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Prostaglandin D_2 receptor-mediated desensitization of the α isoform of the human thromboxane A_2 receptor

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Abstract

Thromboxane (TX) A_2 and prostaglandin (PG) D_2 mediate opposing actions in platelets and in vascular and non-vascular smooth muscle. Here, we investigated the effects of stimulation of the PGD_2 receptor (DP) on signaling by the TXA_2 receptor (TP) expressed in human platelets and in human embryonic kidney (HEK) 293 cells over-expressing the individual TPα and TPβ isoforms. In platelets, the selective DP agonist BW245C abolished TP-mediated mobilization of intracellular calcium ($[Ca^{2+}]_i$) and inhibited platelet aggregation in response to the TXA_2 mimetic U46619. DP-mediated desensitization of TP signaling in platelets was prevented by pretreatment with the cAMP-dependent PKA inhibitor, H-89, but was unaffected by the PKC inhibitor GF 109203X. In HEK 293 cells, signaling by TPα, but not TPβ, was subject to DP-mediated desensitization in a PKA-dependent, PKC-independent manner. U46619-induced signaling by $TP^{\Delta 328}$, a truncated variant of TP containing only those residues common to TPα and TPβ, was insensitive to prior DP stimulation, indicating that the carboxyl terminal tail of TPα contains the target site(s) for DP-mediated desensitization. Mutation of Ser^{329} to Ala^{329} within a consensus PKA site in TPα rendered the mutant $TP\alpha^{S329A}$ insensitive to BW245C-mediated desensitization. Whole cell phosphorylation assays established that TPα, but not TPβ or $TP\alpha^{S329A}$, was subject to DP-mediated phosphorylation and that TPα phosphorylation was blocked by the PKA inhibitor H-89. These data establish that TPα, but not TPβ, is subject to DP-mediated cross desensitization, which occurs through direct PKA-mediated phosphorylation of TPα at Ser^{329}.

Keywords: Thromboxane A_2 receptor; Prostaglandin D_2 receptor; Desensitization; Protein kinase A; Phosphorylation; G-protein-coupled receptor

1. Introduction

Thromboxane (TX) A_2 is the major product of arachidonic acid metabolism in platelets and in activated macrophages and together with prostacyclin (prostaglandin I_2) is thought to play a key role in vascular hemostasis [1–3]. Perturbations in the levels of TXA_2, or its synthase or its receptor, have been implicated in various cardiovascular disorders [4,5]. Depending on the cell type, TXA_2 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]; and contraction of glomerular mesangial cells and intrarenal vascular tissue, decreasing glomerular filtration rates [13].

These effects are transduced through activation of its specific TXA_2 receptor, also termed TP, a member of the G-protein-coupled receptor (GPCR) superfamily. In humans, there are two TP isoforms, termed TPα and TPβ [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 TXA_2, 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 IP_3 and

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Abbreviations: cAMP, cyclic adenosine 5′ monophosphate; $[Ca^{2+}]_i$, intracellular calcium; DP, PGD_2 receptor; HA, hemagluttinin; HEK, human embryonic kidney; HEL, human erythroleukaemia; HBS, HEPES-buffered saline; IP, prostacyclin receptor; IP_3, inositol 1,4,5 trisphosphate; PG, prostaglandin; PKA, protein kinase A; PKC, protein kinase C; RT–PCR, reverse transcriptase–polymerase chain reaction; TXA_2, thromboxane A_2; and TP, TXA_2 receptor.

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mobilization of intracellular calcium ($[Ca^{2+}]_i$) [17,18]. Functional coupling of TPα to G_q and G_{11} has been demonstrated in vivo in response to the TXA_2 mimetic U46619 (9,11-dideoxy-9.alpha.,11.alpha.-methanoepoxy Prostaglandin F_2.alpha) and the F_2-isoprostane, 8-epi-$PGF_{2\alpha}$ [19]. Whereas the functional significance for 2 receptors for TXA_2 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 G_{12} family [20–22], they may oppositely regulate adenylyl cyclase activity [23], and TPα, but not TPβ, has been proposed to couple to Gh [24].

Prostaglandin (PG) D_2, like prostacyclin, is a potent inhibitor of platelet aggregation [25,26]. Two distinct PGD synthases catalyse the isomerisation of PGH_2 to PGD_2: one is the lipocalin type that was previously known as the brain-type enzyme or gluthathione-independent type, and the other is the hematopoietic type or gluthathione-dependent type [27]. PGD_2 can be further converted to 9 alpha, 11 beta-PGF_2, or the J series of prostanoids, such as PGJ_2, Δ12-PGJ_2, and 15-deoxy-Δ 12,14 PGJ_2, which act as endogenous ligands for the peroxisome proliferator-activated receptor (PPAR)γ family of nuclear receptors [28,29]. Peripherally, PGD_2 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]. PGD_2 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 PGD_2, 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 $G\alpha_s$, leading to increases in cAMP. The human DP, when stably expressed in HEK 293(EBNA) cells, gave a transient rise in $[Ca^{2+}]_i$ in response to the DP agonist (4*S*)-(3-[(3*R*,*S*)-3-cyclohexyl-3-hydroxypropyl]-2,5-dioxo)-4-imidazolidineheptanoic acid (BW245C), but without an accompanying rise in intracellular IP_3 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 PGD_2, 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 Ser^{329} [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 Ser^{329} 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-Carboxyozazol-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 (8000–9000 Ci/mmol) was from DuPont NEN. [^{3}H]IP_3 (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:Gα_{11} and pCMV:Gα_q have been previously described [19]. Stable HEK 293 cell lines overexpressing TPα (HEK.α10 cells), TPβ (HEK.β3 cells), $TP^{\Delta 328}$ (HEK.$TP^{\Delta 328}$ cells), and hemagglutinin (HA) epitope-tagged forms of HA:TPα (HEK.HATPα cells), HA:TPβ (HEK.HATPβ cells), HA:$TP\alpha^{S329A}$ (HEK.HATPα^{S329A} cells) have been previously described [22,38]. Cells were transiently transfected as previously described [19] and were harvested 48 hr posttransfection.

