The α, but Not the β, Isoform of the Human Thromboxane A$_2$ Receptor Is a Target for Prostacyclin-mediated Desensitization*

Received for publication, September 27, 1999, and in revised form, March 17, 2000

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In this study, we examined the effects of prostacyclin receptor (IP) agonist cicaprost exhibited on U46619-mediated thromboxane A$_2$ receptor (TP) signaling in platelets and compared it to that which occurs in human embryonic kidney (HEK) 293 cells stably overexpressing the individual TPα or TPβ isoforms. Consistent with previous studies, cicaprost abrogated U46619-mediated platelet aggregation and mobilization of intracellular calcium ([Ca$^{2+}$]i). In HEK 293 cells, signaling by TPα, but not TPβ, was subject to IP-mediated desensitization in a protein kinase A-dependent, protein kinase C-independent manner. Desensitization of TPα signaling was independent of the nature of the IP agonist used, the level of IP expression, or the subtype of G$_q$ protein. Signaling by TPβ$_{328}$, a truncated variant of TP devoid of the divergent residues of the TPβs, or by TPβ$_{328}$A, a site-directed mutant of TPβ, were insensitive to IP agonist activation. Whole cell phosphorylations established that TPα, but not TPβ or TPβ$_{328}$A, is subject to IP-mediated phosphorylation and that TPα phosphorylation is inhibited by 8-9. Thus, we conclude that TPα, but not TPβ, is subject to cross-desensitization by IP mediated through direct protein kinase A phosphorylation at Ser$_{328}$ and propose that TPα may be the isoform physiologically relevant to TP/IP-mediated vascular hemostasis.

The prostanoids thromboxane A$_2$ (TXA$_2$)¹ and prostacyclin play key, yet opposing, roles in the maintenance of vascular hemostasis (1). TXA$_2$, which is synthesized mainly by platelets, mediates platelet shape change and aggregation and constriction of vascular and bronchial smooth muscle, whereas prostacyclin, which is synthesized mainly by the vascular endothelium, is a potent inhibitor of platelet aggregation and induces vasodilation (2). TXA$_2$ may also induce prostacyclin release from endothelial cells in vivo (3). Perturbations in the levels of TXA$_2$ or prostacyclin, or their synthases or receptors, have been implicated in various cardiovascular disorders (4–8). However, the molecular mechanisms underlying the counter-regulation of TXA$_2$ and prostacyclin signaling are poorly understood.

Both TXA$_2$ and prostacyclin exert intracellular effects by interaction with specific members of the G protein-coupled receptor (GPCR) family, termed TP and IP, respectively (9, 10). There are two isoforms of TP in humans, termed TPα and TPβ, as recommended by the International Union of Pharmacology classification on prostanoid receptors (11, 12). These receptors, which are identical for their first 328 amino acids and differ exclusively in their carboxyl-terminal cytoplasmic tail (C-tail) regions, arise due to alternative splicing in exon 3 of the TP gene (12, 13). The physiologic relevance for the existence of two receptors for TP is currently unknown. Wide cell and tissue distribution of the mRNA for both TP isoforms was recently confirmed by selective reverse transcription-PCR procedures (14). Isoform specific antibodies permitted detection of TPα, but not TPβ, in human platelets, leading to the suggestion that TPα may be the predominant isoform in platelets (15), despite the presence of mRNA for both isoforms in platelets (16). The major signaling pathway used by TP in vivo is G protein-dependent stimulation of the β-isofoms of phospholipase C (PLCβ), resulting in increased intracellular concentrations of diacylglycerol and inositol 1,4,5-trisphosphate (IP$_3$) and mobilization of intracellular calcium ([Ca$^{2+}$]i) (17). Using a variety of in vitro approaches, various investigators have proposed that the platelet TPβs might couple to the heterotrimeric G proteins G$_q$, G$_{12}$, G$_{13}$, G$_{16}$, and G$_{2}$(18–25). It was recently demonstrated that the cloned TPα can functionally couple to both G$_q$ and G$_{12}$ following stimulation with the selective TXA$_2$ mimetic U46619 and the isoprostane 8-epi-prostaglandin F$_{2α}$ to mobilize [Ca$^{2+}$]i (26). Coupling to G$_{12}$ was more efficient than that to G$_q$. Both TP isoforms couple similarly to G$_{12}$ in stably transfected HEK 293 cells (27) but oppositely regulate adenyl cyclase activity in transfected Chinese hamster ovary cells (16), suggesting a possible role for the C-tail in determining G protein specificity. Moreover, G$_{12}$, the novel high molecular weight G protein that may also function as a transglutaminase (28–31) can mediate agonist activation of TPα, but not TPβ, leading to inositol phosphate production due to PLC activation (32).

A single receptor, termed IP, appears to mediate the actions of prostacyclin leading to activation of adenyl cyclase via G$_s$ and elevation of intracellular cAMP (33), a signaling system thought to be important in control of both vascular tone and platelet aggregation (34). However, IP may also couple to multiple G protein/effector systems including phosphoinositide turnover via a pertussis toxin insensitive G protein (35, 36). In human erythroleukemia cells, IP has even been proposed to differentially couple to both G$_s$ and G$_i$ (37). Iloprost, a stable carbacyclin analogue of prostacyclin, can stimulate opening of ATP-sensitive K$^+$ channels, leading to hyperpolarization and relaxation of canine carotid artery (38). IP is unique among the family of GPCRs in that it undergoes posttranslational modification by carbon-15 farnesyl isoprene groups (39). This isoprenylation is absolutely required for receptor activation of...
adenylyl cyclase via Gs and for efficient coupling to PLC via Gq or G11 (39).

