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Research Article

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Thromboxane-insensitive Dog Platelets Have Impaired Activation of Phospholipase C Due to Receptor-linked G Protein Dysfunction

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Abstract

Human platelet thromboxane A_2/prostaglandin H_2 (TXA_2/PGH_2) receptors are linked to phosphoinositide-specific phospholipase C (PI-PLC) via a G protein tentatively identified as a member of the G_q class. In contrast, platelet thrombin receptors appear to activate PI-PLC via other unidentified G proteins. Platelets from most dogs are TXA_2 insensitive (TXA_2-); i.e., they do not aggregate irreversibly or secrete although they bind TXA_2, but they respond normally to thrombin. In contrast, a minority of dogs have TXA_2-sensitive (TXA_2+) platelets that are responsive to TXA_2. To determine the mechanism responsible for TXA_2- platelets, we evaluated receptor activation of PI-PLC. Equilibrium binding of TXA_2/PGH_2 receptor agonists, [^{125}I]BOP and [^{3}H]U46619, and antagonist, [^{3}H]SQ29,548, revealed comparable high-affinity binding to TXA_2-, TXA_2+, and human platelets. U46619-induced PI-PLC activation was impaired in TXA_2- platelets as evidenced by reduced (*a*) phosphorylation of the 47-kD substrate of protein kinase C, (*b*) phosphatidic acid (PA) formation, (*c*) rise in cytosolic calcium concentration, and (*d*) inositol 1,4,5 trisphosphate (IP_3) formation, while thrombin-induced PI-PLC activation was not impaired. GTPase activity stimulated by U46619, but not by thrombin, was markedly reduced in TXA_2- platelets. Antisera to G_q class α subunits abolished U46619-induced GTPase activity in TXA_2-, TXA_2+, and human platelets. Direct G protein stimulation by GTPγS yielded significantly less PA and IP_3 in TXA_2- platelets. Immunotransfer blotting revealed comparable quantities of G_q class α-subunits in all three platelet types. Thus, TXA_2- dog platelets have impaired PI-PLC activation in response to TXA_2/PGH_2 receptor agonists secondary to G protein dysfunction, presumably involving a member of the G_q class. (*J. Clin. Invest.* 1993. 92:2469–2479.)
Key words: G proteins • guanosine triphosphate phosphohydrolase • phospholipase C • platelet activation • receptors • thromboxane

Introduction

Recent biochemical studies of receptor function have determined a significant role for heterotrimeric guanine-nucleotide binding proteins (G proteins)[1] in receptor–effector coupling (see reference 1 for review). Human platelets are activated by agonists that bind to membrane receptors via this stimulus–response coupling mechanism (see reference 2 for review). The α subunits of several heterotrimeric G proteins are present in platelets. These include $G\alpha_s$, $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_z$, and one or more members of the G_q class, ($G\alpha_q$, $G\alpha_{11}$) (2). $G\alpha_s$ and $G\alpha_i$ mediate signal transduction from receptors to adenylylcyclase, $G\alpha_z$ is of unknown function and members of the G_q class, $G\alpha_q$ and/or $G\alpha_{11}$, appear to function in activation of phosphoinositide-specific phospholipase C (PI-PLC) (1, 2). G proteins that activate PI-PLC are commonly referred to as "G_p's." In platelets, activation of high-affinity GTPase and G_p by thrombin has been reported to involve both pertussis toxin–sensitive and –insensitive G proteins (3–8), while activation of GTPase and G_p via the thromboxane A_2/prostaglandin H_2 (TXA_2/PGH_2) receptor appears to be pertussis toxin insensitive (6, 9). Recently, G_p linked to the TXA_2/PGH_2 receptor has been tentatively identified as a member of the G_q class (10). PI-PLC activation, and its control via G proteins, is a significant biochemical event since the products of PI-PLC hydrolysis of phosphatidylinositol 4,5 bisphosphate (PIP_2), namely, inositol 1,4,5 trisphosphate (IP_3) and 1,2 diacylglycerol, are mediators of cellular secretion (6, 11).

Signal transduction via the TXA_2/PGH_2 receptor has been of considerable interest to many investigators in light of the important role of TXA_2-mediated events in cardiovascular disease (12). Although precise definition of this pathway has been delayed by difficulty in isolation of the TXA_2/PGH_2 receptor, unavailability of an agonist radioligand with high receptor affinity and identification of the receptor linked G_p, significant progress has been made recently (10, 13, 14). However, the biochemical pathways responsible for TXA_2-stimulated platelet activation, including G_p-mediated processes, remain to be fully defined.

An animal model of potential utility in the study of TXA_2/PGH_2 receptor stimulus–response coupling is the dog platelet.

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1. *Abbreviations used in this paper:* [^{14}C]5HT, 5-hydroxy-3-indolyl([1-^{14}C]ethyl-2-amine) creatinine sulfate; G proteins, guanine-nucleotide binding proteins; G_p, G protein that activates PI-PLC; GTPγS, guanosine 5′-0-(3-thiotrisphosphate); I-BOP, [1S-[1α,2α(Z), 3β(1E,3S*),4α]]-7-[3[3[hydroxy-4-[4-(iodophenoxy]-1-butenyl]-7-oxabicyclo[2.2.1]hept-2-*yl*]-5-heptenoic acid; IP_3, inositol 1,4,5 trisphosphate; 40K protein; 47-kD substrate of PKC; PA, phosphatidic acid; PI-PLC, phosphoinositide-specific phospholipase C; PIP_2, phosphatidylinositol 4,5 bisphosphate; PKC, protein kinase C; SQ29,548, [1S-[1α,2β(5Z),3β,4α]]-7-[3-[[2-[(phenylamino)-carbonyl]-hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-*yl*]-5-heptenoic acid; TXA_2+ and TXA_2-, TXA-sensitive and TXA-insensitive dog platelets; U46619, (15S)-hydoxy-11α,9α (epoxymethano) prosta-5Z, 13E, dienoic acid.

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Most dogs, mongrel or pure bred, have platelets that form TXA_2, but fail to aggregate irreversibly or to secrete granular contents in response to the TXA_2 formed (15–20). These TXA_2-insensitive (TXA_2-) platelets also fail to aggregate irreversibly or to secrete in response to TXA_2 mimics such as (15S)-hydroxy-11α,9α (epoxymethano) prosta-5Z, 13E, dienoic acid (U46619). However, some mongrel dogs, and a few pure-bred strains, have platelets that are TXA_2 sensitive (TXA_2+) in that they aggregate and secrete in response to the TXA_2 formed (18–20) as do platelets from nearly all humans. Each individual dog's platelet response to TXA_2 is consistent and genetically defined (21). TXA_2- platelets aggregate irreversibly and secrete if exposed to thrombin or to subthreshold concentrations of epinephrine in addition to TXA_2, and they aggregate in response to TXA_2 plus subaggregating concentrations of ADP (18–20). Dogs with TXA_2- platelets do not have a bleeding diathesis; however, they have significantly less platelet deposition on prosthetic vascular grafts and significantly higher rates of graft patency than dogs with TXA_2+ platelets (22). Although very rare, TXA_2 refractory human platelets that resemble dog TXA_2- platelets have been described (see reference 23 for review).

The biochemical basis for the failure of TXA_2- platelets to respond to TXA_2 has not previously been determined. Although decreased TXA_2/PGH_2 receptor number or affinity for agonist could explain the functional behavior of TXA_2- platelets, high-affinity TXA_2/PGH_2 receptors with pharmacological and radioligand binding characteristics similar to human platelets were found to be present on dog platelets (24, 25). This observation suggested the possibility that TXA_2- platelets had a variant form of postreceptor stimulus–response coupling compared to human and TXA_2+ platelets. Therefore, we studied the consequences of agonist-induced activation of PI-PLC in TXA_2- platelets and compared the results with similar studies of TXA_2+ and human platelets. Ultimately our studies centered on the presence and function of G_p. The results of these studies indicate that TXA_2- platelets have a functionally impaired TXA_2/PGH_2 receptor-linked G_p.

Methods

Subjects. All dogs studied were part of a colony maintained in an AAALAC-certified animal care facility at the Minneapolis VA Medical Center. The animals' health was monitored by frequent examinations conducted by the facility's Veterinary Medical Officer. All dogs were healthy at the time of study. Most of the dogs studied were the offspring of animals bred for the purpose of defining the mode of inheritance of dog TXA_2- and TXA_2+ platelets (21). Platelets were obtained from normal healthy human volunteers who had taken no medication within the previous week. This study protocol was approved by the Animal Studies and Human Studies Subcommittees of the Research Committee of the Minneapolis VA Medical Center.

