously (6, 7). Human α-thrombin (1200 NIH units/mg protein) was a gift from Dr. J. oh (Division of Laboratory and Research, New York State Department of Health, Albany, NY). Fibrinogen used in labeling of platelets by 125I-fibrinogen was obtained from Sigma. In all other experiments fibrinogen used was purchased from KabiVitrum (Franklin, OH).

Synthesis and Characterization of 8-BDB-TADP—8-BDB-TADP was synthesized and characterized by chemical and physical methods as described previously (24). Briefly, ADP was treated with Br2 to yield 8-bromoadenosine-5′-diphosphate (8-Br-ADP). 8-Bromo-ADP was converted to 8-thioadenosine-5′-diphosphate (8-TADP) by reaction with LiHS. After purification, 8-TADP was coupled with 1,4-dibromobutane-dione to yield 8(4-bromo-2,3-dioxobutylthio)adenosine-5′-diphosphate (8-BDB-TADP). 8-BDB-TADP was stored dried at −80°C. Solutions of 8-BDB-TADP were prepared in 50 mM MES, pH 6.0 and the concentration was determined spectrophotometrically by using a molar absorption of 19 mε cm−1.

The 32P-labeled compound, [β-32P]8-BDB-TADP, was synthesized from 8-thioadenosine-5′-monophosphate, prepared, and purified as reported previously (29), by phosphorylation using [32P]jophosphoric acid (5 mCi added to 500 μmol of the unlabeled phosphoric acid). Tributylammonium [32P]phosphate was prepared after passage of the radioactive sodium phosphate through a column of AG50-X4 (pyridinium form) as described (30) and was dissolved in dimethylformamide. Tributylammonium salt of 8-thioadenosine-5′-monophosphate was purified similarly (30) and was dissolved in dimethylformamide. The phosphorylation was carried out by the method of Kozarich et al. (31). A solution of 1.1-carbonyldimimidazole (200 μmol) in dimethylformamide (1.5 ml) was added to a solution of 100 μmol tributylammonium-8-thioadenosine-5′-monophosphate in dimethylformamide (5 ml) and stirred for 45 min. Methanol (450 μmol) was added, and the reaction mixture was stirred for an additional 30 min. Tributylammonium[32P]jophosphosphate (250 μmol in 2.5 ml of dimethylformamide) was added with stirring and was allowed to stand at room temperature for 20 h. Methanol (9 ml) was then added and the reaction mixture evaporated to dryness. 8-Thioadenosine-5′-diphosphate was purified by chromatography on DEAE-cellulose column using a linear gradient from 10 to 500 mM NH4HCO3, essentially as described for 2-thioadenosine-5′-diphosphate (32). Purified 8-TADP was converted to the free acid form by application to a column of AG-50WX4 (H+1) and elution with distilled water. 8-Thioadenosine-5′-[β-32P]diphosphate was converted into [β-32P]8-BDB-TADP by reaction with 1,4-dibromobutane-dione, as described for the unlabeled 8-BDB-TADP (24).

Platelet Isolation and Washing—Fresh human blood was obtained from healthy volunteers with informed consent. Platelets were isolated by differential centrifugation and washed as described previously (33). Washed platelets were suspended in Tyrode's buffer (18.4 mM HEPES, 0.42 mM NaH2PO4, 136 mM NaCl, 2.7 mM KCl, 11.9 mM d-glucose; 12 mM NaHCO3, and 3.5 mg/ml bovine serum albumin, pH 7.35).

Shape Change and Aggregation of Platelets—Shape change and aggregation of washed platelets were performed in a Lumi-Aggrometer (Chromagard, Haverstown, PA) under constant stirring conditions at a concentration of 1 × 1010/ml and 5 × 109/ml, respectively. Washed platelets (1 × 109/ml) and suspension buffer were used in reference cuvettes in shape change and aggregation experiments, respectively. ADP–induced platelet shape change was performed in the presence of 2 mM EGTA while platelet aggregation was performed in the presence of 1 mM Ca2+ and 1 mg/ml fibrinogen.