A commonly observed phenomenon among GPCRs is desensitization, defined as reduced receptor responsiveness to repeated agonist challenge (40). GPCR desensitization consists of two key mechanisms, namely phosphorylation of the receptor by specific serine/threonine kinases and sequestration or internalization of receptors to intracellular vesicles where they are unavailable for interaction with G proteins.GPCRs can be subject to either homologous (41, 42) or heterologous (42–47) desensitization, mediated via phosphorylation by G protein-coupled receptor kinases or the second messenger-activated protein kinases, including cAMP-dependent PKA and PKC. Such desensitizations provide mechanisms for feedback regulatory loops following receptor activation and signaling and also for crosstalk between different second messenger systems (43). TPα may be phosphorylated in vitro, in the third extracellular loop and the C-tail, by both PKA and PKC (48). Differences in the complement and distribution of serine (Ser) and threonine (Thr) residues within the divergent C-tails of TPα and TPβ could affect their sensitivity to phosphorylation. Both TPα may be phosphorylated in response to stimulation with the TXA2a mimetic U46619 in transfected HEK 293 cells (49), and recent studies indicate that TPβ but not TPα undergoes agonist internalization (50). Like TP, IP is sensitive to desensitization by second messenger kinases following stimulation with the IP agonist iloprost, with a single PKC phosphorylation site being critical for its desensitization (51, 36).

Thus, both TP and IP are potentially vulnerable to “heterologous desensitization” by elements of intracellular cascades induced by activation of other receptors; for example, the IP: adenyl cyclase system is essential to the control of platelet responses and may be manifested at different levels of the signaling system (52). Indeed, cross-talk occurs between TP and IP in human platelets, with prior U46619 stimulation enhancing iloprost-mediated generation of cAMP (52). Similarly, in the megakaryoblastic cell line MEG-01, TXA2a mimetics U46619 and STAa dose-dependently augment subsequent iloprost-induced cAMP formation in a PKC-dependent manner (53). In view of the interplay between TXA2a and prostacyclin in the maintenance of vascular homeostasis, we considered the potential influence that the intracellular signaling processes induced by IP may have on TP function. In particular, we examined the effect that the selective, high affinity IP agonists cicaprost or iloprost exhibited on U46619-mediated TP responses in platelets. More specifically, we investigated whether crosstalk between IP and TP signaling exists and considered whether such cross-talk may have differential impacts on signaling by the individual TPα and TPβ isoforms. Our results indicate that TPα, but not TPβ, appears to be a direct target for cross-talk between IP and TP responses. Furthermore, H-89, a selective inhibitor of PKA (54, 55), but not the PKC inhibitor GF 109203X (56), reduced IP-mediated desensitization of TPα and platelet TPα, whereas TPβ was insensitive to this desensitization pathway. Prior exposure of HEK.TPα cells, stably expressing a variant of TP truncated at amino acid 328 at the point of divergence of TPα and TPβ, to cicaprost or iloprost did not affect subsequent U46619-mediated TP signaling, implying that the C-tail region is a crucial determinant of heterologous desensitization of TPα by IP-mediated signaling. TPα and TPβ are predicted to contain 9 and 10 putative PKA sites, respectively; however, 8 are conserved between both isoforms, and thus, TPα and TPβ contain 1 and 2 putative PKA sites, respectively, within their unique C-tail sequences. Thus, TPα is predicted to contain a unique PKA consensus site within its divergent C-tail, where Ser429 represents the putative target residue for phosphorylation. U46619-mediated [Ca2+] mobilization by HEK.TPαSR232A cells stably overexpressing a site-directed mutant of TPα was insensitive to IP (cicaprost or iloprost)-mediated desensitization, confirming that Ser429 is a target for IP-mediated desensitization. Finally, whole cell phosphorylation assays established that TPα, but not TPβ or TPαSR232A, is subject to IP-mediated phosphorylation and that phosphorylation of TPα is abrogated in the presence of H-89. Thus, taken together, our results establish that TPα, but not TPβ, is subject to cross-desensitization by IP that is mediated through direct PKA phosphorylation of Ser429 and therefore imply that TPα may be the isoform physiologically relevant to the maintenance of vascular hemostasis.

**EXPERIMENTAL PROCEDURES**

**Materials**—The following chemicals were obtained from Cayman Chemical Co.: 5-heptenoic acid, 7-(6-(3-hydroxy-1-oxetanyl)-2-oxacyclo [2,2,1]-hept-5-yl)-1R-[1α,4,5β(5)],6R(1E,3S)9-11-dideoxy-9α,11-lmethanoepoxy prostaglandin F1 (U46619); 5-heptenoic acid, 7-[[3-[Z-phenylamino carbonyl] hydrazino]methyl]-7-oxacyclo [2,2.1].1-hept-2-yl-11S-[1α,2α(Z),3α,4α] (SQ29,548); thromboxane B2 enzyme immunology kit. G418, 1-[2-(5-carboxyoxazol-2-yl)-6-aminoazobenzofuran-2-oxyl-2 (2-amino-3-methylphenyl) ethane-N,N,N′,N′′-tetraacetic acid, pentaoxymethylene ethyl ester (Fura2/AM), D-phosphatase antibody (5–7 mg/ml) was obtained from Babco; horseradish peroxidase-conjugated goat anti-mouse secondary antibody was from Santa Cruz Biotechnology; protein G-Sepharose 4B Fast Flow was obtained from Amersham Pharmacia Biotech. Ultraspec total RNA isolation system was obtained from Biotec Laboratories (Houston, TX); Moloney murine leukemia virus reverse transcriptase, RNasin, deoxyribonucleotides, and Taq DNA polymerase were obtained from Promega. Expand High Fidelity® Taq DNA polymerase, Chemiluminescence Western blotting kit, polynylidiene difluoride membrane, and rat monoclonal 3F10 anti-HA- horseradish peroxidase-conjugated antibody were obtained from Roche Molecular Biochemicals. Mouse monoclonal 101R anti-HA-peroxidase antibody (5–7 mg/ml) was obtained from Babco; horseradish peroxidase-conjugated goat anti-mouse secondary antibody was from Santa Cruz Biotechnology; protein G-Sepharose 4B Fast Flow was obtained from Sigma. All oligonucleotides were synthesized by Genosys Biotechnologies.

**Subcloning and Site-directed Mutagenesis of TPα and TPβ**—The plasmids pCMV5, pCMV:TXR (26), pcDNA3:TPβ (27) have been previously described. To facilitate amino-terminal epitope tagging of proteins with the hemagglutinin (HA) epitope tag (57), cDNAs encoding TPα and TPβ were subcloned in-frame into the HindIII-BamHI sites of the pHm6 (Roche Molecular Biochemicals) to generate pHm:TPα and pHm:TPβ, respectively.

