



Title	Prostaglandin D2 receptor-mediated desensitization of the alpha isoform of the human thromboxane A2 receptor
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Publication date	2001-07-15
Publication information	Foley, John F., Leanne P. Kelley-Hickie, and B. Therese Kinsella. "Prostaglandin D2 Receptor-Mediated Desensitization of the Alpha Isoform of the Human Thromboxane A2 Receptor." Elsevier, July 15, 2001. https://doi.org/10.1016/S0006-2952(01)00661-X .
Publisher	Elsevier
Item record/more information	http://hdl.handle.net/10197/3164
Publisher's statement	This is the author's version of a work that was accepted for publication in Biochemical Pharmacology. Changes resulting from the publishing process, such as peer review, editing, corrections, structural formatting, and other quality control mechanisms may not be reflected in this document. Changes may have been made to this work since it was submitted for publication. A definitive version was subsequently published in Biochemical Pharmacology Volume 62, Issue 2, 15 July 2001, Pages 229-239 DOI 10.1016/S0006-2952(01)00661-X.
Publisher's version (DOI)	10.1016/S0006-2952(01)00661-X

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Prostaglandin D₂ receptor mediated desensitization of the α isoform of the human thromboxane A₂ receptor

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Classification: Molecular and Cellular Pharmacology

Abbreviations: cAMP, cyclic adenosine 5' mono phosphate; $[Ca^{2+}]_i$, intracellular calcium; DP, PGD₂ receptor; HA, hemagglutinin; HEK, human embryonic kidney; HEL, human erythroleukaemia; HBS, Hepes buffered saline; IP, prostacyclin receptor; IP₃, inositol 1,4,5 trisphosphate; PG, prostaglandin; PKA, protein kinase A; PKC, protein kinase C; RT-PCR, reverse transcriptase polymerase chain reaction; TXA₂, thromboxane A₂; TP, TXA₂ receptor.

¹ This research was supported by grants to B.T.K from The Wellcome Trust, The Irish Heart Foundation, The Health Research Board of Ireland and Enterprise Ireland.

Abstract

Thromboxane (TX) A₂ and prostaglandin (PG) D₂ mediate opposing actions in platelets and in vascular and non-vascular smooth muscle. Here, we investigated the effects of stimulation of the PGD₂ receptor (DP) on signaling by the TXA₂ receptor (TP) expressed in human platelets and in human embryonic kidney (HEK) 293 cells over-expressing the individual TP α and TP β isoforms. In platelets, the selective DP agonist BW245C abolished TP-mediated mobilization of intracellular calcium ([Ca²⁺]_i) and inhibited platelet aggregation in response to the TXA₂ mimetic U46619. DP-mediated desensitization of TP signaling in platelets was prevented by pre-treatment with the cAMP-dependent PKA inhibitor, H-89, but was unaffected by the PKC inhibitor GF 109203X. In HEK 293 cells signaling by TP α , but not TP β , was subject to DP mediated desensitization in a PKA dependent, PKC independent manner. U46619-induced signaling by TP Δ^{328} , a truncated variant of TP containing only those residues common to TP α and TP β , was insensitive to prior DP stimulation indicating that the carboxyl terminal tail of TP α contains the target site(s) for DP-mediated desensitization. Mutation of Ser³²⁹ to Ala³²⁹ within a consensus PKA site in TP α rendered the mutant TP α^{S329A} insensitive to BW245C-mediated desensitization. Whole cell phosphorylation assays established that TP α , but not TP β or TP α^{S329A} , was subject to DP-mediated phosphorylation and that TP α phosphorylation was blocked by the PKA inhibitor H-89. These data establish that TP α , but not TP β , is subject to DP mediated cross desensitization, which occurs through direct PKA mediated phosphorylation of TP α at Ser³²⁹.

Keywords: Thromboxane A₂ receptor, prostaglandin D₂ receptor, desensitisation, protein kinase A, phosphorylation, G protein coupled receptor.

1. Introduction

Thromboxane (TX) A₂ is the major product of arachidonic acid metabolism in platelets and in activated macrophages and together with prostacyclin (prostaglandin I₂) is thought to play a key role in vascular hemostasis [1-3]. Perturbations in the levels of TXA₂, or its synthase or its receptor, have been implicated in various cardiovascular disorders [4,5]. Depending on the cell type, TXA₂ can induce different cellular responses including platelet shape change and aggregation [6,7]; constriction of vascular and bronchial smooth muscle cells [8]; potentiation of mitogenic and hypertrophic growth of vascular smooth muscle cells [8-10]; stimulation of prostacyclin release by vascular endothelial cells [11]; apoptosis of immature thymocytes [12]; contraction of glomerular mesangial cells and intrarenal vascular tissue, decreasing glomerular filtration rates [13].

These effects are transduced through activation of its specific TXA₂ receptor, also termed TP, a member of the G protein coupled receptor (GPCR) superfamily. In humans, there are two TP isoforms termed TP α and TP β [14,15], which arise due to alternative splicing, and are identical for the first 328 amino acid residues but differ in their carboxyl terminal tail (C-tail) regions. Consistent with the diverse role of TXA₂, wide cell and tissue distribution and possible differential expression of the mRNAs encoding the human TPs was reported [16]. The main signaling pathway of TP is activation of phospholipase C through members of the G_q family of G proteins resulting in increased intracellular concentrations of diacylglycerol and IP₃ and mobilization of intracellular calcium ([Ca²⁺]_i) [17,18]. Functional coupling of TP α to G_q and G₁₁ has been demonstrated *in vivo* in response to the TXA₂ mimetic U46619 (9,11-dideoxy-9. α .,11. α .-methanoepoxy Prostaglandin F₂. α) and the F₂-isoprostane, 8-epi-PGF_{2 α} [19]. Whereas the functional significance for 2 receptors for TXA₂ in humans, but not in other species, is not fully understood, there is increasing evidence that the TP isoforms may mediate differential signaling within cells [20-24]. Whereas recent evidence indicates that both TP isoforms may couple to members of the G₁₂ family [20-22], they may oppositely regulate adenylyl cyclase activity [23] and TP α , but not TP β , has been proposed to couple to Gh [24].

Prostaglandin (PG) D₂, like prostacyclin, is a potent inhibitor of platelet aggregation [25,26]. Two distinct PGD synthases catalyse the isomerisation of PGH₂ to PGD₂; one is the lipocalin type that was previously known as the brain-type enzyme or glutathione independent type and the other is the hematopoietic type or glutathione dependent type [27]. PGD₂ can be further converted to 9 α , 11 β -PGF₂ or the J series of prostanoids, such as PGJ₂, Δ 12-PGJ₂ and 15-deoxy- Δ 12,14 PGJ₂, which act as endogenous ligands for the peroxisome proliferator-activated receptor (PPAR) γ family of nuclear receptors [28,29]. Peripherally, PGD₂ causes vasorelaxation, inhibition of platelet aggregation, glycogenolysis [30,31] and, as a major prostanoid produced in mast cells, may also function in immune challenge [32]. PGD₂ is also produced at high levels in the central nervous system [33]. Here it exerts a number of effects including sleep induction, modulation of body temperature, olfactory function, hormone release, nociception and neuromodulation [31]. More

recently, it has been discovered that the cyclopentenone PGs, including PGD₂, can also induce anti-inflammatory properties [34] mediated through direct inhibition of IκB kinase [35,36] rather than through the presumed PPARγ signaling pathway.

