Thromboxane A_2 signalling in humans: *a 'tail' of two receptors.*

B. Therese Kinsella*

Department of Biochemistry, Conway Institute of Biomolecular and Biomedical Research, Merville House, University College Dublin, Belfield, Dublin 4, Ireland.

*Correspondence: Tel: 353-1-7161507; Fax 353-1-2837211;
Email: Therese.Kinsella@UCD.ie

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Abbreviations: 8-epi$PGF_{2\alpha}$, 8-epi prostaglandin $F_{2\alpha}$; ERK, extracellular signal-regulated protein kinase; GPCR, G protein coupled receptor; MAPK, mitogen activated protein kinase; PG, prostaglandin; PK, protein kinase; PTX, pertussis toxin; VSM, vascular smooth muscle; TXA_2, thromboxane A_2; TP, TXA_2 receptor.

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Abstract:

Since its discovery in 1975, we now have a wealth of knowledge relating to the biochemical, pharmacological and physiologic actions of thromboxane (TX) A_2 and its related metabolites. These molecular insights have been greatly expedited by the molecular cloning and characterisation of a complementary (c) DNA for the human TXA receptor, now termed T Prostanoid or TP receptor, from a megakaryocytic / placental cDNA library in 1991 and later through the discovery of a cDNA encoding a second isoform of the human TP receptor in 1994. The requirement for two TP receptors in primates, but not in other species thus far investigated, is unclear but points to potential species-specific physiologic differences. In this review, I will describe some recent advances in the research field of TXA_2/TP receptor signalling, focussing particularly on studies pertaining to the human TP receptor isoforms.

Introduction:

The prostanoids mediate a diversity of cellular processes under various physiologic and patho-physiologic settings [1,2]. Derived mainly from arachidonic acid liberated from membrane phospholipids, through the actions of phospholipase A_2, the 5 primary prostanoids include the prostaglandins (PGs), PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 (prostacylin) and the thromboxanes (TXs) including TXA_2. PGG/H synthase 1 and 2, also known as cyclooxygenase 1 and 2 (COX1 and 2), catalyse the first enzymatic step in the conversion of arachidonic acid into PGG_2, and onward into the common precursor PGH_2, and it is this enzymatic step that is the target for inhibition by aspirin and other non steroidal anti-inflammatory inhibitory drugs (NSAIDs) and by the more recently developed COX1/2 selective inhibitors [1, 3-5]. PGH_2 is thereafter converted to the various prostanoids, in a cell and tissue specific manner, through the actions of their respective PG and TXA synthases [1].

The prostanoids TXA_2 and PGI_2 (prostacyclin) play key yet opposing roles in the local control of vascular hemostasis [1,6,7]. TXA_2 was first described by Hamberg *et al*., in 1975 as an unstable platelet-aggregating factor with a novel bicyclic oxane ring and with a half-life of 30 s [8]. Within the vascular system, it is a potent stimulator of platelet aggregation and causes vasoconstriction; on the other hand, PGI_2 inhibits platelet aggregation and causes vasodilation [1,6]. TXA_2 also stimulates PGI_2 release from endothelial cells [9] and, under certain clinical or pathological situations, mediates mitogenic and / or hypertrophic responses in vascular smooth muscle (VSM) [10, 11]. Perturbations in the levels of these two prostanoids, their synthases or their receptors have been implicated in a number of cardiovascular disorders including myocardial infarction, unstable angina, atherosclerosis, pregnancy-induced hypertension and ischemic heart disease [12-16]. Moreover, a number of genetic bleeding disorders have been described in humans where the individuals' platelets were unresponsive to TXA_2 [17]. Both TXA_2 and PGI_2 signal through their signature receptors, each members of the G protein coupled receptor (GPCR) superfamily [1,2,6,7]. TXA_2 interacts with the TXA_2 receptor, also termed TP or T Prostanoid receptor, whereas PGI_2 interacts with the PGI_2 receptor, also termed IP or I Prostanoid receptor [18,19]. Greater understanding of the molecular mechanisms governing the interaction between TXA_2, PGI_2 and their receptors as well as a greater understanding of the interplay between their signal transduction pathways should lead to a greater appreciation of their involvement in vascular hemostasis under normal and patho-physiologic conditions. In this review, I will describe some recent advances in the field of TXA_2/TP signalling and how they may impinge on our current understanding of prostanoid- regulated vascular hemostasis.

Expression of the TP isoforms:

A cDNA for the human TXA_2 receptor (TP) was originally cloned from placenta and the platelet like MEG-01 cell line [20] and since then cDNAs for TPs from a number of species have been cloned and characterised [21-25]. All TPs are predicted to share the seven α-helical transmembrane domain arrangement typical of other members of the GPCR superfamily [26]. Despite earlier, extensive pharmacological and biochemical evidence suggesting the existence of inter and intra-species variants of TPs [27-30], genomic cloning confirmed the existence of a single TP gene which, in humans, is located on chromosome 19p13.3 [31]. Despite this, Raychowdhury *et al*., [32] later isolated a cDNA encoding a second form (isoform) of the TP from a human umbilical vein endothelial cell (HUVEC) cDNA library. Thus, in humans, there are 2 receptors for TXA_2, termed TPα and TPβ [20,31,32], where the TP originally cloned from platelet/placenta is commonly referred to as the TPα isoform and that from HUVECs referred to as the TPβ isoform. The TP isoforms are identical for their N-terminal 328 amino acid (aa) residues but differ exclusively in their carboxyl terminal cytoplasmic (C-tail) domains such that TPα has 15 amino acid residues within its unique C-tail sequence and TPβ has 79 residues within its C-tail sequence [20, 31, 32; **Figure 1**]. TPα and TPβ are encoded by the single TP gene, on chromosome 19p13.3 [31], and arise by a novel differential splicing mechanism within Exon 3 whereby nucleotides 984 – 1642 of the TPα mRNA behave as an Intron (Intron 2b) within the TPβ mRNA [20, 31, 32; **Figure 1**]. Reverse transcriptase polymerase chain reaction (RT PCR) experiments indicated that HUVECs express only TPβ [32] whereas human platelets were reported to express both TPα and TPβ isoforms [33]. The physiologic significance for the existence of 2 receptors for TXA_2 in humans, but not in other species thus far investigated [21-25], is currently unknown but is an area of extensive research interest within my laboratory.

Thus, as an essential prerequisite into studies investigating the potential differential roles of the TPα and TPβ receptors, we initially investigated the expression and tissue distribution of the TP isoforms in cells and tissues of relevance to TXA_2 biology [34]. Whereas Northern blot analyses had previously confirmed the existence of TP mRNA in human MEG-01 cells, placenta and lung [20], in human erythroleukemic (HEL) cells [35-37], these studies did not discriminate between the expression of the TPα and TPβ isoforms. Thus, through a series of studies involving RT PCR, Southern blot and phosphorimage analyses and radioligand binding studies, we found that both TPα and TPβ were expressed in the 17 tissues and cell types studied, with rare exceptions [34]. Whereas the levels of TPα expression predominated and were similar in most of the cell /tissue types examined, extensive differences in the levels of TPβ expression were

observed [34]. Thus, the relative expression of TPα / TPβ varied considerably due to extensive differences in the level of TPβ expression. Most strikingly, contrary to previous reports that HUVECs expressed only TPβ mRNA sequences [32], our studies established that primary HUVECs expressed only low levels of TPβ but rather expressed 6-fold greater levels of TPα than TPβ mRNA [34]. Expression of TP mRNAs in the various cell / tissue types examined correlated with protein expression, as assessed by radioligand binding using the selective TP antagonist [^{3}H]SQ29,548 [34]. Additional immunofluorescence studies, employing TP isoform specific antibodies directed to peptide sequences within the unique C-tail domains of TPα and TPβ, also confirmed the expression and cellular localisation of the TP receptors [38,39]. Taken together, these studies investigating the expression and tissue distribution of the TPα and TPβ receptors indicate that they are subject to differential expression and regulation [34]. The molecular basis of this differential expression is currently unknown and whether it is solely attributable to cell/tissue specific differential splicing, giving rise to the TPα and TPβ mRNA's, or whether it may be due to other factors, such as alternative promoter utilisation [31,40,41], remains to explored at the molecular level. Moreover, whether there is any correlation between the differential levels of TPα versus TPβ expression and TXA_2 associated disease status is currently unknown but, given that the molecular tools are now in place, this important and interesting question could be readily addressed.

