Thromboxane (Tx) A2 is formed in platelets by the sequential metabolism of arachidonic acid by cyclooxygenases and TxA2 synthase (1) following activation by agonists, such as arachidonic acid, thrombin, collagen, or ADP. Although a weak agonist itself, TxA2 plays an important role in amplifying the response to other, more potent platelet agonists (2). Studies with pharmacological ligands in human platelets have suggested the presence of two distinct populations of receptors (3, 4). Distinct functions have been attributed to these subtypes: (i) aggregation and granule secretion appear to be mediated by receptors with low affinity for the agonist ligand I-BOP, which are bound irreversibly by the antagonist GR 32191; and (ii) shape change appears to be mediated by receptors with high affinity for I-BOP, which are bound reversibly by GR 32191 (3, 4). Despite these observations, the molecular basis for this functional segregation of pharmacological TP subtypes is unknown. TP purified from human platelets consists of a broad protein band of 57 kDa (5, 6). Initial cloning of the TP, from megakaryoblastic cell lines and human placental cDNA libraries (referred to as the placental TP or the TPs isofrom) implied its membership in the G protein-coupled receptor superfamily (GPCR) (7–10). Only one TP gene has been cloned to date (11). However, an alternatively spliced form of TP, TPβ, was cloned from an endothelial cDNA library (12). The mRNA for both splice variants have been demonstrated in platelets (13). Because no pharmacological ligand can presently distinguish between these two isoforms, it is still unknown how they relate to the pharmacological subtypes of the TP in human platelets. We (14, 15) and others (16) have also shown that the isoprostane, iP20,III induces platelet shape change but not TPα phosphorylation. Heterologous TP phosphorylation was observed in aspirin-treated platelets exposed to thrombin, high concentrations of collagen, and the calcium ionophore A 23187. Both homologous and heterologous agonist-induced phosphorylation of endogenous TPα was blocked by protein kinase C inhibitors. TPα was the only isoform detectably translated in human platelets. This appeared to correspond to the activation of the low-affinity site defined by the antagonist GR 32191 and not activated by the high affinity agonist, iP20,III. Protein kinase C played a more important role in agonist-induced phosphorylation of native TPα in human platelets than in human embryonic kidney 293 cells overexpressing recombinant TPα.

Phosphorylation of the Thromboxane Receptor α, the Predominant Isoform Expressed in Human Platelets*

A single gene encodes the human thromboxane receptor (TP), of which there are two identified splice variants, α and β. Both isoforms are rapidly phosphorylated in response to thromboxane agonists when overexpressed in human embryonic kidney 293 cells; this phenomenon is only slightly altered by inhibitors of protein kinase C. Pharmacological studies have defined two classes of TP in human platelets; sites that bind the agonist I-BOP with high affinity support platelet shape change. Low affinity sites, which irreversibly bind the antagonist GR 32191, transduce platelet activation and aggregation. Isoform-specific antibodies permitted detection of TPα, but not TPβ, from human platelets, although mRNA for both isoforms is present. A broad protein band of 50–60 kDa, reflecting the glycosylated receptor, was phosphorylated upon activation of platelets for 2 min with I-BOP. This was a rapid (~30 s) and transient (maximum, 2–4 min) event and was inhibited by TP antagonists. Both arachidonic acid and low concentrations of collagen stimulated TPα phosphorylation, which was blocked by cyclooxygenase inhibition or TP antagonism. Blockade of the low affinity TP sites with GR 32191 prevented I-BOP-induced TPα phosphorylation. This coincided with agonist-induced platelet aggregation and activation but not shape change. Also, activation of these sites with the isoprostane iP20,III induced platelet shape change but not TPα phosphorylation. Heterologous TP phosphorylation was observed in aspirin-treated platelets exposed to thrombin, high concentrations of collagen, and the calcium ionophore A 23187. Both homologous and heterologous agonist-induced phosphorylation of endogenous TPα was blocked by protein kinase C inhibitors. TPα was the only isoform detectably translated in human platelets. This appeared to correspond to the activation of the low-affinity site defined by the antagonist GR 32191 and not activated by the high affinity agonist, iP20,III. Protein kinase C played a more important role in agonist-induced phosphorylation of native TPα in human platelets than in human embryonic kidney 293 cells overexpressing recombinant TPα.

