The α, but Not the β, Isoform of the Human Thromboxane A₂ Receptor Is a Target for Nitric Oxide-mediated Desensitization

INDEPENDENT MODULATION OF TPa SIGNALING BY NITRIC OXIDE AND PROSTACYCLIN*

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In humans, thromboxane A₂ signals through two thromboxane A₂ receptor (TP) isoforms termed TPa and TPβ. Signaling by TPa, but not TPβ, is subject to prostacyclin-induced desensitization mediated by direct protein kinase (PK) A phosphorylation where Ser³²⁹ represents the phosphotarget (Walsh, M. T., Foley, J. F., and Kinsella, B. T. (2000) J. Biol. Chem. 275, 20412–20423). In the current study, the effect of the vasodilator nitric oxide (NO) on intracellular signaling by the TP β receptor was investigated. The NO donor 3-morpholinosydnonimine, HCl (SIN-1) and 8-bromo-guanosine 3'5'-cyclic monophosphate, HCl, at concentrations to desensitize TPβ mediated U46619-mediated calcium mobilization and inositol monophosphate (8-Br-cGMP) functionally desensitized TPβ signaling. In contrast, TPa underwent both NO- and prostacyclin-mediated desensitization that occur through two independent mechanisms involving direct PKG phosphorylation of Ser³³¹, in response to NO, and PKA phosphorylation of Ser³³¹, in response to prostacyclin, within the unique carboxy-terminal tail domain of TPa. On the other hand, signaling by TPβ was unaffected by either NO or prostacyclin.

The local control of hemostasis is regulated by a variety of vasoconstrictory and vasodilatory autacoids that act to modulate platelet, vascular endothelium, and vascular smooth muscle (VSM) function (1). The prostanoids thromboxane (TX)A₂ and prostacyclin (prostaglandin (PG)L₂) play key, yet opposing, roles in the maintenance of vascular hemostasis (2); TXA₂, synthesized primarily by platelets and activated macrophages, mediates platelet aggregation and acts as a potent vasocon-strictor whereas prostacyclin, mainly synthesized by the vascular endothelium, inhibits platelet activation and aggregation and induces vasodilation (2). TXA₂ and prostacyclin signal through their respective G protein-coupled receptors (GPCRs) termed TP and IP and are primarily coupled to Gq-dependent activation of phospholipase (PLC)β and to Gs-dependent activation of adenylyl cyclase, respectively (2). The main inhibitory effects of TXA₂ on platelet activation and aggregation are believed to be the result of cAMP-dependent PKA substrates (2). TXA₂ activation of PLCβ results in the formation of the second messengers diacylglycerol (DAG) and inositol phosphate (IP₃), which activate PKC and IP₃ receptors on the platelet surface (2). The primary mediator of the vasodilatory effects of prostacyclin is not yet fully characterized, but it is now apparent that prostacyclin can directly activate soluble guanylate cyclase (5).

This article has been retracted by the publisher. In Fig. 5B, lanes 1 and 3 were duplicated. Fig. 8A did not accurately represent the results of the experimental conditions. In Fig. 8B, lanes 1 and 3 were duplicated, and lanes 4 and 5 did not accurately represent the results of the experimental conditions.

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† To whom correspondence should be addressed. Tel.: 353-1-7166727; Fax: 353-1-2837211; E-mail: Therese.Kinsella@UCD.IE.
‡ The abbreviations used are: VSM, vascular smooth muscle; 8-BrcGMP, guanosine 3',5'-cyclic monophosphate, 8-bromo-, sodium salt; C-tail, carboxy-terminal tail; GPCR, G protein-coupled receptor; HA, hemagglutinin; HEK, human embryonic kidney; IP₁, inositol 1,4,5-trisphosphate; MβP, malse-binding protein; NO, nitric oxide; eNOS, endothelial nitric-oxygen synthase; PG, prostaglandin; PK, protein kinase; PL, phospholipase; sGC, soluble guanylyl cyclase; TX, thromboxane; PVDF, polyvinylidene difluoride; HBS, HEPES-buffered saline.

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...acylation (13, 20), TPβ, but not TPα, is subject to internalization and down-regulation following prolonged exposure to the TXA2 mimetic U46619 (21, 22). In studies investigating cross-talk between TXA2 and other prostanoids, we have established that TPα, but not TPβ, is subject to prostacyclin- and PGI2-mediated desensitization/inhibition of signaling involving direct PKA phosphorylation of TPα itself within its unique C-tail domain (11, 23). Hence, these studies identified TPα as a novel target of prostacyclin-induced PKA phosphorylation and inhibition of platelet activation/vasodilation and imply an essential role for TPα in prostaglandin-regulated vascular hemostasis but point to a redundant, or an as yet unidentified, role for TPβ in this essential physiologic process (11, 23).

In addition to prostacyclin, nitric oxide (NO), also referred to as endothelial-derived relaxing factor or EDRF (24), acts as a second endogenous vasodilator and further protects the blood vessel wall by inhibiting platelet aggregation, secretion, adhesion, and fibrinogen binding to its receptor glycoprotein (Gp)IIb/IIIa (24–29). Endothelium-derived NO, synthesized by the endothelial isoform of NO synthase (eNOS/NOS III) in response to diverse agonists, such as bradykinin and acetylcholine (24, 30–32) and various hemodynamic stimuli, especially fluid shear stress (33), activates its target receptor soluble guanylyl cyclase (sGC) leading to increases in intracellular cGMP concentrations (34, 35). One of the key molecular targets of cGMP are the cGMP-dependent PKG 1α and 1β isoforms that are abundantly expressed in platelets and various types of smooth muscle (36, 37). Although the precise molecular mechanisms of NO-mediated vasodilation and inhibition of platelet activation is currently unknown, like prostacyclin, inhibitory activities are mediated through its second messenger, regulated kinase PKG (4). Key molecular targets of the regulated PKG thus far identified include the IP3 receptor (38), regulated kinase PKG (4). Key molecular targets of NO-regulated desensitization of TPα signaling in human platelets were previously (23). Conversion of Ser329, in response to cGMP implying that the TP(s) themselves may be direct targets of NO-regulated vascular hemostasis (44).

