Defective Platelet-Fibrinogen Interaction in Hereditary Canine Thrombopathia

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A unique, intrinsic, hereditary canine platelet disorder attributable to abnormal fibrinogen receptor availability is described. Thrombopathic platelets from 13 severely affected basset hounds failed to aggregate in response to all agonists tested except thrombin. Normal platelet interaction with the various stimuli was inferred on the basis of their ability to elicit unimpaired shape change in thrombopathic platelets. No quantitative differences in major platelet membrane glycoproteins, intraplatelet fibrinogen, adenine nucleotides, or serotonin uptake were detected. Dense granule secretion was impaired. The ultrastructural appearance of thrombopathic platelets was normal. Fibrinogen-platelet interaction was evaluated by reacting platelet-rich plasma (PRP) with fibrinogen coupled to polymeric acrylonitrile beads and scoring the extent of stimulus-induced agglutination. The aggregatory responses of normal and thrombopathic platelets were closely correlated with fibrinogen receptor availability. In contrast to human platelets, epinephrine-stimulated canine platelets did not interact with immobilized fibrinogen, and arachidonate generally induced only weak agglutination. Thrombopathic platelets agglutinated fibrinogen beads at reduced rates when stimulated with physiologic doses of thrombin and high-dose calcium ionophore, A23187. Our data suggest that thrombin-mediated induction of canine platelet fibrinogen receptors may proceed by pathway[s] alternate to those shared by other platelet agonists, and/or that secreted granule constituents may act synergistically with thrombin to overcome inhibition of signal-response-coupled reactions mediating the interaction of fibrinogen with its receptor. This congenital platelet defect provides further evidence, in a species other than human, for the pivotal role of fibrinogen receptor induction in platelet aggregation.

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FIBRINOGEN appears to play a central role in platelet aggregation. The requirement for plasma-derived fibrinogen in ADP-mediated aggregation is well established,1-3 and there is evidence to suggest that platelet-derived fibrinogen also supports aggregation. Several groups have demonstrated that under the appropriate physiologic conditions, specific and saturable receptors for the molecule are exposed on the platelet surface.5-11 A good correlation between fibrinogen binding and platelet aggregability9 but not shape change14 exists. The surface receptor for fibrinogen is induced by ADP,5 epinephrine,6 collagen,7 arachidonic acid,13,15 or thrombin.14 Prostaglandin I2 has been reported to inhibit receptor exposure,16 presumably in response to elevated levels of cyclic AMP.17

The putative receptor for fibrinogen, present on the surface of unstimulated platelets,18-20 is a calcium-dependent heterodimer complex formed by the association of two major platelet membrane glycoproteins, GPIIb and GPIIIa.24,25 When platelets are stimulated, presumed changes in the conformation and/or microenvironment of the complex occur, through as yet undefined mechanism(s), leading to expression of specific binding sites for fibrinogen and other adhesive proteins including fibronectin, von Willebrand factor, and thrombospondin.26

The functional significance of the GPIIb-IIIa complex is underscored in the rare congenital bleeding disorder, Glanzmann's thrombasthenia, in which the platelets fail to aggregate in response to all known physiological stimuli.27 This severe platelet dysfunction is attributed to the absence or marked reduction of both membrane glycoproteins28-31 and the consequent failure of thrombasthenic platelets to bind fibrinogen.5,9 A hereditary platelet defect having some features in common with human thrombasthenia is quite prevalent in basset hounds.32,33 However, the specific defect in canine thrombopathia appears to be unique and may represent a platelet disorder as yet unrecognized in humans.

This article is the first detailed characterization of canine thrombopathia. Our results suggest that the severe platelet dysfunction may be due to the failure of select platelet agonists to initiate expression of sites on the platelet surface capable of normal interaction with fibrinogen. As such, it would be the first report of a congenital hereditary platelet defect attributable to abnormal fibrinogen receptor induction.

