Investigation of the role of the carboxyl terminal tails of the α and β isoforms of the human thromboxane A_2 receptor (TP) in mediating receptor : effector coupling.

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Abbreviations: cAMP, Adenosine 3', 5' -cyclic Monophosphate; $[Ca^{2+}]_i$, intracellular calcium; EDTA, ethylene diamine tetraacetic acid; FBS, Fetal bovine serum; HEK, human embryonic kidney; HEL, human erythroleukemia; HEPES, (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid); IP, prostacyclin receptor; IP_3, inositol 1,4,5 trisphosphate; PG, prostaglandin; PLC, phospholipase C; RT-PCR, reverse transcriptase -polymerase chain reaction; TP, thromboxane A_2 receptor; TXA_2, thromboxane A_2. Thromboxane A_2 receptor is abbreviated to TP and its splice variants are designated by the Greek letters α and β, as recommended by the IUPHAR classification on prostanoid receptors.

Summary

We have investigated the functional coupling of α and β isoforms of the human thromboxane A_2 receptor (TP) to $G\alpha_{16}$ and $G\alpha_{12}$ members of the G_q and G_{12} families of heterotrimeric G proteins in human embryonic kidney (HEK) 293 cell lines HEK.α10 or HEK.β3, stably over-expressing TPα and TPβ, respectively. Moreover, using HEK.$TP^{\Delta 328}$ cells which over-expresses a variant of TP truncated at the point of divergence of TPα and TPβ, we investigated the requirement of the C-tail *per se* in mediating G protein coupling and effector activation. Both TPα and TPβ couple similarly to $G\alpha_{16}$ to affect increases in IP_3 and mobilization of intracellular calcium ($[Ca^{2+}]_i$) in response to the TP agonist U46619. Whilst both TP isoforms mediated $[Ca^{2+}]_i$ mobilization in cells co-transfected with $G\alpha_{12}$, neither receptor generated corresponding increases in IP_3 indicating that the $G\alpha_{12}$ mediated increases in $[Ca^{2+}]_i$ do not involve PLC activation. Verapamil, an inhibitor of voltage dependent Ca^{2+} channels reduced $[Ca^{2+}]_i$ mobilization in TPα and TPβ cells co-transfected with $G\alpha_{12}$ to approximately 40% of that mobilized in its absence whereas 3,4,5-trimethyloxybenzoic acid 8-(diethylamino)octyl ester (TMB-8), an antagonist of intracellular Ca^{2+} release, had no effect on $[Ca^{2+}]_i$ mobilization by either receptor isoform co-transfected with $G\alpha_{12}$. Despite the lack of differential coupling specificity by TPα and TPβ, $TP^{\Delta 328}$ signaled more efficiently in the absence of a co-transfected G protein compared to the wild type receptors but, on the other hand, displayed an impaired ability to couple to co-transfected $G\alpha_{11}$, $G\alpha_{12}$ or $G\alpha_{16}$ subunits. In studies investigating the role of the C-tail in influencing coupling to the effector adenylyl cyclase, similar to TPα but not TPβ, $TP^{\Delta 328}$ coupled to $G\alpha_S$, leading to increased cAMP, rather than to $G\alpha_i$. Whereas $TP^{\Delta 328}$ signaled more efficiently in the absence of co-transfected G protein compared to the wild type TPα, co-transfection of $G\alpha_s$ did not augment cAMP generation by $TP^{\Delta 328}$. Hence, from these studies involving the wild type TPα, TPβ and $TP^{\Delta 328}$, we conclude that the C-tail sequences of TP are not a major determinant of G protein coupling specificity to $G\alpha_{11}$ and $G\alpha_{16}$ members of the G_q family or to $G\alpha_{12}$; it may play a role in determining G_S versus G_i coupling and may act as a determinant of coupling efficiency.

Introduction

Heterotrimeric G proteins couple signals from seven transmembrane domain, G protein coupled receptors (GPCRs) to a host of intracellular effectors [1]. Agonist-activated GPCRs interact with the GDP-bound heterotrimer leading to GDP-GTP exchange and dissociation of the Gα subunit from the $G_{\beta}\gamma$ subunit. In addition to their regulatory effects on the Gα subunits, the $G_{\beta}\gamma$ dimers may also transmit various intracellular signals [1-3]. However, despite the many activities of the $G_{\beta}\gamma$ dimers, it is believed that it is the Gα subunit of the heterotrimeric G protein which principally dictates its interaction with a specific receptor. On the basis of the amino acid identities of their α subunits, heterotrimeric G proteins are divided into four subfamilies; G_s, G_i, G_q and G_{12} [4]. G protein : effector coupling has been studied in most detail for G_s, G_i, G_q families whereas the main effector targets of the G_{12} family are unclear. The G_{12} subfamily consists of the ubiquitously expressed members $G\alpha_{12}$ and $G\alpha_{13}$. They share a 67% sequence identity and exhibit least homology (45%) to other G protein α-subunits [5]. Whereas $G\alpha_q$ and $G\alpha_{11}$ are widely expressed and are primarily responsible for pertussis toxin (PTX) -insensitive PLC-β activation, the expression of $G\alpha_{14}$ and $G\alpha_{15}$/$G\alpha_{16}$ is restricted to a few tissues [6, 7]. For example, $G\alpha_{16}$ is particularly abundant in and primarily expressed in hematopoietic cells [7, 8].

In the case of the GPCR for thromboxane (TX) A_2, termed TP, the major mode of signaling is activation of β isozymes of phospholipase C leading to phosphatidylinositol turnover and release of calcium from intracellular stores ($[Ca^{2+}]_i$) [9]. There are two TP isoforms in humans, TPα and TPβ, which are identical for their first 328 amino acids and differ exclusively in their carboxyl terminal cytoplasmic tails (C-tails) due to alternative splicing within exon 3 of the TP gene [10, 11]. The prostaglandin (PG) E_2 receptor subtype EP_3 from a number of species also has several splice variants which differ only in their C tails [12-16]. In the case of the bovine EP_3 receptors, differences in the C-tails have been shown to affect G-protein coupling specificity and intracellular signaling [12]. Using a variety of approaches involving either reconstitution studies [17, 18], co-purification or co-immunoprecipitation experiments [19-21], photo-cross linking studies with GTP analogs [22] or co-expression studies [23-26] various investigators have proposed that the platelet TPs might couple to the heterotrimeric 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 [23]; whereas co-expression of G_{12} alone had no effect on affinity, being only able to augment the G_{13} effect. 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 and the isoprostane, 8-epi prostaglandin $F_{2\alpha}$, to mobilize $[Ca^{2+}]_i$ [26]. Coupling to G_{11} was more efficient than that to G_q. Whereas both TP isoforms couple similarly to G_{11} in stably transfected HEK 293 cells [27], they oppositely regulate adenylyl cyclase activity in transfected Chinese hamster ovary cells [28], suggesting a possible role for the C-tail of TP in determining G protein specificity. Moreover, G_h, the novel high molecular weight G protein which may also function as a transglutaminase [29-32], can mediate agonist activation of TPα, but not TPβ, leading to inositol phosphate production due to PLC activation [33].

Whereas many of these studies have implicated various G protein α subunits in mediating TP activation and the latter studies [28, 33] indicate that the TP isoforms may indeed differentially couple to G_s, G_i and G_h, few studies have directly compared the effect of members of the G_q or G_{12} family on signaling by the individual TPα or TPβ isoforms. Thus, in this study, we have investigated the effect of co-expression of the hematopoietic $G\alpha_{16}$ and the ubiquitous $G\alpha_{12}$ on agonist mediated second messenger generation in HEK 293 cells stably over-expressing TPα (HEK.α10 cells) or TPβ (HEK.β3 cells) and compared it to TP signaling in the presence of $G\alpha_{11}$. Moreover, in similar studies using HEK.$TP^{\Delta 328}$ cells, which over-expresses a variant of TP ($TP^{\Delta 328}$) truncated at amino acid 328 at the point of divergence of TPα and TPβ, we investigated the requirement of a C-tail *per se* in mediating G protein coupling and effector activation. Our results indicated that both TPα and TPβ couple similarly to $G\alpha_{16}$ to affect increases in IP_3 and mobilization of $[Ca^{2+}]_i$ in response to the TXA_2 mimetic U46619. Whilst both TPα and TPβ mediated $[Ca^{2+}]_i$ mobilization in cells co-transfected with $G\alpha_{12}$, there was no corresponding elevation of IP_3 for either cell type indicating that G_{12} mediated increases in intracellular Ca^{2+} levels are by a mechanism which does not involve PLCβ activation. Verapamil, an inhibitor of voltage dependent Ca^{2+} channels, principally the L-type channels [34] reduced $[Ca^{2+}]_i$ mobilization in HEK.α10 or HEK.β3 cells co-transfected with $G_{\alpha 12}$ whereas TMB-8, which selectively blocks release of Ca^{2+} from intracellular stores [35] had no significant effect on $[Ca^{2+}]_i$ mobilization by either receptor isoform when co-transfected with $G\alpha_{12}$. Despite the apparent lack of G protein coupling selectivity conferred by the different C-tails of TPα and TPβ, $TP^{\Delta 328}$ appeared to signal in the absence of co-transfected G protein and displayed an impaired ability to couple to co-transfected $G\alpha_{11}$, $G\alpha_{16}$ or $G\alpha_{12}$ subunits. Taken together, these data indicate that although the C-tail of TP is apparently not a major determinant of G protein coupling specificity to G_q ($G\alpha_{11}$ or $G\alpha_{16}$) or $G\alpha_{12}$ members, it is required for efficient G protein interaction. To extend these studies, as TPα and TPβ differ exclusively in their C-tail sequences and they oppositely regulate adenylyl cyclase, we investigated $TP^{\Delta 328}$ signaling to the effector adenylyl cyclase. Similar to the TPα, but not the TPβ isoform, $TP^{\Delta 328}$ exhibited U46619-mediated activation of adenylyl cyclase, with concomitant increases in cAMP generation. However, $TP^{\Delta 328}$ exhibited significantly ($p = 0.002$) higher cAMP generation than TPα in the absence of exogenous G protein, yet displayed an impaired ability to couple to co-transfected $G\alpha_S$.

Materials and Methods

Materials.