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° for 2 min before addition of the

aggregating agent (1 μM U46619, 1 μM 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′ CTCTGAATTC*ACA GACTGGATTC-CATGT* 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 ($[Ca^{2+}]_i$) mobilization

Measurements of $[Ca^{2+}]_i$ in FURA2/AM preloaded cells and platelets were carried out as previously described [19]. Cells were stimulated with 1 μM U46619 or 1 μM 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 μM) or 2-[1-(3-dimethylaminopropyl)-1*H*-indol-3-yl]-3-(1*H*-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 μL 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 (sec) 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 $\Delta[Ca^{2+}]_i \pm$ SEM (nM).

2.6. Measurement of IP_3 levels

Intracellular IP_3 levels were measured as described previously [38,39]. Briefly, cells were harvested, washed twice in ice-cold PBS, and then resuspended at approximately 5×10^6 cells/mL in HBS [38] containing 10 mM LiCl. Cells (200 μL) were then preincubated at 37° for 10 min. Where appropriate, the kinase inhibitors (10 μM H-89 or 50 nM GF 109203X) were added and cells were further incubated for 5 min at 37°. Cells were stimulated for 1 min at 37° in the presence of U46619 (1 μM), BW245C (1 μM) or in the presence of BW245C (1 μM) for 1 min followed by U46619 (1 μM) for 1 min or, to determine basal IP_3 levels in cells, in the presence of an equivalent volume (50 μL) of the vehicle HBS. The level of IP_3 produced was quantified essentially as described [38,39]. Levels of IP_3 produced by ligand-stimulated cells over basal stimulation, in the presence of HBS, were expressed in pmol $IP_3/10^6$ cells ± standard error of the mean (pmol/10^6 cells ± SEM) and as fold stimulation over basal (fold increase ± SEM). 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 ± SE). 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 .HATPα, HEK.HATPβ, and HEK.HATPα^{S329A} cells was performed essentially as described previously [38]. Briefly, cells were washed once in phosphate-free Dulbecco's modified Eagle's media (DMEM), containing 10% dialysed foetal bovine serum (FBS) and were metabolically labelled for 1 hr in the same media (1.5 mL per 60-mm dish) containing 100 μCi/mL of [^{32}P]orthophosphate (8000–9000 Ci/mmol) at 37°, 5% CO_2. Where appropriate, H-89 (10 μM) or the vehicle HBS [38] were added for the duration of the labelling period. Thereafter, specific ligands, or vehicle, were added for 10 min at 37°, 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 were screened by immunoblot analysis using the anti-HA 3F10 horseradish 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

TXA_2 and PGD_2 mediate opposing actions in platelets and in vascular and non-vascular smooth muscle. To inves-

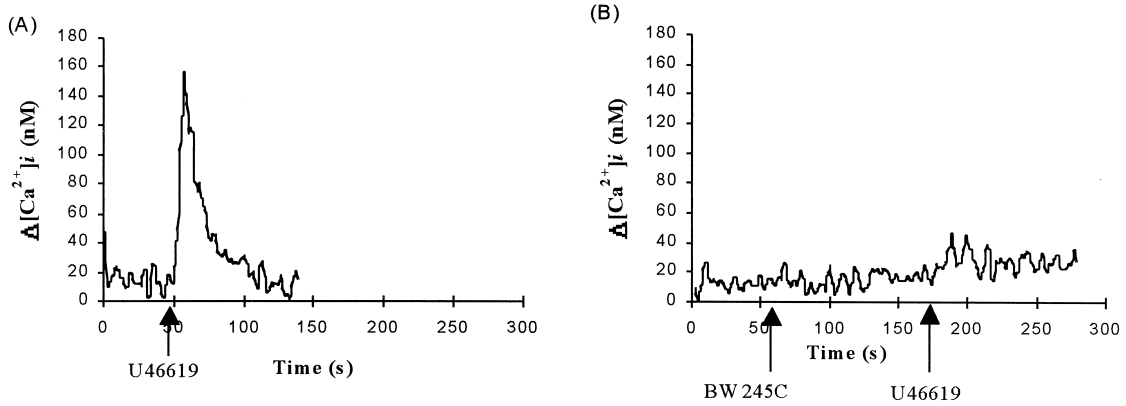

Fig. 1. Effect of BW245C on U46619-induced signaling in platelets. Human platelets were stimulated with 1 μM U46619 (Panel A) or with 1 μM BW245C followed by 1 μM U46619 (Panel B). Actual changes in $[Ca^{2+}]_i$ mobilized were: Panel A: (1 μM U46619, $\Delta[Ca^{2+}]_i = 156 \pm 6.35$ nM); Panel B: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

tigate 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 TPα and TPβ 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 $[Ca^{2+}]_i$ in response to 1 μM U46619 (Fig. 1A). Whereas BW245C at 1 μM (Fig. 1B) or 10 μM (data not shown) failed to mobilize $[Ca^{2+}]_i$, it almost completely abolished mobilization of $[Ca^{2+}]_i$ in response to secondary stimulation of cells with U46619 (Fig. 1B; compare $\Delta[Ca^{2+}]_i = 156 \pm 6.35$ nM, Fig. 1A versus $\Delta[Ca^{2+}]_i = 29.4 \pm 1.1$ nM, Fig. 1B; $P < 0.0001$). Platelet aggregation studies indicated that whilst platelets aggregated irreversibly in response to 1 μM U46619, this aggregation was completely blocked by prior stimulation with 1 μM BW245C (data not shown).