MATERIALS AND METHODS

Reagents. Sodium salts of ADP and ATP, type I bovine collagen, hirudin, (-)-epinephrine bitartrate, imipramine-HCl, N-2-hydroxy-ethyl-piperazine-N'-2-ethanesulfonic acid (HEPES), phosphoenolpyruvate, apyrase, n-ethylmalamide, orosomucoid, Triton X-114 (Rohm and Haas, Philadelphia) and pyruvate kinase were purchased from Sigma Chemical Co, St. Louis. Purified bovine albumin (Pentex fraction V) was purchased from Miles Laboratories, Elkhart, Ind; calcium ionophore, A23187, from Cal Biochem, Behring Corp, LaJolla, Calif; sodium arachidonate (SA; 99%) from Nu Check Prep, Elysian, Minn; human fibrinogen, grade L, from AB Kabi, Stockholm, Sweden; rabbit immunoglobulin to human fibrinogen from Dako, Denmark; [14C]-serotonin (as 5-hydroxytryptamine creatinine sulfate, 53 mCi/mmol) from Amersham Corporation, Arlington Heights, Ill; Sepharose 4B from Pharmacia Fine Chemicals, Sweden; and polymerized acrylonitrile beads, 1 to 3 μm in diameter, from Amicon Corporation, Lexington, Mass. Highly purified human α- and γ-thrombins were generous gifts from Dr John W. Fenton II, New York State Department of Health. All other reagents were of the highest purity available.

A fibrillar suspension of bovine collagen was prepared as described previously.24 ADP and epinephrine bitartrate were dis-
solved in 0.15 mol/L of NaCl; SA was dissolved in 0.05 mol/L Tris-0.15 mol/L NaCl, pH 7.4, and diluted prior to use. SA solutions appeared cloudy even after dilution. α-Thrombins and γ-thrombins were diluted with gel filtered platelet elution buffer containing albumin (0.35%). [14C]-serotonin was dissolved in 70% ethanol to a final activity of 8.8 μCi/mL. Ionophore A23187 was dissolved in Me2SO. All solutions were kept frozen at −60 °C until used. Epinephrine bitartrate was freshly prepared.

Animals. The study population consisted of 16 purebred basset hounds referred to our kennel facility because of their congenital bleeding diathesis.13 Thirteen of the dogs (six males, seven females) were clinically severely affected with thrombopathia; another 3 were asymptomatic and had an intermediate level of platelet dysfunction. The latter group were presumed to be heterozygous for the defect by virtue of having an affected parent or having produced one or more affected offspring. Some of the affected dogs were either littermates or closely related; others were more distantly related or appeared unrelated. Because the inheritance pattern of the defect within these inbred and linebred families of basset hounds is unclear at present,13 the clinically affected animals could be either homozygous for the trait or severely penetrant heterozygotes. Mortality is high in some litters within affected bloodlines, so homozygosity may be lethal, as seen in some breeds with canine von Willebrand's disease.13

The 16 basset hounds and the 30 healthy dogs of various breeds, including basset hounds or mongrels that served as controls for the study, were young adults of both sexes, housed in a kennel environment. None of the bitches were in estrus or pregnant when studied, and all animals received the same diet of maintenance dog meal supplemented with raw meat; water was provided ad libitum. None of the animals had received any medication within at least 2 weeks prior to study. Data for the three putative heterozygotes are not reported here because there were too few such animals for detailed studies of their platelet function.

Platelets. Blood was drawn from the cephalic vein of the adult fasting normal or thrombopathic dogs into plastic syringes containing ½ volume final volume of 3.8% trisodium citrate, acid citrate dextrose (4.5% Na citrate, 2.73% citric acid, 2% glucose) or 54 mmol/L of EDTA in 0.15 mol/L of NaCl. Platelet-rich plasma (PRP) was prepared at room temperature by centrifuging the blood for three 2-minute intervals at 590 g; platelet recovery exceeded 88%. For aggregation studies, the resulting supernatants were pooled and adjusted with autologous platelet-poor plasma (PPP) to a platelet count of 300,000/L. PPP and platelet-free plasma (PFP) were prepared at room temperature by centrifugation at 2,200 g for 15 minutes and at 16,000 g for 10 minutes, respectively.