TP isoform signalling:

As previously stated, the TP isoforms are identical for their N-terminal 328 aa residues but differ exclusively in their C-tail domains [20,32; **Figure 1**]. From structure/ function relationship studies carried out with other prototypical GPCRs, including the β_2 adrenergic receptor [26,42,43], it is widely accepted that while the C-tail domains of GPCRs do not appreciably influence ligand binding, they can indeed play an essential role in determining the specificity and / or efficiency of receptor: heterotrimeric G protein coupling and effector regulation, and may also play an essential role in GPCR desensitization following ligand activation [26,42,43]. The major mode of signalling of TXA_2 and its receptor is activation of the β isozymes of phospholipase (PL) C leading to phosphatidylinositol (PI) turnover and release of calcium from intracellular stores ($[Ca^{2+}]_i$) [44]. Using a variety of approaches involving either reconstitution studies [45,46], co-purification or co-immunoprecipitations [47-49], photo-cross linking studies with GTP analogs [50] or co-expression studies [51-54], various investigators have proposed that the platelet TPs might couple to the G proteins G_q, G_{12}, G_{13}, G_{16} and G_{i2}. In studies involving the cloned receptor, co-expression of the TPα isoform with either G_q or G_{13} increased its affinity for

I-BOP in COS-7 cells [51]. It has also been demonstrated that TPα can functionally couple to both G_q and G_{11} following stimulation with the selective TXA_2 mimetic, U46619 to mobilize $[Ca^{2+}]_i$ [54,55]. Recently, Hirata *et al*., [33] demonstrated that the TP isoforms over-expressed in Chinese hamster ovary cells oppositely regulate adenylyl cyclase activity with TPα activating adenylyl cyclase, through $G\alpha_s$ and TPβ inhibiting it, through $G\alpha_i$ [33], suggesting a possible role for the C-tail of TP in determining G protein specificity. Moreover, TPα, but not TPβ, mediates agonist activation of G_h, the novel high molecular weight G protein [56-59], leading to PLC activation and PI turnover [60].

Whereas many of the latter reported studies have implicated various G protein α subunits in mediating TP activation [45-55] and the latter studies [33,60] indicate that the TP isoforms may indeed differentially couple to G_s, G_i and G_h, these studies had not assessed possible differential coupling of TPα and TPβ to G_q or G_{12} family members. Thus, we investigated the specificity of TP signalling focussing on members of the G_q and G_{12} families [61]. Moreover, we investigated the requirement of a C-tail *per se* in mediating TP: G protein coupling and effector activation [61].

Using mammalian human embryonic kidney (HEK) 293 cells as a suitable host, HEK.TPα10 and HEK.TPβ3 cell lines exclusively over-expressing the TPα and TPβ isoforms, respectively, were established [61-63]. Radioligand binding studies indicated high level expression of TPα and TPβ (B_{max}) in their respective stable cell lines and, consistent with other reports [32, 33], confirmed that the TP isoforms did not exhibit any difference in their affinity (K*d*) for SQ29,548 [61-63]. Both TPα and TPβ exhibited efficient U46619-mediated $[Ca^{2+}]_i$ mobilization which was completely dependent on co-expression of a G protein alpha (α) subunit [54, 61-63]. In control cells or in the absence of a co-expressed Gα, neither TPα or TPβ exhibited an appreciable rise in $[Ca^{2+}]_i$ in response to U46619 stimulation; however, both TPα and TPβ exhibited efficient U46619-induced $[Ca^{2+}]_i$ mobilization in their respective cell lines co-transfected with $G\alpha_{11}$ and $G\alpha_{16}$ [61; **Figure 2A**]. Similarly, both TPα and TPβ exhibited a U46619-mediated rise in IP_3 generation in cells co-transfected with $G\alpha_{11}$ and $G\alpha_{16}$, but not in control cells [61]. Thus, both TPα and TPβ couple to the G_q family members $G\alpha_{11}$ and $G\alpha_{16}$ to mediate activation of PLCβ leading to increases in IP_3 generation and concomitant rises in $[Ca^{2+}]_i$ [61].

In similar studies investigating TP coupling to G_{12} family members, both TPα and TPβ exhibited efficient U46619-induced Ca^{2+} mobilization in cells co-transfected with $G\alpha_{12}$ but not in cells co-transfected with the vector pCMV5, serving as a control [61; **Figure 2B**]. However,

unlike that previously observed with G_q members, neither TPα nor TPβ exhibited U46619-induced rises in IP_3 generation in cells co-transfected with $G\alpha_{12}$. These data indicated that G_{12} subfamily members did not mediate TP activation of PLCβ isozymes and therefore that the source of Ca^{2+} mobilization in the presence of G_{12} members was not from IP_3-operated intracellular $[Ca^{2+}]_i$ stores [61]. In follow up studies, [8-(*N,N*-diethylamino)-octyl-3,4,5-trimethoxybenzoate, hydrochloride] (TMB-8), an antagonist of IP_3-operated $[Ca^{2+}]_i$ stores, had no effect on Ca^{2+} mobilization by either TP isoform in the presence of G_{12} [61]. Furthermore, Verapamil, an inhibitor of voltage sensitive L-type Ca^{2+} channels, reduced $G\alpha_{12}$ mediated Ca^{2+} mobilization by TPα and TPβ indicating that the TP isoforms may mediate opening of L-type channels in a $G\alpha_{12}$ dependent signalling mechanism [61]. Taken together, these studies investigating the signalling by the TP isoforms indicate that both TPα and TPβ couple to both the G_q and G_{12} family with no apparent isoform specific differences in their signalling behaviour.

To further investigate the role of the C-tail *per se* in signalling by the TP receptors, we established a stable cell line over-expressing $TP^{\Delta 328}$, a deletion mutant of TPα and TPβ devoid of their divergent residues and, therefore, contains only those residues common to both TP isoforms. Consistent with the lack of involvement of the C-tail in influencing ligand binding, $TP^{\Delta 328}$ exhibited identical radioligand binding to that of the wild type TP receptors [61]. However, in the absence of a co–transfected G protein, $TP^{\Delta 328}$ signalled more efficiently and mediated significantly greater U46619-induced $[Ca^{2+}]_i$ mobilization than did either the wild type TPα or TPβ receptors [61; **Figure 2C**]. However, unlike that previously observed for the wild type TP receptors, $TP^{\Delta 328}$ exhibited an impaired ability to couple to co-transfected G_q or G_{12} members as co-expression of either $G\alpha_{11}$, $G\alpha_{16}$ or $G\alpha_{12}$ did not augment U46619-induced $[Ca^{2+}]_i$ mobilization by $TP^{\Delta 328}$[61; **Figure 2D**]. Based on these observations, it appears that the C-tail region of the TP receptors does not act as a major determinant of G protein coupling specificity to members of the G_q or G_{12} families but may, however, act as a determinant of G protein coupling efficiency.