Deletion of the amino acids carboxyl to Arg232, at the point of divergence between TPα and TPβ was achieved by conversion of Ser codon 329 to a stop codon (Ser429TCG to stop429TTA). Site-directed mutagenesis was performed by PCR mutagenesis using pCMV:TXR as template and oligonucleotides 5′-CTCTAAAGCTTATG TGG CCC AAC GGC AGT-3′ (sense primer; nucleotides +1 to +18 of TP sequences are underlined) and 5′-CTCTGATCTT7CTATCGGCGCGGCTGTCAGAG′ (antisense primer; sequences complementary to nucleotides +967 to +984 of TP sequences are underlined, and the mutant in-frame stop codon is in boldface italics). The resulting PCR-amplified cDNA was subcloned into the HindIII-BamHI site of pHm6 (Roche Molecular Biochemicals) to generate pHm:TPα and pHm:TPβ, respectively.

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mouse prostanoid receptor (IP), respectively, have been described previously (26, 39).

**Cell Culture and Transfections—**Human embryonic kidney (HEK) 293 cells were obtained from the American Type Culture Collection and were grown in minimal essential medium containing 10% fetal bovine serum.

Cells were transfected with 10 μg of pADVA (58) and 25 μg of pCMV- pcDNA, or pcDNA-based vectors using the calcium phosphate/DNA co- precipitation procedure (26). For transient transfections, cells were harvested 48 h after transfection. To create stable cell lines, HEK 293 cells were transfected with 10 μg of ScramLinized pADVA plus 25 μg of pCMV- pcDNA or pcDNA-based vectors, as described previously (39). Levels of cAMP produced by each ciguatoxin-stimulated cells over basal stimulation, in the presence of HBS, were expressed in pmol of cAMP/mg of cell protein ± S.E. and as fold stimulation over basal (fold increase ± S.E.). The data presented are representative of four independent experiments, each performed in duplicate.

**Measurement of cAMP—**cAMP production was quantified by radiomunnoassay using the cAMP-binding protein from bovine adrenal medulla. Levels of IP3 produced by ciguatoxin-stimulated cells over basal stimulation, in the presence of HBS, were expressed in pmol of IP3/mg of cell protein ± S.E. and as fold stimulation over basal (fold increase ± S.E.). The data presented are representative of four independent experiments.

**Reverse Transcriptase-Polymerase Chain Reaction—**Total RNA isolated from human erythroblasts 92.1.7 or HEK 293 cells using the Ultrasep RNA isolation procedure was converted to first strand cDNA with Moloney murine leukemia virus reverse transcriptase, as described previously (14). Aliquots (3.5 μl) of each first strand cDNA were used as templates in PCRs (25 μl) using the following primers specific for the human IP3 receptor: primer A, 5′-GCTCCGCTGCTACTCAGTACGGCTGCTACCC-3′ (sense primer); and primer B, 5′-GTGCTGGAGTCAGGAGGAGCAGGG-3′. PCR products were designed to span across intron 2 of the human IP3 gene (63) to distinguish products derived from first strand cDNA from trace genomic DNA present in the total RNA.

**Measurement of Agonist-mediated TP Phosphorylation—**Whole cell phosphorylation assays were performed essentially as previously (49) with certain modifications. Briefly, cells (2 × 107 cells in 60-mm dishes) were washed once in phosphate-free Dulbecco's modified Eagle's medium, 10% dialyzed fetal bovine serum and were metabolically labeled for 60 min in the same medium (1.5 ml/60-mm dish) containing 100 μCi/ml [3H]phosphatidylcholine (8000–9000 Ci/mmol) at 37 °C, 5% CO2. Where appropriate, kinase inhibitor (H-89, 10 μM) or vehicle was added during the labeling period. Thereafter, specific ligand or vehicle was added for 10 min. Reactions were terminated by transferring the dishes to ice and aspirating the labeling medium. Cells were washed once in ice-cold phosphate-buffered saline (2 ml/dish) and were lysed with 0.6 ml of radioimmunoprecipitation buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate. Following 15 min of incubation on ice, cells were harvested using a rubber policeman and disrupted by sequentially passing through hypodermic needles of decreasing bore size (G20, G21, G23, and G26), and soluble cell lysates were harvested by centrifugation for 15 min at 13,000 × g at room temperature. HA epitope TP receptors were immunoprecipitated using those cells, and immunoblots were screened using the anti-HA antibody (1:300 dilution) at room temperature. HA epitope TP receptors were immunoprecipitated using the anti-HA antibody (1:300 dilution) at room temperature for 2 h followed by the addition of 10 μl of protein G-Sepharose 4B (Sigma) and further incubation at room temperature for 1 h. Immune complexes were collected by centrifugation at 13,000 × g at room temperature for 5 min and were washed three times in 0.5 ml of radioimmunoprecipitation buffer and finally resuspended in 1× solubilization buffer (10 μM β-mercaptoethanol, 1% Triton X-100, 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM KCl, 5.5 mM glucose, 1 mM phenylmethylsulfonyl fluoride, 10 μM sodium orthovanadate). cAMP produced was quantified by radioimmunoassay using cAMP-producing cells over basal stimulation, in the presence of HBS, were expressed in pmol of cAMP/mg of cell protein ± S.E. and as fold stimulation over basal (fold increase ± S.E.). The data presented are representative of four independent experiments, each performed in duplicate.

**Data Analyses—**Radioligand binding data were analyzed using GraphPad Prism V2.0 (GraphPad Software Inc.) to determine the Kd and Bmax values. Statistical analyses were carried out using the un-
mobilization of [Ca\(^{2+}\)], in response to U46619, it was necessary to co-transfect the cells with the α subunit of a member of the G\(_{\alpha}\) family of heterotrimeric G proteins (G\(_{\alpha11}\), for example) (Fig. 2, A and E).