Gel-filtered platelets (GFP) were prepared from citrated PRP (3 to 5 mL; 7.0 × 109 platelets per milliliter) essentially as described by Tangen and colleagues31 on a column (1.5 × 30 cm) packed with aceton-washed Sepharose 4B and equilibrated with modified Tangen-HEPES buffer 4B (147 mmol/L of NaCl, 5 mmol/L KCl, 0.05 mmol/L of CaCl2, 0.1 mmol/L of MgCl2, 5 mmol/L of HEPES, 5.5 mmol/L of glucose, pH 7.4) containing 0.35% bovine albumin. Platelet recovery was >90%. GFP were diluted with elution buffer to a final count of 300,000/μL. When required, the platelet suspension was made 1 mmol/L with respect to CaCl2. In some experiments, platelets were gel-filtered in dicalcium free-Tyrodes albumin (0.3%) containing 5 U per milliliter of heparin. Washed platelets were prepared from citrated PRP by the Stracian gradient technique of Corash and colleagues.34 For electrophoretic studies, platelets were washed three times and obtained as described by Norden and co-workers.34

Platelet function. Platelet aggregation was measured turbidimetrically35 in a dual-channel aggregometer (Payton Associates, Buffalo, NY) equipped with an OmniScribe recorder (Houston Instruments, Austin, Tex). PPP or platelet suspension buffer was used to represent 100% aggregation. In a typical experiment, 0.45 mL of PRP (300,000/μL) was pipetted into each 1-mL siliconized glass cuvette and warmed to 37 °C for one minute with stirring (850 rpm); then 0.05 mL of the appropriate agonist was added. When Me2SO was the agonist solvent, not more than 5 μL was added to the platelet suspension. The extent of platelet aggregation was measured after 3 minutes. When dual stimuli were used, they were applied one minute apart.

Formation of microaggregates in response to extremely high doses of ADP (>150 μmol/L) was confirmed by phase-contrast microscopy. Shape change events were monitored photometrically and verified by electron microscopy. [14C]-Serotonin was used as a marker for release of dense granule constituents, and release was measured essentially as described by Jerushalmi and Zucker,36 with inclusion of a formalin fixation step prior to centrifugation to minimize reported secretion artifacts associated with rapid centrifugation of A23187-treated platelets. Imipramine (3–10 μmol/L) was also included to inhibit serotonin reuptake.42

Retention of canine platelets on glass bead surfaces was measured by using whole blood as previously described.40 The average number of platelets eluted in the fourth and fifth milliliter of blood was used to calculate percentage of retention. Collagen-platelet interaction was evaluated by using noncovalently immobilized collagen.44 The ability of thrombopathic platelets to support normal clot retraction was determined according to the method of Taylor and Muller-Eberhard.45

Electron microscopy. For ultrastructural studies, citrated PRP (300,000/μL) was warmed with stirring (850 rpm) for 1 minute in an aggregometer; then 50 μL of the appropriate agonist or vehicle was introduced. The samples were fixed by rapidly adding one part 0.1% glutaraldehyde to the cuvette when the point of maximal optical density (platelet shape change) was reached. After ten minutes, the platelets were pelleted (800 g for five minutes); the supernatant was decanted; 1 mL of 3% glutaraldehyde was added; and the samples were sent to Dr James G. White, University of Minnesota, for processing.

Adenine nucleotides. ADP and ATP were quantitated in ethanolic extracts of EDTA PRP by the firefly luciferase method of Holmsen et al37 using an Aminco microphotometer (American Instruments Co, Silver Springs, Md) interfaced with a digital integrator.

