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Inhibition of ADP-Induced Platelet Activation by 7-Chloro-4-nitrobenz-2-oxa-1,3-diazole: Covalent Modification of Aggregin, a Putative ADP Receptor

Rajinder N. Puri and Robert W. Colman

The Sol Sherry Thrombosis Research Center, Temple University School of Medicine, Philadelphia, Pennsylvania 19140

Abstract ADP-induced platelet responses play an important role in the maintenance of hemostasis. There has been disagreement concerning the identity of an ADP receptor on the platelet surface. The chemical structure of 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-Cl) shows considerable resemblance to that of the adenine moiety of adenine-based nucleotides. The reagent has been previously used by other investigators as an affinity label for adenine nucleotide-requiring enzymes, such as mitochondrial ATPase and the catalytic subunit of cAMP-dependent protein kinase. Since ADP-induced platelet responses depend on the binding of ADP to its receptor, we investigated the effect on ADP-induced platelet responses and the nature of ADP-binding protein modified by NBD-Cl. NBD-Cl inhibited ADP-induced shape change and aggregation of platelets in platelet-rich plasma in a concentration- and time-dependent manner. NBD-Cl also inhibited ADP-induced shape change, aggregation, exposure of fibrinogen binding sites, secretion, and calcium mobilization in washed platelets. NBD-Cl did not act as an agonist for platelet shape change and aggregation. Covalent modification of platelets by NBD-Cl blocked the ability of ADP to antagonize the increase in intracellular levels of cAMP mediated by iloprost (a stable analogue of prostaglandin I_2). NBD-Cl was quite specific in inhibiting platelet aggregation by those agonists, e.g., ADP, collagen, and U44619 (a thromboxane mimetic), that completely or partially depend on the binding of ADP to its receptor. Autoradiogram of the gel obtained by SDS–PAGE of solubilized platelets modified by [^{14}C]-NBD-Cl showed the presence of a predominant radiolabeled protein band at 100 kDa corresponding to aggregin, a putative ADP receptor. The intensity of this band was considerably decreased when platelets were either preincubated with ADP and ATP or covalently modified by a sulfhydryl group modifying reagent before modification by [^{14}C]-NBD-Cl. These results (1) indicate that covalent modification of aggregin by NBD-Cl contributed to loss of the ADP-induced platelet responses, and (2) suggest that there is a sulfhydryl group in the ADP-binding domain of aggregin. © 1996 Wiley-Liss, Inc.

Key words: aggregin, chemical modification, ADP-induced platelet responses, NBD-Cl, cAMP

ADP is one of the first known and most important of the established agonists of platelet aggregation [Garrder et al., 1961; Born, 1962]. ADP-induced platelet responses are mediated by a unique P_{2T} receptor [Burnstock, 1990; Hourani and Cusack, 1991]. Previous investigations from our laboratory demonstrated that binding of ADP to aggregin (100 kDa), a putative ADP receptor, is required for platelet aggregation by those agonists that use ADP-dependent mechanisms [Bennett et al., 1978; Figures et al., 1981; Morinelli et al., 1983; Colman et al., 1986; Puri et al., 1995]. In recent years, the presence of a platelet-like P_{2T} receptor has been demonstrated in cells of megakaryocytic lineage [Murgo et al., 1994] and human erythroleukemia (HEL) cells [Shi et al., 1995].

Recent investigations using photoaffinity labeling to identify the ADP receptor on the platelet surface have produced conflicting results [Greco et al., 1991; Mills et al., 1993] that do not agree with our previous findings [Bennett et al., 1978; Figures et al., 1981; Morenelli et al., 1983; Colman et al., 1986; Puri et al., 1995]. Photoaffinity labeling has been used to probe the structure–

Abbreviations used: NBD-Cl, 7-chloro-4-nitro-2-oxa-1,3-diazole; 8-BDB-TADP, 8-(4-bromo-2,3dioxobutylthio) adenosine-5′-diphosphate; FSBA, 5′-p-flurosulfonylbenzoyladenosine; OPTH, o-phthalaldehyde; A23187, a calcium ionophore; U46619, 9,11-dideoxy-9α,11α-methanoepoxy prostaglandin $F_{2\alpha}$; PMA, phorbol-12-myristate-13-acetate; p-APMS, p-aminophenylmethylsulfonylfluoride; GPIIb, glycoprotein IIb; GPIIIa, glycoprotein IIIa; PRP, platelet-rich plasma; SDS–PAGE sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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Address reprint requests to Dr. Rajinder N. Puri, The Sol Sherry Thrombosis Research Center, Temple University School of Medicine, 3400 N. Broad Street, Philadelphia, PA 19140.

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function relationship of purified enzymes as well as functional proteins in intact cellular systems. However, photolabeling suffers from the following disadvantages: (1) indeterminate nature of the ligands actually participating in the photolytic reaction and the products formed [cf. Greco et al., 1991; Cristalli and Mills, 1993], and (2) activation of signal transduction pathways that activate and/or regulate cellular functions [Devary et al., 1992; Schvien and Ledbetter, 1993; Schvien et al., 1993]. 7-Chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-CI) (Fig. 1B) is an electrophilic reagent that forms well-defined covalent fluorogenic adducts with S-amino acids (e.g., cysteine), O-amino acids (e.g., tyrosine), and N-amino acids (e.g., glycine) (Fig. 1C) [Ghosh and Whitehouse, 1968; Shipton et al., 1976; Baines et al., 1977]. Moreover, the chemical structure of NBD-CI resembles that of the adenine moiety of adenine nucleotides, such as ADP (Fig. 1A). Therefore, NBD-CI has been used to probe the structure–function relationship of purified mitochondrial ATPase [Ferguson et al., 1975] and the catalytic subunit of cAMP-dependent protein kinase [Hartle and Roskoski, 1982]. Our previous investigations have concluded that aggregin is a putative ADP receptor [Colman, 1990, 1992; Puri et al., 1995]. In order to gain further insight into the chemical characteristics of aggregin, we investigated the effect on ADP-induced platelet responses and the nature of the ADP-binding protein modified by NBD-CI. Preliminary reports of this work have previously appeared [Puri and Colman, 1994; Colman and Puri, 1995].

MATERIALS AND METHODS

NBD-CI (a pale yellow crystalline solid) was obtained from Sigma Chemical Co. (St. Louis, MO) and used without further purification. [U-^{14}C]-NBD-CI (spec radioact 108 μCi/ml; 0.83 μmol/ml in ethanol) was obtained from Research Products International Corp. (Mount Prospect, IL). Solutions of NBD-CI for investigation of its effect on responses induced by ADP and other agonists of platelets in platelet-rich plasma and washed platelets were prepared in ethanol and DMSO, respectively. Freshly prepared solutions were stored in the dark at 25°C.

