Rapid, Agonist-dependent Phosphorylation in Vivo of Human Thromboxane Receptor Isoforms

MINIMAL INVOLVEMENT OF PROTEIN KINASE C*

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Thromboxane A2 (TxA2) is a potent vasoconstrictor and platelet agonist. Its biological function is tightly regulated. G protein-coupled membrane receptors transduce the effects of TxA2. However, although a single thromboxane receptor (TP) gene has been identified, two splice variants have been cloned from human placenta and megakaryocytic lines (TPα) and from human endothelial cells (TPβ). These differ in the length of their carboxyl-terminal extensions (15 versus 79 residues), which contain multiple potential sites for receptor phosphorylation. Given that TP agonists activate protein kinase C (PKC), it would seem possible that PKC-dependent phosphorylation of TPs might play a central role in homologous desensitization of these receptors.

To determine if the TP isoforms were differentially phosphorylated in response to agonist in vivo, human embryonic kidney (HEK) 293 cells were stably transfected with TPα and TPβ. Isoform-specific anti-peptide antibodies were developed and used to immunoprecipitate the phosphorylated receptors. U46619, a PGH2/TxA2 mimetic, induced specific phosphorylation of both isoforms. Phosphorylation of the two isoforms was similar in dose and time dependence, reaching a plateau at around 100 nM U46619. Inhibition of PKC with either GF 109203X (5 μM) or RO 31–8220 (5 μM) of protein kinase A with H-89 (50 μM) marginally influenced agonist-dependent phosphorylation of either isoform and failed to modulate homologous desensitization of agonist-induced stimulation of inositol phosphate formation. Similar results were obtained when PKC was down-regulated by long term incubation with the phorbol ester, phorbol myristate acetate. Although short term stimulation with phorbol myristate acetate caused PKC-dependent phosphorylation of TPs in vivo, thrombin stimulation of the TP-transfected HEK cells in vivo failed to phosphorylate either of the TP isoforms. Thus, despite the capacity of PKC to phosphorylate TPs in HEK 293 cells and the likely activation of PKC by TP stimulation, this enzyme, like protein kinase A, contributes marginally to rapid, agonist-induced phosphorylation of either TP isoform.

Thromboxane A2 (TxA2) is a product of the sequential metabolism of arachidonic acid by the cyclooxygenases and TxA2 synthase (1). It is formed upon activation of a variety of cells, including platelets, macrophages, and vascular smooth muscle cells and exhibits potent biological activity, causing platelet aggregation and secretion, mitogenesis, and vasoconstriction (2, 3). These effects are transduced via membrane receptors, identified initially with a variety of diverse structural ligands (4). Although such pharmacological studies suggested diversity among thromboxane receptors (TPs), a single gene, encoding a member of the heptahelical G-protein-coupled receptor (GPCR) family, has been cloned (5). However, two variants, based on alternative splicing of the carboxyl-terminal tail of the receptor, have been identified. The first, TPα, was cloned from a cDNA library derived from placental (6) and also from megakaryocytic cell lines (7, 8). The second, TPβ, was cloned from a human umbilical endothelial cell cDNA library (9). The precise biological functions subserved by these isoforms is presently unknown. For example, mRNAs for both isoforms exist in human platelets (10); however, it is unknown whether they represent the two forms of functional TPs identified by ligand binding in human platelets (11). Similarly, the functional response to TP stimulation in endothelial cells remains ill defined. The capacity of the isoforms to subserve distinct biological functions is illustrated by differential coupling of the expressed isoforms to adenylate cyclase in COS cells (10).

One aspect of TP function that, a priori, may differ between the isoforms is agonist-dependent desensitization. TPβ differs from the α isoform in having a longer (79 versus 15 amino acids) carboxyl-terminal extension, which contains an additional 11 serine and 4 threonine residues. It also contains a tyrosine residue, absent from the TPα isoform (9). These amino acids are potential targets for phosphorylation, which is likely to be intrinsic to the process of desensitization. Given the rapid formation of TxA2 by activated cells and its potency, regulation of the response to this eicosanoid by homologous receptor desensitization is of likely biological importance. Biosynthesis of additional ligands, such as thrombin and growth factors (12–14), also suggests that homologous receptor desensitization is an important regulatory event.
16) may amplify the response to TxA<sub>2</sub>, perhaps via cross-talk with TP<sub><i>b</i></sub>. Similarly, TxA<sub>2</sub> may evoke formation of counterregulatory ligands, such as prostacyclin, which may also modulate TP function (17).

Presently, our understanding of the molecular events that underlie TP desensitization in intact cells is limited. These have largely been confined to pharmacological studies (18), which suggest differences between cells but have not discriminated between the cloned isoforms. We have previously demonstrated (19) that a fusion protein, including the carboxy-terminal tail of the TP<sub><i>a</i></sub>, may be phosphorylated by purified PKC and, to a lesser extent, PKA, in vitro. Okwu et al. (20) have provided evidence that TP<sub><i>b</i></sub> in human platelets may be subject to phosphorylation.