Thus, in view of our findings that the TPα, but not the TPβ, isoform of the human TP is subject to direct PKA phosphorylation and desensitization of signaling in response to the inhibitory prostaglandin prostacyclin (23), in the current study we sought to investigate NO-mediated regulation of TPα signaling and to establish whether TPα or TPβ or both are subject to NO-induced desensitization in whole cells. Hence, this study was designed to investigate the intracellular cross-talk mediated by the NO donor 3-morpholinosydnonimine (SIN-1) or the non-hydrolyzable analogue of cGMP, 8-Br-cGMP, on signaling by the individual TPα and TPβ isoforms stably over-expressed in human embryonic kidney (HEK) 293 cells, and to identify the molecular mechanism of that desensitization, should it occur. Our results demonstrate that signaling by TPα is subject to desensitization in response to both SIN-1 and 8-Br-cGMP and that these effects are mediated by direct PKG phosphorylation of TPα whereby Ser331 was identified as the specific phosphorylation residue. On the other hand, signaling by TPβ remains unaffected by either SIN-1 and 8-Br-cGMP, and TPβ was not a substrate for PKG phosphorylation either in vitro or in whole cells. Furthermore, we established that prostacyclin- and NO-regulated desensitization of TPα signaling are mediated through distinctly independent mechanisms involving direct PKA phosphorylation of Ser329, in response to prostacyclin, and PKG phosphorylation of Ser331, in response to NO, respectively, within the unique C-tail domain of TPα.

**EXPERIMENTAL PROCEDURES**

Materials—The following chemicals were obtained from Cayman Chemical Co.: 5-heptenoic acid, 7-[6-(3-hydroxy-1-octenyl)-2-oxabicyclo [2.2.1] hept-5-yl]-[1R-[1-

...-methanoepoxy prostaglandin F1α (U46619); 5-heptenoic acid, 7-[3-

...-epoxy prostaglandin F 2α (U46619); 5-heptenoic acid, 7-[3-

...-amino-5,11,15-trihydroxy-16-methyl-17-oxoheptadecan-10-xyl]-1,2-

...-amino-5,11,15-trihydroxy-16-methyl-17-oxoheptadecan-10-xyl]-1,2-

...-amino-5,11,15-trihydroxy-16-methyl-17-oxoheptadecan-10-xyl]-1,2-

...-[40 Ci/mmol), [32P]orthophosphate in radioactive assay grade, was obtained from American Radiolabeled Chemicals Inc. Deoxyribonucleotides and Taq DNA polymerase were obtained from Promega. Expand High Fidelity® Taq DNA polymerase, a chemiluminescence Western blotting kit, polyvinylidene fluoride (PVDF) membrane, rat monoclonal 3F10 anti-HA horseradish peroxidase-conjugated antibody were obtained from Amersham Applied Science. Mouse monoclonal 101R anti-HA peroxidase antibody was obtained from Covance Research Products; horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies were from Santa Cruz Biotechnology, Inc.; protein G-Sepharose 4B Fast Flow, 5-heptenoic acid, 7-(1H-indol-3-yl)-3-(1H-indol-3-yl)-methyl diphenyl phosphate A-8, and peak phosphatase; 1 unit/μl (P891) were anti-maltose-binding protein polyclonal antibodies were obtained from New England Biolabs. Thromboxane A2-directed mutagenesis kit was purchased from Schering AG (Berlin, Germany). NAD+ coenzymes were synthesized by Genosys.

Site-directed Mutagenesis of TPα and TPβ—The site-directed mutagenesis of TPα and TPβ was performed using the QuikChange™ site-directed mutagenesis kit employing pHM6:TPα as template and mutator oligonucleotides 5′-TGG TGG CCT GGC AGT TCC-3′ (sense primer) and 5′-ATG TGG CCC GGC AGT TCC-3′ (antisense primer) were subcloned in-frame into the HindIII-BamHI sites of the pHiM6 (Roche Applied Science) to generate pHM6:TPα and pHM6:TPβ, respectively, as described previously (15).

Conversion of S329A of TPα to generate TPαS329A has been described previously (23). Conversion of S331A of TPα to generate TPαS331A was performed using the QuikChange™ site-directed mutagenesis kit employing pHM6:TPαS329A as template and mutator oligonucleotides 5′-GGG CCT GGC CAG CAG CGA CCT GGG-3′ (antisense primer), where the sequence corresponding/complementary to mutator Ser (TCC)-Ala (GCC) codon are in italics, to generate the plasmid pHM6:TPαS331A. Similarly, conversion of S329A, S331A to generate TPαS329A,S331A was performed using the QuikChange™ site-directed mutagenesis kit employing pHM6:TPαS329AS331A as template and mutator oligonucleotides 5′-CC CGG CAC GCG GGG CCT GCC CTC C-3′ (sense primer) and 5′-GGG CCT GGC CAG CGA CCT GGG-3′ (antisense primer), where the sequence corresponding/complementary to mutator Ser (TCC)-Ala (GCC) codon is in italics, to generate the plasmid pHM6:TPαS329AS331A.

Deletion of the amino acids carboxyl to Lys588 of TPα was achieved by conversion of Thr337 codon to a Stop codon (Thr337, ACG to Stop 337, ATG). The plasmids pHM6:TPαS329A,S331A and pHM6:TPα were verified by automated double-stranded DNA sequencing. The plasmid pCMV-Gag, coding for Gagα, has been described previously (45, 47).

Cell Culture and Transfections—HEK 293 cells were obtained from the American Type Culture Collection and were grown in minimal essential medium containing 10% fetal bovine serum. Routinely, −48 h
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Prior to transfection, cells were plated in 10-cm culture dishes at a density of 2 × 10^5 cells/dish in 8 ml of medium; thereafter, cells were transiently transfected with 10 μg of pADVA (48) and 25 μg of pCMV- pcDNA- or pHM-based vectors using the calcium phosphate/DNA co-precipitation procedure essentially as described previously (45). For transient transfections, cells were harvested 48 h after transfection. To create stable cell lines, HEK 293 cells were transfected with 10 μg of Scal-linearized pADVA plus 25 μg of pHM-based vectors. Forty-eight h post-transfection, G418 (0.8 mg/ml) was added; individual G418-resistant colonies were selected after ~21 days, and clonal cell lines were expanded and were assessed for TP receptor expression by radioligand binding assay using the radioligand [3H]S29,548. In this way, HEK.TPαS329A (pHM6:TPαS329A) and HEK.TPβS331A (pHM6:TPβS331A) cell lines stably over-expressing HA-tagged forms of TPαS329A and TPβS331A were established using the respective plasmids (in parentheses). HEK.TPα10 and HEK.TPβ3 stable cell lines over-expressing TPα and TPβ, respectively, have been described (14). HEK.HATPα, HEK.HATPβ, and HEK.TPαS290A cell lines stably over-expressing HA-tagged forms of TPα, TPβ, and TPαS290A, respectively, have been described previously (15, 23).

Radioligand Binding Studies—TP radioligand binding assays were carried out at 30°C for 30 min in 100-μl reactions in the presence of 0–40 nM [3H]S29,548, for saturation radioligand binding isotherms, or in the presence of 20 nM [3H]S29,548 essentially as described previously (45). Protein determinations were carried out using the Bradford assay (49).