U46619 (9,11-dideoxy-9α,11α-methanoepoxy prostaglandin $F_{2\alpha}$) was from Cayman Chemicals (Ann Arbor, MI). A23187 (a calcium ionophore), collagen, and p-APMSF (p-aminophenyl-methylsulfonylfluoride) were from Calbiochem (San Diego, CA). Prestained molecular weight standards used as markers to estimate molecular weight of proteins by gel electrophoresis were from Bio-Rad (Hercules, CA). Fura-2/AM was obtained from Molecular Probes (Eugene, OR). cAMP binding protein assay kit was purchased from Diagnostic Corp. (Los Angeles, CA). Human α-thrombin (3,203 NIH U/mg protein) was a gift from Dr. John W. Fenton II (Division of Laboratories and Research, New York State Department of Health, Albany, NY). Fibrinogen used in labeling of platelets by ^{125}I-fibrinogen was obtained from Sigma. In all other experiments, fibrinogen used was purchased from KabiVitrum (Franklin, OH). ADP, ATP, p-chloromercuribenzenesulfonate, benzamidine hydrochloride, FSBA (5′-p-fluorosulfonylbenzoyladenosine), leupeptin, PMA (phorbol-12-myristate-13-acetate), apyrase (from potato), adenosine deaminase (from calf intestinal mucosa, type VII), bovine serum albumin (BSA), and luciferase-luciferin reagent (in glycine buffer) were obtained from Sigma. 8-(4-Bromo-2,3-dioxobutylthio)adenosine-5′-diphosphate [8-BDB-TADP] was a kind gift from Dr. Roberta F. Colman (Department of Chemistry and Biochemistry, University of Delaware, Newark, DE).

Platelet Isolation and Washing

Fresh human blood was obtained from healthy volunteers with written informed consent. PRP was prepared by centrifugation (120*g*, 30 min, 23°C) of fresh whole human blood drawn into acid/citrate/dextrose (citric acid, 0.079 M; sodium citrate, 0.085 M; and dextrose 0.180 M) in a ratio of 1 ml of anticoagulant solution per 9 ml blood. For investigations of the effect of NBD-CI on responses in PRP of platelets exposed to various agonists, the pH was adjusted to 7.4 with 0.1 N NaOH solution. Washed platelets were prepared by differential centrifugation of PRP as described previously [Puri et al., 1989]. Washed platelets were suspended in Tyrode's buffer (Hepes, 18.4 mM; NaH_2PO_4, 0.42 mM; NaCl, 136 mM; KCl, 2.7 mM; D-glucose, 11.9 mM; $NaHCO_3$, 12 mM, and BSA, 3.5 mg/ml, pH 7.35).

Shape Change and Aggregation of Platelets

Shape change and aggregation of platelets in PRP and washed platelets were performed in a Lumi-Aggregometer (Chronolog, Havertown, PA) under constant stirring conditions at 37°C. PRP and PPP (platelet-poor plasma) were used

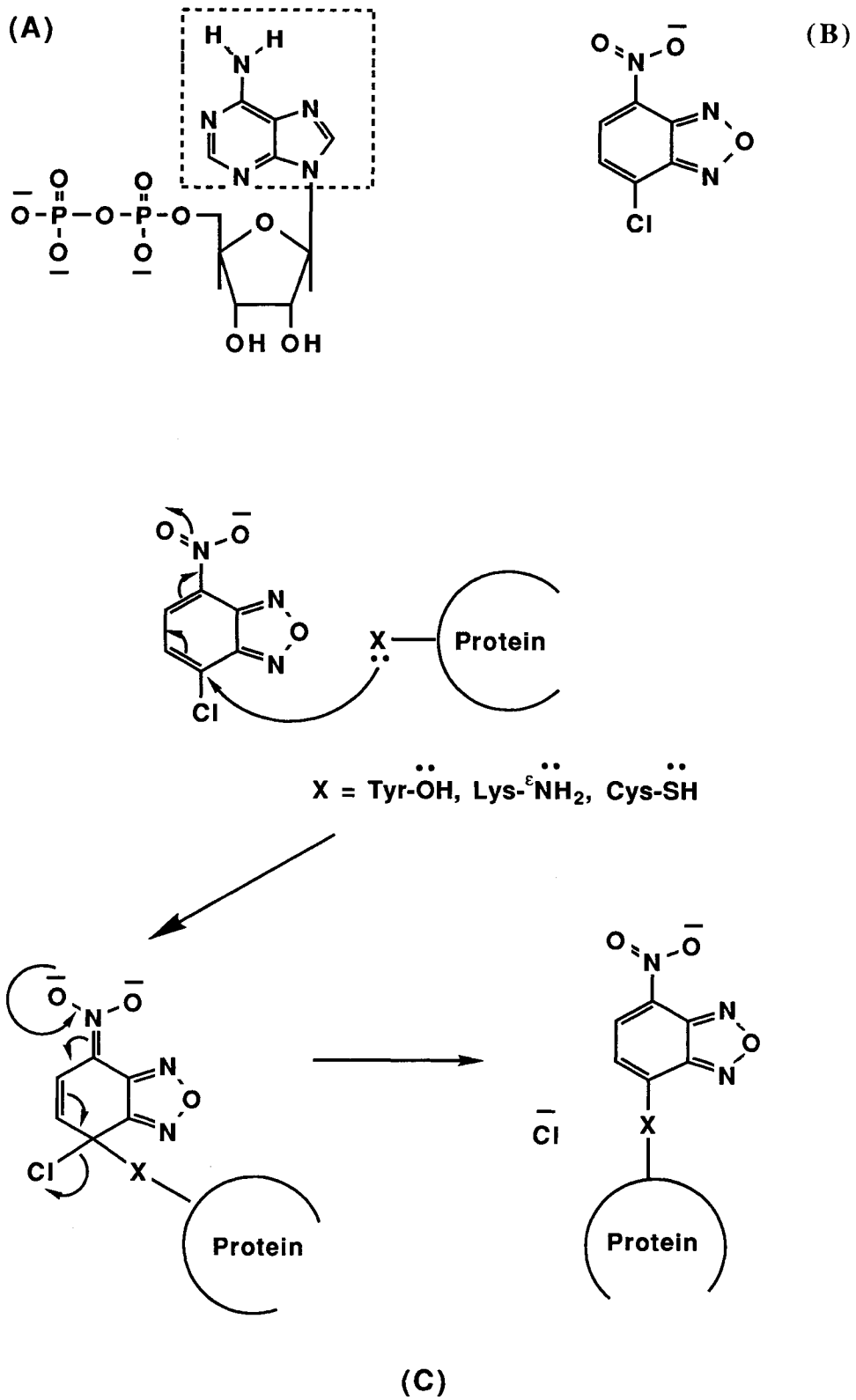

Fig. 1. Representations of NBD-Cl (**A**) and ADP (**B**) and schematic representation of the chemical modification of proteins by NBD-Cl (**C**) were created by the CHEMDRAW computer program (Cambridge Scientific Computing, Cambridge, MA). The adenine moiety in the representation of ADP (**B**) is highlighted by a dashed rectangle to emphasize its structural resemblence to NBD-Cl. Covalent adduct formation between NBD-Cl and proteins (**C**) involves nucleophilic attack by the lone pair of electrons in hetero atoms of the side chains of critical residues (e.g., tyrosine, lysine, and cysteine), present in the functional domains of a variety of proteins, on the carbon atom bearing chlorine in the aromatic ring of NBD-Cl. The cumulative negative inductive effect of nitro group and oxadiazole ring system facilitates the nucleophilic attack on the carbon atom bearing chlorine in NBD-Cl.

in the reference cuvette for platelet aggregation and shape change, respectively. Shape change and aggregation of washed platelets were performed at a concentration of 1×10^8/ml and 5×10^8/ml, respectively. Washed platelets (1×10^8/ml) and suspension buffer were used in reference cuvettes in shape change and aggregation experiments, respectively. ADP-induced platelet shape change in PRP was performed in the presence of 3–6 mM EGTA, while platelet aggregation was performed in the presence of 1 mM Ca^{2+}, but in the absence of externally added fibrinogen. ADP-induced platelet shape change of washed platelets was performed in the presence of 2 mM EGTA while platelet aggregation was performed by preincubating washed platelets with 1 mM Ca^{2+} and 1 mg/ml fibrinogen at 37°C. The data are expressed as percent of rate of maximum shape change (LAU/min; LAU = light absorption units, arbitrary scale) and maximum aggregation (LTU/min; LTU = light transmission units, arbitrary scale) compared with an identical control (100%).