We now report the characterization of specific, peptide-based antibodies to TP<sub><i>a</i></sub> and TP<sub><i>b</i></sub> and demonstrate that both receptor isoforms are predominantly localized at the plasma membrane of human embryonic kidney (HEK) 293 cells stably expressing both isoforms as described previously (22). Briefly, cells were plated at 0.7 × 10<sup>5</sup> cells/100-mm culture dish and incubated for 1 h at 37°C with 5 × 10<sup>-5</sup> M Fura-2/AM (Molecular Probes, Eugene, OR) in phenol red free RPMI 1640 culture media. Cells were further washed and incubated for 5 min in PBS, containing 1 mM EDTA and 5 mM EGTA, harvested, washed again, and resuspended at 10<sup>6</sup> cells/ml in ice-cold phosphate-buffered saline (PBS), containing 0.2% BSA and lysed in 0.5 N NaOH. The protein content was determined by micro-BCA<sup>®</sup> assay (Pierce) with the microbichinonic acid reagent and BSA as a standard. Cell number was assessed in parallel wells. Nonspecific binding was determined by the addition of 10 μM unlabeled SQ29,548 and did not exceed 5–10% of the total binding.

Alternatively, crude membrane fractions were prepared as described (19) with minor modifications. Briefly, cells at confluence in 100-mm dishes were scraped in Heps buffer (10 mM Heps, pH 7.6, containing 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, and 10 μM indomethacin), sonicated, and centrifuged for 15 min at 1,000 × g at 4°C. Supernatants were further centrifuged at 125,000 × g at 4°C for 1 h, and pellets were resuspended in the same buffer, containing 10% glycerol and 100 mM NaCl. Radioligand binding was performed using 50 μM of membrane protein and increasing concentrations of [3H]SQ29,548. Reactions were carried out in a total volume of 100 μl at 4°C for 2 h. The reaction was stopped with 1 ml of 10 mM Tris, pH 7.4, followed by filtration through Whatman GF/C glass filters. Filters were dried, washed twice with water, and counted for radioactivity. Nonspecific binding was determined in the presence of excess unlabeled SQ29,548 (25 μM). It did not exceed 15% of the total binding. For competition binding experiments, 50 μM of membrane protein was incubated with 40 nM [3H]SQ29,548 and increasing concentrations of U46619 (0.1–30 μM). Reactions were carried out under the same conditions as above. The data were subject to Scatchard analysis, and an apparent K<sub>a</sub> and B<sub><i>a</sub></b> were determined using a computer program (Radlig Biosoft<sup>®</sup>, Cambridge, UK). The same program was used for analysis of the competition experiments and for calculation of K<sub>a</sub> values for U46619.

**Materials—** The cDNAs for the TP<sub><i>a</i></sub> isoforms were kindly provided by Dr. Colin Funk and Dr. J. Anthony Ware, respectively. The cDNA and antibodies to TP<sub><i>a</i></sub> and TP<sub><i>b</i></sub> were purchased from Amersham Corp. [3H]SQ29,548 (46 Ci/mmol) was obtained from DuPont NEN. pcDNA3 was obtained from Invitrogen (San Diego, CA). HEK 293 cells were obtained from ATCC (Rockville, MD). CNBr-activated Sepharose and E-Z-SEP<sup>®</sup> polyclonal kit was obtained from Pharmacia Biotech Inc. Genitc (G418, specific activity at 750 μCi/mg) and all tissue culture media were purchased from Life Technologies, Inc. Phorbol 12-myristate 13-acetate (PMA), bovine α-thrombin (285 units/mg of protein), forskolin (FK), dibutyryl cAMP (2 μM), formate/formic acid buffer, pH 5. Total inositol phosphate formation was measured as described previously (23). Briefly, confluent cells (4–5 × 10<sup>5</sup> cells in 12-well plates) were labeled with 2 μCi of myo-[<sup>3</sup>H]-inositol (18.3 Ci/mmol) for 24–24 h in serum-free, insulin-free DMEM, containing 0.5% BSA, 20 mM Heps. The culture medium was changed, and cells were incubated in the same medium containing 20 mM LiCl. After a 10-min incubation, U46619 was added for 10 min unless otherwise indicated. When SQ29,548 was used, it was added for 10 min prior to the addition of U46619. The reaction was stopped by aseptically harvesting the cells, incubating the cell pellets in 5 mM ATP, and then washing the pellet twice with ice-cold phosphate-buffered saline before analyzing the increase in agonist-stimulated total inositol phosphate formation compared with unstimulated cells.