The main intercellular signaling cascade of DP is its activation of adenylyl cyclase, via Gα_s, leading to increases in cAMP. The human DP, when stably expressed in HEK 293(EBNA) cells gave a transient rise in [Ca²⁺]_i in response to the DP agonist (4S)-(3-[(3R,S)-3-cyclohexyl-3-hydroxypropyl]-2,5-dioxo)-4-Imidazolidineheptanoic acid (BW245C), but without an accompanying rise in intracellular IP₃ levels, indicating a lack of coupling of DP to phospholipase Cβ [37]. The order of affinities of the human DP showed very similar ligand binding affinities for PGD₂, BW245C and BW868C [37].

We have recently established that TPα, but not TPβ, is subject to prostacyclin receptor (IP) induced cross talk or heterologous desensitization in a PKA-dependent, PKC-independent manner mediated through direct phosphorylation of TPα at Ser³²⁹ [38]. The suggestion from this study was that TPα, but not TPβ, may be the TP isoform physiologically relevant to TP:IP mediated vascular hemostasis. IP and DP, both members of the relaxant group of prostanoid receptors, share signal transduction pathways involving agonist-induced activation of adenylyl cyclase, a pathway thought to be relevant to their inhibitory actions in platelets [2]. In view of the findings of IP-mediated differential desensitization of the TP isoforms, we sought to extend these studies to investigate potential cross-talk between DP and the individual TPα and TPβ isoforms, stably expressed in HEK 293 cells, comparing it to that which occurs in platelets. Our results established that signaling by TPα, but not TPβ, was subject to BW245C mediated desensitization in a PKA dependent, PKC independent manner, through direct phosphorylation of TPα by PKA at Ser³²⁹ within its unique C-tail. Thus, taken in the context of previous studies involving IP mediated regulation of TP signaling, these studies further support the notion that TPα, but not TPβ, may be the TP isoform relevant to prostanoid regulation of vascular hemostasis.

2. Materials and methods

2.1. Materials.

BW245C and U46619 were obtained from Cayman Chemical Company. 1[2-(5-Carboxyazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2'-amino-5-methylphenoxy)-ethane-N,N,N',N'-tetraacetic Acid, Pentaacetoxymethyl Ester (FURA2/AM), D-myo-inositol 1,4,5-trisphosphate, 3-deoxyhexasodium salt was from Calbiochem. [32 P]orthophosphate (8,000-9,000 Ci/mmol) was from DuPont NEN. [3 H]IP₃ (20-40 Ci/mmol) and [3 H]cAMP (15-30 Ci/mmol) were obtained from American Radiolabelled Chemicals Inc. Monoclonal antibody HA.11 (MMS-101R), clone 16B12 was obtained from BAbCO. Anti-HA-Peroxidase, High Affinity (3F10), clone BMG-rat immunoglobulin G (IgG), was obtained from Roche.

2.2. Cell culture and transfections

The plasmids pCMV:G α_{i1} and pCMV:G α_q have been previously described (19). Stable HEK 293 cell lines over-expressing TP α (HEK. α 10 cells), TP β (HEK. β 3 cells), TP Δ^{328} (HEK.TP Δ^{328} cells) and hemagglutinin (HA) epitope tagged forms of HA:TP α (HEK.HATP α cells), HA:TP β (HEK.HATP β cells), HA:TP α^{S329A} (HEK.HATP α^{S329A} cells) have been previously described [22,38]. Cells were transiently transfected as previously described [19] and were harvested 48 hr post transfection.

2.3. Preparation of platelets.

Platelets were prepared from normal human volunteers, as previously described [19]. For aggregation studies, platelets in platelet rich plasma were diluted to approximately 10^8 platelets/ml in platelet poor plasma; 0.5-ml aliquots were preincubated at 37 °C for 2 min before addition of the aggregating agent (1 μ M U46619, 1 μ M BW245C) or vehicle, and aggregations were monitored in a Biodata Pap 4 aggregometer.

2.4. RT-PCR.

Total RNA isolation and RT-PCR was performed as previously described (16), using the human DP primers Primer A: 5' TCCTCGCCACCGTGCTG 3' (sense primer); and Primer B: 5' CTCTGAATTCACA *GACTGGATTCCATGT* 3' (antisense primer; where sequences complementary to DP mRNA are in italics) which span across Intron 2 of the human DP gene [37].

2.5. Measurement of intracellular calcium ([Ca²⁺]_i) mobilization

Measurements of [Ca²⁺]_i in FURA2/AM preloaded cells and platelets were carried out as previously described [19]. Cells were stimulated with 1 μ M U46619 or 1 μ M BW245C unless otherwise specified, or for dose response studies, with 10^{-12} – 10^{-6} M BW245C. The kinase inhibitors

{N-[2-((*p*-Bromocinnamyl)amini)ethyl]-5-isoquinolinesulfonamide, 2HCL} (H-89, 10 μ M) or 2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide] (GF 109203X; 50 nM) were added 1 to 2 min prior to the addition of ligand. Drugs and inhibitors (in stock solutions containing ethanol or DMSO) were diluted in HBSSHB [38] at the appropriate concentration such that addition of 20 μ l of the diluted drug/inhibitor to 2 ml of cells resulted in the correct working concentration. Results are representative data from at least three independent experiments and are plotted as changes in $[Ca^{2+}]_i$ mobilized ($\Delta[Ca^{2+}]_i$ (nM)) as a function of time (s) upon ligand stimulation. Changes in $[Ca^{2+}]_i$ mobilization were determined by measuring peak rises in intracellular $[Ca^{2+}]_i$ mobilized ($\Delta[Ca^{2+}]_i$) and are presented as mean changes in $\Delta[Ca^{2+}]_i \pm$ S.E.M (nM).

2.6. Measurement of IP₃ levels.

Intracellular IP₃ levels were measured as described previously [38,39]. Briefly, cells were harvested, washed twice in ice-cold PBS and were then resuspended at approximately 5×10^6 cells/ml in HBS [38] containing 10 mM LiCl. Cells (200 μ l) were then pre-incubated at 37 °C for 10 min. Where appropriate, the kinase inhibitors (10 μ M H-89 or 50 nM GF 109203X) were added and cells were further incubated for 5 min at 37 °C. Cells were stimulated for 1 min at 37 °C in the presence of U46619 (1 μ M), BW245C (1 μ M) or in the presence of BW245C (1 μ M) for 1 min followed by U46619 (1 μ M) for 1 min or, to determine basal IP₃ levels in cells, in the presence of an equivalent volume (50 μ l) of the vehicle HBS. The level of IP₃ produced was quantified essentially as described [38,39]. Levels of IP₃ produced by ligand-stimulated cells over basal stimulation, in the presence of HBS, were expressed in pmol IP₃ / 10^6 cells \pm standard error of the mean (pmol / 10^6 cells \pm S.E.M) and as fold stimulation over basal (fold increase \pm S.E.M). The data presented are representative of 4 independent experiments, each performed in duplicate.

2.7. Measurement of cAMP

Ligand mediated cAMP measurements were carried out, in the presence of the phosphodiesterase inhibitor 1 mM 3-isobutyl-1-methylxanthine, essentially as previously described [40]. Levels of cAMP produced by BW245C stimulated cells were expressed as fold stimulation over basal (fold increase \pm S.E). Data presented are representative of 4 independent experiments, each carried out in duplicate.