**Calcium Measurements**—Measurements of intracellular calcium ([Ca²⁺]) mobilization in transfected HEK 293 cells was carried out in FURA2/AM preloaded cells essentially as described previously (45). Cells were stimulated with 1 μM U46619, 1 μM cicaprost, 10 μM 8-Br-cGMP, or 10 μM SIN-1 unless otherwise specified. The PKG inhibitor KT5823 (50 μM), the PKA inhibitor H89 (10 μM), or the PRC inhibitor GF 109203X (50 nM) were added at times and concentrations specified in the figure legends. In all cases, the drug (agonist/kinase inhibitors in ethanol or Me2SO) was diluted 1:1000 in the vehicle HBSS (Ca²⁺ and Mg²⁺ free, 110 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 1.0 mM MgCl₂, 137 mM NaCl, 5.9 mM KCl, 1.25 mM MgCl₂, 11 mM glucose, 5 mM HEPES, pH 7.4; HBS) or HBSS-buffered saline (140 mM NaCl, 4.7 mM KCl, 2.2 mM CaCl₂, 1.2 mM KH₂PO₄, 11 mM glucose, 15 mM HEPES-NaOH, pH 7.4; HBS) in 2% FBS, and 1 μM bicine in HBS buffered with 150 mM NaCl, 5 mM MgCl₂, 0.1 mM cGMP, and 5 μCi of [γ-³²P]ATP (600 Ci/mmol, 10 mCi/ml). In vitro phosphorylation reactions were terminated by acetone precipitation; protein precipitates were collected by centrifugation, re-suspended and solubilized in Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% SDS-PAGE, on 10% polyacrylamide gels, followed by Western transfer onto PVDF membrane. Membranes were subject to Xomat XAR film autoradiographic exposure for 24–48 h to detect phosphorylated proteins. Thereafter, membranes were successively screened using anti-MBP antibody, anti-TpP antibody, and TPβ antibody (53) as primary antibodies followed by screening with horseradish peroxidase-conjugated goat anti-rabbit IgG, as secondary antibody, and chemiluminescence detection, as described by the supplier (Roche Applied Science).

**Measurement of Agonist-mediated TP Phosphorylation**—Whole cell phosphorylation assays were performed essentially as described previously (23) with certain modifications. Briefly, cells (2–3 × 10⁶ cells in 60-mm dishes) were washed twice in phosphate-free Dulbecco’s modified Eagle’s medium, and 200 μl of serum-free medium was added. At time zero, the drug (agonists) was added and the sample was incubated in the absence or presence of 10 μM U2005 8-Br-cGMP, 1 μM 8-CPT-cAMP, 343, and MBP:TPα conjugate; immunoreactive proteins were visualized using the chemiluminescence detection system, as described by the manufacturer (Roche Applied Science).

**Data Analyses**—Radioligand binding data was analyzed using GraphPad Prism V2.0 (GraphPad Software Inc.) to determine the Kᵦ and B_max values. Statistical analyses were carried out using the unpaired Student’s t test using the Statworks Analysis Package. p values of less than than 0.05 or equal to 0.05 were considered to indicate a statistically significant difference.
Effect of the NO/cGMP Pathway on U46619-mediated Signaling in HEK 293 Cells—In the current study we sought to establish whether TPα or TPβ or both are subject to NO-induced desensitization by investigating the effect of the NO donor SIN-1 or the cGMP analogue 8-Br-cGMP on U46619-mediated signaling (Ca2+ mobilization and IP3 generation) by the individual TPα (HEK.TPα10 cells) and TPβ (HEK.TPβ3 cells) isoforms stably over-expressed in HEK 293 cells.

Consistent with previous studies (15), stimulation of both HEK.TPα10 and HEK.TPβ3 cells, each transiently co-transfected with pCMV-Gq, showed efficient Ca2+ mobilization in response to the TXA2 mimetic U46619 (1 μM; see Fig. 1, A and E, respectively). Although neither SIN-1 (10 μM) nor 8-Br-cGMP (10 μM) yielded significant rises in [Ca2+]i in either cell type, both SIN-1 (see Fig. 1B, and compare Fig. 1, A versus B, ∆[Ca2+]i = 186 ± 8.2 nM versus ∆[Ca2+]i = 60 ± 10.6 nM, respectively; p < 0.0007) and 8-Br-cGMP (see Fig. 1C, and compare Fig. 1, A versus C, ∆[Ca2+]i = 186 ± 8.2 nM versus ∆[Ca2+]i = 94.1 ± 9.5 nM, respectively; p < 0.0019) significantly reduced [Ca2+]i mobilization by TPα in response to secondary stimulation of HEK.TPα10 cells with U46619. The IC50 of SIN-1 and 8-Br-cGMP for inhibition of U46619-mediated signaling in HEK.TPα cells were 5.8 and 5.5 μM, respectively. On the other hand, neither SIN-1 (see Fig. 1F, and compare Fig. 1, E versus F, ∆[Ca2+]i = 151 ± 16.7 nM versus ∆[Ca2+]i = 140 ± 10.8 nM, respectively; p = 0.61) nor 8-Br-cGMP (see Fig. 1G, and compare Fig. 1, E versus G, ∆[Ca2+]i = 151 ± 16.7 nM versus ∆[Ca2+]i = 169 ± 23.8 nM, respectively; p = 0.57) affected U46619-mediated signaling by TPβ.

To further investigate the differential effects of the NO/cGMP pathway on TPα and TPβ signaling, the effect of 8-Br-cGMP and SIN-1 on U46619-mediated IP3 generation was also examined. Stimulation of HEK.TPα10 and HEK.TPβ3 cells with U46619 (1 μM) yielded 5- to 7-fold increases in IP3 levels (Fig. 1, D and H, respectively). Although 8-Br-cGMP did not yield a significant increase in IP3 generation in either cell type, pre-incubation of HEK.TPα10 cells with 8-Br-cGMP significantly reduced U46619-mediated IP3 generation by TPα (Fig. 1D, p < 0.005). In contrast, 8-Br-cGMP had no significant effect on U46619-mediated IP3 generation in HEK.TPβ3 cells (Fig. 1H, p = 0.41). Similarly, SIN-1 reduced U46619-mediated IP3 generation by TPα but had no significant effect on IP3 generation by TPβ (data not shown).

To investigate the effect of the second messenger PKs on NO/cGMP-mediated cross-desensitization of TP signaling, the effect of KT5823, a PKG inhibitor (54), H-89, a PKA inhibitor (55, 56), and GF 109203X, a PKC inhibitor (57), on U46619-mediated [Ca2+]i mobilization and IP3 generation was examined. Pre-incubation of HEK.TPα10 cells with KT5823 (50 nM) blocked 8-Br-cGMP cross-desensitization of U46619-mediated [Ca2+]i responses (Fig. 2A, compare ∆[Ca2+]i = 94.1 ± 9.5 nM without KT5823 versus ∆[Ca2+]i = 186 ± 22.0 nM with KT5823; p = 0.0185). In contrast, neither H-89 (10 μM) nor GF 109203X (50 nM) had any significant effect on 8-Br-cGMP-mediated desensitization of [Ca2+]i mobilization in HEK.TPα10 cells (Fig. 2, B and C, respectively). Moreover, pre-incubation of HEK.TPα10 cells with KT5823 prior to 8-Br-cGMP stimulation
restored U46619-mediated IP₃ levels to basal levels (Fig. 2D, p = 0.001), whereas neither H-89 nor GF 109203X had any effect (Fig. 2D). KT5823 (Fig. 2E, H-89, or GF 109203X (data not shown) did not affect U46619-mediated [Ca²⁺]ᵢ mobilization or IP₃ generation (Fig. 2F) by TPβ expressed in HEK.TPβ3 cells.