Fibrinogen Binding

Human ^{125}I-fibrinogen (specific radioactivity 2.12 mCi/mg protein) was prepared by the iodogen method as described previously [Puri et al., 1991], except that the equilibration and elution buffer contained 184 mM Hepes, 136 mM NaCl, 2.7 mM KCl, and 0.35 mg/ml BSA, pH 7.35. The preparation of radioiodinated fibrinogen was found to be 93 ± 5% (n = 4) clottable. The radioiodinated fibrinogen was diluted with unlabeled fibrinogen (20 mg/ml) in a 1:1 ratio before using it in binding experiments. Binding of ^{125}I-fibrinogen to washed platelets was measured as reported previously by Puri et al. [1991]. For total binding of ^{125}I-fibrinogen, platelets (1×10^8/200 μl) were placed in an Eppendorf tube at 25°C and incubated with increasing amount of ^{125}I-fibrinogen in the presence of 1 mM Ca^{2+} for 1 min. The reaction mixture was then incubated with 30 μM ADP for another 5 min. The reaction was terminated by removing a 50-μl aliquot from each sample and layering over 200 μl of silicone oil mixture in a microtube (Sarsdet, Princeton, NJ) [Puri et al., 1991] and centrifuging in a Beckman model E microfuge for 3 min. The pellet at the bottom of the microtube was excised, and radioactivity in the pellet was measured by counting in an automatic γ-counter (model 1470 WIZARD, Wallac Oy, Turku, Finland) equipped with RIaCalc WIZ 2.2 software. Nonspecific binding of ^{125}I-fibrinogen to platelets was determined identically in the presence of 2 mM EDTA and 10-fold molar excess of unlabeled fibrinogen. Binding in the presence of NBD-CI was determined similarly by preincubating the platelets in the dark with NBD-CI at 25°C for 5 min.

Platelet Secretion

Secretion by platelets in PRP following exposure to ADP in the absence and presence of NBD-CI was estimated, simultaneously with aggregation, by measuring the intensity of chemiluminescence of commercial luciferase-luciferin reagent (80 μl/ml of PRP, freshly constituted as described in the protocol provided by the suppliers) in a dual-channel Lumi Aggregometer (Havertown, PA). The ATP release in the incubation mixtures was computed from a standard curve prepared by estimating chemiluminescence from solutions of known concentration of ATP in the absence and presence of NBD-CI as described above. NBD-CI, as high as 0.4 mM, had no effect on the assay.

Measurement of Intracellular Calcium Levels

Platelets were loaded with Fura-2/AM by incubating PRP with the fluorescent indicator (5 μM) at 37°C for 20 min. Stock solutions of the indicator were prepared in DMSO. Maximum fluorescence (F_{max}), reflecting total content of Ca^{2+} in storage granules, was estimated by treating the Fura-2 (λ_{ex} and λ_{em}, 340 and 510 nm, respectively) loaded platelets (2×10^8/ml) with 2 μM ionomycin (in DMSO), and autofluorescence was determined by quenching the above incubation mixture with 10 mM $MnCl_2$. Platelets loaded with the fluorophore 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. The intracellular level of Ca^{2+} was calculated by using the methods described in [Cobbold and Rink, 1987, and references cited therein]. The loaded platelets were then incubated with NBD-CI at 25°C in the dark and ADP-induced release of Ca^{2+} determined as described above.

Measurement of Intracellular cAMP Levels

Intracellular levels of cAMP in washed and NBD-CI-modified platelets (1×10^8/ml) were determined by the commercial cAMP-binding as-

say as outlined by the manufacturer and described previously [Puri et al., 1991].

Labeling of Platelets by [^{14}C]-NBD-Cl

Washed platelets (1–2 × 10^8/0.25 ml) were incubated in the dark with a solution of NBD-Cl (5 μM, final) and [^{14}C]-NBD-Cl (10 μl or 1.1 μCi of the commercial sample) in the absence or presence of various reagents for 5 min at 25°C 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 sec in Beckman model E microfuge. The pellet was suspended in a solution (35 μl) containing TBS and 1 mM EDTA and solubilized by the 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 (1970) with the modification that the gels were prepared from a solution of acrylamide–bisacrylamide mixture containing leupeptin (10 μM), 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, Boston, MA) at −70°C for 7 days before developing.

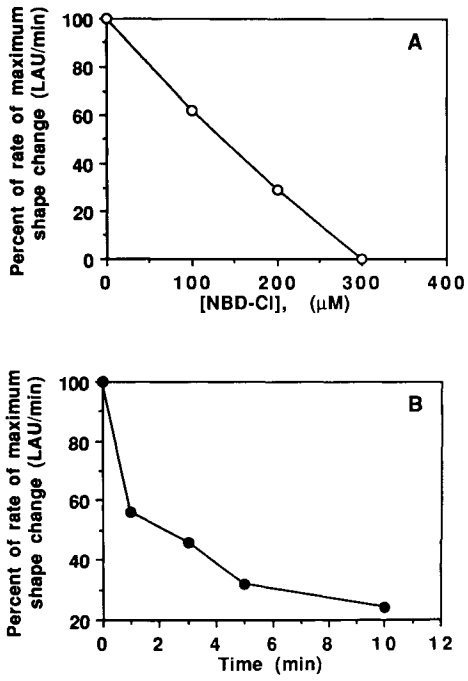

Fig. 2. Effect of NBD-Cl on ADP-induced shape change of platelets in PRP. **A:** Concentration dependence: PRP was incubated in the dark with various concentrations of NBD-Cl for 15 min at 25°C. Incubation mixtures were challenged by 4 μM ADP, monitored for shape change (○), and data plotted as described in MATERIALS AND METHODS. **B:** Time course: PRP was incubated with 100 μM NBD-Cl as described above. Aliquots were withdrawn at various times and challenged by 4 μM ADP to monitor residual shape change (●). The results are representative of the data obtained with PRP from the blood of three different donors.