**Ca<sup>2+</sup>-Release—** Intracellular Ca<sup>2+</sup> levels ([Ca<sup>2+</sup>]<sub>i</sub>) were measured in TPA- or TPβ-transfected HEK 293 cells in suspension as described previously (24). Briefly, cells were washed twice with PBS, loaded for 1 h at 37°C with 5 μM Fura-2/AM (Molecular Probes, Eugene, OR) in phenol red free-RPMI 1640 culture media. Cells were further washed and incubated for 5 min in PBS, containing 1 mM EDTA and 5 mM EGTA, harvested, washed again, and resuspended at 10<sup>6</sup> cells/ml in RPMI 1640. Cells were allowed to sit for 1 h. Fluorescence was detected in suspended cells diluted at 10<sup>6</sup> cells/ml, using an SLM/Aminco AB2 spectrophotometer (Urbana, CA), and approximate values of [Ca<sup>2+</sup>]<sub>i</sub> were calculated using a K<sub>a</sub> of 224 nM for Fura-2.

Cells (1 × 10<sup>5</sup> cells/ml, 1.5 ml) were incubated with 1 μM U46619 for the indicated times in the desensitization experiments. They were then washed and further stimulated with 300 nM U46619. Control cells were treated with vehicle (i.e. ethanol, 0.01%) alone and further assessed in parallel for mobilization of [Ca<sup>2+</sup>].

**Generation of Specific Anti-peptide Antibodies—** Rabbit polyclonal antibodies were raised to a synthetic peptide to appreciate the TP receptor isoforms. These were directed against the last 15 amino acids of the carboxyl-terminal tail of the mammalian expression vector pcDNA3. The TP<sub><i>a</i></sub> and TP<sub><i>b</i></sub> cDNAs for the TP<sub><i>a</i></sub> isoforms were kindly provided by Dr. Colin Funk and Dr. J. Anthony Ware, respectively. The cDNA and antibodies to TP<sub><i>a</i></sub> and TP<sub><i>b</i></sub> were purchased from Amersham Corp. [3H]SQ29,548 as well as by the agonist (U46619)-stimulated increase in total inositol phosphate formation.

**Binding Assays—** Binding of [3H]SQ29,548 was performed on intact cells as follows. Briefly, subconfluent adherent cells in 24-well plates (2.5 × 10<sup>5</sup> cells) were incubated for 30 min at 37°C in 250 μl of serum-free DMEM, 0.2% bovine serum albumin (BSA) in the presence of increasing concentrations of [3H]SQ29,548. Cells were washed twice in ice-cold phosphate-buffered saline (PBS), containing 0.2% BSA and lysed in 0.5 N NaOH. The protein content was determined by micro-BCA<sup>®</sup> assay (Pierce) with the microbichinonic acid reagent and BSA as a standard. Cell number was assessed in parallel wells. Nonspecific binding was determined by the addition of 10 μM unlabeled SQ29,548 and did not exceed 5–10% of the total binding.
Phosphorylation of Human Thromboxane Receptor α and β Isoforms

TABLE I

| Isoforms | \( B_{\text{max}} \) (pmol/mg protein) | \( K_d \) (nM) |
|----------|--------------------------------------|----------------|
| TPα-5    | 1.64 ± 0.2                           | 21.9 ± 2.15    |
| TPβ-17   | 1.7 ± 0.26                           | 24.4 ± 1.8     |

Crude cell membranes were incubated in the presence of increasing amounts of \([\text{H}]\text{SQ29,548}\) for 2 h at 4°C, and binding analysis was performed as described under "Experimental Procedures." Nonspecific binding was determined in the presence of 25 μM unlabeled SQ29,548. Results represent the mean ± S.E. of eight or nine different experiments. When binding experiments were performed on whole cells, TPα-5 and TPβ-17 expressed 2.8 ± 1 × 10^6 sites/cell \((n = 5)\) and 4.6 ± 0.9 × 10^6 sites/cell \((n = 3)\), respectively.

RESULTS

Characterization of the HEK 293 Cells Overexpressing the TP Isoforms—To determine whether TPα or TPβ couples differentially to downstream signals, we developed HEK 293 cells stably overexpressing both isoforms. Normal HEK 293 cells as well as cells transfected with the pcDNA3 vector alone (HEK 293-VEC) showed no detectable amounts of endogenous TPs in binding assays. Among several HEK 293 clones stably overexpressing the receptors, we selected clones that exhibited similar levels of receptors as expressed per number of binding sites per cell or per pmol of receptor per mg of total protein (Table I). The stably overexpressing clones, TPα-5 and TPβ-17, bind \([\text{H}]\text{SQ29,548}\) in a saturable manner. Scatchard analysis revealed similar dissociation constants \((K_d)\) of 21.9 ± 2.2 nM and 24.4 ± 1.8 nM for the TPα and TPβ receptor isoforms, respectively (Table I). The binding of \([\text{H}]\text{SQ29,548}\) was displaced with high concentrations of U46619. The \(K_i\) values for U46619 were 1.95 ± 0.32 μM \((\text{mean} ± \text{S.E., } n = 4)\) for TPα and 1.17 ± 0.17 μM \((\text{mean} ± \text{S.E., } n = 3)\) for TPβ \((p \text{ value not significant).}]

Total Inositol Phosphate Formation and Calcium Release—We assessed the functionality of the expressed isoforms by measurement of agonist-induced total inositol phosphate (IP) formation and calcium mobilization. Both the TPα-5 and TPβ-17 cell lines exhibited an increase in IP formation in the presence of U46619, a prostaglandin H2/TxA2 mimetic. IP formation was similar in the presence (883 ± 33.2 dpm) and the absence (U46619) of U46619 (901 ± 180 dpm) in HEK 293 cells transfected with the pcDNA3 vector alone and corresponded to values observed in unstimulated TPα-5 and TPβ-17 clones.

