Homologous desensitization of signalling by the beta (β) isoform of the human thromboxane A₂ receptor.

Running Title: Agonist-mediated desensitization of TPβ.

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Abbreviations: C-tail, carboxyl-terminal tail; [Ca²⁺], intracellular calcium; FBS, foetal bovine serum; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; HA, hemagglutinin; HEK, human embryonic kidney; IP, prostacyclin receptor; IP₃, inositol 1, 4, 5-trisphosphate; PAGE, polyacrylamide gel electrophoresis; PG, prostaglandin; PK, protein kinase; PL, phospholipase; TP, TXA₂ receptor; TX, thromboxane.

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**SUMMARY:** Thromboxane (TX) A$_2$ is a potent stimulator of platelet activation/aggregation and smooth muscle contraction and contributes to a variety of pathologies within the vasculature. In this study, we investigated the mechanism whereby the cellular responses to TXA$_2$ mediated through the TP$\beta$ isoform of the human TXA$_2$ receptor (TP) are dynamically regulated by examining the mechanism of agonist-induced desensitization of intracellular signalling and second messenger generation by TP$\beta$. It was established that TP$\beta$ is subject to profound agonist-induced homologous desensitization of signalling (intracellular calcium mobilization and inositol 1,3,5 trisphosphate generation) in response to stimulation with the TXA$_2$ mimetic U46619 and this occurs through two key mechanisms: TP$\beta$ undergoes partial agonist-induced desensitization that occurs through a GF 109203X-sensitive, protein kinase (PK)C mechanism whereby Ser$^{145}$ within intracellular domain (IC)$_2$ has been identified as the key phospho-target. In addition, TP$\beta$ also undergoes more profound and sustained agonist-induced desensitization involving G protein-coupled receptor kinase (GRK)2/3-phosphorylation of both Ser$^{239}$ and Ser$^{357}$ within its IC$_3$ and carboxyl-terminal C-tail domains, respectively. Inhibition of phosphorylation of either Ser$^{239}$ or Ser$^{357}$, through site directed mutagenesis, impaired desensitization while mutation of both Ser$^{239}$ and Ser$^{357}$ almost completely abolished desensitization of signalling, GRK phosphorylation and $\beta$-arrestin association, thereby blocking TP$\beta$ internalization. These data suggest a model whereby agonist-induced PKC phosphorylation of Ser$^{145}$ partially impairs TP$\beta$ signalling while GRK2/3 phosphorylation at both Ser$^{239}$ and Ser$^{357}$ within its IC$_3$ and C-tail domains, respectively, sterically inhibits G-protein coupling, profoundly desensitizing signalling, and promotes $\beta$-arrestin association and, in turn, facilitates TP$\beta$ internalization.
INTRODUCTION:
G protein-coupled receptors (GPCRs) constitute the largest and most versatile family of cell surface receptors, regulating many physiologic processes including vision, olfaction, taste, neurotransmission and cardiovascular function [1]. A critical aspect of the GPCR signalling paradigm is the regulation or desensitization of second messenger generation and signalling that occurs in response to the continued presence of the ligand, dampening or terminating the specific cellular response. Such desensitization is initiated by GPCR phosphorylation, uncoupling the receptor from its cognate G-protein [2, 3]. Two classes of serine/threonine kinases are associated with GPCR desensitization and typically may target specific residue(s) within one/all of their intracellular loop (IC) 1-3 and/or their carboxyl terminal (C) tail domains [2, 3]. The second messenger-regulated kinases, such as cAMP-dependent protein kinase (PK) A and diacylglycerol-regulated PKC, can phosphorylate the GPCR even in the absence of its agonist resulting in so-called heterologous desensitization [2, 3]. The G protein-coupled receptor kinases (GRKs), on the other hand, can only phosphorylate and desensitize GPCRs in their agonist-occupied, activated state and, hence, mediate homologous desensitization [2-4]. GRK phosphorylation alone does not desensitize the G protein: GPCR interaction. Rather, the GRK-phosphorylated GPCR recruits the non-visual β-arrestin1/2 adaptor(s) which binds with high affinity and, in turn, sterically hinders G-protein coupling, desensitizing further GPCR signalling [1, 3, 5]. Moreover, depending on the GPCR, further interaction of the recruited β-arrestin adaptor with components of the endocytic machinery can induce the sequestration and internalization of the desensitized GPCR from the plasma membrane into intracellular compartments, such as through clathrin-coated pit- or caveolin-dependent mechanisms [1, 3, 5], which may ultimately lead to either GPCR dephosphorylation and recyclization (resensitization), GPCR degradation (down-regulation) or indeed GPCR recruitment into scaffolds/subcellular microdomains for participation in other novel/distinct signalling cascades [1, 3, 5-7]. Of the 7 GRKs thus far identified, GRK1 and GRK7 are retinal enzymes exclusively regulating desensitization of opsin signalling; GRK4 expression is restricted to the brain, kidney and testes while GRK2 (also referred to as β-adrenergic receptor kinase (β-ARK(1)), GRK3 (β-ARK2), GRK5 and GRK6 are ubiquitously expressed [3] and are generally thought to participate in agonist-induced homologous desensitization of the broader superfamily of GPCR excluding the opsins [1, 3].

The prostanoid thromboxane (TX) A₂ mediates activation and aggregation of platelets and is a potent vaso- and broncho-constrictor as well as an inducer of vascular smooth muscle cell proliferation and hypertrophy [8]. Moreover, TXA₂ has been implicated in a number of cardiovascular, bronchial and renal diseases resulting from defects in TXA₂ synthesis or in its receptor function [9-11]. In humans TXA₂ actually signals through two TP receptor isoforms termed TPα and TPβ that are identical for their N-terminal 328 amino acids but differ within their carboxyl terminal (C)-tail domains [12-14]. On the other hand, in other species including in bovine and rodents, TXA₂ signals through a single species-specific TP receptor that display a high degree of identity (~85%) throughout the length of their sequences but whose sequences diverge completely from that of either TPα or TPβ at residues beyond amino acid 328 [8]. Thus, as TPα and TPβ diverge from the TPs from other species within their C-tail domains, it can be argued that neither TPα nor TPβ is the actual human orthologue of the TP, but rather that humans have evolved to have two separate
TP orthologues in the form of TPα and TPβ. Whereas the relevance of two receptors for TXA₂ is unclear, there is increasing evidence that TPα and TPβ have distinct physiologic/pathophysiologic roles greatly adding to the complexity of TXA₂ signalling in humans. Whilst both TPα and TPβ exhibit identical ligand binding and Gq-dependent activation of PLCβ, their primary effector [13, 15-17], they oppositely regulate adenylyl cyclase activity [18] and TPα, but not TPβ, mediates activation of the Gh/tissue transglutaminase [19]. TPα and TPβ also display distinct patterns of expression in a variety of cell/tissue types of vascular origin, including platelets [20-22] and such differences are due to the fact that TPα and TPβ are not only products of differential splicing but are also regulated by two distinct promoters within the single human TP gene [23-26]. In studies investigating cross-talk between TXA₂ and other prostanoids, it was established that signalling by TPα, but not TPβ, is subject to prostacyclin- and prostaglandin (PG)D₂-mediated desensitization of signalling involving direct PKA phosphorylation of TPα within its unique C-tail domain [27, 28]. Moreover, consistent with the latter, it is now also evident that TPα, but not the TPβ, is a target for nitric oxide-induced desensitization that occurs through a mechanism involving its direct PKG phosphorylation at Ser³³¹ within its unique C-tail domain [29]. These latter studies point to an essential role for TPα in vascular hemostasis and point to a redundant or an, as yet, unidentified role for TPβ in this essential physiologic process [27-29]. While both TPs are reported to undergo agonist-induced phosphorylation [15, 22], TPβ, but not TPα, is subject to both agonist-induced and tonic internalization [30, 31]. Moreover, agonist-induced internalization of TPβ was inhibited by dominant negative forms of dynamin-, GRK2/3-, and β-arrestins, suggesting their involvement in this process [30]. Through additional studies, it was established that agonist-induced endocytosis of TPβ is dependent on a dynamic actin cytoskeleton [32] and that the intracellular trafficking of TPβ may also be regulated by its direct interaction with Rab 11 [33].

Despite these studies investigating the regulation of TPβ internalization in response to tonic or agonist-induced conditions, to date little is understood about the mechanisms of homologous desensitization of second messenger generation and intracellular signalling in response to TXA₂ or its synthetic mimetics. In view of the fact that, as stated, TXA₂ signals in humans through TPα and TPβ that exhibit significant differences in their signalling behaviour, such studies are critical if we are to understand how TXA₂ signalling is dynamically regulated through two distinct receptor isoforms. Herein, we investigated the mechanisms of homologous desensitization of signalling by the TPβ isoform in response to the TXA₂ mimetic U46619. Our data established that TPβ is subject to homologous desensitization of signalling through two key mechanisms involving receptor phosphorylation: one involving a partial and transient PKC-dependent mechanism and another involving a profound and sustained GRK-dependent mechanism what also involves receptor internalization.
MATERIALS & METHODS:

Materials
U46619, SQ29,548 and I-BOP were from the Cayman Chemical Company. Anti-Gαq (C15; SC-392), β-Arrestin-2 (C-18; SC-6387) and βARK1/GRK2 (C-15; SC-562) antibodies were obtained from Santa Cruz Laboratories. FURA2/AM, D-myo-inositol 1,4,5-trisphosphate, 3-deoxyhexasodium salt, U46619, N-[2-(p-bromocinnamyl)amino]ethyl]-5-isoquino-linesulphonamide, 2HCL (H-89) and 2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide (GF 109203X) were from Calbiochem. [32P]orthophosphate (8,000-9,000 Ci/mmol) and [3H]SQ29,548 (50.4 Ci/mmol) were from NEN Life Sciences Products. [3H]IP3 (20-40 Ci/mmol) was from American Radiolabelled Chemicals Inc. Anti-HA 101R monoclonal antibody and anti-HA-3F10-peroxidase conjugated antibody were obtained from BABCO and Roche Molecular Biochemicals, respectively. Dithiobis succinimidylpropionate (DSP) was obtained from Pierce.

Subcloning and site-directed mutagenesis.
The plasmids pBluescript:TPβ, pCMV:Gαq [34], pHM:TPβ, pHM:TPα, pHM:TP328, pHM:TP367 [16, 35], pRK5:βARK1495-689 [36], pcDNA:βArrestin1 and pcDNA:βArrestin2 [30] have been previously described.