Platelet fibrinogen. The amount of fibrinogen in normal and thrombopathic platelets was quantitated by rocket immunoelectrophoresis.42 The procedure of Nurden34 was adapted to measure canine platelet fibrinogen. In brief, washed normal and thrombopathic platelets (2 × 109 cells) were resuspended in 200 μL of 0.033 mol/L of Tris and 0.1 mol/L of glycine, pH 8.6, containing 1% (vol/vol) Triton X-100 and extracted for 30 minutes at 4 °C. Triton insoluble material was removed by centrifugation. The extracts were then electrophoresed as described in Fig 1. The assay was standardized using canine fibrinogen. Peak heights for fibrinogen standards were linear with respect to fibrinogen concentration from 50 to 500 ng per well. Assays were performed in duplicate. A standard curve was constructed from which platelet fibrinogen levels were determined. Maximal receptor sharpness using canine antigen with rabbit anti-human fibrinogen immunoglobulins could be achieved only when the plate temperature was maintained at 28 °C during electrophoresis. Paired normal and thrombopathic platelet extracts were run on the same plates.

Fibrinogen receptor interaction. Canine fibrinogen was isolated from ACD plasma as described by Regoecci and Stannard38 and was further purified39 to remove possible traces of plasminogen, fibronectin, and factors VIII and XIII. Highly purified canine fibrinogen, judged to be free of trace contaminants by sensitive and specific methods described elsewhere,39 was 95% thrombin-clottable. When
quantitation of intraplatelet fibrinogen by rocket immunoelectrophoresis. Triton soluble platelet proteins were diluted at least fourfold with Triton-free extraction buffer prior to well application to minimize Triton staining artifacts (dark spots in wells 8 and 9), then electrophoresed at 10 mA per plate, overnight at 25 °C into a 0.7% agarose gel containing 62 μL rabbit anti-human fibrinogen immunoglobulins. Samples 8 μL in volume were added to each well. Contained in wells were: (1 through 3), 500, 250, and 125 ng purified canine fibrinogen; (4, 6) Triton-soluble platelet proteins from control normal dog 1 and dog 2; (5, 7, 11) platelet extracts from three thrombopathic dogs; and (8 through 10) the previous thrombopathic platelet extracts to which 200 ng of purified canine fibrinogen was added.

subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the preparation was homogeneous under nonreduced conditions. When reduced, it showed the presence of intact Aα, Bβ, and γ chains.

The capacity of normal and thrombopathic canine platelets to interact with immobilized fibrinogen was assessed by the bead agglutination technique of Coller. For most studies, highly purified canine fibrinogen was immobilized to polymeric acrylonitrile beads.

In preliminary studies, crude (Kabi) or purified human fibrinogen was used. Beads to which BSA was similarly coupled served as controls. The extent of agglutination after platelet exposure to a stimulus was scored at three minutes, and with some agonists, at 15 seconds on a scale of 0 to 4+. A slight granuloy reaction was considered 1+; a 4+ value was assigned to samples in which the beads were in relatively few but large clumps. Canine platelet interaction with bead-immobilized human fibrinogen was similar to that observed for canine fibrinogen.

Platelet glycoproteins. Washed normal or thrombopathic platelets were resuspended, solubilized, and reduced (5% vol/vol 2-mercaptoethanol) as described for human platelets. Nonreduced and reduced samples containing 100 to 400 μg of protein were analyzed on 6% or 7% polyacrylamide slab gels, overlaid with a 3.5% stacking gel and electrophoresed according to Laemmli, and fixed, washed, and stained with periodic-acid Schiff (PAS) reagent as detailed by Clementson. Orosomucoid, 5 μg per sample, was used as an internal glycoprotein standard. Gels were scanned at 550 nanometers using a model 2400 Gilford spectrophotometer. Some gels were counterstained with Coomassie brilliant blue R 250.