As TPβ and TPβ diverge exclusively within their C-tail domains (Fig. 3), and results presented herein show that TPβ, but not TPβ, is sensitive to NO-mediated desensitization, we then investigated the effect of SIN-1 and 8-Br-cGMP on signaling by TPβ. A variant of TPβ devoid of those residues unique to TPβ and TPβ (15). Stimulation of HEK.TPβ cells, transiently co-transfected with pCMV:Gαq, showed efficient U46619-mediated [Ca²⁺]ᵢ mobilization (Fig. 2, G and H). However, neither SIN-1 (Fig. 2G, compare Δ[Ca²⁺]ᵢ = 190 ± 12.1 nM without SIN-1 versus Δ[Ca²⁺]ᵢ = 178 ± 14.0 nM with SIN-1; p = 0.37) nor 8-Br-cGMP (Fig. 2H, compare Δ[Ca²⁺]ᵢ = 190 ± 12.1 nM without 8-Br-cGMP versus Δ[Ca²⁺]ᵢ = 214 ± 16.8 nM with 8-Br-cGMP; p = 0.17) had any effect on U46619-mediated [Ca²⁺]ᵢ mobilization by TPβ. Taken together, these results indicate that TPβ, but not TPβ, is subject to NO-mediated desensitization of signaling that occurs through a KT5823-sensitive, PKG mechanism and that the target sites of this desensitization are located within the unique C-tail regions of TPα at site(s) distal to Arg⁴²⁸.

**NO/cGMP-mediated Phosphorylation of TPα**—To investigate whether TPα or TPβ are direct targets for PKG phosphorylation, *in vitro* phosphorylation assays were carried out using recombinant forms of TPα (MBP:TPα) and TPβ (MBP:TPβ) expressed and purified from *E. coli* as fusion proteins to the MBP. MBP:TPα and MBP:TPβ, encoding amino acids 220–343 and 220–407 spanning from the beginning of the third intracellular loop through to the end of the unique C-tail domains of TPα and TPβ, respectively, were used as substrates in PKG and, as controls, in PKA *in vitro* phosphorylation assays (Fig. 4). MBP:TPα was efficiently phosphorylated *in vitro* by both PKG (Fig. 4B, lane 2) and PKA (Fig. 4A, lane 2). In contrast, neither MBP:TPβ (Fig. 4, A and B, lane 3) nor MBP (Fig. 4, A and B, lane 1) were phosphorylated by either kinase. The presence of the equimolar concentrations of the MBP and MBP:TP fusion proteins in the phosphorylation assays was confirmed by Western blotting using anti-MBP (Fig. 4C) whereas the identities of the recombinant MBP:TP fusion proteins to be that of TPα (lane 2) and TPβ (lane 3) were confirmed using affinity purified anti-TPα (Fig. 4D) and anti-TPβ (Fig. 4E) antibodies, respectively.

Thereafter, [³²P]orthophosphate metabolic labeling studies were performed to investigate whether TPα and/or TPβ are direct targets for NO/cGMP-induced PKG phosphorylation *in vivo* in whole cells. Initially, the ability of the anti-HA 101R antisera to immunoprecipitate the HA epitope-tagged TPα or TPβ from their respective HEK.TPα and HEK.TPβ cells stable
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(A) $	ext{TP}_\alpha$ PRLSTRPSLSTPOLTORSGLO

(B) $	ext{TP}_\beta$ PRLSTRPRSLTLWPSLEYSGTSIAHNCNLRIPGSSDSRASASRAAGITGVSHCARPMLFDPRFDILAVQLLPFEPTGKalSRKD

Fig. 3. Amino acid sequence of the carboxyl-terminal (C)-tail domains of TPα and TPβ. The amino acid sequence of the C-tail domains of TPα (residues 321–343) and of TPβ (residues 321–407) are illustrated in panels A and B, respectively, where residues unique to TPα (residues 329–343) and TPβ (residues 329–407) are underlined in each case. Amino acid residues that were mutated in TPα to generate TPαS329A (S329A) or TPαS331A (S331A) are indicated by the solid arrows in panel A, whereas those residues that were converted to stop codons to generate the truncation mutants (indicated by the Δ symbol) following amino acids TPαD336 (Arg328), at the point of divergence of TPα/TPβ, or TPαS336 (Leu336) within TPα and/or TPβ are indicated by the open arrowheads in panels A and B, respectively. An additional mutant TPαS329A,S331A was established whereby both Ser329 and Ser331 were converted to Ala329 and Ala331 in TPα (not shown in panels A and B).

Fig. 4. Phosphorylation of recombinant TPα and TPβ by PKG and PKA in vitro. Purified MBP (lane 1) or recombinant MBP:TPα (lane 2) and MBP:TPβ (lane 3) fusion proteins were used as substrates in PKA (panel A) and PKG (panel B) in vitro phosphorylation assays. Phosphorylated proteins were analyzed by SDS-PAGE and electroblotted onto PVDF membranes, which were subsequently exposed to Xomat XAR-5 film (Kodak) for 24–48 h. Thereafter, membranes were successively screened with either anti-MBP antiserum (panel C) or affinity purified anti-TPα (panel D) or anti-TPβ (panel E) antisera, using anti-rabbit peroxidase-conjugated antibody as the secondary antibody. Immunoreactive bands were visualized by chemiluminescence detection. The relative position of the 45-kDa molecular mass marker (kDa) is indicated to the right of panels A–E.