The following chemicals were obtained from Cayman Chemical Company: 5-Heptenoic acid, 7-[6-(3-hydroxy-1-octenyl)-2-oxabicyclo [2,2,1] hept-5-yl]-[1R-[1α,4α,5β(z), 6α(1E,3S*)]-9,11-dideoxy-9α,11α-methanoepoxy prostaglandin $F_{2\alpha}$ (U46619); 5-Heptenoic acid, 7-[3-[[Z-[phenylamino) carbonyl] hydrazino] methyl] -7- oxabicyclo [2.2.1] hept -2-yl]-,[1S-[1α,2α(Z),3α,4α]] (SQ29,548) [1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-2-oxy}-2-(2'-amino-5'-methylphenoxy)-ethane-N,N,N'N'-tetraacetic acid, pentaacetoxymethyl ester] (FURA2/AM), verapamil, nifedipine, [8-(N,N-Diethylamino)-octyl-3,4,5-trimethoxybenzoate, hydrochloride] (TMB-8) and D-myo-inositol 1,4,5 triphosphate, 3-deoxy-hexa sodium salt (stable analogue of IP_3) were purchased from Calbiochem. [^{3}H]SQ29,548 (50.4 Ci/mmol) was obtained from DuPont NEN. [^{3}H]cAMP (15 - 30 Ci / mmol) and [^{3}H]IP_3 (20 - 40 Ci / mmol) were obtained from American Radiolabeled Chemicals Inc. Antibodies directed against the α subunits of: $G\alpha_{12}$ (AS 233) was kindly donated by Dr Karstan Spicher, Freie Universitat Berlin, Germany; to $G\alpha_S$ (SC-823), $G\alpha_{16}$ (SC - 7416) and $G\alpha_q$ /α_{11} (C19) antibodies, in addition to horse radish peroxidase conjugated anti-mouse, anti-rabbit or anti-goat secondary antibodies were obtained from Santa Cruz. Chemiluminescence Western Blotting Kit, polyvinylidene difluoride (PVDF) membrane was obtained from Roche.

Methods.

Plasmid construction.

The plasmids pCMV5 and pCMV:$G\alpha_{11}$, containing the full length coding sequence for $G\alpha_{11}$ have been previously described [26]. The plasmids pMOB:$G\alpha_{12}$ and pBluescript(KS^+):$G\alpha_{16}$, coding for the α subunits of the mouse G_{12} [36] and human G_{16} heterotrimeric G protein α subunits were kindly donated by Dr G. Schultz. Institut fur Pharmakologie, Freie Universitat Berlin, Germany and Dr M. Simon, California Institute of Technology, Pasadena, California. The plasmids pCMV:$G\alpha_{12}$ and pCMV:$G\alpha_{16}$ were constructed by subcloning the full length coding sequence for $G\alpha_{12}$ and $G\alpha_{16}$ from pMOB:$G\alpha_{12}$ and pBluescript(KS^+):$G\alpha_{16}$, respectively into the Kpn1- BamH1 sites of pCMV5 [37]. The plasmid pDp3: $G\alpha_S$ coding for human $G\alpha_S$ was kindly donated by Dr David Manning, University of Pennsylvania, USA. The plasmid pCMV:$G\alpha_S$ was constructed by subcloning the full length coding sequence for $G\alpha_S$ into the Xba I - Hind III sites of pCMV5 [37].

Cell Culture and transfections.

HEK 293 cells were obtained from the American Type Culture Collection. The following HEK.α10 and HEK.β3 cell lines, stably over-expressing TPα and TPβ respectively have been previously described [27]. Construction of HEK.$TP^{\Delta 328}$ cells which over-express $TP^{\Delta 328}$, a truncated variant of TP devoid of amino acids carboxyl to Arg^{328} at the point of divergence of TPα and TPβ, was carried out essentially as previously described [27]. Independent isolates were characterised by saturation radioligand binding and

those isolates exhibiting [^{3}H]SQ29,548 binding were expanded. For transient transfection studies, HEK 293 cell lines, cultured in MEM, 10% FBS were plated in 100mm culture dishes at a density of 1.8 - 2 x 10^6 cells/dish approximately 48 h prior to transfection. Cells were transfected with 10μg of pADVA [38] and 25μg pCMV:Gα_{11}, pCMV:Gα_{12}, pCMV:Gα_{16}, pCMV:Gα_S or pCMV5 using the calcium phosphate /DNA co-precipitation procedure essentially as previously described [26] and were harvested forty eight hours after transfection.

Calcium measurements.

Measurements of intracellular calcium ($[Ca^{2+}]_i$) in HEK 293 cells were made by monitoring the intensity of FURA2 fluorescence essentially as previously described [26]. Briefly, cells were harvested by scraping, washed twice in PBS, resuspended in modified Ca^{2+} / Mg^{2+} -free Hank's buffered salt solution, containing 10mM HEPES, pH 7.67, 0.1% bovine serum albumin (HBSSHB buffer) at 10^7 cells/ml and incubated in the dark with 5 μM FURA2/AM for 45 min at 37°C. Cells were subsequently collected by centrifugation (900 × g, 5 min), washed once in an equal volume of HBSSHB and finally resuspended in HBSSHB buffer at 10^7 cells/ml and kept at room temperature in the dark until use. For each measurement of $[Ca^{2+}]_i$, aliquots of HEK 293 cells were diluted to 0.825 × 10^6 cells/ml in HBSSHB buffer containing 1 mM $CaCl_2$. FURA2 fluorescence was recorded in gently stirred HEK 293 cells (2 ml aliquots) at 37 °C using a Perkin Elmer-Cetus LS50-B spectrofluorimeter at excitation wavelengths of 340 nm and 380 nm and emission wavelengths of 510 nm [26, 39]. Cells were stimulated with 1 μM U46619, unless otherwise specified. To examine the effect of Ca^{2+} channel blockers, cells were pre-incubated in the presence of verapamil (10 μM), nifedipine (1 μM) or in the presence of TMB-8 (50 μM) for 15 min at 37 °C prior to stimulation with U46619 (1 μM). The calibration of the signal was performed in each sample by adding 0.2% Triton X-100 to obtain the maximal fluorescence (Fmax) and then adding 1 mM EGTA to obtain the minimal fluorescence (Fmin). A rapid, transient rise and fall in $[Ca^{2+}]_i$ levels in response to ligand stimulation was interpreted as receptor-mediated $[Ca^{2+}]_i$ mobilization. The ratio of the fluorescence at 340 nm to that at 380 nm is a measure of $[Ca^{2+}]_i$ [39], assuming a K*d* of 225 nM Ca^{2+} for FURA2/AM. The results presented in the figures are representative data from at least four independent experiments and are plotted as changes in intracellular Ca^{2+} mobilised ($\Delta[Ca^{2+}]_i$ (nM)) as a function of time (sec) upon ligand stimulation.

Radioligand binding and Western blot analysis.

HEK 293 cells were harvested by centrifugation at 500 x g at 4°C for 5 min, were washed three times in Dulbecco's phosphate-buffered saline (PBS) and were resuspended in modified Ca^{2+} / Mg^{2+} -free Hank's buffered salt solution, containing 10 mM HEPES, pH 7.67, 0.1% bovine serum albumin (HBSSHB buffer). Protein determinations were carried out using the Bradford assay [40]. For ligand binding studies, cells were diluted to 1 mg/ml in HBSSHB buffer. Radioligand binding assays were carried out on whole cell fractions (100 μg) at 30 °C for 30 min in 100 μl reactions in the presence of 0 - 40 nM [^{3}H]SQ29,548 for Scatchard analyses, for calculation of K*d* and B_{max} values, or in the presence of 20 nM [^{3}H]SQ29,548, for

routine radioligand binding assays, as previously described [26, 27]. Non-specific binding was determined in the presence of excess non labelled SQ29,548 (10 μM). Reactions were terminated by the addition of 4 ml of ice-cold 10 mM Tris-HCl, pH 7.4, followed by filtration through Whatman GF/C glass filters, and subsequent washing of the filters 3 times with 10 mM Tris-HCl, pH 7.4, followed by liquid scintillation counting of the filters in 5 ml of scintillation fluid. Radioligand binding data was analyzed using GraphPad Prism V2.0 (GraphPad Software Inc.) to determine the K*d* and B_{MAX} values.

For Western blot analysis, equivalent aliquots of whole non-fractionated cells, transiently co- transfected with pCMV5 or pCMV:Gα_{11}, pCMV:Gα_{12}, pCMV:Gα_{16} or pCMV:Gα_S were solubilized by boiling at 100 ^oC x 5 min in solubilization buffer (10% β-mercaptoethanol, 2% sodium dodecyl sulphate, 30% glycerol, 0.025% bromophenol blue, 50mM Tris-HCl, pH 6.8) and were resolved on a 10% SDS-polyacrylamide gel (SDS-PAGE) followed by Western blot transfer onto poly vinylidene membranes essentially as previously described [26]. Thereafter, Western blots were screened with the specific anti Gα-antibodies followed by chemiluminescence detection, using the relevant HRP conjugated secondary antibodies, according to the suppliers' instructions.

Measurement of IP_3 levels.

Measurement of IP_3 levels in HEK 293 cells was made on the basis of competition between unlabelled IP_3 and a fixed concentration of [^{3}H] IP_3 for binding to an IP_3 binding protein derived from bovine adrenal glands, essentially as described by Godfrey [41]. Briefly, cells were harvested by scraping, washed twice in ice-cold PBS, and 2 x 10^6 cells were resuspended in 200 μl Hepes-buffered saline (HBS; 140 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 11 mM glucose, 15 mM HEPES-NaOH, pH 7.4) supplemented with 10 mM LiCl. Cells were pre-equilibrated in this buffer at 37 ^{0}C for 10 min and stimulated at 37 ^{0}C for 1 min or 5 min in the presence of U46619 (1 μM) or, to determine basal IP_3 levels in cells, in the presence of an equivalent volume (50 μl) of HBS vehicle. IP_3 was extracted by the addition of 50 μl ice-cold 30 % trichloroacetic acid (TCA). After vortexing and centrifugation (3500 rpm, 5 min), 250 μl of each supernatant were removed to glass tubes, 1.0 ml H_2O-saturated diethyl ether was added for extraction of TCA and the samples were placed in a methanol-dry ice bath until the aqueous layers froze and the ether layers could be poured off. Ether extraction was repeated 3 times and the samples were checked for complete extraction of TCA (pH of sample > 5.0). Nitrogen was bubbled through the sample to remove diethyl ether and the pH raised to 7.0 by addition of $NaHCO_3$ (approx. 20 mM final concentration).

Levels of IP_3 were determined by binding assays carried out using [^{3}H] D-myo-Inositol 1,4,5-trisphosphate (IP_3). Analytical samples (100 μl) were added to 100 μl assay buffer (0.1 M Tris Cl, 4 mM EDTA., pH 9.0 containing 4 mg / ml BSA). [^{3}H] IP_3 (100 μl, 20 - 40Ci/mmol, 10 mCi/ml diluted 1: 147 in H_20) was added and the tubes vortexed before addition of 100 μl Binding Protein from bovine adrenal medulla and further vortexing. The level of IP_3 produced was quantified by radioimmunoassay essentially as described by Godfrey, [41]. 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 ± S.E) and as fold

stimulation over basal (fold increase ± S.E). The data presented are representative of 4 independent experiments.