In view of the fact that TP isoforms were reported to oppositely regulate adenylyl cyclase activity [33], we extended our studies to investigate whether $TP^{\Delta 328}$ coupled to $G\alpha_s$ or $G\alpha_i$ to activate or inhibit adenylyl cyclase, respectively. Moreover, we investigated the effect of co-expression of $G\alpha_s$ or $G\alpha_i$ on U46619-induced second messenger generation by $TP^{\Delta 328}$. Similar to the TPα isoform, $TP^{\Delta 328}$ mediated activation of adenylyl cyclase to bring about increases in cAMP generation but, unlike TPβ, it failed to couple to $G\alpha_i$. However, in the absence of co-transfection of $G\alpha_s$, cAMP generation by $TP^{\Delta 328}$ was significantly greater than that of the wild-

type TPα. Over-expression of $G\alpha_S$, significantly augmented cAMP generation by TPα but had no effect on cAMP generation by $TP^{\Delta 328}$ in response to U46619 [61]. Taken together, these data indicate that whilst the C-tail *per se* may not determine G-protein specificity to members of G_q or G_{12} family, it may play a role in determining G_s versus G_i coupling and it may be necessary for controlled, efficient G protein coupling and intracellular signalling, acting as a determinant of G protein coupling efficiency.

Isoprostanes and TP receptor activation:

In addition to the prostanoids, arachidonic acid may also be metabolised by free radical induced mechanisms to generate a novel class of lipid mediators referred to as the isoprostanes (iPs). The isoprostanes were originally discovered in 1990 by Morrow *et al*., [64] and are now believed to act as potent mediators of oxidative injury [64-68]. As such, the isoprostanes are mainly generated from arachidonic acid non-enzymatically and are synthesised *in situ* in phospholipids and then released through the actions of phospholipases, such as phospholipase A_2 [64,65]. A wide range of prostanoid-like isoprostanes is generated including the $PGF_{2\alpha}$-, the PGD_2-, and PGE_2- series [64,69]. The F_2 series are the most common and one of these compounds 8-epi $PGF_{2\alpha}$, also recently termed $iPF_{2\alpha}$-III [69], is the most abundant isoprostane generated in situations of oxidative injury [64-69]. More recently, similar free-radical derived isomers of other polyunsaturated fatty acids have been described [69,70]. Generation of 8-epi $PGF_{2\alpha}$ can be readily demonstrated experimentally in animal models of free radical injury, such as following administration of diaquat or carbon tetrachloride (CCl_4) where it is generated in abundance in a COX independent manner [64,65]. Moreover, circulating isoprostane levels are elevated in several disease states involving oxidative stress, including atherosclerosis, chronic pulmonary disease, Alzheimer's disease and diabetes mellitus [69,71].

Among its biologic actions, 8-epi $PGF_{2\alpha}$ is a potent vascular and renal vasocostrictor and is a potent stimulator of vascular smooth muscle (VSM) mitogenesis. In platelets, it induces shape change (1 – 10 μM) and at higher concentrations (100 μM), it induces reversible, but not irreversible, aggregation [65] and may augment the actions of weaker platelet agonists, such as collagen, ADP and arachidonic acid. Most surprisingly, it was found that many of the actions of 8-epi $PGF_{2\alpha}$ were blocked by the highly selective TP antagonist SQ29,548 [65,72] leading to the suggestion that this isoprostane may act as an alternative ligand for TP(s) other than TXA_2 itself. However, it was also suggested that 8-epi $PGF_{2\alpha}$ may have partial agonist activity mediated through the platelet TPs [73]. Moreover, it was also suggested that in VSM, 8-epi $PGF_{2\alpha}$ may act

at receptor sites related to but distinct from TPs [74,75]. Thus, in view of the controversy surrounding the actions of 8-epi $PGF_{2\alpha}$, we sought to address whether the TP receptors indeed mediate the actions of 8-epi $PGF_{2\alpha}$ in human platelets and in mammalian cell lines that over-express the human TP isoforms.

In human (h.) platelets, both the TXA_2 mimetic U46619 and 8-epi $PGF_{2\alpha}$ mediated efficient mobilization of $[Ca^{2+}]_i$; however, the magnitude of the response to 8-epi $PGF_{2\alpha}$ activation (58.8 ± 13.5 nM $[Ca^{2+}]_i$; **Figure 3A**) was considerably lower than that induced by U46619 (211 ± 25.9 nM $[Ca^{2+}]_i$) [54]. The selective TP antagonist SQ29,548 completely blocked TP mediated $[Ca^{2+}]_i$ mobilization in response to both agonists [54] confirming that 8-epi $PGF_{2\alpha}$ is acting through SQ29,548 sensitive TP(s) or through closely related prostanoid / isoprostane receptors in h.platelets [54; **Figure 3B**]. We then examined 8-epi $PGF_{2\alpha}$ mediated signalling in mammalian HEK293 cells that exclusively over-express the human TP isoforms. In control HEK293 cells, 8-epi $PGF_{2\alpha}$ did not induce a rise in $[Ca^{2+}]_i$. However, in cells over-expressing the TPα or the TPβ isoform, in the presence of $G\alpha_{11}$, 8-epi $PGF_{2\alpha}$ resulted in efficient mobilization of $[Ca^{2+}]_i$ which was blocked by the TP antagonist SQ29,548 [54; **Figure 3C & 3D**]. Thus, taken together, these data provided convincing evidence that 8-epi $PGF_{2\alpha}$ signals through the human TP receptors and that the TP isoforms mediate the actions, at least in part, of the free radical derived 8-epi $PGF_{2\alpha}$ [54].

Both TXA_2 and 8-epi $PGF_{2\alpha}$ are known to stimulate mitogenic growth of VSM cells under certain clinical and experimental conditions [76-81]. Thus, we extended our investigations to establish whether the TP isoforms may mediate 8-epi $PGF_{2\alpha}$ induced mitogenesis in VSM cells [38]. Activation of the mitogenic cascades, particularly through GPCR signalling, is a complex process involving the participation of a large number of cellular intermediates and the activation of diverse signalling pathways including activation of the mitogen activate protein kinase (MAPK) cascades [82-85]. *Non–the-less*, despite this complexity, all of these pathways converge to activate a final penultimate activation step, namely the phosphorylation and activation of p44 and p42 isoforms of the extracellular regulated kinases (ERK) 1 and 2 isoforms, respectively [82]. Thus, we investigated mitogenesis in the human (h) VSM cell line [ULTR cells**;** 86] grown in culture in response to the TXA_2 mimetic U46619 and compared it to that induced by the isoprostane 8-epi $PGF_{2\alpha}$ [38]. Consistent with the established mitogenic actions of TXA_2 in h.VSM cells, U46619 mediated efficient activation of ERK 1 and 2 in growth arrested cells as evidenced by detection of increased levels of the phosphorylated forms of ERK 1 and 2 (ppERK 1 and 2) with no apparent changes in the overall levels of ERK 1 and ERK 2 expression (ERK 1

and 2) [38; **Figure 3E**]. Additionally, U46619 mediated phosphorylation of ERK 1 / 2 occurred in a time and concentration dependent manner and was blocked by the TP antagonist SQ29,548 and by the MAPK kinase (MEK) inhibitor PD98059 [38; **Figure 3E**]. Similarly, 8-epi $PGF_{2\alpha}$ mediated efficient time and concentration dependent phosphorylation and activation of ERK 1 / 2 in growth arrested h.VSM cells with no apparent changes in the overall levels of ERK 1 / ERK 2 expression [38; **Figure 3F**]. However, 8-epi $PGF_{2\alpha}$ mediated ERK 1 / 2 activation was blocked by MEK inhibitor PD98059 but was only partially inhibited by the TP antagonist SQ29,548 [38]. Thus, it appears that the TP isoforms expressed in h.VSM cells mediates 8-epi $PGF_{2\alpha}$ induced mitogenesis [38; **Figure 3F**]; however, 8-epi $PGF_{2\alpha}$ appears to be also acting, at least partially, through SQ29,548 insensitive receptors in these h.VSM cells.