The effect of DP activation on TP signaling in HEK.α10 and HEK.β3 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 (Fig. 2A). BW245C (1 μM) stimulation of HEK 293 and HEL cells resulted in significant increases in cAMP generation relative to vehicle-treated cells (Fig. 2B), thereby confirming functional expression of DP in these cells.

Consistent with previous studies, HEK.α10 cells, co-transfected with Gα_{11}, showed efficient $[Ca^{2+}]_i$ mobilization in response to U46619 (1 μM, Fig. 3A). Whereas BW245C (1 μM) did not stimulate significant increases in $[Ca^{2+}]_i$ mobilization in these cells, it significantly reduced subsequent U46619-induced $[Ca^{2+}]_i$ mobilization (Fig. 3B; compare $\Delta[Ca^{2+}]_i = 158 \pm 6.96$ nM, Fig. 3A versus $\Delta[Ca^{2+}]_i = 12.3 \pm 7.3$ nM, Fig. 3B; $P < 0.0007$) with the IC_{50} for BW245C-mediated inhibition determined to be $3.2 \pm 0.44 \times 10^{-8}$ M. In HEK.β3 cells transfected with Gα_{11}, stimulation with U46619 (1 μM) gave rise to efficient

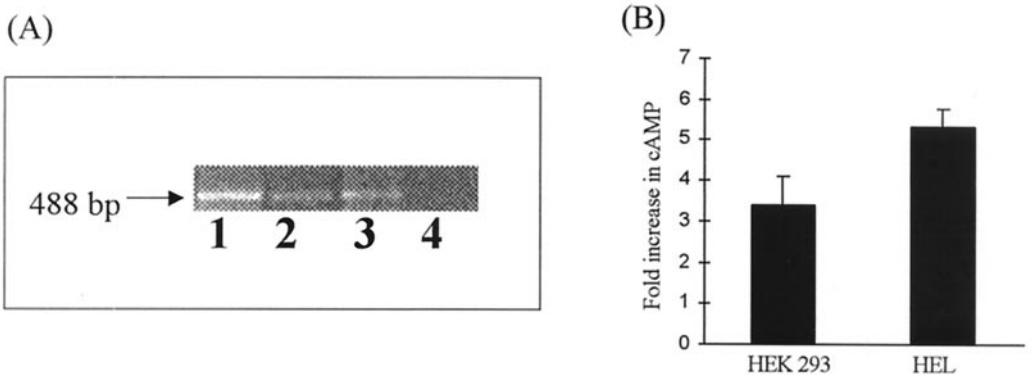

Fig. 2. Analysis of DP expression in HEK 293 cells. (Panel A) RT–PCR analysis of the human DP cDNA (488 bp) amplified from HEK 293 (lane 1) or HEL 92.1.7 (lane 2) cell mRNA. The negative control PCR, where amplification primers were added to the reaction without any template cDNA, is shown in lane 4. 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 μM BW245C or with vehicle HBS for 10 min at 37°. 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 ± 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.