Measurement of cAMP.

HEK 293 cell lines were washed three times in ice-cold phosphate-buffered saline and approximately 1 - 2 X 10^6 cells were resuspended in 200 µl HEPES-buffered saline (HBS; 140 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 11 mM glucose, 15 mM HEPES-NaOH, pH 7.4) containing 1 mM 3-isobutyl-1-methylxanthine and pre-incubated at 37 ^{0}C for 10 min. Thereafter, cells were stimulated in the presence of 1 µM U46619 or in the presence of HBS (50µl) at 37 ^{o}C for 10 min. Alternatively, to assess TP coupling to inhibition of adenylyl cyclase, cells were stimulated in the presence of U46619 (10^{-10} - 10^{-5} M) plus 10 µM forskolin or, as a control, in the presence of 10 µM forskolin alone at 37 ^{o}C for 10 min. In all cases ligands were diluted in HBS and were added in a final volume of 50µl. Reactions were terminated by heat inactivation (100 ^{o}C, 5 min) and the level of cAMP produced was quantified by radioimmunoassay using the cAMP binding protein from bovine adrenal medulla essentially as described by Farndale *et al.* [42]. Protein determinations were carried out using the Bradford assay [40]. Levels of cAMP produced by U46619 stimulated cells over basal stimulation, in the presence of HBS or in the presence of 10 µM forskolin, were expressed in pmol cAMP / mg cell protein ± standard error of the mean (pmol/ mg ± S.E) and as fold stimulation over basal (fold increase ± S.E) or as percentage cAMP produced in U46619 stimulated cells relative to cAMP levels produced in control forskolin treated cells (cAMP content; % of control ± S.E). The data presented are representative of 3 independent experiments, each carried out in duplicate.

Data analyses

Radioligand binding data was analyzed using GraphPad Prism V2.0 (GraphPad Software Inc.) to determine the K*d* and B_{max} values. Statistical analyses were carried out using the unpaired Student's *t* test using the Statworks Analysis Package. *P*-Values of less than or equal to 0.05 were considered to indicate a statistically significant difference.

Results:

TP signaling via $G\alpha_{16}$.

We have previously established that the control, non-transfected human embryonic kidney (HEK) 293 cells lack sufficient levels of TP to support measurable intracellular signaling in response to stimulation with the TXA_2 mimetic U46619 [26]. Moreover, in studies involving $G\alpha_{11}$ and $G\alpha_q$, efficient U46619-mediated intracellular signaling in HEK 293 cells either transiently or stably transfected with TPα or TPβ was dependent on co-expression of the α subunit of the coupling G protein [26, 27]. Thus, in this study, in order to assess whether the TP isoforms couple to $G\alpha_{16}$ to mediate activation of PLCβ, HEK 293 cell lines stably over-expressing TPα (HEK.α10 cells) or TPβ (HEK.β3 cells) were transiently co-transfected with $G\alpha_{16}$. TP expression was monitored by Scatchard analyses using the selective radioligand [^{3}H]SQ29,548 and PLCβ activation was monitored by measurement of IP_3 generation and mobilization of $[Ca^{2+}]_i$ in response to U46619. TPα and TPβ signaling in the presence of $G\alpha_{16}$ was compared to that observed in the presence of $G\alpha_{11}$ or, as a control, in the presence of the vector pCMV5. Co-expression of HEK.α10 cells (K*d*: 5.56 ± 0.98 nM, B_{MAX} 3.38 ± 0.08 pmol/mg cell protein) or HEK.β3 cells (K*d*: 8.44 ± 1.44 nM, B_{MAX} 3.24 ± 0.33 pmol/mg cell protein) with $G\alpha_{16}$ or $G\alpha_{11}$ or pCMV5 produced no significant change in the affinities of either TPα or TPβ for SQ29,548 (data not shown). Confirmation of G protein over-expression in the various HEK 293 cell lines was routinely performed by Western blot analysis using antibodies specific to $G\alpha_{11}$ and $G\alpha_{16}$ (Figure 1B & 1D).

To assess whether TPα or TPβ couple to $G\alpha_{16}$ to mediate changes in intracellular signaling, cells were stimulated with either U46619 (1 μM) or vehicle (HBS) for 1 min or 5 min; levels of IP_3 generation were measured and expressed as fold increase over of basal IP_3 levels in HBS - treated cells (Figure 2A & 2B). Consistent with previous data [26, 43], dose response studies established that 1 μM U46619 elicited maximal IP_3 generation and $[Ca^{2+}]_i$ mobilization by both TPα and TPβ. For both HEK.α10 and HEK.β3 cells, co-transfection of pCMV5 did not support significant increases in IP_3 generation in response to U46619 over basal levels whereas co-transfection of cells with either $G\alpha_{11}$ or $G\alpha_{16}$ generated significant increases in IP_3 levels (Figure 2A & 2B). In general, elevation of IP_3 levels was transient, with maximal levels observed following 1 min stimulation with U46619. (Figure 2A & 2B). The level of IP_3 generation compared well to previously published IP_3 data following stimulation of the mouse TP with 1 μM U46619 [44]. In the case of HEK.α10 cells, both $G\alpha_{11}$ and $G\alpha_{16}$ co-transfection facilitated stimulation of IP_3 levels in response to U46619 to similar extents (Figure 2A). In the case of HEK.β3 cells, levels of U46619-mediated IP_3 generation in cells co-transfected with $G\alpha_{16}$ were not significantly different than those observed in cells co-transfected with $G\alpha_{11}$ (Figure 2B; $p = 0.125$ for 1 min of treatment). Moreover, comparing HEK.α10 to HEK.β3 cells, the IP_3 levels generated with either $G\alpha_{11}$ or $G\alpha_{16}$ were not significantly different between the two cell types ($p = 0.329$ for TPα versus TPβ in the presence of $G\alpha_{11}$, $p = 0.093$ for TPα versus TPβ in the presence of $G\alpha_{16}$ for 1 min U46619 treatment) indicating that TPα and TPβ couple similarly to these members of the G_q family in this system.

Coupling of TPα and TPβ to $G\alpha_{11}$ and $G\alpha_{16}$ was also evaluated by measurement of intracellular calcium ($[Ca^{2+}]_i$) mobilization in response to stimulation of cells with U46619. HEK.α10 or HEK.β3 cells were transiently co-transfected with the cDNA for the appropriate Gα protein or, as a control, with pCMV5 and cells were loaded with FURA2/AM before measurement of $[Ca^{2+}]_i$ mobilization in response to U46619. Dose response studies confirmed that 1 μM U46619 was necessary to elicit maximal Ca^{2+} mobilization, consistent with previously published data [26]. Co-transfection of cells with $G\alpha_{11}$ and $G\alpha_{16}$ significantly increased U46619 (1 μM) mediated $[Ca^{2+}]_i$ mobilization by both HEK.α10 (Figure 3A & 3B) and HEK.β3 (Figure 3C & 3D) cells over levels obtained with pCMV5 transfected cells (Figure 3) or non-transfected cells (data not shown). However, for example, in the case of HEK.α10 cells co-transfected with $G\alpha_{11}$, raising the agonist concentration to 10 μM U46619 did not significantly increase $[Ca^{2+}]_i$ mobilization ($p= 0.712$) whilst lowering the concentration to 0.1 μM significantly ($p<0.05$) reduced $[Ca^{2+}]_i$ mobilization. Similar results were observed for $G\alpha_{16}$ and in HEK.β3 cells. However, there was no significant difference in signaling between HEK.α10 and HEK.β3 cells in the presence of either G protein indicating that TPα or TPβ do not differentially couple to $G\alpha_{11}$ or $G\alpha_{16}$.

Signaling via $G\alpha_{12}$

A number of independent studies have indicated that the TP(s) may couple to members of the G_{12} family [21-24] although the intracellular effectors influenced by these G proteins are unclear. To assess whether TPα or TPβ couple to $G\alpha_{12}$, HEK.α10 and HEK.β3 cells were transiently co-transfected with the cDNA for $G\alpha_{12}$ and U46619 mediated intracellular signaling was monitored. Co-expression of $G\alpha_{12}$ did not significantly affect the K*d* or B_{max} of HEK.α10 or HEK.β3 cells for SQ29,548. Confirmation of G protein over-expression in the various HEK 293 cell lines was routinely performed by Western blot analysis using antibodies specific to $G\alpha_{12}$ (Figure 1C). For either cell type co-transfected with $G\alpha_{12}$, stimulation of cells with 1 μM U46619 (Figure 4A & 4B) or 10 μM U46619 (data not shown) failed to generate significant elevations of IP_3 levels, despite the fact that 1 μM U46619 has been established to elicit maximal TP responses [42, 26]. In fact, U46619 mediated IP_3 levels in HEK.α10 or HEK.β3 cells in the presence of $G\alpha_{12}$ were not significantly greater than those generated by cells co-transfected with pCMV5 control (Figure 4A & 4B), confirming that neither TPα or TPβ couple via $G\alpha_{12}$ to activate PLCβ isoforms and increase IP_3 levels. However, HEK.α10 and HEK.β3 cells co-transfected with $G\alpha_{12}$ each exhibited significantly greater mobilization of Ca^{2+} compared to those cells co-transfected with pCMV5 (Figure 5). Dose-responses confirmed that maximal $[Ca^{2+}]_i$ mobilization was stimulated by 1 μM U46619; for example, in HEK.α10 cells co-transfected with $G\alpha_{12}$, raising the concentration of U46619 to 10 μM did not significantly ($p=0.67$) increase $[Ca^{2+}]_i$ mobilization whereas lowering the concentration to 0.1 μM U46619 significantly reduced Ca^{2+} mobilization ($p <0.05$). Similar results were obtained in HEK.β3 cells. These results implied that the maximal Ca^{2+} mobilization observed in HEK.α10 and HEK.β3 cells co-transfected with $G\alpha_{12}$ was the result

of a separate mechanism to that of $G\alpha_{11}$ or $G\alpha_{16}$ and did not involve PLCβ activation and phosphoinositide turnover.