2.8. Measurement of TP phosphorylation in whole cells.

Agonist mediated TP phosphorylation in intact HEK.HATP α , HEK.HATP β and HEK.HATP α^{S329A} cells was performed essentially as described previously [38]. Briefly, cells were washed once in phosphate-free dulbecco's modified eagles media (DMEM), containing 10% dialysed foetal calf serum (FCS) and were metabolically labelled for one hour in the same media (1.5 ml per

60-mm dish) containing 100 $\mu\text{Ci/ml}$ [^{32}P]orthophosphate (8,000 – 9,000 Ci/mmol) at 37°C, 5% CO_2 . Where appropriate, H-89 (10 μM) or the vehicle HBS [Walsh *et al.*, 2000b] were added for the duration of the labelling period. Thereafter, specific ligands, or vehicle, were added for 10 min at 37°C, 5% CO_2 . Reactions were terminated and HA-tagged TP receptors were immunoprecipitated using the anti-HA 101R antibody, blotted and analysed by autoradiography and phosphor image analysis, essentially as previously described [38]. In parallel experiments, cells were incubated under identical conditions in the absence of [^{32}P]orthophosphate; HA-TP receptors were immunoprecipitated (101R antibody) and immunoblots were screened using the anti-HA antibody to check for quantitative recovery of each receptor type. Thereafter, membranes are screened by immunoblot analysis using the anti-HA 3F10 horse radish peroxidase conjugate; immunoreactive proteins were visualized using the chemiluminescence detection system [38].

2.9 Data analyses

Statistical analyses were carried out using the unpaired Student's *t* test using the Statworks Analysis Package. *P*-Values ≤ 0.05 indicated statistically significant differences.

3. Results

3.1. Effect of BW245C on U46619-mediated signaling in human platelets and HEK 293 cells.

TXA₂ and PGD₂ mediate opposing actions in platelets and in vascular and non-vascular smooth muscle. To investigate the effect of activation of DP on TP signaling and to ascertain whether the TP(s) themselves may be direct targets in DP-mediated cross talk, we examined the effect of DP activation by the agonist BW245C on U46619-mediated signaling by the individual TP α and TP β isoforms expressed in HEK 293 cells and compared it to that which occurs in platelets. Consistent with previous reports, the platelets exhibited efficient mobilization of [Ca²⁺]_i in response to 1 μ M U46619 (Figure 1A). Whereas BW245C at 1 μ M (Figure 1B) or 10 μ M (data not shown), failed to mobilize [Ca²⁺]_i, it almost completely abolished mobilization of [Ca²⁺]_i in response to secondary stimulation of cells with U46619 (Figure 1B; compare Δ [Ca²⁺]_i = 156 \pm 6.35 nM, Figure 1A versus Δ [Ca²⁺]_i = 29.4 \pm 1.1 nM, Figure 1B; p < 0.0001). Platelet aggregation studies indicated that whilst platelets aggregated irreversibly in response to 1 μ M U46619, this aggregation was completely blocked by prior stimulation with 1 μ M BW245C (data not shown).

The effect of DP activation on TP signaling in HEK. α 10 cells and HEK. β 3 cells was then investigated. The presence of mRNA encoding DP in HEK 293 cells and in the platelet like megakaryocytic human erythroleukaemia 92.1.7 (HEL) cell line was initially confirmed by RT-PCR (Figure 2A). BW245C (1 μ M) stimulation of HEK 293, and HEL cells, resulted in significant increases in cAMP generation relative to vehicle-treated cells (Figure 2B), thereby confirming functional expression of DP in these cells.

Consistent with previous studies, HEK. α 10 cells, co-transfected with G α ₁₁ showed efficient [Ca²⁺]_i mobilization in response to U46619 (1 μ M, Figure 3A). Whereas BW245C (1 μ M) did not stimulate significant increases in [Ca²⁺]_i mobilization in these cells, it significantly reduced subsequent U46619-induced [Ca²⁺]_i mobilization (Figure 3B; compare Δ [Ca²⁺]_i = 158 \pm 6.96 nM, Figure 3A versus Δ [Ca²⁺]_i = 12.3 \pm 7.3 nM, Figure 3B; p < 0.0007) with the IC₅₀ for BW245C mediated inhibition determined to be 3.2 \pm 0.44 \times 10⁻⁸ M. In HEK. β 3 cells, transfected with G α ₁₁, stimulation with U46619 (1 μ M) gave rise to efficient [Ca²⁺]_i mobilization (Figure 3C). Exposure to BW245C (1 μ M) did not support [Ca²⁺]_i mobilization (Figure 3D). However, pre-incubation of HEK. β 3 cells with BW245C at 1 μ M did not significantly reduce subsequent U46619-induced [Ca²⁺]_i mobilization (Figure 3D; compare Δ [Ca²⁺]_i = 145 \pm 16.4 nM, Figure 3C versus Δ [Ca²⁺]_i = 138 \pm 15.9 nM, Figure 3D; p > 0.78).

To investigate factors that mediate the DP-induced differential desensitization of the TP isoforms, we examined the effects of pre-incubation of HEK. α 10 cells transfected with G α ₁₁, with GF 109203X, a specific inhibitor of PKC [41], and H-89, a specific PKA inhibitor [42] and compared it to

that which occurs in platelets. Pre-incubation of platelets with 50 nM GF 109203X for 2 min prior to agonist stimulation had no effect on BW243C induced desensitization of U46619 mediated $[Ca^{2+}]_i$ mobilization. (Figure 4A). In contrast, pre-treatment of platelets with 10 μ M H-89 for 2 min prior to BW245C (1 μ M) stimulation almost completely restored subsequent U46619 (1 μ M) mediated $[Ca^{2+}]_i$ mobilization to normal, pre-BW245C levels (compare $\Delta[Ca^{2+}]_i = 156 \pm 6.35$ nM, Figure 1A versus $\Delta[Ca^{2+}]_i = 144 \pm 7.5$ nM, Figure 4B; $p > 0.27$). Pre-treatment of HEK. α 10 cells with GF 109203X (50 nM) for 2 min prior to incubation with BW245C (1 μ M) did not alleviate the DP-mediated desensitization in subsequent U46619-induced $[Ca^{2+}]_i$ mobilization (Figure 4C). However, pre-treatment of HEK. α 10 cells with H-89 (10 μ M) for 2 min prior to incubation with BW245C (1 μ M) and then U46619 (1 μ M), significantly prevented DP-mediated desensitization of TP α signaling, restoring U46619-induced $[Ca^{2+}]_i$ mobilization to 85% of that generated by 1 μ M U46619 only (Figure 4D; compare $\Delta[Ca^{2+}]_i = 158 \pm 6.96$ nM, Figure 3A versus $\Delta[Ca^{2+}]_i = 138 \pm 2.0$ nM, Figure 4D; $p < 0.0005$). In the case of HEK. β 3 cells prior incubation with either GF 109203X (50 nM) or H-89 (10 μ M) had no effect on U46619-induced $[Ca^{2+}]_i$ mobilization (data not shown). To rule out the possibility that H-89 may act as an antagonist of the DP, BW245C (1 μ M) mediated cAMP generation was measured in HEK 293 cells in the absence and presence of 10 μ M H-89. No significant difference ($p > 0.7$) was observed in cells stimulated in the absence (1 μ M BW245C, fold increase in cAMP = 2.29 ± 0.01) or presence (1 μ M BW245C, 10 μ M H-89, fold increase in cAMP = 2.34 ± 0.06) of H-89 confirming that H-89 does not function as an antagonist of DP.