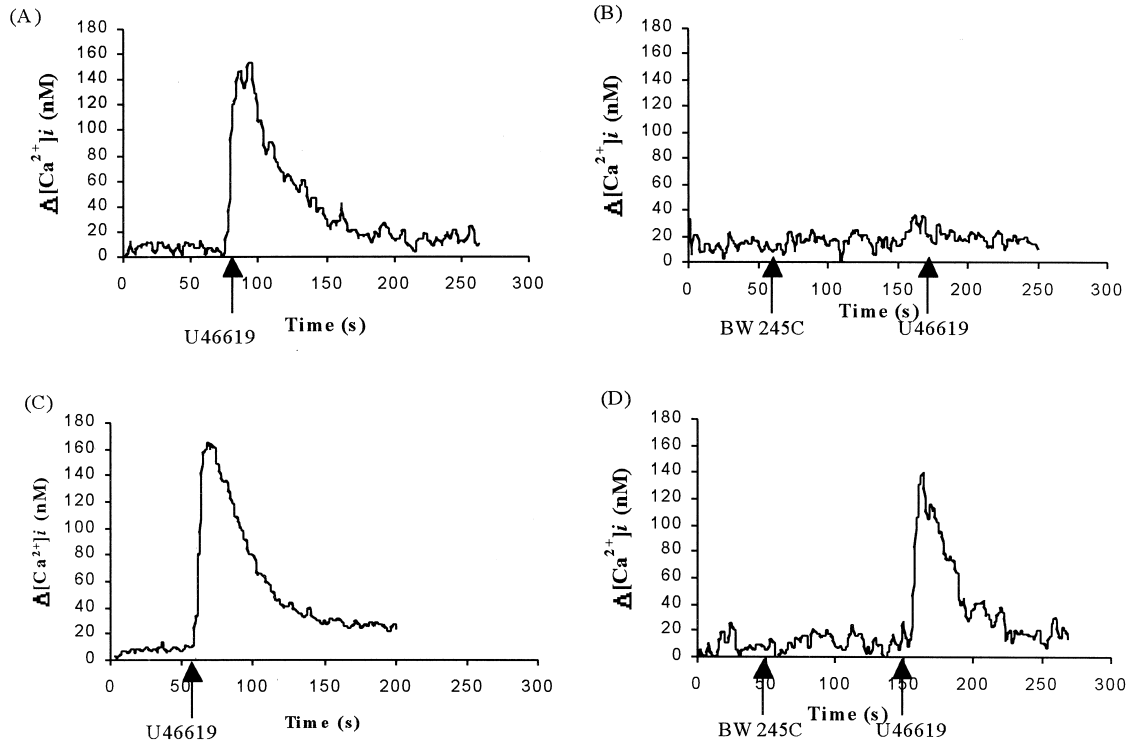

Fig. 3. Effect of BW245C on U46619-induced $[Ca^{2+}]_i$ mobilization in HEK.α10 and HEK.β3 cells. HEK.α10 cells (Panels A and B) and HEK.β3 cells (Panels C and D), transiently co-transfected with $G\alpha_{11}$, were stimulated with 1 μM U46619 (Panels A and C) or with 1 μM BW245C followed by 1 μM U46619 (Panels B and D), respectively. Actual changes in $[Ca^{2+}]_i$ mobilized were: Panel A: (1 μM U46619, $\Delta[Ca^{2+}]_i$ = 158 ± 6.96 nM); Panel B: (1 μM BW245C, $\Delta[Ca^{2+}]_i$ = 0 nM; 1 μM U46619, $\Delta[Ca^{2+}]_i$ = 12.3 ± 7.3 nM); Panel C: (HEK.β3 cells, 1 μM U46619, $\Delta[Ca^{2+}]_i$ = 145 ± 16.4 nM); Panel D: (HEK.β3 cells, 1 μM BW245C, $\Delta[Ca^{2+}]_i$ = 0 nM; 1 μM U46619, $\Delta[Ca^{2+}]_i$ = 138 ± 15.9 nM).

$[Ca^{2+}]_i$ mobilization (Fig. 3C). Exposure to BW245C (1 μM) did not support $[Ca^{2+}]_i$ mobilization (Fig. 3D). However, preincubation of HEK.β3 cells with BW245C at 1 μM did not significantly reduce subsequent U46619-induced $[Ca^{2+}]_i$ mobilization (Fig. 3D; compare $\Delta[Ca^{2+}]_i$ = 145 ± 16.4 nM, Fig. 3C versus $\Delta[Ca^{2+}]_i$ = 138 ± 15.9 nM, Fig. 3D; $P > 0.78$).

To investigate the factors that mediate the DP-induced differential desensitization of the TP isoforms, we examined the effects of preincubation of HEK.α10 cells transfected with $G\alpha_{11}$, 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. Preincubation 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 (Fig. 4A). In contrast, pretreatment of platelets with 10 μM H-89 for 2 min prior to BW245C (1 μM) stimulation almost completely restored subsequent U46619 (1 μM)-mediated $[Ca^{2+}]_i$ mobilization to normal, pre-BW245C levels (compare $\Delta[Ca^{2+}]_i$ = 156 ± 6.35 nM, Fig. 1A versus $\Delta[Ca^{2+}]_i$ = 144 ± 7.5 nM, Fig. 4B; $P > 0.27$). Pretreatment of HEK.α10 cells with GF 109203X (50 nM) for 2 min prior to incubation with BW245C (1 μM) did not alleviate the DP-mediated desensitization in subsequent U46619-induced $[Ca^{2+}]_i$ mobilization (Fig. 4C). However, pretreatment of HEK.α10 cells with H-89 (10 μM) for 2 min prior to incubation with BW245C (1 μM) and then U46619 (1 μM) significantly prevented DP-mediated desensitization of TPα signaling, restoring U46619-induced $[Ca^{2+}]_i$ mobilization to 85% of that generated by 1 μM U46619 only (Fig. 4D; compare $\Delta[Ca^{2+}]_i$ = 158 ± 6.96 nM, Fig. 3A versus $\Delta[Ca^{2+}]_i$ = 138 ± 2.0 nM, Fig. 4D; $P < 0.0005$). In the case of HEK.β3 cells, prior incubation with either GF 109203X (50 nM) or H-89 (10 μM) 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 μM)-mediated cAMP generation was measured in HEK 293 cells in the absence and presence of 10 μM H-89. No significant difference ($P > 0.7$) was observed in cells stimulated in the absence (1 μM BW245C, fold increase in cAMP = 2.29 ± 0.01) or presence (1 μM BW245C, 10 μM 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 IP_3 generation via TPα and TPβ isoforms

To further investigate the differential effects of DP activation on TPα and TPβ signaling, U46619-induced IP_3 generation was measured in HEK.α10 and HEK.β3 cells in