Thus, our studies involving the isoprostane 8-epi $PGF_{2\alpha}$ confirm that the h.TP receptor isoforms mediate signalling and mitogenesis in response to 8-epi $PGF_{2\alpha}$ in human platelets, in HEK293 cells over-expressing either TPα or TPβ and in h.VSM cells. Whereas high concentrations of 8-epi $PGF_{2\alpha}$ (in the μM range) are required for TP activation, high local concentrations of the isoprostanes, particularly 8-epi $PGF_{2\alpha}$ are found in clinical situations of oxidative injury [71]. Thus, through our studies, we conclude that 8-epi $PGF_{2\alpha}$ represents an alternative ligand for TP activation and therefore, it is likely that incidental activation of the TP receptors by 8-epi $PGF_{2\alpha}$ may indeed contribute to and exacerbate the pathology associated with oxidative injury. In keeping with these data, Audoly *et al*., [87] recently reported that transgenic mice over-expressing the TPβ isoform in the vasculature, but not in platelets, exhibited an exaggerated pressor response to infused 8-epi $PGF_{2\alpha}$ ($iPF_{2\alpha}$-III) compared to wild type mice, an effect that was blocked by the selective TP antagonist SQ29,548. Moreover, in TP knockout mice, both pressor responses to 8-epi $PGF_{2\alpha}$ and its effect on platelet function were abolished [87].

TP receptor desensitization:

A commonly observed phenomenon among GPCRs is desensitization [26]. GPCRs can be subject to either homologous [88,89] or heterologous desensitization [89-94], largely mediated via phosphorylation by the G-protein coupled receptor kinases (GRKs) or the second messenger regulated protein kinases (PKs), including cAMP dependent PKA and PKC [26,95]. Such desensitizations provide mechanisms for feedback regulatory loops following receptor activation and also for cross talk between different second messenger systems [90]. Differences in the complement of Ser and Thr residues in their unique C-tail domains imply that the TPα and TPβ

isoforms may be subject to differential homologous / heterologous desensitization [20,32]. Both TPα and TPβ have recently been established to undergo agonist-induced homologous desensitization and phosphorylation in transfected HEK 293 cells [96]. In addition, recent studies indicate that TPβ, but not TPα, may undergo agonist-induced internalization [97,98] and we have established that the TP isoforms are subject to differential prostanoid EP_1 receptor-induced desensitization mediated at PKC sites unique to the individual TPs [63].

Intermolecular cross talk and / or heterologous desensitization have been widely documented to occur between the anti-aggregatory IP/ adenylyl cyclase system and the pro-aggregatory TP/ phospholipase C system within platelets and vascular smooth muscle [99-101]. The counter regulatory roles of TXA_2 and PGI_2 may be illustrated experimentally if one examines the effect of PGI_2 on TXA_2 /U46619-induced platelet aggregation and signalling [62] whereby pre-exposure of platelets to PGI_2 or its mimetic cicaprost completely inhibits platelet aggregation and $[Ca^{2+}]_i$ mobilization in response to their secondary stimulation with the TP agonist U46619 [62; **Figure 4A & 4B**]. The main inhibitory actions of PGI_2 / adenylyl cyclase within platelets are believed to be mediated through its activation of cAMP-dependent PKA [101]. Many of the molecular targets of PGI_2/ PKA mediated inhibition of platelet aggregation have been identified and include PLC, thrombolamban, myosin light chain kinase and $G\alpha_{13}$ [62, 99-101]. However, in view of the essential role of PGI_2 and its receptor in modulating or counter regulating TXA_2 mediated signalling in platelets and VSM, we sought to investigate whether the TP receptors themselves may be direct targets in this desensitization process. Additionally, in view of the existence of 2 receptors for TXA_2 in humans, namely the TPα and TPβ isoforms, we sought to investigate whether the TP isoforms themselves may be subject to differential regulation or desensitization by PGI_2 mediated signalling.

Thus, we examined the effect of the selective IP agonist cicaprost on the counter regulation or desensitization of signalling by the h.TP(s) endogenously expressed in h.platelets and compared it to that which occurred in mammalian HEK293 cells stably over-expressing the individual TPα (HEK.TPα10 cells) and TPβ (HEK.TPβ3 cells) isoforms [62; **Figure 4**]. Whereas pre-incubation of HEK.TPα10 or HEK.TPβ3 cells with cicaprost did not result in a measurable rise in $[Ca^{2+}]_i$ (Figure 4D & 4F) or IP_3 generation *per se,* cicaprost pre-stimulation completely blocked TPα mediated $[Ca^{2+}]_i$ mobilization and IP_3 generation in response to secondary stimulation of cells with the TP agonist U46619 [62; **Figure 4C & 4D**]. However, unlike that which occurred in platelets and in HEK.TPα10 cells, cicaprost had no effect on TPβ mediated

$[Ca^{2+}]_i$ mobilization or IP_3 generation in response to stimulation of cells with U46619 [62; **Figure 4E & F**].

Whereas the PKC inhibitor GF109203X had no effect on cicaprost mediated desensitization of U46619-induced $[Ca^{2+}]_i$ mobilization or IP_3 generation in both h.platelets and in HEK.TPα10 cells, the PKA inhibitor H-89 completely inhibited cicaprost mediated desensitization of TPα signalling [62; **Figure 5A & 5B**]. Moreover, unlike that which occurred in platelets and in HEK.TPα10 cells, cicaprost pre-stimulation had no effect on $TP^{\Delta 328}$ mediated mobilization of $[Ca^{2+}]_i$ or IP_3 generation in response to secondary stimulation of HEK.$TP^{\Delta 328}$ cells with U46619 [62; **Figure 5C**]. Thus, taken together these data indicate that the TPα, but not the TPβ, isoform is subject to IP induced desensitization, mediated through H-89 sensitive PKA phosphorylation sites located within the unique C-tail sequences of TPα.

Computational analyses of the unique C-tail domains of the TPα isoform indicated the presence of a putative PKA phosphorylation site within the sequence $RS^{329}LSL$ where Ser^{329} is predicted to be the target residue for phosphorylation [62]. Site directed mutagenesis was employed to convert Ser^{329} to Ala^{329}, to generate $TP\alpha^{S329A}$. Initial characterisation of $TP\alpha^{S329A}$ established that it displayed identical pharmacological properties, in terms of ligand binding and agonist mediated signalling, as the wild type TPα [62]. In HEK.$TP\alpha^{S329A}$ cells, mammalian HEK293 cells which stably over-express $TP\alpha^{S329A}$, the TP agonist U46619 mediated efficient mobilization of $[Ca^{2+}]_i$ and IP_3 generation in a G_q dependent manner [62]. However, unlike that which occurred in platelets or in HEK.TPα10 cells, pre-incubation of HEK.$TP\alpha^{S329A}$ cells with cicaprost had no effect on $TP\alpha^{S329A}$ mediated mobilization of $[Ca^{2+}]_i$ in response to secondary stimulation of cells with U46619 [62; **Figure 5D**]. Subsequent, whole cell phosphorylation assays confirmed that TPα, but not TPβ or $TP\alpha^{S329A}$, is indeed a direct target of cicaprost / IP-mediated PKA phosphorylation [62]. Thus, these studies confirm that TPα, but not TPβ, is subject to counter regulation or heterologous desensitization by IP, mediated through direct PKA phosphorylation within the unique C-tail of TPα whereby Ser^{329} has been identified as the target residue for phosphorylation. An important implication of these studies is that TPα, but not TPβ, may be the TP isoform physiologically relevant to TP:IP mediated vascular hemostasis and implies that TPβ may have a redundant role in prostanoid-regulated vascular hemostasis. Consistent with this hypothesis, based on observations that TP isoform specific antibodies permitted detection of TPα, but not TPβ in human platelets, Habib *et al*., [39] have proposed that TPα may be the predominant isoform in platelets, despite the presence of mRNA for both isoforms [33].