3.2. Differential effects of BW245C on U46619 mediated IP₃ generation via TP α and TP β isoforms.

To further investigate the differential effects of DP activation on TP α and TP β signaling, U46619 induced IP₃ generation was measured in HEK. α 10 and HEK. β 3 cells in the presence or absence of pre-stimulation with BW245C. Stimulation of HEK. α 10 and HEK. β 3 cells with U46619 (1 μ M) resulted in a 2.2 -3 fold increase in IP₃ levels (Figure 5A). Pre-incubation of HEK. α 10 cells with BW245C (1 μ M) significantly reduced U46619 mediated IP₃ generation by TP α (Figure 5A, $p < 0.012$). In contrast, pre-incubation of HEK. β 3 cells with BW245C (1 μ M) did not significantly ($p > 0.6$) reduce U46619 mediated IP₃ generation by TP β (Figure 5B). Moreover, H-89 (10 μ M), but not GF 109203X (50 nM), blocked BW245C mediated desensitization of TP α signaling (Figure 5A; $p < 0.03$) but had no effect on TP β signaling (data not shown). Consistent with previous reports (37), stimulation of HEK 293 cells with U46619 or HEK. α 10 and HEK. β 3 cells with BW245C alone failed to generate any increase in IP₃, further indicating that endogenous DP receptors in HEK 293 cells do not couple to PLC (Figure 5B & data not shown).

3.3. The role of the unique C-tail in BW245C-mediated desensitization of TP α signaling.

We then investigated the effect of DP-agonist stimulation on signaling by TP^{Δ328}, a truncated variant of TP devoid of the divergent C-tail residues between TPα and TPβ (22). Consistent with previous studies [22], stimulation of HEK.TP^{Δ328} cells, co-transfected with Gα₁₁, with U46619 (1 μM) resulted in efficient [Ca²⁺]_i mobilization (Figure 6A), while BW245C (1 μM) did not (Figure 6B). In contrast to HEK.α10 cells, BW245C (1 μM) exposure of HEK.TP^{Δ328} cells did not reduce subsequent [Ca²⁺]_i mobilization in response to U46619 (Figure 6B; compare Δ[Ca²⁺]_i = 161 ± 13.2 nM, Figure 6A versus Δ[Ca²⁺]_i = 163 ± 7.9 nM, Figure 6B; *p* > 0.91).

We have previously constructed a variant TPα isoform, TPα^{S329A}, in which Ser³²⁹ was mutated to Ala³²⁹, thereby disrupting the potential PKA phosphorylation site (RPRS³²⁹LSL) unique to TPα [38]. Stimulation of HEK.HATPα^{S329A} cells transfected with Gα₁₁, with U46619 (1 μM) led to efficient [Ca²⁺]_i mobilization (Figure 6C). Pre-incubation with BW245C (1 μM) did not result in [Ca²⁺]_i mobilization and also did not reduce subsequent U46619-induced [Ca²⁺]_i mobilization (Figure 6D; compare Δ[Ca²⁺]_i = 249 ± 5.2 nM, Figure 6C versus Δ[Ca²⁺]_i = 260 ± 2.9 nM, Figure 6D; *p* > 0.15). Similarly, stimulation of HEK.HATPα^{S329A} cells with U46619 (1 μM) resulted in a 2.2 fold increase in IP₃ levels (Figure 5B). Pre-incubation of HEK.HATPα^{S329A} cells with BW245C (1 μM) did not significantly (*p* > 0.1) reduce U46619 mediated IP₃ generation by TPα^{S329A} (Figure 5B). Moreover, neither H-89 (10 μM) or GF 109203X (50 nM) had any effect on TPα^{S329A} signaling (data not shown).

3.4. DP-mediated phosphorylation of TPα.

To investigate whether TPα, TPβ, or TPα^{S329A} were direct targets for DP-mediated phosphorylation, whole-cell phosphorylation assays were performed using cell lines over-expressing HA-epitope tagged TPα, TPβ or TPα^{S329A} receptors [38]. Discrete protein bands of approximately 39 kDa and broad protein bands of 46-60 kDa were present in the TPα and TPα^{S329A} immunoprecipitates which were previously confirmed to correspond to the non-glycosylated and glycosylated forms, respectively, of TPα and TPα^{S329A} (Figure 7D, lanes 1 and 3). A discrete protein band of approximately 46 kDa and a broader band of 50-60 kDa respectively, representing the non-glycosylated and glycosylated forms of TPβ, were immunoprecipitated from the HEK.HATPβ cell line (Figure 7D, lane 2). No protein bands were immunoprecipitated from control HEK 293 cells (Figure 7D, lane 4). Pre-treatment of HEK.HATPα cells with BW245C (1 μM) resulted in a significantly higher level of TPα phosphorylation than that observed in vehicle-treated (basal level) cells (Figure 7A, lanes 1 and 3), which in turn was blocked by pre-treatment with H-89 (Figure 7A, lane 2). Pre-treatment of HEK.HATPβ cells with BW245C (1 μM) did not increase phosphorylation of TPβ relative to vehicle-treated cells (Figure 7B, lanes 1 and 3). Pre-treatment of HEK.HATPβ cells with H-89 (10 μM) had no significant effect on the basal level of TPβ phosphorylation (Figure 7B,

lane 2). Prior incubation of HEK.HATP α^{S329A} cells with BW245C (1 μ M) did not result in any significant increase in TP α^{S329A} phosphorylation relative to vehicle-treated cells (Figure 7C, lanes 1 and 3). Pre-treatment of HEK.HA:TP α^{S329A} cells with H-89 (10 μ M) made no significant difference to the basal level of TP α^{S329A} phosphorylation (Figure 7C, lane 2). Consistent with previous studies [38], stimulation of cells with U46619 (1 μ M, 10 min) led to 5-7 fold increases in the phosphorylation of TP α , TP β and TP α^{S329A} confirming that each of these receptors are subject to homologous desensitization (data not shown). These studies confirm that TP α , in contrast to TP β , is subject to DP-mediated desensitization at a PKA sensitive site located at S³²⁹ within its unique C-tail.

4. Discussion

Individually, both TXA₂ and PGD₂ mediate a range of physiologic responses in a diversity of cell and tissue types [1-3]. Whereas many of those responses occur in non-overlapping or distinct tissue types, many also occur in common cell types, such as platelets and vascular and non-vascular smooth muscle cells. TXA₂ is a potent stimulator of platelet aggregation and constrictor of vascular smooth muscle whereas PGD₂, like prostacyclin, inhibits platelet aggregation [3]. Thus, the actions of PGD₂ in the vascular system mimic those of prostacyclin, a prostanoid widely associated with the counter regulation of vascular hemostasis. While prostacyclin is primarily produced by the vascular endothelium, PGD₂, like TXA₂, is synthesised by platelets and as such, serves as a platelet derived inhibitor of platelet aggregation [3]. Homologous and heterologous desensitization of DP and TP(s) have been previously investigated [43-47]. However, little is known about how the responses to TXA₂ and PGD₂ are counter-regulated in cell or tissue types where their receptors are co-expressed, such as platelets, in various types of smooth muscle and in the brain.