Materials. Sodium arachidonate was obtained from Nu Chek Prep, Elysian, MN. Iloprost was obtained from Berlex Laboratories, Wayne, NJ. ADP, bovine thrombin, and guanosine 5'-0-(3-thiotrisphosphate) (GTPγS) were from Sigma Chemical Co., St. Louis, MO, and epinephrine from Parke-Davis, Morris Plains, NJ. U46619 was a gift from The Upjohn Company, Kalamazoo, MI. [^{3}H]U46619, 6.3 Ci/mmol, was prepared by Amersham Corp., Arlington Heights, IL., by custom labeling of cold U46619 (26). The TXA_2/PGH_2 receptor antagonist, [1S-[1α,2β(5Z),3β,4α]]-7-[3-[[2-[(phenylamino)-carbonyl]hydrazino]-methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid (SQ29,548), and SQ29,548-[5,6-^{3}H], ([^{3}H]SQ29,548), 40 Ci/mmol, were obtained from New England Nuclear, Boston, MA. The TXA_2/PGH_2 receptor agonist, [1S-[1α,2α(Z),3β(1E,3S*),4α]]-7-[3[3[hydroxy-4-[4-(iodophenoxy]-1-butenyl]-7-oxabicyclo[2.2.1]hept-2-*yl*]-5-heptenoic acid (I-BOP), and [^{125}I]BOP, 2,000 Ci/mmol, were obtained from Cayman Chemicals, Ann Arbor, MI. Phosphatidylinositol-4,5-biphosphate [inositol-2-^{3}H(N)]-, 4 Ci/mmol, was obtained from New England Nuclear. Carrier-free $H_3[^{32}P]O_4$ was obtained from ICN Biomedicals, Irvine, CA. 5-Hydroxy-3-indolyl([1-^{14}C]ethyl-2-amine)creatinine sulfate([^{14}C]5HT), 55 mCi/mmol, guanosine 5'-[γ-^{32}P] triphosphate (γ-^{32}P-GTP), 5,000 Ci/mmol, and D-myoinositol, 1,4,5-trisphosphate [^{3}H-IP$_3$] assay system were obtained from Amersham Corp. Gamma Prep A kit was obtained from Promega Corp., Madison, WI, and used for [γ-^{32}P]ATP synthesis.

Platelet preparation. Blood was obtained without anesthesia from dogs conditioned to the blood drawing procedure by external jugular venipuncture using a 19-gauge needle and a multiple syringe technique (21). Blood (9 ml) was added to 3.8% sodium citrate (1 ml), and after thorough mixing, platelet-rich plasma was obtained by centrifugation at 180 *g* for 15 min at room temperature. Human blood was obtained from an antecubital venipuncture, and platelet-rich plasma obtained as described above. Buffer suspended platelets were obtained from platelet-rich plasma by centrifugation (1,000 *g*, 20 min, 15°C in the presence of 10 mM EDTA [26]) followed immediately by resuspension in an appropriate buffer. In aggregation, [^{14}C]5HT secretion and phosphorylation studies the buffer was Hepes Tyrode's buffer (134 mM NaCl, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.36 mM NaH_2PO_4, 1 mM $MgCl_2$, 5 mM Hepes, 5.5 mM glucose [pH 7.1]) or Lindon's buffer (19).

Platelet aggregation and platelet [^{14}C]5HT secretion. Platelet aggregation and secretion were performed as previously described (18, 26). Maximal responses to agonists were determined in each study. Since the amount of agonist required to achieve maximum responses varied among subjects, a concentration range is recorded.

Radioligand binding to TXA_2/PGH_2 receptors. Equilibrium binding of TXA_2/PGH_2 receptor agonists or antagonists to buffer-suspended platelets was performed as previously described (26). Briefly, platelets were incubated with labeled and unlabeled ligand over a concentration range 10-fold on either side of its expected K_d: [^{3}H]U46619 (14 nM to 1.43 μM, ~ 0.06–0.6 μCi/assay, 1×10^9 platelets/ml, 10 min, room temp.); [^{125}I]BOP (0.03–32 nM, ~ 0.05–0.5 μCi/assay, 0.3–0.5 $\times 10^9$ platelets/ml, 20 min, room temp.) or [^{3}H]SQ 29,548 (0.2–30 nM, ~ 0.01–1.2 μCi/assay, 0.5–0.8 $\times 10^9$ platelets/ml, 20 min, room temp.). The binding reaction was terminated by the addition of 10 ml of ice-cold Hepes-citrate buffer followed immediately by rapid filtration through Whatman GF/C filters under reduced pressure with a 15-ml cold buffer wash. The entire filtration was completed within 15 s. Specific binding was defined as the difference between binding in the absence and in the presence of 1,000-fold molar excess of unlabeled ligand. Nondisplaceable binding was observed to be [^{3}H]-U46619 ≤ 20%, [^{125}I]BOP ~ 5%, and [^{3}H]SQ29,548 ~ 5%. Binding analyses were performed using the computer programs, LIGAND (27) and CURFIT 4 (28).

Protein phosphorylation. Phosphorylation of the 47-kD substrate (40K protein) of protein kinase C (PKC) was studied by SDS-PAGE (29) and scintillation counting as previously described (26).

PKC. Washed platelets were resuspended to 1×10^{12} platelets/ml in breaking buffer (200 mM Tris HCl [pH 7.5], 250 mM sucrose, 0.02% leupeptin, 1 mM PMSF, 10 mM β-mercaptoethanol, 2 mM EDTA, 5 mM EGTA), sonicated in two 15-s bursts on ice, and spun at 100,000 *g* for 90 min. The PKC activity was then measured in the platelet extracts using a modification of the assay system of Go et al. (30). The 200-μl reaction mixture contained 5 μmol of Tris-HCl (pH 7.5), 5 mM $Mg(NO_3)_2$, 40 μg of H1 histone, 0.4 μg of phosphatidylserine + 0.2 μg 1-stearoyl-2-arachidonyl-sn-glycerol in 20 mM Tris, 2.0 nmol of [γ-^{32}P]ATP (> 100 cpm/pmol), and $CaCl_2$ (500 μM Ca^{2+} or 200 nM Ca^{2+}). After 3 min at 30°C the assay was terminated by the addition of an equal volume of ice-cold 20% TCA. The precipitated protein was trapped on 0.45-μm cellulose nitrate filters, using a 10% cold TCA wash, and counted.

Phosphatidic acid formation. The formation of [^{32}P]phosphatidic acid (PA) in permeabilized platelets stimulated by U46619 or GTPγS was studied by the method of Brass et al. (6). Washed platelets were resuspended in K^+-Hepes buffer (160 mM KCl, 5.3 mM $MgCl_2$, 3.3 mM ATP [disodium], 6.7 mM creatine phosphate [disodium], 6.7 U/ml creatine phosphokinase, 13.3 mM Hepes [monosodium]) to a concentration of 8×10^8/ml. The platelets (0.5 ml) were incubated in an aggregometer with 15 μg/ml saponin for 3 min, 37°C, followed by the addition of 6–8 μCi [^{32}P]ATP. After 1 min, U46619 or GTPγS was added and serial samples were removed and mixed with methanol/1N HCl (25:1.25) and put on ice. The samples were extracted with 1.5 ml of chloroform/0.4 ml water, vortexed, and centrifuged at 2,000 rpm for 10 min. The chloroform layer was removed, dried, redissolved (chloroform/methanol/10 mM HCl [20:10:1]) and analyzed against standards on silica plates impregnated with K^+ oxalate-EDTA by TLC (31) in chloroform/methanol/H_2O/concn. NH_4OH (17:13.2:2.8:1).

Cytosolic ionized calcium. The cytosolic ionized calcium concentration ($[Ca^{2+}]_i$) was measured using fura-2, as previously described (26). Platelets, loaded with fura-2 AM, were resuspended at 3×10^8 platelets/ml in Rink's buffer (145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 0.5 mM Na_2HPO_4, 10 mM Hepes, 5 mM glucose, pH 7.4), containing 1 mM Ca^{2+}, or no added Ca^{2+} ($[Ca^{2+}] \sim 1$ μM), stirred in a Spex Fluorlog (Spex Industries, Inc., Edison, NJ), and allowed to equilibrate to 37°C for 2 min. Data for basal $[Ca^{2+}]_i$ was recorded for 1 min. U46619 or thrombin was added and repeat excitation scans were performed every 30 s for up to 5 min. $[Ca^{2+}]_i$ was calculated from the spectral excitation ratio of 340 nm/380 nm (32) using a K_d of 634 nM (26). The rise in $[Ca^{2+}]_i$ was calculated by subtraction of the basal from the stimulated results. The effect of IP_3 on the release of Ca^{2+} from internal stores was evaluated in fura-2 AM–labeled, saponin–permeabilized cells in K^+ Hepes buffer (see *Phosphatidic acid formation*).