Washed normal human, canine and thrombopathic platelets were also solubilized and phase extracted with Triton X-114 to obtain detergent phase fractions selectively enriched for integral membrane proteins (predominantly IIb and IIIa). Reduced and nonreduced SDS-solubilized detergent phase proteins, 25 to 100 μg per lane, were electrophoresed, stained (PAS), and scanned under identical conditions and often on the same slab gel as SDS-solubilized platelets.

RESULTS

Aggregation. Aggregation profiles typical for severe hereditary canine thrombopathia are presented in Fig 2. Stimulation of thrombopathic platelets with physiologic concentrations of ADP, collagen, or SA failed to induce aggregation. Unlike human platelets, canine platelet sensitivity to exogenous arachidonate was variable, as others have pre-
Previously reported, normal basset hound platelets (N = 15) responded to SA with consistent weak reversible aggregation. Heparinized (10 U per milliliter) PRP was used in a few experiments; the aggregation profiles obtained for all agonists were similar to those shown for the citrated system.

In marked contrast to the other physiologic stimuli, thrombin induced aggregation of thrombopathic platelets. When quantitated with GFP, thrombopathic platelets aggregated to the same maximal extent as their normal counterparts over a wide range of thrombin concentrations (Fig 3A). However, the rate of the response was impaired (Fig 3C). In heparinized-GFP, no significant differences in the rate of α-thrombin-induced aggregation were observed. This may be due to the reported proaggregatory effects of heparin.

Bell and co-workers and Patterson and co-workers have reported that thrombopathic platelets fail to aggregate in response to thrombin; however, they used a single dose of thrombin (0.2 U per milliliter PRP).

In PRP, stimulation of thrombopathic platelets with high-dose calcium ionophore A23187 failed to induce aggregation (Fig 4). In GFP made 1 mmol/L with respect to CaCl₂ (Fig 4: Inset) aggregation occurred but only in response to ionophore doses eight- to tenfold higher than required by normal platelets (Fig 3B). Likewise, the rate of aggregation was markedly impaired (Fig 3D).

In other experiments, increasing the concentration of extraplatelet ionic calcium had no demonstrable effect on the reactivity of thrombopathic platelets, even in response to agonist concentrations 20- to 50-fold higher than those shown. Likewise, the severe platelet dysfunction could not be corrected by resuspending Stractan-washed platelets for up to two hours in normal canine plasma. When equal volumes of normal and thrombopathic PRP (300,000 platelets per microliter) were mixed and incubated for up to 10 minutes at 37 °C, the presence of thrombopathic platelets had no effect on platelet reactivity. GFP was made 1 mmol/L with respect to CaCl₂.
on the aggregatory response of normal platelets stimulated with ADP, collagen or γ-thrombin.

**Shape change and morphology.** Thrombopathic platelets underwent dose-dependent shape change in response to all stimuli. For all agonists examined, the onset and degree of shape change (quantitated photometrically on the basis of slope and extent of increased light scatter) paralleled those observed for normal canine platelets.

Aggregometric evidence for shape change events was confirmed by ultrastructural analysis of platelets fixed at the point of maximal light scatter (Fig 2, open arrow). Morphological changes consistent with shape change were evident upon platelet stimulation. Platelet ultrastructural reorganization in response to ADP, collagen, or thrombin was similar. The general features of thrombopathic platelet morphology appeared normal. In comparison to normal canine platelets, thrombopathic platelets appeared to be the same size and to contain the same number of dense bodies and other organelles, whose size and shape were also within normal limits.

**Potentiation by epinephrine.** The sensitivity of thrombopathic platelets to ADP appeared to be enhanced immediately after the animals were exposed to the mild stress of a change in kennel environment. Stress-induced activation of the sympathoadrenal system is often associated with an increase in epinephrine secretion. Because epinephrine has been proposed as a "gain controller" in canine platelet function,64 due to its ability to increase markedly without apparent activation, platelet sensitivity to various agonists, we examined its possible potentiating effect on thrombopathic platelets. Epinephrine (5.4 μmol/L) did not activate thrombopathic platelets (Fig 5) or normal controls (not shown). It has no apparent potentiating effect on the response of thrombopathic platelets to collagen. However, preexposure to epinephrine enhanced the sensitivity of thrombopathic platelets to physiologic concentrations of ADP. Such platelets reacted with irreversible aggregation; although at rates four- to fivefold lower than those of non-epinephrine-exposed, ADP-stimulated, normal canine platelets. For thrombopathic platelets, aggregation rates approaching normal could not be achieved even when epinephrine and ADP concentrations were increased to 20 and 150 μmol/L, respectively.