RESULTS

Shape Change of Platelets in PRP

NBD-Cl inhibited ADP-induced shape change of platelets in PRP in a concentration-dependent manner with an IC_{50} of 120 μM (Fig. 2A). Complete inhibition of ADP-induced shape change required 300 μM of NBD-Cl. The time course of inhibition of ADP-induced platelet shape change by 150 μM NBD-Cl showed almost complete inhibition of shape change in 10 min (Fig. 2B). However, loss of 50% of the rate of shape change occurred in 5 min. NBD-Cl, up to 400 μM, did not induce platelet shape change. The results show that NBD-Cl was a potent inhibitor of ADP-induced shape change in PRP.

Aggregation of Platelets in PRP

NBD-Cl inhibited ADP-induced aggregation of platelets in PRP in a concentration-dependent manner with an IC_{50} of about 20 μM (Fig. 3A). Inhibition of ADP-induced aggregation was also time dependent, and NBD-Cl (50 μM) decreased the rate of ADP-induced aggregation by 50% in about 5 min and 100% in 25 min. (Fig. 3B). NBD-Cl, as high as 400 μM, did not act as an agonist of platelet aggregation. The results demonstrate that NBD-Cl was an effective inhibitor of ADP-induced platelet aggregation in PRP.

Shape Change and Aggregation of Washed Platelets

NBD-Cl inhibited ADP-induced shape change of washed platelets in a concentration-dependent manner with an IC_{50} of 5 μM (Fig. 4A). Complete inhibition of ADP-induced shape change required 40 μM of NBD-Cl. NBD-Cl also inhibited ADP-induced aggregation of washed platelets in a concentration-dependent manner with an IC_{50} of 1.5 μM (Fig. 4B). Complete inhibition of ADP-induced aggregation required 4 μM of NBD-Cl. Previous work from our labo-

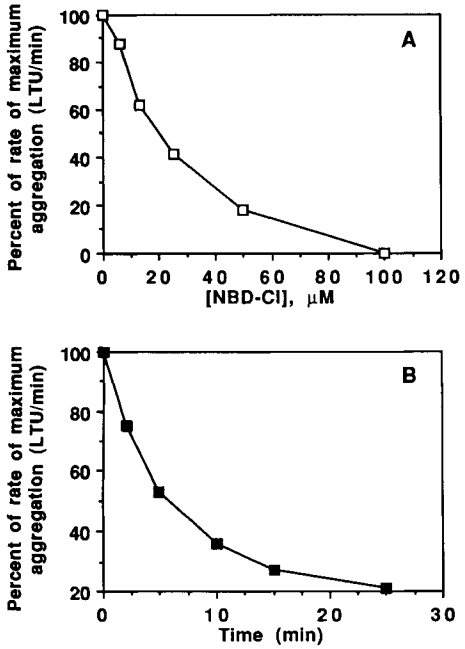

Fig. 3. Effect of NBD-Cl on ADP-induced aggregation of platelets in PRP. **A:** Concentration dependence: PRP was incubated in the dark with various concentrations of NBD-Cl for 15 min at 25°C, challenged by 4 μM ADP, and monitored for aggregation (□), and data plotted as described in Materials and Methods. **B:** Time course: PRP was incubated with 50 μM NBD-Cl. Aliquots were withdrawn and challenged by 30 μM ADP and monitored for shape change (■). The data are typical of those obtained with PRP from the blood of three donors.

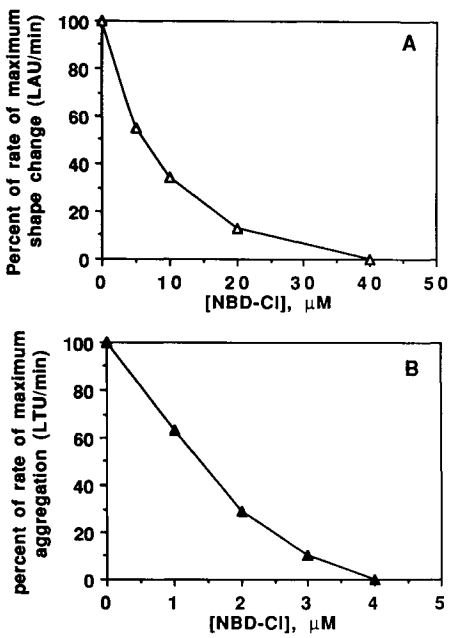

Fig. 4. Effect of NBD-Cl on ADP-induced shape change and aggregation of washed platelets. **A:** Concentration dependence of inhibition of shape change: Platelets were incubated in the dark with various concentrations of NBD-Cl for 5 min at 25°C, challenged by 10 μM ADP, and monitored for shape change (△), and data plotted as described in MATERIALS AND METHODS. **B:** Concentration dependence of inhibition of aggregation: Platelets were incubated in the dark with NBD-Cl for 5 min at 25°C. Aliquots were withdrawn and challenged by 30 μM ADP and monitored for aggregation (▲). The data are typical of those obtained with platelets from the blood of three donors.

ratory demonstrated that concentrations of ADP required to elicit shape change and aggregation of washed platelets are higher than those needed to elicit the same responses in platelets in PRP [Puri et al., 1993]. Results show that NBD-Cl inhibited ADP-induced shape change and aggregation of washed platelets in a concentration-dependent manner.

Fibrinogen Binding

When washed platelets were exposed to 30 μM ADP, total binding of ^{125}I-fibrinogen was concentration dependent (Fig. 5, top curve). Nonspecific binding of ^{125}I-fibrinogen to platelets (Fig. 5, middle curve) remained at about 26 ± 3% level (mean ± SEM) over the 16-fold concentration range of the radiolabeled fibrinogen. When NBD-Cl-modified platelets were incubated with increasing concentration of the radiolabeled fibrinogen followed by exposure to 30 μM ADP, the binding of fibrinogen was diminished to a value of 20 ± 3% (Fig. 5, bottom curve) compared with that of unmodified platelets, similar to nonspecific binding values. The results show that NBD-Cl completely blocked exposure by ADP of fibrinogen binding sites (the GPIIb–IIIa complex) on the platelet surface.

Aggregation Induced by Various Agonists

When washed platelets were partially modified by 5 μM NBD-Cl for 1 min at 25°C, washed and resuspended in Tyrode's buffer, pH 7.35, the rate of the ADP-induced aggregation was 40% of that of a corresponding control. The rates of collagen- and U46619-induced aggregation of platelets modified by NBD-Cl were lower compared with the corresponding controls (Table I). By contrast, the rate of aggregation of platelets induced by thrombin, PMA, and A23187 + PMA were minimally affected, because aggregation induced by these agonists proceed by ADP-independent mechanisms [Nishizuka, 1984; Kaibuchi et al., 1986; Puri et al., 1989]. The results show that the rates of aggregation of platelets by ADP, U46619, and collagen—agonists that completely or partially use ADP-dependent mecha-

