Characterization of the Platelet Prostaglandin D_2 Receptor

LOSS OF PROSTAGLANDIN D_2 RECEPTORS IN PLATELETS OF PATIENTS WITH MYELOPROLIFERATIVE DISORDERS

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ABSTRACT Prostaglandin (PG) D_2 is synthesized in platelets at concentrations which could inhibit aggregation via activation of adenylate cyclase. To more directly define platelet-PG interactions, a binding assay has been developed for platelet PG receptors with [^{3}H]PGD_2 as ligand. [^{3}H]PGD_2 binding to intact platelets was saturable and rapid with the ligand bound by 3 min at 20°C. PG competed with the [^{3}H]PGD_2 binding site with a potency series: PGD_2 (IC_{50} = 0.08 μM) $\gg$ PGI_2 (IC_{50} = 2 μM) > PGE_1 (IC_{50} = 6 μM) > $PGF_{2\alpha}$ (IC_{50} = 8 μM). Scatchard analysis of binding data from six normal subjects showed a single class of binding sites with a dissociation constant (K_d) of 53 nM and 210 binding sites per platelet. This PGD_2 receptor assay was then used to study platelets from five patients with myeloproliferative disorders (polycythemia vera, essential thrombocythemia, and chronic myelogenous leukemia), as over 90% of these patients have platelets resistant to the effects of PGD_2 on aggregation and adenylate cyclase activity (1978. *Blood.* **52**: 618–626.). In the presence of 50 nM [^{3}H]PGD_2, the patients' platelets bound 7.1±2.9 fmol ligand/10^8 platelets compared with 15.1±1 fmol/10^8 platelets in normals, a decrease of 53% ($P < 0.01$). Scatchard analysis showed that the K_d of [^{3}H]PGD_2 binding (33 nM) was comparable to normal platelets, which indicates that the decreased PGD_2 binding in these platelets represented fewer receptors rather than altered affinity of the ligand for the binding site. The 53% decrease in [^{3}H]PGD_2 binding correlated with a 48% decrease in PGD_2-activated platelet adenylate cyclase. The characterization of the platelet PGD_2 binding site provides further direct evidence that there are at least two PG receptors on platelets, one for PGE_1 and PGI_2, and a separate receptor for PGD_2. Direct binding analysis will be a useful tool for studying the role of PG in regulating platelet function, as demonstrated by the selective loss of PGD_2 binding sites in patients with myeloproliferative disorders.

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INTRODUCTION

There is increasing evidence that prostaglandins and their derivatives may regulate platelet aggregation (1). Whereas certain prostaglandin (PG)[1] intermediates such as PGG_2 and PGH_2, as well as thromboxanes, may induce platelet aggregation, other stable PG such as PGI_2, PGE_1, and PGD_2 are potent inhibitors of platelet aggregation via activation of adenylate cyclase (2–9). PGI_2 is synthesized by endothelial cells and may play an important role in mediating platelet-endothelial cell interactions (2). However, of these PG only PGD_2 is produced by platelets at sufficient concentrations to potentially inhibit platelet aggregation (9). As such, a binding assay characterizing the platelet PGD_2 receptor would be a useful tool to further our understanding of platelet-PG interactions. With the availability of [^{3}H]PGD_2 for use as a radioligand, such binding studies are now feasible.

Data from other investigators (10–12) and our laboratory (13) have suggested that there is a common platelet receptor for PGE_1 and PGI_2, which is distinct from

[1] *Abbreviations used in this paper*: IC_{50}, concentration of unlabeled prostaglandin needed to displace 50% of [^{3}H]PGD_2; PG, prostaglandin(s).

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the PGD_2 binding site. This hypothesis is also supported by the recent report that platelets from patients with myeloproliferative disorders are resistant to PGD_2 but respond normally to PGI_2 and PGE_1 (14). Platelets from these patients require higher than normal concentrations of PGD_2 to inhibit collagen-induced serotonin release, and stimulation of platelet adenylate cyclase by PGD_2 is blunted. These studies provide the initial characterization of the platelet PGD_2 receptor and demonstrate its use in studying the PGD_2 receptor in platelets from patients with myeloproliferative disorders.