Fibrinogen Binding—Human 125I-fibrinogen (specific radioactivity 2.12 mCi/mg protein) was prepared by the iodogen method as described previously by Puri et al. (34) except that the equilibration and elution buffer contained 184 mM HEPES, 136 mM NaCl, 2.7 mM KCl, and 0.35 mg/ml bovine serum albumin, pH 7.35. The preparation of radiolabeled fibrinogen was found to be 93 ± 5% (n = 4) clottable. The radiolabeled fibrinogen was diluted with unlabeled fibrinogen (20 mg/ml) in a 1:1 ratio before using it in binding experiments. Binding of 125I-fibrinogen to washed platelets was measured as reported previ-
Labeling of Platelets by 8-BDB-TADP and NaB[3H]4—

Covalent Labeling of a Platelet P2T Receptor by an ADP Analog

... 8-BDB-TADP was determined similarly by preincubating the platelets in the dark with 8-BDB-TADP at 25 °C for 30 min.

Platelet Secretion—Secretion of ADP by platelets (5 × 10^8/ml) following exposure to ADP in the absence and presence of 8-BDB-TADP, was estimated, simultaneously with aggregation, by measuring the intensity of chemiluminescence from solutions of known concentration of ATP in the absence and presence of 8-BDB-TADP as described above. 8-BDB-TADP, as high as 0.6 mM, had no effect on the assay.

Measurement of Intracellular Calcium Levels—Platelets were loaded with fluorescent indicators by incubating platelet-rich plasma with Fura-2/AM (5 μM) or Quin-2/AM (20 μM) at 37 °C for 20 min. Stock solutions of the indicators were prepared in MeSO. Maximum fluorescence (F_max), reflecting total content of Ca^{2+} in storage granules, was estimated by treating the Fura-2: (λ_exc and λ_conv, 340 and 510 nm, respectively) loaded platelets (2 × 10^8/ml) with 2 μM iomycin (in MeSO), and autofluorescence (AF) was determined by quenching the above incubation mixture with 10 mM MnCl_2. Platelets loaded with the fluorophores were then incubated with 1 mM CaCl_2 at 37 °C for 1 min and then stirred with an increasing concentration of ADP to monitor the fluorescence emission intensity (F_em). The intracellular level of Ca^{2+} ([Ca^{2+}]_i), was calculated (35, 36) by using the equation F_em/F_max = F_{max} - F_em / [F_{max} - F_{min}](where F_{min} = AF + F_{fluor-AF}, and K_d = 228 for Fura-2/AM).

The loaded platelets were then incubated with 8-BDB-TADP at 25 °C in the dark and ADP-induced release of Ca^{2+} determined as described above. When Quin-2/AM was used as the fluorophore, the protocol used for determining [Ca^{2+}]_i was similar to the one described above except that the λ_conv and λ_exc used were 393 and 492 nm, respectively, and a value of 114 was used for K_d (37).

Measurement of Intracellular cAMP Levels—Intracellular levels of cAMP in washed and 8-BDB-TADP-modified platelets (1 × 10^9/ml) were determined by the commercial cAMP-binding assay as outlined by the manufacturer and described previously by Puri et al. (34).

Labeling of Platelets by [3H]FSBA—Washed platelets (5 × 10^9/ml) were incubated with 200 μM [3H]FSBA and adenosine deaminase at 25 °C for 40 min. An ice-cold solution containing TBS and 1 mM EDTA (1 ml) was added to the incubation mixture and the mixture centrifuged for 30 s in a microfuge. The labeled platelets were resuspended, dissolved, and electrophoresed as described above. Gels were carefully; shaken with ENLIGHTENER (NEN-DuPONT) for 20 min at 25 °C and dried immediately. Autoradiography required exposure of the gels to x-ray film for 2–4 weeks.