Figure 1 illustrates the C-tail sequence of TPβ and its mutated variants that were generated and used in this study. Site-directed mutagenesis was performed using QuickChange™ (Stratagene), unless specified otherwise. Mutation of Ser145 to Ala145 of TPβ and TP328 to generate the plasmids pHM:TPβS145A and pHM:TPS145A,A328 was achieved using pHM:TPβ and pHM:TP328 as templates, respectively, and the sense/antisense primer pair (5'-GC CCG GCG GTC GCC GCG CAG CGC GCC-3'). Mutation of Ser239 to Ala239 of TPβ to generate pHM:TPβS239 was achieved using pHM:TPβ as template and sense/antisense primer pair (5'-G CTG CCC CGG GAC GCC GAG GTG GAG A-3'). Mutation of Ser357 to Ala357 of TPβ to generate pHM:TPβS357A was achieved using pHM:TPβ as template and sense/antisense primer pair (5'-GGT TCA AGC GAT GCT GTG GCC TCA GCC-3'). Conversion of both Ser239, Ser357 to Ala239, Ala357 of TPβ to generate pHM:TPβS239,357A was achieved using pHM:TPβS239A as template and sense/antisense primer pair (5'-GGT TCA AGC GAT GCT GTG GCC TCA GCC-3'). For each primer pair above, sequences shown correspond to the sense primer and in each case the identity of the mutator codon is in boldface italics.

The plasmid pHM:TPβA259 was generated by conversion of Ser360 (TCA) codon to a Stop (TGA) codon. Briefly, PCR site-directed mutagenesis was performed using pBluescript:TPβ as template and primers 5'-GAGAAGCTTG ATG TGG CCC AAC GGC AGT TCC-3' (where nucleotides +1 to +21 of TPβ sequence are underlined) and 5'-TCTC GAAT TCA GGC AGC AGA ATC GCT TGA-3' (nucleotides complementary to +1062 to +1077 of TPβ sequence are underlined, and the mutator in-frame stop codon is in boldface italics). Similarly, the plasmid pHM:TPβA356 was generated by conversion of Ser357 (TCT) codon to a Stop (TGA) codon using pBluescript:TPβ as template and primers 5'-GAGAAGCTTG ATG TGG CCC AAC GGC AGT TCC-3' (where nucleotides +1 to +21 of TPβ sequence are underlined) and 5'-TCTC
GAAT TCA ATC GCT TGA ACC CGG GAG-3' (nucleotides complementary to +1056 to +1068 of TPβ sequence are underlined, and the mutator in-frame stop codon is in boldface italics). In each case, PCR amplifications were carried out using Pfu Turbo (R) DNA polymerase (Strategene) and products were subcloned into the HindIII-EcoRI sites of pHM6 to generate pHM:TPβΔ359 and pHM:TPβΔ356.

**Cell culture and transfections**

Human embryonic kidney (HEK) 293 cells were cultured in minimal essential medium with Earle’s salts (MEM) supplemented with 10% FBS and maintained at 37 °C in 5% CO₂. The following HEK 293 cell lines stably over-expressing hemagglutinin (HA) epitope-tagged forms of TPβ (HEK.TPβ), TPα (HEK.TPα), TPΔ328 (HEK.TPΔ328) and TPΔ367 (HEK.TPΔ367) have been previously described [16, 35].

For transient transfections, routinely HEK 293 cells were plated in 10 cm dishes at a density of 2 X 10⁶ cells/dish in 8 ml medium approximately 48 h prior to transfection. Cells were transiently transfected with 10 μg pADVA [37] and 25 μg of pcDNA-, pCMV- or pHM- based vectors using the calcium phosphate/DNA co-precipitation procedure, as previously described [34]. Routinely, cells were harvested 48 h post transfection. The plasmid pADVA encodes adenovirus type 2 virus-associated RNA genes VAI and VAII and its co-transfection into mammalian cells, such as in adenovirus-transformed HEK 293 cells, enhances protein expression by overcoming a block in translation due to the production of vector-derived double stranded RNA by RNA polymerase III during transcription [37]. To create HEK 293 cell lines stably over-expressing HA-epitope tagged forms of TPβS239A (HEK.TPβS239A), TPβΔ359 (HEK.TPβΔ359), TPβΔ356 (HEK.TPβΔ356), TPβS145 (HEK.TPβS145), TPβS145AΔ328 (HEK.TPβS145AΔ328), TPβS357A (HEK.TPβS357A) and TPβS239,S357A (HEK.TPβS239,S357A), cells were transfected with 10 μg of Sca1-linearised pADVA plus 25 μg of the appropriate PvuI-linearised pHM6-based recombinant plasmid. Forty-eight h post-transfection, G418 (0.8 mg/ml) was applied and after approximately 21 days, individual G418-resistant colonies were selected and individual pure clonal stable cell lines/isolates were examined for TP expression by analysis of radioligand binding.

**Radioligand Binding Studies**

Cells were harvested by centrifugation at 500 X g at 4 °C for 5 min and washed three times with ice-cold Ca²⁺/Mg²⁺ -free phosphate-buffered saline (PBS). 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]SQ29,548 for Scatchard analysis or in the presence of 20 nM [³H]SQ29,548 for saturation radioligand binding experiments as previously described [34]. Protein determinations were carried out using the Bradford assay [38].

**Measurement of intracellular calcium ([Ca²⁺]ᵢ) mobilization**

Measurement of intracellular calcium mobilization ([Ca²⁺]ᵢ) in FURA2/AM preloaded HEK 293 cell lines, each transiently co-transfected with pCMV:Gαq, was carried out as previously described [34]. To investigate the effect of GRK2/βARK1 on TP-mediated [Ca²⁺]ᵢ mobilization, HEK.TPβ cells (2 X 10⁶
cells/dish) were co-transfected with pADVA (10 µg), pCMV:Gαq (25 µg) along with pRK5:βARK1 (25 µg), encoding a dominant negative form of GRK2/βARK1, approximately 48 h prior to harvesting.

Thereafter, cells were harvested by scraping, washed twice in ice-cold PBS and resuspended in HBSSHB (modified Ca²⁺/Mg²⁺ -free Hank’s buffered salt solution, containing 10 µM HEPES, pH 7.67, 0.1 % bovine serum albumin (BSA)) buffer at 10⁷ cells/ml and incubated in the dark with 5 µM FURA2/AM for 45 min at 37 °C. Cells were collected by centrifugation (900 x g, 5 min), washed once in an equal volume of HBSSHB, and were finally resuspended in HBSSHB buffer at 10⁷ cells/ml and kept at room temperature in the dark for 40 min. For each measurement of [Ca²⁺], mobilization, aliquots of cells were diluted to 0.825 x 10⁶ cells/ml in HBSSHB, 1 mM CaCl₂ and FURA2 fluorescence was recorded (2 ml aliquots of cells) at 37 °C with gentle stirring with a Perkin Elmer-Cetus LS50-B spectrofluorometer at excitation wavelengths of 340 nm and 380 nm and emission wavelength of 510 nm, respectively.

Cells were stimulated with the TP agonist U46619 (1 µM) and changes in [Ca²⁺], mobilization was monitored as a function of time. To assess agonist-mediated homologous desensitization, cells (0.825 x 10⁶ cells/ml; 2 ml per assay) were stimulated with 1 µM U46619 for 4 min (Primary stimulation); thereafter, cells were collection by centrifugation (900 x g, 5 min); were washed twice in 2 ml HBSSHB, 1 mM CaCl₂; and finally resuspended in 2 ml HBSSHB, 1 mM CaCl₂ and were left to recover over various time intervals (ranging from 0-60 min); thereafter, cells re-stimulated with 1 µM U46619 (Secondary stimulation) and changes in [Ca²⁺], mobilization was monitored as a function of time. To assess the role of protein kinase (PK) A or PKC on U46619-mediated desensitization of TPβ signalling, cells were pre-incubated with the PKA inhibitor H-89 (10 µM) or the PKC inhibitor GF 109203X (50 nM) for 15 min and respective kinase inhibitors were maintained throughout the washes and primary/secondary U46619-stimulations.

In all cases, the drugs (agonist or kinase inhibitors in 0.01% ethanol) were diluted in the vehicle HBSSHB such that addition of 20 µl of the diluted drug/inhibitor to 2 ml of cells resulted in the correct working concentration. The vehicle had no effect on U46619-mediated [Ca²⁺], mobilization. Calibration of the signal was performed in each sample by adding 0.2% Triton X-100 to obtain the maximal fluorescence ratio (Rₘₐₓ) and then 1 mM EGTA to obtain the minimal fluorescence ratio (Rₘᵢₙ). The ratio of the fluorescence at 340 nm to that at 380 nm is a measure of [Ca²⁺], [39], which assumes a Kᵅ of 225 nM Ca²⁺ for FURA2/AM. The results presented in the figures are representative data from at least three or four independent experiments and are plotted as changes in Ca²⁺ mobilized (Δ[Ca²⁺], (nM)) as a function of time (s) upon ligand stimulation. Changes in [Ca²⁺], mobilization were determined by measuring peak rises in [Ca²⁺], mobilized (Δ[Ca²⁺],) and were calculated as mean changes in Δ[Ca²⁺], ± S.E.M (nM).

Measurement of IP₃ levels

Intracellular IP₃ levels were measured as previously described [27, 40]. Briefly, cells were harvested by scraping, washed twice in ice-cold PBS and resuspended at approximately 2 x 10⁶ cells/200 µl in HEPES-buffered saline (HBS; 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), 10 mM LiCl. Cells (200 µl) were pre-incubated in HBS buffer at 37 °C for 10
min; where appropriate kinase inhibitors (H-89, 10 μM; GF 109203X, 50 nM) or vehicle (HBS) were added to the cells and pre-incubated at 37 °C for 15 min. Thereafter, cells were stimulated with 1 μM U46619 at 37 °C for 2 min. To assess agonist-mediated homologous desensitization, cells (2 x 10^6 cells/200 μl) were stimulated with 1 μM U46619 at 37 °C for 2 min (Primary stimulation); thereafter, cells were collected by centrifugation (900 x g, 5 min); were washed once in 200 μl HBS, 10 mM LiCl and finally resuspended in 200 μl HBS, 10 mM LiCl and were left to recover for 60 min; thereafter, cells were re-stimulated with 1 μM U46619 at 37 °C for 2 min (Secondary stimulation). All ligands and kinase inhibitors were pre-diluted in HBS such that 50 μl added to 200 μl of cell suspension in HBS gave the desired final concentration. To determine basal IP$_3$ level, an equivalent volume (50 μl) of the vehicle HBS was added instead of ligand. The level of IP$_3$ produced was quantified by radio competition assay essentially as previously described [27, 40]. Levels of IP$_3$ produced by ligand stimulated cells over basal stimulation, in the presence of HBS, were expressed in pmol IP$_3$/mg protein ± S.E.M. and as fold stimulation over basal (fold increase ± S.E.M.). In all cases, 4 independent experiments were performed, each in duplicate.