METHODS

Binding assay. Platelet-rich plasma was obtained from 200 ml of venous blood anticoagulated with 13.5% acid citrate dextrose (National Institutes of Health Formula A) by centrifugation at 160 *g* for 10 min. The platelet-rich plasma was adjusted to pH 6.5 with additional acid citrate dextrose, and a platelet pellet was prepared by centrifugation at 1,500 *g* for 10 min. The pellet was suspended in 10 ml of buffer containing 8 mM Na_2HPO_4, 2 mM NaH_2PO_4, 10 mM EDTA, 5 mM KCl, and 135 mM NaCl, pH 7.2, and recentrifuged at 1,500 *g* for 10 min. The supernate was discarded, and the platelet pellet was resuspended in 3.5 ml of assay buffer (138 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, and 25 mM Tris-HCl, pH 7.5). If necessary, additional assay buffer was added to obtain a platelet concentration of $1-1.5 \times 10^8$ platelets/0.10 ml.

Incubations were carried out in a total volume of 200 μl which included 100 μl of platelet suspension. Unless otherwise stated, the platelets were incubated at 20°C for 20 min with indicated concentrations of [^{3}H]PGD_2. Separate tubes containing 10 μM of nonradioactive PGD_2 were included to determine "nonspecific" binding. "Specific binding" was defined as the difference between radioactivity bound in the presence and absence of 10 μM PGD_2 and is referred to in all figures. Specific binding amounted to 50–60% of total bound counts. After the incubation, 4 ml of ice-cold buffer (50 mM Tris/HCl, pH 7.4) was added to each tube, and the contents were rapidly filtered through a Whatman GF/C glass microfiber filter (Whatman, Inc., Clifton, N. J.) under reduced pressure. Each assay tube and the filter were then washed successively with four 5-ml portions of ice-cold buffer. Whereas nonspecific binding decreased from 50 to 30% with serial washings, specific binding did not decrease. The filter was then dried, suspended in 10 ml of Beckman Ready-Solv liquid scintillation cocktail (Beckman Instruments, Inc., Fullerton, Calif.), and counted in a Packard Tri-carb scintillation spectrometer (Packard Instrument Co., Downers Grove, Ill.) with an overall efficiency of 60%. [^{3}H]PGD_2 (70 Ci/mmol) was provided by New England Nuclear (Boston, Mass.) with a radiochemical purity of >98% by high-pressure liquid chromatography. Other PG were a gift from Dr. John Pike (The Upjohn Co., Kalamazoo, Mich.). Platelet counts were performed with an electronic particulate counter (Coulter model ZF, Coulter Electronics, Inc., Hialeah, Fla.).

Calculations. The interaction of [^{3}H]PGD_2 with platelets was analyzed by the method of Scatchard (15). The dissociation constant (K_d) for [^{3}H]PGD_2 binding was then determined by linear regression analysis of equilibrium binding data. The concentration of unlabeled PG needed to displace 50% of the [^{3}H]PGD_2 (IC_{50}) was estimated by visual inspection of the competition curves. The differences between means were assessed by Student's *t* test.

Adenylate cyclase assay. Platelets from 20 ml of blood were prepared and washed twice in buffer as described above. The platelet pellet was then frozen and thawed in a dry ice-acetone bath and suspended in 2 ml of ice-cold Tris-saline (15 mM Tris-HCl, 138 mM NaCl, pH 7.6). Enzyme activity of the platelet suspension was measured immediately after thawing as previously described (14).

Patients. Five patients with myeloproliferative disorders were selected from the Outpatient Hematology Clinic population of the Peter Bent Brigham Hospital and the West Roxbury Veterans Administration Medical Center. Patients studied included two with polycythemia vera, two with essential thrombocythemia, and one with chronic myelogenous leukemia. Four of these patients were included in a prior report of 23 patients with myeloproliferative disorders having diminished PGD_2-sensitive adenylate cyclase activity (14). Patients were not taking any drugs known to interfere with platelet function. The two patients with essential thrombocythemia had elevated platelet counts ($>10^6$ platelets/mm^3), but the other three patients had platelet counts in the normal range. Five patients with reactive thrombocytosis were also studied. These patients averaged 56 yr of age and included one splenectomized patient with beta thalassemia intermedia; three infected, febrile patients; and one patient with Crohn's disease. These patients had platelet counts between 550,000 and 800,000/mm^3. The experimental protocol was reviewed and approved by the hospital committee for the protection of human subjects. Platelets from six normal volunteers were also studied, and a normal control was included along with each patient sample for both binding studies and measurement of adenylate cyclase activity.