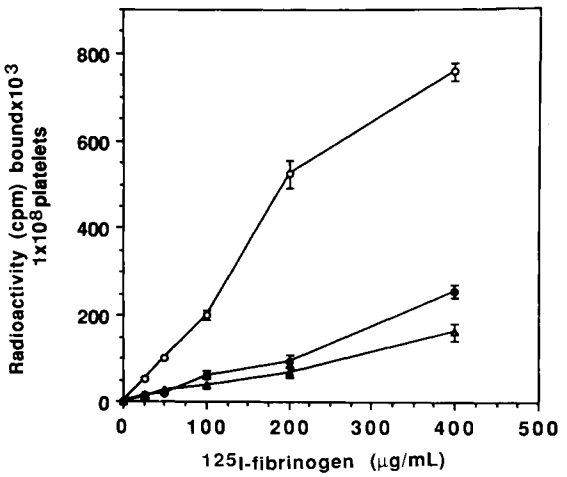

Fig. 5. Effect of NBD-CI on ADP-induced exposure of fibrinogen binding sites in washed platelets. For total binding (○), washed platelets (1×10^8/200 μl) in the presence of 1 mM Ca^{2+} were incubated with increasing concentrations of ^{125}I-fibrinogen (0.2 mCi/mg protein) for 1 min followed by 30 μM ADP for 3 min at 25°C. Three aliquots (50 μl) were withdrawn from each incubation mixture and layered over a mixture of silicon oils as described in Materials and Methods 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 of ^{125}I-fibrinogen to platelets in the presence of NBD-CI (△) was performed as described in the case of total binding except that the washed platelets were preincubated in the dark with 5 μM NBD-CI at 25°C for 5 min. The data are expressed as mean ± SEM. (n = 3) of the radioactivity bound/10^8 platelets versus concentration of ^{125}I-fibrinogen.

nisms [Figures et al., 1981; Morinelli et al., 1983; Colman et al., 1990]—were affected as a result of covalent modification of the ADP-binding site on the platelet surface.

Secretion

ADP caused secretion of 2 μM ATP from the storage granules of platelets in PRP. Only 8 μM NBD-CI was enough to block completely secretion of nucleotides by platelets in PRP (data not shown). Similar results have been obtained by us during our investigations of the inhibition of ADP-induced platelet responses by 8-BDB-TADP [Puri et al., 1995].

Intracellular Calcium Mobilization

Intracellular levels of calcium in washed platelets exposed to ADP (30 μM) rose from a basal value of 100 nM to 553 nM, as measured by using Fura-2/AM (Fig. 6). Preincubation of platelets with 5 μM NBD-CI for 5 min at 25°C, completely blocked ADP-induced elevation of levels of intracellular Ca^{2+}. NBD-CI, itself, did not cause any change in the basal levels of Ca^{2+} in platelets.

Intracellular Levels of cAMP

There is little or no intracellular cAMP present in resting platelets (Table II, 1) [Puri et al., 1991]. Iloprost (a stable carbocyclic derivative of prostaglandin I_2) and papaverine (a cAMP 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 [Brass and Shattil, 1986; Puri and Colman, 1991] to a larger extent and ADP [Mills and Smith, 1972; Puri and Colman, 1991] to a lesser extent antagonize increase in intracellular levels of cAMP in platelets induced by prostaglandins. ADP, at concentrations of 1 and 10 mM, blocked by 38% and 82%, respectively, the increase in intracellular levels of cAMP induced by iloprost (Table II, 3 and 4). NBD-CI, as high as 100 μM, neither raised (Table II, 5 and 6), nor did it affect the ability of iloprost to raise intracellular levels of cAMP (Table II, 7 and 8). Covalent modification of washed platelets by NBD-CI completely blocked the ability of ADP to antagonize increase in cAMP levels in platelets exposed to iloprost (Table II, 9). These results show that covalent modification of the ADP-binding site on the platelet surface results in the loss of the ability of ADP to modulate stimulated adenylate cyclase activity. The inability of NBD-CI to raise intracellular levels of cAMP at a concentration (100 μM) (Table II, 6) far more than that needed (4 μM) to inhibit ADP-induced aggregation of washed platelets (5 μM; Fig. 4A) strongly suggests that NBD-CI is not internalized.

Identification of NBD-CI 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 [^{3}H]FSBA-labeled membranes obtained from [^{3}H]FSBA-labeled platelets, corresponds to a putative ADP-receptor, aggregin, on the platelet surface [Colman, 1992, and references cited therein]. We recently demonstrated that an autoradiogram of the gel obtained by SDS–PAGE of solubilized [^{3}H]FSBA-labeled platelets also showed the presence of a

TABLE I. Effect of Chemical Modification of Washed Platelets by NBD-Cl on Platelet Aggregation Induced by Various Agonists*

Agonist	Concn	Rate of aggregation: Control (LTU/min)	Rate of aggregation: of NBD-Cl modified (LTU/min)	% of control
ADP	20 μM	20	8	40
Collagen	6 μg/ml	54	38	70
U46619	1 μM	28	22	79
Thrombin	2 nM	60	60	100
PMA	16 nM	60	53	88
A23187 + PMA	8 nM + 0.3 μM	43	48	112

*Platelets were chemically modified by incubating them with 5 μM NBD-Cl 25°C for 1 min in the dark. The rate of residual ADP-induced aggregation was 40% of a control identically carried through the same protocol in the absence of the inhibitor and this value was normalized to 100%. The platelets were then centrifuged and resuspended in Ca^{2+} free Tyrode's buffer, pH 7.35. The control platelets and NBD-Cl-modified platelets were challenged by various agonists. Initial slope of the aggregation tracing was computed as LTU (light transmission units, arbitrary scale)/min. The data are expressed as percent of the rate of aggregation of NBD-Cl-modified platelets by an agonist compared with unmodified platelets as the control (set at 100%).