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† This paper is dedicated to the memory of Dr. Jacques Maclouf, deceased on July 14, 1998. Jacques Maclouf was a precious mentor and friend.
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kinases in the phosphorylation of a particular GPCR may vary according to cell type, study of this process has largely been confined to heterologous expression systems (23–25). There are actually few reported studies of agonist-induced phosphorylation of endogenous receptors (26), probably because they are usually expressed in relatively low abundance. We have previously described isofrom-specific antibodies for TPα (27). Using these reagents, we now report that only TPα is detected in human platelets. Upon activation of the platelets with a TP agonist or arachidonic acid as a source of endogenous TxA2, a rapid, transient, and PKC-dependent phosphorylation of the TPα occurs. This involves the low affinity form of the TPα, as defined by irreversible binding of GR 32191. Furthermore, TPα may also be phosphorylated in a PKC-dependent manner in response to platelet activation by thrombin in aspirin-treated platelets. The major role of PKC in rapid, agonist-dependent phosphorylation of endogenous TPα in platelets contrasts with our previous observations when recombinant TPα was overexpressed in HEK-293 cells (27).

EXPERIMENTAL PROCEDURES

Materials—Re-43-5054 and Re-44-9883 were a kind gift of Dr. B. Steiner (Hoffmann-La Roche, Basel, Switzerland). GR 32191 was kindly provided by Dr. B. Bain (Glaxo, Greenford, Middlesex, United Kingdom). Adenosine 5-triphosphate, arachidonic acid, bovine α-thrombin (285 units/mg of protein), benzamidine hydrochloride, calcium ionophore A23187, deoxycholic acid, ferruliprobe, forskolin, leupeptin, phorbol 12-myristate 13-acetate (PMA), H-Arg-Gly-Asp-Ser-Oh (RGDS), H-Arg-Gly-Glu-Seer-Oh (RGES), sodium orthovanadate, sodium fluoride, sodium pyrophosphate, and acetyl-Salicylic acid were purchased from Sigma. Hydrogen peroxide (H2O2) was from Aldrich. Sodium fluoride, sodium pyrophosphate, and acetylsalicylic acid were obtained from all donors in conformity with the French Etablissement d’Echanges. Sodium fluoride, sodium pyrophosphate, and acetylsalicylic acid were obtained from all donors in conformity with the French Etablissement d’Echanges. Bisindolylmaleimide I or GP190230X, Ro-31–8220, and okadaic acid were from Calbiochem (San Diego, Ca). Collagen was from Diagnostica Stago (Asnie`res, France) and was prepared according to the manufacturer’s instructions. Prostaglandin (PG) E1, PGE2, iPF2α, mPGES-1, and PGH2 were obtained from Cayman Chemical Co. (Ann Arbor, MI). [32P]Orthophosphate (5000–6000 Ci/mmol) was from ICN (Costa Mesa, CA). ECL chemiluminescence reagents, CNBr-activated Sepharose, and E-Z-SEP® polyclonal kit were purchased from Amsham Pharmacia Biotech. P-Tyr monoclonal antibody (4 G 10) was from UBI (Lake Placid, Ny). All other reagents were obtained from J. T. Baker (Phillipsburg, NJ).

Platelet Preparation and Labelling—Peripheral blood from healthy volunteers, who had not received any medication for at least 10 days, was collected into ACD-A (National Institute of Health formula: 0.8% citric acid, 2.2% trisodium citrate, 2H2O, 2.45% glucose) and platelet-rich plasma was collected, as described by Westergren, Philipson-Reich, and Platelet Research. Blood was obtained from all donors in conformity with the French Etablissement d’Echanges. The blood was centrifuged at 120 g for 20 min at 20 °C, and platelet-rich plasma was collected, acidified with ACD-A, and further centrifuged at 1200 g for 20 min. The platelets were washed in a phosphate-free modified tyrode buffer without calcium (Buffer A, pH 6.8: 136 mM NaCl, 2.7 mM KCl, 12 mM NaHCO3, 2 mM MgCl2, and 5 mM glucose) in the presence of 0.1 mM PGE1. Platelets were resuspended at 10 5/ml in the same buffer and labeled with 1 nCi/ml of [32P]Orthophosphate for 1 h 30 min at room temperature (28). After further washing in the same buffer, platelets were resuspended in the reaction buffer (Buffer A containing 2 mM CaCl2 and 0.4 mM NaH2PO4, pH 7.4) at 4 × 10 9/ml and allowed to rest at room temperature for 1 h before aggregation was performed.