RESULTS

Kinetics and specificity of [^{3}H]PGD_2 binding. Fig. 1 illustrates that [^{3}H]PGD_2 binding to intact platelets was rapidly saturable and reversible. At 20°C most of the ligand was bound within 3 min. Binding was stable for at least 40 min, and the addition of 10 μM of unlabeled PGD_2 at equilibrium resulted in rapid displacement of [^{3}H]PGD_2. Because of the rapid binding and dissociation of the ligand, accurate calculation of a rate constant or kinetic K_d was not possible. Fig. 2 illustrates the

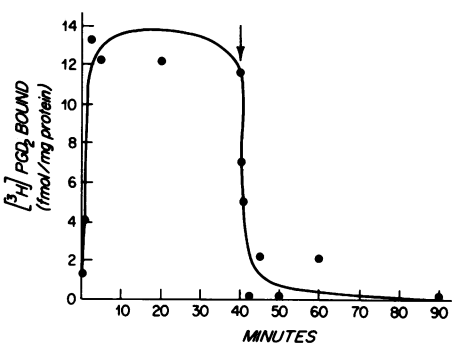

FIGURE 1 Time-course of binding and dissociation of [^{3}H]-PGD_2 to intact platelets at 20°C. Platelets were incubated with 3 nM [^{3}H]PGD_2, and samples were withdrawn at the indicated time. At 40 min (indicated by arrow) 10 μM of unlabeled PGD_2 was added to initiate dissociation of [^{3}H]PGD_2 from the platelets. Each point represents mean of duplicate determinations and is expressed as specific binding.

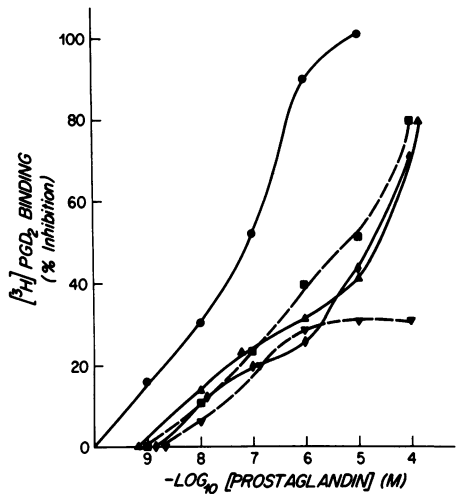

FIGURE 2 Inhibition of [^{3}H]PGD_2 binding to platelets by PGD_2 (●), PGI_2 (■), PGE_1 (◆), $PGF_{2\alpha}$ (▲), and PGE_2 (▼). [^{3}H]PGD_2 at a concentration of 4 nM was incubated for 20 min at 20°C with PG at the concentrations indicated. Results are expressed as percent inhibition of [^{3}H]PGD_2 binding in the absence of added unlabeled PG. Each point represents the mean of two experiments with duplicate determinations at each point.

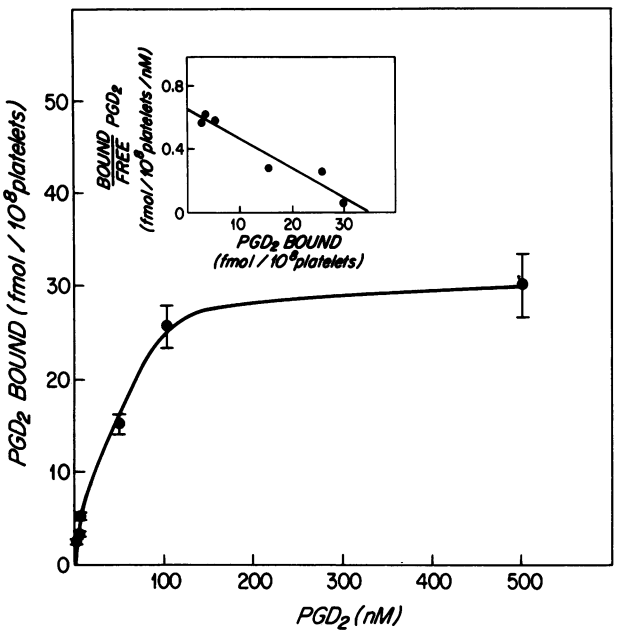

FIGURE 3 The binding of [^{3}H]PGD_2 to intact platelets is depicted. Mean values±SEM of triplicate determinations from six separate experiments are shown. Platelets were incubated with 4 nM [^{3}H]PGD_2 plus 0 to 10 μM of unlabeled PGD_2 for 20 min at 20°C. Total binding was determined for each point by dividing counts per minute by the calculated specific activity obtained by diluting 4 nM [^{3}H]PGD_2 with a known concentration of unlabeled PGD_2. The inset shows the Scatchard analysis of this data. The line represents the least squares fit of the data points and has a slope of -0.18×10^9 M ($r = 0.96$). The reciprocal of the slope, the K_d, equals 54 nM. The intercept of the line with the ordinate gives an estimate of 35 fmol receptor/10^8 platelets.

