BJP BRITISH JOURNAL OF PHARMACOLOGY

BPS BRITISH PHARMACOLOGICAL SOCIETY

British Journal of Pharmacology (2009), 158, 830–847
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0007-1188/09
www.brjpharmacol.org

RESEARCH PAPER

Intermolecular cross-talk between the prostaglandin E_2 receptor $(EP)_3$ subtype and thromboxane A_2 receptor signalling in human erythroleukaemic cells

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Background and purpose: In previous studies investigating cross-talk of signalling between prostaglandin (PG)E_2 receptor (EP) and the TPα and TPβ isoforms of the human thromboxane (TX)A_2 receptor (TP), 17-phenyl trinor PGE_2-induced desensitization of TP receptor signalling through activation of the AH6809 and SC19220-sensitive EP_1 subtype of the EP receptor family, in a cell-specific manner. Here, we sought to further investigate that cross-talk in human erythroleukaemic (HEL) 92.1.7 cells.
Experimental approach: Specificity of 17-phenyl trinor PGE_2 signalling and its possible cross-talk with signalling by TPα/TPβ receptors endogenously expressed in HEL cells was examined through assessment of agonist-induced inositol 1,4,5-trisphosphate $(IP)_3$ generation and intracellular calcium ($[Ca^{2+}]_i$) mobilization.
Key results: While 17-Phenyl trinor PGE_2 led to activation of phospholipase (PL)Cβ to yield increases in IP_3 generation and $[Ca^{2+}]_i$, it did not desensitize but rather augmented that signalling in response to subsequent stimulation with the TXA_2 mimetic U46619. Furthermore, the augmentation was reciprocal. Signalling by 17-phenyl trinor PGE_2 was found to occur through AH6809- and SC19920-insensitive, *Pertussis* toxin-sensitive, $G_i/G_{\beta\gamma}$-dependent activation of PLCβ. Further pharmacological investigation using selective EP receptor subtype agonists and antagonists confirmed that 17-phenyl trinor PGE_2-mediated signalling and reciprocal cross-talk with the TP receptors occurred through the EP_3, rather than the EP_1, EP_2 or EP_4 receptor subtype in HEL cells.
Conclusions and Implications: The EP_1 and EP_3 subtypes of the EP receptor family mediated intermolecular cross-talk to differentially regulate TP receptor-mediated signalling whereby activation of EP_1 receptors impaired or desensitized, while that of EP_3 receptors augmented signalling through TPα/TPβ receptors, in a cell type-specific manner.
British Journal of Pharmacology (2009) **158,** 830–847; doi:10.1111/j.1476-5381.2009.00351.x; published online 20 August 2009

Keywords: thromboxane; receptor; agonist; human

Abbreviations: C-tail, carboxyl-terminal tail; $[Ca^{2+}]_i$; intracellular calcium; EP, PGE_2 receptor; FBS, foetal bovine serum; GPCR, G protein-coupled receptor; HEK, human embryonic kidney; HEL, human erythroleukaemic; IP_3, inositol 1,4,5-trisphosphate; PG, prostaglandin; PK, protein kinase; PL, phospholipase; TP, TXA_2 receptor; TXA_2, thromboxane A_2

Introduction

The prostanoids, comprising the prostaglandins (PG) and thromboxane (TX), mediate a diversity of physiological responses under both normal and pathological conditions (Narumiya *et al.*, 1999; Bos *et al.*, 2004; Matsuoka and Narumiya, 2007). The five primary prostanoids PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and TXA_2 signal through their cognate prostanoid (P) receptors termed DP, EP (EP_1–EP_4 subtypes), FP, IP and TP (nomenclature follows Alexander *et al.*, 2008), respectively, to primarily regulate activation (DP, EP_2, EP_4 and IP) or inhibition (EP_3) of adenylyl cyclase or activation of phospholipase (PL)Cβ [EP_1, FP and TP (Coleman *et al.*, 1994; Narumiya *et al.*, 1999; Bos *et al.*, 2004)]. In addition, the effects of certain prostanoids may also be mediated by the more recently described chemoattractant receptor CRTH2, or DP2, and by certain nuclear receptors including the peroxisome proliferator-activated receptors α,δ,γ (Norel, 2007). Further diversity of prostanoid signalling occurs because of overlapping ligand specificities and/or due to the coexistence of more than one prostanoid receptor type or subtype within a given cell or tissue (Narumiya *et al.*, 1999). Moreover, many of the

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Received 5 March 2009; accepted 21 April 2009

prostanoid receptors, including EP_1, EP_3, FP and TP, occur as isoforms greatly adding to the complexity of their signalling, in certain species at least (Coleman *et al.*, 1994; Narumiya *et al.*, 1999; Breyer *et al.*, 2001; Narumiya and FitzGerald, 2001; Bos *et al.*, 2004).

PGE_2, the most versatile and abundantly produced PG, exhibits multiple and, often times, opposing actions on a target cell or tissue type largely due to the existence of the structurally and functionally divergent EP_1, EP_2, EP_3 and EP_4 receptor subtypes (Coleman *et al.*, 1994; Narumiya *et al.*, 1999; Breyer and Breyer, 2001; Breyer *et al.*, 2001; Dey *et al.*, 2006; Sugimoto and Narumiya, 2007). Further complexity of EP receptor signalling is compounded by the existence of eight distinct isoforms, referred to as $EP_{3.I–VI}$, $EP_{3.e}$ and $EP_{3.f}$, of the human EP_3 receptor subtype, that diverge exclusively in their respective carboxyl-terminal (C)-tail domains (Breyer *et al.*, 2001; Dey *et al.*, 2006; Sugimoto and Narumiya, 2007). While EP_3 receptor signalling is primarily associated with G_i-mediated inhibition of adenylyl cyclase, it may also to lead to mobilization of intracellular calcium ($[Ca^{2+}]_i$) in an EP_3 isoform-specific manner, through a mechanism involving *Pertussis* toxin (PTX)-sensitive $G_i/G_{\beta\gamma}$-dependent activation of PLCβ (Coleman *et al.*, 1994; Narumiya *et al.*, 1999).

In humans, but not in non-primates, TXA_2 also signals through distinct receptor isoforms, termed TPα and TPβ, to mediate platelet aggregation and constriction of various types of smooth muscle (Hirata *et al.*, 1991; Raychowdhury *et al.*, 1994a; 1995; Narumiya *et al.*, 1999). TPα and TPβ receptors are identical for their N-terminal 328 amino acids and also diverge exclusively within their C-tail domains (Raychowdhury *et al.*, 1994a; 1995; Narumiya *et al.*, 1999; Kinsella, 2001). TPα and TPβ receptors arise by differential splicing (Raychowdhury *et al.*, 1994a; 1995) but display distinct patterns of mRNA and protein expression (Miggin and Kinsella, 1998; Habib *et al.*, 1999), being transcriptionally regulated by different promoters within the single human TP gene (Coyle *et al.*, 2002; 2005; Coyle and Kinsella, 2005; 2006; Gannon and Kinsella, 2008). While both TPα and TPβ receptors exhibit identical G_q-dependent PLCβ activation, their primary effector (Raychowdhury *et al.*, 1994b; Habib *et al.*, 1997; Walsh *et al.*, 2000a,b), they also differentially regulate other effectors including adenylyl cyclase (Hirata *et al.*, 1996) and tissue transglutaminase (Vezza *et al.*, 1999). Furthermore, TPα and TPβ receptors also display important differences in their mechanistic responses to agonist/homologous- and non-agonist/heterologous-induced desensitization (Walsh and Kinsella, 2000; Walsh *et al.*, 2000b; Foley *et al.*, 2001; Reid and Kinsella, 2003; Kelley-Hickie and Kinsella, 2004; 2006; Kelley-Hickie *et al.*, 2007). For example, in studies investigating cross-talk between TXA_2 and other prostanoids, it was established that signalling by TPα, but not by TPβ receptors, undergoes PGI_2/prostacyclin- and PGD_2-induced desensitization involving direct protein kinase (PK)A phosphorylation of TPα at Ser^{329} within its unique C-tail domain (Walsh *et al.*, 2000b; Foley *et al.*, 2001). Moreover, 17-phenyl trinor PGE_2, acting through AH6809- and SC-19220-sensitive EP_1 receptors, partially desensitized both TPα- and TPβ-mediated signalling in transfected human embryonic kidney (HEK) 293 cells and in primary renal mesangial cells through direct PKC-induced phosphorylation of TPα and TPβ receptors within their C-tail domains, where Thr^{337} and Thr^{399} were identified as the specific phosphorylation targets respectively (Walsh and Kinsella, 2000; Kelley-Hickie and Kinsella, 2004).

However, despite the latter findings, it was also suggested that those effects may be entirely cell type-specific whereby in platelets, for example, 17-phenyl trinor PGE_2 did not significantly affect TPα/TPβ receptor-mediated signalling (Walsh and Kinsella, 2000; Kelley-Hickie and Kinsella, 2004). While the nature of the apparent cellular differences of 17-phenyl trinor PGE_2 on TP receptor signalling were not established, they could in principle occur due to its differential activation of other EP/prostanoid receptors, due to differential expression and activation of the coupling G protein(s) or downstream effector(s), or due to the differentiation status of the cell type(s) themselves. In view of the critical role of TXA_2 and PGE_2 signalling within the vasculature, we have here sought to investigate the effect of 17-phenyl trinor PGE_2 and other EP_1 receptor agonists on signalling by TPα/TPβ receptors expressed in human erythroleukaemic (HEL) 92.1.7 cells, which exhibit megakaryocytic and platelet properties (Martin and Papayannopoulou, 1982; Papayannopoulou *et al.*, 1983, Long *et al.*, 1990; Wu *et al.*, 1991; 1992). In contrast to the findings in anucleate platelets (Walsh and Kinsella, 2000), we found, in HEL cells, that 17-phenyl trinor PGE_2 induced a large increase in PLCβ-mediated $[Ca^{2+}]_i$ mobilization. However, in contrast to that which occurred in the primary renal mesangial cells and transfected HEK 293 cell lines, 17-phenyl trinor PGE_2 did not desensitize but, rather, significantly augmented TP receptor signalling in HEL cells. Detailed pharmacological investigation involving a range of more selective EP receptor subtype agonists and antagonists established that 17-phenyl trinor PGE_2-mediated signalling in HEL 92.1.7 cells does not occur through an EP_1 receptor-dependent mechanism but rather through PTX-sensitive G_i-dependent activation of PLCβ involving EP_3 receptor isoform signalling. Taken together, these data establish that selective activation of the EP receptor subtypes may impair or augment TPα/TPβ receptor signalling in a cell type-specific manner and, hence, any pharmacological agents targeting the EP receptor subtypes/isoforms must not only be carefully evaluated for their EP receptor specificity but also for their relative ability to counter-regulate signalling by the TP receptors.