Influx of Calcium via verapamil sensitive Ca^{2+} channels is a major element in $G\alpha_{12}$ coupling in HEK 293 cells

To further investigate the mechanism of U46619-mediated increases in $[Ca^{2+}]_i$ in HEK.α10 and HEK.β3 cells co-transfected with $G\alpha_{12}$, we examined the effect of verapamil, an inhibitor of voltage dependent Ca^{2+} channels, principally the L-type channels [34] on $[Ca^{2+}]_i$ mobilization in cells co-transfected with $G\alpha_{12}$ and compared it to its effect on those cells co-transfected with $G\alpha_{11}$. U46619 mediated Ca^{2+} mobilization was assessed in FURA2/AM-loaded HEK.α10 and HEK.β3 cells in the presence or absence of pre-treatment with verapamil (10 μM, 15 min pre-treatment). Initially, it was established that this treatment did not significantly ($p=0.269$) affect basal Ca^{2+} levels. Pre-treatment of HEK.α10 cells co-transfected with $G\alpha_{11}$ with verapamil did not significantly reduce U46619 mediated $[Ca^{2+}]_i$ mobilization ($p = 0.254$; Figure 6A & 6E). However, verapamil pre-treatment of HEK.α10 cells co-transfected with $G\alpha_{12}$ reduced Ca^{2+} mobilization (Figure 6B & 6E) to 43.3 ± 3.26 % of untreated levels, a significantly ($p< 0.05$) greater reduction than in those cells transfected with $G\alpha_{11}$. The extent of U46619 mediated $[Ca^{2+}]_i$ mobilization remaining in the presence of verapamil was not significantly ($p=0.31$) different from the small basal level observed in HEK.α10 cells in the absence of co-transfected G protein (Figures 6G, 3 & 5). This implies that $[Ca^{2+}]_i$ mobilization mediated due to TP coupling to $G\alpha_{12}$ triggers influx of Ca^{2+} due to verapamil sensitive channel opening. In HEK.β3 cells co-transfected with $G\alpha_{11}$, verapamil did not affect U46619 mediated $[Ca^{2+}]_i$ mobilization (Figure 6C & 6F). However, verapamil reduced Ca^{2+} mobilization in HEK.β3 cells co-transfected with $G\alpha_{12}$ to 37.8 ± 12.9 % of that mobilized in its absence (Figure 6D & 6F). Similar to HEK.α10 cells, the extent of U46619 mediated $[Ca^{2+}]_i$ mobilization remaining in the presence of verapamil was not significantly ($p=0.877$) different from that in HEK.β3 cells in the absence of co-transfected G protein (Figures 6G, 3 & 5). These results imply that the Ca^{2+} mobilization observed in both HEK.α10 or HEK.β3 cells co-transfected with $G\alpha_{12}$ was largely due to influx of Ca^{2+} via opening of verapamil sensitive membrane Ca^{2+} channels.

To further investigate the mechanism of U46619-mediated increases in $[Ca^{2+}]_i$ in HEK.α10 and HEK.β3 cells co-transfected $G\alpha_{12}$, we also examined the effect of 3,4,5-trimethyloxybenzoic acid 8-(diethylamino)octyl ester (TMB-8), which selectively blocks the release of Ca^{2+} from intracellular stores [35] on $[Ca^{2+}]_i$ mobilization in cells co-transfected with $G\alpha_{12}$ and compared it to its effect on those cells co-transfected with $G\alpha_{11}$. HEK.α10 or HEK.β3 cells, transiently co-transfected with either $G\alpha_{11}$ or $G\alpha_{12}$ were loaded with FURA2/AM prior to treatment with TMB-8 (50 μM) for 15 min. At the concentrations used, TMB-8 partially reduced the mobilization of $[Ca^{2+}]_i$ in both HEK.α10 (Figure 7A & 7E) and HEK.β3 (Figure 7C & 7F) cells co-transfected with $G\alpha_{11}$ to 59.1 ± 12.2 % and 76.7 ± 11.1%, respectively. However, TMB-8 did not reduce $[Ca^{2+}]_i$ mobilization in either cell type co-transfected with $G\alpha_{12}$ (Figure 7B,

7D - 7F). The effects of TMB-8 on $G\alpha_{11}$, but on not $G\alpha_{12}$, coupled signaling further confirms that TPα and TPβ mediated $[Ca^{2+}]_i$ mobilization via $G\alpha_{11}$ is dependent on sources of intracellular Ca^{2+} but that neither TP isoform mobilizes Ca^{2+} from intracellular sources upon coupling to $G\alpha_{12}$.

The carboxyl terminal cytoplasmic tail of TPs contains determinants of G protein coupling efficiency.

TPα and TPβ differ exclusively in their C-tail regions and previous studies have indicated that the TP isoforms may differentially couple to $G\alpha_S$, $G\alpha_i$ and G_h [28, 33]. Thus, to assess whether a C-tail *per se* is important in mediating TP coupling to the G_q or G_{12} family of heterotrimeric G proteins, we investigated U46619-mediated intracellular signaling and G protein coupling by HEK.$TP^{\Delta 328}$ cells which over-express a truncated variant of TP ($TP^{\Delta 328}$) lacking the C-tail sequences distal to amino acid 328 at the point of divergence of TPα and TPβ. $TP^{\Delta 328}$ exhibited identical [^{3}H]SQ29,548 radioligand binding characteristics (K*d*, 6.96 ± 0.88 nM; B_{MAX}, 1.54 ± 0.28 pmol / mg cell protein) to those of the wild type TPα and TPβ receptors. Thereafter, the effect of U46619-mediated $[Ca^{2+}]_i$ mobilization by $TP^{\Delta 328}$ was compared to that of the wild type TPα and TPβ receptors in the presence or absence of co-transfection of $G\alpha_{11}$, $G\alpha_{12}$ or $G\alpha_{16}$. Non-transfected HEK.$TP^{\Delta 328}$ cells (data not shown) or cells co-transfected with the vector pCMV5 exhibited a significantly greater change in $[Ca^{2+}]_i$ mobilization in response to U46619 than did HEK.α10 ($p < 0.02$) or HEK.β3 ($p < 0.02$) cells transfected with pCMV5 (Figure 8A). However, unlike that observed for HEK.α10 or HEK.β3 cells, co-transfection of HEK.$TP^{\Delta 328}$ cells with either $G\alpha_{11}$, $G\alpha_{12}$ or $G\alpha_{16}$ did not significantly enhance the levels of $[Ca^{2+}]_i$ mobilization in either case (Figure 8B - 8E). To confirm these data, we investigated the effect of co-expressing Gα subunits on U46619 mediated $[Ca^{2+}]i$ in a second, independent isolate (isolate B) of HEK.$TP^{\Delta 328}$ cells which over-expresses higher levels of $TP^{\Delta 328}$ (B_{MAX} = 2.33 ± 0.096 pmol / mg cell protein, n = 4) than the previously described HEK.$TP^{\Delta 328}$ cells (isolate A). Consistent with previous data, whereas HEK.$TP^{\Delta 328}$ isolate B exhibited slightly higher U46619 mediated signaling than the original isolate A in both non-transfected cells or in cells co-transfected with pCMV5, co-expression of either $G\alpha_{11}$, $G\alpha_{12}$ or $G\alpha_{16}$ did not significantly augment $[Ca^{2+}]_i$ mobilization by that cell line (Figure 9). Taken together, these results imply that although the C-tail of TP does not appear to dictate specificity of G protein coupling to the G_q or G_{12} members examined, the presence of a C-tail may be important for controlled and efficient G protein coupling and that it may actually confer negative regulatory effects on receptor: G protein interaction.

To extend these studies, as TPα and TPβ differ exclusively in their C-tail sequences and they oppositely regulate adenylyl cyclase, we also investigated $TP^{\Delta 328}$ signaling to the effector adenylyl cyclase. Whereas stimulation of HEK.α10 cells with U46619 (1 μM) led to an approximately 1.5 fold increases in cAMP, stimulation of HEK.$TP^{\Delta 328}$ cells led to a significantly ($p = 0.002$) higher generation of cAMP (Figure 10A). On the other hand, stimulation of HEK.β3 cells failed to generate an increase in cAMP but did reduce forskolin mediated cAMP generation, consistent with its coupling to $G\alpha_i$ in these cells (Figure 10B). Neither TPα nor $TP^{\Delta 329}$ showed coupling to $G\alpha_i$ whereas TPβ showed significant coupling to $G\alpha_i$ ($p < 0.05$, 1 μM

U46619; Figure 10B). Whilst co-transfection of $G\alpha_S$ with TPα facilitated a significant ($p < 0.02$) augmentation in U46619 (1 μM) mediated cAMP generation, it did not lead to a significant ($p = 0.58$) augmentation in cAMP by $TP^{\Delta 328}$. Confirmation of G protein over-expression in the various HEK 293 cell lines was routinely performed by Western blot analysis using antibodies specific to $G\alpha_S$ (Figure 1A). Thus, similar to the TPα, but not the TPβ isoform, $TP^{\Delta 328}$ exhibited U46619-mediated activation of adenylyl cyclase, with concomitant increases in cAMP generation. However, $TP^{\Delta 328}$ exhibited significantly higher cAMP generation than TPα in the absence of exogenous G protein yet displayed an impaired ability to couple to co-transfected $G\alpha_S$.

Discussion

In this study, we have investigated the functional coupling of the α and β isoforms of the human TP to $G\alpha_{16}$,$G\alpha_{12}$ and $G\alpha_S$ in HEK 293 cells over-expressing the individual TP isoforms in order to determine whether divergence in the C-tail sequences between the receptor isoforms influences G protein coupling specificity. HEK 293 cells lack sufficient endogenous levels of TP expression to permit measurement of intracellular signaling, despite the presence of mRNAs encoding both TP isoforms (Miggin & Kinsella., unpublished data). Furthermore, HEK 293 cells either transiently or stably transfected with TP do not exhibit significant agonist mediated $[Ca^{2+}]_i$ mobilization unless they were co-transfected with $G\alpha_q$ or $G\alpha_{11}$ [26, 27], similar to the *alpha*-2 adrenoreceptor stimulation of PLCβ activity in HEK 293 cells which is completely dependent on the co-expression of $G\alpha_q$ [45].

Our results demonstrated that TPα and TPβ each exhibited similar U46619-mediated coupling to $G\alpha_{11}$ and $G\alpha_{16}$ to effect increases in IP_3 and mobilization of $[Ca^{2+}]_i$. Coupling of the TP isoforms to $G\alpha_{16}$ is consistent with its expression in platelets and other megakaryocytic cells [20, 46]. In fact, it has been suggested that the expression of $G\alpha_{16}$ mRNA and protein in various megakaryoblastic cell lines of increasing maturation corresponds to the appearance of TXA_2 induced signaling ($[Ca^{2+}]_i$ mobilization and stimulation of PGI_2 - induced cAMP formation) in megakaryocytic cell lineages [20].