HEK\(\alpha10\) cells co-transfected with G\(_{\alpha11}\) showed efficient mobilization of [Ca\(^{2+}\)], in response to 1 μM U46619 (Fig. 2A). Cicaprost (1 μM) stimulated a 2-fold elevation of intracellular cAMP levels over vehicle-treated cells (Fig. 2C), thereby confirming the presence of IP in HEK\(\alpha10\) cells. The presence of mRNA encoding IP in HEK 293 cells was also confirmed by reverse transcription-PCR (Fig. 2D). Cicaprost at 1 μM (Fig. 2B) or 10 μM (data not shown) did not stimulate [Ca\(^{2+}\)] mobilization in HEK\(\alpha10\) cells transiently co-transfected with G\(_{\alpha11}\), indicating that the endogenous IP receptors present in these cells lack the ability to couple to PLC\(\beta\). However, following prior stimulation with 1 μM cicaprost, mobilization of [Ca\(^{2+}\)] induced by U46619 was significantly reduced to 44.3 ± 7.3% of that generated by U46619 stimulation only (Fig. 2B, p < 0.001). This implies that, as in human platelets, activation of IP leads to desensitization of TP\(\alpha\) signaling in HEK 293 cells, confirming cross-talk between the cAMP signaling system induced by IP activation and the IP\(_{\alpha}\) dependent Ca\(^{2+}\) mobilization system induced by TP\(\alpha\) activation.

To investigate whether the “cross-talk” between the IP and TP signaling systems extended to TP\(\beta\), the effects of cicaprost on subsequent U46619-induced mobilization of [Ca\(^{2+}\)], by HEK\(\beta3\) cells was monitored. HEK\(\beta3\) cells stimulated with 1 μM U46619 showed efficient mobilization of [Ca\(^{2+}\)], which was dependent on the presence of G\(_{\alpha11}\) (Fig. 2E). Similar to that observed in platelets and HEK\(\alpha10\) cells, HEK\(\beta3\) cells co-transfected with G\(_{\alpha11}\) did not support [Ca\(^{2+}\)] mobilization in response to cicaprost (Fig. 2F). However, in contrast to both platelets and HEK\(\alpha10\) cells, prior stimulation with 1 μM cicaprost showed no reduction in U46619-mediated mobilization of [Ca\(^{2+}\)] (Fig. 2F). To determine whether the difference in IP-mediated desensitization of TP\(\alpha\) and TP\(\beta\) could be accounted for by an inability of HEK\(\beta3\) cells to support elevation in cAMP in response to cicaprost, the presence of functional endogenous IP receptors in HEK\(\beta3\) cells was confirmed by analyzing cicaprost-mediated increases in cAMP formation (Fig. 2G). The
observed elevation of cAMP levels was not significantly \((p > 0.1)\) different between HEKβ3 and HEKα10 cells (Fig. 2, C and G). The higher levels of cAMP elevation observed using 10 \(\mu M\) cicaprost were still insufficient to reduce subsequent U46619-induced \([Ca^{2+}]]\), mobilization in HEKβ3 cells (data not shown).

Similar to the results with cicaprost, prior exposure of HEKα10 cells with iloprost (1 \(\mu M\)) reduced subsequent U46619-mediated mobilization of \([Ca^{2+}]]\), by TPα to 58.4 \(\pm\) 4.1% (Fig. 3A), whereas mobilization of \([Ca^{2+}]]\), by TPβ was unaffected by iloprost (Fig. 3B). There was no significant difference in iloprost- or cicaprost-mediated desensitization of TPα \((p = 0.168)\). Moreover, whereas HEK 293 cells do contain endogenous IP (Table I), which is coupled to activation of adenylyl cyclase (Fig. 2, C and G), it is formally possible that the levels of endogenous IP expressed are not sufficiently high to mediate efficient desensitization of the TP isoforms, which might also account for the failure to observe desensitization of the TPβ isoform in these cells. Thus, to address this, HEKα10 or HEKβ3 cells were transiently co-transfected with the cDNA encoding the mouse IP. Overexpression of IP was initially confirmed by saturation radioligand binding studies using \([3H]IP\) (Fig. 5A). Thereafter, the effect of overexpression of IP on iloprost-mediated (Fig. 3C) or cicaprost-mediated (data not shown) desensitization of the TP isoforms was investigated. Similar to previous data involving endogenous IPs, prior stimulation of HEKα10 cells with iloprost significantly reduced U46619-mediated \([Ca^{2+}]]\), mobilization by TPα to 49.8 \(\pm\) 5.78% (Fig. 3C), whereas iloprost had no effect on U46619-mediated \([Ca^{2+}]]\), mobilization in HEKβ3 cells (Fig. 3D). Moreover, overexpression of IP in HEKα10 cells did not significantly enhance iloprost-mediated \((p = 0.29)\) or cicaprost-mediated (data not shown) desensitization of TPα signaling, indicating that the endogenous levels of IPs expressed in HEK 293 cells are sufficient to mediate efficient desensitization of TPα in those cells.

To investigate whether IP-mediated desensitization of TP signaling in HEK 293 cells may be dependent on the nature of the coupling G protein, we extended our studies to investigate the effect of co-expression of the α subunit of \(G_i\) as a substitute to \(G_{111}\). Prestimulation of cells with iloprost (Fig. 3E) or cicaprost (data not shown) reduced subsequent U46619-mediated \([Ca^{2+}]]\), mobilization in HEKα10 cells to 55.1 \(+\) 2.2% (Fig. 3E), whereas U46619-mediated \([Ca^{2+}]]\), mobilization in HEKβ3 cells was unaffected (Fig. 3F). Moreover, IP-mediated desensitization of TPα was not significantly different in the presence of \(G_i\) compared with \(G_{111}\) \((p = 0.52)\). Thus, we conclude that TPα but not TPβ is subject to IP-mediated desensitization in HEK 293 cells and that this desensitization is independent of the nature of the IP agonist, is independent of the level of IP expression, and is independent of the coupling G protein.

\section*{Differential Effects of Cicaprost on U46619-mediated IP3 Generation via TPα and TPβ Isoforms—}To further investigate the differential effects of IP activation on TPα and TPβ signaling, \([3H]IP\) binding to IP γ isoforms was measured in HEKα10 and HEKβ3 cells in the presence or absence of prestimulation with cicaprost. Stimulation of HEKα10 and HEKβ3 cells with U46619 (1 \(\mu M\)) resulted in 1.5- and 1.4-fold increases in IP3 levels, respectively, comparable with previously reported data (16). However, preincubation of HEKα10 cells with cicaprost (1 \(\mu M\)) significantly \((p = 0.024)\) reduced U46619-mediated IP3 generation by TPα (Fig. 4A). However, in contrast to HEKα10 cells, preincubation of HEKβ3 cells with cicaprost (1 \(\mu M\)) did
not significantly (p = 0.42) reduce U46619-mediated IP₃ generation by TPβ (Fig. 4B). Stimulation of HEK 293 cells with U46619 or HEK.a10 and HEK.b3 cells with cicaprost alone failed to generate any increase in IP₃, further indicating that endogenous IP receptors in HEK 293 cells do not couple to PLC.