Cell lines (23), but not from the control HEK 293 cell line, was confirmed (Fig. 5D). Broad protein bands of ~39–60 kDa (Fig. 5D, lane 2) and ~46–60 kDa (Fig. 5D, lane 3) representing the non-glycosylated and glycosylated forms of TPα and TPβ were immunoprecipitated from HEK.TPα and HEK.TPβ cells, respectively, but were not present in the immunoprecipitates from control HEK 293 cells (Fig. 5D, lane 1). Consistent with previous reports (23), stimulation of HEK.TPα (Fig. 5A, lanes 1 and 2) and HEK.TPβ cells (Fig. 5B, lanes 1 and 2) with U46619 significantly increased the level of TPα and TPβ phosphorylation relative to their basal phosphorylation observed in vehicle-treated control cells. Incubation of cells with SIN-1 significantly increased TPα phosphorylation (Fig. 5A, lane 3) but not TPβ phosphorylation (Fig. 4B, lane 3). Compared to 85.2 ± 8.81 nM) nor 8-Br-cGMP had any effect on signaling by TPβ (Fig. 4B, lane 3). In contrast, 0.001 significantly reduced [Ca²⁺]i (Fig. 3) and that this occurs through a KT5823-dependent mechanism involving direct phosphorylation of a unique C-tail domain. On the other hand, neither SIN-1 nor 8-Br-cGMP had any effect on signaling by TPβ (Fig. 4B, lane 3). Therefore, these data demonstrate that signaling by TPβ is subject to desensitization in response to secondary stimulation of HEK.TPβ cells, transiently co-transfected with Gαi and HEK 293 stable cell line (Fig. 3).

Identification of the Mechanism of NO/cGMP-desensitization of TPα Signaling—Analysis of the amino acid sequence of the unique C-tail domain of TPα reveals the presence of 4 Ser/Thr residues (Ser329, Ser331, Thr337, Ser340) that might represent putative target residues for PKG phosphorylation (Fig. 3). Thus, site-directed/deletion mutagenesis, in combination with metabolic labeling studies, were employed to investigate whether any of these putative phosphorylation sites within TPα represent potential target sites for NO/cGMP-mediated desensitization (Fig. 3). Initially, TPαS329A, a truncated variant of TPα devoid of all C-tail residues distal to Leu336, including the putative phosphatase Thr337 and Ser340, was generated by deletion mutagenesis (Fig. 3), and a HEK 293 stable cell line over-expressing TPαS329A was established (Table I). Stimulation of HEK.TPαS329A cells, transiently co-transfected with Goαi, showed efficient [Ca²⁺]i mobilization in response to U46619 (Δ[Ca²⁺]i) = 173 ± 9.91 nM) and also stimulated TPαS329A cells, both SIN-1 (10 μM) nor 8-Br-cGMP (10 μM) yielded significant rises in [Ca²⁺]i (Δ[Ca²⁺]i) = 173 ± 9.91 nM) without SIN-1 versus Δ[Ca²⁺]i) = 85.2 ± 8.81 nM) with SIN-1, respectively; p = 0.0014) and 8-Br-cGMP (compare Δ[Ca²⁺]i) = 173 ± 9.91 nM) without 8-Br-cGMP versus Δ[Ca²⁺]i) = 98.3 ± 7.72 nM) with 8-Br-cGMP, respectively; p = 0.001) significantly reduced [Ca²⁺]i, mobilization by TPαS329A in response to secondary stimulation of HEK.TPαS329A cells with U46619. These data indicate that the site(s) of NO/PKG desensitization lie proximal to Leu336 and that neither Thr337 nor Ser340 are the target(s) for that phosphorylation.

Thereafter, site-directed mutagenesis was used to generate the variants TPαS329A (23), TPαS331A, and TPαS329A,S331A...
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Fig. 5. SIN-1 induced phosphorylation of TPα and TPβ in [32P]orthophosphate metabolically labeled whole cells. Panels A–C, HEK 293 cells stably over-expressing HA epitope-tagged TPα (panel A) and TPβ (panel B), or, as controls, non-transfected HEK 293 cells (panel C) were metabolically labeled with [32P]orthophosphate for 1 h at 37 °C, 5% CO₂ in the presence of KT5823 (50 nM), H-89 (10 μM), GF 109203X (50 nM), or vehicle HBS. Thereafter, cells were stimulated for 10 min with 1 μM U46619, 10 μM SIN-1, or vehicle alone, as indicated. HA epitope-tagged TP receptors were immunoprecipitated using the anti-HA 101R antibody, and immunoprecipitates were subjected to SDS-PAGE/Western blot using the anti-HA 3F10 peroxidase-conjugated antibody and immunoreactive bands were visualized by chemiluminescence detection. The relative positions of the molecular mass markers (kDa) are indicated to the left and right of panels A and D, respectively.

Table I
Radioligand binding studies

Saturation radioligand binding isotherms were carried out on HEK 293 cells stably over-expressing HA epitope-tagged forms of wild type TPα or TPβ or their respective variant receptors using the TP antagonist [3H]SQ29,548 (50.4 Ci/mmol, 0.4 nM) and 75 μg of whole cell homogenate. Radioligand binding data were analyzed using GraphPad Software Inc. to determine the Kₐ and Bₘax. The results presented are the mean values of four independent experiments. For each cell line, Kₐ and Bₘax and S.E. of the mean are presented.

| Cell lines | Kₐ (nM) | Bₘax (pmol/mg protein) | S.E. pmol/mg protein |
|------------|---------|------------------------|---------------------|
| HEK.TPₐ   | 5.56    | 20.1 ± 0.15            | 0.19                |
| HEK.TPₐ⁢S₃₂⁹⁹ | 9.65   | 20.1 ± 0.15            | 0.19                |
| HEK.TPₐ⁢S₃₂⁹⁹, S₃₃₁ₐ | 7.11 ± 0.03 | 19.0 ± 0.19      | 0.14                |
| HEK.TPₐ⁢S₃₂⁹⁹, S₃₃₃₁ₐ | 8.22 ± 0.14 | 19.0 ± 0.19      | 0.14                |
| HEK.TPₐ⁢S₃₂⁹⁹, S₃₃₃₁ₐ | 5.66 ± 0.14 | 19.0 ± 0.19      | 0.14                |
| HEK.TPₐ⁢S₃₂⁹⁹ | 8.44 ± 0.14 | 19.0 ± 0.19      | 0.14                |