**Measurement of Agonist-mediated TP Phosphorylation**

Agonist-mediated TP phosphorylation in whole cells (1.8-2 X10$^6$ cells/10 cm dish) was performed essentially as previously described [27]. Briefly, cells were transiently co-transfected approximately 48 h prior to labelling with pADVA (10 μg), pCMV:Gα$q$ (25 μg) in the presence of either (i) pRK:βARK1$_{495-689}$ (25 μg), (ii) pcDNA:βArrestin2 (25 μg) or (iii) an equivalent concentration of the vector control pRK5/pcDNA3 (25 μg), as indicated in the figure legends. Thereafter, cells were washed once in phosphate-free DMEM, 10% dialysed FBS and were metabolically labelled for 60 min in the same medium (2 ml/10 cm dish) containing 100 μCi/ml [32P]orthophosphate (8000-9000 Ci/mmol) at 37 °C, 5% CO$_2$. Thereafter, U46619 (1 μM) or an equivalent volume of vehicle (0.01% ethanol in HBS; 20 μl) were added and cells were incubated for 10 min at 37 °C, 5% CO$_2$. Reactions were terminated and HA- epiptope tagged TP receptors were processed and immunoprecipitated using the anti-HA 101R antibody as previously described [27]. Electroblots were then exposed to Xomat XAR (Kodak) film to detect 32P-labelled proteins. Alternatively, blots were subject to phosphorimage analysis, and the intensities of agonist-induced phosphorylation were expressed in arbitrary units relative to basal (vehicle-stimulated) levels. In parallel experiments, cells were incubated under identical conditions in the absence of [32P]orthophosphate; HA-tagged TP receptors were immunoprecipitated from the same cell lines using the anti-HA 101R antibody and immunoblots were screened using the anti-HA 3F10 horseradish peroxidase-conjugated antibody [27].

**βArrestin Co-immunoprecipitations**

HEK 293 cells or the respective TP stable cell line equivalents (2 X 10$^6$ cells/ 10 cm dish) were transiently co-transfected with pADVA (10 μg), pCMV:Gα$q$ (25 μg) plus pcDNA:βArrestin2 (25 μg). Approximately, 48 h post transfection, cells were serum starved 48 h post transfection for 1 h in 3 ml DMEM at 37 °C in 5% CO$_2$ prior to stimulation for 10 min with 1 μM U46619 or an equivalent volume of its vehicle (0.01%
ethanol in HBS). Cells were then washed once in ice cold Dulbecco’s phosphate buffered saline containing 10 mM HEPES, pH 7.4 and then 1 ml cross-linking buffer (Dulbecco’s phosphate buffered saline containing 10 mM HEPES, pH 7.4, 10% DMSO, 2.5 mM of the cell permeant homobifunctional cross-linking agent dithiobis succinimidylpropionate (DSP)) was added and cells incubated for 30 min with continuous mixing, essentially as described previously [41]. Cross linking buffer was then replaced with 1 ml radioimmune precipitation (RIP) buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 % Nonidet P-40 (v/v), 0.5 % deoxycholate (w/v), 0.1 % SDS (w/v), 0.5 % sodium fluoride, 25 mM sodium pyrophosphate, 1 μg/ml leupeptin, 0.5 mM phenyl-methysulfonyl fluoride (PMSF), 10 μg/ml aprotintin, 10 μg/ml antipain, and 1 mM sodium orthovanadate). Reactions were terminated by disruption of cells in RIP buffer and HA-tagged TP receptors were immunoprecipitated using the anti-HA 101R antibody, blotted and analysed essentially as described previously [27]. Immunoblots were screened with the anti-βarrestin2 (C-18: SC-6387) antibody and visualised by chemiluminescence [27] or the Odyssey Infrared Imaging System (Li-Cor, Bioscience).

**Agonist-induced Internalization Assays**

For quantitation of TP receptor internalization, ELISAs (enzyme linked immunosorbent assays) were performed as described [42]. Briefly, HEK.TPα, HEK.TPβ, HEK.TPβS239A, HEK.TPβS357A and HEK.TPβS239,357A cells, encoding HA-epitope tagged forms of TPα/TPβ, were plated at 2 X 10^6 cells per 10-cm dish and transiently transfected with pCMV:Gαq (25 μg) plus pADVA (10 μg) approximately 24 h prior to trypsinization. Cells were replated into poly-L-lysine (0.1 mg/ml; Sigma) pre-coated wells of a 96 well plate at a density of 1 X 10^3 cells / well in 100 μl MEM, 10% FBS. After 24 h, cells were washed once with PBS and pre-incubated in serum free-MEM for 10 min prior to incubation with the agonist U46619 (1 μM) for 0 – 4 hr at 37 °C. The reactions were stopped, cells were fixed and surface expression of HA-tagged TPs was determined by ELISA using the anti-HA 101R primary and goat anti-mouse IgG conjugated alkaline phosphatase secondary antibodies, as described [42]. Results were calculated as the percentage (%) cell surface receptor expression in U46619-stimulated cells relative to vehicle (0.01% ethanol in HBS)-treated cells and are presented as mean data of three independent experiments each carried out in triplicate (mean ± SEM, n = 3), where vehicle-treated control cells were assigned a value of 100%.

**Data analysis**

Radioligand binding and Scatchard analysis data was analysed using GraphPad Prisim V4.0 programme (GraphPad Software Inc., San Diego CA, U.S.A.). Statistical analyses were carried out using the unpaired Students’ t test using the Statworks Analysis Package. P-Values ≤ 0.01 indicated statistically significant differences.
RESULTS:

**Effect of primary and secondary agonist stimulation on TPβ-mediated intracellular signalling**

The current study sought to investigate the mechanism of agonist-induced, homologous desensitization of signalling ([Ca$^{2+}$], mobilization and IP$_3$ generation) by the TPβ isoform of the human thromboxane (TX) A$_2$ receptor.

Stimulation of HEK.TPβ cells, a HEK 293 cell line stably over-expressing TPβ and transiently co-transfected with pCMV:Gαq to augment signalling, with the TXA$_2$ mimetic U46619 (1 µM) yielded efficient mobilization of intracellular calcium ([Ca$^{2+}$], Figure 2A). Pre-stimulation of HEK.TPβ cells with U46619 almost completely desensitized signalling following agonist-washout and secondary restimulation with U46619 at 15 min following the initial stimulation (Figure 2B, $p = 0.0001$). Moreover, U46619-mediated desensitization of TPβ signalling was sustained and did not show any significant recovery even at 60 min following the primary stimulation (Figure 2C, $p = 0.0001$).

Stimulation of HEK.TP$^{A328}$ cells, stably over-expressing the truncated TP$^{A328}$ variant devoid of divergent C-tail residues of TPα and TPβ, also led to efficient U46619-induced [Ca$^{2+}$], mobilization (Figure 3A) consistent with previous reports [17, 27]. On the other hand, pre-stimulation of TP$^{A328}$ with U46619 only partially desensitized [Ca$^{2+}$], mobilization in response to its secondary stimulation with U46619 (Figure 3B). Specifically, approximately 81% of the initial primary U46619-induced response occurred following secondary restimulation of TP$^{A328}$ at 15 min (Compare Figure 3A versus Figure 3B, $p = 0.2$). Moreover, restimulation of TP$^{A328}$ at 60 min revealed that U46619-mediated [Ca$^{2+}$], mobilization was transient and signalling had fully recovered, showing no desensitization, at 60 min (Compare $\Delta$[Ca$^{2+}$], = 160 ± 1.5 nM, Figure 3A versus $\Delta$[Ca$^{2+}$], = 156 ± 3 nM, Figure 3C; $p = 0.22$).

Consistent with these data, stimulation of HEK.TPβ and HEK.TP$^{A328}$ cells with U46619 also resulted in efficient increases in IP$_3$ levels (Figure 2D & 3D, respectively). While pre-incubation with U46619 almost completely inhibited IP$_3$ generation by TPβ in response to secondary restimulation with U46619 (Figure 2D, $p = 0.005$), it had no significant effect on IP$_3$ generation by TP$^{A328}$ at 60 min following the primary stimulation (Figure 3D, $p = 1$). Moreover, similar agonist-induced desensitizations of IP$_3$ generation and [Ca$^{2+}$], mobilization by TPβ and TP$^{A328}$ were obtained when the TP agonist I-BOP was used as the stimulatory ligand, replacing U46619 (data not shown). Taken together, these data confirm that TPβ is subject to prolonged, nearing complete agonist-induced homologous desensitization. Conversely, TP$^{A328}$ undergoes partial and transient desensitization suggesting that the major target site(s) involved in homologous desensitization of TPβ is located within its unique C-tail domain.

*Investigation of the role of the second messenger kinases in homologous desensitization of TPβ*

To investigate whether the second messenger protein kinases (PKs) may be involved in mediating homologous desensitization of TPβ, the effects of the PKA and PKC inhibitors H-89 and GF 109203X, respectively, on U46619-induced [Ca$^{2+}$], mobilization were investigated. Pre-incubation of HEK.TPβ or HEK.TP$^{A328}$ cells with H-89 had no significant effect on [Ca$^{2+}$], mobilizations in response to their primary or
secondary stimulations with U46619 (Figure 4C & 3E, respectively). Whilst pre-incubation with GF 109203X had no significant effect on [Ca\(^{2+}\)], mobilization by TP\(\beta\) or TP\(^{A328}\) in response to primary stimulation (Figure 4E & 3F, respectively), it partially inhibited desensitization of TP\(\beta\) such that in the presence of GF 109203X secondary restimulation with U46619 yielded a response of 27% relative to that of the primary response (Compare Figure 4B & 4F). In addition, pre-incubation of HEK.TP\(^{A328}\) cells with GF 109203X fully impaired agonist-induced desensitization in response to secondary U46619 stimulation (Compare Figure 3B versus Figure 3F, secondary response).

**Investigation of the role of Ser\(^{145}\) within the intracellular loop (IC)\(_2\) in mediating GF 109203X-sensitive, PKC-mediated homologous desensitization of TP\(\beta\) and TP\(^{A328}\).**

Hence, it is evident that TP\(^{A328}\) undergoes partial and transient desensitization that occurs through a GF 109203X-sensitive, PKC mechanism, whilst TP\(\beta\) undergoes extensive, almost complete and more sustained desensitization that is only partially sensitive to GF 109203X/ PKC inhibition. Moreover, as TP\(^{A328}\) is devoid of C-tail residues of both TP\(\alpha\) and TP\(\beta\), it is evident that the target PKC-sensitive site(s) is located at sites other than within the C-tail domain of TP\(\beta\). Analysis of the amino acid sequence of TP\(^{A328}\) / TP\(\beta\) identified a putative PKC site within the second intracellular loop (IC)\(_2\) where Ser\(^{145}\) represents the predicted phospho-target residue [43]. Thus, to investigate the importance of this putative PKC site in mediating the partial GF 109203X-sensitive, agonist-induced desesitization of TP\(^{A328}\) and TP\(\beta\), site-directed mutagenesis was used to generate TP\(^{S145A,A328}\) and TP\(^{\beta S145A}\). Primary stimulation of HEK.TP\(^{S145A,A328}\) (K\(_{d}: 6.1 \pm 3.7\) nM SQ29,548; B\(_{max}: 3.8 \pm 0.03\) pmol/mg protein) and HEK.TP\(^{\beta S145A}\) (K\(_{d}: 6.6 \pm 2.4\) nM SQ29,548; B\(_{max}: 3.8 \pm 0.03\) pmol/mg protein) cells with U46619 each resulted in efficient [Ca\(^{2+}\)], mobilization (Figure 5B & 5D, respectively) and compared well to that of their respective wild type TP\(^{A328}\) (Figure 5A, primary stimulation; \(p = 0.3\)) and TP\(\beta\) (Figure 5C, primary stimulation; \(p = 0.7\)) equivalents. Pre-stimulation of TP\(^{S145A,A328}\) with U46619 did not desensitize [Ca\(^{2+}\)], mobilization in response to its secondary stimulation with U46619 at 15 min following the initial stimulation (Figure 5B, compare primary & secondary stimulations; \(p = 0.55\)). Moreover, unlike the wild type TP\(\beta\) (Figure 5C), pre-stimulation with U46619 did not fully desensitize signalling by TP\(^{\beta S145A}\), such that secondary restimulation with U46619 yielded a response of 23% relative to the primary response (Figure 5D). Similar data was obtained when agonist-induced IP\(_3\) generation was analysed (data not shown).