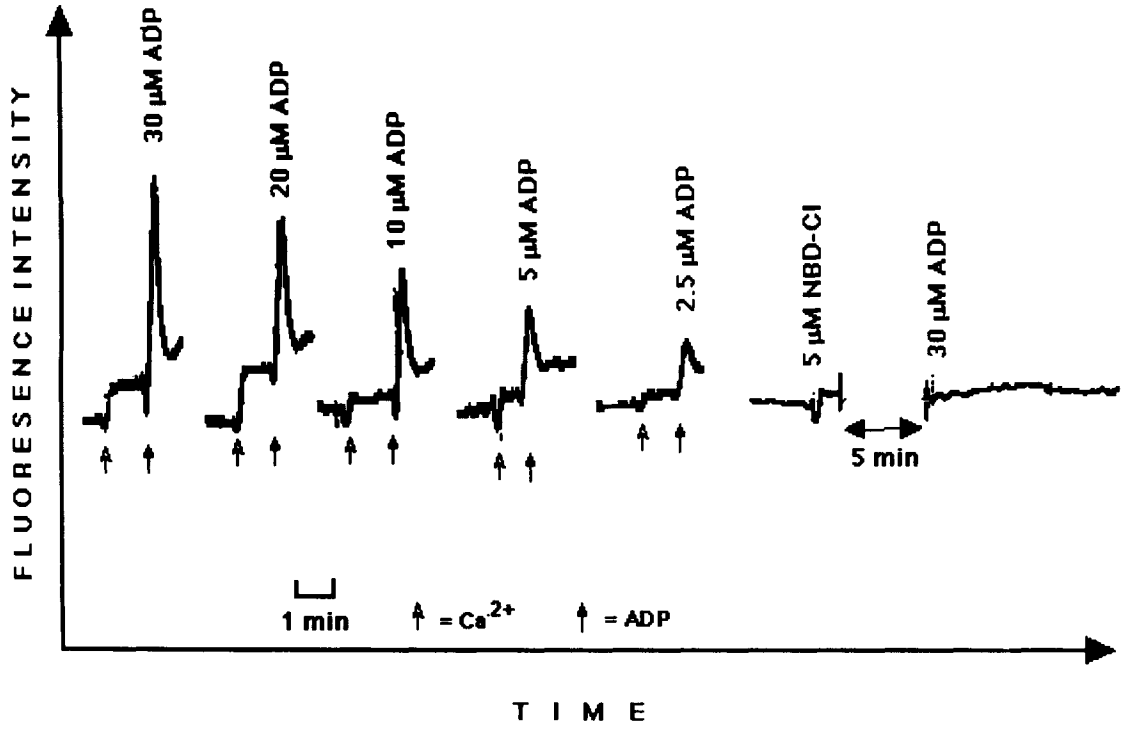

Fig. 6. Effect of NBD-Cl on ADP-induced increase in intracellular calcium levels in washed platelets. Platelets were loaded with Fura 2/AM fluorophore as described in Materials and Methods. The platelets loaded with the fluorophore were then stirred with various concentrations of ADP in the presence of 1 mM external Ca^{2+}. Ca^{2+} release from the platelets was monitored as described in Materials and Methods. To evaluate the effect of NBD-Cl, washed platelets were preincubated in the dark with 5 μM NBD-Cl for 5 min at 25°C, followed by treatment with 1 mM Ca^{2+} and 30 μM ADP.

single protein band corresponding to radiolabeled aggregin (100 kDa) [Puri et al., 1995]. [^{14}C]-NBD-Cl labeled predominantly a protein at 100 kDa on the platelet surface (Fig. 7, lane a). Other minor bands at 200 kDa and 30 kDa in the autoradiogram in Figure 7 are also visible. When FSBA-modified platelets (Fig. 7, lanes b and c) or platelets exposed to 30 mM ATP (lane d) or 30 mM ADP (lane e) were incubated with [^{14}C]-NBD-Cl either no label or very little of it was incorporated into the protein corresponding to aggregin (Fig. 7). We previously demonstrated that aggregin appears to contain lysine and cysteine residues at the ADP-binding do-

TABLE II. Effect of Chemical Modification of Platelets by NBD-Cl on Intracellular cAMP Levels

Addition	Intracellular levels of cAMP (pmol/10^8 platelet)	% of control
1. None	<3	
2. Papaverine (2 mM) + iloprost (2.5 μM)	634 ± 25	100 ± 4
3. ADP (1 mM) + papaverine (2 mM) + iloprost (2.5 μM)	394 ± 28	62 ± 7
4. ADP (10 mM) + papaverine (2 mM) + iloprost (2.5 μM)	114 ± 10	18 ± 2
5. NBD-Cl (10 μM)	<2	
6. NBD-Cl (100 μM)	9 ± 1	1
7. NBD-Cl (10 μM) + papaverine (2 mM) + iloprost (2.5 μM)	584 ± 70	92 ± 12
8. NBD-Cl (100 μM) + papaverine (2 mM) + iloprost (2.5 μM)	634 ± 59	100 ± 9
9. NBD-Cl (10 μM) + ADP (1 mM) + papaverine (2 mM) + iloprost (2.5 μM)	734 ± 112	116 ± 15

Intracellular levels of cAMP in washed platelets in the absence and presence of NBD-Cl were estimated by using commercial protein binding assay as described by Puri et al. [1991]. cAMP level in unmodified platelets following exposure to iloprost was set at 100% (control). Experiments were performed in triplicate and data are expressed as the mean ± SEM (n = 3). In 5, 6, 7, 8, and 9, washed platelets were preincubated in the dark with 5 μM NBD-Cl for 5 min at 25°C before subsequent treatments.

main in aggregin and covalent and simultaneous modification of such residues by o-phthaladehyde led to loss of ADP-induced platelet responses [Puri and Colman, 1991]. Results showing that platelets exposed to p-chloromercuribenzenesulfonate (a sulfhydryl group modifying reagent) and 8-BDB-TADP were not labeled by [^{14}C]-NBD-CI (data not shown) are consistent. The results show that NBD-CI covalently modifies aggregin, a putative ADP-receptor, and such chemical modification is blocked by preincubation of platelets either by ADP/ATP or covalent modification by FSBA and 8-BDB-TADP.

DISCUSSION

NBD-CI inhibited ADP-induced shape change and aggregation of platelets in PRP in a concentration- and time-dependent manner. NBD-CI also inhibited ADP-induced shape change and aggregation of washed platelets in a concentration dependent manner. These results suggest that NBD-CI covalently modifies an ADP-binding site on the platelet surface. NBD-CI completely blocked ADP-induced binding of ^{125}I-fibrinogen to washed platelets. These results are consistent with those of the experiments that show that NBD-CI inhibited ADP-induced platelet aggregation. The rate of ADP-induced platelet aggregation was diminished by 40% under conditions of partial modification. Under the same experimental conditions, rates of collagen- and U46619-induced platelet aggregation were also reduced in NBD-CI-modified platelets. These results are in accord with the previous findings that covalent and selective modification of aggregin by FSBA and 8-BDB-TADP partially inhibited U46619- (a thromboxane mimetic) [Morinelli et al., 1983; Puri et al., 1995] and collagen- [Colman et al., 1986; Puri et al., 1995] induced platelet aggregation. Furthermore, we found that NBD-CI completely blocked ADP-induced platelet secretion of nucleotides by platelets in PRP.

Binding of ADP to its receptor releases from the dense tubular system Ca^{2+}, which serves to signal pathways that mediate platelet responses elicited by this agonist [Rink and Hellem, 1984]. Our results show that chemical modification of washed platelets by NBD-CI completely blocked mobilization of intracellular Ca^{2+} when the modified platelets were exposed to ADP. NBD-CI itself did not raise levels of intracellular calcium in washed platelets. ADP has been shown to antagonize elevation of intracellular levels of cAMP induced by prostaglandins [Mills and Smith, 1972]. Covalent modification of washed platelets by NBD-CI completely blocked the ability of ADP to inhibit stimulated adenylate cyclase activity.