Platelet Activation and Aggregation—Unless otherwise indicated, the platelet suspension was incubated at 4 × 10 9/ml (0.4 ml per sample) in an aggregometer cuvette for 1 min at 37 °C. Different agonists or vehicle were then added, with constant stirring for 2 min. Platelets were incubated without stirring in the presence or absence of the TP agonist for various periods of time (0.5–90 min) in the kinetic experiments. Platelets were incubated for 1 min prior to platelet activation. PKC inhibitors were incubated for 30 min at 37 °C. Me2SO and ethanol concentrations did not exceed 0.05% and did not modify platelet function or the pattern of phosphorylation of TPα. The reaction was stopped using 1 volume of 2× radioimmune precipitation buffer (1× radioimmune precipitation buffer: 50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Nonidet-P40 (v/v), 0.5% sodium deoxycholate (w/v), 0.1% SDS (w/v), containing 10 mM sodium fluoride, 10 mM sodium orthovanadate, 25 mM sodium pyrophosphate, 10 mM ATP, 1 mg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 1 mM benzamidine hydrochloride, and 0.5 mM phenylmethylsulfonyl fluoride).

Platelets were further treated to immunoprecipitate the TPα as described below. The phosphorylation of pleckstrin p-47, a PKC substrate, was assessed by SDS-PAGE of 20 μl of total platelet lysate (29, 30). TxB2, the inactive hydrolysis product of TxA2, was measured in the platelet lysates by enzyme immunoassay (31).

Immunoprecipitation of Human TPα from Platelets or Cells Overexpressing TPα—Immunoprecipitation of TPα was performed using specific polyclonal antibodies for human TP isoforms (27). Briefly, these antibodies were directed against peptides located at the end of the carboxyl-terminal tail of either TPα or TPβ. NH-SSLQPQGLR-TGRSGLQ-COOH (referred to as Aba) for TPα and NH2-C-PFEPEPP-GKALSRKD-COOH (referred to as Abb) for TPβ. Immunoaffinity columns with each antibody were prepared as follows. Briefly, antibodies were first partially purified using the E-Z-SEP® kit and further incubated with CNBr-activated Sepharose, according to the manufacturer’s instructions. Immunoglobulins derived from nonimmune rabbit serum, coupled to CNBr-activated Sepharose, were used to preclot the platelet lysates. After preclotting for 1 h at 4 °C, using 50 μl of normal rabbit IgG covalently coupled to Sepharose CL-4B, samples were immunoprecipitated overnight at 4 °C using 50 μl of immunoprecipitated antibody. The beads were washed four times with 1 ml of radioimmune precipitation buffer and resuspended in 100 μl of 1× Laemml buffer (4% SDS (w/v), 0.5% sodium dodecyl sulfate (w/v), 0.05% benzyl alcohol, and 0.005% bromphenol blue) under nonreducing conditions. Samples were vigorously vortexed for 15 min, centrifuged for 5 min at 10 000 × g, and loaded on SDS-polyacrylamide gels as described previously (27). Analysis of radioactivity in the samples was performed using a Fuji Bioimaging Analyzer (Fuji, Tokyo, Japan) after the gels were dried.

In some cases, immunoblot analysis of the TPα isoforms was performed. SDS-polyacrylamide gels were transferred onto nitrocellulose membranes. The TPα were visualized using the Aba or Abb followed by a donkey anti-rabbit antibody coupled to horseradish peroxidase (Jack- son-ImmunoResearch, West Grove, Pa). Positive protein bands were revealed by ECL according to the manufacturer’s instructions.