Labeling of Platelets by [3H]-8-BDB-TADP—Washed platelets (1–2 × 10^8/0.25 ml) were incubated in the dark with 200 μM [3H]-8-BDB-TADP (5.91 mCi/mol) for 30 min followed by addition of an ice-cold solution (1 ml) containing Tris-buffered saline (TBS; 15 mM Tris-HCl and 139 mM NaCl, pH 7.4) and EDTA (10 mM). The reaction mixture was centrifuged for 30 s in a Beckman model E microcentrifuge. The pellet was suspended in a solution (35 μl) containing TBS and 1 mM EDTA and solubilized by addition of an equal volume of a solution containing SDS (4%, w/v), β-mercaptoethanol (12%, v/v), leupeptin (20 μM), p-APMSF (60 μM), and benzamidine (2 mM). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 10% gels according to the method of Laemmli (38) with the modification that the gels were prepared from a solution of acrylamide-bisacrylamide mixture containing 1% SDS (luminophore), p-APMSF (30 μM), and benzamidine (1 mM). Gels were calibrated with prestained molecular weight standards. The dried gels were exposed to CRONEX x-ray film (NEN-DuPONT) at −70 °C on 7–14 days before developing.

Labeling of Platelets by 8-BDB-TADP and NaNi[32P]8-BDB-TADP—Platelets were modified by unlabeled 8-BDB-TADP as described above, washed once with ice-cold TBS buffer containing 10 mM EDTA (1 ml), and centrifuged in a microfuge for 30 s. 8-BDB-TADP-modified platelets were suspended in Tyrode's buffer, pH 7.35 (0.2 ml). The suspension was treated with a NaNi[32P]8-BDB-TADP solution (25 μl; 50–70 Ci/ml) for 30 min at 25°C with gentle shaking. The NaNi[32P]8-BDB-TADP solution was used with occasional gentle shaking. The NaNi[32P]8-BDB-TADP solution was centrifuged for 30 s in a microfuge. The pellet was suspended in the TBS buffer and solubilized and subjected to electrophoresis as described in the previous section. Gels were carefully; shaken with ENLIGHTENER (NEN-DuPONT) for 20 min at 25 °C and dried immediately. Autoradiography required exposure of the gels to x-ray film for 2–4 weeks.

RESULTS

Shape Change—8-BDB-TADP inhibited ADP-induced shape change in a concentration-dependent manner with an IC_50 of about 75 μM (Fig. 2A). Complete inhibition of ADP-induced shape change required 400 μM of the 8-BDB analog. The inhibition of shape change by the 8-BDB analog followed biphasic kinetics. The time course of inhibition of ADP-induced platelet shape change by 200 μM 8-BDB-TADP showed complete inhibition of shape change in 70 min (Fig. 2B). However, loss of 50% of the rate of shape change occurred in a relatively short time, 10 min. 8-BDB-TADP, at concentrations as high as 400 μM, did not induce platelet shape change. The results show that 8-BDB-TADP was a potent inhibitor of ADP-induced shape change.

Aggregation—8-BDB-TADP blocked ADP-induced platelet aggregation in a concentration-dependent manner with an IC_50 of about 75 μM (Fig. 3A). Inhibition of ADP-induced aggregation was also time-dependent, and the 8-BDB analog (150 μM) inhibited the rate of ADP-induced aggregation by 50% in 10 min and 90% in 40 min (Fig. 3B). 8-BDB-TADP, as high as 400 μM, did not act as an agonist of platelet aggregation. The results demonstrate that 8-BDB-TADP is an effective inhibitor of ADP-induced platelet aggregation.
Nonspecific binding of $^{125}$I-fibrinogen to platelets was concentration dependent. A challenge by 30 $\mu M$ ADP, total binding of $^{125}$I-fibrinogen was concentration dependent. A, non-specific binding of $^{125}$I-fibrinogen to platelets that completely or partially utilize ADP-degrading activities and aggregation are synchronous events, both reaching maximum within a minute, ADP-induced secretion only starts after maximum aggregation has occurred. This lag may be due to the presence of a higher concentration of ADP (some released from the platelets) in the localized environment of platelets.