Experimental methods

Cell culture and transfections

All mammalian cells were grown at 37°C in a humid environment with 5% CO_2. HEL 92.1.7 cells were cultured in RPMI 1640, 10% foetal bovine serum (FBS). HEK 293 cells, obtained from the American Type Culture Collection, were grown in minimal essential medium containing 10% FBS. HEK.TPα and HEK.TPβ stably overexpressing haemagglutinin-tagged forms of TPα and TPβ receptors, respectively, have been previously described (Walsh *et al.*, 2000b).

Routinely, approximately 48 h prior to transfection, cells were plated at a density of 2×10^6 cells per 10 cm culture dish in 8 mL media. Thereafter, cells were transiently transfected with 10 μg of pADVA (Gorman *et al.*, 1990) and 25 μg of

pcDNA- or pCMV-based vectors using the calcium phosphate/DNA co-precipitation procedure, as previously described (Kinsella *et al.*, 1997). For transient transfections, cells were harvested 48 h after transfection, unless otherwise indicated.

Calcium measurements

Measurements of agonist-induced $[Ca^{2+}]_i$ mobilization were carried out in FURA2/AM-preloaded HEL, HEK 293, HEK.TPα and HEK.TPβ cells, essentially as described (Kinsella *et al.*, 1997). Cells were stimulated with 1 μM U46619, 1 μM 17-phenyl trinor PGE_2, 1 μM sulprostone, 1 μM PGE_2, 5 μM UK14304 or 1 μM cicaprost as indicated in the figures or for concentration response studies with 10^{-12}–10^{-5} M ligand. The EP receptor antagonists, AH6809 (10 μM) and SC19220 (10 μM), the PKA inhibitor H-89 (10 μM), the PKC inhibitor GF 109203X (50 nM), the PLCβ inhibitor U73122 (10 μM), the phosphoinositide-3-kinase (PI3K) inhibitors wortmannin (400 nM) or LY294002 (50 μM) and PTX (50 ng·mL^{-1}) were added at times and concentrations specified in the figure legends. In all cases, the agonists and antagonists were diluted in the vehicle, modified Ca^{2+}/Mg^{2+}-free Hank's buffered salt solution, containing 10 mM HEPES, pH 7.67, 0.1% bovine serum albumin; HBSSHB) and 20 μL was added to 2 mL cells (1.5×10^6 cells·mL^{-1} for HEL cells or 0.8×10^6 cells·mL^{-1} for HEK 293 cells) to achieve the desired working concentration. The vehicle, DMSO or ethanol had no effect on $[Ca^{2+}]_i$ mobilization by either agonist and had no effect on experimental data. The ratio of the fluorescence at 340–380 nm is a measure of $[Ca^{2+}]_i$ (Grynkiewicz *et al.*, 1985), 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+} mobilized ($\Delta[Ca^{2+}]_i$ (nM)) as a function of time (s) following agonist stimulation.

Measurement of inositol 1,4,5-trisphosphate (IP_3) levels

Measurement of agonist-mediated IP_3 generation in HEL cells was performed by anion exchange chromatography essentially as described (Berridge *et al.*, 1983; Liu *et al.*, 1997; Benjamin *et al.*, 2004). Briefly, 24 h prior to experiment, HEL cells were seeded at approximately 1.5×10^6 cells·mL^{-1} in 1.5 mL serum-free RPMI media in six-well plates, and subsequently incubated overnight at 37°C in 1.5 mL serum-free RPMI media supplemented with 5 μCi [^{3}H]myo-inositol. Thereafter, the cells were washed three times in lithium chloride in assay buffer (127 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 700 μM NaH_2PO_4, 2 mM $CaCl_2$, 5 mM $NaHCO_3$, 8 mM HEPES, 10 mM glucose, 10 mM LiCl, pH 7.4) and resuspended in a final volume of 1.5 mL assay buffer. Agonists or as control, vehicle, diluted in assay buffer, were added in 15 μL volume and incubated for 20 min at room temperature. The reaction was stopped by the addition of ice-cold 4% perchloric acid (PCA) and incubated on ice for 30 min. The PCA was extracted using 1:1 trioctylamine/trichloro-trifluoroethane, containing 1 mM EDTA. Following centrifugation, the pH was adjusted with 1 M Tris–HCl, pH 8.5 to achieve a light pink colour in the presence of phenol red. Thereafter, samples were placed onto freshly prepared Dowex AG-1x8 anion-exchange resin (resin/H_20, 1:1 v/v) packed 10 mL columns. The stepwise elution of the different inositol components was performed by increasing levels of formate: water (free/unconverted inositol); 60 mM sodium formate/5 mM dissodium tetraborate (glycerophosphoinositol); 0.2 M ammonium formate in 0.1 M formic acid (inositol monophosphate; IP_1); 0.4 M ammonium formate in 0.1 M formic acid (inositol bisphosphate; IP_2); 0.8 M ammonium formate in 0.1 M formic acid (inositol bisphosphate; IP_3); 1.2 M ammonium formate in 0.1 M formic acid (inositol tetrakisphosphate; IP_4). Radioactivity of each fraction was determined by adding 500 μL aliquots, in duplicate to 10 mL scintillation fluid, followed by liquid scintillation counting. Levels of IP_3 were expressed as a mean fold increase in IP_3 levels generated in agonist-treated cells relative to basal levels determined in vehicle-treated cells.

Isolation of human platelets

Blood (50 mL) was drawn via venepuncture from human volunteers who had not taken any medication for at least 10 days into syringes containing indomethacin (10 mM) and one-sixth volume of ACD buffer (10 mL; 38 mM citric acid, 75 mM sodium citrate, 124 mM glucose). Thereafter, the blood was centrifuged at room temperature for 10 min at 160× *g* and the top 50–70% platelet-rich plasma carefully removed. Contaminating leukocytes and red blood cells were reduced by two further centrifugations of the platelet-rich plasma (10 min at 160× *g*), and their efficient removal was confirmed by phase-contrast microscopy where samples containing less than 1×10^3 leukocytes per millilitre were used for RNA extraction. Platelet yields were typically $1–3 \times 10^9$ from 50 mL blood, as assessed by Coulter counting in the relevant size thresholds for platelets.

Reverse-transcriptase-polymerase reaction (RT-PCR)

Total RNA was extracted from HEL and HEK 293 cells using TRI reagent (Sigma) and was subject to RT-PCR using oligonucleotide primers designed to specifically amplify EP_3 receptor isoforms I–VI, e and f, EP_1 receptor and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA sequences (Table 1). Human platelets were collected by centrifugation at 750× *g* for 10 min at room temperature prior to resuspension of the pellets in TRI reagent allowing 1 mL per 1×10^9 platelets. Total RNA was extracted according to the manufacturer's instructions. Thereafter, platelet RNA was subject to RT-PCR using the previously outlined oligonucleotide primers in addition to primers specific for platelet GPIIb mRNA (Table 1).

Briefly, total RNA (1.4 μg) was converted to first strand cDNA with mouse moloney leukaemia virus (MMLV) RT in the presence of random hexamers (100 pM) in a 25 μL reaction, containing 1 mM dNTPs, 40 U RNasin, 1 U RT MMLV, 50 mM Tris–HCl; pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT. Thereafter, 3.5 μL of first strand cDNA was used as template in each PCR reaction in the presence of 10 mM Tris–HCl; pH 8.3, 50 mM KCl, 2 mM $MgCl_2$ 0.2 mM dNTPs, 6.7% glycerol, 1 μM sense primer, 1 μM antisense primer and 1 U Taq DNA polymerase. Primer sequences and expected product sizes are indicated in Table 1.

Table 1 Oligonucleotide primers used for reverse-transcriptase PCR analysis

Primer		*Sequence*	*Product size (bp)*
EP_3	5′	5′ GCATCTCAGTCCAGGCCCAGTG 3′	
$EP_{3.I}$	3′	5′ TCATCTTTCCAAATGGTCGCTCC 3′	377
$EP_{3.II}$	3′	5′ TCATGCTTCTGTCTGTATTATTTCATTAG 3′	371
$EP_{3.III}$	3′	5′ TTAATTTCCCCAAAATTCCTCCTGGC 3′	302
$EP_{3.IV}$	3′	5′ TTAATTTCCCCAAAATTCCTCTTGCTC 3′	329
$EP_{3.V}$	3′	5′ TCAGCTTAGCTGGACACTGCAG 3′	386
$EP_{3.VI}$			482
$EP_{3.f}$			461
$EP_{3.e}$	3′	5′ TATTCTGTCTTTACTGTTGAGATTCTG 3′	413
EP_1	5′	5′ GACGCCGCTCCCGACG 3′	392
EP_1	3′	5′ AGAGGCGAAGCAGTTGGCG 3′	
GPIIb	5′	5′ GTCAGCTGGAGCGACGTCA 3′	585
GPIIb	3′	5′ CTGAATGCCCAAAATACGACG 3′	
GAPDH	5′	5′ CCACAGTCCATGCCATCAC 3′	467
GAPDH	3′	5′ CATGTGGGCCATGAGGTC 3′	

Sequences of 5′ and 3′ primers used for amplification of EP_3, EP_1, GPIIb and glyceraldehyde-3-phosphate dehydrogenase cDNA sequences are indicated. The bracket indicates the common 3′ primer used to amplify $EP_{3.V}$, $EP_{3.VI}$ and $EP_{3.f}$ cDNA sequences.
EP, prostaglandin E_2 receptor.

Assessment of Akt phosphorylation

HEL cells were incubated for 30 min with either vehicle, 400 nM wortmannin or 50 μM LY294002 followed by stimulation for 10 min with 1 μM U46619 or 1 μM 17-phenyl trinor PGE_2. Samples were resolved by SDS-PAGE (50 μg protein per lane) on 10% acrylamide gels, and blots were initially screened with *anti*-phospho-Akt (Ser^{473}) antibody. Thereafter, blots were rescreened with *anti*-Akt antibody to confirm uniform expression of AKT. Results presented are representative of three independent experiments.

Data analyses

Statistical analyses were carried out using the unpaired Student's *t*-test employing the GraphPad Prism (version 4.00) package. *P*-values of less than or equal to 0.05 were considered to indicate a statistical significant difference.