PNgase F Digestion—[32P]Pi-labeled platelets were incubated with 10 nM I-BOP for 2 min. Labeled HEK-TPα or HEK-TPβ was incubated for 10 min with 300 nM U 46619 as defined previously (27). TPα or TPβ were immunoprecipitated as described above. Immunoprecipitates were further denatured for 10 min at 90 °C with SDS 0.5% and β-mercaptoethanol 1% prior to the addition of 1250 units of PNGase F per reaction according to the manufacturer’s instructions. The reaction was carried on for 1 h at 37 °C and then stopped with 1× Laemml buffer. Samples were subjected to SDS-PAGE, and dried gels were exposed to Biomax M3 films.

Stimulation of Tyrosine Phosphorylation—Platelets were exposed to high or low concentrations of I-BOP, thrombin, or pervanadate, an inhibitor of phosphatases, which induced strong tyrosine phosphorylation. After a 4 min incubation, platelets were lysed as described above, and tyrosine phosphorylation was assayed in total platelet lysates or after immunoprecipitation of the TPα receptor isoform by immunoblot analysis, using a specific P-Tyr monoclonal antibody.

RESULTS

Immunodetection of TPα in Human Platelets—We used polyclonal antibodies raised against specific sequences of TPα or TPβ, to isolate TPα from human platelets. Polyclonal antibodies specific for TPα (Abα) were used to immunoprecipitate 1 mg of human platelet lysate, which corresponds to 0.3 pmol/mg of protein, as assessed by binding of [3H]SQ 29548. A broad band with a molecular weight of 50–60 was detected after immunoblot analysis with the same antibody (Fig. 1A). Abα also immunoprecipitated the TPα from HEK-293 cells stably transfected with the corresponding cDNA (Fig. 1A) as described previously (27). However, immunoprecipitation of 1 mg of total platelet protein lysate using the TPβ isofrom-specific antipeptide antiserum failed to reveal any detectable band (Fig. 1A), although these antibodies were able to immunoprecipitate the TPβ receptor isoform from HEK-293 overexpressing these receptors (Fig. 1A). These antibodies were able to immunoprecipitate as little as 50 fmol of receptors/mg of protein from
HEK-293 overexpressing either TPα or TPβ. Using these cells, we have previously shown that these antibodies were both isoform-specific by Western blotting and by immunofluorescence analysis (data not shown).

We next checked that the broad protein band isolated from platelets with Abα was glycosylated. Results were compared with the digestion of TPs in HEK-293-TPα or TPβ. To avoid technical problems subsequent to deglycosylation of the antibodies used for immunoprecipitation, we performed these experiments on phosphorylated TPs, which are obtained after activation with TP agonist, as demonstrated below. Deglycosylation with PNGase F resulted in a shift in the broad protein band from 50–60 kDa in platelets and from 55–70 kDa HEK-293 cells, to an apparent molecular weight of 28. Deglycosylation of TPβ also revealed a shift in the molecular weight to 32.5. These results indicate that the broad protein band of 50–60 kDa observed in platelets corresponds to glycosylated TPα and that TPs in HEK-293 cells and human platelets are differentially glycosylated. The difference in the apparent molecular weight between deglycosylated TPs and TPβ corresponds to the difference in the number of amino acids between the two isoforms (343 amino acids for TPα and 407 for TPβ).

Phosphorylation of the TPα Isoform in Human Platelets—Homologous and heterologous desensitization of human platelets in response to U46619, a TXA2 mimetic, or to thrombin has been reported previously (32, 33). Incubation of aspirin-treated platelets with I-BOP, a TX analog, for increasing periods of time, resulted in the phosphorylation of a broad protein band of 50–60 kDa (Fig. 2A). Phosphorylation was rapid (<0.5 min) but transient (maximum, 2–4 min). We regularly observed a phosphorylated band of 68 kDa in these samples, with a stronger signal in activated platelets. Detection of this band was not modified when immunoprecipitation of TPα was performed in the presence of the specific Abα-peptide used for immunization, whereas immunoprecipitation of the broad protein band of 50–60 kDa was completely abolished (data not shown), suggesting that it is not related to TP receptors.

Low concentrations of I-BOP (≤0.3 mM) that induced only shape change in the absence of platelet aggregation (32, 34) failed to phosphorylate plexkstrin or induce aggregation (Fig. 2B). Under these conditions, no phosphorylation of TPα was observed (Fig. 2B). Only higher concentrations of I-BOP (>1–30 mM) induced reproducible phosphorylation of TPs (Fig. 2B). SQ 29548, a TP antagonist, suppressed phosphorylation of TPα. (Fig. 2C).