Taken together, these data indicate that TP\(\beta\) and TP\(^{A328}\) are subject to partial U46619-induced, GF 109203X -sensitive homologous desensitization that occurs through a PKC-dependent mechanism where Ser\(^{145}\) within IC\(_2\) represents the phospho-target residue. On the other hand, these data also indicate that whilst Ser\(^{145}\) within TP\(\beta\) plays a minor, though significant role in its homologous desensitization, the major determinant(s) of U46619-induced desensitization of TP\(\beta\) signalling are located within its unique C-tail domain.

**The role of GRKs in homologous desensitization of TP\(\beta\)**

11
In addition to the second messenger protein kinases, the family of G protein coupled receptor kinases (GRKs) can play a key role in homologous desensitization of various GPCRs [1]. Hence, in the current study, we sought to investigate the role of the ubiquitously expressed GRK2/3 members, also referred to as the β adrenergic kinases (β-ARK) 1/2, in the homologous desensitization of signalling by TPβ. To this end, we examined the effect of over-expressing a dominant-negative form of GRK2/βARK1 on U46619-induced desensitization of TPβ and TPβ^{A328} signalling. GRK2/βARK1^{495-689} encodes the carboxyl-terminal 495-689 residues of GRK2/βARK1 and can be effectively used as a dominant negative GRK2/3 sequestering Gβγ subunits and inhibiting GRK2/3 membrane translocation and activation [36]. Transient over-expression of βARK1^{495-689} (Figure 6E) had no significant effect on primary U46619-induced [Ca^{2+}]; mobilization by TPβ (Figure 6A) or TPβ^{A328} (Figure 6C). However, over-expression of βARK1^{495-689} almost fully impaired desensitization of TPβ signalling following its secondary stimulation with U46619 (Compare Figure 2B versus Figure 6B, \( p = 0.0001 \)). On the other hand, over-expression of βARK1^{495-689} had no effect on the partial agonist-induced desensitization of signalling by TPβ^{A328} (Figure 6D). Over-expression of βARK1^{495-689} was confirmed by western blot analysis using the anti-βARK1/GRK2 (C-15; SC-562) antibody (Figure 6E). Thus, the GRK2/3 (βARK1/2) appears to play a major role in the homologous desensitization of TPβ.

The role of the unique C-tail and intracellular loop (IC) 3 in homologous desensitization of TPβ

To further investigate the role of the unique C-tail region of TPβ in its homologous desensitization and to identify the target site(s) involved in this GRK-mediated desensitization, a combination of deletion and site-directed mutagenesis was employed. Owing to the number of Ser (11) and Thr (4) residues within the C-terminal domain of TPβ, deletion mutagenesis was initially used to generate TPβ^{A467}, TPβ^{A539} and TPβ^{A356} (Figure 1). Values obtained for the affinity (\( K_d; 6.7 \pm 1.9; 7.7 \pm 2.6 \& 7.5 \pm 3.4 \) nM SQ29,548; respectively) and maximal binding (\( B_{max}; 3.08 \pm 0.03; 3.77 \pm 0.03 \& 4 \pm 0.03 \) pmol/mg protein, respectively) by the respective HEK.TPβ^{A467}, HEK.TPβ^{A539} & HEK.TPβ^{A356} cell lines for the radioligand \([^{3}H]\)SQ29,548 compared well to values previously reported for wild type HEK.TPβ cells [27]. Stimulation of TPβ^{A467}, TPβ^{A539} and TPβ^{A356} yielded efficient U46619-induced increases in [Ca^{2+}]; mobilization (Figure 7A, 7D & 7G, respectively) and IP_{3} generation (Figure 7C, 7F & 7I, respectively). Moreover, similar to TPβ, both TPβ^{A467} and TPβ^{A539} underwent U46619-induced homologous desensitization as determined by analysis of both [Ca^{2+}]; mobilization (Figure 7B & 7E, \( p < 0.0001 \)) and IP_{3} generation (Figure 7C & 7F, \( p < 0.001 \& p < 0.0001 \), respectively). In contrast, pre-incubation of HEK.TPβ^{A356} cells with U46619 only partially reduced secondary U46619-mediated [Ca^{2+}]; mobilization (Figure 7G & 7H) or IP_{3} generation (Figure 7I) by TPβ^{A356}. Thus, taken together, these data suggest that a major target site(s) involved in U46619-induced homologous desensitization of TPβ lies between residues Arg^{366} and Ala^{399}.

Further analysis of the C-tail sequence of TPβ identified a putative GRK phosphorylation site with the sequence DS^{357}R whereby Ser^{357} represents the predicted phospho-target [44]. Hence, site-directed
mutagenesis was used to generate the variant TPβS357A thereby destroying this potential GRK phosphorylation site. Stimulation of HEK.TPβS357A cells (K_i: 7.6 ± 3.0 nM SQ29,548; B_max: 4 ± 0.04 pmol/mg protein) with U46619 yielded efficient increases in \([\text{Ca}^{2+}]_i\), mobilization (Figure 7J) and IP_3 generation (Figure 7L). Pre-stimulation of HEK.TPβS357A cells with U46619 did not substantially reduce \([\text{Ca}^{2+}]_i\), mobilization (Figure 7K; p < 0.14) or IP_3 generation (Figure 7L; p = 0.5) following secondary stimulation of TPβS357A with U46619. More specifically, it was evident that TPβS357A maintained approximately 72% of its original U46619-induced \([\text{Ca}^{2+}]_i\), mobilization (Figure 7J & 7K; Compare \(\Delta[\text{Ca}^{2+}]_i = 160 \pm 10\) nM versus \(\Delta[\text{Ca}^{2+}]_i = 130 \pm 13\) nM, respectively). Thus, from these data it is evident that Ser^{357} located within the C-tail of TPβ appears to be a major GRK phospho-target site involved in U46619-mediated homologous desensitization of TPβ.

Analysis of the amino acid sequences of TPβ also revealed a second putative GRK phosphorylation site, with the sequence DS^{239}E [44], located in the third intracellular loop (IC_3) of TPβ where Ser^{239} represents the predicted phospho-target residue. Hence, site-directed mutagenesis was used to generate TPβS239A and TPβS239,357A thereby destroying the putative GRK phosphorylation site(s) within the IC_3 and/or C-tail domains. Stimulation of HEK.TPβS239A cells (K_i: 7.2 ± 3.3 nM SQ29,548; B_max: 4.3 ± 0.04 pmol/mg protein) with U46619 yielded efficient increases in \([\text{Ca}^{2+}]_i\), mobilization (Figure 8A) and IP_3 generation (Figure 8C). Upon secondary stimulation, TPβS239A only underwent partial agonist-induced desensitization and maintained approximately 30% of its primary U46619-induced \(\Delta[\text{Ca}^{2+}]_i\), mobilization (Figure 8A & 8B) and IP_3 generation (Figure 8C) following its secondary restimulation. Moreover, stimulation of HEK.TPβS239,357A cells (K_i: 6.1 ± 1.9 nM SQ29,548; B_max: 3.5 ± 0.03 pmol/mg protein) with U46619 yielded efficient increases in \([\text{Ca}^{2+}]_i\), mobilization (Figure 8D) and IP_3 generation (Figure 8F). However, pre-stimulation of HEK.TPβS239,357A cells with U46619 did not significantly reduce U46619-induced \([\text{Ca}^{2+}]_i\), mobilization (Figure 8E) or IP_3 generation (Figure 8F) following secondary agonist stimulation. More specifically, it was evident that TPβS239,357A maintained approximately 99% of its original U46619-induced \([\text{Ca}^{2+}]_i\), mobilization (Figure 8D & 8E; Compare \(\Delta[\text{Ca}^{2+}]_i = 174 \pm 7\) nM versus \(\Delta[\text{Ca}^{2+}]_i = 173 \pm 6.5\) nM; p = 0.9). Hence, it is evident that U46619-mediated homologous desensitization of TPβ occurs through a GRK-dependent mechanism and involves both Ser^{239} and Ser^{357} within its IC_3 and C-tail domains, respectively.

**Agonist-induced phosphorylation, β-arrestin binding and internalization of TPβ**

Thereafter, the role of GRK2 in agonist-induced phosphorylation of TPβ and TPβS357A was investigated by examining the effect of over-expression of GRK2/βARK1^{495-689} on that phosphorylation. Stimulation of HEK.TPβ cells with U46619 led to a 5-fold increase in TPβ phosphorylation relative to that in vehicle-treated cells (Figure 9A). Moreover, over-expression of βARK1^{495-689} reduced the level of U46619-induced phosphorylation of TPβ by approximately 2.5-fold (Figure 9A), further confirming the role of GRK2/3 in TPβ desensitization. On the other hand, stimulation of HEK.TPβS357A cells with U46619 only led to a modest (1.8-fold) increase in TPβS357A phosphorylation and that phosphorylation was only
marginally affected by over-expression of βARK1495-689 (Figure 9B). The presence of HA-tagged TPβ and TPβS357A in the respective immunoprecipitates was confirmed through parallel western blots studies (Figure 9C) where, in each case, a discrete band of approximately 46 kDa and a broad diffuse band of 50-66 kDa representing the non-glycosylated and glycosylated forms of TPβ and TPβS357A were evident (Figure 9C, lanes 1 & 2, respectively). On the other hand, no immunoreactive bands were present in immunoprecipitates from non-transfected HEK 293 cells (Figure 9C, Lane 3).