**Serotonin release.** When the release reaction of thrombopathic platelets was evaluated in PRP (Table 1), <6% of the total platelet 14C-serotonin was secreted in response to collagen (100 μg per milliliter of PRP) or calcium ionophore A23187 (40 μmol/L). Thrombin at 5 U per milliliter of PRP induced secretion exceeding 70%. ADP (8 to 10 μmol/L)-induced secretion was variable for individual thrombopathic dogs, ranging from 2% to 20%. Release was maximal at 30 seconds in contrast to platelets from normal dogs which maximally released (<6%) 14C-serotonin between one to three minutes. At high ADP concentrations (68 to 100 μmol/L), secretion by thrombopathic platelets was impaired. For 12 dogs examined, the early 14C-serotonin release induced by ADP represents, at its highest, less than one-third of the total secretable pool. This atypical-release response was also observed in thrombopathic platelets by Patterson and colleagues.61,63 They suggested that thrombopathic platelets secrete the bulk of their dense granule constituents in response to 10 μmol/L of ADP and that the early release of large amounts of platelet ATP may explain why thrombopathic platelets fail to aggregate. The fact that several of the bassets (N = 5) in this study released <4% of their 14C-serotonin in response to 10 μmol/L of ADP and failed to aggregate does not support this hypothesis.

Dose–response curves typical for 14C-serotonin release by normal and thrombopathic GFP in response to α-thrombin and calcium ionophore A23187 are shown in Fig 3E and F. No significant differences in release were observed over a wide range of thrombin concentrations. In contrast, A23187 induced maximal secretion by thrombopathic platelets only

| Table 1. Serotonin Release in Citrated PRP Obtained From Normal and Thrombopathic Dogs |
|-----------------|-----------------|
| **Agonist**     | **Final Concentration** | **Normal Mean ± SD** | **Thrombopathic Mean ± SD** |
| ADP             | 8–10 μmol/L      | 4 ± 2                | 10 ± 6                |
| Collagen        | 68–100 μmol/L    | 51 ± 9               | 23 ± 11               |
| Thrombin        | 100 μg           | 66 ± 9               | 5 ± 1                 |
| α-Thrombin      | 5 U              | 75 ± 3               | 70 ± 4                |
| γ-Thrombin      | 0.12 μmol/L      | 81 ± 9               | 79 ± 7                |
| A23187          | 40 μmol/L        | 50 ± 16              | 5 ± 1                 |

PRP, platelet-rich plasma.

*14C-Serotonin uptake, after 30 minutes, 21 °C, was 92% ± 2% and 91% ± 1% for normal and thrombopathic platelets, respectively; release at three minutes.

†Canine platelets exhibit irreversible aggregation in response to doses of ADP ranging from 8 to 12 μmol/L.
at ionophore concentrations three- to fivefold higher than required by normal canine platelets.

Adenine nucleotides. The abnormal release reaction of thrombopathic platelets could not be attributed to low concentrations of platelet adenine nucleotides. No significant differences in either ADP, 1.44 ± 0.34 and 1.42 ± 0.37 μmol/10^11 platelets or ATP, 7.07 ± 0.91 and 6.75 ± 45 μmol/10^11 platelets were detected for thrombopathic (N = 8) and normal (N = 5) platelets, respectively.