[^{14}C]-NBD-CI predominantly labeled one protein (100 kDa). The presence of this protein band was noticeably absent when FSBA- and 8-BDB-TADP-modified platelets were labeled by [^{14}C]-NBD-CI. Since selective and covalent modification of aggregin by FSBA [Bennett et al., 1978; Figures et al., 1981] and the ADP analogue 8-BDB-TADP [Puri et al., 1995] leads to inhibition of all ADP-induced platelet functions, it seems highly probable that the major [^{14}C]-NBD-CI-labeled protein band corresponds to aggregin, a putative ADP receptor. These results are further strengthened by the fact that 30 mM

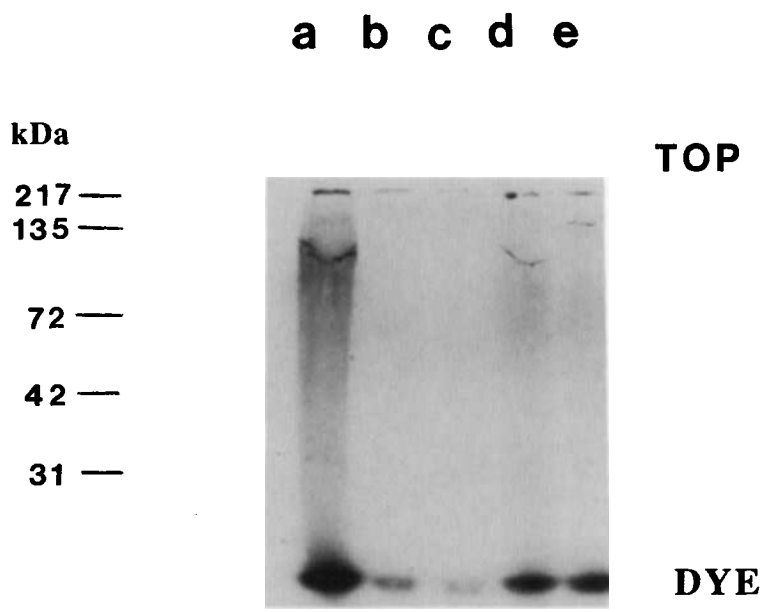

Fig. 7. SDS–PAGE of platelets labeled with [^{14}C]-NBD-CI: Platelets were labeled with various reagents, solubilized and subjected to SDS–PAGE on 10% gels according to the procedure of Laemmli [1970] as described in Materials and Methods. Platelets (2×10^8/0.2 ml) were incubated in the dark with [^{14}C]-NBD-CI (5 μM) for 5 min at 25°C (*lane a*), platelets were modified by 100 μM FSBA in the presence of adenosine deaminase [Puri et al., 1989] and FSBA-modified platelets incubated with [^{14}C]-NBD-CI as described above (*lanes b,c*), platelets preincubated with 30 mM ATP for 2 min at 25°C followed by exposure to [^{14}C]-NBD-CI (*lane d*), and platelets preincubated with 30 mM ADP for 2 min at 25°C before labeling by [^{14}C]-NBD-CI (lane e). The gel was calibrated with prestained molecular-weight standards (BioRad), each of which was covalently attached to a dye of different color and consisted of the following: Myosin, 217 kDa; β-galactosidase, 135 kDa; bovine serum albumin, 72 kDa; carbonic anhydrase, 42 kDa, and soybean trypsin inhibitor, 31 kDa. The values of the apparent molecular weight are those supplied by BioRad and correspond to those of the proteins derivatized by the dyes and are different from those of the underivatized proteins. The dense band of radioactivity at the bottom of the gel is due to unbound radioactivity and long time of exposure of the gel to X-ray film.

ADP or ATP almost completely blocked incorporation of [^{14}C]-NBD-CI into aggregin. We previously showed that concentrations of ADP and ATP, as high as 10 mM, were required to prevent labeling of aggregin completely by 8-BDB-TADP [Puri et al., 1995]. Covalent modification of the ADP-binding site by NBD-CI is a kinetically controlled. Such chemical reactivity stems from the high degree of electrophillic character of NBD-CI (Fig. 1C) [Shipton et al., 1976; Baines et al., 1977], which is manifested in rapid reactions with nucleophiles, such as thiol functions in proteins [Blockhurst et al., 1981]. On the other hand, reversible and equilibrium binding of ADP to its receptor, aggregin, is a thermodynamically controlled reaction. It is therefore reasonable to expect that although a small concentration of ADP is adequate to cause platelet activation, it is not enough to prevent incorporation of NBD-CI into aggregin. These results are consistent with the recent findings that demonstrated that a concentration of ATP, as high as 30 mM, was needed to block ADP-induced mobilization of intracellular Ca^{2+} in HEL cells [Shi et al., 1995]. Similar results have been obtained during investigations of the protection of yeast hexokinase from inactivation due to covalent modification by OPTH [Puri et al., 1988] and Cibacron blue 3G-A [Puri and Roskoski, 1994].

The results of this investigation, demonstrating that NBD-CI blocked all of the ADP-induced platelet responses by covalently modifying aggregin, a putative ADP receptor, are consistent with previous investigations identifying aggregin as an ADP receptor on the platelet surface [Bennett et al., 1978; Figures et al., 1981; Morinelli et al., 1983; Colman et al., 1986; Puri et al., 1995]. Our results make it unlikely that GPIIbα [Greco et al., 1991] or a 43-kDa protein [Cristalli and Mills, 1993] are candidates for an ADP receptor. However, these findings do not rule out the possibility that aggregin contains two ADP-binding sites on aggregin, differing in their affinity for ADP, that mediate shape change and antagonize the stimulated adenylate cyclase activity [Mills et al., 1985, 1992]. Indeed, the

data show that lower concentrations of ADP are adequate to cause shape change of platelets but much higher concentrations of ADP inhibit stimulated adenylate cyclase activity. Another possibility is that the same receptor uses different signal-transducing mechanisms mediated by different G-proteins for ADP-induced shape change and inhibition of stimulated adenylate cyclase activity [cf. Burnstock, 1990; Dubyak, 1991; El-Maotassin et al., 1992].

In summary, we have demonstrated that NBD-CI inhibits all the ADP-induced platelet responses by covalently modifying aggregin, a putative ADP-receptor, on the platelet surface. The chemical modificatio of aggregin by NBD-CI probably involves covalent modification of a sulfhydryl group in the ADP-binding domain of aggregin.

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REFERENCES

Baines BS, Allen G, Blockhurst K (1977): The highly electrophilic character of 7-chloro-4-nitrobenzfurazan and possible consequences for its application as a protein-labelling reagent. Biochem J 163:189–192.

Bennett JS, Colman RF, Colman RW (1978): Identification of adenine nucleotide proteins in human platelet membranes by affinity labeling by 5′-p-fluorosulfonylbenzoyladenosine. J Biol Chem 253:7346–7354.

Blockhurst K, Baines BS, Malthouse JPG (1981): Differences in the interactions of catalytic groups of the active centers of actinidin and papain. Biochem J 197:739–746.

Born GVR (1962b): Aggregation of blood platelets by adenosine diphosphate and its reversal. Nature 194:927–929.

Brass LF, Shattil SJ (1986): Inhibition of thrombin-induced platelet activation by leupeptin. J Biol Chem 263:5210–5216.

Burnstock G (1990): Purinergic mechanisms. Ann NY Acad Sci 603:1–17.

Cobbold PH, Rink TJ (1987): Fluorescence and bioluminescence measurements of cytoplasmic free calcium. Biochem J 248:313–328.

Colman RW (1990): Aggregin: a plate ADP receptor that mediates activation. FASEB J. 4:1425–1435.

Colman RW (1992): Platelet ADP receptors stimulating shape change and inhibiting adenylate cyclase. News Physiol Sci 7:274–278.

Colman RW, Puri RN (1995): Covalent modification of aggregin, an ADP receptor, by 7-chloro-4-nitrobenz-2-oxa-1,3-diazole results in a loss of ADP-induced platelet functions. FASEB J 9:1421.