Phospholipase C assay. Platelet PI-PLC activity was assayed by the method of Banno et al. (33). Platelet membranes were prepared as described below (see *GTPase activity*). Phosphatidylethanolamine (10 mg/ml) and PIP_2 (1 mg/ml) were mixed (143 and 200 μl, respectively) with 0.5 μCi of [^{3}H]PIP_2, dried under nitrogen, and dispersed into 1.0 ml of 200 mM Tris-maleate (pH 6.8) by vigorous vortexing. The sample was sonicated in an ultrasonic bath for 2 h to produce small unilamellar vesicles to yield 2 nmol of [^{3}H]PIP_2 with 20 nmol of phosphatidylethanolamine per assay. The small unilamellar vesicles were stored at room temperature and used within 48 h. 50 μl of small unilamellar vesicles ($\sim 1.5 \times 10^4$ cpm) were incubated with 100 μl of 0.1 M Tris-maleate buffer, (pH 6.8) plus 40 μl EGTA (final concn. 2 mM) and 10 μl of the membrane preparation. Free Ca^{2+} concentrations were adjusted using Ca^{2+} + EGTA as determined by the computer program FREECAL. The reaction was terminated by the addition of 1 ml of chloroform/methanol/1N HCl (10:10:0.6 by volume). EDTA (5 mM) in 1N HCl (200 μl) was added, the mixture was centrifuged (250 *g* for 5 min), and 400 μl of the [^{3}H]IP_3-rich water phase was counted.

IP_3 formation. Platelets resuspended in Hepes-Tyrode's buffer to a concentration of $6–8 \times 10^8$/ml were warmed to 37°C and stimulated with U46619 or thrombin. Permeabilized platelets were prepared by incubation with saponin (16 μg/ml) as described above (see *Phosphatidic acid formation*). 20 s after the addition of U46619 or thrombin to intact platelets, or GTPγS to permeabilized platelets, 200-μl aliquots were removed, and perchloric acid was extracted, neutralized with 10 N KOH to pH 7.5–8.0, and assayed for IP_3 by RIA.

GTPase activity. Platelet membranes were prepared as described by Baldassare et al. (7). Platelets, $7–10 \times 10^9$ platelets/ml, suspended in TEA buffer with inhibitors (10 mM triethanolamine, 5 mM EDTA, 1.8 mM EGTA [pH 6.8], 0.5 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, 100 ng/ml leupeptin, 10 mM dithiothreitol) were sonicated at 90 W on ice (5 s five times) and centrifuged 100,000 *g* for 30 min (Beckman TL-100 ultracentrifuge) and the resultant membranes were resuspended by sonication (5–10 μg/μl). GTPase activity was measured as per Cassel and Selinger (34) as adapted by Houslay et al. (9). The reaction mixture (100 μl) contained 0.56 μM GTP[$\sim$ 0.4 μCi γ^{32}P], 5 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT, 0.5 mM EGTA, 1 mM ATP, 12.5 mM creatine phosphate, 5 U creatine phosphokinase, 50 mM Tris, (pH 7.4) plus U46619, thrombin, or iloprost. Platelet membranes (12–25 μg) were added to the reaction mixture (37°C), and aliquots were taken immediately and at 4 min after the addition of membranes. The reaction was terminated with 0.5 ml 5% (wt/vol) Norit-A charcoal (20 mM sodium phosphate buffer, pH 7.4). The tubes were vortexed and centrifuged at 8,000 *g* for 2 min, and a 200-μl aliquot of supernatant was removed for scintillation counting. Low-affinity (high K_m) GTPase activity was assayed in the presence of 100 μM GTP. High-affinity (low K_m) GTPase activity was calculated from the cpm difference after subtraction of low-affinity GTPase cpm and represented $\geq$ 71% of the ^{32}P released for stimulated responses above 2 pmol. Blank values for this assay never exceeded 2% of the total radioactivity. In studies performed to evaluate the effect of antisera to α subunits of the G_q class, G_i, or G_z on GTPase activity, membrane suspensions were mixed 9:1 (vol/vol) with antisera (generally 10 μl) and kept on ice for 60 min before assay.

Antisera. Antisera to α subunits of the G_q class (W082, X384, and Z811) were kindly provided by Dr. Paul C. Sternweis, University of Texas Southwestern Medical School, Dallas, TX. W082 antiserum recognizes a unique internal sequence in $G\alpha_q$ (35), while antisera X384 and Z811 recognize the carboxyl terminus of $G\alpha_q$ and $G\alpha_{11}$ (36, 37). Antisera to $G\alpha_z$ (2921), $G\alpha_i$ (8730 and 1398) (38) were kindly provided by Dr. David R. Manning, University of Pennsylvania School of Medicine, Philadelphia, PA.

Immunotransfer blotting. The presence of the G_q class of G proteins was evaluated using standard methodology (39, 40). Solubilized whole-cell platelet samples (10 μl of $0.06–1.0 \times 10^8$ platelets per 150 μl) or platelet membrane samples (10 μl of 0.1–1.0 mg/ml) were run on 11% SDS-PAGE gels and the resolved proteins were transferred to nitrocellulose paper using Mini-PROTEAN techniques (Bio-Rad Laboratories, Richmond, CA). Nonspecific binding was blocked by incubation with 7.5% milk in Tris-buffered saline containing goat serum (1:100) for 1 h at room temperature. Primary antibody incubation with antisera to α subunits of the G_q class was carried out in the presence of 1.5% milk for 4 h at room temperature. Antiserum X384 was diluted 1:400 and antiserum WO82 was diluted 1:500 with Tris-Tween–buffered saline (100 mM Tris, 0.9% NaCl, 0.1% Tween 20, pH 7.5). After incubation, bound antibody was detected with a biotinylated affinity-purified goat anti–rabbit immunoglobulin (1:200) and the strepavidin alkaline phosphatase–conjugated development system of Vector Laboratories, Burlingame, CA.

Membrane protein. Platelet membrane protein was determined by the Bradford protein assay (Bio-Rad Laboratories) with ovalbumin as the standard.

Statistical analysis. Means, standard deviations and tests of significance (Student's *t* test for paired or unpaired values or ANOVA-Fisher's PLSD) were calculated with the programs STATWORKS or STATVIEW II. Data are expressed as mean±SD unless otherwise specified. Significance is defined as $P < 0.05$.

Results

Platelet aggregation and secretion. Consistent with our previous studies utilizing sodium arachidonate (18, 19), TXA_2– platelets demonstrated markedly impaired aggregation and secretion in response to the TXA_2 mimics, U46619 and I-BOP. TXA_2– platelets failed to demonstrate the concentration dependence characteristic of TXA_2+ or human platelets (Fig. 1). Under maximally stimulating conditions, buffer-suspended TXA_2+ platelets aggregated irreversibly and secreted [^{14}C]5HT when stirred with U46619 (80.0±10.2% aggregation; 30.4±11.7% secretion) or I-BOP (75.6±5.5% aggregation; 28.1±12.4% secretion) (Table I). Similar results were obtained with human platelets exposed to either agonist, although lower U46619 concentrations resulted in maximum responses (Fig.