H-89, an Inhibitor of PKA, Prevents Cicaprost-induced Inhibition of TP Signaling—Second messenger protein kinases, such as PKA and PKC, have been implicated in heterologous desensitization and in mediating cross-talk between different G-protein-coupled receptor signaling systems (42–47). Moreover, both IP and TP are subject to phosphorylation by these kinases (48, 49, 51). Thus, to investigate whether PKA and/or PKC is involved and provide a potential mechanism whereby IP activation cross-desensitizes TP signaling in platelets and HEK.a10 cells, we used H-89, a specific inhibitor of PKA (54, 55), and GF 109203X, a specific PKC inhibitor (56). Preincubation of platelets with 50 nM GF 109203X for 2 min prior to agonist stimulation had no effect on cicaprost induced desensitization of U46619-mediated [Ca²⁺], (Fig. 5, A and B). On the other hand, pretreatment of platelets with 10 μM H-89 for 1 min prior to cicaprost (1 μM) stimulation completely restored subsequent U46619-mediated (1 μM [Ca²⁺],₁) mobilization to normal, precicaprost levels (Δ[Ca²⁺],₁ = 195 ± 45.9 nM; Fig. 5C). Similarly, in HEK.a10 cells, whereas pretreatment with 50 nM GF 109203X for 2 min had no effect on cicaprost inhibition of U46619-induced [Ca²⁺], mobilization (Fig. 6, A and B), pretreatment with H-89 (10 μM, 1 min) significantly (p < 0.001) rescued cicaprost-induced inhibition of U46619-induced [Ca²⁺], mobilization from 45 to 86% (Fig. 6C). In HEK.b3 cells, in which the U46619-induced changes in [Ca²⁺], mobilization are impervious to prestimulation by cicaprost, addition of GF 109203X or H-89 had no effect on signaling by TPβ (Fig. 6, D–F). These results indicate that the observed reduction of U46619-induced changes in [Ca²⁺], by cicaprost or iloprost in HEK.a10 cells is largely due to activation of PKA and subsequent phosphorylation, either of TPβ directly or of some other element of its signaling pathway. Mobilization of [Ca²⁺], via TPβ is, on the other hand, unaffected by PKA in this cross-desensitization pathway.

It has recently been reported that H-89 may act as antagonist of certain GPCRs, thereby calling into question its utility as a selective PKA inhibitor (65). Thus, to rule out the possibility that H-89 may act as an antagonist of the IP, cicaprost-mediated (1 μM) cAMP stimulation was measured in HEK 293 cells over expressing the IP in the absence and presence of 10 μM H-89. No significant difference (p > 0.84) was observed in cells stimulated in the absence (1 μM cicaprost; fold increase in cAMP = 22.2 ± 3.74) or presence (1 μM cicaprost, 10 μM H-89; fold increase in cAMP = 20.9 ± 4.71) of H-89, confirming that H-89 does not function as an antagonist of IP.

### Table I

| Cell type | [³H]Iloprost bound fmol/mg cell protein ± S.E. |
|-----------|------------------------------------------|
| HEK 293   | 15.4 ± 4.1                              |
| HEK.a10 + IP | 244 ± 32                             |
| HEK.b3 + IP | 234 ± 19                              |
| HEK TPαS253A + IP | 229 ± 12                           |
| HEK TPαS253A + IP | 203 ± 5                           |

**FIG. 3.** IP-mediated desensitization of TPα signaling is independent of IP agonist, the level of IP expression, and the G subunit. HEK.a10 cells (A, C, and E) or HEK.b3 cells (B, D, and F) were transiently co-transfected with pCMV:Gα₁₁ (A–D), pCDNA3.mIP (C and D), or pCMV:Gα₁₁ (E and F). After 48 h, cells were harvested, preloaded with Fura2/AM, and stimulated either with U46619 (1 μM) or with iloprost (1 μM) followed by U46619 (1 μM), as indicated. The ligands were added at the times indicated by the arrows. The results are representative of at least three independent experiments and are plotted as changes in intracellular Ca²⁺ mobilized as a function of time following ligand stimulation. Actual changes in [Ca²⁺], mobilization were as follows. Panel A: (1 μM U46619, Δ[Ca²⁺],₁ = 160 ± 10 nM; 1 μM iloprost, Δ[Ca²⁺],₁ = 0 nM; 1 μM U46619, Δ[Ca²⁺],₁ = 93.5 ± 6.5 nM). Panel B: (1 μM U46619, Δ[Ca²⁺],₁ = 130 ± 10 nM; 1 μM iloprost, Δ[Ca²⁺],₁ = 207 ± 21.4 nM). (1 μM iloprost, Δ[Ca²⁺],₁ = 0 nM; 1 μM U46619, Δ[Ca²⁺],₁ = 107 ± 12.5 nM). Panel D: (1 μM U46619, Δ[Ca²⁺],₁ = 203 ± 8.82 nM). Panel E: (1 μM U46619, Δ[Ca²⁺],₁ = 126 ± 4 nM; 1 μM iloprost, Δ[Ca²⁺],₁ = 0 nM; 1 μM U46619, Δ[Ca²⁺],₁ = 216 ± 4 nM). Panel F: (1 μM U46619, Δ[Ca²⁺],₁ = 186 ± 4 nM; 1 μM iloprost, Δ[Ca²⁺],₁ = 0 nM; 1 μM U46619, Δ[Ca²⁺],₁ = 189 ± 1 nM.).
Cicaprost-induced Desensitization of TP Signaling Is Mediated by the TP C-tail—In order to establish whether the C-tail of the TPα contains the target regulatory site for phosphorylation by PKA, deletion mutagenesis was utilized to generate a truncated version of TP (TPA328), which is devoid of the C-tail sequences carboxy-terminal to amino acid 328 at the point of divergence of TPα and TPβ. A stable HEK 293 cell line overexpressing TPA328 was established, and cells were characterized by Scatchard analysis using [3H]SQ29,548 as the specific ligand. Values obtained for the affinity (Kd) and maximal binding (Bmax) for TPA328 (Kd = 6.99 ± 0.88 nM; Bmax = 1.54 ± 0.28 pmol/mg; n = 5) compared well to values previously reported for the wild type TPα and TPβ receptors (26). It is noteworthy that TPA328 exhibited identical affinity for its ligand and retained the ability to mediate specific agonist-induced intracellular signaling, albeit at somewhat reduced levels relative to those of the wild type TPs. Intracellular signaling by HEK.TP328 cells transiently co-transfected with Gα11 was investigated by analyzing [Ca2+]i mobilization and IP3 generation in response to the TXA2 mimetic U46619 and the effect of cicaprost on TP signaling was assessed. Stimulation of HEK.TP328 cells with U46619 (1 μM) led to mobilization of [Ca2+]i (Fig. 7A), whereas stimulation with cicaprost (1 μM) did not (Fig. 7B). Unlike platelets or HEK.a10 cells, pretreatment with cicaprost did not reduce subsequent U46619-induced [Ca2+]i mobilization (Fig. 7B). Moreover, these effects were independent of the agonist used or the level of IP expression (Fig. 7, C and D; Table I). Similarly, U46619 stimulation of HEK.TP328 cells generated increases in IP3 levels, which were not significantly reduced by prior stimulation with cicaprost (data not shown). These data confirm that the increased sensitivity to cicaprost or iloprost observed for TPα, as opposed to TPβ and TPS328, is due to unique elements in the C-tail of TPα, most likely, given that pretreatment with H-89 alleviated cicaprost induced inhibition of TPα signaling, at an important PKA-sensitive phosphorylation site(s).