whereby the critical Ser³²⁹⁹ and Ser³₃₃₁ were mutated to Ala³²⁹⁹ and Ala³₃₃₁ either individually or collectively (Fig. 3), and the relative sensitivities of the latter receptors to NO/PKG and prostacyclin/PKA signaling was investigated. Initial saturation radioligand binding studies confirmed that the mutations per se did not affect the ligand properties of the variant TPα receptors stably expressed in the respective HEKTPα⁢S₃₂⁹⁹, HEK.TPₐ⁢S₃₃₁ₐ, and HEK.TPₐ⁢S₃₂⁹⁹, S₃₃₁ₐ, and HEK.TPₐ⁢S₃₂⁹⁹, S₃₃₃₁ₐ cells (Table I). Thereafter, the effect of the 8-Br-cGMP (10 μM) and the NO donor SIN-1 (data not shown) on U46619-mediated signaling by TPα⁢S₃₂⁹⁹, TPα⁢S₃₃₁ₐ, and TPα⁢S₃₂⁹⁹, S₃₃₃₁ₐ was investigated and was compared with the effect of the prostacyclin receptor (IP) agonist cicaprost on TP signaling. Stimulation of HEK.TPα⁢S₃₂⁹⁹ and HEK.TPα⁢S₃₃₁ₐ cells, each transiently co-transfected with Goα, with U46619 led to efficient [Ca²⁺]i mobilization (Fig. 6, A and E, respectively). Pre-incubation of cells with 8-Br-cGMP significantly reduced U46619-mediated [Ca²⁺]i mobilization in HEK.TPα⁢S₃₂⁹⁹ cells (see Fig. 6B, and compare Fig. 6, A versus B, Δ[Ca²⁺]i = 159 ± 17.3 nM versus Δ[Ca²⁺]i = 57.4 ± 10.2 nM, respectively; p < 0.0002) but had no effect on U46619-mediated signaling in HEK.TPα⁢S₃₃₁ₐ cells (see Fig. 6F, and compare Fig. 6, E versus F, Δ[Ca²⁺]i = 201 ± 9.0 nM versus Δ[Ca²⁺]i = 181 ± 19.6 nM; p = 0.17). Conversely, pre-incubation with the prostacyclin mimetic cicaprost (1 μM) did not affect U46619-mediated [Ca²⁺]i mobilization, whereas KT5823; p = 0.0001) but significantly reduced U46619-mediated IP₃ generation in HEK.TPα⁢S₃₂⁹⁹ and HEK.TPα⁢S₃₃₁ₐ cells (see Fig. 6D, and compare Fig. 6C, and compare Fig. 6, A versus G, Δ[Ca²⁺]i = 201 ± 9.0 nM versus Δ[Ca²⁺]i = 159 ± 17.3 nM; p < 0.0001), respectively. As a consequence, the effect of 8-Br-cGMP and cicaprost on IP₃ generation in HEK.TPα⁢S₃₂⁹⁹ and HEK.TPα⁢S₃₃₁ₐ cells were investigated. Stimulation of HEK.TPα⁢S₃₂⁹⁹ and HEK.TPα⁢S₃₃₁ₐ cells with U46619 yielded 7-fold increases in IP₃ generation above basal levels (Fig. 6D, and compare Fig. 6H, respectively). Pre-stimulation of HEK.TPα⁢S₃₂⁹⁹ cells with 8-Br-cGMP significantly reduced U46619-mediated IP₃ generation (Fig. 6D, p < 0.05), whereas cicaprost had no significant effect on U46619-mediated IP₃ generation by TPα⁢S₃₃₁ₐ (p = 0.13). In contrast, pre-stimulation of HEK.TPα⁢S₃₃₁ₐ cells with 8-Br-cGMP had no significant effect U46619-mediated IP₃ generation (Fig. 6H, p = 0.16) whereas cicaprost significantly impaired IP₃ generation by TPα⁢S₃₃₁ₐ (Fig. 6H, p < 0.004).

To further characterize the nature of 8-Br-cGMP- and cicaprost-induced desensitization, the effect of the protein kinase inhibitors KT5823, H-89, or GF 109203X on U46619-mediated signaling by TPα⁢S₃₂⁹⁹ and TPα⁢S₃₃₁ₐ was investigated. Pre-incubation with KT5823 blocked 8-Br-cGMP-induced desensitization of U46619-mediated [Ca²⁺]i mobilization by HEK.TPα⁢S₃₂⁹⁹ cells (Fig. 7A, compare Δ[Ca²⁺]i = 57.4 ± 10.2 nM without KT5823 versus Δ[Ca²⁺]i = 151 ± 23.9 nM plus KT5823; p = 0.02). In contrast, neither H-89 nor GF 109203X had any effect on 8-Br-cGMP-induced desensitization of U46619-mediated [Ca²⁺]i mobilization by TPα⁢S₃₂⁹⁹ (Fig. 7, B and C, respectively). Consistent with the latter, KT5823, but not H-89 or GF 109203X, blocked 8-Br-cGMP-induced desensitization of U46619-mediated IP₃ generation by TPα⁢S₃₂⁹⁹ (Fig. 7D). In HEK.TPα⁢S₃₃₁ₐ cells, pre-incubation with H-89 blocked cicaprost-induced desensitization of U46619-mediated [Ca²⁺]i mobilization (see Fig. 7F, and compare Fig. 6G versus Fig. 7F, Δ[Ca²⁺]i = 58.2 ± 18.4 nM versus Δ[Ca²⁺]i = 190 ± 14.3 nM, respectively; p = 0.005). On the other hand, neither KT5823 nor GF 109203X affected cicaprost-induced desensitization of

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U46619-mediated \([Ca^{2+}]_i\), mobilization in HEK.TP\(_{\alpha/S329A}\) cells (Fig. 7, E and G, respectively). Moreover, H-89, but not KT5823 or GF 109203X, blocked cicaprost-induced desensitization of U46619-mediated IP\(_3\) generation by TP\(_{\alpha/S331A}\) (Fig. 7H). Whole cell phosphorylation assays established that TP\(_{\alpha/S329A}\) underwent increased phosphorylation in response to U46619 and SIN-1, but not in response to cicaprost, and that SIN-1-induced phosphorylation of TP\(_{\alpha/S329A}\) was blocked by KT5823 but not by H-89 (Fig. 8A). In contrast, TP\(_{\alpha/S331A}\) underwent increased phosphorylation in response to U46619 and cicaprost, but not in response to SIN-1, and cicaprost-induced TP\(_{\alpha/S331A}\) phosphorylation was inhibited by H-89 but not by KT5823 (Fig. 8B).