Materials

Cicaprost was obtained from Schering AG (Berlin, Germany). 17-Phenyl trinor PGE_2, SQ29,548, I-BOP and U46619 were obtained from Cayman Chemical Company; FURA2/AM, H-89, PTX, U73122 and LY294002 were from Calbiochem; AH6809, G418 sulphate, GF 109203X, PCA, PGE_2, SC19220, sulprostone, trichlorotrifluoroethane, trioctylamine, UK14304 and wortmanin were obtained from Sigma. [^{3}H]myo-inositol (20 Ci·mmol^{-1}) was obtained from Sigma. [^{3}H]SQ29,548 (50.4 Ci·mmol^{-1}) was obtained from DuPont NEN. EP-receptor subtype-specific agonists (ONO-DI-004, ONO-AE1-259-01, ONO-AE-248, ONO-AE1-329) and antagonists (ONO-8713, ONO-AE3-240, ONO-AE3-208) were a gift from N. Shigeta, ONO Pharmaceutical Co. Ltd., Osaka, Japan. Rabbit polyclonal antibodies a*nti*-phospho-Akt (Ser^{473}) and *anti*-Akt were obtained from Cell Signalling Technology; horse radish peroxidase-conjugated goat *anti*-rabbit (400 μg·mL^{-1}) secondary antibody was from Santa Cruz.

Results

Effects of 17-phenyl trinor PGE_2 on U46619-mediated signalling in HEL 92.1.7 cells

In previous studies investigating possible intermolecular cross-talk/heterologous desensitization between TP and EP receptor signalling, it has been established that 17-phenyl trinor PGE_2 acting through the AH6809- and SC19220-sensitive EP_1 receptor subtype partially impairs agonist-induced signalling (IP_3 generation and $[Ca^{2+}]_i$ mobilization) by both TPα or TPβ receptors endogenously expressed in primary human mesangial cells and in HEK.TPα and HEK.TPβ cells, clonal HEK 293 cells stably overexpressing TPα and TPβ receptors respectively (Walsh *et al.*, 2000b; Kelley-Hickie and Kinsella, 2004). Here, we sought to investigate the possible cross-talk between EP_1 and TP receptor signalling in HEL 92.1.7 cells, a widely used model to study megakaryocyte/platelet development and known to endogenously express TPα, TPβ and EP_1 receptors (Long *et al.*, 1990; Funk *et al.*, 1993, Schwaner *et al.*, 1995; Coyle and Kinsella, 2005; 2006).

Consistent with previous studies (Walsh *et al.*, 2000b; Kelley-Hickie and Kinsella, 2004), stimulation of HEK.TPα and HEK.TPβ cells with either the TXA_2 mimetic U46619 or with 17-phenyl trinor PGE_2 led to significant, transient rises in $[Ca^{2+}]_i$ mobilization (Figure 1A,B,D,E, respectively) and IP_3 generation (data not shown), while pre-stimulation with 17-phenyl trinor PGE_2 led to approximately 70% and 60% reductions in the U46619-mediated $[Ca^{2+}]_i$ response in HEK.TPα (compare Figure 1A,B, $P < 0.0001$) or HEK.TPβ (compare Figure 1D,E, $P < 0.0001$) cells respectively. Stimulation of HEL cells with the TP receptor agonist U46619 induced modest increases in $[Ca^{2+}]_i$ mobilization (Figure 1G, EC_{50} = 23 nM) and IP_3 generation (Figure 1K, 1.3-fold over basal), while 17-phenyl trinor PGE_2 yielded significantly greater increases in both $[Ca^{2+}]_i$ mobilization (Figure 1H; EC_{50} = 186 nM) and IP_3 generation (Figure 1K, 1.7-fold over basal). However, in contrast to that observed in HEK.TPα and HEK.TPβ cells, 17-phenyl trinor PGE_2 did not impair but rather substantially augmented U46619-induced $[Ca^{2+}]_i$ mobilization (Figure 1I; threefold augmentation, $P < 0.0001$) in HEL cells. Moreover, in reciprocal studies, pre-stimulation of HEL cells with U46619 led to a 50% augmentation of the 17-phenyl trinor PGE_2-induced $[Ca^{2+}]_i$ response (Figure 1J, P = 0.027) while co-stimulation with U46619 and 17-phenyl trinor PGE_2 also significantly augmented, as opposed to desensitized or impaired, IP_3 generation, relative to that generated following stimulation of cells with either agonist alone (Figure 1K; twofold to threefold augmentations, $P < 0.0001$). Because the U46619- and 17-phenyl trinor PGE_2-induced $[Ca^{2+}]_i$ mobilization was inhibited by the PI-PLCβ inhibitor U73122, we subsequently focused on $[Ca^{2+}]_i$ measurements, rather than on IP_3 generation.

In light of the reciprocal augmentation of EP and TP receptor-mediated signalling in HEL cells, it was also sought to clarify whether U46619-mediated signalling by TPα and TPβ receptors in the HEK.TPα and HEK.TPβ cell lines may

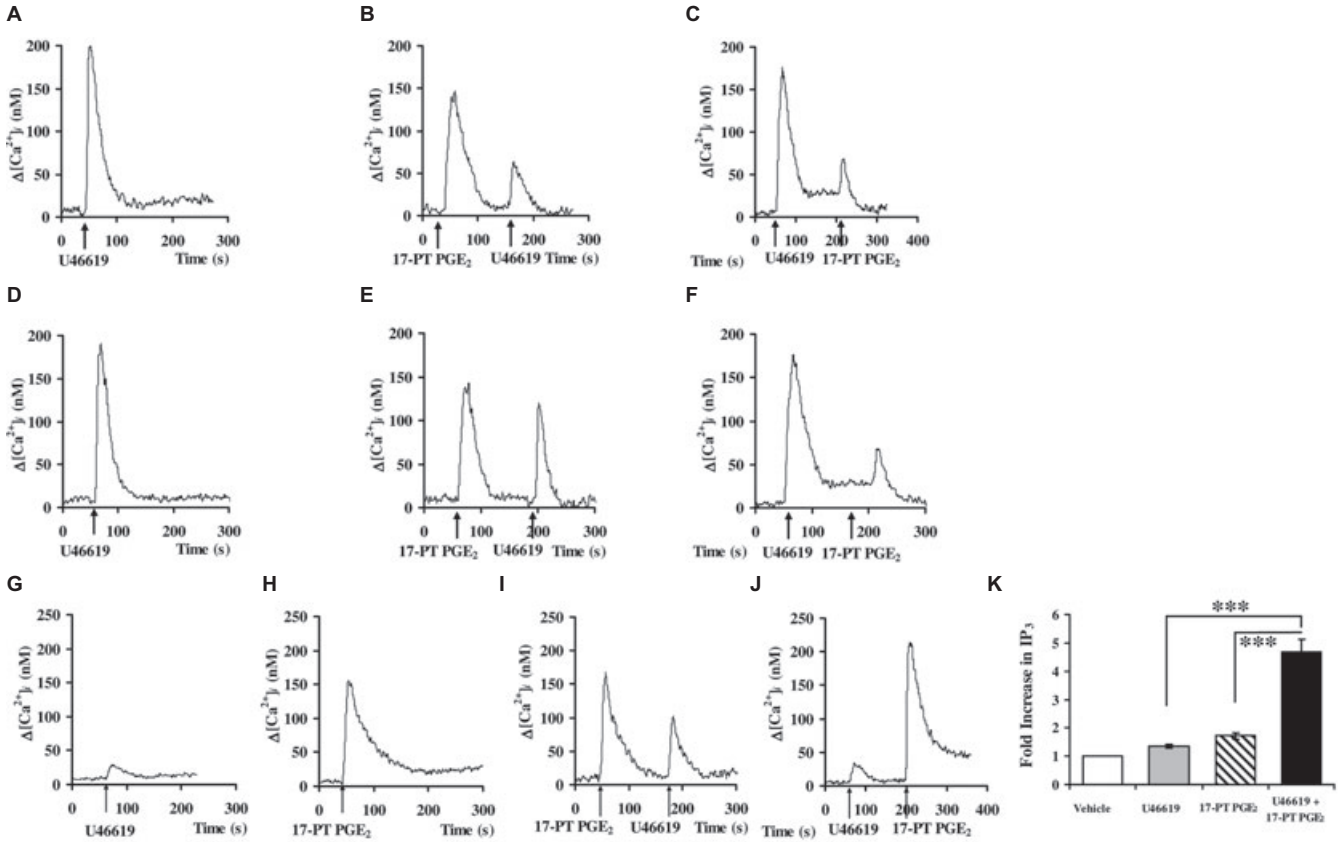

Figure 1 Effect of the EP agonist 17-phenyl trinor (17-PT) PGE_2 on TP receptor signalling in HEK 293 and in HEL 92.1.7 cells. HEK.TPα (A–C) and HEK.TPβ (D–F) cells, transiently transfected with pCMV5.$G\alpha_q$ and preloaded with FURA2/AM, were stimulated with 1 μM U46619 alone (A,D) or in sequential combination with 1 μM 17-phenyl trinor PGE_2 (B,C,E,F), where agonists were added at times indicated by the arrows. (G–J) HEL cells, preloaded with FURA2/AM, were stimulated with 1 μM U46619 or 1 μM 17-phenyl trinor PGE_2, either alone or in sequential combination, as indicated by the arrows. Results presented are representative of at least five independent experiments, where actual mean changes in intracellular Ca^{2+} mobilized ($\Delta[Ca^{2+}]_i$, nM) are: (A) $\Delta[Ca^{2+}]_i = 197 \pm 15.2$; (B) 17-phenyl trinor PGE_2, $\Delta[Ca^{2+}]_i = 134 \pm 13.9$, U46619, $\Delta[Ca^{2+}]_i = 52.2 \pm 13.0$; (C) U46619, $\Delta[Ca^{2+}]_i = 148 \pm 3.0$, 17-phenyl trinor PGE_2, $\Delta[Ca^{2+}]_i = 32.4 \pm 9.3$; (D) $\Delta[Ca^{2+}]_i = 199 \pm 5.4$; (E) 17-phenyl trinor PGE_2, $\Delta[Ca^{2+}]_i = 142 \pm 12.8$, U46619, $\Delta[Ca^{2+}]_i = 88.2 \pm 7.5$; (F) U46619, $\Delta[Ca^{2+}]_i = 211 \pm 9.5$, 17-phenyl trinor PGE_2, $\Delta[Ca^{2+}]_i = 38.2 \pm 1.5$; (G) $\Delta[Ca^{2+}]_i = 36.8 \pm 6.2$; (H) $\Delta[Ca^{2+}]_i = 159 \pm 18.1$; (I) 17-phenyl trinor PGE_2, $\Delta[Ca^{2+}]_i = 156 \pm 9.1$, U46619, $\Delta[Ca^{2+}]_i = 103 \pm 6.2$; (J) U46619, $\Delta[Ca^{2+}]_i = 37.2 \pm 7.8$, 17-phenyl trinor PGE_2, $\Delta[Ca^{2+}]_i = 237 \pm 23.8$. (K) HEL cells were stimulated at 37°C for 20 min with 1 μM U46619 (U46619), 1 μM 17-phenyl trinor PGE_2 (17-PT PGE_2), either alone or in combination. In each case, basal IP_3 levels were determined by exposing the cells to the vehicle under identical conditions. Levels of IP_3 generated by agonist- or vehicle-treated cells are expressed as mean fold increases in IP_3 levels generated in agonist-treated cells relative to vehicle-treated cells (Fold increase in $IP_3 \pm$ SEM, $n = 4$), where *** indicates $P \leq 0.001$. $[Ca^{2+}]_i$; intracellular calcium; EP, PGE_2 receptor; HEK, human embryonic kidney; HEL, human erythroleukaemic; IP_3, inositol 1,4,5-trisphosphate; PG, prostaglandin; TP, thromboxane A_2 receptor.