The addition of increasing amounts of U46619 (3–3000 nM) for 10 min resulted in a 5–8-fold increase in IP formation, which reached a plateau at around 100 nM for TPα and 300 nM for TPβ (Fig. 1). The EC_{50} values for U46619 were higher for TPβ than for TPα (57 ± 6.7 nM versus 11.1 ± 2 nM, respectively, \(p < 0.002\)). U46619 rapidly increased IP formation via both isoforms \((< 1 \text{ min})\). The increase in IP formation was linear up to 60 min \((\text{data not shown).}]

Preincubation of either TPα-5 or TPβ-17 with the antagonist SQ29,548 abolished the ability of either to transduce IP formation in response to U46619 stimulation \((\text{data not shown).}]

When TPα-5 and TPβ-17 were loaded with Fura-2, U46619 induced a rapid increase in intracellular \([\text{Ca}^{2+}]\), (Fig. 2). In TPα-5 (Fig. 2A, left part), the increase in \([\text{Ca}^{2+}]\), mobilization
The increase in [Ca^{2+}]_{i} was sustained up to 10 min and was dependent on extracellular sources, since chelation of extracellular Ca^{2+} by pretreating the cells with 2 mM EGTA for 45 s led to a rapid return to the baseline. In contrast, U46619 induced a more transient Ca^{2+} mobilization in TPβ-17 (Fig. 2B, left part), although the response did not return completely to baseline. Basal [Ca^{2+}]_{i} was 46 ± 13 nM (mean ± S.D., n = 21) for TPα and 51 ± 18 nM (mean ± S.D., n = 13). For both isoforms, the presence of EGTA depressed Ca^{2+} mobilization by roughly 60–70%. Thus, Ca^{2+} mobilization is dependent on extracellular, as well as intracellular, pools of Ca^{2+}. Similarly, preincubation with 1 μM U46619 abolished the increase of Ca^{2+} in response to a second addition of U46619 in both cell lines (Fig. 2, A and B, right parts). Homologous desensitization of the Ca^{2+} response to U46619 was demonstrable for concentrations of U46619 as low as 10 nM and for periods of preincubation as short as 2 min (data not shown). No significant increase was observed when cells were incubated with vehicle alone or when they were pretreated with SQ29,548. HEK 293-VEC showed no Ca^{2+} mobilization (data not shown).

**Immunoblotting and Immunoprecipitation of the Thromboxane Receptors—Anti-peptide antibodies were raised against amino acid sequences unique to either the human TPα or the human TPβ isofrom. Both antibodies demonstrated specific immunoreactivity toward the receptors, as assessed by immunoblot analysis and immunoprecipitation of TPα or TPβ-17.** Fig. 3A represents an immunoblot analysis of thromboxane receptors in crude membrane fractions of the TPα-5 and TPβ-17 cells. The antisera Abo reacted with a broad protein band ranging from 55 to 65 kDa in TPα-5 cellular membrane fractions (Fig. 3A, lane 1), whereas the antisera Abβ showed no immunoreactivity (Fig. 3A, lane 2). No immunoreactivity was revealed in membranes from cells transfected with vector alone, by either Abo (Fig. 3A, lane 1) or Abβ (data not shown). Preincubation of the Abo or Abβ with the corresponding peptides suppressed the immunoreactivity in TPα-5 (Fig. 3A, lane 3) or TPβ-17 (Fig. 3A, lane 5), respectively. The Abo and Abβ antibodies did not cross-react (data not shown), indicating their specificity. The capability of these antibodies to immunoprecipitate the TP isoforms was demonstrated using immunooaffinity columns prepared with either Abo or Abβ as described under “Experimental Procedures.” Immunooaffinity columns prepared with these antibodies immunoprecipitated a broad protein band from TPα-5 and TPβ-17 (Fig. 3B, lanes 3 and 4, respectively), with an apparent molecular weight similar to that obtained by direct immunoblotting of the membrane fractions. It is important to note that since Abo and Abβ are different sera and could have different characteristics, it is not

![Fig. 1. Total inositol phosphate formation in response to U46619 in TPα and TPβ-expressing cells.](image1)

**Fig. 1.** Total inositol phosphate formation in response to U46619 in TPα and TPβ-expressing cells. HEK 293 cells were stably transfected with TPα and TPβ isoforms. Two clones expressing comparable levels of the isoforms were studied in the presence of the prostaglandin H₂/Thromboxane antagonist U46619. TPα-5 (●—●) and TPβ-17 (○—○) cells were labeled with 2 μCi of myo-[2-3H]inositol for 18–24 h. Cells were further incubated with 20 mM LiCl for 10 min prior to the addition of U46619. The dose-response relationship of U46619 with IP₆ was analyzed after 10 min of incubation. IP₆ formation was analyzed as described under “Experimental Procedures.” Results represent the mean ± S.E. of three to five experiments.