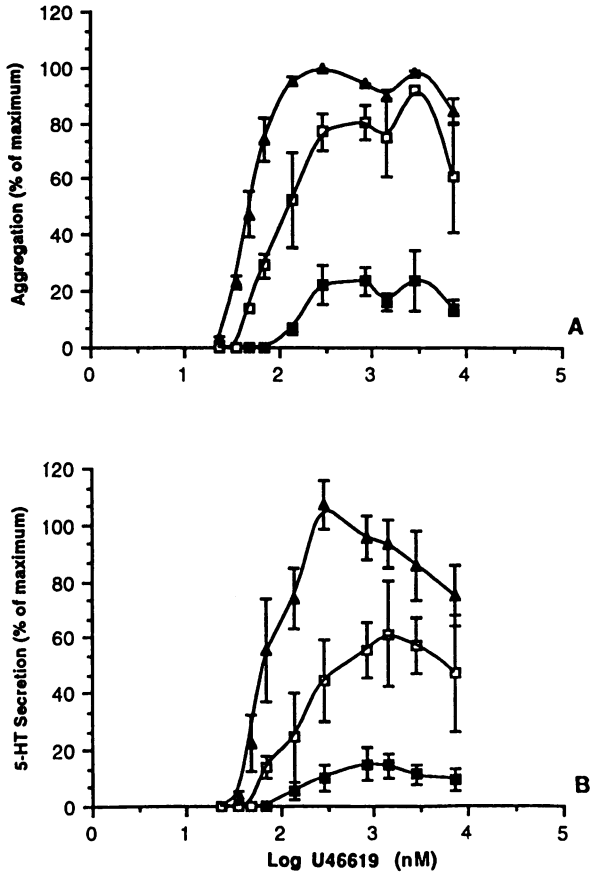

Figure 1. (*A*) Aggregation and (*B*) [^{14}C]-5HT secretion profiles for washed (■) TXA_2-, (□) TXA_2+, and (▲) human platelets in response to the TXA_2 mimic, U46619. Buffer was modified Lindon's with BSA. Concentration range observed was 14.4 nM to 1.43 μM. Maximal aggregation was defined at 2 min after agonist to be 90% transmittance. Maximal secretion was defined at 3 min after agonist to be 50%. The data are presented as mean±SD (n = 4–8) of the log U46619 concentration (nM). EC_{50} for aggregation: 126 nM for TXA_2+ platelets; 56 nM for human platelets. EC_{50} for secretion: 210 nM for TXA_2+ platelets; 90 nM for human platelets.

1, Table I). In contrast, washed TXA_2- platelets demonstrated significantly impaired responses when stirred in the aggregometer with U46619 (24.0±17.6% aggregation; 6.7±3.7% secretion), or I-BOP (32.1±12.5% aggregation; 7.8±2.4% secretion) (Table I). The initial aggregation response was often followed by deaggregation. Although TXA_2- platelets demonstrated markedly impaired aggregation and secretion in response to U46619 or I-BOP, these platelets aggregated (72.3±7.2%), and secreted (84.5±9.0%) as well as TXA_2+ platelets or human platelets when they were stimulated with 0.1–0.5 U of thrombin (Table I).

Radioligand binding to TXA_2/PGH_2 receptors. Equilibrium binding studies of TXA_2/PGH_2 receptor agonist, [^{125}I]BOP, to intact platelets demonstrated the presence of high- and low-affinity binding sites on TXA_2-, TXA_2+, and human platelets. Computer analysis of the binding data, using either LIGAND (27) or CURFIT 4 (28) consistently favored a two-site analysis. High-affinity binding sites (n_1) of 545±239 sites/platelet were observed on TXA_2- platelets (Table II). This was not different from the number found on TXA_2+ platelets (n_1 = 866±443 sites per platelet) or human platelets (758±296 sites per platelet). The high-affinity site K_d's for [^{125}I]BOP binding to TXA_2- platelets (0.21±0.06 nM) and TXA_2+ platelets (0.22±0.08 nM) were similar to that of human platelets (0.25±0.07 nM). The proportion of high- and low-affinity receptors was approximately equal on TXA_2- and TXA_2+ platelets (TXA_2-, n_1 = 545±239 and n_2 = 778±181; TXA_2+, n_1 = 866±443 and n_2 = 720±319 sites per platelet), but human platelets demonstrated a higher proportion of low-affinity binding sites (n_1 = 758±296 and n_2 = 4,138±1,830 sites per platelet). The low-affinity site K_d was ~ 6 nM for all three types of platelets. High-affinity binding sites identified by use of the TXA_2/PGH_2 agonist, [^{3}H]U46619, also did not differ among the three types of platelets (TXA_2- 1,010±477 sites per platelet; TXA_2+ 1,587±557 sites per platelet; human 999±206 sites per platelet) and their K_d's were equivalent (58±28, 68±57, 60±11 nM). A low-affinity [^{3}H]U46619 binding site (micromolar range) was also observed for all three types of platelets. No significant differences were noted among TXA_2-, TXA_2+, or human platelets in regard to binding of the TXA_2/PGH_2 receptor antagonist, [^{3}H]SQ 29,548, that bound to only a single site (TXA_2- 989±40; TXA_2+ 1,132±57; human 1,246±76 sites per platelet) with an ~ 3 nM K_d (Table II).

PKC activation. Maximal 40K protein phosphorylation in TXA_2- platelets stimulated by U46619 or sodium arachidonate (TXA_2 precursor) was only ~ 50% of that observed in TXA_2+ platelets, while thrombin-induced 40K protein phosphorylation was equal in TXA_2- and TXA_2+ platelets (Fig. 2). The 40K protein phosphorylation pattern observed in TXA_2+ platelets was very similar to that which we found previously in human platelets (26). To ensure that the altered phosphorylation profile of TXA_2- platelets was not the result of PKC deficiency, we evaluated the PKC activity in platelet extracts from both types of dogs. The supernatant PKC activity (maximal at 500 μM Ca^{2+}) observed in sonicated extracts of TXA_2- platelets was 3.8±0.6 nmol/min per mg protein, while that seen with TXA_2+ platelets was 3.4±0.6 nmol/min per mg protein. When the assay was performed under Ca^{2+}-restrictive conditions (200 nM Ca^{2+}), we observed 400–500% stimulation of PKC activity with 1.5 μM 1-stearoyl-2-arachidonyl-sn-glycerol in both types of dog platelets, indicating no difference in activation sensitivity to 1,2-diacylglycerol. Direct activation of PKC with 810 nM phorbol myristate acetate resulted in similar [^{14}C]5-HT secretion from TXA_2- (62.8±22.3%; n = 13) and TXA_2+ (68.6±19.9%; n = 11) platelets (P = 0.5).

Calcium flux. The increase in platelet $[Ca^{2+}]_i$ stimulated by U46619 in the presence of 1 mM external Ca^{2+} was lower in TXA_2- platelets (160±28 nM) than in TXA_2+ platelets (230±41 nM; P = 0.004) or human platelets (376±122 nM; P = 0.001) (Table III). In a low-Ca^{2+} (~ 1 μM) buffer the rise in $[Ca^{2+}]_i$ was appropriately lowered in all three types of platelets, though only the difference between TXA_2- platelets and human platelets was significant (Table III). In contrast to the results obtained with U46619, thrombin stimulated a comparable rise in platelet $[Ca^{2+}]_i$ in all three types of platelets (Table III). To evaluate the response of TXA_2- platelets to IP_3 directly, we observed fura-2 AM–loaded platelets permeabilized with saponin and exposed to IP_3. The elevation in $[Ca^{2+}]_i$ stimulated by IP_3 was not different in TXA_2- platelets and TXA_2+ platelets (125±74 nM vs. 174±58 nM; P = 0.26).

PA formation. The time course of PA formation in response to maximally stimulating concentrations of U46619

Table I. Platelet Aggregation and 5HT Secretion Stimulated by U46619, I-BOP, or Thrombin

	Agonist					
	U46619		I-BOP		Thrombin	
Platelets	Aggregation	Secretion	Aggregation	Secretion	Aggregation	Secretion
			%			
TXA_2-	24.0±17.6* (16)	6.7±3.7*	32.1±12.5* (8)	7.8±2.4*	72.3±7.2 (13)	84.5±9.0
TXA_2+	80.0±10.2 (8)	30.4±11.7	75.6±5.5 (11)	28.1±12.4	76.0±8.1 (18)	84.0±10.9
Human	80.4±8.5 (23)	47.2±14.6	73.5±11.0 (6)	40.3±9.4	82.2±6.2 (9)	84.0±8.4

Dog TXA_2-, TXA_2+, and human platelets were labeled with [^{14}C]5HT, washed, and resuspended in modified Lindon's or Hepes Tyrode's buffer. Aggregation is expressed as percentage change in light transmission observed 4 min after the addition of agonist relative to the maximum light transmission attainable with buffer. [^{14}C]5HT secretion is expressed as percentage of maximum (cpm in the platelet supernatant, corrected for dilution, over the cpm in the ^{14}C-labeled platelet suspension, after blank subtraction, multiplied by 100). *n* indicated in parentheses. The data are presented as mean±SD of maximum aggregation and secretion responses obtained using a range of concentrations for each agonist in each individual study. U46619 concentration 0.3–1.4 μM (dog platelets); 0.057–0.095 μM (human platelets). I-BOP concentration 6–12 nM. Thrombin concentration 0.1–0.5 U/ml. * $P < 0.005$ compared to TXA_2+ and human platelets.