**Fig. 3. Effect of 8-BDB-TADP on ADP-induced platelet aggregation.** A, concentration dependence: platelets were incubated in the dark with various concentrations of 8-BDB-TADP for 30 min at 25 $^\circ$C, challenged by 30 $\mu M$ ADP, and monitored for aggregation ($\bigcirc$), and data plotted as described under "Experimental Procedures." B, time course: platelets were incubated with 150 $\mu M$ 8-BDB-TADP. Aliquots were withdrawn and challenged by 30 $\mu M$ ADP and monitored for aggregation ($\bigcirc$). The data are expressed as percent of rate of maximum aggregation (LTU/min: LTU = light transmission units, arbitrary scale) compared with an identical control (100%). The data are typical of those obtained with platelets from the blood of three donors.

ADP, total binding of $^{125}$I-fibrinogen was concentration dependent (Fig. 4). Nonspecific binding of $^{125}$I-fibrinogen to platelets remained at about 10% level over the 16-fold concentration range of the radiolabeled fibrinogen. When 8-BDB-TADP-modified platelets were incubated with an increasing concentration of the radiolabeled fibrinogen followed by exposure to 30 $\mu M$ ADP, the total binding of fibrinogen was diminished to a mean (±S.E.) value of 21 ± 5% compared with that of unmodified platelets. The results show that specific binding of $^{125}$I-fibrinogen to platelets (or exposure of GPIIb-IIIa complex) induced by ADP was blocked by 80% when platelets were exposed to the 8-BDB-TADP.

**Fig. 4. Effect of 8-BDB-TADP on ADP-induced exposure of fibrinogen binding sites in platelets.** For total binding washed platelets (1 × 10$^8$/200 $\mu l$) in the presence of 1 mM Ca$^{2+}$ were incubated with increasing concentration of $^{125}$I-fibrinogen (0.2 mCi/mg protein) for 1 min followed by 30 $\mu M$ ADP for 3 min at 25 $^\circ$C. Three aliquots (50 $\mu l$) were withdrawn from each incubation mixture and layered over a mixture of silicon oils as described under "Experimental Procedures" and centrifuged in a microfuge for 3 min. The pellet at the bottom of the centrifuge tube was excised and assayed for radioactivity. Nonspecific binding of $^{125}$I-fibrinogen to washed platelets was determined as described above except that the incubation mixtures contained 2 mM EDTA and 10-fold molar excess of unlabeled fibrinogen. Binding to platelets in the presence of 8-BDB-TADP was performed as described in the case of total binding except that the washed platelets were preincubated in the dark with 300 $\mu M$ 8-BDB-TADP at 25 $^\circ$C for 30 min. Specific binding of $^{125}$I-fibrinogen to platelets in the absence (●) and presence (○) of 8-BDB-TADP were computed by subtracting nonspecific binding from the total binding in each case. The data are expressed as mean of the molecules of $^{125}$I-fibrinogen bound ± S.E./platelet versus concentration of $^{125}$I-fibrinogen.