TPαS329A Is Not Subject to IP-mediated Desensitization—Computational analysis of the amino acid sequence of the C-tail of TPα for putative protein kinase phosphorylation sites using the PhosphoBase program for sequence analysis (66) identified the presence of a unique consensus PKA phosphorylation site within the sequence RPRSLSL, where Ser329 was identified as the target residue for phosphorylation. Therefore, to investigate whether this consensus PKA phosphorylation site may represent a target site for IP-mediated desensitization of TPα, the critical Ser329 was mutated Ala329 to generate the variant TPαS329A. A stable HEK 293 cell line overexpressing a HA epitope-tagged TPαS329A was established, and cells were characterized by Scatchard analysis using [3H]SQ29,549 (Kd = 9.63 ± 0.94 nM; Bmax = 6.01 ± 0.16 pmol/mg; n = 3). Stimulation of HEK.TPαS329A cells with U46619 (1 μM) led to efficient mobilization of [Ca2+]i, whereas stimulation of cells with iloprost did not (Fig. 8, A and B). However, initial stimulation of cells with iloprost did not reduce subsequent U46619-induced [Ca2+]i mobilization. Moreover, these effects were independent of the agonist used (cicaprost or iloprost), the coupling G protein (Gα11 or Gα11), of the level of IP expression (Fig. 8; Table I).

IP-mediated Desensitization Involves Phosphorylation of TPα at Ser329—To further investigate whether the TP isoforms or TPαS329A are subject to IP-mediated phosphorylation, additional stable cell lines overexpressing HA epitope-tagged variants of TPα and TPβ were established. HA.β receptors were characterized by radioligand binding studies, and each cell line exhibited similar levels of [3H]SQ29,548 binding (approximately 3 pmol of [3H]SQ29,548 bound/mg of cell protein); moreover, consistent with previous reports (50), the presence of the HA epitope tag did not affect the ligand binding characteristics or the cell signaling properties of the TPs (data not shown). Initially, the specificity of the anti-HA antibodies to immunoprecipitate TPs from the respective cell lines, but not from the parental HEK 293 cells, was confirmed (Fig. 9D). The presence of a discrete band of approximately 39 kDa and a broad diffuse band of 46–66 kDa was evident in TPα and TPαS329A immunoprecipitates; the narrow and broad bands may represent the nonglycosylated and glycosylated forms, respectively, of TPα and TPαS329A. Similarly, bands of 46 kDa and 50–66 kDa, possibly representing the nonglycosylated and glycosylated forms of TPβ, respectively, were immunoprecipitated from cells overexpressing TPβ. On the other
Fig. 6. Effect of kinase inhibitors on cicaprost-mediated desensitization of TP signaling in HEK.a10 and HEK.β3 cells. HEK.a10 (A–C) or HEK.β3 (D–F), transiently co-transfected with pCMV-Gα11i, were preloaded with Fura2/AM and stimulated with 1 μM cicaprost followed by 1 μM U46619 (A and D). Alternatively, cells were preincubated with 50 nM GF 109203X and then stimulated with 1 μM cicaprost followed by 1 μM U46619 (B and E) or were preincubated with 10 μM H-89 and then stimulated with 1 μM cicaprost followed by 1 μM U46619 (C and F). The ligands were added at the times indicated by the arrows. The results presented are representative of at least four independent experiments and are plotted as changes in intracellular Ca2+ mobilized (n = 4) as a function of time following ligand stimulation. Actual changes in [Ca2+]i, mobilization were as follows: 1 μM U46619, Δ[Ca2+]i = 114 ± 12.3 nM (data not shown). A, 1 μM cicaprost, Δ[Ca2+]i = 0 nM; 1 μM U46619, Δ[Ca2+]i = 50.0 ± 5.8 nM. B, 1 μM cicaprost, Δ[Ca2+]i = 0 nM; 1 μM U46619, Δ[Ca2+]i = 51.0 ± 11.5 nM. C, 1 μM cicaprost, Δ[Ca2+]i = 0 nM; 1 μM U46619, Δ[Ca2+]i = 98.3 ± 11.3 nM. D, 1 μM cicaprost, Δ[Ca2+]i = 0 nM; 1 μM U46619, Δ[Ca2+]i = 117 ± 9.6 nM. E, 1 μM cicaprost, Δ[Ca2+]i = 0 nM; 1 μM U46619, Δ[Ca2+]i = 112 ± 3.1 nM. F, 1 μM cicaprost, Δ[Ca2+]i = 0 nM; 1 μM U46619, Δ[Ca2+]i = 110 ± 3.7 nM.