(Fig. 3 *A*) and GTPγS (Fig. 3 *B*) was observed in permeabilized TXA_2- platelets, and the results were compared to permeabilized TXA_2+ and human platelets. At 0.5, 1, and 2 min after the addition of U46619, TXA_2- platelets formed less PA than either TXA_2+ or human platelets (Fig. 3 *A*). At 2 min, PA production stimulated by U44619 in TXA_2- platelets was decreased ~ 40% compared with TXA_2+ and human platelets (TXA_2- vs. TXA_2+, $P = 0.042$). PA formation by TXA_2- platelets in response to GTPγS (Fig. 3 *B*) was also reduced (~ 50%) at 2 min compared with TXA_2+ and human platelets (TXA_2- vs. TXA_2+, $P = 0.002$). To evaluate the source of PA, TXA_2-, TXA_2+, and human platelets were ^{32}P-labeled and extracted, and their respective phospholipid components were visualized by autoradiography after TLC. The amounts of endogenously labeled PIP_2 and PI were similar for both types of dog and human platelets (data not shown).

To evaluate directly PI-PLC activity, crude membrane preparations from TXA_2-, TXA_2+, and human platelets were prepared and their basal activities evaluated using synthetic vesicles of phosphatidylethanolamine/[^{3}H]PIP_2 (10:1 mole ratio). Both types of dog platelet membranes exhibited higher levels of basal PI-PLC activity (TXA_2-, 63.9±26.9; TXA_2+, 44.3±9.3 nmol/min per mg) than human platelet membranes (6.1±1.8 nmol/min per mg; $n = 4$). Under these conditions we could not demonstrate stimulation of PI-PLC activity by TXA_2 mimics.

IP_3 formation. Since the studies above suggested impaired PI-PLC activation after TXA_2/PGH_2 receptor binding, we assessed in situ PI-PLC activation after agonist binding by assaying IP_3 production. Basal IP_3 production was similar in dog platelets and human platelets (Fig. 4). Stimulation of intact platelets with U46619 resulted in less IP_3 (pmol/2×10^8 platelets) produced by TXA_2- platelets (0.50±0.66) than by TXA_2+ platelets (3.9±2.0; $P = 0.001$) or human platelets (4.1±2.4; $P = 0.003$). Basal IP_3 formation was not significantly different in permeabilized TXA_2- and TXA_2+ platelets ($P = 0.078$), but IP_3 formation stimulated by direct G protein activation with GTPγS was reduced in TXA_2- platelets (2.2±1.1) in comparison to TXA_2+ platelets (6.8±3.0; $P = 0.016$) or human platelets (10.22±5.5; $P = 0.015$). However, intact platelet stimulation by thrombin resulted in IP_3 formation by TXA_2- platelets (11.3±2.6) that was not less than that formed by TXA_2+ platelets (8.3±1.5) or human platelets (7.6±4.1) (Fig. 4).

Table II. Platelet TXA_2/PGH_2 Receptor Binding

	TXA_2-		TXA_2+		Human	
	K_d	Sites per platelet	K_d	Sites per platelet	K_d	Sites per platelet
[^{125}I]BOP						
n_1 (*nM*)	0.21±0.06	545±239	0.22±0.08	866±443	0.25±0.07	758±296
n_2 (*nM*)	5.8 ±1.8 (3)	778±181	6.9 ±5.4 (3)	720±319	5.7 ±2.3 (4)	4138±1830
[^{3}H]SQ29,548						
(*nM*)	2.9 ±1.1 (3*)	989±40	2.9 ±1.4 (3*)	1132±57	3.4 ±0.6 (2)	1246±76

Equilibrium binding of [^{125}I]BOP ($0.3–0.5 \times 10^9$ cells/ml, 20 min) or [^{3}H]SQ29,548 ($0.5–0.8 \times 10^9$ cells/ml, 20 min) to washed platelets was studied at room temperature in combination with unlabeled ligand. The data are presented as mean±SD. *n* indicated in parentheses. n_1 designates high-affinity binding site; n_2 designates low-affinity binding site. * These studies required pooled platelets from three different donor dogs.

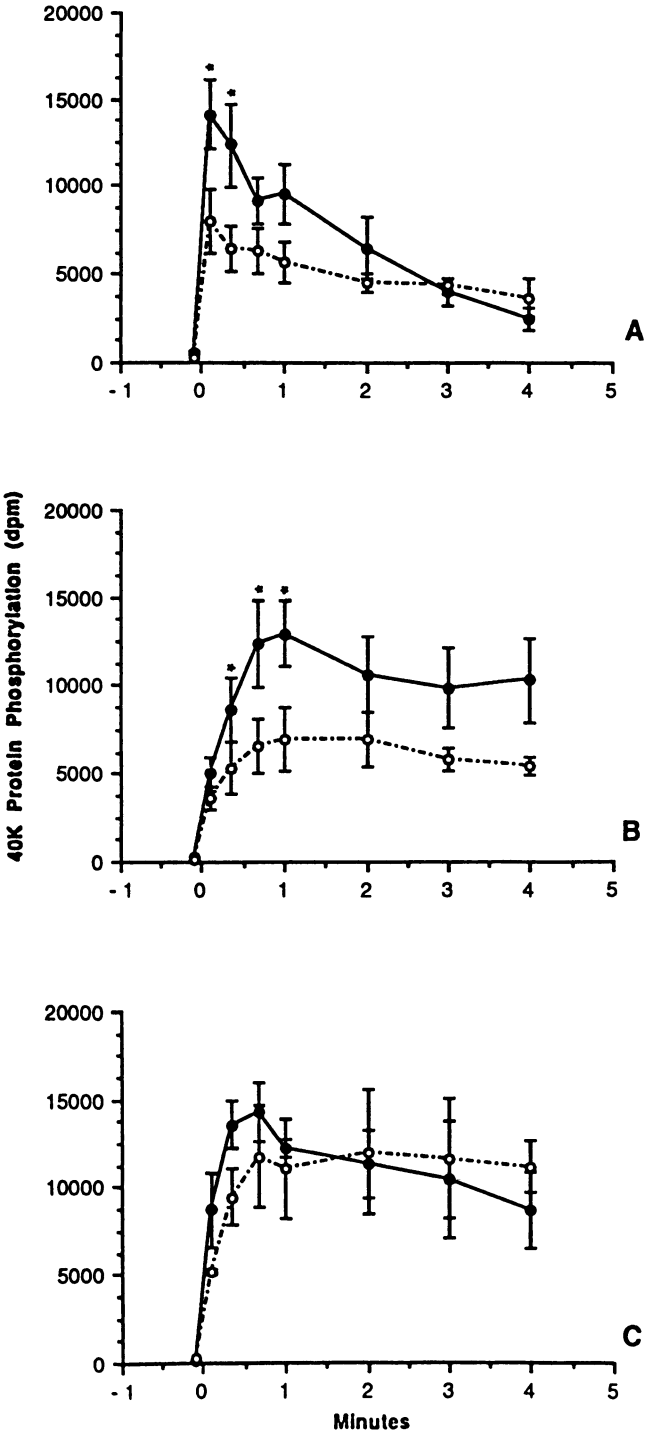

Figure 2. Phosphorylation profiles of 40 K protein stimulated by (*A*) 572 nM U46619, (*B*) 60 μM sodium arachidonate, or (*C*) 0.25 U thrombin in washed (○) TXA_2- and (●) TXA_2+ platelets. Radioactivity was determined by liquid scintillation counting of solubilized SDS-PAGE gel slices obtained from 3×10^7 stimulated ^{32}P-labeled platelets at 5, 20, and 40 s and 1, 2, 3, and 4 min after agonist addition. The data are presented as mean±SEM ($n = 4$). *$P < 0.05$ vs. TXA_2-.

GTPase activity. In light of the reduced ability of TXA_2- platelets to activate PI-PLC upon exposure to U46619, despite high-affinity agonist binding, we evaluated the integrity of the G_p linkage to the TXA_2/PGH_2 receptor by measuring GTPase activity after U46619 exposure. Basal GTPase activity (pmol P_i/min per mg) was higher in TXA_2- and TXA_2+ platelets (31.4±13 and 32.7±15, respectively [$n = 22$]) than in human platelets (17.7±6 [$n = 13$]). Exposure to 715 nM U46619 resulted in minimal elevation of GTPase activity in TXA_2- platelets (1.2±5.1) that was less (paired t-test, $P < 0.001$) than that observed in TXA_2+ platelets (6.4±3.7) and human platelets (7.4±4.1). GTPase activity was elevated in TXA_2- platelets (8.1±4.6) to a level comparable to that of TXA_2+ platelets (8.4±4.3), and human platelets (8.8±3.1) following exposure to 50 μM iloprost ($n = 5$). Thrombin (1 U/ml) stimulated comparable GTPase activity in both TXA_2- (6.9±3.6) and TXA_2+ platelets (5.7±2.9) ($n = 19$). A somewhat higher response to thrombin was observed in human platelets (11.9±6.1, $n = 20$).