**TABLE I**

| Agonist | Conc. | LTU/min | Rate of aggregation of control | Rate of aggregation of 8-BDB-TADP-modified platelets | % of control |
|--------|------|---------|-----------------------------|---------------------------------|-------------|
| ADP    | 20 $\mu M$ | 22 | 2 | 9 |
| Collagen | 6 $\mu g/ml$ | 21 | 4 | 19 |
| U46619 | 1 $\mu M$ | 18 | 7 | 39 |
| Thrombin | 2 $\mu M$ | 22 | 23 | 105 |
| A23187 | 0.8 $\mu M$ | 27 | 28 | 99 |
| PMA | 16 $\mu M$ | 15 | 10 | 67 |
| A23187 + PMA | 8 $\mu M$ | 28 | 19 | 68 |
| + PMA | 0.3 $\mu M$ | | | |

The concentration of 8-BDB-TADP (50 $\mu M$) which minimally affected ADP-induced platelet aggregation completely blocked ADP-induced secretion. The results show that (a) thrombin- and ADP-induced secretion of nucleotides from dense granules proceed by two different mechanisms, and (b) ADP-induced secretion does require binding of the agonist to the receptor, and chemical modification of this receptor by 8-BDB-TADP blocks this response.

**Intracellular Calcium Mobilization**—Intracellular levels of calcium in platelets exposed to ADP rose from a basal value of 100 to 703 nm and 683 nm as measured by using Fura-2/AM and Quin-2/AM, respectively (Fig. 6, data for measurements by using Quin-2/AM not shown). Preincubation of platelets with 230 $\mu M$ 8-BDB-TADP for 30 min at 25 $^\circ$C completely blocked ADP-induced elevation of [Ca$^{2+}$]. 8-BDB-TADP itself did not cause any change in the basal levels of Ca$^{2+}$ in platelets.
Iloprost (a stable carbocyclic derivative of prostaglandin I₂) and extracellular cAMP present in resting platelets (Table II, 1) (39, 40).

Similar results were obtained with platelets from the blood of two different donors.

The loaded platelets were then stirred with various concentrations of ADP in the presence of 1 mM external Ca²⁺. Ca²⁺ release from the platelets was monitored as described under “Experimental Procedures.” To evaluate the effect of 8-BDB-TADP, washed platelets were preincubated in the dark with 230 μM 8-BDB-TADP for 30 min at 25°C followed by treatment with 1 mM ADP. 8-BDB-TADP had no effect on the intensity of chemiluminescence produced by solutions of known concentration of ATP (used for calibration of the assay) treated with luciferase-luciferin reagent under identical conditions. Thrombin- and ADP-induced secretion were computed from two separate calibrations employing standard solutions of ATP. Similar results were obtained with platelets from the blood of two different donors.

Table II

| Addition                        | Intracellular levels of cAMP (pmol/10⁸ platelet) | % of control |
|---------------------------------|-------------------------------------------------|-------------|
| 1. None                         | <2                                              | <2          |
| 2. Papaverine (2 mM) + iloprost (2.5 μM) | 538 ± 38                                      | 100         |
| 3. 8-BDB-TADP (0.2 mM)          | <2                                              | <2          |
| 4. 8-BDB-TADP (0.2 mM) + papaverine (2 μM) + iloprost (2.5 μM) | 578 ± 22                                      | 107         |
| 5. ADP (1 mM) + papaverine (2 μM) + iloprost (2.5 μM) | 398 ± 17                                      | 74          |
| 6. ADP (10 mM) + papaverine (2 μM) + iloprost (2.5 μM) | 178 ± 20                                      | 33          |
| 7. 8-BDB-TADP (0.2 mM) + ADP (1 mM) + papaverine (2 μM) + iloprost (2.5 μM) | 522 ± 40                                      | 97          |
| 8. 8-BDB-TADP (0.2 mM) + ADP (10 mM) + papaverine (2 μM) + iloprost (2.5 μM) | 338 ± 38                                      | 63          |

Intracellular Levels of cAMP—There is little or no intracellular cAMP present in resting platelets (Table II, 1) (39, 40). Iloprost (a stable carbocyclic derivative of prostaglandin I₂) and papaverine (a phosphodiesterase inhibitor) in combination raise and sustain intracellular levels of cAMP by stimulating membranous adenylate cyclase which converts ATP to cAMP (Table II, 2). Thrombin (40, 41) to a larger extent and ADP (Table II, 5 and 6) (42) to a lesser extent antagonize the increase in intracellular levels of cAMP in platelets induced by prostaglandins. 8-BDB-TADP neither raised (Table II, 3) nor affected the ability of iloprost to raise intracellular levels of cAMP (Table II, 4). However, the 8-BDB analog impaired the ability of ADP to antagonize the increase in cAMP levels in platelets exposed to iloprost (Table II, 8). These results show that covalent modification of ADP-binding sites on platelet surface results in the loss of the ability of ADP to modulate stimulated adenylate cyclase activity.