GRK phosphorylation of GPCRs is thought to recruit and/or stabilize the binding of β-arrestin proteins to the agonist-occupied GRK phosphorylated receptor and, in turn, mediate receptor internalization such as through clathrin-coated pits [1]. Moreover, it is also proposed that β-arrestin binding can, in turn, stabilize the phosphorylation status of the GPCR [1, 45], potentially enhancing detection of the GRK-phosphorylated receptor in experimental settings for example. Hence, through co-immunoprecipitation experiments, we initially investigated β-arrestin2 binding to TPβ, TPβS239A, TPβS357A and TPβS239,357A and thereafter examined the effect of β-arrestin2 expression on agonist-induced phosphorylation of TPβ and its mutated derivatives. β-arrestin2 was efficiently co-precipitated along with TPβ in U46619-stimulated cells with trace levels also detected in the absence of agonist stimulation (Figure 10, lanes 1 & 2). However, the level of β-arrestin2 in the co-immunoprecipitates from vehicle- or U46619-treated HEK.TPβS239A and HEK.TPβS357A cells was significantly reduced relative to that of the wild type TPβ (Figure 10, lanes 3 - 6) while β-arrestin2 was not detected in the co-immunoprecipitates from HEK.TPβS239,357A cells either in the absence or presence of U46619 stimulation (Figure 10, lanes 7 & 8).

Consistent with these data, whole cell phosphorylations confirmed that detection of U46619-induced TPβ phosphorylation was significantly increased in the presence of β-arrestin2 (Figure 9D). Moreover, the overall level of U46619-induced TPβS239A, TPβS357 and TPβS239,357 phosphorylation was significantly reduced relative to that of the wild type TPβ (Figure 9D). The presence of HA-tagged TPβ, TPβS239A, TPβS357A and TPβS239,357A in the immunoprecipitates was confirmed in parallel western blots (data not shown). Hence, these data confirm that TPβ undergoes agonist-induced GRK2/3 phosphorylation whereby Ser239 and Ser359 within the IC3 and C-tail domain represent the phospho-targets. Moreover, impairment of phosphorylation at either Ser239 or Ser359 independently reduce high affinity β-arrestin2 binding, while mutation of both Ser239 and Ser359 reduced agonist-induced TPβ phosphorylation to near basal levels and led to substantial reductions in β-arrestin2 binding.

TPβ is widely reported to undergo agonist-induced internalization that occurs through GRK2/β-arrestin and Rab11 dependent mechanisms [30, 33, 46]. Hence, in the current study, we investigated the involvement of the GRK sites at Ser239, Ser357 or both Ser239 & Ser357 in mediating internalization of TPβ by comparing U46619-induced internalization of TPβ, TPβS239A, TPβS357A and TPβS239,357A. As a control for these experiments, we also examined U46619-mediated internalization of TPα, a TP receptor isoform that is known not to undergo significant agonist-induced internalization [30].

Consistent with previous reports [30], TPβ underwent efficient U46619-mediated internalization as evidenced by a 40% decrease in its surface expression relative to vehicle-treated cells following 4 h
incubation at 37 °C (Figure 1A & 1B). The t½ of TPβ internalisation in response to 1 μM U46619 was approximately 45 min. On the other hand, TPα did not undergo significant agonist-induced internalization (Figure 1A). U46619-induced internalization of TPβS239A and TPβS357A were each significantly impaired (p > 0.02) when compared to TPβ while TPβS239,357A (p > 0.0001) failed to undergo significant internalization (Figure 1B), even following prolonged agonist exposure (data not shown).
DISCUSSION:

Due to the critical role of TXA₂ as a potent biological mediator, tight controls mechanisms must exist to regulate its signalling capacity in a rapid and efficient manner, such as within the vasculature where the actions of TXA₂ are most acutely manifest [8, 47, 48]. One such mechanism is the rapid hydrolysis of TXA₂ to its inactive metabolite TXB₂ within 30 sec of its generation [8]. However, in such cases as thrombosis, vascular occlusion and hypertension where TXA₂ is produced in large quantities over prolonged periods [9, 10], turning off the TXA₂/TP signal may also occur through either homologous and heterologous desensitization leading to uncoupling of the GPCR from its cognate G-protein(s): second messenger generating effector system and/or activation of the internalization pathway, sequestering the receptor away from the PM [1-3]. The existence of two TP receptor isoforms, termed TPα and TPβ, adds greatly to the complexity of TXA₂ signalling in humans providing a mechanism whereby the responses to this potent lipid mediator may be potentially regulated through two receptor-dependent mechanisms. Whilst both TPα and TPβ exhibit identical ligand binding and Gq/PLCβ coupling [11, 13, 15, 17], they display distinct patterns of expression in a variety of cell/tissue types of vascular origin [20-24] and are now known to be subject to a host of isoform-specific regulatory mechanisms such as cross-talk or heterologous desensitization in response to counter-regulatory autocoids including prostacyclin [27, 28] and nitric oxide [29]. Moreover, several studies focussing on post-receptor signalling events have established that TPβ undergoes agonist-induced internalization through mechanism(s) involving GRK/β-arrestin and/or Rab 11 [30, 33], while both TP isoforms undergo rapid agonist-induced phosphorylation [15]. However, despite these reports, to date no studies have investigated in detail the molecular mechanism(s) of ligand-induced homologous desensitization of signalling by either of the TP isoforms. Hence, the aim of the current study was to investigate the mechanism of homologous desensitization of signalling by the TPβ isoform in response to the TXA₂ mimic U46619. Through an independent series of studies, we also aim to investigate the mechanism of homologous desensitization of TPα signalling (on-going investigations).

Initially it was confirmed that TPβ undergoes complete agonist-induced homologous desensitization of signalling as assessed by analysis of U46619-mediated increases in IP₃ generation and [Ca²⁺], mobilization and that TPβ desensitization was sustained, showing no significant, measurable recovery some 60 min following the primary stimulation. On the other hand, the truncated derivative TPα328, devoid of those residues unique to TPβ within its C-tail domain, only exhibited partial (20%) homologous desensitization and that desensitization was transient where the magnitude of the secondary signal had fully recovered to that of the primary response within 60 min. Thereafter, to investigate the mechanism involved in agonist-induced homologous desensitization of TPβ, and indeed of TPα328, we initially investigated the possible role of the second messenger- kinases PKA and PKC in that desensitization. The PKA inhibitor H-89 had no effect on either the primary signalling response or on level of agonist-induced desensitization by either TPβ or TPα328. On the otherhand, the PKC inhibitor GF 109203X completely inhibited the partial, transient homologous desensitization of TPα328. Moreover, GF 109203X also had a marginal though significant effect on the level of desensitization of TPβ, restoring its signalling to approximately 20% of those levels generated
in response to primary U46619 stimulation. These data suggest that TPβ may be subject to two types of desensitization where: (i) the major determinant(s) of the sustained TPβ desensitization lies within its C-tail domain and that desensitization is insensitive to either PKA or PKC inhibition and (ii) both TPβ and TPβS328 are also subject to a GF 109203X-sensitive-, PKC-dependent partial and transient desensitization and that this occurs within the N-terminal 328 residues of TPβ, also found in TPβA328.

In keeping with these results, computational sequence analysis indicated the presence of a unique PKC recognition site within TPβS328, where Ser145 within IC₂ represents the putative phospho-target [43]. Initially it was established that mutation of that putative PKC site in either TPβ or TPβA328 had no significant effect on the basic ligand binding or agonist-induced IP₃ generation- or [Ca²⁺], mobilization-capacity of the resulting TPβS145A and TPβS145AΔ328 receptors, respectively. Furthermore, secondary stimulation of TPβS145A with U46619 showed impaired agonist-induced homologous desensitization relative to the wild type TPβ such that following secondary stimulation with U46619, it signalled to approximately 20% of its primary response. Moreover, the mutant TPβS145AΔ328 failed to undergo any significant agonist-induced desensitization such that the level of the secondary U46619 response was not significantly different to that of its primary response. These data indicate that Ser145 within IC₂ is a common PKC target site within both TPβS328 and TPβ and that it is the site responsible for the GF 109203X-sensitive, partial and transient desensitization of TPβA328 and plays a minor though significant role in the partial PKC induced homologous desensitization of TPβ. A role for PKC in the homologous desensitization of TXA₂ has been reported in the case of the single mouse (m) TP where it was demonstrated that both U46619-induced desensitization and phosphorylation of the mTP is attenuated by pre-incubation with GF 109203X resulting in the inhibition of more than 70% of its agonist-induced phosphorylation [49-51]. Moreover, the PKA inhibitor H-89 had no apparent effect on U46619-induced phosphorylation of the mTP. In another study, consistent with data presented herein involving the human TP isoforms, Habib et al., [15] found no role for PKA and only a minimal role for PKC in the agonist-induced phosphorylation of TPα or TPβ thereby, by deduction, potentially implicating the GRKs in homologous desensitization of one or both TP isoforms. As stated we are currently investigating agonist-induced desensitization of TPα and whether it displays a similar level of homologous desensitization and second-messenger kinase dependent sensitivity awaits further detailed investigation.

Through numerous studies GRKs have been shown to promote agonist-induced desensitization of various GPCRs [1, 2, 4], such as exemplified in the case of the β₂-adrenergoreceptors where GRKs have an important role in modulating myocardial contraction in mice ([4, 52-54]. Furthermore, levels of GRK2 are increased in human heart failure [55] and hypertension [56]. GRKs are a family of novel protein kinases of which there are 7 members including rhodopsin kinase (GRK1), β-adrenergic receptor kinase (βARK) 1 and 2 (GRK2 and GRK3, respectively) and GRK4 – GRK7 [3]. They have the unique ability to recognize and phosphorylate their GPCR substrates only in their active conformations (i.e., agonist-occupied GPCR). GRK involvement in agonist-induced phosphorylation of TP has been suggested by Parent et al.,[30]. They showed that TPβ, but not TPα, underwent agonist-induced internalization and, moreover, transient co-
transfection of a dominant negative GRK$^{2K200R}$ reduced internalization of TP$\beta$. Herein, we initially investigated the role of the ubiquitously expressed GRK2/3 in the homologous desensitization of signalling by TP$\beta$ by examining the effect of over-expressing GRK2/βARK1$^{495-689}$, a dominant negative form that impairs GRK2/3 membrane translocation and activation [36]. While expression of βARK1$^{495-689}$ had no effect on the primary signalling responses by either TP$\beta$ or TP$^{A328}$, it almost fully impaired agonist-induced desensitization of TP$\beta$ signalling such that the level of the secondary [Ca$^{2+}$], or IP$_3$ responses was not significantly different to that of the respective primary responses in the presence of βARK1$^{495-689}$. On the other hand, βARK1$^{495-689}$ expression had no significant effect on the partial, transient desensitization of TP$^{A328}$. Collectively, these data confirmed that GRK2/3 plays a major role in the homologous desensitization of TP$\beta$ signalling and that some or all of the determinants of GRK-mediated desensitization rely on sequences within the C-tail domain of TP$\beta$.