To further disentangle the apparent independent roles of Ser\(^{259}\) and Ser\(^{331}\) in mediating differential desensitization of TP\(_{\alpha}\) in response to cicaprost and NO/cGMP, we also investigated the effect of cicaprost and SIN-1/8-Br-cGMP on signaling by TP\(_{\alpha/S329A,S331A}\) (see Fig. 3 and Table I). Stimulation of HEK.TP\(_{\alpha/S329A,S331A}\) cells, transiently co-transfected with Go\(_{q}\) with U46619 led to efficient \([Ca^{2+}]_i\) mobilization (Fig. 9A). Pre-incubation of cells with either the NO donor SIN-1 (see Fig. 9B, and compare Fig. 9, A versus B, \(\Delta[Ca^{2+}]\), = 206 \(\pm\) 9.8 nM versus \(\Delta[Ca^{2+}]\), = 190 \(\pm\) 24.9 nM; \(p = 0.38\)) or 8-Br-cGMP (see Fig. 9C, and compare Fig. 9, A versus C, \(\Delta[Ca^{2+}]\), = 206 \(\pm\) 9.8 nM versus \(\Delta[Ca^{2+}]\), = 187 \(\pm\) 20.8 nM; \(p = 0.25\)) or the IP agonist cicaprost (see Fig. 9C, and compare Fig. 9, A versus B, \(\Delta[Ca^{2+}]\), = 206 \(\pm\) 9.8 nM versus \(\Delta[Ca^{2+}]\), = 179 \(\pm\) 28.6 nM; \(p = 0.20\)) did not affect signaling by TP\(_{\alpha/S329A,S331A}\) in response to its secondary stimulation with U46619 (Fig. 9, B-D, respectively). Moreover, the PKG inhibitor KT5823 had no effect on SIN-1 or 8-Br-cGMP desensitization of U46619-mediated signaling by TP\(_{\alpha/S329A,S331A}\) (Fig. 9, E and F, compare \(\Delta[Ca^{2+}]\), = 190 \(\pm\) 24.9 nM without KT5823 versus \(\Delta[Ca^{2+}]\), = 180 \(\pm\) 35.7 nM with KT5823; \(p = 0.76\), and compare \(\Delta[Ca^{2+}]\), = 187 \(\pm\) 20.8 nM without KT5823 versus \(\Delta[Ca^{2+}]\), = 193 \(\pm\) 16.4 with KT5823; \(p = 0.79\), respectively) whereas the PKA inhibitor H-89 had no effect on cicaprost desensitization of U46619-mediated signaling (Fig. 9G, compare \(\Delta[Ca^{2+}]\), = 179 \(\pm\) 28.6 nM without H-89 versus \(\Delta[Ca^{2+}]\), = 171 \(\pm\) 20.9 nM with H-89; \(p = 0.78\)). Whole cell phosphorylation assays established that TP\(_{\alpha/S329A,S331A}\) did not undergo increased phosphorylation in response to either SIN-1 or cicaprost stimulation (data not shown).

Hence, taken together, these data indicate that TP\(_{\alpha}\) is subject to NO/8-Br-cGMP-induced desensitization of signaling mediated through its direct PKG-mediated phosphorylation whereby Ser\(^{331}\), but not Ser\(^{259}\), has been identified as the phosphorylation site for this desensitization. On the other hand, consistent with our previous findings (23), TP\(_{\alpha}\) is also subject to independent cicaprost-induced desensitization that occurs through a mechanism involving PKA phosphorylation of Ser\(^{259}\), without targeting Ser\(^{331}\).
The TXA₂ and NO/cGMP signaling pathways exert opposing actions in platelets and in smooth muscle cells within the vasculature to regulate hemostasis and blood vessel tone (2, 24). The physiological significance for the existence of two TP isoforms in humans is unknown, but they exhibit distinct functional differences, particularly in terms of their modes of regulation and desensitization of signaling (12). For example, TPα, but not TPβ, is desensitized in response to signaling by the inhibitory prostanooids prostacyclin and PGD₂ and has led to the suggestion that TPα is the TP isofrom involved in prostanooid-regulated vascular hemostasis whereas the role of TPβ, if any, in this critical physiologic process remains to be identified (11, 23). Moreover, Wang et al. (44) reported that 8-Br-cGMP inhibited TXA₂-stimulated GTPase in human platelets, that the TP(s) expressed in megakaryocytic human erythroleukemia cells showed increased phosphorylation in response to cGMP, and that recombinant glutathione S-transferase fusion proteins encoding domains of TPα and TPβ were substrates for PKG phosphorylation in vitro. These latter observations imply that, in addition to their established differential regulation by prostacyclin and PGD₂, the TP isofrom(s) may also be direct targets for NO-regulated desensitization. Thus, the primary aim of the current study was to investigate whether TPα or TPβ or both are subject to NO-induced desensitization in vivon in whole cells and to establish whether they may be differentially sensitive to such regulation.

Hence, to this end, the effect of NO donor SIN-1 and 8-Br-cGMP on signaling by the individual TPα and TPβ isoforms stably over-expressed in HEK 293 cells was investigated. Both SIN-1 and 8-Br-cGMP impaired U46619-mediated [Ca²⁺]i mobilization and IP₃ generation by TPα, but neither agent affected signaling by TPβ. Pre-incubation with the PKG inhibitor KT5823, but not the PKA or PKC inhibitors H-89 or GF 109203X, blocked SIN-1- and 8-Br-cGMP-induced desensitization of TPα signaling but had no effect on signaling by TPβ. Moreover, neither SIN-1 nor 8-Br-cGMP had any effect on U46619-mediated signaling by TPα or TPβ devoid of the divergent C-tail domains of TPα and TPβ (15).

To establish whether TPα or TPβ are substrates for PKG, initially in vitro phosphorylation assays were carried out using MBP:TPα and MBP:TPβ recombinant fusion proteins that contain amino acids 220–343 and 220–407, which include the third intracellular loop through to the end of the divergent C-tail domains of TPα (6) and TPβ (7, 8), respectively. Consistent with previous reports, TPα was efficiently phosphorylated...
in vitro by both PKG (44) and PKA (52) within its C-tail domain. On the other hand, MBP:TP[H9252 protein was not phosphorylated by either PKG or PKA. Although the observation that TP[H9252 is not phosphorylated by PKA is in keeping with earlier studies (11, 23), the finding that TP[H9252 is not phosphorylated by PKG is actually contrary to previous reports (44). The reason for the apparent discrepancy between data generated within the current study and those reported previously (44) can be rationalized by the fact that the recombinant TP[H9252 protein (residues 331–369) employed by the latter investigators is unlikely to truly correspond to correct TP[H9252 sequences (44). Because of an initial cloning artifact, it was originally reported

Fig. 8. SIN-1 induced phosphorylation of TP[32P] and TP[32P] in [32P]orthophosphate metabolically labeled whole cells. Panels A and B, HEK 293 cells stably over-expressing HA epitope-tagged TP[32P] and TP[32P] (panel A) and TP[32P] (panel B) were metabolically labeled with [32P]orthophosphate for 1 h at 37°C, 5% CO2 in the presence (+) or absence (–) of KT5823 (50 nM) and H-89 (10 μM) or in the presence of the vehicle (HBS) alone, as indicated. Thereafter, cells were stimulated at 37°C for 10 min with 1 μM U46619, 10 μM SIN-1, 1 μM ciaeprost, or vehicle alone, as indicated. HA epitope-tagged TP receptors were immunoprecipitated using the anti-HA antibody 101R; immunoprecipitates were resolved by SDS-PAGE and electrophotolled onto polyvinylidene difluoride membranes, which were then exposed to Xomat XAR-5 film for 15 days.