![Fig. 2. Effect of U46619 on the [Ca^{2+}]_{i}, mobilization in cells expressing TPα or TPβ.](image2)

**Fig. 2.** Effect of U46619 on the [Ca^{2+}]_{i}, mobilization in cells expressing TPα or TPβ. A, TPα-5 cells; B, TPβ-17 cells. Left parts, EGTA (2 mM) was added 45 s prior to the addition of U466619. Right parts, cells were incubated for 10 min with 1 μM U46619, washed once, and resuspended at 10⁶ cells/ml. Cells in suspension were incubated with 0.2 μM U46619, and [Ca^{2+}]_{i} release was measured as described under “Experimental Procedures." Basal [Ca^{2+}]_{i} was 46 ± 13 nM (mean ± S.D., n = 21) for TPα and 51 ± 18 nM (mean ± S.D., n = 13). The increase in [Ca^{2+}]_{i} was 126 ± 37 nM (mean ± S.D., n = 15) for TPα and 256 ± 82 nM (mean ± S.D., n = 13) for TPβ. These results are representative of four experiments.

![Fig. 3. Immunoblot analysis of TPα and TPβ receptors using anti-peptide antibodies.](image3)

**Fig. 3.** Immunoblot analysis of TPα and TPβ receptors using anti-peptide antibodies. A, crude membrane fractions (50 μg) of HEK 293-VEC (lane 1), TPα-5 (lanes 2 and 3), and TPβ-17 (lanes 4 and 5) cells were subjected to SDS-PAGE and electrotransferred to nitrocellulose. The membranes were further saturated in Tris-buffered saline buffer, containing 2% BSA. They were incubated with specific antibodies for TPα (lanes 1–3) or TPβ (lanes 4 and 5). Immunoreactivity was revealed as described under “Experimental Procedures.” The antisera were preadsorbed with the corresponding peptides prior to the incubation with the membranes in lanes 3 and 5. B, HEK-293 cells or washed human platelets were lysed, and 0.8 mg of total cell protein were immunoprecipitated using the immunooaffinity columns of Abo and Abβ for TPα and TPβ isoforms. Samples were processed as described above. Lanes 1, 3, and 5 correspond to HEK 293-VEC, TPα-5, and human platelets, respectively, and were blotted with Abo; lanes 2 and 4 correspond to HEK 293-VEC and TPβ, respectively, and were blotted with Abβ.
possible to compare the quantities of the two isoforms that are immunoprecipitated. Similarly, immunoprecipitation of a human platelet cell lysate with the Abα revealed a broad protein band with an apparent molecular weight of 45–50 kDa (Fig. 3B, lane 5). No immunoreactivity could be detected when immunoprecipitation of the platelet cell lysate was performed with the Abβ.

Membrane Localization of the TP Receptor Isoforms—The antibodies Abα and Abβ recognized the receptors in situ as assessed by immunofluorescence staining of TPα-5 and TPβ-17 clones (Fig. 4, A and C, respectively). Almost all of the cells stained positively for the TPs. Immunofluorescence staining was uniformly distributed over the cell surface. No staining was detected in the absence of Abα or Abβ (Fig. 4, B and D, respectively). Furthermore, no immunofluorescence was observed when anti-peptide antibodies were substituted with nonimmune rabbit serum or when the antibodies were saturated with the corresponding peptides (data not shown).

Specific Phosphorylation of Human TPs by U46619—Incubation of [32P]orthophosphate-prelabeled TPα-5 or TPβ-17 with 1 μM U46619 resulted in phosphorylation of a broad radioactive protein band that migrated at the same molecular weight as that of the TP isoforms described above (Fig. 5, A and B, for TPα-5 and TPβ-17, respectively). A very weak signal was detected in the unstimulated cells (Control) or when HEK 293-VEC were incubated with U46619 (data not shown). The increase in U46619-dependent phosphorylation over unstimulated cells was 4–5-fold (n = 12) and 3–4-fold (n = 8) for TPα and TPβ, respectively. Preincubation of the cells with 50 μM SQ29,548 prevented phosphorylation of the TPs, whereas SQ29,548 alone did not induce any phosphorylation. The nature of the weakly phosphorylated band observed in the unstimulated cells is difficult to determine, since preclearing of the cell lysates with the nonimmune rabbit serum eliminated a nonspecific but strongly radioactive doublet that migrates exactly at the same molecular weight as that of TPα and TPβ. Immunoblot analysis of the immunoprecipitated receptors in these samples showed no difference in the quantity of the TP isoforms immunoprecipitated (data not shown). This indicates that the antisera may recognize both the phosphorylated and the unphosphorylated isoforms of the receptors. This is important, since the antibodies are directed against a polypeptide sequence present in the carboxyl-terminal end, where phosphorylation of the receptors may occur (9).