Whereas both TPα and TPβ did exhibit increases in $[Ca^{2+}]_i$ mobilization in cells co-transfected with $G\alpha_{12}$, neither receptor generated IP_3 increases in the presence of $G\alpha_{12}$. Verapamil, an inhibitor of voltage dependent Ca^{2+} channels, principally the L-type Ca^{2+} channels [34], significantly reduced $[Ca^{2+}]_i$ mobilization by TPα and TPβ in cells co-expressing $G\alpha_{12}$, but not $G\alpha_{11}$, implying that in this system TPα and TPβ couple via $G\alpha_{12}$ to activate an endogenous, verapamil-sensitive voltage-dependent membrane calcium channel. The possible involvement of L-type channels was also confirmed with nifedipine, an alternative L-type Ca^{2+} channel blocker, which caused significant reductions in $G\alpha_{12}$ coupled mobilization of $[Ca^{2+}]_i$ by either TPα or TPβ in response to U46619. However, this compound significantly ($p<0.005$) perturbed basal Ca^{2+} levels making it less suitable for studies involving Fura 2 based Ca^{2+} measurements (results not shown). It has been reported that the endogenous Ca^{2+} channels in HEK 293 cells differ somewhat from L-type channels in terms of kinetic properties and pharmacological features [47]. However, they are sensitive to the effects of the L-type channel inhibitor isradipine, a dihydropyridine and nimodipine but somewhat insensitive to blockers of N-, P- and Q- type channels affirming the existence of L-type Ca^{2+} channels in HEK 293 cells [47]. Consistent with these studies, Tosun *et al*., [51] reported that TP mediated contraction of rat aortic smooth muscle is partially dependent on the influx of extracellular Ca^{2+} from verapamil sensitive L-type Ca^{2+} channels. TMB-8, an antagonist of intracellular Ca^{2+} mobilization widely used to distinguish dependence on intracellular versus extracellular Ca^{2+} [35], reduced $[Ca^{2+}]_i$ mobilization by both TPα and TPβ in the presence of $G\alpha_{11}$ but not in the presence of $G\alpha_{12}$ thereby confirming that $G\alpha_{12}$ mediated mobilization of Ca^{2+} is not from intracellular stores. Whilst TMB-8 has been demonstrated to have effects other than antagonism of intracellular Ca^{2+}, for example in antagonism of muscarinic receptor in the

HT29 epithelial cell line, functional antagonism of nicotinic acetylcholine receptors, and inhibition of transcription factor NF-κB activation [48-50], it seems unlikely, particularly in the context of the verapamil studies, that these effects are relevant in the case of TP mediated signaling. A number of GPCRs have been reported to stimulate Ca^{2+} influx by activation of L-type Ca^{2+} channels including the novel TM4 splice variant of the pituitary adenylate cyclase-activating polypeptide (PACAP) receptor [52] and angiotensin $(AT)_1$ [53]. In the present study, we have established that whereas $G\alpha_{12}$ did not couple TPα or TPβ to PLC activation, that $G\alpha_{12}$ does couple each receptor to verapamil sensitive Ca^{2+} channels, consistent with L-type channels.

A number of independent studies have implicated TP coupling to members of the G_{12} family [21-24, 54] and the presence of both $G\alpha_{12}$ and $G\alpha_{13}$ in platelets and other hematopoietic cells has been widely reported [55, 56, 22]. Both $G\alpha_{12}$ and $G\alpha_{13}$ have been shown to be activated by TP(s) in human platelets [22]. Moreover, activation of human platelets with the TP agonist U46619 leads to phosphorylation of both $G\alpha_{12}$ and $G\alpha_{13}$ by a mechanism involving PKC [56]. In general, the immediate effectors coupled to the G_{12} family are unknown. They have been implicated in transduction of thrombin receptor activation of AP-1 mediated gene expression [57] activation of Jun Kinase / stress activated protein kinase and activation of different isoforms of a growth factor -responsive Na^+/H^+ exchanger (NHE) present in most cells [58, 59].

Thus, our data indicate that TPα and TPβ couple almost identically to $G\alpha_{11}$ and $G\alpha_{16}$ members of the G_q family and to $G\alpha_{12}$, in response to the TP agonist, U46619, in stably transfected HEK 293 cells. In dose-response experiments, we found that 1 μM U46619 was required for maximal IP_3 generation and $[Ca^{2+}]_i$ mobilization in HEK.α10 and HEK.β3 stable cell lines transiently co-transfected with G proteins ($G\alpha_{11}$, $G\alpha_{16}$ and $G\alpha_{12}$), with respective EC_{50} values of approximately 50 nM observed in each case, regardless of the G protein used. This is consistent with previous reports concerning $G\alpha_{11}$-mediated coupling to PLC activation [27]. However, in transfected CHO cells, they oppositely regulate adenylyl cyclase, [28] and have been recently shown to couple differentially to the high molecular weight G protein G_h in COS-7 cells [33]. This implies that the difference in the C-tail may influence specificity of G protein coupling and hence downstream signaling events, as is the case for other GPCR isoforms which diverge exclusively in their C-tail sequences, for example the EP_3 isoform of the PGE_2 receptor [12,60] and a wide variety of other GPCRs [61]. Thus in this study, we also used cells which over-express $TP^{\Delta 328}$, a variant of TP lacking the C-tail sequences at the point of divergence of TPα and TPβ distal to amino acid 328, to investigate whether the C-tail *per se* is important in mediating TP coupling to $G\alpha_{11}$, $G\alpha_{16}$ or $G\alpha_{12}$. Whereas $TP^{\Delta 328}$ exhibited identical ligand binding properties to the wild type TP isoforms, signaling was significantly reduced. However, non-transfected HEK.$TP^{\Delta 328}$ cells or cells co-transfected with the vector pCMV5 exhibited a significantly greater increase in $[Ca^{2+}]_i$ mobilization in response to U46619 than did HEK.α10 or HEK.β3 cells. Unlike TPα or TPβ, however, co-expression of $G\alpha_{11}$, $G\alpha_{12}$, or $G\alpha_{16}$ did not significantly enhance receptor mediated $[Ca^{2+}]_i$ mobilization or IP_3 generation (results not shown) by $TP^{\Delta 328}$. These effects were independent of the level of TP expression, as two independent isolates of HEK.$TP^{\Delta 328}$ cells

which express $TP^{\Delta 328}$ at varying levels exhibited this effect. Thus, to extend our studies on the signaling of the $TP^{\Delta 328}$, we sought to establish whether it coupled to $G\alpha_s$ or $G\alpha_i$ to mediate activation or inhibition of adenylyl cyclase, respectively. Moreover, we sought to investigate the effect of co-expression of the coupling G -protein ($G\alpha_s$ or $G\alpha_i$) on second messenger generation by TP. Our findings were that, like TPα, $TP^{\Delta 328}$ mediated activation of adenylyl cyclase in response to U46619 stimulation to bring about increases in cAMP formation but unlike TPβ, it failed to couple to $G\alpha_i$ to inhibit adenylyl cyclase. However, in the absence of co-transfection of $G\alpha_s$, cAMP generation by $TP^{\Delta 328}$ was significantly greater than that of 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.

These results indicate that whilst the C-tail *per se* may not determine G-protein specificity, it may play a role in influencing G_S versus G_i coupling and it may confer regulatory effects that influence receptor: G protein interaction. In studies involving the mouse TP, Spurney & Coffman [62] previously reported that the C-terminal 20 amino acids of the single mouse TP was required for optimal intracellular signaling and also played a role in mediating homologous and, in part, heterologous desensitisation of TP. In many mammalian species, several C-tail splice variants exist for the PGE_2 receptor subtype 3 (EP_3) [12-16]. For example in mouse there are 4 receptor subtypes with different G protein coupling specificities [13, 14]. The $EP_{3\alpha}$ receptor exhibits some agonist-independent constitutive inhibition of adenylyl cyclase whereas the $EP_{3\beta}$ receptor does not [63]. However, a mutant receptor truncated at the splicing site showed full constitutive activity. A chimeric receptor composed of the N-terminal domain to transmembrane domain 7 of the G_i coupled rat $EP_{3\beta}$ receptor and the C-tail of the G_s coupled human EP_4 receptor was used to test the effect of the C-tail on G-protein coupling specificity and constitutive activity [64]. Whilst there was no agonist-independent receptor signaling, the chimeric receptor behaved as a G_i rather than a G_s coupled receptor indicating that the C-tail did not confer G protein coupling specificity but did maintain the receptor in a state whereby signaling arose only in response to agonist [59]. In this study, we also demonstrate that whilst the C-tail of the TP isoforms apparently does not confer G protein coupling specificity to members of the G_q or G_{12} families in HEK 293 cells, 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 signaling.

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Figures.

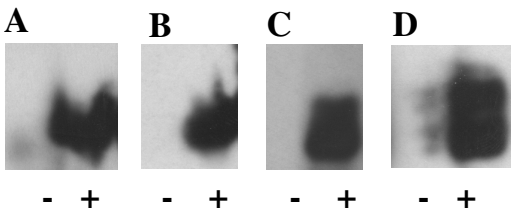

Figure 1. Western blot analysis.

HEK 293 cells were transiently co-transfected with the cDNA encoding $G\alpha_s$ (Panel A, +), $G\alpha_{11}$ (Panel B, +), $G\alpha_{12}$ (Panel C, +), $G\alpha_{16}$ (Panel D, +) or as a control, with the vector pCVM5 (Panels A, B, C, D, -). Forty-eight hours post-transfection, cells were harvested and aliquots (100 μg) of whole cell protein were subjected to SDS-PAGE followed by Western blot analysis. The blots presented are representative of at least four independent experiments. The relative position of the 46 kDa molecular weight marker indicated by an arrow at the left of each panel. Similar levels of G protein expression were observed in HEK.α10, HEK.β3 and HEK.$TP^{\Delta 328}$ cell lines transiently co-transfected with the various G protein α-subunits.

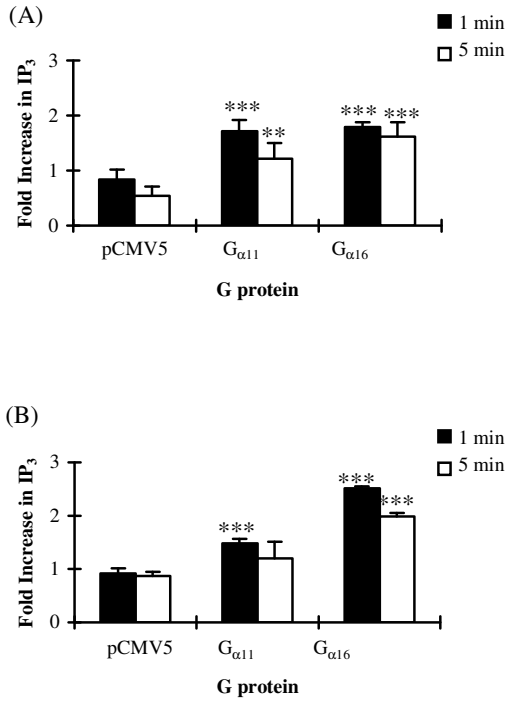

Figure 2. U46619-mediated IP_3 production in HEK.α10 and HEK.β3 cells co-transfected with $G\alpha_{11}$ or $G\alpha_{16}$.