ability of unlabeled PG to compete for the [^{3}H]PGD_2 binding site. There was a reproducible potency series in inhibiting [^{3}H]PGD_2 binding. PGI_2 (IC_{50} = 2 μM) competed closely with PGE_1 (IC_{50} = 6 μM) and $PGF_{2\alpha}$ (IC_{50} = 8 μM), but all required 100-fold greater concentrations than PGD_2 (IC_{50} = 0.08 μM). PGE_2 competed very poorly with only 30% displacement of the label at 0.1 mM.

Saturability and affinity of [^{3}H]PGD_2 binding. The pattern of [^{3}H]PGD_2 binding to intact platelets is shown in Fig. 3. Saturation occurred at 30 fmol ligand bound/10^8 platelets. At a ligand concentration of 50 nM, one-half of the binding sites were occupied. This value gives an estimate of the K_d of [^{3}H]PGD_2 for the binding sites. Analysis of the data by Scatchard plot (Fig. 3, inset) demonstrated a single class of binding sites with a K_d of 54 nM. This analysis gave an estimate of 35 fmol ligand bound/10^8 platelets, which represents 210 binding sites per platelet.

[^{3}H]PGD_2 binding in myeloproliferative disorders. To determine [^{3}H]PGD_2 binding to platelets from patients with myeloproliferative disorders, ligand binding was studied with 54 nM PGD_2. This concentration of PG approximates the K_d of [^{3}H]PGD_2 for the binding sites. In normal platelets, 15.1±1 fmol ligand bound/10^8 platelets was noted, whereas in the patients there was only 7.1±2.9 fmol ligand bound/10^8 platelets, a decrease of 53% ($P < 0.01$). Although altered platelet size in these patients could theoretically account for part of this discrepancy, this variable is unlikely because three of the five patients had normal platelet counts and the average protein per platelet in patients (3.5±0.4 mg/10^9 platelets) was the same as normals (3.5±0.3 mg/10^9 platelets). In addition, five patients with reactive thrombocytosis were studied, and platelets from all these individuals had normal PGD_2 binding with 17.8 ±3.0 fmol ligand bound/10^8 platelets in the presence of 54 nM PGD_2.

To determine whether the diminished PGD_2 binding noted in platelets from patients with myeloproliferative disorders represented decreased numbers of binding sites, rather than an altered affinity of the ligand for receptor, a saturation curve was carried out in one patient. As shown in Fig. 4, Scatchard analysis indicated that, like normal platelets, the patient's platelets contained a single class of binding sites. The K_d of 33 nM is comparable to the K_d of 54 nM of normal platelets, but only 11 fmol ligand/10^8 platelets were bound (66 receptors per platelet) compared with the 35 fmol bound/10^8 platelets in normals (Fig. 3). These

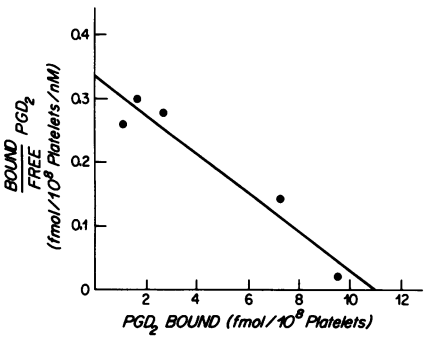

FIGURE 4 Scatchard analysis of [^{3}H]PGD_2 binding to platelets in a patient with myeloproliferative disorder. Platelets were incubated under conditions described in Fig. 2. Each point represents the mean of triplicate determinations. The line represents the least squares fit of the data points (r = 0.97) with a slope of -0.030×10^9 M. The K_d = 33 nM with an estimated 11 fmol receptor/10^8 platelets.

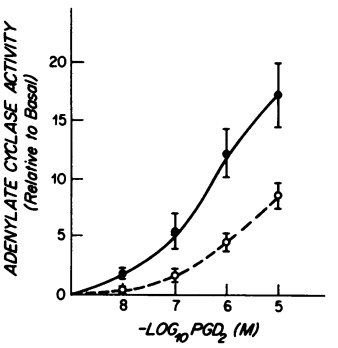

FIGURE 5 Effects of PGD_2 on platelet adenylate cyclase activity in normals (●) and in patients with myeloproliferative disease (○). Enzyme activity is expressed as a ratio of stimulated to basal activity. Mean±SEM for five normals and five patients.

data indicate that the abnormal platelets contain fewer PGD_2 binding sites rather than altered affinity of the ligand for the receptor.