Identification of 8-BDB-TADP-labeled Protein on Platelet Surface—We have previously demonstrated that a single peak of radioactivity at 100 kDa in the radioactivity distribution profile of slices of gels obtained by SDS-PAGE of solubilized [3H]FSBA-labeled membranes obtained from [3H]FSBA-labeled platelets corresponds to an ADP receptor, aggregin, on the platelet surface (Ref. 9, and references cited therein). However, because of the relatively low specific radioactivity of the available [3H]FSBA and the low emitting threshold of tritium, it is cumbersome and time consuming to obtain good autoradiograms by autoradiography of gels obtained by SDS-PAGE of solubilized [3H]FSBA-labeled platelets. Nevertheless, an autoradiograph of the gel obtained by SDS-PAGE of solubilized [3H]FSBA-labeled platelets shows the presence of a single protein band corresponding to radiolabeled aggregin (100 kDa) (data not shown). [β-32P]8-BDB-TADP also labeled a single protein (100 kDa) on the platelet surface; the intensity of this band was reduced when labeling was carried out with platelets preincubated with 2 mM ADP (Fig. 7a). When FSBA-modified platelets or platelets exposed to 10 mM ADP or 10 mM ATP were incubated with [β-32P]8-BDB-TADP, no label was incorporated into the protein corresponding to aggregin (Fig. 7b); whereas 2 mM ADP and 8-bromo-ADP (Fig. 7b, lanes B and D) considerably reduced the intensity of the [β-32P]8-BDB-TADP-labeled aggregin, ATP-α-S, adenosine, AMP, and GDP (Fig. 7c, lanes E–H), at the same concentration, minimally protected aggregin from labeling by [β-32P]8-BDB-TADP (Fig. 7c). Platelets previously exposed to 2 mM p-chloromercuribenzenesulfonate (a sulfhydryl modifying reagent) were not labeled by [β-32P]8-BDB-TADP (Fig. 7c, lane I). This result is consistent with our previous demonstration that aggregin contains lysine and cysteine residues at the ADP-binding domain in aggregin and that
an aliquot of chemical reduction by NaB\(^{3H}\) of the ketogroups in the labels of platelets (40). Labeling of platelets by 8-BDB-TADP followed by covalent and simultaneous modification of such residues by platelets (23 c much larger concentration of platelets used in this experiment. It at the electrophoresis. The gel was calibrated with molecular weight kDa protein band corresponding to aggregin. Incorporation of covalently attached to platelets incorporated tritium into a 100 kDa protein band corresponding to aggregin. Incorporation of the label into aggregin was completely blocked when labeling was carried out by preincubating platelets with 10 mM ADP or 10 mM ATP (Fig. 8). The results are consistent with the fact that \([^{3H}]\)FSBA, \([^{32P}]8\)-BDB-TADP, and NaB\(^{3H}\) covalently and selectively label the same ADP-binding protein, aggregin, on the platelet surface.