Thereafter, in order to localise the target sites involved in this GRK-mediated desensitization, a series of deletions, namely TP$\beta^{A367}$, TP$\beta^{A359}$ and TP$\beta^{A356}$, were generated and their ability to undergo agonist-induced desensitization of signalling was investigated. Similar to the wild type TP$\beta$, both TP$\beta^{A367}$ and TP$\beta^{A359}$ underwent significant agonist-induced desensitization of signalling confirming that the determinant(s)/target site(s) for agonist-induced desensitization is upstream of residue 359. TP$\beta^{A356}$, on the other hand, yielded efficient IP$_3$ generation and [Ca$^{2+}$], mobilization upon secondary U46619 stimulation to levels that were approximately 80% of those observed generated following primary stimulation with U46619. These data established that a major determinant of homologous desensitization is indeed located between residues 356 and 359 within the C-tail of TP$\beta$.

Unlike the second messenger kinases, such as PKA or PKA, the substrate recognition motif of the GRKs is not widely recognizable. However, the GRKs do show a strong preference for acidic residues (aspartic acid or glutamic acid) immediately adjacent to the targeted Ser/Thr residue, particularly in the amino-terminal position [44]. Analysis of the amino acid sequence between Arg$^{356}$ and Ala$^{359}$ of TP$\beta$ identified this region as a putative GRK site with the sequence DS$^{357}$R [44]. Disruption of this putative GRK site by converting Ser$^{357}$ to Ala$^{357}$ to generate TP$\beta^{S357A}$ initially confirmed that the mutation per se did not affect the ligand binding or basic signalling properties relative to those of the wild type TP$\beta$. However, unlike TP$\beta$ or TP$\beta^{A359}$, TP$\beta^{S357A}$ did not undergo substantial agonist-induced desensitization mediating efficient IP$_3$ generation and [Ca$^{2+}$], mobilization following secondary stimulation with U46619. More specifically, TP$\beta^{S357A}$ signalled to approximately 70-75% of its original U46619-mediated primary signal. Thereafter, whole cell (in vivo) phosphorylation assays were used to establish whether TP$\beta$ & TP$\beta^{S357A}$ may be a direct target for agonist-induced GRK phosphorylation. TP$\beta$ underwent significant U66619-mediated phosphorylation. Moreover, that agonist-induced phosphorylation was substantially reduced by over-expression of the dominant negative βARK1$^{495-689}$ confirming the involvement of GRK2/3 in that phosphorylation. On the other hand, TP$\beta^{S357A}$ underwent modest U46619-induced phosphorylation although not to the same degree as the native TP$\beta$, suggesting that mutation of Ser$^{357}$ to Ala$^{357}$ significantly disrupted
U46619-induced phosphorylation of TPβ^{S357A}. Surprisingly, we also found that agonist-induced phosphorylation of TPβ^{S357A} was further marginally reduced in the presence of the dominant negative βARK1^{495-689}. The low level of U46619-induced phosphorylation of TPβ^{S357A} is indeed consistent with our finding that TPβ may also undergo agonist-induced phosphorylation at other sites, such as at the GF 109203X-sensitive PKC site at Ser^{145} within IC₂ or at other site(s) within TPβ. However, consistent with previous reports [15, 22], we found that PKC had only a minimal involvement in agonist-induced TPβ phosphorylation whereby GF 109203X did not substantially reduce the level of agonist-induced phosphorylation of TPβ or indeed of TPβ^{S357A} or of TP^{S328} (data not shown). These findings led us to hypothesize that TPβ may actually be subject to GRK phosphorylation at sites other than at Ser^{357}.

Analysis of the amino acid sequence of TPβ revealed a second putative GRK phosphorylation site, with the sequence DS^{239}E [44], within the IC₃ of TPβ where Ser^{239} represents the phospho-target residue. Hence, site-directed mutagenesis was used to generate TPβ^{S239A} and TPβ^{S239,357A} destroying the putative GRK phosphorylation site(s) within the IC₃ and C-tail domains, respectively, and it was initially confirmed that, as expected, neither mutation per se affected their ligand binding or basic signalling properties. TPβ^{S239A} only underwent partial agonist-induced desensitization and, following secondary stimulation, maintained approximately 30% of its primary signalling responses (U46619-induced Δ[Ca^{2+}], mobilization and IP₃ generation). However, TPβ^{S239,357A} did not undergo significant agonist-induced desensitization of signalling such that the level of its secondary signalling response (e.g. [Ca^{2+}], mobilization) was approximately 100% of its primary response. Hence, it is evident that U46619-mediated homologous desensitization of TPβ occurs through a GRK-dependent mechanism and involves both Ser^{239} and Ser^{357} within its IC₃ and C-tail domain, respectively.

Phosphorylation of GPCRs by the non-visual GRKs may recruit and stabilize the binding of β-arrestin1/2 to the agonist-occupied phosphorylated receptor, sterically impairing G protein coupling and desensitizing further signalling [1, 3]. Moreover, β-arrestin binding stabilizes the phosphorylation status of the GPCR [1, 3, 45] and may also mediate receptor internalization, such as through clathrin- or caveolin-dependent mechanisms. Hence, herein, we investigated β-arrestin2 binding to TPβ, TPβ^{S239A}, TPβ^{S357A} and TPβ^{S239,357A} and thereafter examined the effect of β-arrestin2 expression on agonist-induced phosphorylation of TPβ and its mutated derivatives. β-arrestin2 was efficiently co-precipitated along with TPβ in U46619-stimulated cells with low levels also detected in the absence of agonist stimulation. However, the level of β-arrestin2 in the TPβ^{S239A} or TPβ^{S357A} co-immunoprecipitates from vehicle- or U46619-treated cells was significantly reduced relative to that of the native TPβ while β-arrestin2 did not co-immunoprecipitate with TPβ^{S239,357A}. Whole cell phosphorylations confirmed that detection of agonist-induced TPβ phosphorylation was somewhat increased in the presence of β-arrestin2 indicating that β-arrestin binding can indeed stabilize the phosphorylation status of TPβ, in keeping with findings with other GPCRs [45]. On the other hand, the overall level of agonist-induced phosphorylation TPβ^{S239A}, TPβ^{S357} and TPβ^{S239,357} phosphorylation was significantly reduced relative to that of the wild type TPβ even in the presence of β-arrestin2 over-
expression. Hence, these data confirm that TPβ undergoes agonist-induced GRK2/3 phosphorylation whereby Ser\textsuperscript{239} and Ser\textsuperscript{359} within the IC\textsubscript{3} and C-tail domains, respectively, represent the phosho-targets. Moreover, impairment of phosphorylation at either Ser\textsuperscript{239} or Ser\textsuperscript{359} independently reduce high affinity β-arrestin2 binding, while mutation of both Ser\textsuperscript{239} and Ser\textsuperscript{359} reduced agonist-induced TPβ phosphorylation to near basal levels and led to substantial reductions in β-arrestin2 binding.

TPβ is widely reported to undergo agonist-induced internalization that occurs through a GRK2/β-arrestin- and Rab11- dependent mechanism [30, 33]. Moreover, Parent et al., [30] established that a cluster of residues between 355 – 366 were required for agonist promoted TPβ internalization by GRK2/β-arrestin while Hamelin et al., [33] established that residues 335 – 343, also within its C-tail domain, are required for Rab11 interaction with TPβ. The identification herein of Ser\textsuperscript{357} as a critical site of agonist-induced GRK phosphorylation, desensitization of signalling and β-arrestin binding is in agreement with previous findings regarding the role of residues 355 – 366 in mediating agonist-induced GRK/β-arrestin internalization of TPβ [30]. However, we have also established that Ser\textsuperscript{239} within IC\textsubscript{3} is also important in agonist-induced desensitization of signalling of TPβ and hence, may also play a role in its agonist-induced internalization. Hence, we investigated agonist-induced internalization of TPβ\textsuperscript{S239A}, TPβ\textsuperscript{S357A} and TPβ\textsuperscript{S239,357A}, comparing it to that of TPβ or, as a control, to TPα. Consistent with previous reports [30], while TPα did not undergo agonist-induced internalization, TPβ was efficiently internalized such that there was a 40% decrease in its surface expression following 4 h incubation. Significantly, U46619-induced internalization of TPβ\textsuperscript{S239A} and TPβ\textsuperscript{S357A} were each substantially impaired relative to TPβ while TPβ\textsuperscript{S239,357A} failed to undergo significant agonist-induced internalization.

Taken together, data presented herein establish that TPβ is subject to significant agonist-induced homologous desensitization of signalling that occurs through two key mechanisms involving receptor phosphorylation (Figure 12). TPβ undergoes partial and transient U46619-induced desensitization that occurs through a GF 109203X-sensitive, PKC mechanism whereby Ser\textsuperscript{145} within IC\textsubscript{2} has been identified as the key phosho-target. In addition, TPβ also undergoes more profound and sustained agonist-induced desensitization involving GRK2/3-phosphorylation of both Ser\textsuperscript{239} and Ser\textsuperscript{357} within its IC\textsubscript{3} and C-tail domains, respectively. Inhibition of phosphorylation of either Ser\textsuperscript{239} or Ser\textsuperscript{357}, such as through site directed mutagenesis, impairs agonist-induced desensitization of TPβ while mutation of both Ser\textsuperscript{239} and Ser\textsuperscript{357} almost completely abolishes desensitization of signalling, GRK phosphorylation, β-arrestin association, and blocks agonist-induced TPβ internalization. This proposed mechanism of agonist-induced homologous desensitization of TPβ herein bears close resemblance to a previously presented model involving agonist-induced GRK phosphorylation, β-arrestin association and desensitization of signalling of the D\textsubscript{1} dopamine receptor [57]. Key features of their model are that under basal conditions, they propose that the IC\textsubscript{3} and C-tail domains are in close association. Agonist occupancy results in an altered conformation allowing G-protein interaction but also renders the D\textsubscript{1} receptor a substrate for GRK-phosphorylation within both its IC\textsubscript{3} and C-tail domains similar to TPβ. In their case, they propose that GRK phosphorylation of the D\textsubscript{1} receptor...
takes place in a hierarchical fashion, occurring first within the C-tail domain and then within IC$_3$ and they do not rule out the possibility of additional phosphorylations within IC$_1$ and/or IC$_2$ [57]. While our data does not necessarily confirm or deny such sequential or hierarchical phosphorylation of Ser$^{357}$ versus Ser$^{239}$ within the C-tail and IC$_3$ domains, respectively, we have indeed found evidence of the role of Ser$^{145}$ within the IC$_2$ in agonist-induced signalling and desensitization of TP$\beta$. Moreover, given that TP$^{328}$ exhibits similar Gq coupling to that of TP$\beta$, and indeed of TP$\alpha$ [17], but simply fails to undergo extensive GRK-mediated desensitization as demonstrated herein, it is likely that the role of GRK2/3 phosphorylation of Ser$^{357}$ within the C-tail domain is to facilitate the recruitment and orientation of GRK2/3 to facilitate phosphorylation of Ser$^{239}$ within IC$_3$ thereby disrupting Gq coupling by TP$\beta$. Hence, GRK2/3 binding is dependent on sequences within the unique C-tail domain of TP$\beta$ whilst Gq uncoupling is due to GRK phosphorylation of Ser$^{239}$ within IC$_3$ and, to a lesser extent, PKC phosphorylation of Ser$^{145}$ within IC$_2$ (Figure 13).