Dose- and Time-dependent Phosphorylation of the TPs and the TPβ Isoforms by U46619—[32P]Labeled TPα-5 and TPβ-17 were stimulated with increasing concentrations of U46619 (1–1000 nM). Phosphorylation of the TP isoforms was detectable in TPα-5 cells at concentrations as low as 3 nM U46619 and reached a plateau at ~100 nM (Fig. 6A). Similar results were obtained for TPβ-17 cells (Fig. 6B). The EC50 values for U46619 for phosphorylation of the TPs and TPβ isoforms were similar (12 ± 2 nM for TPα, n = 3; 11.5 ± 1.3 nM for TPβ, n = 4) (Fig. 6B). Phosphorylation of the TPs and TPβ (Fig. 7, A and B, respectively) receptors was rapidly (1 min) detected at a saturating concentration of U46619 (300 nM) and reached a plateau at ~30 min. Incubation times of up to 90 min resulted in no further change in the phosphorylation pattern. Basal phosphorylation was detected at longer incubation times (>30 min).

Effect of Protein Kinase C Inhibition on U46619-dependent Phosphorylation of the TP Isoforms—To study the role of PKC activation in the U46619-dependent phosphorylation of the TP receptors, we first tested the effect of a specific inhibitor of PKC, the bisindolylmaleimide I (GF109203X) (27). PMA alone (100 nM) induced 2–3-fold phosphorylation of the TPs over unstimulated cells and roughly 2-fold for TPβ-17. No phospho-
Phosphorylation was observed with 4α-PMA, an isomer of PMA that does not activate PKC. Increasing concentrations of GF 109203X (2–10 μM) inhibited the PMA-dependent phosphorylation of the TP isoforms by 70–90% (Fig. 8, A and B). Although high concentrations of GF 109203X reduced basal phosphorylation of the both TP isoforms, they inhibited U46619-dependent phosphorylation by only about 30%. Similar percentages of inhibition were obtained at lower concentrations of U46619.

Prolonged preincubation with GF 109203X for up to 2 h or the use of a distinct PKC inhibitor, RO-31–8220 (28), in TPα-5 cells gave similar results (data not shown). Furthermore, thrombin (2 units/ml) failed to induce phosphorylation of the TP isoforms, although it did cause a significant increase (80–100 nM) in [Ca^{2+}]_i mobilization (data not shown). Similar results were obtained when the cells were transiently transfected with the cDNA for the human thrombin receptor despite a 2-fold increase in thrombin receptor expression as analyzed by flow cytometry using specific anti-peptide antibody against human thrombin receptor (data not shown).

PKC down-regulation was accomplished by incubating the cells for 24 h in the presence of PMA (200 nM). Under these conditions, both TPα or TPβ were still phosphorylated by 1 μM U46619, but not by 100 nM PMA (Fig. 9).

**Effect of Protein Kinase A Inhibition on U46619-dependent Phosphorylation of the TP Receptors**—Incubation of prelabeled cells with 10 μM FK in the presence of 0.5 mM IBMX resulted in a weak phosphorylation of the TPα isoforms. The capacity for PKA-dependent phosphorylation of both TP isoforms was illustrated when Bt_{3}cAMP was incubated for 30 min in the presence of IBMX. The phosphorylation of TPα receptors by FK was strongly inhibited by H-89 (29), a competitive inhibitor of PKA (Fig. 10A). Under these conditions, U46619-dependent TP receptor phosphorylation was not inhibited by H-89.

**Homologous Desensitization of TP Isoform-mediated Inositol Phosphate Formation**— Pretreatment of TPα-5 or TPβ-17 with 300 nM U46619 for 10 min resulted in 70–90% inhibition of IP formation in response to a subsequent addition of U46619 (Fig. 11, A and B). Desensitization was rapid for both receptors, reaching a maximum after 1–2 min of pretreatment (Fig. 11C).
Maximal desensitization was observed when pretreating the cells with 100 nM U46619. Moreover, incubation of cells with 5 μM GF 109203X prior to pretreatment of the cells with U46619 did not modify the pattern of desensitization for either the TPα or TPβ isoform, as assessed by IP formation (Fig. 12, A and B, respectively). Along with these results, the pretreatment of the cells with either 200 nM PMA for 10 min or 0.5 mM Bt2cAMP for 30 min did not modify U46619-mediated IP formation (Table II). Neither PMA or Bt2cAMP pretreatment modified the extent of homologous desensitization of TPs under these conditions.