HEK.α10 cells (Panel A) or HEK.β3 cells (Panel B), transiently co-transfected with the cDNA encoding $G\alpha_{11}$ or $G\alpha_{16}$ or as a control, with the vector pCMV5, were stimulated with 1 μM U46619 at 37 ^{o}C for 1 min or 5 min. In each case, basal IP_3 levels were determined by exposing the cells to the vehicle HBS under identical conditions. Levels of IP_3 produced in ligand stimulated cells relative to vehicle treated cells (basal IP_3) were expressed as fold stimulation of basal (Fold increase in $IP_3 \pm$ S.E). The data presented are the mean values of 4 independent experiments, each carried out in duplicate. ** ($p<0.02$) or *** ($p<0.005$) indicates that U46619-induced IP_3 levels were significantly higher in cells co-transfected with $G\alpha_{11}$ or $G\alpha_{16}$ than in cells co-transfected with pCMV5.

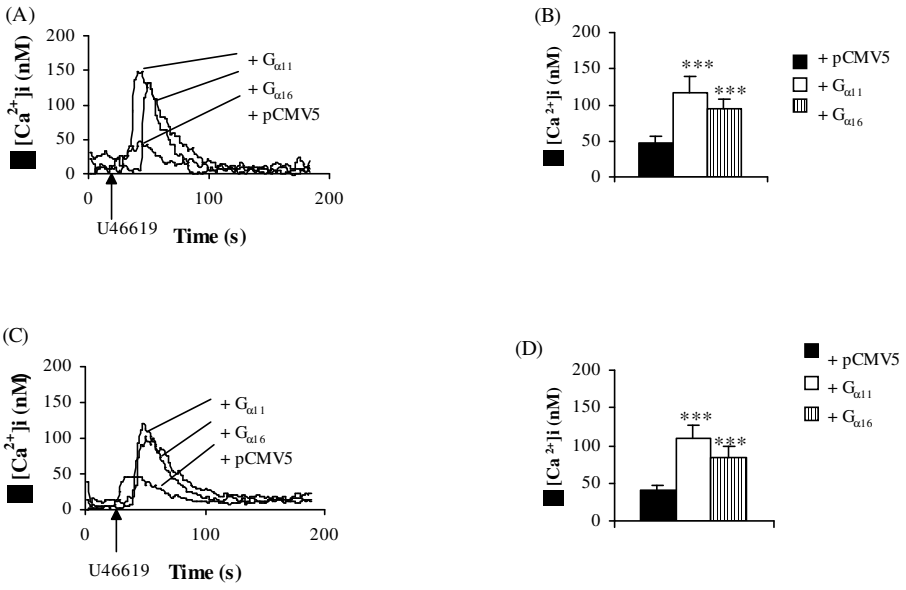

Figure 3. U46619-mediated $[Ca^{2+}]_i$ mobilization in HEK.α10 and HEK.β3 cells co-transfected with $G\alpha_{11}$ or $G\alpha_{16}$. HEK.α10 cells (Panels A & B) or HEK.β3 cells (Panel C & D), transiently co-transfected with the cDNA encoding $G\alpha_{11}$ or $G\alpha_{16}$ or, as a control, with the vector pCMV5, were pre-loaded with FURA2/AM and stimulated with 1 μM U46619. Panels A & C: the data presented are a representative profile from 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 where U46619 was added at the times indicated by the arrows.

Panels B & D: For each experiment, the mean values for changes in U46619 - mediated intracellular Ca^{2+} mobilized from at least 4 independent experiments were calculated; mean data are plotted as changes in intracellular Ca^{2+} mobilized ($\Delta[Ca^{2+}]_i \pm$ S.E, nM; n = 4) versus G protein.

*** ($p<0.005$) indicates that U46619-induced $\Delta[Ca^{2+}]_i$, was significantly higher in cells co-transfected with $G\alpha_{11}$ or $G\alpha_{16}$ than in cells co-transfected with pCMV5.

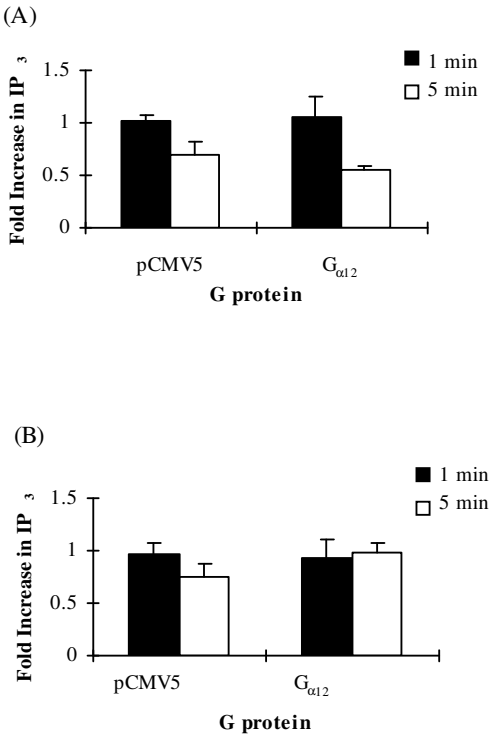

Figure 4. U46619-mediated IP_3 production in HEK.α10 and HEK.β3 cells co-transfected with $G\alpha_{12}$. HEK.α10 cells (Panel A) or HEK.β3 cells (Panel B), transiently co-transfected with the cDNA encoding $G\alpha_{12}$ or as a control, with the vector pCMV5, were stimulated with 1 μM U46619 at 37 ^{o}C for 1 min or 5 min. In each case, basal IP_3 levels were determined by exposing the cells to the vehicle HBS under identical conditions. Levels of IP_3 produced in ligand stimulated cells relative to vehicle treated cells (basal IP_3) were expressed as fold stimulation of basal (Fold increase in IP_3 ± S.E). The data presented are the mean values of 4 independent experiments, each carried out in duplicate.

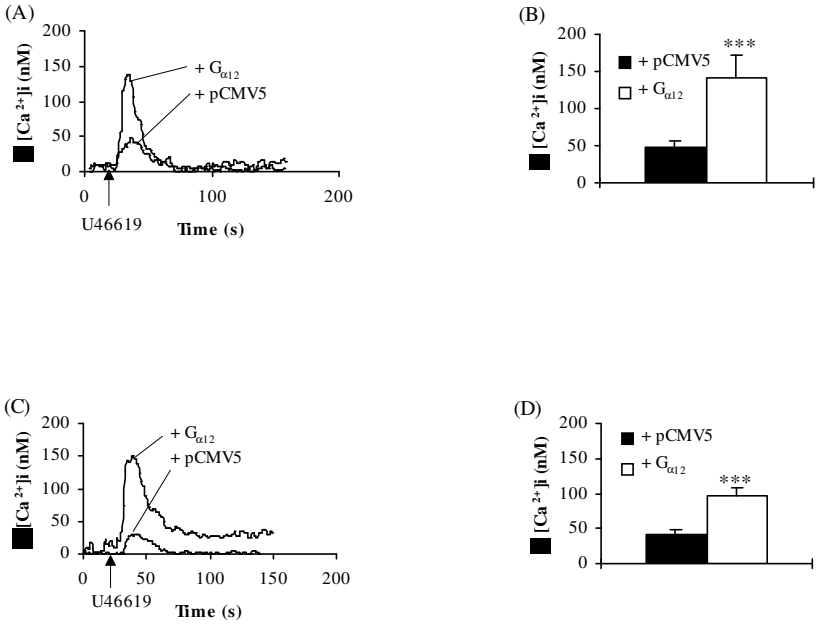

Figure 5. U46619-mediated $[Ca^{2+}]_i$ mobilization in HEK.α10 and HEK.β3 cells co-transfected with $G\alpha_{12}$ HEK.α10 cells (Panels A & B) or HEK.β3 cells (Panel C & D), transiently co-transfected with the cDNA encoding $G\alpha_{12}$ or as a control, with the vector pCMV5, were pre-loaded with FURA2/AM and stimulated with 1 μM U46619. Panels A & C: the data presented are a representative profile from 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 where U46619 was added at the times indicated by the arrows. Panels B & D: For each experiment, the mean values for changes in U46619 - mediated intracellular Ca^{2+} mobilized from at least 4 independent experiments were calculated; mean data are plotted as changes in intracellular Ca^{2+} mobilized ($\Delta[Ca^{2+}]_i \pm$ S.E, nM; n = 4) versus G protein.

*** ($p<0.005$) indicates that U46619-induced $\Delta[Ca^{2+}]_i$, was significantly higher in cells co-transfected with $G\alpha_{12}$ than in cells co-transfected with pCMV5.