In follow up studies, we have recently confirmed that the TP isoforms are also subject to this type of differential counter regulation of signalling by another inhibitory prostanoid, namely PGD_2, thereby adding further credence to the hypothesis that the TP isoforms indeed play essential, though differential, roles in prostanoid regulated vascular hemostasis [102]. Within the vasculature, platelet derived PGD_2 inhibits platelet aggregation and causes relaxation of vascular smooth muscle leading to vasodilation [103,104]. However, PGD_2 is also synthesised within the central nervous system, where is acts as a potent regulator of sleep induction, and also acts as a mediator of nociception (pain perception) and neurotransmitter release [1,105,106]. These actions of PGD_2 are mediated through a single PGD_2 receptor, termed DP, which is widely expressed in platelets, VSM, through out regions of the CNS, such as within the leptomeninges but not in the brain itself, and also within the retina and small intestinal tissue [1,6]. Our finding that the TP isoforms are subject to differential desensitization or counter regulation of signalling by PGD_2 [102], may also point to additional distinct roles of the TP isoforms not only within the vasculature but also in the CNS, where TPs are also abundantly expressed [1].

Concluding remarks:

Our studies investigating the counter regulation of TP responses by PGI_2 / cicaprost and PGD_2 have established that the TPα, but not the TPβ isoform, is subject to IP- and DP- induced heterologous desensitization mediated through direct PKA phosphorylation of Ser^{329} within the unique C-tail of TPα. These findings point to potentially important physiologic differences between the TP isoforms and imply that TPα may be the TP isoform involved in prostanoid-regulated vascular hemostasis. In addition, these findings also imply that signalling by the TPβ isoform remains active or unchecked in response to the prostanoids PGI_2 and PGD_2. Such lack of desensitization of TPβ signalling by the inhibitory prostanoids may have important physiological and / or clinical implications that are currently unappreciated. It is particularly noteworthy that it is the very first divergent amino acid residue between the TP isoforms, namely Ser^{329} of TPα, that is the target for phosphorylation by PKA and, hence, the key mediator of regulation by the inhibitory prostanoids $PG1_2$ and PGD_2. Whether this is a simple coincidence or a distinct incident of nature will remain an open, unresolved question.

In summary, in this review I have outlined some of our recent studies investigating TP expression and signalling, particularly as pertaining to the human TPα and TPβ isoforms. Our studies investigating the expression, tissue distribution and regulation of the TPα and TPβ isoforms have confirmed that they are subject to differential expression. Whereas the TP

isoforms exhibit identical coupling to G_q and G_{12} family members, their unique C-tail sequences may play a role in determining G_s versus G_i coupling and may act as a determinant of receptor:G protein coupling efficiency. Our studies have provided convincing evidence that the TP isoforms mediate the actions, at least in part, of the free-radical derived isoprostane 8-epi $PGF_{2\alpha}$ leading to activation of TP signalling and TP mediated mitogenesis in VSM. Finally, our studies investigating the counter regulation of TP signalling by PGI_2, have indeed confirmed that the TP receptor itself is a direct target for IP/PKA mediated desensitisation; however, the TP isoforms display distinct patterns of regulation, pointing to essentially differential roles for TPα and TPβ in the fundamental physiologic process of vascular hemostasis.

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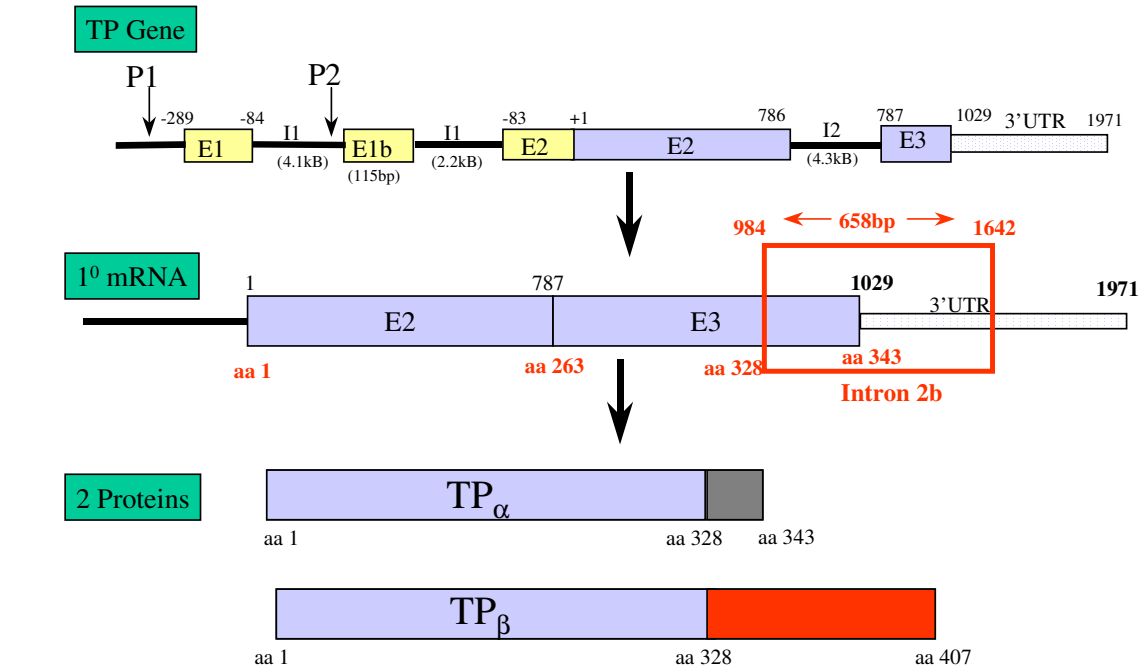
(A)

FIGURES:


TP Gene
P1
P2
-289
-84
I1
(4.1kB)
E1
E1b
(115bp)
I1
(2.2kB)
-83
+1
E2
E2
786
I2
(4.3kB)
787
1029
3'UTR
1971
E3
984
658bp
1642
1⁰ mRNA
1
787
1029
1971
E2
E3
3'UTR
aa 1
aa 263
aa 328
aa 343
Intron 2b
2 Proteins
TPα
aa 1
aa 328
aa 343
TPβ
aa 1
aa 328
aa 407

(B)