Cross-talk, or counter regulation of responses, has been widely documented to occur between the anti-aggregatory adenylyl cyclase system and the pro-aggregatory phospholipase C system in platelets and vascular smooth muscle [48]. The main inhibitory actions of adenylyl cyclase signaling within platelets is believed to be mediated through its activation of cAMP dependent PKA [48]. Whereas many of the molecular targets of adenylyl cyclase/ PKA have been identified within platelets, such as phospholipase C, myosin light chain kinase, thrombolamban and Gα13 [48-50], it is also possible that the receptors themselves, such as the TP(s), may be direct targets of adenylyl cyclase / PKA. In this context, we have recently established that the TPα, but not the TPβ, isoform of the human TXA₂ receptor, is indeed a target for prostacyclin desensitization, mediated through direct PKA dependent phosphorylation of TPα within its unique C-tail sequence [38]. Thus, in the current study, we sought to establish whether the TPs may be subject to DP-mediated desensitization and if so, to establish whether this desensitization may be directed to TPα or TPβ or both. Consistent with previous studies [2,3], stimulation of platelets with the selective DP agonist BW235C inhibited TP (U46619) mediated platelet aggregation and activation of phospholipase C, as assessed by measurement of [Ca²⁺]_i mobilization. Moreover, this occurred in a dose dependent manner with the IC₅₀ determined to be 2 nM BW245C. Expression of DP in HEK 293 cells, and in cell line derivatives, was confirmed by RT-PCR and functional expression was confirmed by measurement of BW235C mediated cAMP generation, indicating the endogenous DP expressed in HEK 293 cells couples to activation of adenylyl cyclase. Endogenous DP expressed in HEK 293 cells did not lead to significant changes in [Ca²⁺]_i mobilization in response to BW245C, consistent with studies in HEK 293(EBNA) cells (37). Pre-stimulation of endogenous DPs in HEK.α10 cells, stably transfected with the TPα isoform, significantly desensitized subsequent TP (U46619) mediated [Ca²⁺]_i mobilization and IP₃ generation and this occurred in a dose dependent manner with the IC₅₀ for BW245C determined to

be $3.2 \pm 0.44 \times 10^{-8}$ M. In contrast, signaling by TP β was unaffected by pre-stimulation of endogenous DP expressed in HEK. β 3 cells indicating that the TP α , but not TP β , is a target for DP mediated cross desensitization of TP responses.

Pre-incubation of platelets or HEK. α 10 cells with the PKA inhibitor H-89 almost completely blocked BW245C mediated inhibition of TP signaling. Failure to completely desensitize TP α in HEK. α 10 cells is possibly due to the relatively high TP receptor density in those cells [51]. On the other hand, H-89 had no effect on TP β signaling and the PKC inhibitor GF 109203X had no appreciable effect on DP inhibition of TP signaling in platelets or on TP α or TP β signaling in HEK 293 cells. Whereas stimulation of HEK.TP Δ^{328} cells with U46619 led to efficient mobilization of $[Ca^{2+}]_i$, TP Δ^{328} was not subjected to BW245C mediated desensitization. These data indicate that BW245C induced desensitization of TP α is mediated at unique elements within its C-tail which, coupled to the H-89 effects, most likely correspond(s) to PKA phosphorylation site(s). Computational analysis of the C-tail sequences of TP α identified the presence of a unique consensus PKA phosphorylation site within the sequence RPRSLSL, where S³²⁹ was predicted to represent the target residue for phosphorylation [52; 38]. While stable cell lines over-expressing TP α^{S329A} exhibited identical U46619-mediated intracellular signaling to that of the wild type TP α , U46619 mediated signaling by TP α^{S329A} was insensitive to pre-stimulation of DP with BW245C. Whereas platelets and other hematopoietic cells are reported to express G α_q and G α_{16} , they are reported not to express significant levels of G α_{11} [53]. Substitution of G α_{11} with G α_q also supported DP mediated desensitization of TP α but not TP β , TP Δ^{328} , or TP α^{S329A} in response to BW245C indicating that DP-mediated differential desensitization of TPs was independent of the coupling G protein (data not shown).

Finally, to establish whether the TP(s) may be direct targets for DP mediated phosphorylation, HEK 293 cell lines stably over-expressing HA-epitope tagged forms of TP α , TP β or TP α^{S329A} were used in whole cell phosphorylation assays. Whereas each of the TPs underwent U46619-mediated phosphorylation [38], stimulation of cells with BW249C resulted in agonist dependent phosphorylation of TP α but not TP β or TP α^{S329A} . Moreover, the PKA inhibitor H-89 blocked BW245C mediated phosphorylation of TP α . Taken together, these studies confirm that TP α , but not TP β , is subject to DP-mediated desensitization and that this desensitization involves direct PKA phosphorylation of TP α , where Ser³²⁹ is the target residue for DP mediated phosphorylation of TP α .

The results presented in this study, involving DP mediated desensitization of TP responses, closely resemble those previously obtained by us involving IP mediated desensitization of TP, using the selective IP agonists cicaprost and iloprost [38] and further support the concept that it is the TP α isoform that is subject to counter-regulation by the anti-platelet prostanoids prostacyclin and PGD₂. Should this type of counter-regulation occur in platelets and in other vascular cell types such as

vascular smooth muscle and/or endothelial cells, it is tempting to suggest that TP α may be the TP isoform physiologically relevant to regulated vascular hemostasis. Consistent with this Habib *et al.*, [45] detected immunoreactive protein corresponding to TP α , but not TP β , within platelets and established that TP α is phosphorylated in platelets in response to the TP agonist [1S-[1 α ,2 α (Z),3 β (1E,3S*),4 α)]-7-{3[{3-hydroxy-4-4(indophenoxy)-1-butenyl]-7-oxabicyclo[2,2,1]hept-2-yl]-5-Heptenoic acid (I-BOP; 45). Whereas the latter studies and those reported herein point to important mechanisms whereby the action of TP α may be subject to homologous [45] and heterologous [38] desensitization, they also suggest that the TP β isoform is not subject to this type of regulation. Consistent with this, we have also established that signaling by the TP α , but not the TP β , isoform was subject to partial forskolin induced desensitization of U46619 mediated signaling (data not shown). On the otherhand, Parent *et al.*, [54] established that the TP β , but not the TP α , isoform is subject to agonist (U46619) mediated internalisation. In studies investigating the expression and tissue distribution of the TP isoforms, mRNA for both isoforms have been found to be co-expressed in a variety of cell and tissue types of non-vascular and vascular origin, including platelets [23,16] supporting the notion that the TP receptors may indeed co-exist within the same cell type, albeit at different levels. However, our data sheds further light that signaling by the TP isoforms is subject to differential heterologous regulation and indicates that TXA₂ mediated signaling by TP α may be counter-regulated by the inhibitory prostanoids prostacyclin and PGD₂; on the other hand TXA₂ mediated signaling by the TP β isoform remains unaffected by these autocooids. The current study specifically considered the roles of the prostanoids TXA₂ and PGD₂ within the vascular system; however, the findings have wider implications with respect to the possible counter regulation of TXA₂ and PGD₂ responses in other cell types, for example within the brain where both TP and DP protein expression and mRNA expression [55,56,2,16] are widely detected. The findings reported here also imply that the TP β isoform is not subject to PKA dependent heterologous desensitization and such lack of desensitization may have important physiological consequences which are currently unappreciated.

Acknowledgements: This research was supported by grants to B.T.K from The Wellcome Trust, The Irish Heart Foundation, The Health Research Board of Ireland and Enterprise Ireland.