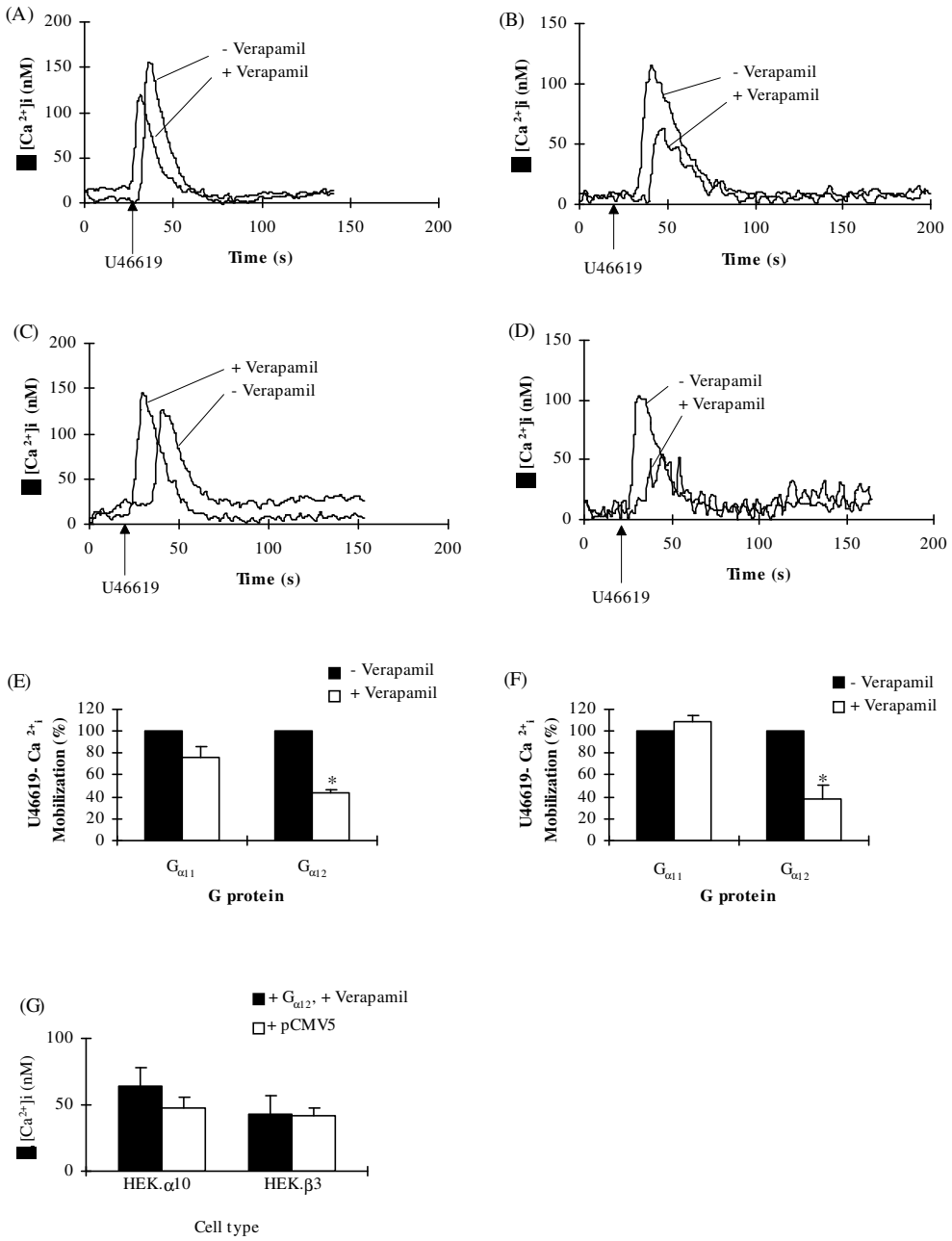

Figure 6. Effect of Verapamil on U46619 -mediated $[Ca^{2+}]_i$ mobilization by TPα and TPβ.

HEK.α10 cells (Panels A, B & E) and HEK.β3 cells (Panels C, D & F), transiently co-transfected with the cDNA encoding $G\alpha_{11}$ (Panels A, C, E, F) or $G\alpha_{12}$ (Panels B, D, E, F) were pre-loaded with FURA2/AM and stimulated with either 1 μM U46619 or were pre-incubated in the presence of verapamil (10 μM) at 37 °C for 15 min prior to stimulation with 1 μM U46619.

Panels A - D: the data presented are a representative profile from 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 where U46619 was added at the times indicated by the arrows.

Panels E & F: Changes in U46619 - mediated intracellular Ca^{2+} mobilized ($\Delta[Ca^{2+}]_i \pm$ S.E, nM; n = 4) were calculated; those levels of $[Ca^{2+}]_i$ mobilized following stimulation with U46619 only (- Verapamil) were set to represent 100 % and thereafter, the level of U46619-mediated $[Ca^{2+}]_i$ mobilized subsequent to prior stimulation with verapamil (+ Verapamil) were calculated as a percentage of that value. Data are plotted as U46619 mediated $Ca^{2+}{}_i$ Mobilization, Percentage (U46619 - $Ca^{2+}{}_i$ Mobilization (%)) versus G protein. *($p<0.05$) indicates that U46619-induced $\Delta[Ca^{2+}]_i$, was significantly lower in cells co-transfected with $G\alpha_{12}$ treated with verapamil than in untreated cells.

Panel G: HEK.α10 cells and HEK.β3 cells transiently co-transfected with the cDNA encoding $G\alpha_{12}$ or with pCMV5 were pre-loaded with FURA2/AM. Cells co-transfected with $G\alpha_{12}$ were pre-incubated in the presence of verapamil (10 μM) at 37 ^{o}C for 15 min prior to stimulation with 1 μM U46619. Cells co-transfected with pCMV5 were stimulated with 1 μM U46619. Data are plotted as U46619 mediated $Ca^{2+}{}_i$ Mobilization (nM) ($\Delta[Ca^{2+}]_i \pm$ S.E, nM; n = 3) versus cell type.

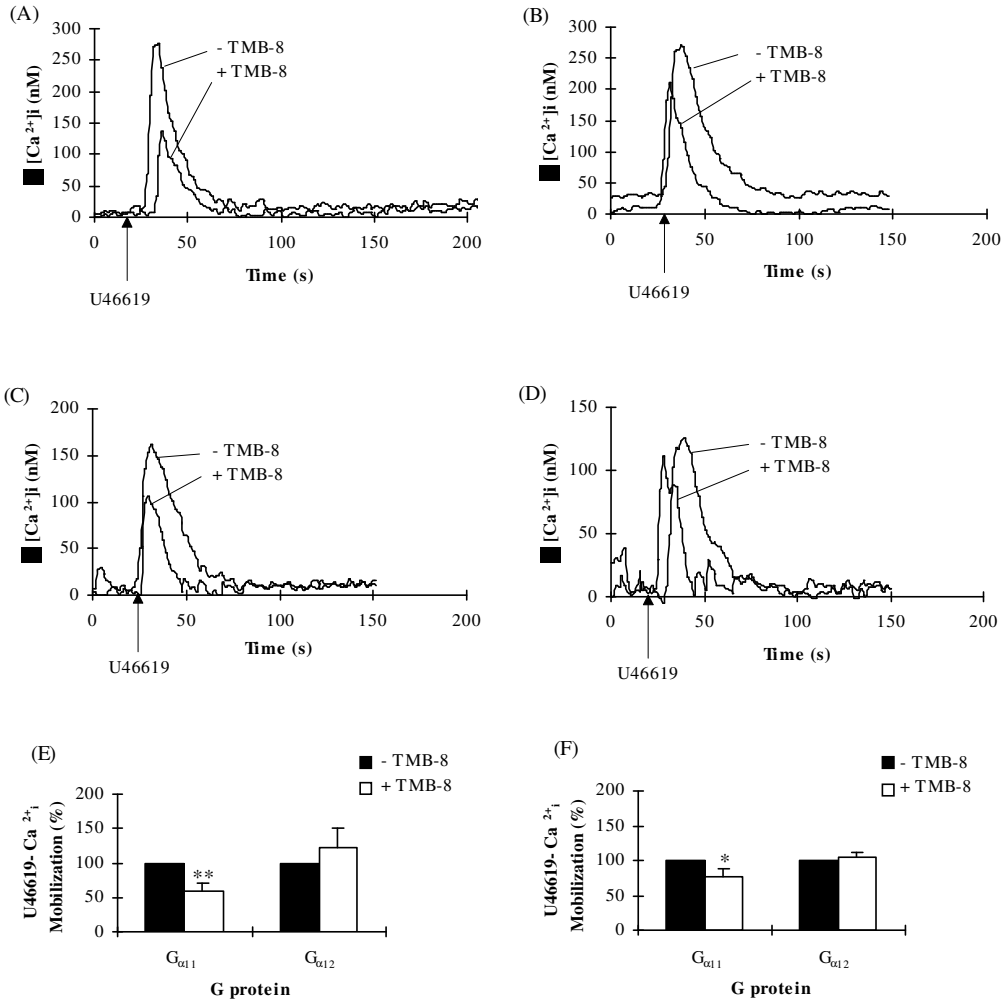

Figure 7. Effect of TMB-8 on U46619 -mediated $[Ca^{2+}]_i$ mobilization by TPα and TPβ.

HEK.α10 cells (Panel A, B & E) and HEK.β3 cells (Panel C & D & F), transiently co-transfected with the cDNA encoding $G\alpha_{11}$ (Panels A, C, E, F) or $G\alpha_{12}$ (Panels B, D, E, F) were pre-loaded with FURA2/AM and stimulated with either 1 μM U46619 or were pre-incubated in the presence or TMB-8 (50 μM) at 37 °C for 15 min prior to stimulation with 1 μM U46619. Panels A - D: the data presented are a representative profile from 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 where U46619 was added at the times indicated by the arrows. Panels E & F: Changes in U46619 - mediated intracellular Ca^{2+} mobilized ($\Delta[Ca^{2+}]_i \pm$ S.E, nM; n = 4) were calculated; those levels of $[Ca^{2+}]_i$ mobilized following stimulation with U46619 only (- TMB-8) were set to represent 100 % and thereafter, the level of U46619-mediated $[Ca^{2+}]_i$ mobilized subsequent to prior stimulation with TMB-8 (+ TMB-8) were calculated as a percentage of that value. Data are plotted as U46619 mediated $Ca^{2+}{}_i$ Mobilization, Percentage (U46619 - $Ca^{2+}{}_i$ Mobilization (%)) versus G protein. * ($p<0.05$) or ** ($p<0.02$) indicates that U46619-induced $\Delta[Ca^{2+}]_i$, was significantly lower in cells co-transfected with $G\alpha_{11}$ treated with TMB-8 than in untreated cells.