actually affect 17-phenyl trinor PGE_2-mediated signalling therein. Pre-stimulation with U46619 significantly impaired the subsequent 17-phenyl trinor PGE_2-induced $[Ca^{2+}]_i$ response in HEK.TPα (compare Figure 1A,C, $P < 0.001$) and HEK.TPβ (compare Figure 1D,F, $P = 0.0002$) cells respectively. Moreover, pre-stimulation with U46619 impaired the 17-phenyl trinor PGE_2-mediated IP_3 generation of HEK.TPα and HEK.TPβ cells and vice versa (data not shown). Hence, taken together these data suggest that in HEK 293 cells, there was a reciprocal cross-talk between TP- and EP receptor-mediated signalling whereby 17-phenyl trinor PGE_2 desensitizes/impairs U46619-mediated signalling and vice versa. Conversely, while such a reciprocal relationship exists in HEL cells, 17-phenyl trinor PGE_2-augmented, rather than desensitized or impaired, U46619-mediated signalling and vice versa.

Effect of sulprostone and PGE_2 on TP-mediated signalling in HEL cells

In order to exclude the possibility that the augmentation of U46619- and 17-phenyl trinor PGE_2-induced signalling observed in HEL cells may be a simple phenomenon of the agonists employed, the effect of the general EP receptor agonist PGE_2 and of the EP_1/EP_3 receptor agonist sulprostone (Coleman *et al.*, 1994) on U46619-mediated $[Ca^{2+}]_i$ mobilization was investigated, as was the effect of 17-phenyl trinor PGE_2 on signalling by the TP receptor agonist, I-BOP, and vice versa. Stimulation of HEL cells with sulprostone induced a large increase in $[Ca^{2+}]_i$ mobilization (Figure 2A, $EC_{50} = 439$ nM) and induced a threefold increase in the subsequent U46619-mediated response (compare Figures 1G & 2B, $P < 0.0001$), while pre-stimulation with U46619 led to a 70% increase in $[Ca^{2+}]_i$ mobilization in response to secondary

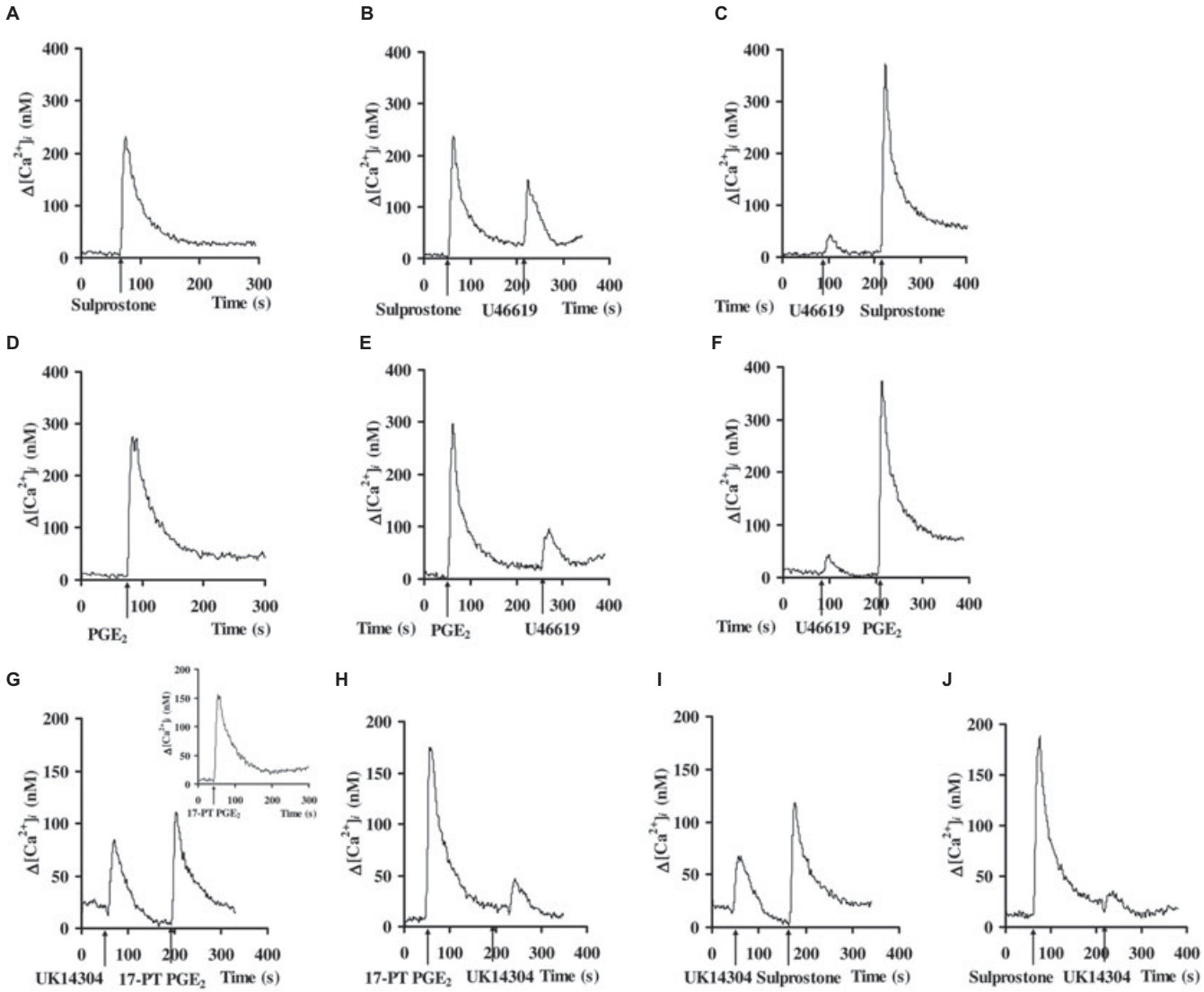

Figure 2 Effect of EP receptor agonists sulprostone and PGE_2 on signalling in HEL cells. HEL cells, preloaded with FURA2/AM, were stimulated with 1 μM sulprostone (A–C) or 1 μM PGE_2 (D–F) either alone or in sequential combination with 1 μM U46619, where agonists were added at times indicated by the arrows. (G–J) HEL cells were stimulated with 10 μM UK14304 in sequential combination with either 1 μM 17-phenyl trinor (17-PT) PGE_2 (G,H) or 1 μM sulprostone (I,J), where the agonists were added at times indicated by the arrows. Results presented are representative of at least five independent experiments, where actual mean changes in intracellular Ca^{2+} mobilized ($\Delta[Ca^{2+}]_i$, nM) are: (A) $\Delta[Ca^{2+}]_i = 228 \pm 3.8$; (B) sulprostone, $\Delta[Ca^{2+}]_i = 227 \pm 5.0$, U46619, $\Delta[Ca^{2+}]_i = 118 \pm 14.3$; (C) U46619, $\Delta[Ca^{2+}]_i = 42.4 \pm 9.5$, sulprostone, $\Delta[Ca^{2+}]_i = 381 \pm 13.2$; (D) $\Delta[Ca^{2+}]_i = 246 \pm 12.5$; (E) PGE_2, $\Delta[Ca^{2+}]_i = 246 \pm 12.5$, U46619, $\Delta[Ca^{2+}]_i = 95.7 \pm 8.5$; (F) U46619, $\Delta[Ca^{2+}]_i = 47.6 \pm 7.9$, PGE_2, $\Delta[Ca^{2+}]_i = 400 \pm 18$; (G) UK14304, $\Delta[Ca^{2+}]_i = 72.5 \pm 10.1$, 17-phenyl trinor PGE_2, $\Delta[Ca^{2+}]_i = 127 \pm 22.2$; (G inset) $\Delta[Ca^{2+}]_i = 159 \pm 18.1$; (H) 17-phenyl trinor PGE_2, $\Delta[Ca^{2+}]_i = 175 \pm 42.1$, UK14304, $\Delta[Ca^{2+}]_i = 27.9 \pm 3.6$; (I) UK14304, $\Delta[Ca^{2+}]_i = 69.0 \pm 14.9$, sulprostone, $\Delta[Ca^{2+}]_i = 127 \pm 7.6$; (J) sulprostone, $\Delta[Ca^{2+}]_i = 171 \pm 19.6$, UK14304, $\Delta[Ca^{2+}]_i = 17.7 \pm 4.7$. $[Ca^{2+}]_i$; intracellular calcium; EP, PGE_2 receptor; HEL, human erythroleukaemic; PG, prostaglandin.

stimulation with sulprostone (compare Figure 2A,C, $P = 0.0001$). Similarly, PGE_2 mediated a large increase in $[Ca^{2+}]_i$ mobilization in HEL cells (Figure 2D, $EC_{50} = 192$ nM) and caused a twofold augmentation of the subsequent U46619 response (compare Figures 1G & 2E, $P < 0.0001$) while pre-stimulation with U46619 also caused a 60% increase in the subsequent PGE_2 response (compare Figures 2D & 2F, $P > 0.0001$). Moreover, while stimulation of HEL cells with I-BOP mediated a modest rise in $[Ca^{2+}]_i$ mobilization ($\Delta[Ca^{2+}]_i = 33.7 \pm 12.6$ nM; $EC_{50} = 5.0$ nM), pre-stimulation with 17-phenyl trinor PGE_2 induced a threefold increase in $[Ca^{2+}]_i$ mobilization in response to secondary stimulation with I-BOP ($P < 0.0001$). Similarly, pre-stimulation with I-BOP led to a 55% augmentation of the $[Ca^{2+}]_i$ response following secondary stimulation with 17-phenyl trinor PGE_2 ($P = 0.023$).