In conclusion, the existence of two TP isoforms within humans and their extensive expression throughout cells and tissues of the vasculature leads to and requires complicated regulation of their functions in mediating the actions of the potent autocoid TXA$_2$. Characterization of the molecular events that underlie TP desensitization in platelets and other cell types is of great importance in our understanding of the mechanisms involved in vascular haemostasis and in elucidating the physiological relevance of two TP isoforms, such as in clinical syndromes of thrombosis and vascular occlusion, myocardial infarction and stroke. Ultimately, such understandings may afford therapeutic opportunities to differentially or selectively modulate the responses to TXA$_2$ by targeting the individual TP$\alpha$ and/or TP$\beta$ isoforms in vivo. While data presented herein deals exclusively with that of TP$\beta$, studies are underway to ascertain the mechanisms of agonist-induced desensitization of signalling by the TP$\alpha$ isoform and given that TP$\alpha$ does not undergo significant agonist-induced GRK/\beta-arrestin-dependent internalization, such mechanisms are likely to be substantially different to those of TP$\beta$.

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REFERENCES:


FIGURES:

Figure 1.

Figure 1: Carboxyl terminal (C)-tail domain of TPβ.

The amino acid sequence of the carboxyl terminal (C)-tail domain of TPβ (residues 321-407) are shown, where residues unique to TPβ (residues 329-407) are underlined. Codons converted to Stop codons to generate the truncation (Δ) mutants following amino acids R328 (TPβΔ328), D356 (TPβΔ356), A359 (TPβΔ359) or I367 (TPβΔ367) are indicated by the open arrow heads while that residue mutated to generate TPβS357A (S357A) is indicated by the solid arrow.

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PRLSTRPRSLTLWPSLEYSGTISAHCNLRLPGSSD
321 330 340 350 360 370 380 390 400
Δ328 Δ356 Δ359 Δ367
S357A
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Figure 2: U46619-mediated desensitization of signalling by TPβ.

HEK.TPβ cells, co-transfected with pCMV:Gαq and pre-loaded with FURA2/AM, were stimulated with 1 μM U46619 for 4 min (Panel A); thereafter, cells were washed to remove the U46619 (indicated by the horizontal arrows) and then re-stimulated with 1 μM U46619 either 15 min (Panel B) or 60 min (Panel C) following the primary stimulation, where ligands were added at the times indicated by the vertical arrows. Data presented are representative profiles from at least three independent experiments and are plotted as changes in intracellular Ca²⁺ mobilization (Δ[Ca²⁺]ᵢ, nM) as a function of time (second, s). Actual mean changes in U46619-induced [Ca²⁺]ᵢ mobilization (nM ± S.E.M.) were: Panel A: Δ[Ca²⁺]ᵢ = 155 ± 9.6 nM; Panel B: Δ[Ca²⁺]ᵢ = 0 nM; Panel C: Δ[Ca²⁺]ᵢ = 0 nM. Panel D: HEK.TPβ cells, co-transfected with pCMV:Gαq, were pre-stimulated with 1 μM U46619 for 2 min (U46619); cells were washed and re-stimulated 60 min following the primary stimulation with 1 μM U46619 for 2 min (U4, U4). As controls, HEK 293 cells were stimulated with 1 μM U46619 for 2 min (HEK 293). In each case, basal levels of IP₃ were determined by exposing cells to the vehicle HBS under identical conditions. Levels of IP₃ produced in ligand-stimulated cells relative to the vehicle-treated cells (basal IP₃) were expressed as fold stimulation of basal (fold increase in IP₃ ± S.E.M; n = 3). The asterisks indicate that U46619-mediated IP₃ generation by HEK.TPβ cells was significantly reduced following secondary stimulation compared to the primary stimulation and that U46619-mediated IP₃ generation was significantly lower in HEK 293 cells than in HEK.TPβ cells where *, ** and *** indicates p < 0.05, p < 0.01 and p < 0.001, respectively. Panel E: HEK.TPβ cells co-transfected with the control vector pCMV5 (lane 1) or with pCMV:Gαq (lane 2) were analysed by SDS PAGE (75 μg whole cell protein analysed/lane) followed by western blot analysis using anti-Gαq antibody (Gαq/11 (C-19): S.c 392). Data presented is a representative immunoblot from four independent experiments. The relative position of the 46 kDa molecular size marker is indicated to the right of Panel E.
Figure 3

**Figure 3**: U46619-mediated desensitization of signalling by TP$^{A328}$.

HEK.TP$^{A328}$ cells, co-transfected with pCMV:G$\alpha_{q}$, were stimulated with 1 $\mu$M U46619 for 4 min (Panel A); thereafter, cells were washed and re-stimulated with 1 $\mu$M U46619 at 15 min (Panel B) or 60 min (Panel C) following the primary stimulation. Alternatively, cells were pre-incubated for 10 min with 10 $\mu$M H-89 (Panel E) or 50 nM GF 109203X (Panel F) prior to stimulation with 1 $\mu$M U46619 for 4 min (Primary stimulations); cells were then washed and re-stimulated at 15 min following the primary stimulation with 1 $\mu$M U46619 in the presence of 10 $\mu$M H-89 or 50 nM GF 109203X (Panels E & F, respectively; Secondary stimulations). Vertical arrows in the panels indicate the time of addition of the U46619. Actual mean changes in U46619-induced $[Ca^{2+}]_i$ mobilization (nM ± S.E.M.; n = 3) were: Panel A: $\Delta[Ca^{2+}]_i = 160 \pm 1.5$ nM; Panel B: $\Delta[Ca^{2+}]_i = 130 \pm 7.7$ nM; Panel C: $\Delta[Ca^{2+}]_i = 156 \pm 2.9$ nM; Panel E: Primary signal, $\Delta[Ca^{2+}]_i = 169 \pm 16.2$ nM; Secondary signal, 155 ± 4.3; Panel F: Primary signal, $\Delta[Ca^{2+}]_i = 190 \pm 7.5$ nM; Secondary signal, 200 ± 16 nM.

Panel D: HEK.TP$^{A328}$ cells, co-transfected with pCMV:G$\alpha_{q}$, were stimulated with 1 $\mu$M U46619 for 2 min (U46619); cells were washed and re-stimulated 60 min following the primary stimulation with 1 $\mu$M U46619 for 2 min (U4, U4). As controls, HEK 293 cells were stimulated with 1 $\mu$M U46619 for 2 min (HEK 293). Levels of IP$_3$ produced in ligand-stimulated cells relative to the vehicle (HBS)-treated cells (basal IP$_3$) were expressed as fold stimulation of basal (fold increase in IP$_3$ ± S.E.M; n = 3). The asterisks indicate that U46619-mediated IP$_3$ generation by TP$^{A328}$ cells was not significantly reduced following secondary stimulation compared to the primary stimulation and that U46619-mediated IP$_3$ generation was significantly lower in HEK 293 cells than in HEK.TP$^{A328}$ cells where *, ** and *** indicates $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.
Figure 4: Effect of H-89 and GF 109203X on U46619-mediated desensitization of TPβ signalling.

HEK.TPβ cells, co-transfected with pCMV:Gαq, were pre-incubated for 10 min with either vehicle (0.001% DMSO; Panel A), 10 μM H-89 (Panel C) or 50 nM GF 109203X (Panel E) prior to stimulation with 1 μM U46619 for 4 min. Cells were then washed and re-stimulated at 15 min following the primary stimulation with 1 μM U46619 in the presence of vehicle (Panel B), 10 μM H-89 (Panel D) or 50 nM GF 109203X (Panel F). Actual mean changes in U46619-induced [Ca2+]i mobilization (nM ± S.E.M.; n = 3) were: Panel A: Δ[Ca2+]i = 177 ± 9.2 nM; Panel B: Δ[Ca2+]i = 0 nM; Panel C: Δ[Ca2+]i = 240 ± 12 nM; Panel D: Δ[Ca2+]i = 0 nM; Panel E: Δ[Ca2+]i = 199 ± 7.9 nM; Panel F: Δ[Ca2+]i = 45 ± 4.4 nM.
Figure 5. U46619-mediated desensitization of signalling by TP^{S145A, Δ328} and TPβ^{S145A}.

HEK.TP^{Δ328} (Panel A), HEK.TP^{S145A, Δ328} (Panel B), HEK.TPβ (Panel C) and HEK.TPβ^{S145A} (Panel D) cells, each transiently co-transfected with pCMV:Gαq, were stimulated for 4 min with 1 μM U46619 (Primary stimulation; Panels A – D); thereafter, cells were washed and re-stimulated at 15 min following the primary stimulation with 1 μM U46619 (Secondary stimulation; Panels A – D) where ligands were added at the times indicated by the arrows. Actual mean changes in U46619-induced primary and secondary [Ca^{2+}]; mobilizations (nM ± S.E.M.; n = 3) were: Panel A: Primary signal, Δ[Ca^{2+}]; = 166 ± 3.7 nM; Secondary signal; Δ[Ca^{2+}]; = 144 ± 2.9; Panel B: Primary signal, Δ[Ca^{2+}]; = 174 ± 4.7 nM; Secondary signal, Δ[Ca^{2+}]; = 170 ± 3.9 nM; Panel C: Primary signal, Δ[Ca^{2+}]; = 177 ± 4.2 nM, Secondary signal, Δ[Ca^{2+}]; = 0 nM; Panel D: Primary signal, Δ[Ca^{2+}]; = 175 ± 3.6 nM; Secondary signal, Δ[Ca^{2+}]; = 40 ± 1.8 nM.
Figure 6: Effect of over-expression of βARK1\(^{495-689}\) on U46619-mediated desensitization of signalling by TP\(\beta\) and TP\(^{\Delta328}\).

HEK.TP\(\beta\) (Panels A & B) or HEK.TP\(^{\Delta328}\) (Panels C & D) cells, each transiently co-transfected with pCMV:Go\(_{q}\) and pRK5:βARK1\(^{495-689}\), were stimulated with 1 µM U46619 for 4 min (Panels A & C). Thereafter, cells were washed and re-stimulated at 15 min following the primary stimulation with 1 µM U46619 (Panels B & D), where ligands were added at the times indicated by the vertical arrows. Actual mean changes in U46619-induced [Ca\(^{2+}\)]\(_{i}\) mobilization (nM ± S.E.M.; n = 3) were: Panel A: \(\Delta[Ca^{2+}]_i = 199 \pm 5.5\) nM; Panel B: \(\Delta[Ca^{2+}]_i = 190 \pm 9\) nM; Panel C: \(\Delta[Ca^{2+}]_i = 198 \pm 7.8\) nM; Panel D: \(\Delta[Ca^{2+}]_i = 177 \pm 15\) nM. Panel E: HEK.TP\(\beta\) transiently co-transfected with pRK5:βARK1\(^{495-689}\) (Lane 1) or the vector pRK5 (Lane 2) were analysed by SDS PAGE (75 µg whole cell protein analysed/lane) followed by western blot analysis using \textit{anti-} GRK2/βARK1 (GRK 2(C-15): Sc562) antibody (1:2000). Data are representative immunoblots from at least four independent experiments. The relative position of the 33 kDa molecular size marker is indicated to the left of Panel E.
Figure 7: U46619-mediated desensitization of signalling TPβΔS67, TPβΔS59, TPβΔS56 and TPβS357A.