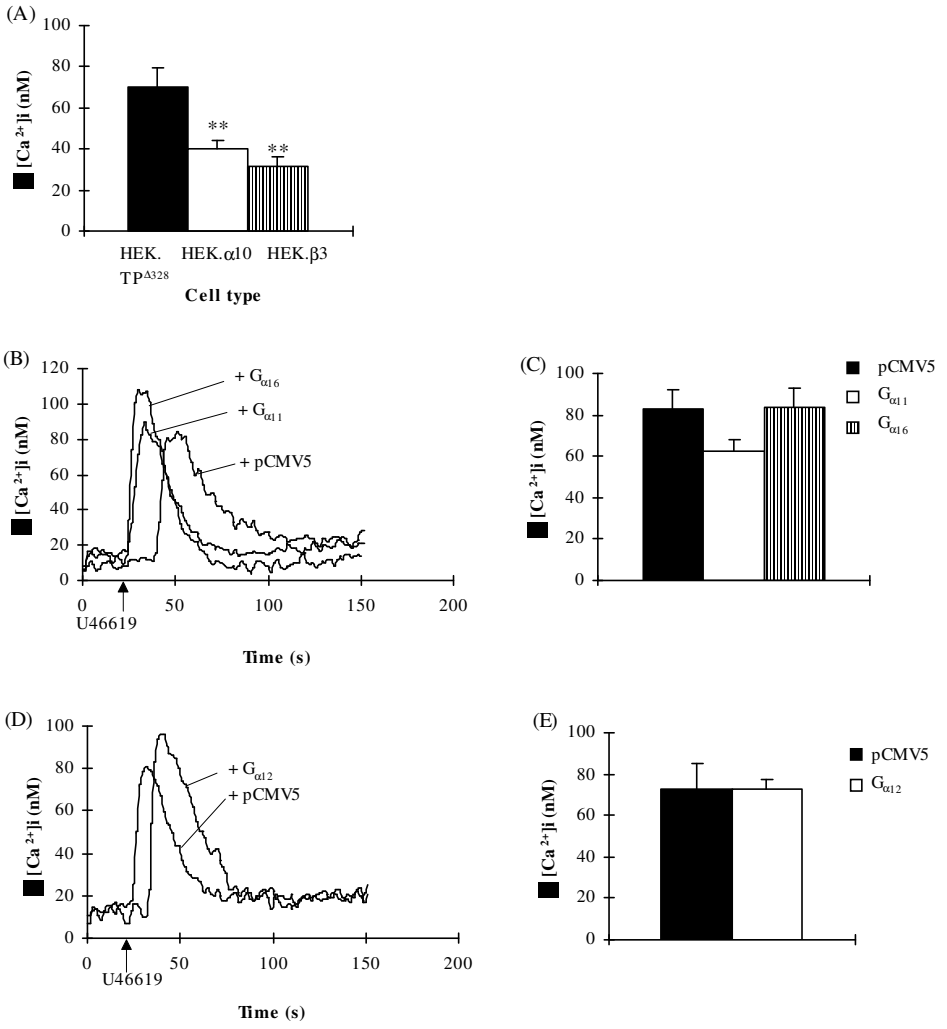

Figure 8. U46619-mediated $[Ca^{2+}]_i$ mobilization in HEK.TP$^{\Delta 328}$ cells co-transfected with Gα_{11} , Gα_{12} or Gα_{16}.

HEK.TP$^{\Delta 328}$, HEK.α10 or HEK.β3 cells, transiently co-transfected with pCMV5 (Panel A) or HEK.TP$^{\Delta 328}$, transiently co-transfected with the cDNA encoding Gα_{11} or Gα_{16} (Panels B & C) or Gα_{12} (Panels D & E) or as controls, with the vector pCMV5 (Panels A-E), were pre-loaded with FURA2/AM and stimulated with 1 μM U46619. Panels B & D: the data presented are a representative profile from at least 4 independent experiments and are plotted as changes in intracellular Ca^{2+} mobilized ($\Delta[Ca^{2+}]_i \pm$ S.E, nM) as a function of Time (second, s) following ligand stimulation where U46619 was added at the times indicated by the arrows. Panels A, C & E: For each experiment, the mean values for changes in U46619 - mediated intracellular Ca^{2+} mobilized from at least 4 independent experiments were calculated; mean data are plotted as changes in intracellular Ca^{2+} mobilized ($\Delta[Ca^{2+}]_i \pm$ S.E, nM; n = 4) versus G protein.

** ($p<0.02$) indicates that U46619-induced $\Delta[Ca^{2+}]_i$, was significantly higher in HEK.TP$^{\Delta 328}$ cells co-transfected with pCMV5 than in HEK.α10 or HEK.β3 cells co-transfected with pCMV5 .

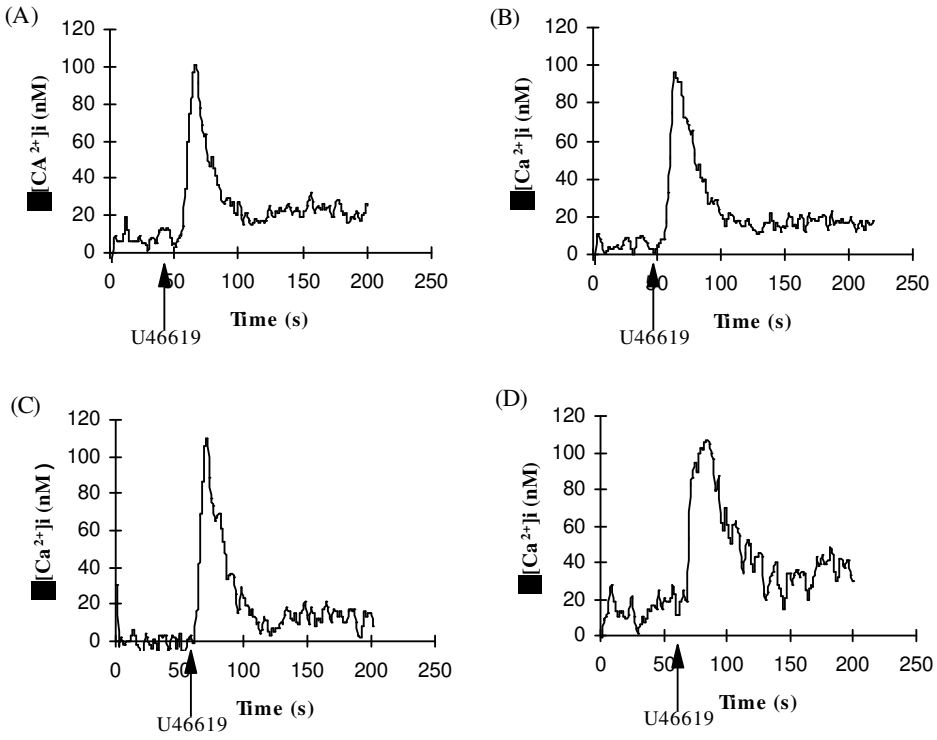

Figure 9. Effect of G protein α-subunits on U46619-mediated $[Ca^{2+}]_i$ mobilisation in an independent isolate of HEK.TP$^{\Delta 328}$ cells.

HEK.TP$^{\Delta 328}$ cells (isolate B), were transiently co-transfected with pCMV5 (*A*), pCMV5:$G_{\alpha 11}$ (*B*), pCMV5:$G_{\alpha 12}$ (*C*), or pCMV5:$G_{\alpha 16}$ (*D*). After forty-eight hours, cells were harvested, pre-loaded with FURA2/AM and stimulated with U46619 (1 μM). The ligand was added at the times indicated by the arrows. The results are representative of at least three independent experiments and are plotted as changes in intracellular Ca^{2+} mobilized ($\Delta[Ca^{2+}]_i$ (nM)) as a function of time (s) following ligand stimulation. Levels of U46619 mediated $[Ca^{2+}]_i$ mobilization in HEK.TP$^{\Delta 328}$ cells were not significantly different in the presence of co-transfected G proteins than in cells co-transfected with the vector pCMV5.

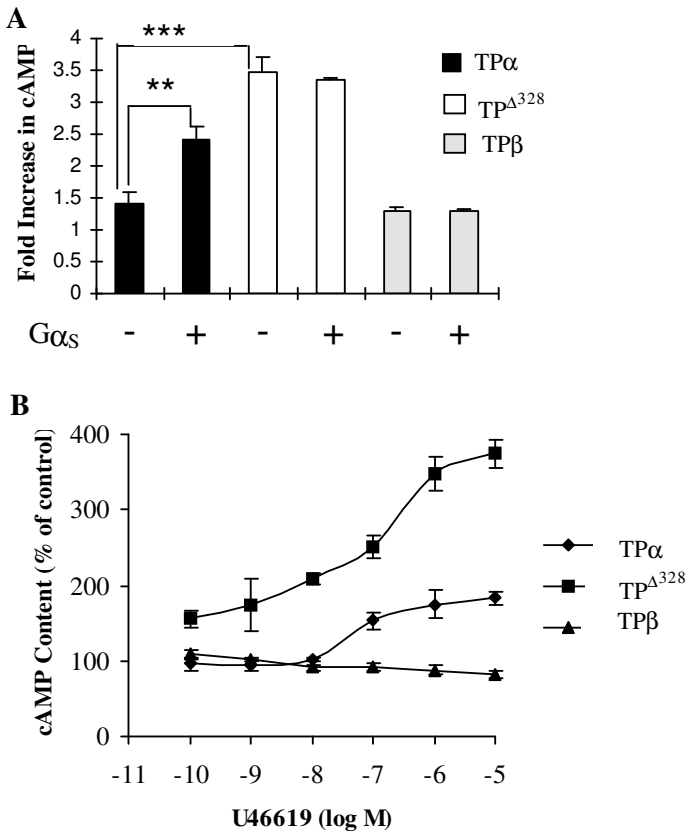

Figure 10. $TP^{\Delta 328}$ couples to activation, rather than inhibition, of adenylyl cyclase.

Panel A: HEK.α10 (TPα), HEK.β3 (TPβ) or HEK.$TP^{\Delta 328}$ ($TP^{\Delta 328}$) cells, transiently co-transfected with pCMV5 (- $G\alpha_S$) or with pCMV:$G\alpha_S$ (+ $G\alpha_S$), were either stimulated with 1 μM U46619 or, as a control, with the vehicle HBS at 37 °C for 10 min. Levels of cAMP produced in ligand or vehicle treated cells were calculated as the mean value per mg cell protein ± S.E, n = 3 (pmol cAMP / mg cells ± S.E) and are presented as levels of cAMP produced in U46619 stimulated cells relative to basal cAMP levels produced by vehicle treated cells (Fold Increase in cAMP ± S.E). ** ($p<0.02$) indicates that U46619-induced $\Delta[Ca^{2+}]_i$, was significantly higher in HEK.α10 cells co-transfected with pCMV:$G\alpha_S$ than in HEK.α10 cells co-transfected with pCMV5. *** ($p<0.005$) indicates that U46619-induced $\Delta[Ca^{2+}]_i$, was significantly higher in HEK.$TP^{\Delta 328}$ cells co-transfected with pCMV5 than in HEK.α10 co-transfected with pCMV5 . Panel B: HEK.α10 (TPα), HEK.β3 (TPβ) or HEK.$TP^{\Delta 328}$ ($TP^{\Delta 328}$) cells were either stimulated with U46619 (10^{-10} - 10^{-5} M) in the presence of 10 μM forskolin or, as a control, in the presence of 10 μM forskolin at 37 °C for 10 min. Levels of cAMP produced in ligand stimulated or control cells were calculated as the mean value per mg cell protein ± S.E, n = 3 (pmol cAMP / mg cells ± S.E) and are presented as a percentage cAMP produced in U46619 stimulated cells relative to cAMP levels produced by forskolin treated cells (cAMP content, % of control ± S.E). Only TPβ showed significant reduction in forskolin induced cAMP ($p < 0.05$, 1 μM U46619).