To eliminate the possibility that the augmentation between the EP and TP receptor agonists observed in HEL cells may be due to non-specific sensitization of the $[Ca^{2+}]_i$ release mechanism, the effect of the α_2-adrenoceptor agonist UK14304 on EP receptor-mediated signalling was investigated. While UK14304 mediated a significant, transient rise in $[Ca^{2+}]_i$ mobilization in HEL cells ($EC_{50} = 59$ nM), it did not augment the subsequent 17-phenyl trinor PGE_2-induced $[Ca^{2+}]_i$ response (Figure 2G). On the contrary, in the presence of UK14304

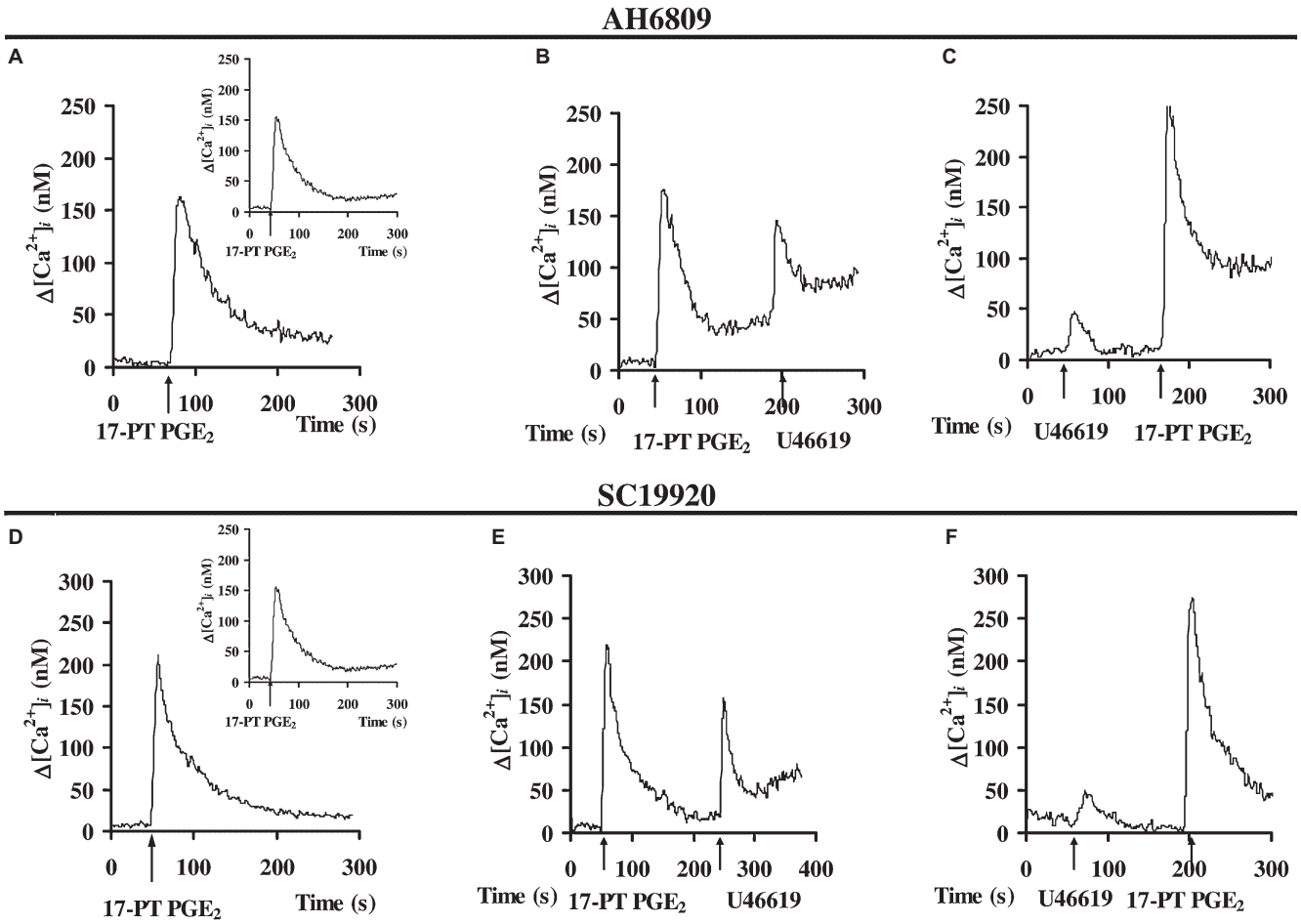

Figure 3 Effect of AH6809 and SC19220 on EP receptor signalling in HEL cells. HEL cells, preloaded with FURA2/AM, were pre-incubated for 10 min with either 10 μM AH6809 (A–C) or 10 μM SC19920 (D–F) prior to stimulation with 1 μM 17-phenyl trinor (17-PT) PGE_2 alone (A,D) or in sequential combination with 1 μM U46619 (B,C,E,F), where agonists were added at times indicated by the arrows. Results presented are representative of at least five independent experiments, where actual mean changes in intracellular Ca^{2+} mobilized ($\Delta[Ca^{2+}]_i$, nM) are: (A) $\Delta[Ca^{2+}]_i = 175 \pm 0.7$; (A inset) $\Delta[Ca^{2+}]_i = 159 \pm 18.1$; (B) 17-phenyl trinor PGE_2, $\Delta[Ca^{2+}]_i = 175 \pm 0.7$, U46619, $\Delta[Ca^{2+}]_i = 89.0 \pm 12.5$; (C) U46619, $\Delta[Ca^{2+}]_i = 42.4 \pm 11.6$, 17-phenyl trinor PGE_2, $\Delta[Ca^{2+}]_i = 279 \pm 14.9$; (D) $\Delta[Ca^{2+}]_i = 209 \pm 39.7$; (D inset) $\Delta[Ca^{2+}]_i = 159 \pm 18.1$; (E) 17-phenyl trinor PGE_2, $\Delta[Ca^{2+}]_i = 209 \pm 39.7$, U46619, $\Delta[Ca^{2+}]_i = 126 \pm 17.6$; (F) U46619, $\Delta[Ca^{2+}]_i = 56.4 \pm 14.7$, 17-phenyl trinor PGE_2, $\Delta[Ca^{2+}]_i = 291 \pm 6.3$. $[Ca^{2+}]_i$; intracellular calcium; EP, PGE_2 receptor; HEL, human erythroleukaemic; PG, prostaglandin.

there was a modest reduction in the secondary 17-phenyl trinor PGE_2-induced $[Ca^{2+}]_i$ response (Figure 2G & inset, $P = 0.0005$). Moreover, 17-phenyl trinor PGE_2 induced desensitization of the subsequent UK14304 response (Figure 2H, $P = 0.024$). Likewise, sulprostone-induced $[Ca^{2+}]_i$ mobilization was subject to UK14304-mediated desensitization (Figure 2I, $P < 0.0001$) and vice versa (Figure 2J, $P < 0.01$). Together, these data indicate that the augmentation observed between EP and TP receptor signalling in HEL cells is specific and not a more general Ca^{2+} sensitizing phenomenon found in HEL cells.

Effect of EP_1 receptor antagonists on EP receptor-mediated augmentation of TP receptor signalling in HEL cells

As stated, 17-phenyl trinor PGE_2-mediated desensitization of U46619-induced signalling in HEK.TPα and HEK.TPβ cells was sensitive to the more general EP/DP receptor antagonist AH6809 and to the more selective EP_1 receptor antagonist SC19220 (Walsh *et al.*, 2000b; Kelley-Hickie and Kinsella, 2004). Hence, to establish whether 17-phenyl trinor PGE_2-mediated signalling in HEL cells also occurs through activation of an EP_1 receptor-mediated pathway, as opposed to another EP receptor subtype, and to identify whether its augmentation of TP receptor signalling is EP_1 receptor-dependent, HEL cells were pre-incubated with AH6809 (Figure 3A–C) or SC19220 (Figure 3D–F) prior to stimulation with 17-phenyl trinor PGE_2 alone or in sequential combination with U46619. AH6809 did not reduce the 17-phenyl trinor PGE_2-mediated $[Ca^{2+}]_i$ response (Figure 3A, $P = 0.24$) or indeed affect U46619-mediated signalling ($P = 0.64$). The more specific EP_1 receptor antagonist SC19220 did not inhibit the 17-phenyl trinor PGE_2-mediated $[Ca^{2+}]_i$ response (Figure 3D, $P = 0.22$). Similarly, signalling by sulprostone was insensitive to AH6809 ($P = 0.21$) and to SC19920 ($P = 0.22$). Furthermore, neither AH6809 nor SC19220 reduced or further enhanced the 17-phenyl trinor PGE_2-mediated augmentation of U46619-induced $[Ca^{2+}]_i$ mobilization (Figure 3B,E) or vice versa (Figure 3C,F). Collectively, these data suggest that