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FIGURES

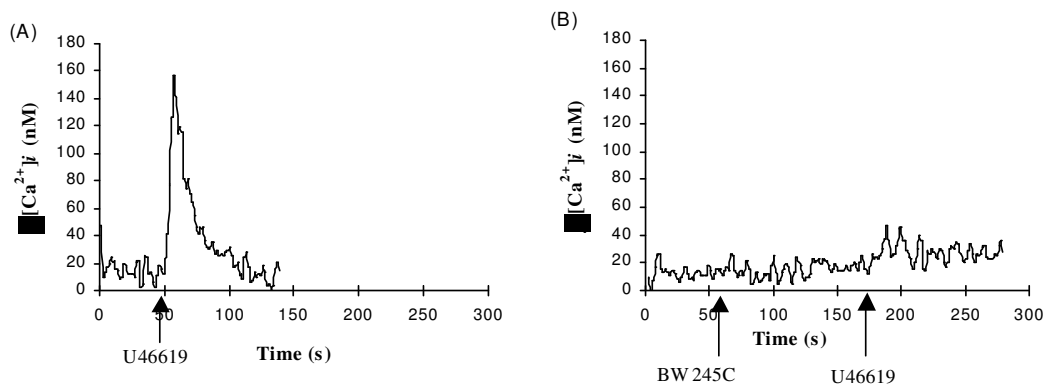


Figure 1. Effect of BW245C on U46619-induced signaling in platelets.

Human platelets were stimulated with 1 μ M U46619 (*Panel A*) or with 1 μ M BW245C followed by 1 μ M U46619 (*Panel B*). Actual changes in $[Ca^{2+}]_i$ mobilized were: *Panel A*: (1 μ M U46619, $\Delta[Ca^{2+}]_i = 156 \pm 6.35$ nM); *Panel B*: (1 μ M BW245C, $\Delta[Ca^{2+}]_i = 0$ nM; 1 μ M U46619, $\Delta[Ca^{2+}]_i = 29.4 \pm 1.1$ nM).

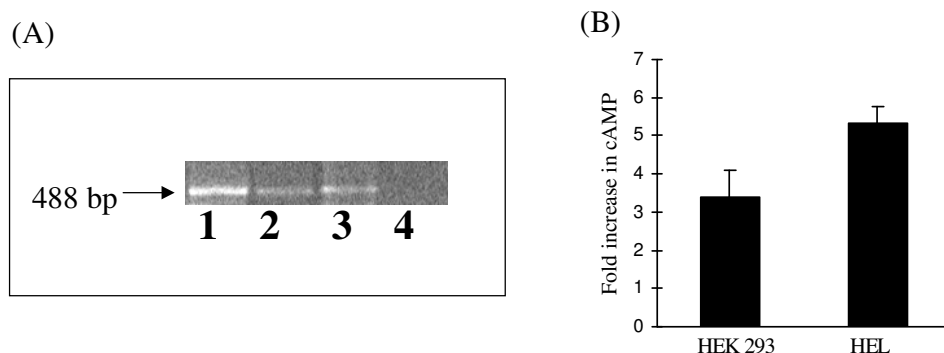


Figure 2. Analysis of DP expression in HEK 293 cells.

Panel A: RT-PCR analysis of the human DP cDNA (488 bp) amplified from HEK 293 (lane 1) or HEL 92.1.7 (lane 2) cell mRNA. The negative control PCR, where amplification primers were added to the reaction without any template cDNA, is shown in lane 4. A molecular size marker of 517 bp is shown in lane 3. *Panel B:* HEK 293 cells or HEL 92.1.7 (HEL) cells were stimulated with 1 μ M BW245C or with vehicle HBS for 10 min at 37°C. Levels of cAMP generated in ligand stimulated cells relative to vehicle treated cells (basal cAMP) were expressed and are presented as fold stimulation of basal (Fold increase in cAMP \pm SEM, n = 4). Basal levels of cAMP in HEK 293 cells were 0.53 ± 0.05 nmol / mg cell protein. Basal levels of cAMP in HEL cells were 0.9 ± 0.08 nmol / mg cell protein.

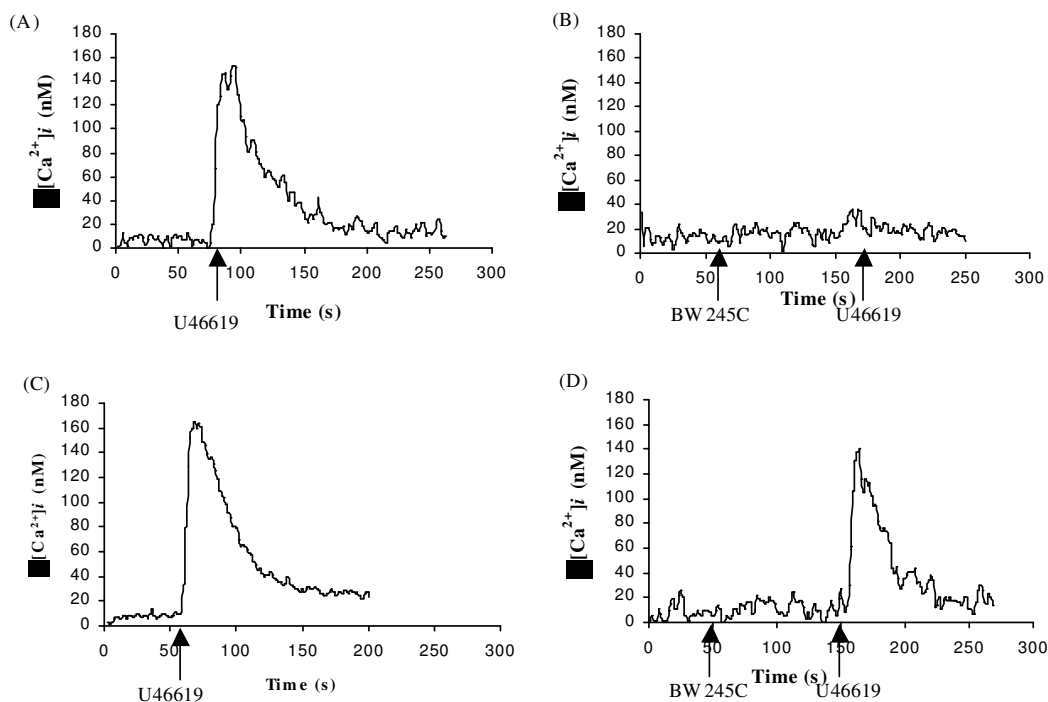


Figure 3. Effect of BW245C on U46619-induced $[Ca^{2+}]_i$ mobilization in HEK.α10 and HEK.β3 cells.

HEK.α10 cells (*Panels A & B*) and HEK.β3 cells (*Panels C & D*), transiently co-transfected with $G\alpha_{11}$, were stimulated with 1 μ M U46619 (*Panels A & C*) or with 1 μ M BW245C followed by 1 μ M U46619 (*Panels B & D*), respectively. Actual changes in $[Ca^{2+}]_i$ mobilized were: *Panel A*: (1 μ M U46619, $\Delta[Ca^{2+}]_i = 158 \pm 6.96$ nM); *Panel B*: (1 μ M BW245C, $\Delta[Ca^{2+}]_i = 0$ nM; 1 μ M U46619, $\Delta[Ca^{2+}]_i = 12.3 \pm 7.3$ nM); *Panel C*: (HEK.β3 cells, 1 μ M U46619, $\Delta[Ca^{2+}]_i = 145 \pm 16.4$ nM); *Panel D*: (HEK.β3 cells, 1 μ M BW245C, $\Delta[Ca^{2+}]_i = 0$ nM; 1 μ M U46619, $\Delta[Ca^{2+}]_i = 138 \pm 15.9$ nM).