Fig. 7. Effect of IP agonists on U46619-mediated signaling by TPαS329A. HEK.TPαS329A cells were transiently co-transfected either with pCMV-Gα11i (A and B) or with pCMV-Gα11i plus pDNA3.miP (C and D). After 48 h, cells were preloaded with Fura2/AM and were stimulated with 1 μM U46619 alone (A and C), with 1 μM cicaprost followed by 1 μM U46619 (B), or with 1 μM iloprost followed by 1 μM U46619 (D), as indicated. The ligands were added at the times indicated by the arrows. The results presented are representative of at least four independent experiments and are plotted as changes in intracellular Ca2+ mobilized as a function of time following ligand stimulation. Actual changes in [Ca2+]i, mobilization were as follows. A, 1 μM U46619, Δ[Ca2+]i = 55.1 ± 6.53 nM. B, 1 μM cicaprost, Δ[Ca2+]i = 0 nM; 1 μM U46619, Δ[Ca2+]i = 59.9 ± 8.23 nM. C, 1 μM U46619, Δ[Ca2+]i = 95 ± 15 nM. D, 1 μM iloprost, Δ[Ca2+]i = 0 nM; 1 μM U46619, Δ[Ca2+]i = 104 ± 13.5 nM.

hand, no immunoreactive bands were evident in immunoprecipitates prepared from nontransfected HEK 293 cells (Fig. 9D).

Stimulation of cells with U46619 (1 μM, 10 min) led to 5–7-fold increases in the phosphorylation of TPα, TPβ, and TPαS329A, confirming that each of these receptors is subject to homologous desensitization (Fig. 9A–C). Stimulation of cells with iloprost (1 μM, 10 min) led to a 5-fold increase in the phosphorylation of TPα (Fig. 9A) but no increase in the phosphorylation of TPβ or TPαS329A (Fig. 9A–C) but no increase in the phosphorylation of TPβ or TPαS329A. Moreover, H-89 blocked iloprost-mediated phosphorylation of TPα (Fig. 9A). Similar findings were observed when cicaprost was used as the stimulating ligand. Thus, taken together, these studies confirm that TPα, but not TPβ, is subject to IP-mediated desensitization at a PKA-sensitive site within its unique C-tail, that this desensitization involves direct IP-induced PKA phosphorylation of TPα, and that Ser329 is the target phosphoamino acid.

Discussion

The prostanoioids TXA2 and prostacyclin play central yet opposing roles in the maintenance of vascular hemostasis (1), and thus, it is essential that their activities be tightly regulated. Individually, both the TXA2 (TP) and prostacyclin (IP) receptors are subject to specific agonist-induced, homologous desensitization mediated, in part, by the second messenger kinases PKA and/or PKC (15, 36, 49, 51, 67–69). Additionally in humans, TP signaling is mediated through two receptor isoforms, TPα and TPβ, providing a potential mechanism(s) for more complex regulation of responses to TXA2 in cells (11, 12). Whereas both isoforms exhibit identical ligand binding (8, 12, 27) and activation of PLCβ (8, 27), they oppositely regulate adenyl cyclase activity (8) and may be subject to differential expression (19, 14) and agonist-mediated desensitization (67, 50).

Intermolecular cross-talk has been extensively demonstrated between different GPCRs and their intracellular signaling pathways (42–47) and between GPCRs and members of the tyrosine kinase receptor family (42, 43). For example, in A7r5 vascular smooth muscle cells transfected with TPα, stimulation with the TXA2 mimetic I-BOP led to activation of the mitogen-activated protein kinase cascade with concomitant ty-
vested, preloaded with Fura2/AM, and stimulated either with 1 mM PCNA3:mIP (NO on TP responses. Moreover, both TP by PKG may provide a mechanism for the inhibitory effects of the human platelet TP(s) as a substrate for cGMP-dependent platelet GpIIbIIIa receptor (80). A recent study (81) identified endogenous vasodilator (72, 73), which further protects the endothelium secretes nitric oxide (NO), the most important known 71). Similarly, in addition to prostacyclin, the vascular endo-

system within platelets and vascular smooth muscle (51, 34, 57). The data indicated that TPα, but not TPβ, is a target for IP-mediated cross-desensitization of TP responses. Moreover, these effects were independent of the IP agonist used, the coupling G protein, or the level of IP expression. In investigating the kinetics of TPα desensitization, prestimulation of cells with cicaprost from times ranging from 30 s to 5 min prior to U46619 stimulation resulted in no significant, time-dependent difference in IP-mediated desensitization of TPα signaling over that period of time (n = 12, p = 0.662). As the IP itself is widely reported to undergo agonist-mediated internalization, leading to loss of surface receptors (64), longer preincubations with IP agonists make results difficult to interpret.

We have previously reported that in order to support efficient mobilization of [Ca^{2+}], in HEK cells overexpressing TP, it was necessary to overexpress the β subunits of a member of the Gq family such as Goq or Go11 (26). In the absence of the co-transfected G protein, the levels of [Ca^{2+}], mobilization are low and may reflect the endogenous pool of Go subunits, which may not be sufficient to support signaling due to relative abundance of overexpressed TP receptors. Our findings for a requirement of exogenous G protein to mediate efficient receptor signaling are not unique. In the case of the Gα-adrenergic receptor, stimulation of PLC activity in HEK 293 cells was completely dependent on co-expression of Gq (83). Whereas platelets and other hematopoietic cells are reported to express Goq and Gβq, these cells are reported not to express significant levels of Go11 (84–86). To investigate whether IP-mediated desensitization of TP signaling in HEK 293 cells may be dependent on the nature of the coupling G protein, we extended our studies to investigate the effect of co-expression of the β subunit of Go as opposed to Gα11. Substitution of Go11 with Goq also supported IP-mediated desensitization of TPα but not TPβ in response to cicaprost or iloprost.