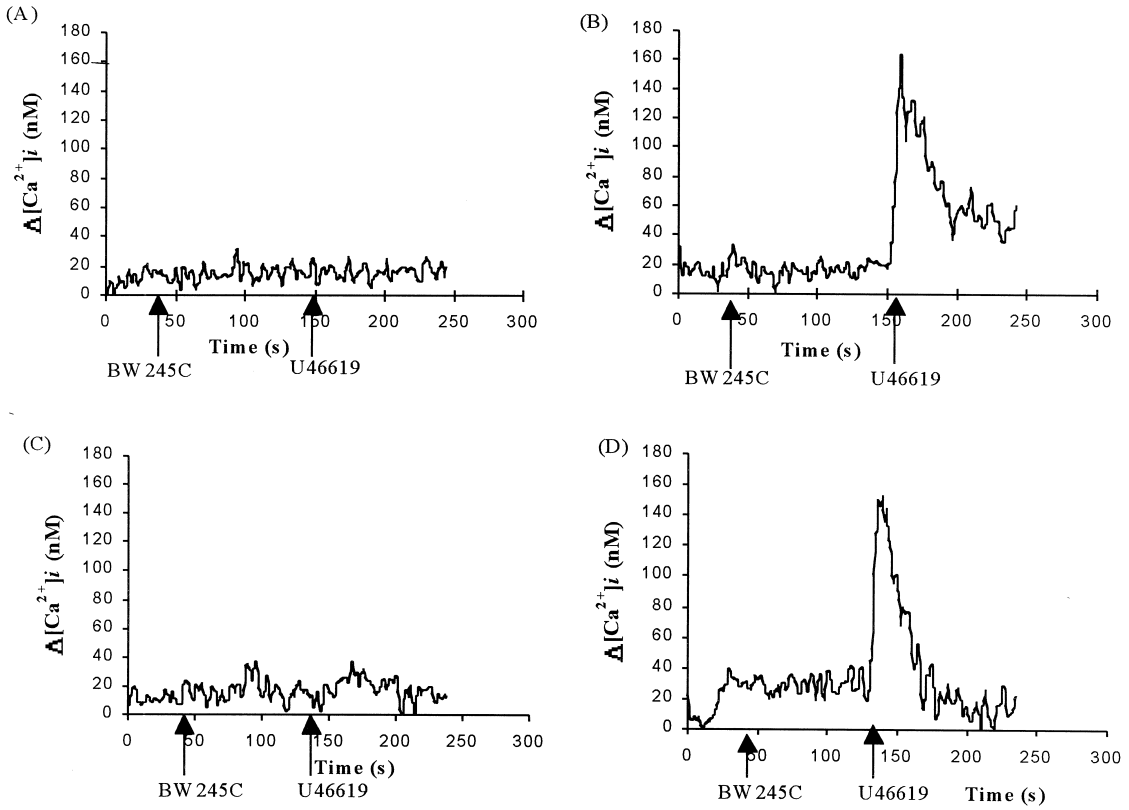

Fig. 4. Effect of BW245C on U46619-induced $[Ca^{2+}]_i$ mobilization in human platelets and in HEK.α10 cells. Human platelets (Panels A and B) or HEK.α10 cells (Panels C and D), transiently co-transfected with Gα_{11}, were preincubated with 50 nM GF109203X (Panels A and C) or 10 μM H-89 (Panels B and D) for 2 min prior to stimulation by 1 μM BW245C and then 1 μM U46619. Actual changes in $[Ca^{2+}]_i$ mobilized were: Panel A: (platelets, 1 μM BW245C, $\Delta[Ca^{2+}]_i$ = 0 nM; 1 μM U46619, $\Delta[Ca^{2+}]_i$ = 0 nM); Panel B: (platelets, 1 μM BW245C, $\Delta[Ca^{2+}]_i$ = 0 nM; 1 μM U46619, $\Delta[Ca^{2+}]_i$ = 144 ± 7.5 nM); Panel C: (HEK.α10 cells, 1 μM BW245C, $\Delta[Ca^{2+}]_i$ = 0 nM; 1 μM U46619, $\Delta[Ca^{2+}]_i$ = 20 ± 9.6 nM); Panel D: (HEK.α10 cells, 1 μM BW245C, $\Delta[Ca^{2+}]_i$ = 0 nM; 1 μM U46619, $\Delta[Ca^{2+}]_i$ = 138 ± 2.0 nM).

the presence or absence of prestimulation with BW245C. Stimulation of HEK.α10 and HEK.β3 cells with U46619 (1 μM) resulted in a 2.2- to 3-fold increase in IP_3 levels (Fig. 5A). Preincubation of HEK.α10 cells with BW245C (1 μM) significantly reduced U46619-mediated IP_3 generation by TPα (Fig. 5A, $P < 0.012$). In contrast, preincubation of HEK.β3 cells with BW245C (1 μM) did not significantly ($P > 0.6$) reduce U46619-mediated IP_3 generation by TPβ (Fig. 5B). Moreover, H-89 (10 μM), but not GF 109203X (50 nM), blocked BW254C-mediated desensitization of TPα signaling (Fig. 5A; $P < 0.03$), but had no effect on TPβ signaling (data not shown). Consistent with previous reports [37], stimulation of HEK 293 cells with U46619 or HEK.α10 and HEK.β3 cells with BW245C alone failed to generate any increase in IP_3, further indicating that endogenous DP receptors in HEK 293 cells do not couple to PLC (Fig. 5B and data not shown).

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

We then investigated the effect of DP-agonist stimulation on signaling by $TP^{\Delta 328}$, 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.$TP^{\Delta 328}$ cells, co-transfected with Gα_{11}, with U46619 (1 μM) resulted in efficient $[Ca^{2+}]_i$ mobilization (Fig. 6A), while BW245C (1 μM) did not (Fig. 6B). In contrast to HEK.α10 cells, BW245C (1 μM) exposure of HEK.$TP^{\Delta 328}$ cells did not reduce subsequent $[Ca^{2+}]_i$ mobilization in response to U46619 (Fig. 6B; compare $\Delta[Ca^{2+}]_i$ = 161 ± 13.2 nM, Fig. 6A versus $\Delta[Ca^{2+}]_i$ = 163 ± 7.9 nM, Fig. 6B; $P > 0.91$).

We have previously constructed a variant TPα isoform, $TP\alpha^{S329A}$, in which Ser^{329} was mutated to Ala^{329}, thereby disrupting the potential PKA phosphorylation site ($RPRS^{329}LSL$) unique to TPα [38]. Simulation of HEK.$HATP\alpha^{S329A}$ cells transfected with Gα_{11} with U46619 (1 μM) led to efficient $[Ca^{2+}]_i$ mobilization (Fig. 6C). Preincubation with BW245C (1 μM) did not result in $[Ca^{2+}]_i$ mobilization and also did not reduce subsequent U46619-induced $[Ca^{2+}]_i$ mobilization (Fig. 6D; compare $\Delta[Ca^{2+}]_i$ = 249 ± 5.2 nM, Fig. 6C versus $\Delta[Ca^{2+}]_i$ = 260 ± 2.9 nM, Fig. 6D; $P > 0.15$). Similarly, stimulation of HEK.$HATP\alpha^{S329A}$ cells with U46619 (1 μM) resulted in