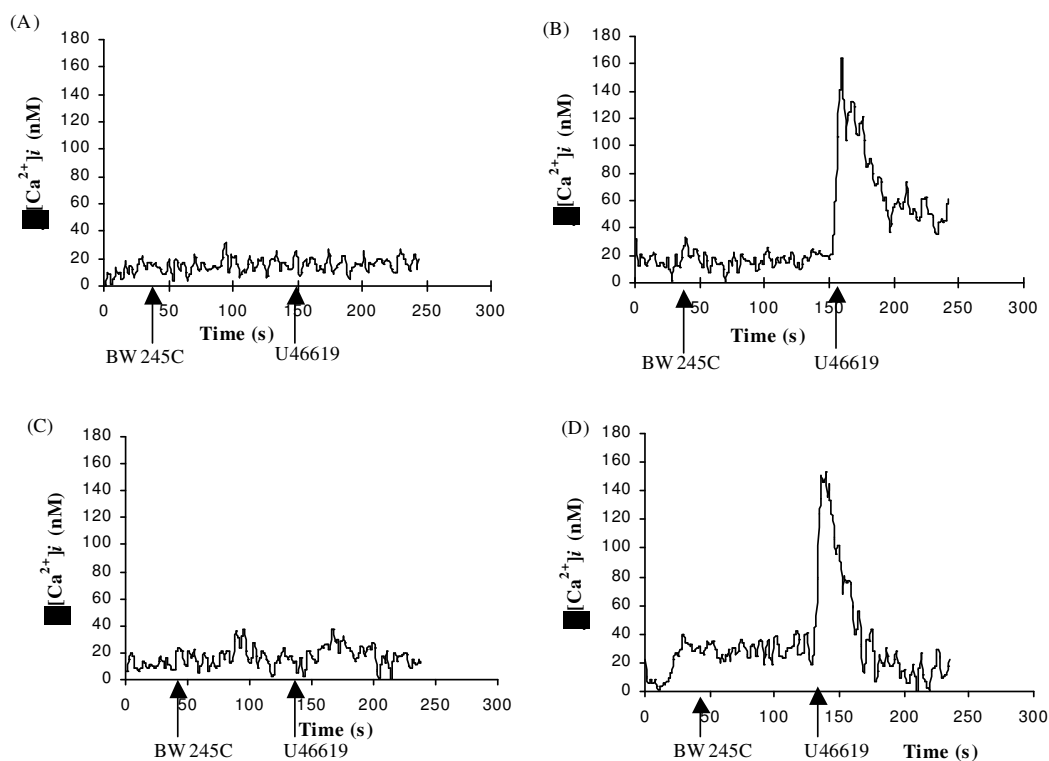


Figure 4. Effect of BW245C on U46619-induced $[Ca^{2+}]_i$ mobilization in human platelets and in HEK.α10 cells.

Human platelets (*Panels A & B*) or HEK.α10 cells (*Panels C & D*), transiently co-transfected with $G\alpha_{11}$, were pre-incubated with 50 nM GF109203X (*Panels A & C*), or 10 μ M H-89 (*Panels B & D*), for 2 min prior to stimulation by 1 μ M BW245C and then 1 μ M U46619. Actual changes in $[Ca^{2+}]_i$ mobilized were: *Panel A*: (Platelets, 1 μ M BW245C, $\Delta[Ca^{2+}]_i = 0$ nM; 1 μ M U46619, $\Delta[Ca^{2+}]_i = 0$ nM); *Panel B*: (Platelets, 1 μ M BW245C, $\Delta[Ca^{2+}]_i = 0$ nM; 1 μ M U46619, $\Delta[Ca^{2+}]_i = 144 \pm 7.5$ nM); *Panel C*: (HEK.α10 cells, 1 μ M BW245C, $\Delta[Ca^{2+}]_i = 0$ nM; 1 μ M U46619, $\Delta[Ca^{2+}]_i = 20 \pm 9.6$ nM); *Panel D*: (HEK.α10 cells, 1 μ M BW245C, $\Delta[Ca^{2+}]_i = 0$ nM; 1 μ M U46619, $\Delta[Ca^{2+}]_i = 138 \pm 2.0$ nM).

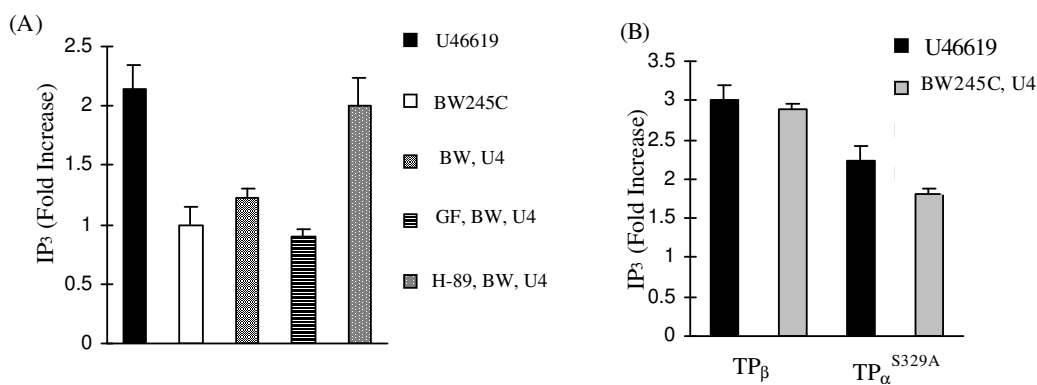


Figure 5. Effect of BW245C on U46619-mediated IP₃ generation in HEK.α10, HEK.β3 and TP α^{S329A} cells.

HEK.α10 (*Panel A*) and HEK.β3 or HEK.TP α^{S329A} (*Panel B*) cells, transiently co-transfected with G α_{i1} , were stimulated at 37°C with 1 μ M U46619 for 1 min (U46619), 1 μ M BW245C for 1 min (BW245C), or 1 μ M BW245C for 1 min followed by 1 μ M U46619 for 1 min (BW, U4). Alternatively, cells were pre-incubated for 5 min with 50 nM GF 109203X prior to stimulation by 1 μ M BW245C for 1 min followed by 1 μ M U46619 for 1 min (GF, BW, U4) or with 10 μ M H-89 for 5 min prior to stimulation with 1 μ M BW245C for 1 min followed by 1 μ M U46619 for 1 min (H-89, BW, U4). In each case, basal levels of IP₃ were determined by exposing cells to the vehicle HBS under identical reaction conditions. Levels of IP₃ produced in response to ligand relative to vehicle-treated cells were expressed as fold stimulation of basal (Fold increase in IP₃ \pm S.E.M; n = 3, 5A & B). Basal levels of IP₃ in HEK.α10 cells was 0.39 \pm 0.09 nmol / mg; in HEK.β3 cells was 0.32 \pm 0.08 nmol / mg and in HEK.TP α^{S329A} cells was 0.27 \pm 0.06 nmol / mg.