**DISCUSSION**

TxA2 is an evanescent biological mediator; it exerts potent effects on platelet function and vascular tone in the immediate microenvironment of its formation (18). Given its critical role in determining vascular patency, it would seem likely that its formation and effects would be tightly regulated. TxA2 is not stored, preformed in cells. Rather, it is formed and released rapidly in response to cellular (e.g. platelet) activation by diverse stimuli. Its effects are limited by its hydrolysis to the inactive thromboxane B2, which has a half-life estimated to be 30 s at physiological pH (2) and by homologous desensitization of its membrane receptor-mediated responses (30, 31). This desensitization appears to result initially from uncoupling of the TP from its attendant G proteins; we have previously estimated that the half-life of such a response (coupling to phospholipase C in human platelets) is approximately 120 s. This phenomenon is followed by more gradual loss of binding sites from platelet membranes (30).

Maximal desensitization was observed when pretreating the cells with ~100 nM U46619. Moreover, incubation of cells with 5 μM GF 109203X prior to pretreatment of the cells with U46619 did not modify the pattern of desensitization for either the TPα or TPβ isoform, as assessed by IP formation (Fig. 12, A and B, respectively). Along with these results, the pretreatment of the cells with either 200 nM PMA for 10 min or 0.5 mM Bt2cAMP for 30 min did not modify U46619-mediated IP formation (Table II). Neither PMA or Bt2cAMP pretreatment modified the extent of homologous desensitization of TPs under these conditions.

FIG. 9. Agonist-dependent phosphorylation of the TP receptors. Effect of down-regulation of PKC. TPα-5 (A) or TPβ-17 (B) cells were incubated for 24 h in the presence of 200 nM PMA to down-regulate PKC. The cells were further washed three times for 5 min with phosphate-free culture media and incubated in the absence or presence of 100 nM PMA or 1 μM U46619. The cells were treated as described under “Experimental Procedures.” Equivalent total cpm were used for immunoprecipitation of the cells preincubated with PMA. The single experiments shown are representative of two replicates.

FIG. 10. Effect of PKA inhibition or activation on the phosphorylation of the TP receptor isoforms. TPα-5 (A) or TPβ-17 (B) cells were labeled with 1Porthophosphate and treated with 50 μM H-89 for 45 min in the presence of 0.5 mM IBMX. The cells were further stimulated with 300 nM U46619 or 10 μM FK for 10 min and treated as indicated in the legend for Fig. 5. Labeled cells were incubated with Bt2cAMP for 30 min to allow maximal activation. The increase in the phosphorylation in this condition should be compared with the control cells obtained at 30 min. The experiments are representative of three replicates for FK and two with Bt2cAMP.

FIG. 11. Effect of agonist pretreatment on TP receptor function. Subconfluent TPα-5 or TPβ-17 cells in 12 well plates were metabolically labeled with 2 μCi of myo-inositol for 20 h in inositol-free DMEM culture medium. TPα-5 (A) or TPβ-17 (B) cells were incubated for 10 min in the presence (●, □) or the absence (○, ○) of 300 nM U46619. They were further washed with glycine buffer, pH 3, and incubated with increasing doses of U46619. C, TPα-5 (●) or TPβ-17 (○) cells were pretreated with 300 nM U46619 for different periods of time and then challenged with 300 nM U46619. Results are the average ± S.E. of three different experiments performed in duplicate.
or Bt2cAMP and untreated cells. Unpaired experiments, cellswere incubated with vehicle, PMA, or Bt2cAMP prior to the pretreatment with 300 nM U46619 (Pretreatment U46619) or vehicle (Control) for 10 min. Cells were washed once with glycine buffer, pH 3, for 30 s and further incubated for 10 min with 300 nM U46619 (black bars) or vehicle alone (gray bars). Total inositol phosphates were analyzed, and cells were further processed as indicated under “Experimental Procedures.” Results are the average ± S.E. of three different experiments performed in duplicate.

**TABLE II**

| Pretreatment       | Total IP | Desensitization |
|--------------------|----------|-----------------|
|                    | TPα      | TPβ             |
|                    | % of control | % of control |
| Vehicle            | 673 ± 96  | 531 ± 40        |
| PMA                | 489 ± 66  | 433 ± 40        |
| Bt2cAMP            | 566 ± 75  | 411 ± 70        |

TP is subject to alternative splicing in the carboxyl-terminal region, as has been previously described for E prostaglandin receptor type 3 (33). Little is known about the functional significance of this observation. However, pharmacological studies have indicated the possibility of tissue-specific differences in the characteristics of TPs and, indeed, differences in TP binding of ligands within a single cell, platelets (11, 34, 35). Observations involving E prostaglandin receptor carboxyl-terminal isoforms indicate that they are capable of coupling to distinct downstream signaling systems and that they may differ in the rate and extent to which they are subject to homologous desensitization (36). Narumiya and colleagues have recently reported that mRNAs for the two TP isoforms are expressed in platelets and the vasculature (11, 34). Again the TP agonist, U46619, could stimulate an increase in [Ca\(^2+\)] and total inositol phosphates via both isoforms. However, the pattern of the calcium response evoked via the two isoforms appeared to differ, at least in the stable cell lines that we established. Thus, whereas U46619 induced a rapid transient, followed by a delayed plateau in cells expressing TPα, the plateau phase was almost absent in the cells expressing TPβ. Activation of both isoforms appeared to involve release of calcium from intracellular stores. However, the plateau phase of the TPα response appeared to derive from an extracellular source, raising the possibility of linkage to a receptor-activated calcium channel (37). Further experiments will address this possibility and whether a similar distinction is evident in other cells transfected with the two isoforms.