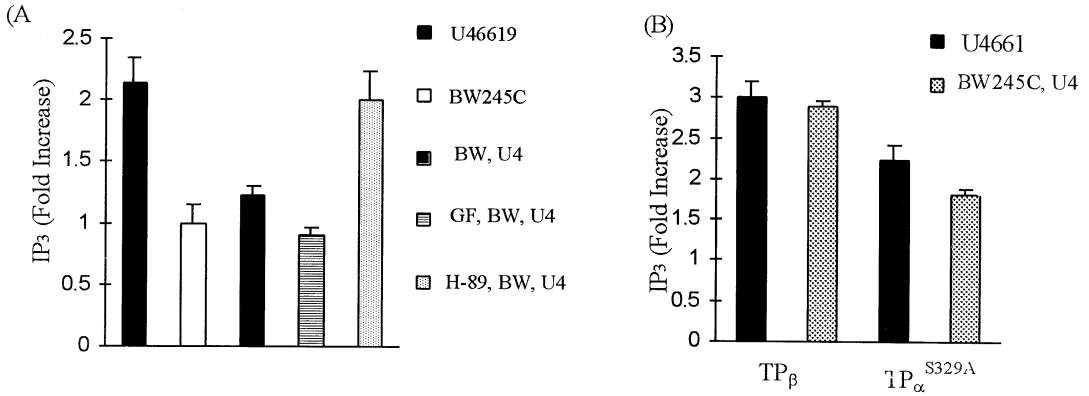

Fig. 5. Effect of BW245C on U46619-mediated IP_3 generation in HEK.α10, HEK.β3, and TPα^{S329A} cells. HEK.α10 (Panel A) and HEK.β3 or HEK.TPα^{S329A} (Panel B) cells, transiently co-transfected with Gα_{11}, were stimulated at 37° with 1 μM U46619 for 1 min (U46619), 1 μM BW245C for 1 min (BW245C), or 1 μM BW245C for 1 min followed by 1 μM U46619 for 1 min (BW, U4). Alternatively, cells were preincubated for 5 min with 50 nM GF 109203X prior to stimulation by 1 μM BW245C for 1 min followed by 1 μM U46619 for 1 min (GF, BW, U4) or with 10 μM H-89 for 5 min prior to stimulation with 1 μM BW245C for 1 min followed by 1 μM U46619 for 1 min (H-89, BW, U4). In each case, basal levels of IP_3 were determined by exposing cells to the vehicle HBS under identical reaction conditions. Levels of IP_3 produced in response to ligand relative to vehicle-treated cells were expressed as fold stimulation of basal (fold increase in IP_3 ± SEM; N = 3, Panels A and B). The basal level of IP_3 in HEK.α10 cells was 0.39 ± 0.09 nmol/mg; in HEK.β3 cells 0.32 ± 0.08 nmol/mg; and in HEK.TP$^{\Delta 328}$ cells 0.27 ± 0.06 nmol/mg.

a 2.2-fold increase in IP_3 levels (Fig. 5B). Preincubation of HEK.HATPα^{S329A} cells with BW245C (1 μM) did not significantly ($P > 0.1$) reduce U46619-mediated IP_3 generation by TPα^{S329A} (Fig. 5B). Moreover, neither H-89 (10 μM) nor GF 109203X (50 nM) had any effect on TPα^{S329A} signaling (data not shown).