HEK.TPβΔS67 (Panels A & B), HEK.TPβΔS59 (Panels D & E), HEK.TPβΔS56 (Panels G & F) and HEK.TPβS357A (Panels J & K) cells, each transiently co-transfected with pCMV:Gαq, were stimulated for 4 min with 1 μM U46619 (Panels A, D, G & J). Thereafter, cells were washed and re-stimulated at 15 min following the primary stimulation with 1 μM U46619 (Panels B, E, H & K), where ligands were added at the times indicated by the vertical arrows. Actual mean changes in U46619-induced [Ca^{2+}]; mobilization (nM ± S.E.M.) were: Panel A: Δ[Ca^{2+}]i = 157 ± 4.1 nM; Panel B: Δ[Ca^{2+}]i = 0 nM; Panel D: Δ[Ca^{2+}]i = 181 ± 16 nM; Panel E: Δ[Ca^{2+}]i = 0 nM; Panel G: Δ[Ca^{2+}]i = 162 ± 10 nM; Panel H: Δ[Ca^{2+}]i = 130 ± 13 nM; Panel J: Δ[Ca^{2+}]i = 160 ± 10 nM; Panel K: Δ[Ca^{2+}]i = 120 ± 13 nM.

Alternatively, HEK.TPβΔS67 (Panel C), HEK.TPβΔS59 (Panel F), HEK.TPβΔS56 (Panel I) cells and HEK.TPβS357A (Panel L) cells, co-transfected with pCMV:Gαq, were stimulated with 1 μM U46619 for 2
min (U46619); cells were then washed and re-stimulated at 60 min following the primary stimulation with 1 μM U46619 for 2 min (U4, U4; Panels C, F, I & L). As controls, HEK 293 cells were stimulated for 2 min with 1 μM U46619 (Panels C, F, I & L; HEK 293). Levels of IP₃ produced in ligand-stimulated cells relative to the vehicle (HBS) treated cells (basal IP₃) were expressed as fold stimulation of basal (fold increase in IP₃ ± S.E.M; n = 4). The asterisks indicate that the level of U46619-mediated IP₃ generation in HEK:TPβΔ367 (Panel C), HEK:TPβΔ359 (Panel F) and HEK:TPβΔ356 (Panel I) cells was significantly reduced following secondary stimulation compared to their respective primary stimulations. Furthermore, the level of U46619-mediated IP₃ generation was significantly lower in HEK 293 cells than in each of the above cell lines where *, ** and *** indicates p < 0.05, p < 0.01 and p < 0.001, respectively.
Figure 8: U46619-mediated desensitization of signalling by TPβS239A and TPβS239,357A.

HEK.TPβS239A (Panels A & B) and HEK.TPβS239,357A (Panels D & E) cells, co-transfected with pCMV:Gαq, were stimulated with 1 μM U46619 for 4 min (Panels A & D); thereafter, cells were washed and re-stimulated at 15 min following the primary stimulation with 1 μM U46619 (Panels B & E), where ligands were added at the times indicated by the vertical arrows. Actual mean changes in U46619-induced [Ca²⁺]i mobilization (nM ± S.E.M.) were as follows: Panel A: Δ[Ca²⁺]i = 197 ± 27 nM; Panel B: Δ[Ca²⁺]i = 54 ± 6.1 nM; Panel D: Δ[Ca²⁺]i = 174 ± 7 nM; Panel E: Δ[Ca²⁺]i = 173 ± 6.5 nM. Alternatively, HEK.TPβS239A (Panel C) and HEK.TPβS239,357A (Panel F) cells, co-transfected with pCMV:Gαq, were stimulated with 1 μM U46619 for 2 min (U46619); cells were then washed and re-stimulated at 15 min following the primary stimulation with 1 μM U46619 for 2 min (U4, U4). As controls, HEK 293 cells were stimulated with 1 μM U46619 for 2 min (HEK 293). Levels of IP₃ produced in ligand-stimulated cells relative to the vehicle (HBS) treated cells (basal IP₃) were expressed as fold stimulation of basal (fold increase in IP₃ ± S.E.M; n = 4). The asterisks indicate that the level of U46619-mediated IP₃ generation in HEK:TPβS239A cells was significantly reduced following secondary stimulation compared to that of the respective primary stimulation (Panel C) whilst the level of U46619-mediated IP₃ generation in HEK:TPβS239,357A cells shows no significant difference following secondary stimulation compared to that of the respective primary stimulation (Panel F). Furthermore, the level of U46619-mediated IP₃ generation was significantly lower in HEK 293 cells than in each of the latter cell lines (Panels C & F). *, ** and **** indicates p < 0.05; p < 0.01 or p < 0.001, respectively.
Figure 9: U46619-mediated phosphorylation of TPβ and TPβS357A.

HEK.TPβ (Panel A) and HEK.TPβS357A cells (Panel B), each transiently co-transfected with pCMV:Gαq along with either pRK5:βARK1495-689 (Lane 3) or the empty vector pRK5 (Lanes 1 & 2) and metabolically labelled with [32P]orthophosphate, were pre-incubated for 10 min with vehicle HBS (Lane 1) or were stimulated for 10 min with 1 μM U46619 (Lanes 2 & 3). Thereafter, HA epitope-tagged TPβ receptors were immunoprecipitated using the anti-HA antibody 101R. Immunoblots were subject to phosphorImage analysis and the intensities of U46619-mediated TPβ phosphorylation relative to basal phosphorylation in the presence of HBS were determined and expressed in arbitrary units as follows: TPβ: 1 μM U46619, 5-fold increase; 1 μM U46619 plus βARK495-689, 2.0-fold increase; TPβS357A: 1 μM U46619, 1.8-fold increase; 1 μM U46619 plus βARK495-689, 1.6-fold increase. Panel C, HEK.TPβ (Lane 1), HEK.TPβS357A (Lane 2) or control HEK 293 (Lane 3) cells were subject to immunoprecipitation using the anti-HA antibody 101R, immunoprecipitates were screened using the anti-HA 3F10 horseradish peroxidase-conjugated antibody and visualised by chemiluminescence detection. The positions of the molecular weight markers (kDa) are indicated to the left and right of the panels. The arrow to the left of the Panel A indicates the position of the phosphorylated TPβ. Data presented are representative of three independent experiments.

Panel D: HEK.TPβ, HEK.TPβS239A, HEK.TPβS357A and HEK.TPβS239,S357A cells, each transiently co-transfected with pCMV:Gαq and pcDNA3:β-arrestin2 and metabolically labelled with [32P]orthophosphate, were incubated for 10 min with 1 μM U46619 (+) or an equivalent volume of the vehicle HBS (-). Thereafter, HA epitope-tagged TPβ receptors were immunoprecipitated, blots were subject to phosphorImage analysis, and the intensities of U46619-mediated TPβ phosphorylation relative to basal phosphorylation in the presence of HBS were determined and expressed in arbitrary units (Phosphorylation; fold increase; n = 3).
Figure 10: Co-immunoprecipitation of β-arrestin 2 with TPβ, TPβ<sub>S239A</sub>, TPβ<sub>S357A</sub> and TPβ<sub>S239,S357A</sub>.

Panel A: HEK.TPβ (Lanes 1 & 2), HEK.TPβ<sub>S239A</sub> (Lanes 3 & 4), HEK.TPβ<sub>S357A</sub> (Lanes 5 & 6) and HEK.TPβ<sub>S239,S357A</sub> (Lanes 7 & 8) cells, each transiently co-transfected with pCMV:Gαq and pcDNA3:β-arrestin2, were incubated for 10 min with 1 μM U46619 (+) or an equivalent volume of the vehicle (-). Thereafter, proteins were crosslinked with DSP, HA epitope-tagged TPβ receptors were immunoprecipitated and immunoblots were screened with anti-β-arrestin2 antibody. Panel B: HEK.TPβ transiently co-transfected with pcDNA3:β-arrestin2 (Lane 1) or the vector pcDNA3 (Lane 2) were analysed by SDS PAGE (75 μg whole cell protein analysed/lane) followed by western blot analysis using anti-β-arrestin2 antibody. Data are representative of four independent experiments. The relative position of the 46 kDa molecular size marker is indicated to the right of Panel B.
Figure 11: U46619-mediated internalisation of TP$\alpha$, TP$\beta$, TP$\beta^{S239A}$, TP$\beta^{S357A}$ and TP$\beta^{S239,S357A}$.

Panel A: HEK.TP$\beta$ (TP$\beta$) and HEK.TP$\alpha$ (TP$\alpha$) cells or Panel B: HEK.TP$\beta$ (TP$\beta$), HEK.TP$\beta^{S239A}$ (TP$\beta^{S239A}$), HEK.TP$\beta^{S357A}$ (TP$\beta^{S357A}$) and HEK.TP$\beta^{S239,357A}$ (TP$\beta^{S239,357A}$) cells were stimulated with U46619 (1 $\mu$M) for 0 – 4 hr. Agonist-induced internalisation of HA-tagged TP receptors was quantified by ELISA. Results are presented as the percentage (%) cell surface receptor expression in U46619-stimulated cells relative to vehicle (0.01% ethanol in HBS)-treated cells and are mean data of three independent experiments each carried out in triplicate (mean ± SEM, n = 3), where vehicle-treated control cells were assigned a value of 100%.
Figure 12: Hypothetical scheme for agonist-induced TPβ phosphorylation, desensitization of signalling, β-arrestin binding and internalization.

Agonist binding and activation of TPβ and TPβ328 leads to GF 109203X-sensitive PKC phosphorylation of Ser145 within intracellular loop (IC)2 leading to a partial (20%) and transient desensitization of PLCβ coupling and second messenger generation (IP3 and [Ca2+]i mobilization), such as is evident experimentally in response to secondary agonist stimulation. The broken line indicates the transient nature of U46619-induced TPβ and TPβ328 desensitization due to PKC-phosphorylation at Ser145. In addition, agonist activation of TPβ, but not TPβ328, leads to GRK2/3 recruitment & activation and phosphorylation of both Ser239 and Ser357 within its IC3 and C-tail domains. This, in turn, leads to: (a) almost complete and prolonged desensitization of PLCβ coupling and signalling, (b) β-arrestin binding and/or stabilization of TPβ phosphorylation at Ser239 and Ser357 and (c) promotion of agonist-induced TPβ internalization. TPβS239A and TPβS357A undergo reduced agonist-induced desensitization of signalling, phosphorylation, β-arrestin binding and internalization, albeit at different levels of sensitivity, while that of TPβS239,357A is almost abolished.