Preincubation of platelets or HEK.α10 cells with the PKA inhibitor H-89 almost completely blocked cicaprost-mediated desensitization of TP signaling. On the other hand, H-89 had no effect on TPβ signaling, and the PKC inhibitor GF 109203X had no appreciable effect on IP desensitization of TP signaling in platelets or on TPα or TPβ signaling in HEK 293 cells. These data indicated that TPα, but not TPβ, is sensitive to IP desensitization mediated by the second messenger kinase PKA. As the α and β isoforms of TP differ exclusively in their carboxy-terminal cytoplasmic tail sequences (11, 12), it is possible that TPα is subject to IP-stimulated PKA phosphorylation at a site distal to the point of divergence of these TP receptor isoforms. Thus, to investigate this possibility, a stable cell line overexpressing a truncated variant of TP (TPα328) was established. Whereas deletion of the divergent carboxy-terminal amino acids of the TP had no effect on ligand binding, intracellular signaling by the deletion mutant TPα328 was significantly reduced relative to that of the wild type TPα/TPβ receptors, thus
supporting the notion that the C-tail of TP may also play a role in G protein coupling (16, 32). Consistent with these findings, Spurney and Coffman (68) reported that deletion of the carboxyl-terminal 22 amino acids of the single mouse TP also yielded a receptor with identical ligand binding properties to the wild type receptor but with diminished agonist-mediated IP formation. Unlike that exhibited by the platelet TP(s) or the TPα isoform, U46619-mediated signaling by TPα328A was insensitive to prestimulation of IPs (endogenous or overexpressed) with cicaprost or iloprost. Taken together, these data imply that IP-induced desensitization of TP responses is mediated, at least in part, via a PKA target site found in the TPβ isoform. TPα328 differs from the wild type TPα in that it is devoid of the carboxyl-terminal 15 amino acid residues of the latter receptor. We have previously established that the C-tail of TPα is phosphorylated in vitro by PKA (48). Computational analysis of the C-tail sequences of TPα for putative protein kinase phosphorylation sites (66) identified the presence of a unique consensus PKA phosphorylation site within the sequence KPRSLSL, where Ser329 was predicted to represent the target residue for phosphorylation. Thus, to investigate whether this consensus PKA site may represent the target site for IP-mediated desensitization of TPα, the critical Ser329 was mutated to Ala329 generating the variant TPα328A. Whereas stable cell lines expressing TPα328A, exhibited identical ligand binding characteristics and agonist-mediated intracellular signaling to that of the wild type TPα, U46619-mediated signaling by TPα328A was insensitive to prestimulation of IPs with cicaprost or iloprost. Moreover, these effects were independent of the agonist used (cicaprost or iloprost), the level of IP expression (endogenous or overexpressed) or the coupling G protein, Goq or Go11. Finally, to establish whether the TP(s) may be direct a target for IP-mediated phosphorylation, stable cell lines expressing HA epitope-tagged forms of TPα, TPβ, or TPα328A were used in whole cell phosphorylation assays. Whereas each of the TP receptors underwent U46619-mediated phosphorylation, stimulation of cells with iloprost specifically resulted in agonist-dependent phosphorylation of TPα but not TPβ or TPα328A. Moreover, the PKA inhibitor H-89 blocked iloprost-mediated phosphorylation of TPα. Taken together, these studies confirm that TPα, but not TPβ, is subject to IP-mediated desensitization at a PKA-sensitive site within its unique C-tail and that this desensitization involves direct IP induced PKA phosphorylation of TPα. It is also evident that Ser329, the very first divergent residue between TPα and TPβ, is the target for IP-mediated phosphorylation of TPα.

In view of the differential desensitization of the TPα by IP-selective agonists coupled to the lack of desensitization of TPβ, TPβ328A, and TPα328A receptors and our demonstration of IP-mediated PKA phosphorylation of TPα and not TPβ, TPβ328A, and TPα328A receptors, the mechanism of desensitization involves direct TPα phosphorylation. Moreover, in view of the selective involvement of TPα and not the other TPβ, TPβ328A, and TPα328A receptors, it is unlikely that other components of the signaling system, such as the Go subunits, are involved. In terms of the structure-function relationships of GPCRs, many of the target desensitization/phosphorylation sites on the GPCR are not actually located within the G protein interacting domains (mainly believed to be intracellular loop 3 with roles also evoked for intracellular loops 2 and 1, and in some cases, the C-tail) but are mainly located within the C-tail regions of GPCRs. Our findings on TPα phosphorylation is consistent with this. Phosphorylation of receptors by the second messenger kinases PKA/PKC are mainly associated with classic feedback mechanisms or in mediating cross-talk whereby phosphorylation is believed to alter the overall conformation of the receptor thereby interfering with receptor: G protein interactions. Other mechanisms are believed to occur for other kinases, such as the G protein-coupled receptor kinases whereby GPCR phosphorylation initiates arrestin binding and may, in turn, lead to GPCR sequestration through dynamin/clathrin-coated pit internalization mechanisms (40).

In summary, our results demonstrate that TPα, but not TPβ, is subject to cross-desensitization by IP in HEK 293 cells and suggest that this occurs in platelets or vascular smooth muscle, TPα may be the isofrom physiologically relevant to the maintenance of vascular hemostasis. Consistent with these
findings, based on observations that TP isoform specific antibodies permitted detection of TPα, but not TPβ in human platelets, Habib et al. (15) have hypothesized that TPα may be the predominant isoform in platelets, despite the presence of mRNA for both isoforms in platelets, which suggests that both isoforms are actually present albeit at different levels (16). It has been recently demonstrated that U46619 induced platelet aggregation required concomitant stimulation of both Gq-coupled TP receptors (leading to Ca2+ mobilization and platelet shape change) and Gq-coupled P2Y12c and α2A receptors (activated upon secretion of granule contents), leading to platelet aggregation (87). Thus, at least two separate signal transduction cascades are required, one TP-dependent and one TP-independent, to result in platelet aggregation following activation with U46619. Whereas our studies have pinpointed an essential requirement of TPα and an apparent redundancy for TPβ in TP-IP-mediated cross-talk with implications for vascular hemostasis, and other studies have identified differences in the expression, signaling, and desensitization between the TP isoforms (12, 14, 16, 32, 50, 66), identification of a physiologic role for the β isoform of TP found in humans but not in other species, such as mouse or rat (88, 89), further investigation is also particularly noteworthy that amino acid Ser299, the target residue for IP-mediated PKA phosphorylation in TPα, is absolutely conserved in both monkey TP (90) and bovine TP (91) but not in the mouse or rat TPs; thus, it is possible that the former receptors are also subject to a similar mechanism of IP-mediated desensitization to that of the human TPα isoform.

Acknowledgments—We thank Schering AG (Berlin, Germany) for the gift of cicaprost. We thank Sinead Migglin and Leanne Kelley for assisting with construction of stable cell lines and with phosphorylations.

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