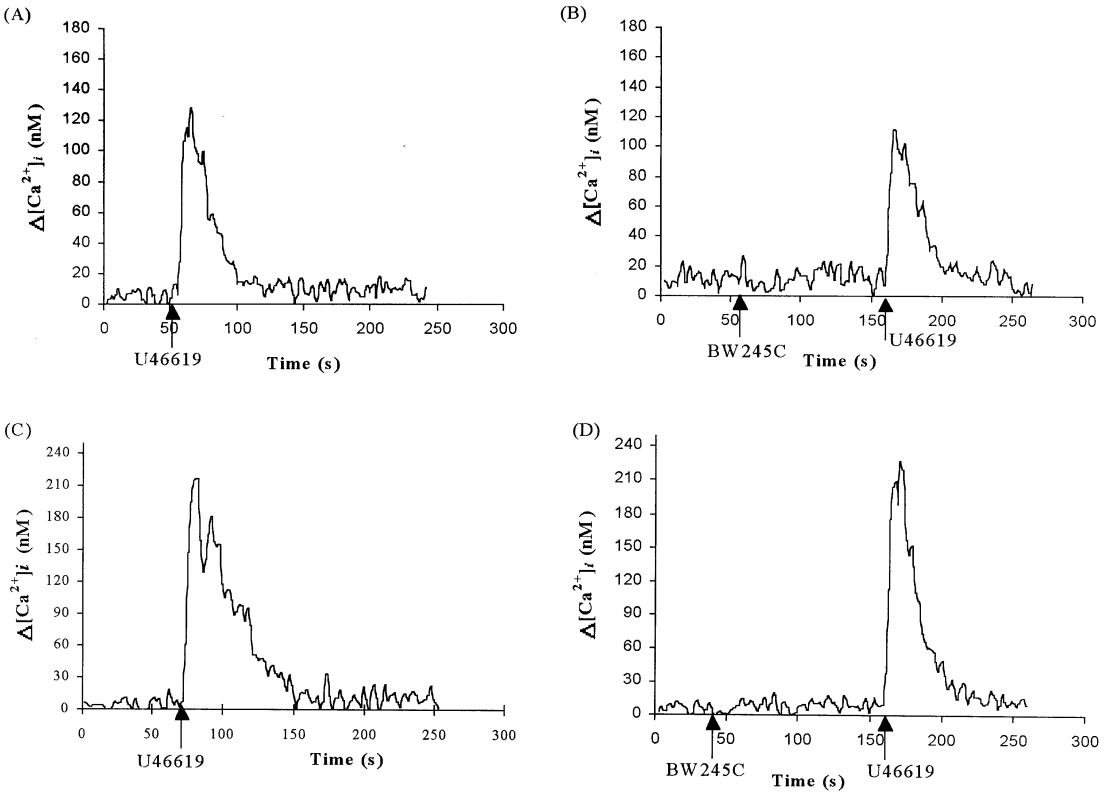

Fig. 6. Effect of BW245C on U46619-induced $[Ca^{2+}]_i$ mobilization in HEK.TP$^{\Delta 328}$ and HEK.TPα^{S329A} cells. HEK.TP$^{\Delta 328}$ cells (Panels A and B) and HEK.TPα^{S329A} cells (Panels C and D), transiently co-transfected with Gα_{11}, were stimulated with either 1 μM U46619 (Panels A and C) or 1 μM BW245C followed by 1 μM U46619 (Panels B and D). Actual changes in $[Ca^{2+}]_i$ mobilized were: Panel A: (HEK.TP$^{\Delta 328}$ cells, 1 μM U46619, $\Delta[Ca^{2+}]_i$ = 161 ± 13.2 nM); Panel B: (HEK.TP$^{\Delta 328}$ cells, 1 μM BW245C, $\Delta[Ca^{2+}]_i$ = 0 nM; 1 μM U46619, $\Delta[Ca^{2+}]_i$ = 163 ± 7.9 nM). Panel C: (1 μM U46619, $\Delta[Ca^{2+}]_i$ = 249 ± 5.2 nM); Panel D: (1 μM BW245C, $\Delta[Ca^{2+}]_i$ = 0 nM; 1 μM U46619, $\Delta[Ca^{2+}]_i$ = 260 ± 2.9 nM).

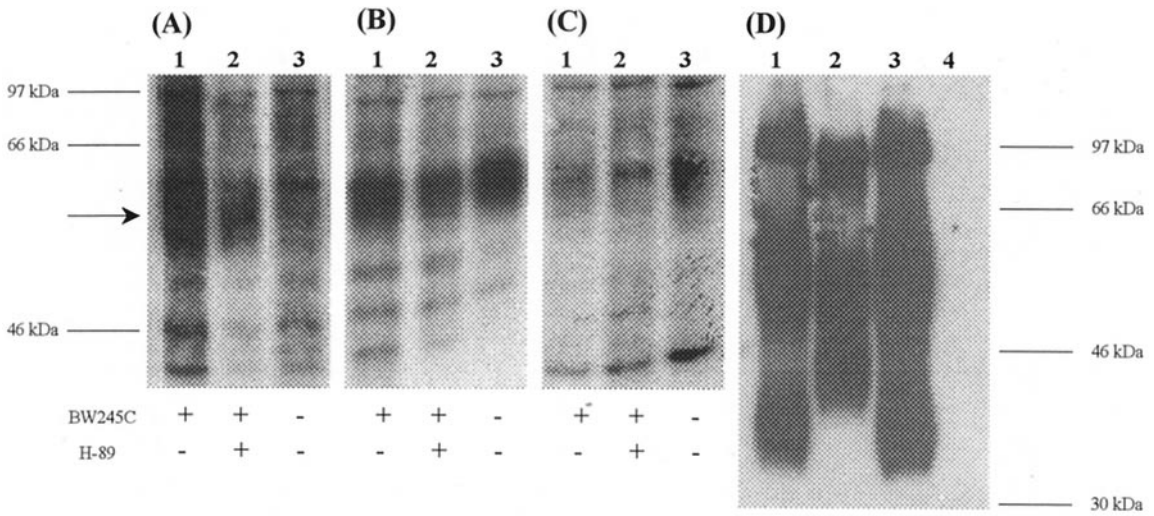

Fig. 7. DP-mediated phosphorylation of TPα, TPβ, and TPα^{S329A}. Panels A–C: HEK.HATPα (Panel A), HEK.HATPβ (Panel B), or HEK.HATPα^{S329A} (Panel C) cells were labelled with [^{32}P]orthophosphate in the presence (Panels A, B, and C, lane 2) or absence (Panels A, B, and C, lane 1) of H-89 (10 μM), or with the vehicle HBS (Panels A, B, and C, lane 3). Cells were then incubated for 10 min with BW245C (1 μM) (Panels A, B, and C, lanes 1 and 2) or vehicle (Panels A, B, and 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: TPα, 1 μM BW245C, 4.8-fold; 10 μM H-89, 1 μM BW245C, 1.8-fold; TPβ, 1 μM BW245C, 1.2-fold; 10 μM H-89, 1 μM BW245C, 0.9-fold; TPα^{S329A}, 1 μM BW245C, 0.7-fold; 10 μM H-89, 1 μM BW245C, 0.8-fold. Panel D: HEK 293 control cells (lane 4) or HEK 293 cells over-expressing HA-epitope tagged TPα (lane 1), TPβ (lane 2), or TPα^{S329A} (lane 3) were immunoprecipitated, subjected to Western blotting followed by 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 TPα. These data are representative of three independent experiments.

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 that were previously confirmed to correspond to the non-glycosylated and glycosylated forms, respectively, of TPα and TPα^{S329A} (Fig. 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 (Fig. 7D, lane 2). No proteins were immunoprecipitated from control HEK 293 cells (Fig. 7D, lane 4). Pretreatment 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 (Fig. 7A, lanes 1 and 3), which in turn was blocked by pretreatment with H-89 (Fig. 7A, lane 2). Pretreatment of HEK.HATPβ cells with BW245C (1 μM) did not increase phosphorylation of TPβ relative to vehicle-treated cells (Fig. 7B, lanes 1 and 3). Pretreatment of HEK .HATPβ cells with H-89 (10 μM) had no significant effect on the basal level of TPβ phosphorylation (Fig. 7B, lane 2). Prior incubation of HEK.HATPα^{S329A} cells with BW245C (1 μM) did not result in any significant increase in TPα^{S329A} phosphorylation relative to vehicle-treated cells (Fig. 7C, lanes 1 and 3). Pretreatment of HEK.HA:TPα^{S329A} cells with H-89 (10 μM) made no significant difference to the basal level of TPα^{S329A} phosphorylation (Fig. 7C, lane 2). Consistent with previous studies [38], stimulation of cells with U46619 (1 μM, 10 min) led to 5- to 7-fold increases in the phosphorylation of TPα, TPβ, and TPα^{S329A}, confirming that each of these receptors is subject to homologous desensitization (data not shown). These studies confirm that TPα, in contrast to TPβ, is subject to DP-mediated desensitization at a PKA-sensitive site located at S^{329} within its unique C-tail.