Since previous pharmacological studies suggested the presence of high and low affinity receptors in human platelets, we investigated the relevance of these observations in the phosphorylation of TPs in human platelets. We used a particular TP antagonist, GR 32191, that dissociates very slowly, if at all, from the low affinity binding sites in human platelets (4). Fig. 2D illustrates this effect. When platelets derived from PRP treated with 1 μM of GR32191, no increase in TPα phosphorylation was observed with I-BOP 10 nM. TPα in platelets derived from untreated-PRP were normally phosphorylated by I-BOP and GR 32191 inhibited this phosphorylation, similarly to SQ 29548.

Further characterization of this phosphorylation showed that okadaic acid, an inhibitor of serine/threonine phosphatases, resulted in an increase in TPα phosphorylation. Under these conditions, receptor phosphorylation was sustained for up to 30 min, compared to 4 min in the absence of okadaic acid (data not shown). Immunoblot analysis of immunoprecipitated TPs using P-Tyr antibodies did not reveal any phosphorylation of TPs in platelets activated with I-BOP, thrombin, or pervanadate, a strong inhibitor of tyrosine-phosphatases (35), despite marked tyrosine kinase-dependent substrate phosphorylation (data not shown).

iPF2α,III Does Not Induce Phosphorylation of the TPα Receptor—Previous studies by our group and others have shown that iPF2α,III induces platelet shape change (14, 16), Ca2⁺ mobilization, and reversible platelet aggregation at high concentrations of the agonist (15, 18). All of these effects were abolished by TP antagonists. However, iPF2α,III-induced inositol phosphate formation in human platelets was not blocked by TP antagonists (14). Consistent with this observation, we failed to observe TPα phosphorylation with 5–50 μM iPF2α,III (Fig. 3A). Although iPF2α,III-induced platelet shape change, neither prolongation of the incubation time (5 min) (Fig. 3A) nor pretreatment with 1 μM okadaic acid induced significant TPα phosphorylation as compared with control unstimulated platelets (data not shown). Therefore, iPF2α,III appears to favor activation of the high affinity sites, which mediate platelet shape change. In contrast, pretreatment of platelets with 50 μM of iPF2α,III reduced I-BOP-induced platelet aggregation (60%) and TPα phosphorylation (Fig. 3B), consistent with a competition between I-BOP and high concentrations of iPF2α,III for the occupancy of the low affinity sites, which mediate agonist-induced phosphorylation of TPs in human platelets. Moreover, activation of HEK-TPα cells with iPF2α,III resulted in TPα phosho-
Endogenously Formed TxA\textsubscript{2} Phosphorylates TP\textsubscript{a}: Effect of arachidonic acid and low concentrations of collagen—We next tested whether endogenously formed TxA\textsubscript{2} induces phosphorylation of the platelet TP\textsubscript{a}. Addition of arachidonic acid (2.5 \textmu M) to platelets resulted in the formation of 200–400 ng/ml of TxB\textsubscript{2} (corresponding to 0.2 \times 10\textsuperscript{9} platelets). Under these conditions, TP\textsubscript{a} was phosphorylated, to a degree similar to platelets, when incubated with 10 nM I-BOP (Fig. 4). When platelets were pretreated with 10 \textmu M of SQ 29548 or flurbiprofen, an inhibitor of cyclooxygenase, TP\textsubscript{a} phosphorylation was inhibited, demonstrating that endogenous TxA\textsubscript{2} (or PGH\textsubscript{2}) formed by cyclooxygenase-1 was responsible for receptor phosphorylation in response to arachidonic acid (Fig. 4). Similar results were obtained with a low concentration of collagen (Fig. 4). These results demonstrate that TP\textsubscript{a} phosphorylation can occur in activated platelets via endogenous TxA\textsubscript{2} generation. In these samples, platelet aggregation and pleckstrin phosphorylation were also inhibited by SQ 29548 and flurbiprofen treatment.