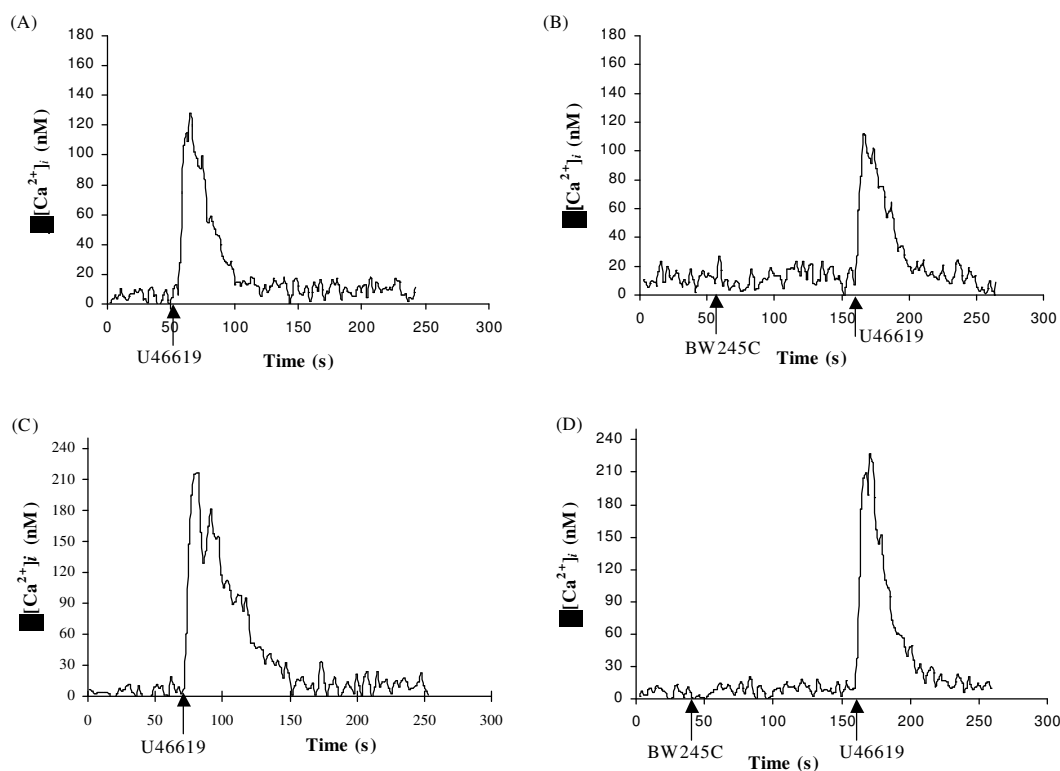


Figure 6. Effect of BW245C on U46619-induced $[Ca^{2+}]_i$ mobilization in HEK.TP Δ^{328} and HEK.TP α^{S329A} cells.

HEK.TP Δ^{328} cells (Panels A & B) and HEK.TP α^{S329A} cells (Panels C & D), transiently co-transfected with $G\alpha_{11}$, were stimulated with either 1 μ M U46619 (Panel A & C), or 1 μ M BW245C followed by 1 μ M U46619 (Panel B & D). Actual changes in $[Ca^{2+}]_i$ mobilized were: Panel A: (HEK.TP Δ^{328} cells, 1 μ M U46619, $\Delta[Ca^{2+}]_i = 161 \pm 13.2$ nM); Panel B: (HEK.TP Δ^{328} cells, 1 μ M BW245C, $\Delta[Ca^{2+}]_i = 0$ nM; 1 μ M U46619, $\Delta[Ca^{2+}]_i = 163 \pm 7.9$ nM). Panel C: (1 μ M U46619, $\Delta[Ca^{2+}]_i = 249 \pm 5.2$ nM); Panel D: (1 μ M BW245C, $\Delta[Ca^{2+}]_i = 0$ nM; 1 μ M U46619, $\Delta[Ca^{2+}]_i = 260 \pm 2.9$ nM).

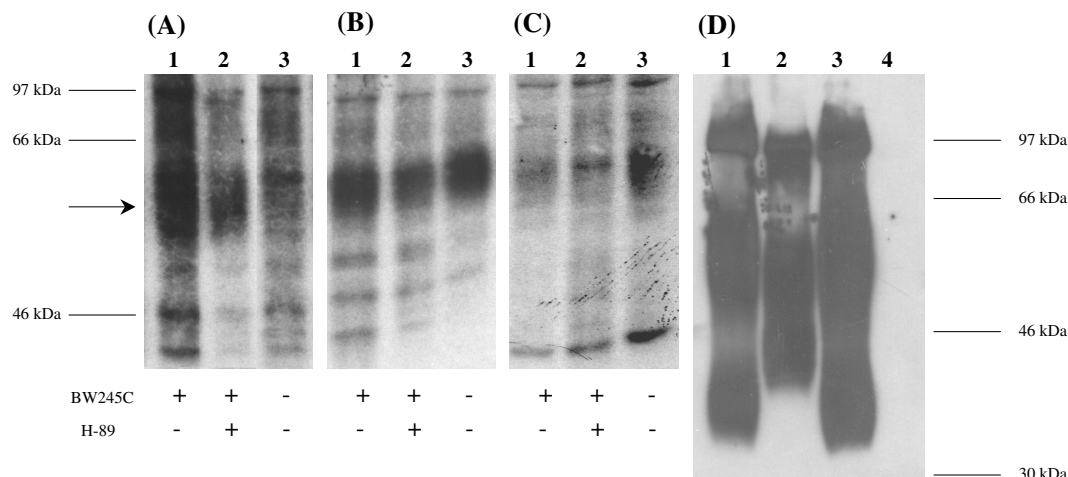


Figure 7. DP mediated phosphorylation of TP α , TP β and TP α^{S329A} .

Panels A – C: HEK.HATP α (*Panel A*), HEK.HATP β (*Panel B*), or HEK.HATP α^{S329A} (*Panel C*) cells were labelled with [32 P]orthophosphate in the presence (*Panels A, B & C, lane 2*) or absence (*Panels A, B & C, lane 1*) of H-89 (10 μ M), or with the vehicle HBS (*Panels A, B & C, lane 3*). Cells were then incubated for 10 min with BW245C (1 μ M) (*Panels A, B & C, lanes 1 and 2*) or vehicle (*Panels A, B & C, lane 3*). HA-tagged TP receptors were immunoprecipitated, subjected to SDS-PAGE and exposed to Xomat XAR-5 film (Kodak) for 15 days. Thereafter, blots were subject to Phosphor Image analysis and the intensities of phosphorylation relative to basal levels were determined and expressed, in arbitrary units, as follows: TP α , 1 μ M BW245C, 4.8 fold; 10 μ M H-89, 1 μ M BW245C, 1.8 fold; TP β , 1 μ M BW245C, 1.2 fold; 10 μ M H-89, 1 μ M BW245C, 0.9 fold; TP α^{S329A} , 1 μ M 1 μ M BW245C, 0.7 fold; 10 μ M H-89, 1 μ M BW245C, 0.8 fold. *Panel D:* HEK 293 control cells (*lane 4*) or HEK 293 cells over-expressing HA-epitope tagged TP α (*lane 1*), TP β (*lane 2*), or TP α^{S329A} (*lane 3*) were immunoprecipitated, subjected to western blotting followed chemiluminescence detection. Molecular weight markers (kDa) are indicated to the left and right of the panels. The arrow to the left of Panel A indicates the position of the phosphorylated TP α . These data are representative of three independent experiments.