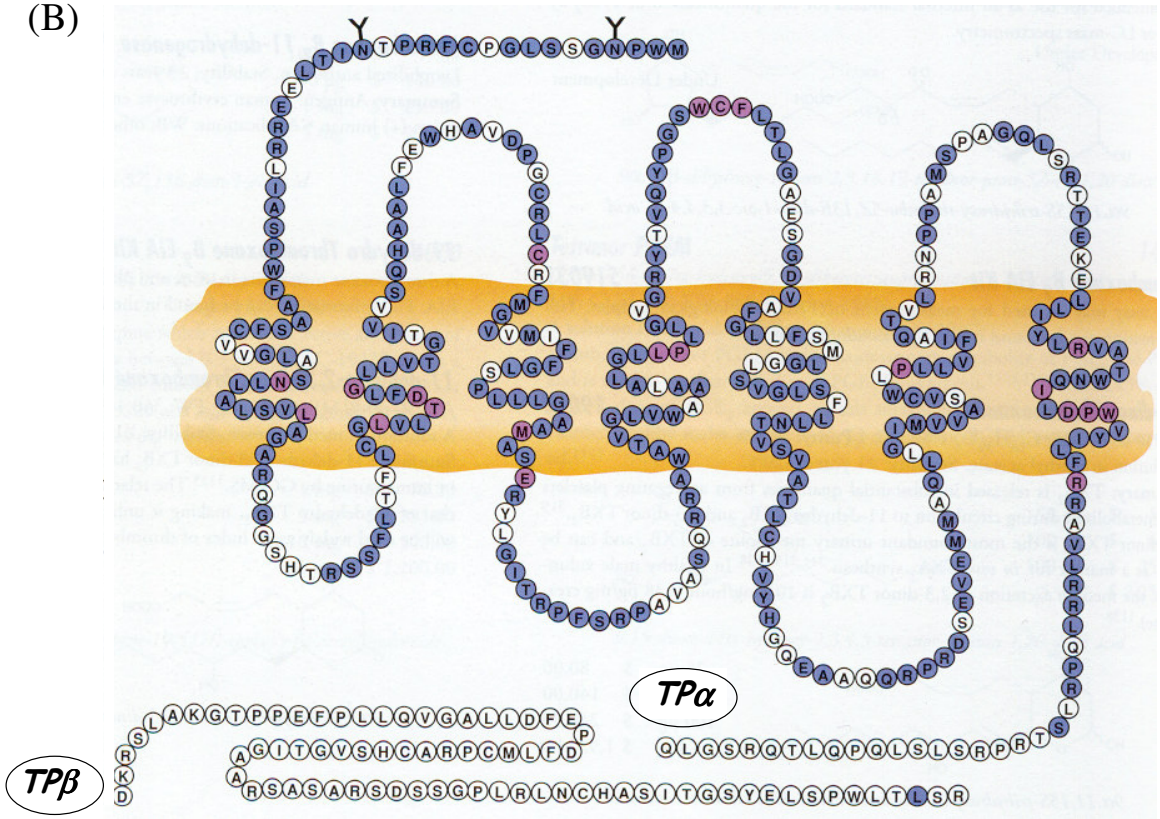

TPα
TPβ

Figure 1A. Organization of the human TP gene.

The intron (I) – exon (E) arrangement of the human TP gene is given (TP gene); thereafter, the TP gene is transcribed and processed to produce a mature primary mRNA (1^{o} mRNA) which is further translated to produce the TPα isoform of 343 amino acid (aa) residues. In the TPβ mRNA, nucleotides 984-1642 behave as Intron 2B; thus, splicing of nucleotides 984/1643 generates a mRNA with an extended open reading frame which is further translated to produce the TPβ isoform of 407 aa residues. Nucleotides encoding the 5' untranslated region (UTR) are given the minus (-) designation and the first nucleotide of the initiation codon is given the +1. P indicates promoter regions on the TP gene; in the 1^{o} mRNA, nucleotide numbers are given above the mRNA and corresponding aa codon numbers are given below. bp; base pair.

Figure 1B. Structural organization of the human TPα and TPβ receptors.

The TPα and TPβ are each predicted to have an amino terminal (N) extracellular domain, 7 alpha-helical transmembrane spanning domains, 3 inter-connecting intracellular loops, 3 inter-connecting extracellular loops and a carboxyl-terminal cytoplasmic tail (C-tail) domain. The TP receptors are identical for their N-terminal 328 aa residues but differ such that TPα and TPβ have 15 aa and 79 aa residues within their unique C-tail domains, respectively. N-linked glycosylation sites at Asn^{4} and Asn^{16} [55] are indicated by the Y symbol. Figure 1B was reproduced with kind permission of the Cayman Chemical Company.

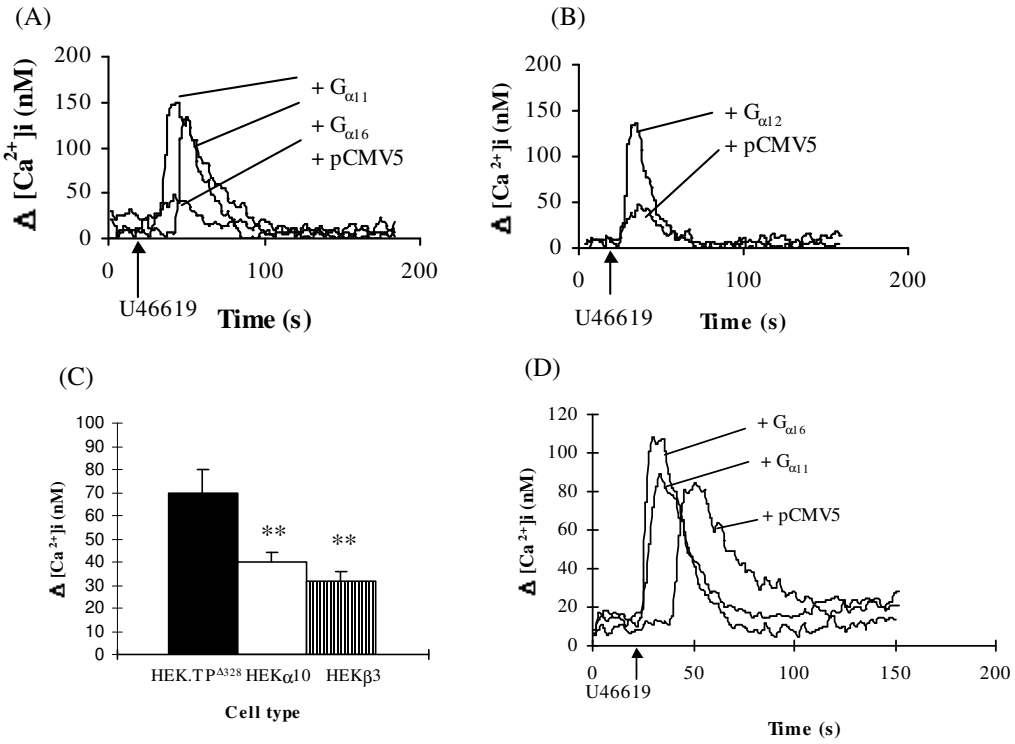


Figure 2. Specificity of TP: G protein coupling.

Panels A, B, & D. HEK 293 cells stably over-expressing TPα (HEK.TPα10 cells; panels A & B) or $TP^{\Delta 328}$ (HEK.$TP^{\Delta 328}$ cells; panel D) were transiently co-transfected with the control vector pCMV5, or with plasmids over-expressing $G\alpha_{11}$, $G\alpha_{12}$ or $G\alpha_{16}$, as indicated in the panels. FURA2/AM pre-loaded cells were stimulated with U46619 (1 μM) at the times indicated by the arrows. Data presented are representative of at least 4 independent experiments and are plotted as changes in intracellular Ca^{2+} mobilised ($\Delta[Ca^{2+}]_i$, nM) as a function of Time (second, s).