Fig. 9. Effect of NO/8-Br-cGMP and ciaeprost on U46619-mediated signaling by TP[32P]. HEK.TP[32P] cells, transiently co-transfected with pCMV.G[32P] and preloaded with FURA2/AM, were stimulated with 1 μM U46619 alone (panel A) or were stimulated with 10 μM SIN-1 (panel B), 10 μM 8-Br-cGMP (panel C), and 1 μM ciaeprost (panel D) followed by 1 μM U46619, where ligands were added at times indicated by the arrows. Alternatively, cells were pre-incubated with 50 nM KT5823 (panels E and F) or 10 μM H-89 (panel G) prior to stimulation with 10 μM SIN-1 (panel E), 10 μM 8-Br-cGMP (panel F), and 1 μM ciaeprost (panel G) followed by 1 μM U46619, where ligands were added at 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 (Δ[Ca2+]i, nM) as a function of time (s) following ligand stimulation. Actual mean changes in U46619-mediated [Ca2+]i mobilization (nM ± S.E.) were as follows: panel A, Δ[Ca2+]i = 206 ± 9.8 nM; panel B, Δ[Ca2+]i = 190 ± 24.9 nM; panel C, Δ[Ca2+]i = 187 ± 20.8 nM; panel D, Δ[Ca2+]i = 179 ± 28.6 nM; panel E, Δ[Ca2+]i = 180 ± 35.7 nM; panel F, Δ[Ca2+]i = 193 ± 16.4 nM; panel G, Δ[Ca2+]i = 171 ± 20.9 nM.
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![Diagram](image.png)

**FIG. 10. Model of nitric oxide- and prostacyclin-mediated regulation of TP signaling.** Ligand (TXA<sub>2</sub>) activation of TPα and TPβ mediates G<sub>q</sub>-dependent coupling to PLCβ, leading to increases in diacylglycerol (DAG) and IP<sub>3</sub> generation and mobilization of [Ca<sup>2+</sup>]<sub>i</sub>. NO signaling through its receptor sGC yields increases in cGMP generation and concomitant activation of cGMP-dependent PKG 1α and/or 1β isozymes. Activated PKG phosphorylates TPα, but not TPβ, where Ser<sup>331</sup> has been identified as the target residue for NO-mediated phosphorylation and thereby desensitizes signaling by TPα. PKG-mediated phosphorylation and desensitization of TPα signaling through its receptor IP primarily couples G<sub>i</sub> with PLC, leading to decreases in cAMP generation and, in turn, activation of cAMP-dependent PKA. Activated PKA, in turn, phosphorylates TPα at the Ser<sup>331</sup> residue and thereby desensitizes signaling by TPα. PKA-mediated phosphorylation of Ser<sup>331</sup> and Ser<sup>329</sup> was identified as the phosphotarget for PKA phosphorylation and desensitization. Hence, signaling by TPα is regulated independently by NO-regulated PKG and PKA, and TPβ signaling is subject to desensitization by PKG-mediated phosphorylation.

PKA-mediated phosphorylation and that TPβ is not a substrate for phosphorylation by PKG in vitro establishes that the cDNA for TPβ encodes a protein of 369 amino acids (protein accession number A56194) and not 369 residues (7). However, it was subsequently established that the cDNA, the TPβ mRNA also devoid of those residues distal to Leu<sup>336</sup> was subject to both NO/PKG phosphorylation and desensitization. As stated previously, it has been reported that the prostacyclin- and PGD<sub>2</sub>-mediated desensitization of TPα signaling occurs through a mechanism involving direct PKA phosphorylation of Ser<sup>329</sup> within the consensus PKA site Arg-Pro-Arg-Ser<sup>329</sup>-Leu-Leu-Ser-Leu located within the C-tail domain of TPα (11, 23). However, in other studies investigating the regulation of TPα by agents that activate PKG (58) or PKA (59), it has been also suggested that the latter sequence Arg-Pro-Arg-Ser<sup>329</sup>-Leu-Leu-Ser-Leu located within the C-tail domain of TPα may act as an overlapping PKA and PKG consensus site and that Ser<sup>329</sup> may represent the predominant site of phosphorylation by both kinases (58, 59). Thus, in the current study, we sought to identify the site of NO-mediated PKG phosphorylation within the C-tail domain of TPα. Moreover, in view of the suggestion the Ser<sup>331</sup>, as opposed to Ser<sup>329</sup>, might act as a phosphotarget for both PKA and PKG, we sought to clarify whether Ser<sup>331</sup> and Ser<sup>331</sup> are targeted independently by the former kinases, respectively, or, on the contrary, whether they act as overlapping PKA and PKG phosphorylation sites.

Hence, to address this issue, the variants TPα<sup>S329A</sup> (23), TPα<sup>S331A</sup> and TPα<sup>S329A,S331A</sup> were generated whereby the critical Ser<sup>329</sup> and Ser<sup>331</sup> were mutated to Ala<sup>329</sup> and Ala<sup>331</sup> either...
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individually or collectively, and the relative sensitivities of the latter receptors to NO/PKG and prostacyclin/PKA signaling was investigated. Initial saturation radioligand binding experiments confirmed that the mutations per se did not affect the ligand binding properties of the latter TP receptors, and each exhibited efficient U46619-mediated intracellular signaling ([Ca²⁺]i mobilization and IP3 generation). Although 8-Br-cGMP and SIN-1 (data not shown) significantly reduced U46619-mediated signaling by TPαS329A, they had no effect on signaling by TPαS331A. Conversely, the prostacyclin mimetic cicaprost did not affect signaling by TPαS329A but significantly reduced signaling by TPαS331A. Moreover, KT5823, but not H-89 or GF 19020X, blocked 8-Br-cGMP-induced desensitization of TPαS331A signaling whereas H-89, as opposed to KT5823 or indeed GF 19020X, blocked cicaprost-induced desensitization of TPαS331A. Whole cell phosphorylation assays established that TPαS329A underwent increased KT5823-sensitive phosphorylation in response to SIN-1 and 8-Br-cGMP (data not shown), but not in response to cicaprost. In contrast, TPαS331A underwent increased H-89-sensitive, KT5823-insensitive phosphorylation in response to cicaprost but not in response to SIN-1 or 8-Br-cGMP (data not shown). Moreover, signaling by TPαS329A,S331A was insensitive to desensitization by either the NO donor SIN-1 or 8-Br-cGMP or by the IP3 agonist cicaprost and did not undergo increased phosphorylation in response to either SIN-1/8-Br-cGMP or cicaprost stimulation (data not shown).

Hence, taken together, these data confirm that TPα, but not TPβ, is subject to independent regulation or desensitization by NO/PKG and prostacyclin/PKA signaling by the endothelium-derived vasodilatory/antithrombotic autacoids NO and prostacyclin and lead us to a model of these desensitizations, as outlined in Fig. 10. In this model, TPα expressed, for example, in the VSM, is subject to desensitization by NO mediated through its direct phosphorylation whereby Ser331, but not Ser 329, acts as the phosphorylation target for that desensitization. Consistent with our previous findings, and subject to independent cicaprost-induced desensitization, Ser329 is unaffected by NO/PKG inhibitory mechanisms, in addition to its established lack of responses to prostacyclin/PKA mechanisms (23), unveils novel insights into the differential, independent modes of regulation of the inhibitory TPα isoforms and points to further potentially critical physiologic differences between the TP isoforms within the vasculature.

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