Heterologous Phosphorylation of the TP\textsubscript{a}—Because heterologous activation of platelets by non-thromboxane agonists may contribute to the desensitization of TP\textsubscript{a} (32), we examined the ability of various agonists to phosphorylate TP\textsubscript{a}. We utilized
and the active phorbol ester PMA, phosphorylated the TPα in the absence or presence of 0.2 units/ml thrombin, 500 nM PMA, or 10 μM forskolin (FK) (for 2 min). Non-aspirin-treated platelets were incubated in the presence or absence of 10 μM of Flurbiprofen or SQ 29548 for 1 min at 37 °C prior to the addition of 100 μg/ml of collagen. Platelet aggregation, TPα or p-47 phosphorylation were assayed on these samples as described in legend for Fig. 2. A, TPα phosphorylation was assayed in platelets incubated with 0.2 units/ml thrombin for increasing periods of time (0.5–60 min) under nonstirring conditions. Data are representative of three similar experiments for A and B.

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Aspirin-treated platelets, thus excluding signaling via endogenous TXα₂ formation, thrombin, calcium ionophore A23187, and the active phorbol ester PMA, phosphorylated the TPα (Fig. 5A). In these experiments, the absence of endogenous TXα₂ was verified by measuring TXB₂ in platelet lysates by enzyme immunoassay (data not shown). Phosphorylation of pleckstrin was also observed (Fig. 5A). In contrast, little phosphorylation of either substrate was obtained with 200 nM PGE₁ or PGE₂ or with 10 μM forskolin (Fig. 5A). Although platelet aggregation induced by low concentrations of collagen is dependent on the formation of TXα₂ (Fig. 4), higher concentrations can bypass this inhibition. When collagen was used at 100 μg/ml, neither flurbiprofen nor SQ 29548 prevented platelet aggregation and phosphorylation of the TPα and pleckstrin (Fig. 5A). Thrombin-induced phosphorylation of TPα was transient (Fig. 5B) and resembled kinetics observed with I-BOP-induced phosphorylation (described in Fig. 2A).

**Effect of PKC on TPα Phosphorylation**—Because we observed that TXα₂ and all other agonists tested induced phosphorylation of TPα and pleckstrin, we utilized specific PKC inhibitors to address the role of this kinase in TPα phosphorylation. Pretreatment of platelets for 30 min at 37 °C with two structurally distinct but specific PKC inhibitors, GF 109203X (Fig. 6, middle panel) and Ro-43–5054 (Fig. 6, right panel), resulted in a dramatic reduction in TPα phosphorylation (~80%) (Fig. 6). Thrombin-induced TPα phosphorylation was also inhibited by GF 109203X (Fig. 6, right panel). The effectiveness of these molecules as inhibitors of PKC was assessed by their capacity to inhibit the PMA-dependent phosphorylation TPα (Fig. 6, right panel). Our recent studies on the phosphorylation of recombinant TP isoforms stably expressed in HEK-293 cells showed little involvement of PKC in response to TXα₂ mimetics, although PMA could readily induce PKC-dependent TP phosphorylation in this system (27). Thus, agonist-induced phosphorylation of TPα in human platelets, in contrast to the HEK-293 expression system, appears largely dependent on PKC.

**DISCUSSION**

Although mRNA detection for the two recognized human TP isoforms has been reported in human platelets (13), it is unknown whether either or both are translated to protein. It is also unknown whether these isoforms relate to the high and low affinity forms of the receptors that have been characterized pharmacologically (4). Using isoform-specific antibodies, we were able to immunoprecipitate TPα as a 50–60-kDa protein band from platelet lysate. TPβ could not be detected. We estimate that ≥ 50 fmol/mg of protein of TPβ could be detected with the specific antibodies from binding experiments in HEK-293 cells transfected with the TPβ isoform. These results suggest that TPβ is expressed at very low levels, if at all, in human platelets.