To define the G protein involved with the receptor, we evaluated the effects of platelet membrane incubation with antisera to $G\alpha_i$, $G\alpha_z$, and G_q class α-subunits on GTPase activity in TXA_2-, TXA_2+, and human platelets. In the presence of preimmune sera (Table IV), basal GTPase values were slightly reduced in both dog platelet types, but the U46619-stimulated responses were not significantly different from those seen in buffer alone. Basal GTPase values observed in the presence of antisera did not differ from buffer control values. In TXA_2+ platelet membranes, U46619-stimulated GTPase activity was significantly decreased from 6.6±1.4 (mean±SEM) to 1.6±1.5 by carboxyl terminus antisera (X384 and Z811), and to 5.1±2.0 below basal by the $G\alpha_q$ internal sequence antiserum (WO82) (Table IV). In TXA_2- platelet membranes, the much lower U46619-stimulated GTPase activity (1.0±0.8) was likewise slightly decreased by the carboxyl terminus antisera (0.6±0.6) and also significantly lowered by the internal sequence antiserum to 2.2±1.1 below basal. U46619-stimulated GTPase activity in either TXA_2- or TXA_2+ platelet mem-

Table III. Elevation of $[Ca^{2+}]_i$ in TXA_2-, TXA_2+, and Human Platelets after Addition of U46619 or Thrombin

	$\Delta[Ca^{2+}]_i$		
	TXA_2-	TXA_2+	Human
		nM	
1 mM Ca^{2+}			
1.43 μM U46619	160±28*	230±41‡	376±122
	(7)	(6)	(8)
≥0.1 U thrombin	663±322	724±281	725±282
	(6)	(6)	(16)
~1 μM Ca^{2+}			
1.43 μM U46619	50±26‡	68±36	157±12
	(2)	(2)	(3)
≥0.1 U thrombin	244±49	399±151	281±160
	(4)	(3)	(6)

Buffer suspended platelets were labeled with fura-2 AM, washed, and resuspended in Rink's buffer containing either 1 mM Ca^{2+} or no added calcium (~1 μM Ca^{2+}). $[Ca^{2+}]_i$ was measured as nanomolar rise above basal at 10 s. The data are presented as mean±SD. Basal values were 73±30, 56±26, and 37±12 nM for TXA_2-, TXA_2+, and human platelets, respectively. n indicated in parentheses. * $P < 0.005$ compared to TXA_2+ and human platelets. ‡ $P < 0.05$ compared to human platelets.

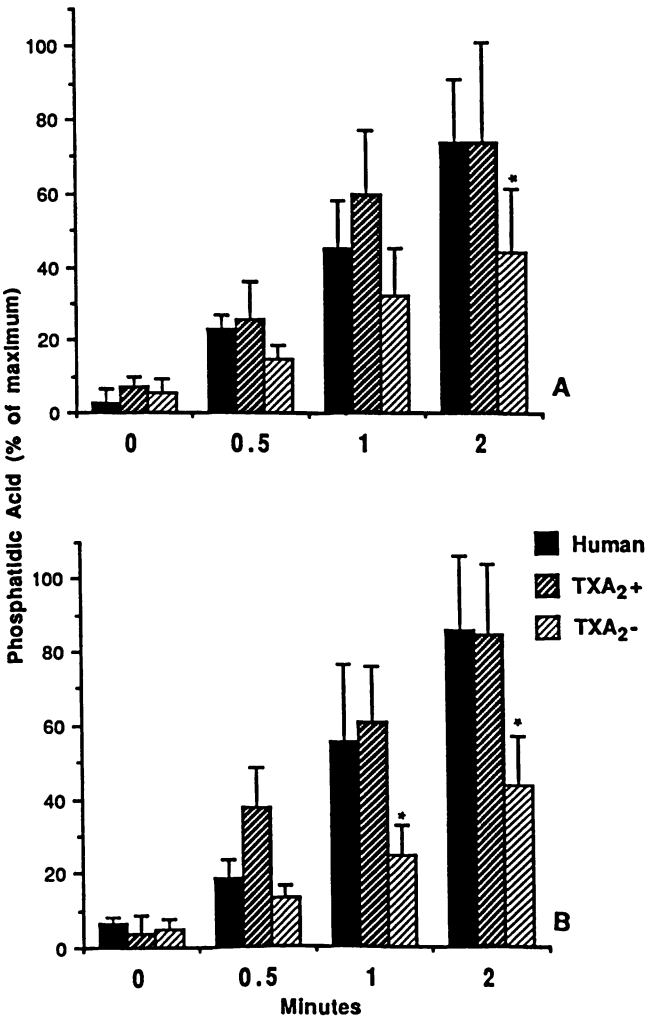

Figure 3. Phosphatidic acid production by saponin-permeabilized platelets in response to (*A*) 1.43 μM U46619 or (*B*) 77 μM GTPγS. Saponin concentration 15 μg/ml; [^{32}P]ATP specific activity 810 dpm/pmol; radioactivity determined by liquid scintillation counting of the PA spot after TLC of aliquots taken at 0, 0.5, 1, and 2 min. Maximum PA = 3.4±1.4 pmol produced/2 min per 10^8 platelets. The data are presented as mean±SD (n = 4). *P < 0.05 vs. TXA_2+.

branes was not significantly affected by antisera specific for $G\alpha_z$ (0.8±1.9; 4.1±1.0) or $G\alpha_i$ (1.4±0.5; 7.4±0.9). Comparable results were obtained with human platelet membranes (Table IV). U46619-stimulated GTPase activity (8.6±2.1) was abolished by both types of G_q class antisera (10.0±2.3 below basal and 4.4±1.6 below basal) but not significantly affected by antisera specific for $G\alpha_z$ (5.7±1.0) or $G\alpha_i$ (6.5±1.3). Thrombin-stimulated GTPase activities of TXA_2−, TXA_2+, or human membranes showed no significant differences when incubated with antisera to $G\alpha_z$, $G\alpha_i$, or $G\alpha_q/G\alpha_{11}$.

Immunotransfer blotting. Since antibodies to α subunits of the G_q class abolished the rise in GTPase activity stimulated by U46619, we investigated the abundance of these α subunits in both types of dog, as well as human platelets. Immunotransfer blotting utilizing antiserum X384 or WO82 demonstrated the presence of $G\alpha_q/G\alpha_{11}$ in comparable quantities in TXA_2−, TXA_2+, and human platelets (data not shown). In an attempt to further evaluate the absolute amounts of these G protein subunits, solubilized whole platelets and platelet membranes were diluted over a 10-fold range and subjected to SDS-PAGE and subsequently immunoblotted. Our results, shown in Fig. 5 for solubilized whole cells, indicate apparently similar concentrations of $G\alpha_q$ in all three platelet types. In the three most concentrated whole-cell samples of dog platelets a distinct minor band of slightly greater molecular weight was observed (Fig. 5, lanes *7* and *8*). The identity of this band is uncertain, but it is possible that this represents a variant of $G\alpha_q$ present in dog platelets that is not present in human platelets. This second band was not seen in washed membrane preparations immunoblotted with the same antisera, nor was it recognized by antiserum X384. When whole cell preparations were diluted over a 10-fold range, electrophoresed, and immunoblotted with antiserum X384, the concentrations of $G\alpha_q/G\alpha_{11}$ also appeared similar (data not shown). Similar results were obtained with both antisera when platelet membranes, rather than solubilized whole platelets, were diluted and immunoblotted (data not shown).