To clarify the role of specific kinases in the desensitization process, we characterized specific, peptide-based antibodies for the two receptors. These confirmed the membrane localization of the two receptor isoforms, as inferred by their sequences.

Interestingly, both isoforms were rapidly phosphorylated following exposure to agonist. The time and dose dependence of this phenomenon seemed similar for the two isoforms. Both isoforms coupled to downstream signaling systems. We focused on phospholipase C-dependent events, since these are thought most relevant to TXA\(_2\)-mediated platelet aggregation and vasoconstriction (11, 34). Again the TP agonist, U46619, could stimulate an increase in [Ca\(^2+\)], and total inositol phosphates via both isoforms. However, the pattern of the calcium response evoked via the two isoforms appeared to differ, at least in the stable cell lines that we established. Thus, whereas U46619 induced a rapid transient, followed by a delayed plateau in cells expressing TPα, the plateau phase was almost absent in the cells expressing TPβ. Activation of both isoforms appeared to involve release of calcium from intracellular stores.

Given the role of phospholipase C-mediated responses in the biological consequences of TP activation, it would seem likely that downstream activation of PKC might play a central role in homologous desensitization. Indeed, we have previously shown that PKC may phosphorylate a fusion protein based on the carboxyl-terminal end of the TPs receptor (residues 321–343) in vitro (19). Peptide competition experiments suggested that this involved sites in the third extracellular loop and the carboxyl-terminal tail (19). Okwu et al. (20) have recently presented data consistent with TP phosphorylation in human platelets. Phosphorylation plays an important role in regulating both homologous and heterologous desensitization of GPCRs like the TPs. Additionally, other serine/threonine kinases such as PKA, G protein-coupled receptor-dependent kinases, and tyrosine kinases have been implicated in GPCR phosphorylation (38–40). We have demonstrated that PKA may phosphorylate TPα to a minor degree in vitro (19). Similarly, we demonstrate the capacity for PKA to phosphorylate
the receptor in the present study in vivo. However, PKA appears to contribute trivially, if at all, to rapid phosphorylation of the TPs induced by U46619. Although there is no information on G protein–coupled receptor–dependent kinase–mediated phosphorylation of eicosanoid receptors, recent studies have demonstrated their ability to phosphorylate angiotensin and adrenoreceptor GPCRs (41, 42).

We also demonstrate that PKC appears to play a modest role in agonist-dependent phosphorylation of either TP isoform. Thus, two specific competitive inhibitors of PKC, GF 109203X and RO 31–8220, failed to modify the rate or extent of agonist-stimulated phosphorylation of either isoform. Similarly, down-regulation of PKC, by prolonged exposure to the phorbol ester, PMA, also failed to alter these phenomena. We demonstrate that homologous desensitization of agonist-evoked increases in IPs is similar for both isoforms. Again, the PKC inhibitor, GF 109203X, failed to modify homologous desensitization of this response evoked via either isoform. Although PKC appears largely irrelevant to the agonist-mediated desensitization response, activation of this enzyme may phosphorylate TPs. Thus, short term exposure to PMA will result in phosphorylation of both isoforms. However, treatment of the cells with thrombin (2 units/ml) did not induce phosphorylation of the TPs, although cells were activated, as assessed by [Ca2+]i mobilization. Transient co-expression of the human thrombin receptor and stimulation with thrombin did not result in the phosphorylation of the TP receptor isoforms. Similarly, we have recently shown that phosphorylation of the prostacyclin receptor (43) can be induced by thrombin in a PKC-dependent manner, confirming that the endogenous thrombin receptor in HEK-293 cells is sufficient to mediate PKC-dependent phosphorylation of a related GPCR.

In contrast to agonist-dependent phosphorylation, the PKC inhibitors appeared to diminish basal phosphorylation of both isoforms. Similar to our findings with PKC, PKA appeared to be of trivial relevance to agonist-dependent phosphorylation of either isoform. Both FK and Bt2cAMP treatment of the stable isoform. Both FK and Bt2cAMP treatment of the stable

In conclusion, we have demonstrated that the rate and extent of agonist-dependent phosphorylation of the two cloned TP isoforms is similar, although their coupling to [Ca2+]i and IP formation is somewhat different. Neither PKC nor PKA plays a major role in such phosphorylation of either isoform, implicating the G protein–coupled receptor–dependent kinases in homologous desensitization of these receptors. However, both TP isoforms are substrates for PKC and PKA in vivo, and these enzymes may play a more important role in their heterologous desensitization or in receptor regulation in other cells. The availability of specific antibodies for TP isoforms is likely to facilitate investigation of their comparative distribution and biology.

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