Colman RW, Figures WR, Scearce ML, Strimmpler AM, Zhou F, Rao K (1986): Inhibition of collagen-induced platelet activation by 5′-p-fluorosulfonylbenzoyladenosine: Evidence for an ADP-requirement and synergistic influence of prostaglandin endoperoxides. Blood 68:565–570.

Cristalli G, Mills DCB (1993): Identification of a receptor for ADP by photoaffinity labelling. Biochem J 291:875–881.

Devary Y, Gottlieb RA, Smeal T, Karin M (1992): The mammalian ultraviolet response is triggered by activation of *src* kinases. Cell 71:1081–1091.

Dubyak GR (1991): Signal transduction by P2-purinergic receptors for extracellular ATP. Am J Respir Cell Mol Biol 4:295–300.

El-Maotassin J, Dorrand Mani JC (1992): Extracellular ATP and cell signalling. Biochim Biophys Acta 1134:31–45.

Ferguson SJ, Lloyd WJ, Radda GK (1975): The mitochondrial ATPase: Selective modification of a nitrogen residue in the β-subunit. Eur J Biochem 54:127–133.

Figures WR, Niewiarowski S, Morinelli TA, Colman RF, Colman RW (1981): Affinity labeling of human platelet membrane protein with 5′-p-fluorosulfonylbenzoyladenosine. J Biol Chem 256:7789–7795.

Garrder AA, Jonsen A, Laland S, Hellem S, Owen PA *(1961)*: Adenosine diphosphate in red cells as a factor in the adhesiveness of human blood platelets. Nature 192:531–532.

Ghosh PB, Whitehouse MW (1968): 7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole: A new fluorigenic reagent for amino acids and other amines. Biochem J 108:55–156.

Greco NJ, Yammamoto N, Jackson BW, Tandon NN, Jamieson GA (1991): Low structural specificity for nucleoside triphosphates as antagonists of ADP-induced platelet activation. J Biol Chem 266:13627–13633.

Hartl FT, Roskoski RR Jr (1982): Adenosine 3′,5′-cyclic monophosphate protein kinase from bovine brain: Inactivation of the catalytic subunit and holoenzyme by 7-chloro-4-nitro-2 1,3-benzoxadiazole. Biochemistry 21:5175–5183.

Hourani SMO, Cusack NJ (1991): Pharmacological receptors on blood platelets. Pharmacol Rev 43:243–298.

Kaibuchi T, Terutaka T, Kikuchi A, Tamimoto T, Yanashita T, Takai Y (1986): Possible involvement of protein kinase C and calcium ion in growth factor-induced expression of c-*myc* oncogene in Swiss 3T3 fibroblasts. J Biol Chem 261:3025–3029.

Laemmli UK (1970): Cleavage of structural proteins during assambly of the head of bacteriophage T4. Nature 227:680–685.

Mills DCB, Smith JB (1972): The control of platelet responsiveness by agents that influence cyclic AMP metabolism. Ann NY Acad Sci 201:391–399.

Mills DCB, Figures WR, Scearce ML, Stewart GJ, Colman RF, Colman RF (1985): Two mechanisms for inhibition of ADP-induced shape change by 5′-p-fluorosulfonylbenzoyladenosine. J Biol Chem 260:8078–8083.

Mills DCB, Puri RN, Hu CJ, Minniti C, Grana G, Freedman MD, Colman RF, Colman RW (1992): Clopedogrel inhibits the binding of ADP analogues to the receptor mediating inhibition of platelet adenylate cyclase. Arterioscler Thromb 12:430–436.

Morinelli TA, Niewiarowski S, Kornecki E, Figures WR, Wachtfogel Y, Colman RW (1983): Platelet aggregation and exposure of fibrinogen receptors by prostaglandin endoperoxide analogs. Blood 61:41–49.

Murgo AJ, Contrera GJ, Sistare FD (1994): Evidence for separate calcium signalling P2t and P2u purinoreceptors in human megakaryocytic Dami cells. Blood 33:1258–1267.

Nishizuka Y (1984): The role of protein kinase C in cell surface signal transduction and tumor promotion. Nature 308:693–698.

Puri RN, Colman RW (1991): Inhibition of ADP-induced platelet shape change and aggregation by o-phthaladehyde: Evidence for covalent modification of cysteine and lysine residues. Arch Biochem Biophys 286:419–427.

Puri RN, Colman RW (1994): Inhibition of ADP-induced platelet activation by 7-chloro-4-nitrobenz-2-oxa-1,3-diazole. Blood (suppl) 81:690a.

Puri RN, Roskoski RR Jr (1994): Inactivation of yeast hexokinase by Cibacron blue 3G-A: Spectral, kinetic and structural investigations. Biochem J 300:91–97.

Puri RN, Bhatnagar D, Roskoski RR Jr (1988): Inactivation of yeast hexokinase by o-phthaladehyde: Evidence for the presence of a cysteine and lysine residue at or near the active site. Biochim Biophys Acta 957:34–46.

Puri RN, Zhou F, Bradford H, Hu CJ, Colman RF, Colman RW (1989): Thrombin-induced platelet aggregation involves an indirect proteolytic cleavage of aggregin by calpain. Arch Biochem Biophys 271:346–358.

Puri RN, Zhou F, Hu CJ, Colman RF, Colman RW (1991): High molecular weight kininogen inhibits thrombin-induced platelet aggregation and cleavage of aggregin by inhibiting binding of thrombin to platelets. Blood 77:500–507.

Puri RN, Matseuda R, Umeyama H, Bradford HN, Colman RW (1993): Modulation of thrombin-induced platelet aggregation by inhibition of calpain by a synthetic peptide derived from the thiol-protease inhibitory sequence of kininogens and *S*-(3-nitro-2-pyridinesulfenyl)-cysteine. Eur J Biochem 214:233–241.

Puri RN, Kumar A, Chen H, Colman RF, Colman RW (1995): Inhibition of ADP-induced platelet responses by covalent modification of aggregin, a putative ADP-receptor, by 8-(4-bromo-2,3-dioxobutylthio)ADP. J Biol Chem 270:24482–24488.

Rink TJ, Hellem TJ (1984): What turns platelets on? Trend Biochem Sci 9:215–219.

Schieven GL, Ledbetter JA (1993): Ultraviolet radiation induces differential calcium signals in human perpheral blood lymphocyte subsets. J Immunother 14:221–225.

Schieven GL, Klrihara JM, Gilliland LK, Uckum FM, Ledbetter JA (1993): Reactive oxygen intermediates activate NF-KB in a tyrosine kinase dependent mechanism and in combination with vanadate activate p^{56lck} and $p59^{fyn}$ tyrosine kinases in human lymphocytes. Mol Biol Cell 4:523–530.

Shi XP, Yin KC, Gardell SJ (1995): Human erythroleukemic (HEL) cells express a platelet P2T-like ADP receptor. Thromb Res 77:235–237.

Shipton M, Stuchbury T, Blockhurst K (1976): 7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole as a reactivity probe for the investigation of the thiol proteinases. Biochem J 159:235–244.