Platelet fibrinogen. Total platelet protein and platelet fibrinogen levels were also determined. Intraplatelet fibrinogen, as illustrated for a typical experiment (Fig 1), was quantitated by rocket immunoelectrophoresis. No significant differences were found in total platelet protein, 1.73 ± 0.21 and 1.62 ± 0.27 mg/10^9 platelets, or platelet fibrinogen, 60 ± 12 and 56 ± 13 μg/10^9 platelets, for normal (N = 6) and thrombopathic (N = 12) platelets, respectively.

Retention, adhesion, and clot retraction. Functional parameters of the basic platelet reaction other than aggregation were also evaluated. No significant differences were observed between normal and thrombopathic dogs (N = 6) in percentage of platelet adherence to fibrillar type I collagen, 51% ± 6% and 52% ± 5%, or their ability to support normal clot retraction, 90% ± 6% and 94% ± 3% retraction, respectively. However, platelets from thrombopathic dogs exhibited a marked impairment in their ability to react with and be retained by glass bead columns, 10% ± 10% vs 94% ± 3% retention for normal platelets; despite normal levels (90% to 120% control) of factor VIII-related antigen.

Fibrinogen receptor interaction. The abnormal retention of thrombopathic platelets on glass-bead surfaces prompted us to assess the interaction of these platelets with canine fibrinogen. The bead agglutination technique of Coller was used to evaluate receptor availability. Results obtained for platelets from arachidonate-insensitive normal or thrombopathic dogs are presented in Table 2.

Unstimulated normal platelets failed to interact with fibrinogen. Stimulation of normal platelets with 10 μmol/L of ADP, 0.1 U per milliliter of PRP α-thrombin, 32 nmol/L of γ-thrombin, 40 μmol/L of A23187, but not 5.4 μmol/L of epinephrine, induced maximal fibrinogen–platelet interaction. In contrast, thrombin and high-dose ionophore were the only agonists capable of initiating fibrinogen receptor availability in thrombopathic platelets. The rate of response (agglutination after 15 seconds) for both agonists was impaired. Stimulation with a combination of epinephrine and ADP induced receptor availability; however, the extent of agglutination was consistently less than that for normal platelets exposed to ADP alone.

Platelet glycoproteins. Normal platelet interaction with fibrinogen requires the presence of GPIIb and GPIIIa in normal amounts. Thrombopathic platelet glycoproteins were therefore analyzed by SDS-PAGE (Fig 6). No quantitative differences in major platelet glycoproteins were detected. Under identical extraction conditions, no differences were observed in the protein content of detergent phases from human, normal canine, or thrombopathic platelets. Mobilities of PAS-staining bands in this fraction (predominantly IIb and IIIa) were identical to glycoprotein reactive bands from whole platelets. Canine PAS reactive bands designated as IIb and IIIa (Fig 6, arrows) comigrated with human IIb and IIIa and had relative mol ws (reduced and nonreduced) similar to those of their human counterparts.

**DISCUSSION**

Canine thrombopathia appears to be a unique, autosomally inherited platelet disorder without a recognized human counterpart. Unlike platelets from human type I Glanzmann’s thrombasthenia, thrombopathic platelets agglomerated in response to thrombin; exhibited impaired dense granule secretion; supported normal clot retraction; and had normal amounts of PAS-reactive GPIIb-GPIIIa and intraplatelet fibrinogen. No support for the proposal that thrombopathia may be a model for the even rarer type II form of Glanzmann’s thrombasthenia was obtained.

The failure of a wide array of platelet stimuli to induce aggregation, dense granule secretion, and fibrinogen receptor exposure suggests that the defect occurs at a locus or loci subsequent to agonist receptor occupancy. Indirect support for this is provided by the unaltered shape-change response of thrombopathic platelets. That thrombopathic membranes have normal collagen receptor density was demonstrated by our studies of platelet adhesion to immobilized collagen.
Thrombopathic platelets also responded abnormally to calcium ionophore A23187, an agonist which bypasses the normal receptor process.