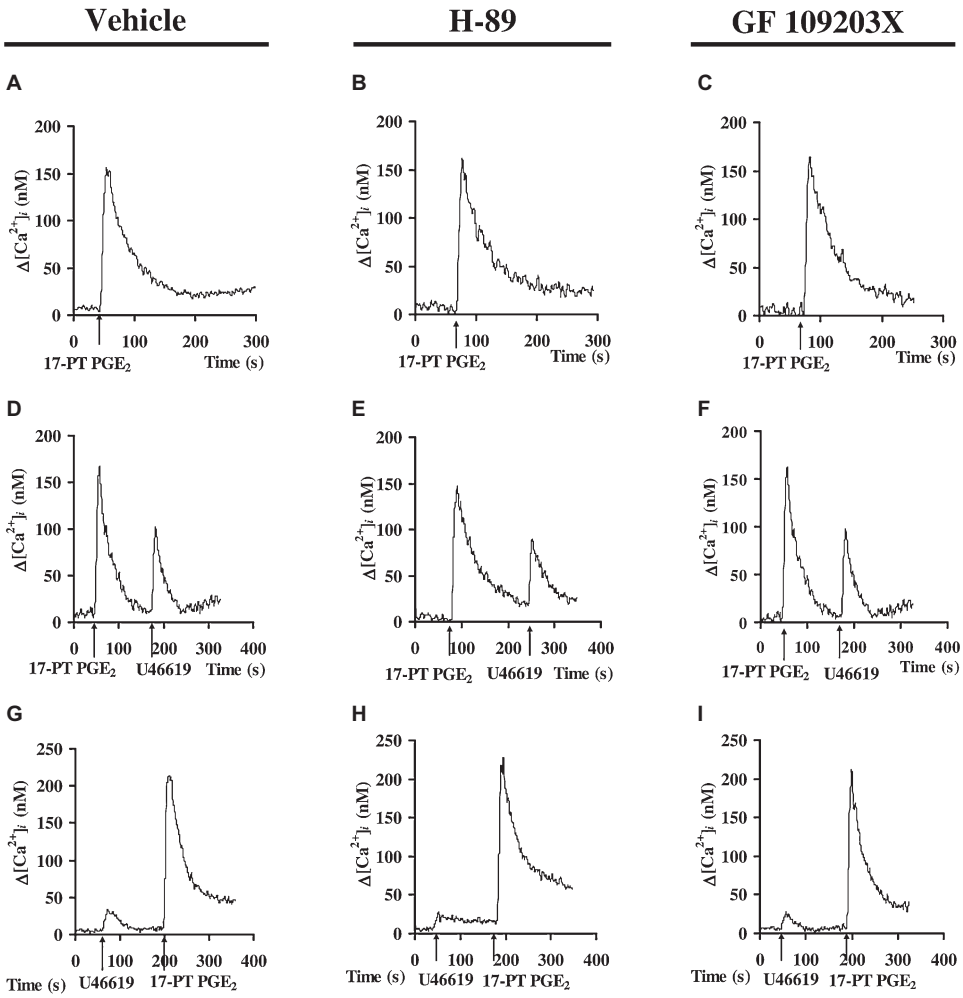

Figure 4 Effect of protein kinase inhibitors on 17-phenyl trinor (17-PT) PGE_2-mediated signalling. HEL cells, preloaded with FURA2/AM, were pre-incubated for 10 min with either vehicle (A,D,G), 10 μM H-89 (B,E,H) or 50 nM GF 109203X (C,F,I) prior to stimulation with 1 μM 17-phenyl trinor PGE_2 alone (A–C), or in sequential combination with 1 μM U46619 (D–I), where agonists were added at times indicated by the arrows. Results presented are representative of at least five independent experiments, where actual mean changes in intracellular Ca^{2+} mobilized ($\Delta[Ca^{2+}]_i$, nM) are: (A) $\Delta[Ca^{2+}]_i = 153 \pm 21.1$; (B) $\Delta[Ca^{2+}]_i = 159 \pm 8.3$; (C) $\Delta[Ca^{2+}]_i = 190 \pm 23.1$; (D) 17-phenyl trinor PGE_2, $\Delta[Ca^{2+}]_i = 156 \pm 9.1$, U46619, $\Delta[Ca^{2+}]_i = 103 \pm 6.2$; (E) 17-phenyl trinor PGE_2, $\Delta[Ca^{2+}]_i = 158 \pm 9.6$, U46619, $\Delta[Ca^{2+}]_i = 95.6 \pm 19.3$; (F) 17-phenyl trinor PGE_2, $\Delta[Ca^{2+}]_i = 205 \pm 4.8$, U46619, $\Delta[Ca^{2+}]_i = 113 \pm 30.0$; (G) U46619, $\Delta[Ca^{2+}]_i = 37.2 \pm 7.8$, 17-phenyl trinor PGE_2, $\Delta[Ca^{2+}]_i = 237 \pm 23.8$; (H) U46619, $\Delta[Ca^{2+}]_i = 38.0 \pm 10.1$, 17-phenyl trinor PGE_2, $\Delta[Ca^{2+}]_i = 266 \pm 37.7$; (I) U46619, $\Delta[Ca^{2+}]_i = 43.4 \pm 10.7$, 17-phenyl trinor PGE_2, $\Delta[Ca^{2+}]_i = 276 \pm 8.5$. $[Ca^{2+}]_i$; intracellular calcium; HEL, human erythroleukaemic; PG, prostaglandin.

17-phenyl trinor PGE_2 may not signal via an EP_1 receptor-mediated pathway to elicit $[Ca^{2+}]_i$ mobilization in HEL cells. Moreover, in contrast to the desensitization observed in the primary human mesangial cells and in the HEK.TPα and HEK.TPβ cell lines, 17-phenyl trinor PGE_2 augmented TP receptor signalling in HEL cells through a pathway that was independent of AH6809- and SC19220-sensitive EP_1 receptor-mediated signalling.

Effect of PK inhibitors of EP receptor-mediated augmentation of TP receptor signalling in HEL cells

Previous studies established that 17-phenyl trinor PGE_2-mediated desensitization of TPα and TPβ receptors in primary human mesangial cells and in the clonal HEK 293 cell lines occurs through a PKC-dependent, PKA-independent mechanism, and Thr^{337} and Thr^{399} within the unique C-tails of TPα and TPβ receptors, respectively, were identified as the specific phosphorylation target residues (Walsh *et al.*, 2000b; Kelley-Hickie and Kinsella, 2004). Hence, we sought to determine whether the augmentation between TP and EP receptor signalling observed in HEL cells may also be subject to regulation by the second messenger kinases PKA and/or PKC. Pre-incubation of HEL cells with either H-89 (compare Figure 4A,B, $P = 0.76$) or GF 109203X (compare Figure 4A,C, $P = 0.28$), inhibitors of PKA and PKC, respectively (Toullec *et al.*, 1991; Geilen *et al.*, 1992), did not significantly affect 17-phenyl trinor PGE_2-induced $[Ca^{2+}]_i$ mobilization. Moreover, neither H-89 nor GF 109203X affected 17-phenyl trinor PGE_2-mediated augmentation of the U46619-induced $[Ca^{2+}]_i$ response (compare Figure 4D with Figure 4E,F, $P = 0.70$ and $P = 0.70$, respectively) or U46619-mediated augmentation of 17-phenyl trinor PGE_2 signalling (compare Figure 4G with Figure 4H,I, $P = 0.56$ and $P = 0.38$, respectively). Taken together, these data demonstrate that the

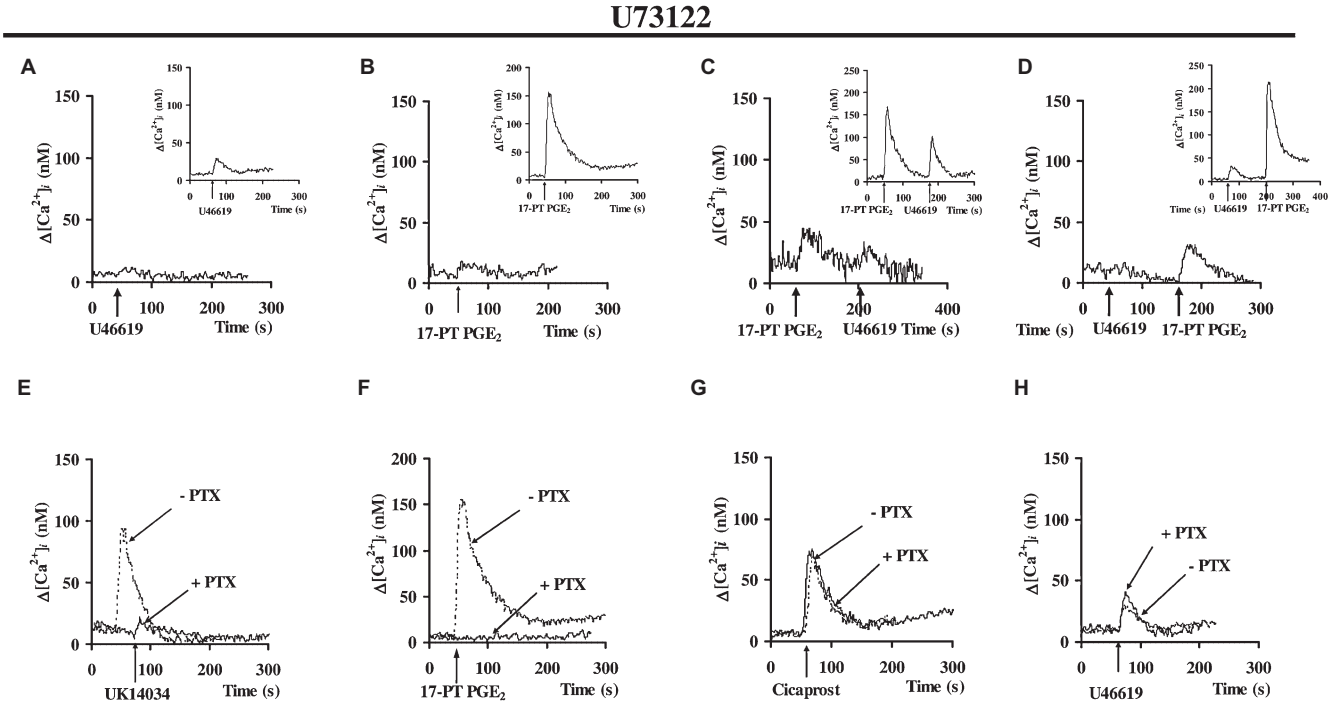

Figure 5 Effect of U73122 and PTX on EP receptor signalling in HEL cells. (A–D) HEL cells, preloaded with FURA2/AM, were pre-incubated for 10 min with 10 μM U73122 (A–D) prior to stimulation with 1 μM U46619 or 1 μM 17-phenyl trinor (17-PT) PGE_2 alone (A,B, respectively) or in sequential combination (C,D), where the agonists were added at times indicated by the arrows. (E–H) HEL cells were incubated overnight at 37°C in the presence of 50 ng·mL^{-1} PTX (+PTX) or its vehicle (–PTX) prior to loading with FURA2/AM and stimulation with 5 μM UK14304, 1 μM 17-phenyl trinor PGE_2, 1 μM cicaprost or 1 μM U46619 (E–H, respectively), where the ligands were added at times indicated by the arrows. Results presented are representative of at least four independent experiments, where actual mean changes in intracellular Ca^{2+} mobilized ($\Delta[Ca^{2+}]_i$, nM) are: (A) $\Delta[Ca^{2+}]_i = 5.10 \pm 7.3$; (A inset) $\Delta[Ca^{2+}]_i = 36.8 \pm 6.2$; (B) $\Delta[Ca^{2+}]_i = 47.6 \pm 13.0$; (B inset) $\Delta[Ca^{2+}]_i = 159 \pm 18.1$; (C) 17-phenyl trinor PGE_2, $\Delta[Ca^{2+}]_i = 47.6 \pm 13.0$, U46619, $\Delta[Ca^{2+}]_i = 35.8 \pm 9.5$; (C inset) 17-phenyl trinor PGE_2, $\Delta[Ca^{2+}]_i = 156 \pm 9.1$, U46619, $\Delta[Ca^{2+}]_i = 103 \pm 6.2$; (D) U46619, $\Delta[Ca^{2+}]_i = 5.10 \pm 7.3$, 17-phenyl trinor PGE_2, $\Delta[Ca^{2+}]_i = 34.6 \pm 3.1$; (D inset) U46619, $\Delta[Ca^{2+}]_i = 37.2 \pm 7.8$, 17-phenyl trinor PGE_2, $\Delta[Ca^{2+}]_i = 237 \pm 23.8$; (E) –PTX, $\Delta[Ca^{2+}]_i = 93.5 \pm 6.5$, +PTX, $\Delta[Ca^{2+}]_i = 3.60 \pm 6.2$; (F) –PTX, $\Delta[Ca^{2+}]_i = 162 \pm 16.3$, +PTX, $\Delta[Ca^{2+}]_i = 0$; (G) –PTX, $\Delta[Ca^{2+}]_i = 68.4 \pm 4.5$, +PTX, $\Delta[Ca^{2+}]_i = 70.8 \pm 4.5$; (H) –PTX, $\Delta[Ca^{2+}]_i = 36.8 \pm 6.2$, +PTX, $\Delta[Ca^{2+}]_i = 48.5 \pm 14.2$. $[Ca^{2+}]_i$; intracellular calcium; EP, PGE_2 receptor; HEL, human erythroleukaemic; PG, prostaglandin; PTX, *Pertussis* toxin.

augmentation between TP- and EP receptor-mediated signalling in HEL cells was independent of the second messenger kinases PKA and PKC.