Adenylate cyclase activity. Fig. 5 compares the PGD_2-sensitive platelet adenylate cyclase activity in the five patients with normal platelets. Dose-response curves show decreased stimulation of the patient's adenylate cyclase at all concentrations of PGD_2. In the presence of 0.1 μM PGD_2 there was 5.8-fold enzyme activation in normal platelets compared with 2.3-fold enhancement in those of patients. At the highest concentration of PGD_2 tested (10 μM) the PGD_2 activated adenylate cyclase 16.5-fold in controls as compared with only 8.6-fold in the patients, a decrease of 48%. In contrast with the decreased stimulation observed with PGD_2, maximal enzyme stimulation by PGE_1 (29 ±2 times basal) was the same as normals (28±2 times basal). In addition, enzyme activation by sodium fluoride (6.7±0.4 times basal) in the patients was in the normal range (7.0±0.6), and basal activity was comparable for the patients and normals (both: 0.1 nM cyclic AMP/mg protein per 10 min). Finally, the concentration of PGD_2 required for half-maximal stimulation of adenylate cyclase in the patients (0.7 μM) was comparable to normal platelets (0.3 μM).

DISCUSSION

These studies demonstrate that the binding of [^{3}H]-PGD_2 to intact platelets is rapid, reversible, and saturable. There is excellent correlation between the binding K_d (54 nM), the previously reported (14) half-maximal concentration of PGD_2 needed to inhibit collagen-induced serotonin release (50 nM), and the concentration of PGD_2 required for half-maximal stimulation of adenylate cyclase (300 nM). The slightly (sixfold) higher concentration of PGD_2 required to produce half-maximal stimulation of adenylate cyclase than to cause half-maximal displacement of [^{3}H]PGD_2 binding has also been noted in β-adrenergic receptor systems (16) and PG receptor systems (17), and is probably because the nucleotide (ATP) present in the enzyme assay, but not in the binding assay, decreases the affinity of the PG for the receptor.

With the characterization of the platelet PGD_2 receptor, there is direct evidence for at least two platelet PG receptors. PGE_1 and PGI_2 compete very poorly with [^{3}H]PGD_2, whereas the previously reported competition of these two PG for [^{3}H]PGE_1 and [^{3}H]PGI_2 correlated with their potent effects on platelet adenylate cyclase and on platelet aggregation (12, 13). In these prior studies PGD_2 competed very poorly for [^{3}H]PGE_1 (IC_{50} = 70 μM), or [^{3}H]PGI_2 (IC_{50} > 100 μM), values over 1,000-fold greater than noted in our assay (54 nM).

The fact that platelets of patients with myeloproliferative disorders have a selective resistance to PGD_2 further supports the presence of at least two platelet PG receptors. We recently studied patients with polycythemia vera, chronic myelogenous leukemia, essential thrombocythemia, and myeloid metaplasia (14) and found that these patients required 10-fold higher than normal concentrations of PGD_2 to inhibit collagen-induced platelet serotonin release. Whereas stimulation of platelet adenylate cyclase by PGE_1, PGI_2, and sodium fluoride was normal, enzyme stimulation by PGD_2 was blunted in 90% of the 23 patients studied and correlated with the serotonin-release data found with intact platelets. We postulated that decreased platelet PGD_2 receptors, or altered affinity of the ligand for the binding site, probably accounted for these observations. With the radioligand binding assay for PGD_2, we now find that these patients have lost 53% of their PGD_2 binding sites but have retained normal

receptor-ligand affinity. Moreover, this loss of PGD_2 receptors correlates with the 50% decrease in maximal platelet adenylate cyclase activation.

The role of PGD_2 in platelet metabolism has not been clarified. Because sufficient quantities of this PG (17 nM) are released into plasma during aggregation to potentially inhibit platelet aggregation (9), it is theoretically possible, although not yet demonstrated, that this PG could play a physiologic role in the regulation of platelet aggregation (6–9). Characterization of the PGD_2 platelet receptor, as well as the identification of certain platelets that have decreased number of these receptors, may lead to a better understanding of platelet-PG interactions.

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