In the present studies, TX analogs induced rapid agonist-induced phosphorylation of a broad protein band in platelets. Many arguments support that this broad phosphorylated protein band appears to correspond to the TPα isoform. Thus, (i) the broad protein band is specifically immunoprecipitated with the Aba antibody and migrates at the same molecular weight as that revealed by immunoblot analysis, (ii) digestion of immunoprecipitated TPα with PNGase F results in an apparent molecular weight similar to that obtained from HEK-293 cells transfected with the TPβ isoform, and (iii) the phosphorylation of the 50–60-kDa protein band is associated with TP receptor activation. SQ 29548, a TP receptor antagonist, suppress agonist-induced phosphorylation of this band. Previous results by different groups (5, 38, 39) have detected TP recep-

**Fig. 5. Phosphorylation of TPα by heterologous agonist stimulation.** A, [32P]P-labeled aspirin-treated platelets were incubated in the absence or presence of 0.2 units/ml thrombin, 500 nM PMA, or 10 μM forskolin (FK) for 2 min. Non-aspirin-treated platelets were incubated in the presence or absence of 10 μM of Flurbiprofen or SQ 29548 for 1 min at 37 °C prior to the addition of 100 μg/ml of collagen. Platelet aggregation, TPα or p-47 phosphorylation were assayed on these samples as described in legend for Fig. 2. B, TPα phosphorylation was assayed in platelets incubated with 0.2 units/ml thrombin for increasing periods of time (0.5–60 min) under nonstirring conditions. Data are representative of three similar experiments for A and B.

**Fig. 6. Effect of PKC inhibition on the phosphorylation of TPα in human platelets.** [32P]P-labeled washed platelets (0.4 × 10⁹/ml, 0.4 ml) were incubated for 30 min in the absence or presence of 5 μM GF 109203X (GF) or Ro-31–8220 (Ro) prior to activation with 10 nM I-BOP, 500 nM PMA, or 0.2 units/ml thrombin (Thr) for 2 min. Analysis of phosphorylated TPα was performed as described in legend for Fig. 2. The data are representative of at least five experiments.

**Involvement of Integrin Gp IIb/IIIa in the Phosphorylation of TPα—**Activation of platelets by U 46619, a stable TX analog, has been shown to result in the association of p90src with the cytoskeleton (36). Such events, related to ligand occupancy of GpIIb/IIIa, may play a role in the phosphorylation of TPα via “inside-out” signaling (37). Thus, the influence of platelet aggregation on TPα phosphorylation was investigated. The active peptide, RGDS, and two peptide mimetics that are antagonists of GpIIb/IIIa, Ro-43-5054 and Ro-44-9883, were used. The phosphorylation of TPα by I-BOP was unaffected in the presence of 50 μM RGDS, 0.1 μM Ro-43-5054, or 0.2 μM Ro-44–9883 (Fig. 7A). Under these conditions, I-BOP-induced platelet aggregation was totally inhibited (Fig. 7B). Also, phosphorylation of TPα induced by I-BOP was unchanged under either stirring or nonstirring conditions (data not shown). In a few blood donors, we observed a small increase (~20%) in TPα phosphorylation in nonaggregating conditions (data not shown). Moreover, TPα phosphorylation induced by low or high concentrations of collagen was not modified by RGDS (Fig. 7C), thus dissociating platelet TP receptor phosphorylation from aggregation. These results suggest that engagement of the GpIIb/IIIa complex is downstream of the events leading to agonist-induced phosphorylation of TPα.
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Differences in affinities, or in the relative abundance of the receptors, or in the amounts of the kinases in different cells could explain this diversity of response.

Another difference involves the absence of response of this receptor to the isoprostanes in human platelets. iPF_{α,III} increased phosphorylation of TPα in the expression system. However, in human platelets, iPF_{α,III} failed to cause a dose-dependent increase in TPα phosphorylation, despite stimulating inositol phosphate formation as described earlier (44).

In conclusion, we have demonstrated that the TPα isofrom exists in human platelets; TPβ is much less abundant, if it is expressed at all. Phosphorylation of TPα is consistent with the activation of the low affinity site defined pharmacologically with GR 32191. Our results suggest that human platelet TPα is phosphorylated by TX₁A₄ analogs and by other platelet agonists, such as thrombin, through activation of PKCs. Differences in the regulation of the Tx-dependent TP phosphorylation in the HEK-293 overexpressing system, where PKC is of marginal importance, could derive from differences in cellular contents of kinases and their affinity for the receptors in the presence of their ligands. Thus, heterologous expression systems afford sufficient levels of protein to simplify the study of posttranslational modifications of GPCRs. However, such observations may not accurately mimic the regulation of all endogenous receptors in their native milieu.

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