Discussion

The role of heterotrimeric G proteins in receptor-activated signal transduction has been delineated for multiple receptors in

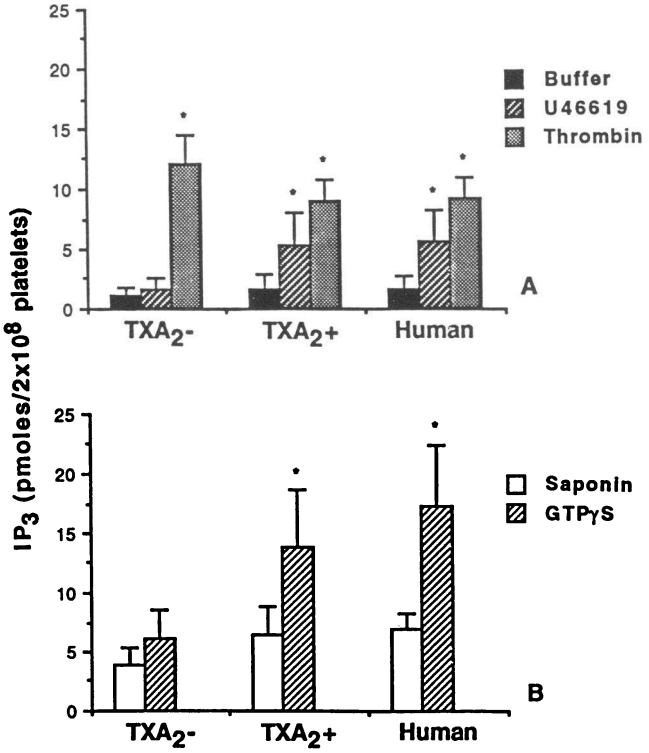

Figure 4. Platelet IP_3 formation after stimulation with (*A*) U46619 or thrombin or (*B*) GTPγS. IP_3 production was measured in buffer-incubated intact control platelets and 20 s after addition of U46619 (715 nM for dog platelets, 143 nM for human platelets) or 1 U/ml thrombin for both human and dog platelets (n = 4–9). GTPγS-stimulated IP_3 production was measured 40 s after addition of 75 μM GTPγS to permeabilized platelets (saponin: 16 μg/ml, 3 min, 37°C, K^+ Hepes buffer, n = 4). The data are presented as mean±SD. Basal IP_3 production in control platelets: TXA_2− 1.07±0.6; TXA_2+ 1.59±1.3; human 1.58±1.08. Basal IP_3 production in permeabilized platelets: TXA_2− 3.9±1.4; TXA_2+ 6.5±2.3; human 7.0±1.2. *P < 0.05 compared to buffer or saponin.

Table IV. Effect of α Subunit Antisera on Stimulated GTPase Activity in TXA_2−, TXA_2+, and Human Platelet Membranes

	Basal GTPase activity				
	Buffer	Preimmune	$G\alpha_q/G\alpha_{11}$	$G\alpha_q$	$G\alpha_z$
	pmol/min per mg				
TXA_{2-}	25.5±1.4	20.7±1.8	21.8±2.0	23.2±2.3	27.8±2.6
TXA_{2+}	28.6±2.6	21.0±1.0	25.5±3.1	26.0±2.6	29.9±1.1
Human	17.7±1.6	17.5±2.3	21.7±2.8	18.6±3.0	17.6±2.0
	U46619 net stimulation				
	Buffer	Preimmune	$G\alpha_q/G\alpha_{11}$	$G\alpha_q$	$G\alpha_z$
	pmol/min per mg				
TXA_{2-}	1.5±0.8	1.0±0.8	0.6±0.6	−2.2±1.1*	0.8±1.9
TXA_{2+}	6.6±0.9	6.6±1.4	1.6±1.5*	−5.1±2.0*	4.1±1.0
Human	7.4±1.1	8.6±2.1	−10.0±2.3*	−4.4±1.6*	5.7±1.0
	Thrombin net stimulation				
	Buffer	Preimmune	$G\alpha_q/G\alpha_{11}$	$G\alpha_q$	$G\alpha_z$
	pmol/min per mg				
TXA_{2-}	7.4±1.0	6.5±1.3	2.4±0.3	5.9±1.5	4.0±0.6
TXA_{2+}	6.5±1.3	4.0±0.2	5.0±1.8	6.3±1.0	5.6±2.2
Human	9.7±1.1	9.5±0.5	9.4±0.7	ND	9.8±1.0

Membrane suspensions (5–10 μg/μl) were mixed 9:1 (vol/vol) with antisera for $G\alpha_q/G\alpha_{11}$ (X384 or Z811, data pooled), $G\alpha_q$ (WO82), $G\alpha_z$ (2921), or preimmune sera, and kept on ice for 60 min before assay. GTPase activity was measured at 5 s and 4 min after the addition of membranes. High-affinity GTPase was determined from the difference of ^{32}P released in the presence of excess unlabeled GTP. Raw data ranged from 1,500 to 10,000 cpm. Data are mean±SEM; n = 3–6 experiments in duplicate; ND, not done. (−) GTPase activity below basal values. * $P < 0.05$ (Student t test and ANOVA Fisher-PLSD) compared to preimmune and buffer controls.

several tissues including platelets (1). The G protein that links platelet TXA_2/PGH_2 receptors to PI-PLC appears to be a member of the G_q class (35, 41), since antisera to the carboxyl terminus of the α subunits of this class of G proteins markedly inhibited the GTPase activity of a platelet membrane preparation stimulated with I-BOP (10). $G\alpha_q$ and $G\alpha_{11}$, activate β isozymes of PI-PLC (see reference 42 for review). Although PI-PLC activation by these G proteins has not been demonstrated in platelets, G_q class G proteins are widely distributed in many tissues (35, 41). G_q and/or G_{11} are the prime candidates for G_p linked to the TXA_2/PGH_2 receptor (2, 10). In view of the importance of PI-PLC activation in platelet secretion and the need to further define the role of members of the G_q class in TXA_2/PGH_2 receptor-mediated activation of PI-PLC, the availability of a naturally occurring model of G_p dysfunction could prove to be highly valuable. Our studies indicate that dog TXA_2− platelets provide such a model.

Our initial efforts to define the mechanism responsible for TXA_2− platelets focused on TXA_2/PGH_2 receptors. Since TXA_2− platelets exhibited markedly impaired functional responses to TXA_2/PGH_2 receptor agonists, we performed a detailed analysis of equilibrium binding to these receptors. Studies performed with the selective receptor agonist, [^{125}I]BOP, revealed binding to two sites on human platelets, as previously described (43, 44), as well as on TXA_2− and TXA_2+ platelets, with binding affinities closely related to physiologically relevant (nM) values. Dorn and DeJesus (44) related the high affinity (~ 0.2 nM) [^{125}I]BOP site to platelet shape change and the lower affinity (~ 4 nM) [^{125}I]BOP site to platelet aggregation. Others (45, 46) have also identified two platelet TXA_2/PGH_2 binding sites, one linked to shape change and the other to aggregation and secretion. In contrast, the antagonist radioligand, [^{3}H]SQ29,548, bound to only a single site on human platelets, as previously described (47), and we observed a single site on TXA_2− and TXA_2+ platelets as well. Overall, we found no significant differences between TXA_2− platelets and either TXA_2+ or human platelets in regard to the characteristics of their high-affinity TXA_2/PGH_2 binding sites evaluated by equilibrium binding of agonist or antagonist radioligands. Mais et al. (24) also identified high-affinity TXA_2/PGH_2 binding sites (K_d 24 nM) on dog platelets, by use of the antagonist radioligand, [^{125}I]PTA-OH. The pharmacological properties of dog TXA_2/PGH_2 receptors were similar to those of human platelets (25). We also observed high and low affinity binding sites for [^{3}H]U46619 that were similar for TXA_2− and TXA_2+ platelets. In addition, we found that [^{125}I]BOP bound similarly to the lower affinity site on both types of dog platelets, thus precluding this site as the source of their differing responses.