Effect of inhibition of Gα protein-coupled signalling on EP receptor signalling in HEL cells

While the primary mode of TXA_2 signalling is G_q-dependent activation of PLCβ (Kinsella, 2001), PGE_2 signalling is more complex not least due to the EP_1–EP_4 receptor subtypes and the existence of multiple EP_3 receptor isoforms (Kotani *et al.*, 1997; Bilson *et al.*, 2004; Bos *et al.*, 2004; Sugimoto and Narumiya, 2007). As we had confirmed here that both U46619 and 17-phenyl trinor PGE_2 increased IP_3 generation in HEL cells and that co-stimulation of cells with either agonist significantly augmented IP_3 levels (Figure 1K), we next sought to investigate the effect of PLCβ inhibition on U46619/TP- and 17-phenyl trinor PGE_2/EP-mediated $[Ca^{2+}]_i$ mobilization and on their reciprocal augmentation thereof in HEL cells. As expected, the PLCβ inhibitor U73122 significantly decreased $[Ca^{2+}]_i$ mobilization by both the TXA_2 mimetic U46619 (Figure 5A, $P = 0.017$; $IC_{50} = 0.2$ μM) and by 17-phenyl trinor PGE_2 (Figure 5B, $P = 0.0051$; $IC_{50} = 0.3$ μM), confirming that 17-phenyl trinor PGE_2-mediated $[Ca^{2+}]_i$ mobilization occurred through activation of PLCβ in HEL cells. Moreover, in the presence of U73122, 17-phenyl trinor PGE_2 did not augment U46619-mediated $[Ca^{2+}]_i$ mobilization or vice versa (Figure 5C,D respectively).

To further investigate the G protein-dependent mechanism of $[Ca^{2+}]_i$ mobilization in response to 17-phenyl trinor PGE_2 in HEL cells, the effect of PTX on signalling was examined. As a control for those studies, the effect of PTX on $[Ca^{2+}]_i$ mobilization by the G_i-coupled α_2-adrenoceptor and by the prostacyclin receptor, a prostanoid receptor that can couple to both G_s/adenyl cyclase and G_q/PLCβ activation in HEL cells (Baltensperger and Porzig, 1997; Miggin and Kinsella, 2002a), was also investigated. Pretreatment of HEL cells with PTX completely prevented $[Ca^{2+}]_i$ mobilization in response to the α_2-adrenoceptor agonist UK14304 (Figure 5E, $P < 0.0001$) while it had no significant effect on signalling in response to the selective prostacyclin receptor agonist cicaprost (Figure 5G, compare + & –PTX, $P = 0.72$). Specifically, 17-phenyl trinor PGE_2-induced $[Ca^{2+}]_i$ mobilization was significantly reduced in the presence of PTX (Figure 5F, $P < 0.0001$), consistent with 17-phenyl trinor PGE_2-coupling to G_i-mediated signalling in HEL cells. Consistent with TP receptor signalling to G_q/PLCβ activation, pretreatment with PTX had no significant affect on U46619-induced $[Ca^{2+}]_i$

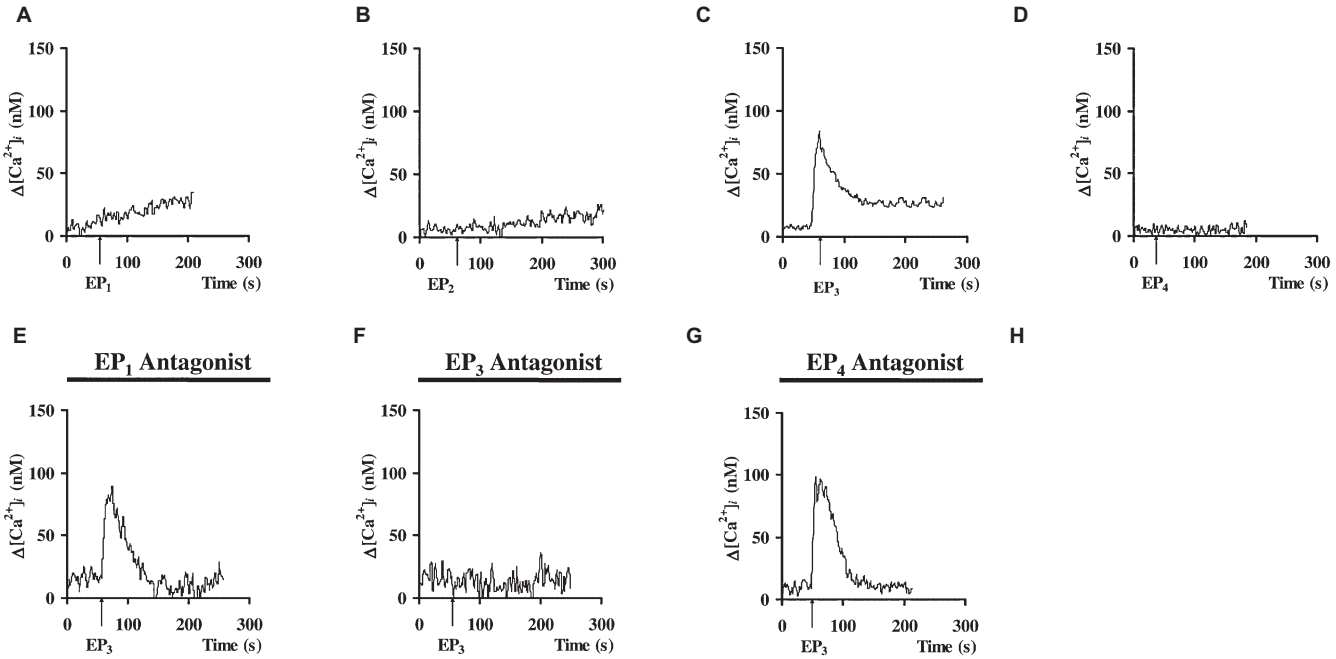

Figure 6 Characterization of EP receptor signalling in HEL cells. (A–D) HEL cells, preloaded with FURA2/AM, were stimulated with 10 μM ONO-D1-004 (EP_1; A), 10 μM ONO-AE1-259 (EP_2; B), 10 μM ONO-AE1-248 (EP_3; C) and 10 μM ONO-AE1-329 (EP_4; D), where the agonists were added at times indicated by the arrows. (E–G) HEL cells were pre-incubated for 10 min with the EP receptor antagonists 10 μM ONO-8713 (EP_1; E), ONO-AE3-240 (EP_3; F) and ONO-AE-208 (EP_4; G) prior to stimulation with the EP_3 receptor agonist 1 μM ONO-AE1-248. Results presented are representative of at least four independent experiments, where actual mean changes in intracellular Ca^{2+} mobilized ($\Delta[Ca^{2+}]_i$, nM) are: (A) $\Delta[Ca^{2+}]_i = 0$; (B) $\Delta[Ca^{2+}]_i = 0$; (C) $\Delta[Ca^{2+}]_i = 83.6 \pm 12.7$; (D) $\Delta[Ca^{2+}]_i = 0$; (E) $\Delta[Ca^{2+}]_i = 80.5 \pm 3.1$; (F) $\Delta[Ca^{2+}]_i = 0$; (G) $\Delta[Ca^{2+}]_i = 95.4 \pm 5.6$. EP, prostaglandin E_2 receptor; HEL, human erythroleukaemic.

mobilization in HEL cells (Figure 5H, compare + & –PTX, $P = 0.41$). Taken together, these data suggest that 17-phenyl trinor PGE_2-mediated $[Ca^{2+}]_i$ mobilization in HEL cells occurs through G_i-coupled, as opposed to G_q-coupled, PLCβ activation in HEL cells consistent with its activation of G_i-coupled EP_3, as opposed to G_q-coupled EP_1 receptor, subtypes in HEL cells.

Specificity of EP receptor-mediated signalling in HEL cells

Due to the overlapping specificities of 17-phenyl trinor PGE_2 and sulprostone for EP_1 and EP_3 receptor subtypes, it is difficult to attribute cellular effects to a particular EP receptor subtype. We therefore, next used the more recently described ONO series of compounds that display increased selectivity for the individual EP_1-EP_4 receptor subtypes (Yamamoto *et al.*, 1999; Yamane *et al.*, 2000; Norel *et al.*, 2004; Baryawno *et al.*, 2008) to establish whether EP_1 and/or EP_3 receptors were responsible for 17-phenyl trinor PGE_2-mediated elevation in $[Ca^{2+}]_i$ mobilization and augmentation of TP receptor-mediated signalling in HEL cells. Stimulation of HEL cells with the EP receptor subtype-specific agonists ONO-D1-004 (EP_1; Figure 6A), ONO-AE1-259 (EP_2; Figure 6B), ONO-AE1-248 (EP_3; Figure 6C) and ONO-AE1-329 (EP_4; Figure 6D) established that only the selective EP_3 receptor agonist ONO-AE1-248 induced a transient rise in $[Ca^{2+}]_i$ in HEL cells (Figure 6C), and was found to have an EC_{50} of 210 nM. The specificity of ONO-AE1-248 for EP_3 receptors was confirmed using the EP receptor subtype-specific antagonists ONO-8713 (EP_1), ONO-AE3-240 (EP_3) and ONO-AE-208 (EP_4). Pre-incubation of HEL cells with ONO-8713 (Figure 6E) or ONO-AE-208 (Figure 6G) did not significantly reduce $[Ca^{2+}]_i$ mobilization response to the EP_3 receptor agonist ONO-AE1-248 (Figure 6E,G, $P = 0.43$ and $P = 0.75$ respectively) while the EP_3 receptor antagonist ONO-AE3-240 completely inhibited ONO-AE1-248-induced $[Ca^{2+}]_i$ mobilization (Figure 6F, $P < 0.0001$; $IC_{50} = 15$ nM). These results confirm that in HEL, cells EP receptor-induced increases in $[Ca^{2+}]_i$ are mediated by the EP_3 receptor subtype only.