Panel C: HEK.$TP^{\Delta 328}$, HEK.TPα10 or HEK.TPβ3 cells, transiently co-transfected with pCMV5 were pre-loaded with FURA2/AM and stimulated with 1 μM U46619. Mean data are plotted as changes in intracellular Ca^{2+} mobilized ($\Delta[Ca^{2+}]_i \pm$ S.E, nM; n = 4). ** ($p<0.02$) indicates that U46619-induced $\Delta[Ca^{2+}]_i$, was significantly higher in HEK.$TP^{\Delta 328}$ cells co-transfected with pCMV5 than in HEK.TPα10 or HEK.TPβ3 cells co-transfected with pCMV5 . Data presented are adapted from reference [61].

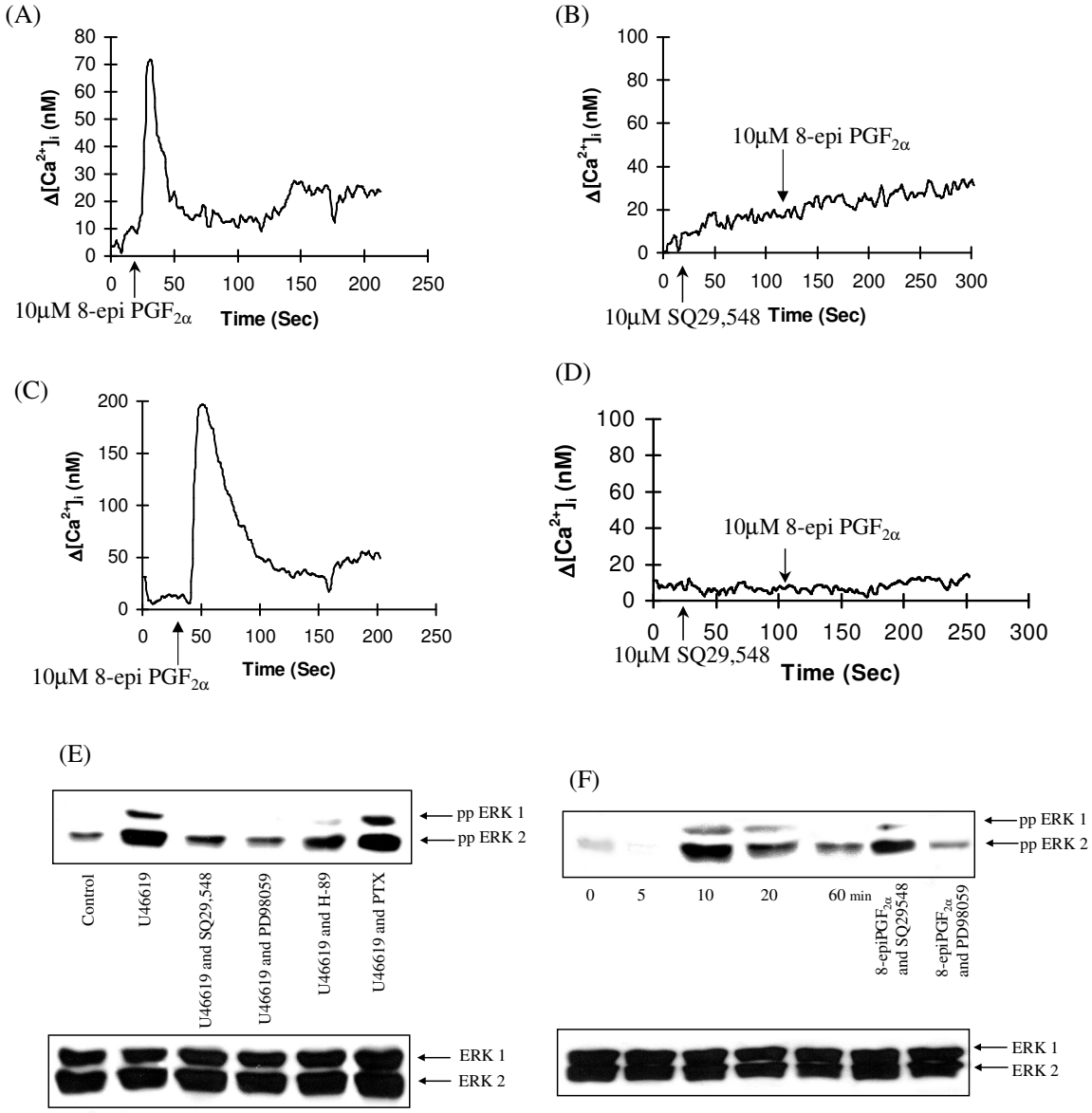


Figure 3: 8-epi PGF$_{2\alpha}$-induced TP signalling.

Panels A-D. Human platelet preparations (Panels A & B) or HEK 293 cells co-transfected with TPα plus Gα_{11} (Panels C & D) were pre-loaded with FURA2/AM and then stimulated with 10 μM 8-epi PGF$_{2\alpha}$ (Panels A & C) or with 10 μM SQ29,548 followed by 10 μM 8-epi PGF$_{2\alpha}$ (Panels B & D), where ligands were added at the times indicated by the arrows. Data presented are representative of at least 4 independent experiments and are plotted as changes in intracellular Ca^{2+} mobilised ($\Delta[Ca^{2+}]_i$, nM) as a function of time (s).

Panel E: Human vascular smooth muscle (ULTR) cells were preincubated with either SQ29,548 (1 μM, 1 min), PD 98058 (10 μM, 30 min), H-89 (10 μM, 5 min), PTX (50 ng/ml, 16 hr).

Subsequently, U46619 (100 nM) was added for 10 min with cells exposed exclusively to U46619 (100 nM for 10 min) or with vehicle (Control) alone serving as references. ***Panel F:*** Time dependent (0 – 60 min) activation of ERK 1/2 in response to 300 nM 8-epiPGF$_{2\alpha}$. Alternatively, cells were preincubated with SQ29,548 (1 μM, 1 min) or with PD 98059 (10 μM, 30 min), prior to stimulation with 8-epiPGF$_{2\alpha}$ (300 nM, 10 min). ***Panels E & F, upper panels:*** Immunoblots were screened with anti-ACTIVE^{TM} ERK to detect the phosphorylated, active forms of ERK (pp ERK1/2) whereas in ***Panels E & F, lower panels:*** blots were screened with anti-ERK antibodies to detect ERK1/2 immunoreactive protein. Results are representative of at least three independent experiments. PTX, pertussis toxin. Data presented are adapted from references [54; panels A-D] and [38; panels E & F].