**DISCUSSION**

8-BDB-TADP inhibited ADP-induced platelet shape change and aggregation in a concentration- and time-dependent manner signifying that the reagent covalently modifies ADP-binding sites on platelet surface. Preincubation of the platelets with 8-BDB-TADP effectively blocked ADP-induced binding of \(^{125I}\) fibrinogen to platelets. These results are consistent with the fact that the 8-BDB analog inhibits ADP-induced platelet aggregation. When 8-BDB-TADP-modified platelets were examined for their ability to aggregate by exposure to various agonists, only ADP-induced aggregation was almost completely blocked. The results that the rates of collagen- and U46619-induced platelet aggregation were significantly reduced in 8-BDB-TADP-modified platelets are in accord with the previous findings that platelet aggregation induced by these two agonists proceeds, at least in part, by ADP-dependent mechanisms (10, 11). The rates of aggregation induced by thrombin A23187, PMA, and A23187-PMA were minimally affected by chemical modification of ADP-binding sites. These results are in accord with the fact that aggregation of platelets induced by the above agonist follow ADP-independent mechanisms (43-45). 8-BDB-TADP also blocked ADP-induced secretion of nucleotides by platelets.

ADP-induced release of \(^{45}Ca^{2+}\) from the dense tubular system plays an important role in the platelet responses mediated by the binding of ADP to its receptor (46). Our results show that 8-BDB-TADP itself was not an agonist of intracellular mobilization of \(^{45}Ca^{2+}\), but it effectively blocked similar mobilization by ADP. ADP is a non-penetrating reagent, and platelet responses elicited by this agonist are mediated through its interaction with specific receptors on platelet surface (9, 47). In addition to inducing intracellular increase in \(^{45}Ca^{2+}\), ADP also has another important function: it antagonizes elevation of intracellular levels of cAMP induced by prostaglandins (42). Our observation that covalent modification of platelets by 8-BDB-TADP blocked the ability of ADP to inhibit stimulated adenylate cyclase activity is consistent with the previous studies.
The ADP-binding site mediating ADP-induced platelet shape change may be different from the one mediating intracellular Ca²⁺ mobilization (48). Other investigators suggested that that ADP-binding site for platelet shape change might be different from those antagonizing stimulated adenylate cyclase activity (49). Savi et al. (50) recently presented experimental evidence for two types of ADP-binding sites that differ in their affinity for ADP. Unlike 8-BDB-TADP, FSBA was not able to antagonize the stimulated adenylate cyclase activity, but this may be due to its lower affinity for aggregin (23).

Although the two-receptor hypothesis for different ADP-induced platelet responses has been widely discussed, the identity of two distinct ADP-binding proteins on platelet surface has never been established. Results presented in this investigation, for the first time, show that [³H]FSBA and [β-³²P]8-BDB-TADP label the same protein, aggregin (100 kDa), the ADP receptor on the platelet surface. Covalent modification of aggregin by 8-BDB-TADP not only inhibits ADP-induced shape change, aggregation, and exposure of fibrinogen-binding sites but also blocks the ability of ADP to antagonize stimulated adenylate cyclase activity suggesting that all of these responses are mediated by aggregin. Complete prevention of incorporation of [β-³²P]8-BDB-TADP into aggregin required either prior covalent modification of aggregin by FSBA or precubation of platelets with a concentration of ADP or ATP as high as 10 mM. Our experience has shown that those concentrations of 2',3'-dialdehyde derivative of ADP (10 mM) that far exceeded those necessary to inhibit ADP-induced platelet shape change and aggregation, could not completely block labeling of platelets by [³H]FSBA. We have recently shown that 7-chloro-4-nitro-2-oxa-1,3-diazole blocks ADP-induced platelet responses by covalently modifying aggregin (51). Precubination of platelets with 30 mM ADP, 30 mM ATP, or covalent modification of platelets by FSBA was necessary to block completely labeling of aggregin by [³H]FSBA. Two investigators have shown that a concentration of ATP, as high as 30 mM, was needed to block ADP-induced mobilization of Ca²⁺ in human erythrocytes cells (5). Cristalli and Mills (17) found that ADP (0.8 mM) reduced the intensity of the 43 kDa radiolabeled protein band and claimed that it was the ADP receptor.