The concentrations of adenine nucleotides in thrombopathic platelets agreed with those reported for canine platelets and are consistent with the metabolic requirements for platelet support of normal clot retraction. The possibility that the thrombin response was an artifact related to fibrin formation was considered; however, it seemed unlikely since 

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g_{\text{thrombin-stimulated thrombopathic platelets agglutinated bead-immobilized fibrinogen whereas } g_{\text{thrombin in the absence of platelets had no effect. For both thrombin and calcium ionophore, the rate of receptor induction was impaired, which correlated with the impaired aggregation rates seen in GFP for both agonists.}}
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Electrophoretic analysis of thrombopathic platelets failed to demonstrate significant quantitative differences in the major platelet glycoproteins, in particular GPIIb-GPIIIa complexes. In contrast to human platelets, 

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\text{epinephrine-stimulated canine platelets did not interact with immobilized fibrinogen, and arachidonate induced weak agglutination. In the presence of apyrase, ADP-induced receptor expression was reduced whereas thrombin-induced exposure was unaffected. Fibrinogen binding sites were exposed on thrombopathic platelets only in response to thrombin and high-dose calcium ionophore. The possibility that the thrombin response was an artifact related to fibrin formation was considered; however, it seemed unlikely since } g_{\text{thrombin-stimulated thrombopathic platelets agglutinated bead-immobilized fibrinogen whereas } g_{\text{thrombin in the absence of platelets had no effect. For both thrombin and calcium ionophore, the rate of receptor induction was impaired, which correlated with the impaired aggregation rates seen in GFP for both agonists.}}
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Stimulation of platelets with thrombin or ADP causes an increase in intracellular ionized calcium, which is a prerequisite for the secretory and aggregatory responses of platelets. The increase appears to result from a release of Ca\textsuperscript{2+} from intracellular sites and influx of extracellular Ca\textsuperscript{2+} in addition, 1,2-diacylglycerol and inositoltrisphosphate may serve as intracellular messengers. Thrombin and collagen induce phosphoinositide breakdown and lead to a transient increase in cellular 1,2-diacylglycerol. It has been proposed that Ca\textsuperscript{2+} and 1,2-diacylglycerol act as coordinate second messengers in platelet activation by greatly increasing the affinity of protein kinase C for Ca\textsuperscript{2+}. Thrombin stimulation results in an increase in both internal ionized calcium and 1,2-diacylglycerol, whereas collagen increases only 1,2-diacylglycerol and has no apparent effect on the concentration of intracellular ionized calcium. Although Ca\textsuperscript{2+} acts as an activator second messenger, cAMP can serve as an inhibitory second-messenger that antagonizes the
mobilization of Ca\(^{2+}\). It is also known that cAMP inhibits the hydrolysis of inositol lipids. The impaired responsiveness of thrombopathic platelets to Ca\(^{2+}\) ionophore A23187 implies that Ca\(^{2+}\) mobilization and/or utilization may be abnormal. This is consistent with our recent observation of elevated cAMP levels in thrombopathic platelets. It appears highly probable that mobilized Ca\(^{2+}\) is rapidly resequestered into intracellular storage sites. The potential by epinephrine of ADP-induced receptor exposure and aggregation is likely related to inhibition of adenylate cyclase in thrombopathic platelets.

The ability of thrombin and high-dose ionophore to initiate thrombopathic platelet fibrinogen receptor availability, although at impaired rates, may be due to their ability to initiate generation of coordinate second messenger(s) which serves to lower requirements for ionic calcium in reactions leading to platelet activation. The failure of thrombopathic platelets to secrete dense granule constituents and express fibrinogen binding sites appears to be related to abnormal second-messenger signal generation. Because the details of reactions leading to expression of fibrinogen binding sites on GPIIb-GPIIIa complexes have yet to be established, thrombopathic platelets may serve as a model system for the assignment of functional significance to biochemical events implicated in receptor induction.

**ACKNOWLEDGMENT**

The authors thank Mr. Gerald P. Meccariello for his expert technical assistance.

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