Since TXA_2− platelets had adequate numbers of TXA_2/PGH_2 binding sites with affinities comparable to TXA_2+ and human platelets, we studied the signal transduction pathway in these platelets and compared the results to those obtained from identical studies of TXA_2+ and human platelets. We observed a significant (~ 50%) reduction in maximal PKC-stimulated phosphorylation of 40K protein after TXA_2/PGH_2 receptor stimulation by TXA_2, formed from sodium arachidonate, in TXA_2− platelets, even though these platelets were previously

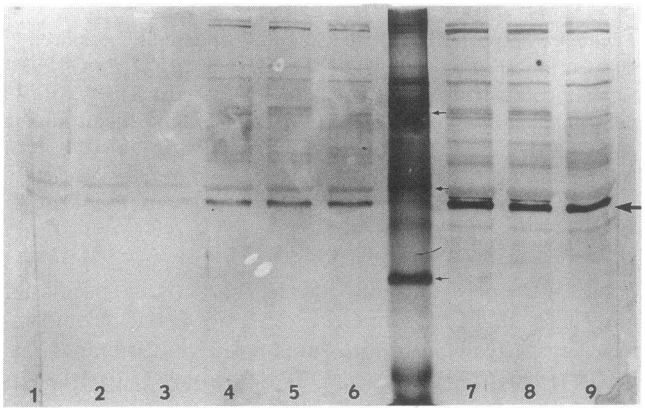

Figure 5. Evaluation of the relative abundance of G_q protein in platelets using immunotransfer blotting. Solubilized whole-cell TXA_2+ (lanes *1,4,7*), TXA_2− (lanes *2,5,8*), and human (lanes *3,6,9*) platelet samples were run on 11% SDS-PAGE gels and the resolved proteins transferred to nitrocellulose paper. Primary incubation with antiserum W082, (internal sequence of $G\alpha_q$) (1:500, Tris, Tween-buffered saline 1.5% milk), preceded detection with a biotinylated goat anti-rabbit and strep-avidin alkaline phosphatase–conjugated development system. Lanes *1–3* contain 0.55 μg of protein (0.031×10^8 platelets); lanes *4–6* contained 2.2 μg of protein (0.125×10^8 platelets); lanes *7–9* contained 8.8 μg of protein (0.5×10^8 platelets). Molecular weight standards are designated at 66,000, 43,000, 31,000 (*small arrows*). The prominent band (*large arrow*) represents $G\alpha_q$.

shown to form TXA_2 from exogenous arachidonate (16, 19) and their intrinsic PKC activity level was equivalent to that of TXA_2+ platelets. The stable endoperoxide and TXA_2 mimic, U46619, was no more effective in stimulating 40K phosphorylation in TXA_2− platelets than arachidonate. We also observed a blunted rise in $[Ca^{2+}]_i$ in TXA_2− platelets after exposure to U46619, while IP_3 stimulated the release of comparable amounts of Ca^{2+} from internal stores in TXA_2− and TXA_2+ platelets. Experiments to evaluate PI-PLC activity in response to TXA_2/PGH_2 agonist stimulation, namely PA and IP_3 formation, again indicated substantially reduced responses in TXA_2− platelets. In contrast, stimulation of TXA_2− platelets with thrombin resulted in Ca^{2+} flux, 40K protein phosphorylation, and IP_3 formation equal to or greater than that observed in TXA_2+ or human platelets. Thus PI-PLC could be receptor-activated in TXA_2− platelets by agonist binding to thrombin receptors, but not by agonist binding to TXA_2/PGH_2 receptors. Therefore, we evaluated the functional integrity of G_p linked to TXA_2/PGH_2 receptors in TXA_2− platelets.

Classical heterotrimeric G proteins are activated by receptor interaction after agonist binding (48). Agonist-bound receptors promote dissociation of GDP from G protein α subunits. GTP binding to the α subunit results in separation of α from the $\beta\gamma$ complex. The GTP bound α subunit activates an effector. Hydrolysis of bound GTP by an intrinsic GTPase activity of the α subunit terminates the effector interaction, and the GDP-bound α subunit rebinds to $\beta\gamma$. Therefore, ligand binding to receptors results in increased GTP hydrolysis.

The GTPase activity of TXA_2− platelets stimulated by U46619 was significantly reduced compared to TXA_2+ and human platelets, while that stimulated by either thrombin or iloprost was comparable in all three platelet types. The reduction in GTPase activity in TXA_2− platelets in response to U46619 was not due to a deficiency of G_q or G_{11} since immunoblot analysis revealed the presence of G_q class α subunits in both types of dog platelets in apparently equivalent amounts. Stimulation of GTPase activity by U46619 in human platelets was blocked by prior exposure to antisera directed against the carboxyl terminus of α subunits of G_q and G_{11}, but not by antisera to $G\alpha_z$ or $G\alpha_i$. These results confirm those of Shenker et al. (10). Similar studies demonstrated that these antisera also abolished the rise in GTPase activity in TXA_2+ platelets stimulated by U46619. An antiserum to a unique internal sequence of $G\alpha_q$ also inhibited U46619-stimulated GTPase activity in TXA_2+ and human platelets, as well as the minimal rise observed in TXA_2− platelets. Antisera to $G\alpha_z$ or $G\alpha_i$ did not significantly inhibit the U46619-stimulated rise in dog platelet GTPase. Therefore, TXA_2/PGH_2 receptors are linked to a member, or members, of the G_q class in dog as well as in human platelets.

Thrombin responses in the presence of antisera to $G\alpha_q/G\alpha_{11}$, $G\alpha_z$, or $G\alpha_i$ were not significantly different for any of the three platelet types. A nonsignificant reduction in mean thrombin-induced GTPase activity was observed in TXA_2− platelets in the presence of antisera to the carboxyl terminus of $G\alpha_q/G\alpha_{11}$, but not in the presence of $G\alpha_q$-specific antiserum. If confirmed by further study, this apparent reduction could be evidence in support of the suggestion (5) that thrombin receptors are linked to PI-PLC via G proteins of the G_q class, and that they activate one or more G_q class proteins other than $G\alpha_q$.

Additional data that strongly suggest that TXA_2− platelets have a dysfunctional G_p linked to the TXA_2/PGH_2 receptor derived from our studies utilizing GTPγS. Both PA and IP_3 formation were significantly reduced in permeabilized TXA_2− platelets, compared to TXA_2+ or human platelets, after direct G protein activation by GTPγS. Permeabilization alone has been shown to result in some IP_3 production (49). We confirmed this observation. However, we found no significant difference between the basal IP_3 levels observed for saponin-permeabilized TXA_2− and TXA_2+ platelets, yet a threefold reduction in the response of TXA_2− platelets to GTPγS was observed.

In summary, dog TXA_2− platelets demonstrate marked impairment of PI-PLC activation in response to agonist stimulation, despite the presence of adequate TXA_2/PGH_2 receptors, that is attributable to dysfunction of G_p. We postulate that TXA_2− platelets have a mutant form of G_p, probably mutant $G\alpha_q$ or $G\alpha_{11}$, that results in impaired signal transduction from the TXA_2/PGH_2 receptor to PI-PLC; however, at least two other alternatives are possible. A mutation in $\beta\gamma$ or in the heterotrimer binding domains of the TXA_2/PGH_2 receptor could result in functional defects consistent with some, but not all, aspects of our findings. $\beta\gamma$ subunits are essential for efficient coupling of α subunits to receptors (48), and they appear to play important roles in modulating activation of effectors, including PI-PLC (40, 50–53). Similarly, a mutation in the second or third cytoplasmic loops of the receptor that interact with the heterotrimer (54), could reduce agonist-induced conformational change that results in α subunit release and activation. However, this would be unlikely to result in impaired activation of PI-PLC by GTPγs.

Naturally occurring examples of G protein dysfunction are uncommon; therefore a close analogy to the postulated defect in the TXA_2− platelet cannot be cited. However, the similarity of some of our findings in TXA_2− platelets to those described in human platelets (23, 55, 56) suggests that the same G_p-related mechanism may be responsible for TXA_2 insensitivity of both dog and human platelets. Another example that bears some resemblance to the dog TXA_2− platelet is the impaired function of G_s in cardiac and skeletal muscle of cardiomyopathic Syrian hamsters (57, 58). Dog TXA_2− platelets and cardiomyopathic hamster myocytes are similar in that they both manifest impaired signal transduction from receptor to effector secondary to apparent G protein malfunction despite the presence of normal quantities of the relevant G protein α subunit. It has been suggested that the reduction in effector activation is likely the result of an alteration in the primary structure of the α subunit or in its post-translational modification that diminishes coupling from the receptor to the effector (57).

The postulated mutation in G_p linked to the TXA_2/PGH_2 receptor of TXA_2− platelets remains to be defined. However, if the mutation exists in the α subunit, a G protein that may be analogous is the $G\alpha_s$ mutation responsible for the H21a phenotype in S49 mouse lymphoma cells. An amino acid substitution of alanine for glycine 226, that results in failure of the α subunit to undergo an appropriate conformational change necessary for α dissociation from $\beta\gamma$, is responsible for its functional impairment (59, 60). This mutant G protein interacts normally with receptors, as evidenced by its ability to promote high-affinity binding of β-adrenergic receptor agonists, but it fails to activate its effector (adenylylcyclase) when stimulated

via the receptor or directly by AlF_4^- or GTP analogues. These characteristics are similar to those we have observed in TXA_2– platelets. Further study of dog TXA_2– platelets should yield new information regarding the regulation of G_p.

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