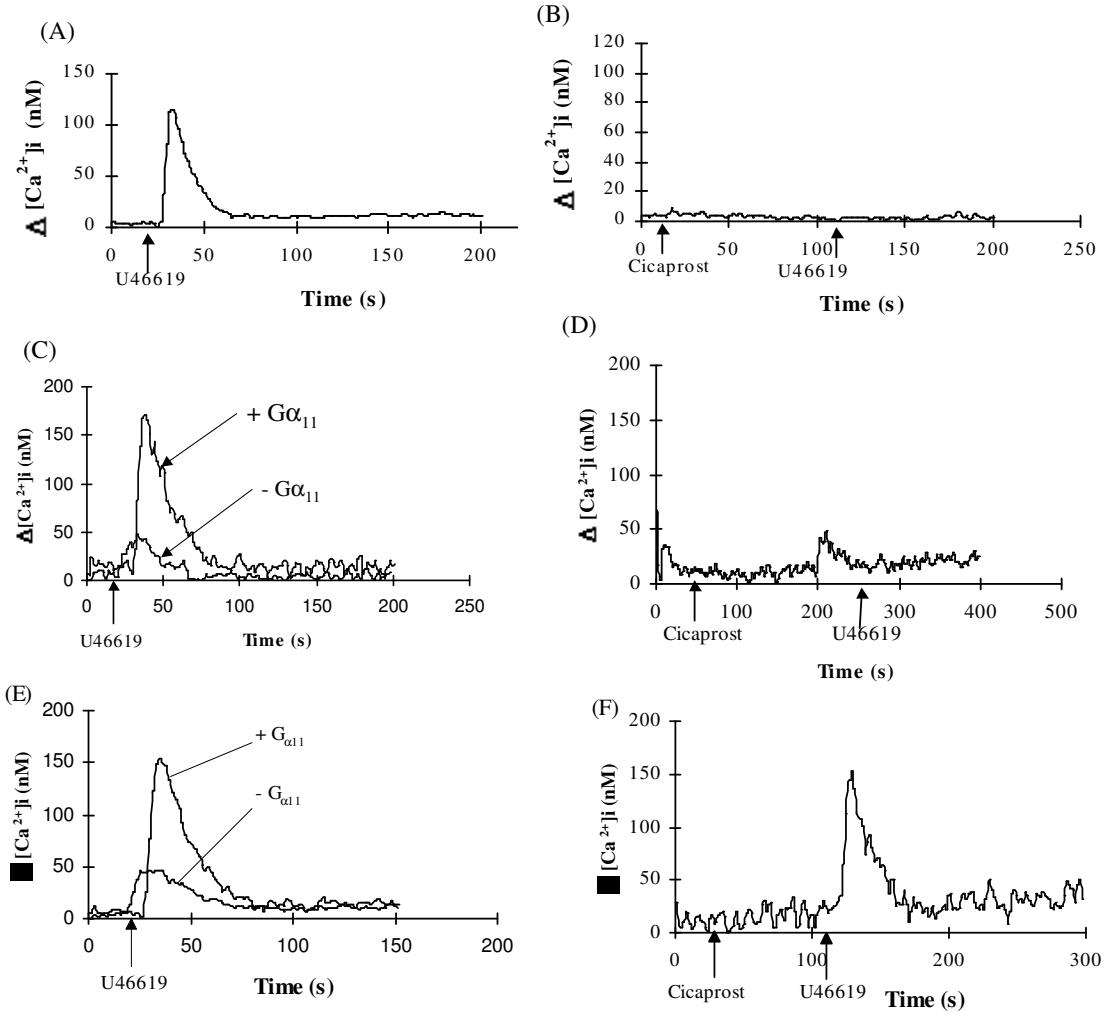


Figure 4. Cicaprost-induced desensitization of TP signalling.

Panels A & B: Platelets were pre-loaded with FURA2/AM and were stimulated with 1 μM U46619 (Panel A) or 1 μM cicaprost followed by 1 μM U46619 (Panel B), where ligands were added at the times indicated by the arrows.

Panels C – F: HEK.TPα10 cells (Panels C & D) or HEK.TPβ3 cells (Panels E & F), transiently co-transfected with pCMV:Gα_{11}, were pre-loaded with FURA2/AM and stimulated with either U46619 (1 μM) or with cicaprost (1 μM) followed by U46619 (1 μM) as indicated in the panels, where ligands were added at the times indicated by the arrows. Data presented are representative of at least 4 independent experiments and are plotted as changes in intracellular Ca^{2+} mobilized ($\Delta[Ca^{2+}]_i$, nM) as a function of Time (second, s) following ligand stimulation. + Gα_{11} / - Gα_{11} in panels C & E indicated that cells were transfected with (+) or without (-)pCMV:Gα_{11}. Data presented are adapted from reference [62].

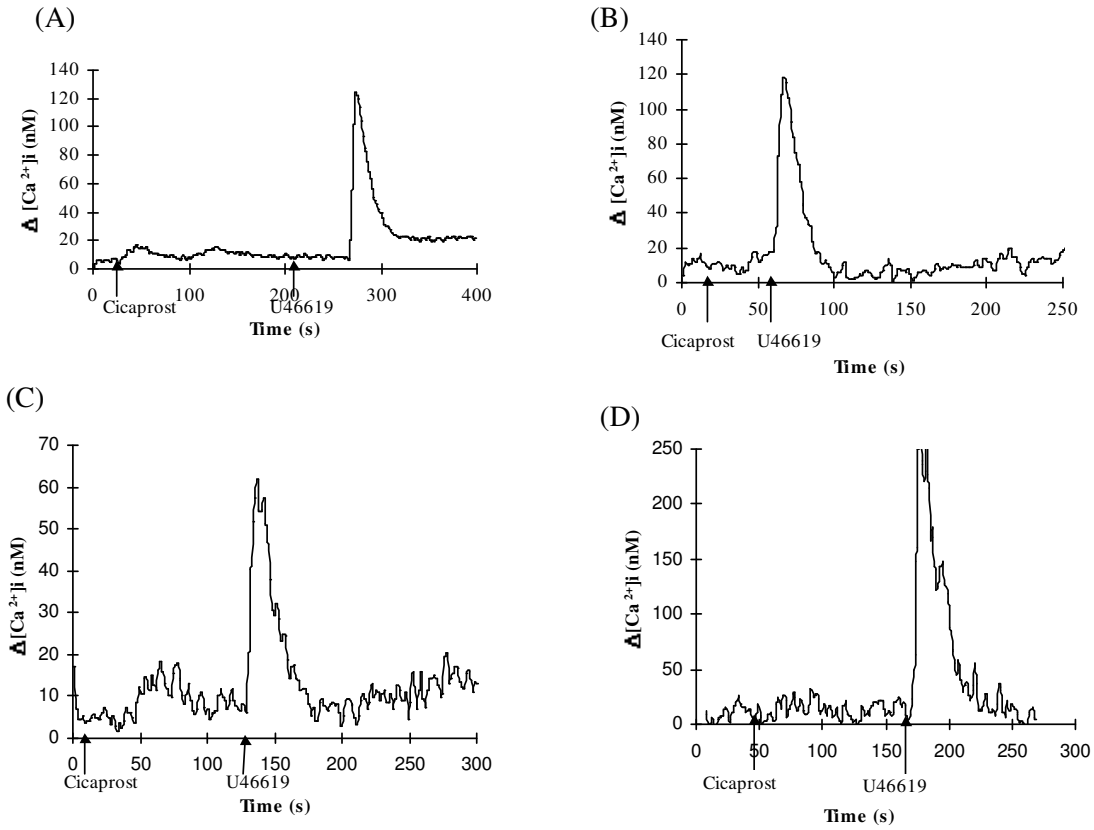


Figure 5. Mechanism of cicaprost-induced desensitization of TP signalling.

Panels A & B: Human platelets (Panel A) or HEK.TPα10 cells, transiently co-transfected with $G\alpha_{11}$ (Panel B) were pre-incubated with 10 μM H-89 and then stimulated with 1 μM cicaprost followed by 1 μM U46619, where ligands were added at the times indicated by the arrows.

Panels C & D: HEK.TP$^{\Delta 328}$ cells (Panel C) or HEK.TPα^{S329A} cells (Panel D), transiently co-transfected with $G\alpha_{11}$ were stimulated with 1 μM cicaprost followed by 1 μM U46619, where ligands were added at the times indicated by the arrows. Data presented are representative of at least 4 independent experiments and are plotted as changes in intracellular Ca^{2+} mobilized ($\Delta[Ca^{2+}]_i$, nM) as a function of Time (second, s) following ligand stimulation. Data presented are adapted from reference [62].