4. Discussion

Individually, both TXA_2 and PGD_2 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. TXA_2 is a potent stimulator of platelet aggregation and constrictor of vascular smooth muscle, whereas PGD_2, like prostacyclin, inhibits platelet aggregation [3]. Thus, the actions of PGD_2 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, PGD_2, like TXA_2, is synthesised by plate-

lets 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 TXA_2 and PGD_2 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 TXA_2 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 $[Ca^{2+}]_i$ mobilization. Moreover, this occurred in a dose-dependent manner with the IC_{50} 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 that 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 $[Ca^{2+}]_i$ mobilization in response to BW245C, consistent with studies in HEK 293(EBNA) cells [37]. Prestimulation of endogenous DPs in HEK.α10 cells, stably transfected with the TPα isoform, significantly desensitized subsequent TP (U46619)-mediated $[Ca^{2+}]_i$ mobilization and IP_3 generation and this occurred in a dose-dependent manner with the IC_{50} for BW245C determined to be $3.2 \pm 0.44 \times 10^{-8}$ M. In contrast, signaling by TPβ was unaffected by prestimulation of endogenous DP expressed in HEK.β3 cells, indicating that the TPα, but not TPβ, is a target for DP-mediated cross-desensitization of TP responses.

Preincubation of platelets or HEK.α10 cells with the PKA inhibitor H-89 almost completely blocked BW245C-mediated inhibition of TP signaling. Failure to completely desensitize TPα in HEK.α10 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 TPβ signaling, and the PKC inhibitor GF 109203X had no appreciable effect on DP inhibition of TP signaling in platelets or on TPα or TPβ signaling in HEK 293 cells. Whereas stimulation of HEK.$TP^{\Delta 328}$ cells with U46619 led to efficient mobilization of $[Ca^{2+}]_i$, $TP^{\Delta 328}$ was not subjected to BW245C-mediated desensitization. These data indicate that BW245C-induced desensitization of TPα 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 TPα identified the presence of a unique consensus PKA phosphorylation site within the sequence RPRSLSL, where S^{329} was predicted to represent the target residue for phosphorylation [38,52]. While stable cell lines over-expressing $TP\alpha^{S329A}$ exhibited identical U46619-mediated intracellular signaling to that of the wild-type TPα, U46619-mediated signaling by $TP\alpha^{S329A}$ was insensitive to prestimulation of DP with BW245C. Whereas platelets and other hematopoietic cells are reported to express $G\alpha_q$ and $G\alpha_{16}$, they are reported not to express significant levels of $G\alpha_{11}$ [53]. Substitution of $G\alpha_{11}$ with $G\alpha_q$ also supported DP-mediated desensitization of TPα but not TPβ, $TP^{\Delta 328}$, or $TP\alpha^{S329A}$ 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 TPα, TPβ, or $TP\alpha^{S329A}$ 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 TPα but not TPβ or $TP\alpha^{S329A}$. Moreover, the PKA inhibitor H-89 blocked BW245C-mediated phosphorylation of TPα. Taken together, these studies confirm that TPα, but not TPβ, is subject to DP-mediated desensitization and that this desensitization involves direct PKA phosphorylation of TPα, where Ser^{329} is the target residue for DP-mediated phosphorylation of TPα.

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 TPα isoform that is subject to counter-regulation by the anti-platelet prostanoids prostacyclin and PGD_2. 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 TPα may be the TP isoform physiologically relevant to regulated vascular hemostasis. Consistent with this Habib *et al.* [45] detected immunoreactive protein corresponding to TPα, but not TPβ, within platelets and established that TPα is phosphorylated in platelets in response to the TP agonist [1*S*-

[1α,2α(Z),3β(1E,3S*),4α]]-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 TPα may be subject to homologous [45] and heterologous [38] desensitization, they also suggest that the TPβ isoform is not subject to this type of regulation. Consistent with this, we have also established that signaling by the TPα, but not the TPβ, isoform was subject to partial forskolin-induced desensitization of U46619-mediated signaling (data not shown). On the other hand, Parent *et al*. [54] established that the TPβ, but not the TPα, 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 [16,23], supporting the notion that the TP receptors may indeed co-exist within the same cell type, albeit at different levels. However, our data shed further light that signaling by the TP isoforms is subject to differential heterologous regulation and indicate that TXA_2-mediated signaling by TPα may be counter-regulated by the inhibitory prostanoids prostacyclin and PGD_2; on the other hand, TXA_2-mediated signaling by the TPβ isoform remains unaffected by these autocoids. The current study specifically considered the roles of the prostanoids TXA_2 and PGD_2 within the vascular system; however, the findings have wider implications with respect to the possible counter-regulation of TXA_2 and PGD_2 responses in other cell types, for example within the brain where both TP and DP protein expression and mRNA expression [2,16,55,56] are widely detected. The findings reported here also imply that the TPβ isoform is not subject to PKA-dependent heterologous desensitization, and such lack of desensitization may have important physiological consequences that are currently unappreciated.

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