Pre-incubation of HEL cells with AH6809 or SC19220 did not significantly inhibit ONO-AE1-248/EP_3-mediated $[Ca^{2+}]_i$ mobilization (compare Figure 7A with Figure 7B,C, $P = 0.09$ and $P = 0.11$ respectively), while 17-phenyl trinor PGE_2-mediated $[Ca^{2+}]_i$ signalling was completely inhibited by the EP_3 receptor antagonist ONO-AE3-240 (Figure 7D; $P < 0.0001$; $IC_{50} = 75$ nM), but not by the EP_1 receptor antagonist ONO-8713 (Figure 7E; $P = 0.81$) further confirming that 17-phenyl trinor PGE_2-induced $[Ca^{2+}]_i$ signalling is by an EP_3 receptor-mediated pathway. To further clarify the mechanism of EP_3 receptor signalling, it was confirmed that both the PLCβ inhibitor U73122 (compare Figure 7A with Figure 7F, $P = 0.005$) and pretreatment with PTX (compare Figure 7A with Figure 7G, $P < 0.0001$) almost completely inhibited ONO-AE1-248-induced $[Ca^{2+}]_i$ mobilization in HEL cells. Hence, taken together, these data further confirm that PLCβ-mediated $[Ca^{2+}]_i$ mobilization in HEL cells in response to EP receptor signalling, can be attributed to EP_3-, and not EP_1 receptor-mediated signalling that occurs through a PTX-sensitive G_i-dependent mechanism leading to PLCβ activation and subsequent elevation in $[Ca^{2+}]_i$.

Having identified EP_3 receptors as the subtype responsible for the observed EP receptor-induced signalling in HEL cells, it

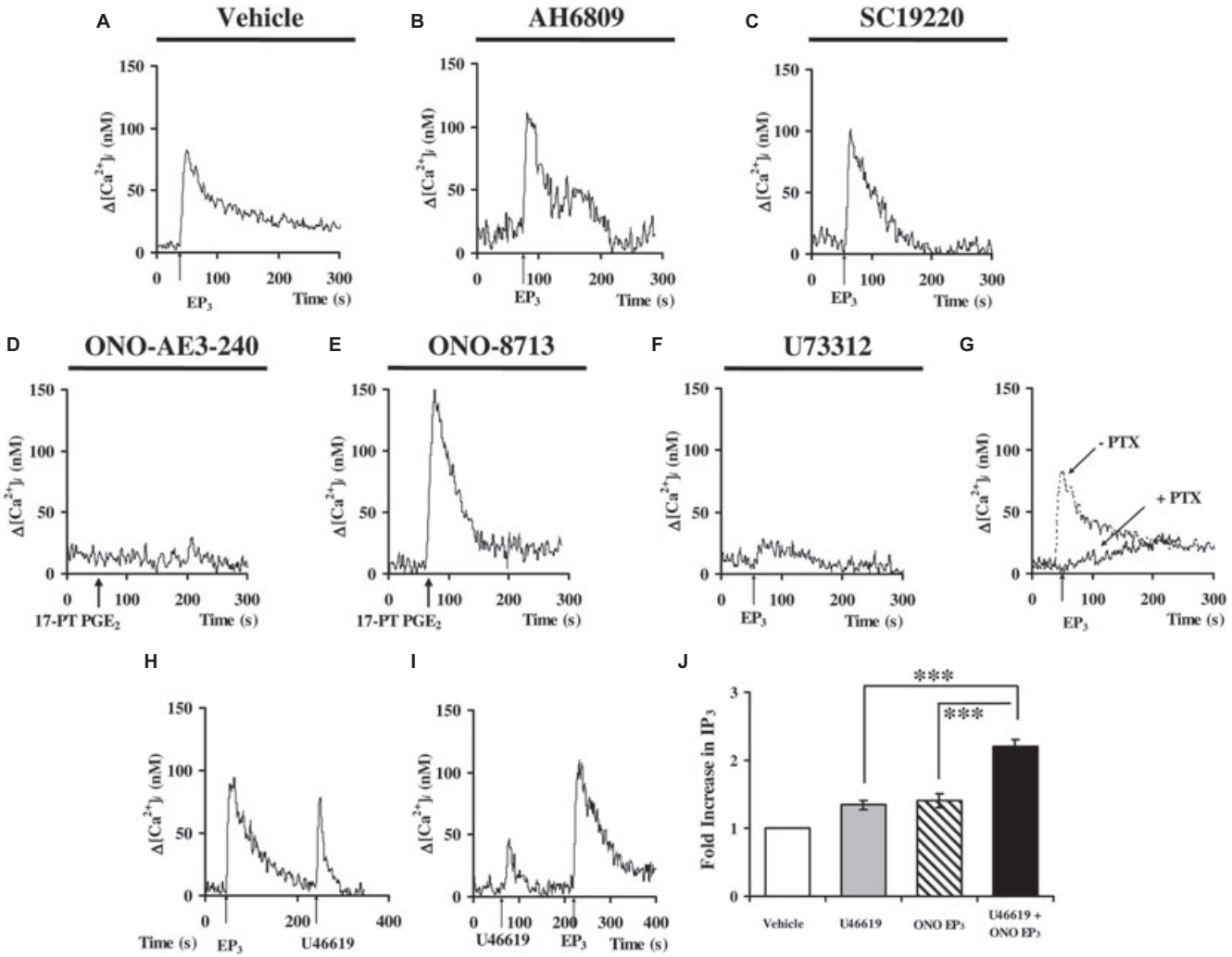

Figure 7 Specificity of EP_3 receptor signalling in HEL cells. (A–I) HEL cells, preloaded with FURA2/AM, were incubated for 10 min with either vehicle (A), 10 μM AH6809 (B), 10 μM SC19920 (C), 10 μM ONO-AE3-240 (D), 10 μM ONO-8713 (E), 10 μM U73122 (F) prior to stimulation with 1 μM ONO-AE1-248 (A–C) or 1 μM 17-phenyl trinor (17-PT) PGE_2 (D,E) where the agonists were added at times indicated by the arrows. (G) HEL cells were incubated overnight at 37°C with 50 ng·mL^{-1} PTX (+PTX) or with its vehicle (–PTX) prior to harvesting and loading with FURA2/AM followed by stimulation with the EP_3 agonist 1 μM ONO-AE1-248, where the ligand was added as indicated by the arrows. (H,I) HEL cells were stimulated with 1 μM ONO-AE1-248 and 1 μM U46619 in sequential combination. Results presented are representative of at least four independent experiments, where actual mean changes in intracellular Ca^{2+} mobilized ($\Delta[Ca^{2+}]_i$, nM) are: (A) $\Delta[Ca^{2+}]_i = 88.3 \pm 7.5$; (B) $\Delta[Ca^{2+}]_i = 113 \pm 7.2$; (C) $\Delta[Ca^{2+}]_i = 119 \pm 20.0$; (D) $\Delta[Ca^{2+}]_i = 0$; (E) $\Delta[Ca^{2+}]_i = 152 \pm 21.1$; (F) $\Delta[Ca^{2+}]_i = 31.7 \pm 15.8$; (G) $\Delta[Ca^{2+}]_i = 0$; (H) ONO-AE1-248 $\Delta[Ca^{2+}]_i = 85.9 \pm 12.7$, U46619, $\Delta[Ca^{2+}]_i = 83.3 \pm 17.9$; (I) U46619, $\Delta[Ca^{2+}]_i = 53.6 \pm 3.8$, ONO-AE1-248 $\Delta[Ca^{2+}]_i = 116 \pm 13.3$. (J) HEL cells were stimulated at 37°C for 20 min with 1 μM U46619 (U46619), 1 μM ONO-AE1-248 (ONO EP_3), either alone or in combination. In each case, basal IP_3 levels were determined by exposing the cells to the vehicle under identical incubation conditions. Levels of IP_3 generated by agonist- or vehicle-treated cells are expressed as a mean fold increases in IP_3 levels generated in agonist-treated cells relative to basal (vehicle)-treated cells (Fold increase in $IP_3 \pm$ SEM, $n = 4$), where *** indicates $P \leq 0.001$. $[Ca^{2+}]_i$; intracellular calcium; EP, PGE_2 receptor; HEL, human erythroleukaemic; IP_3, inositol 1,4,5-trisphosphate; PG, prostaglandin; PTX, *Pertussis* toxin.

was next sought to clarify whether the augmentation in EP and TP receptor-induced $[Ca^{2+}]_i$ mobilization and IP_3 generation also specifically occurred in response to the selective EP_3 receptor agonist ONO-AE1-248, similar to that which occurred with 17-phenyl trinor PGE_2. Pre-stimulation of HEL cells with 1 μM ONO-AE1-248 caused a significant augmentation of U46619-stimulated $[Ca^{2+}]_i$ mobilization (Figure 7H, $P = 0.003$) and in IP_3 generation (Figure 7J, $P < 0.0001$). Likewise, pre-stimulation with U46619 augmented the subsequent ONO-AE1-248 response (compare Figure 7A with Figure 7I, $P = 0.001$). Therefore, the data clearly establish that there is reciprocal augmentation in PLCβ coupling and $[Ca^{2+}]_i$ mobilization between the TPα/TPβ receptor isoforms and the EP_3 receptor subtype in HEL cells.

Finally, to further clarify the 17-phenyl trinor PGE_2/EP_1-mediated signalling in HEK.TPα and HEK.TPβ cells, the effect of the selective EP_1 and EP_3 receptor antagonists from ONO on 17-phenyl trinor PGE_2-induced $[Ca^{2+}]_i$ mobilization was examined. While the EP_1 receptor antagonist ONO-8713 impaired 17-phenyl trinor PGE_2 ($P < 0.0001$ and $P < 0.0001$, respectively; $IC_{50} = 18.3–25$ nM), the EP_3 receptor antagonist ONO-AE3-240 did not significantly inhibit the 17-phenyl trinor PGE_2-induced $[Ca^{2+}]_i$ responses in HEK.TPα and HEK.TPβ cells ($P = 0.77$ & $P = 0.91$, respectively).

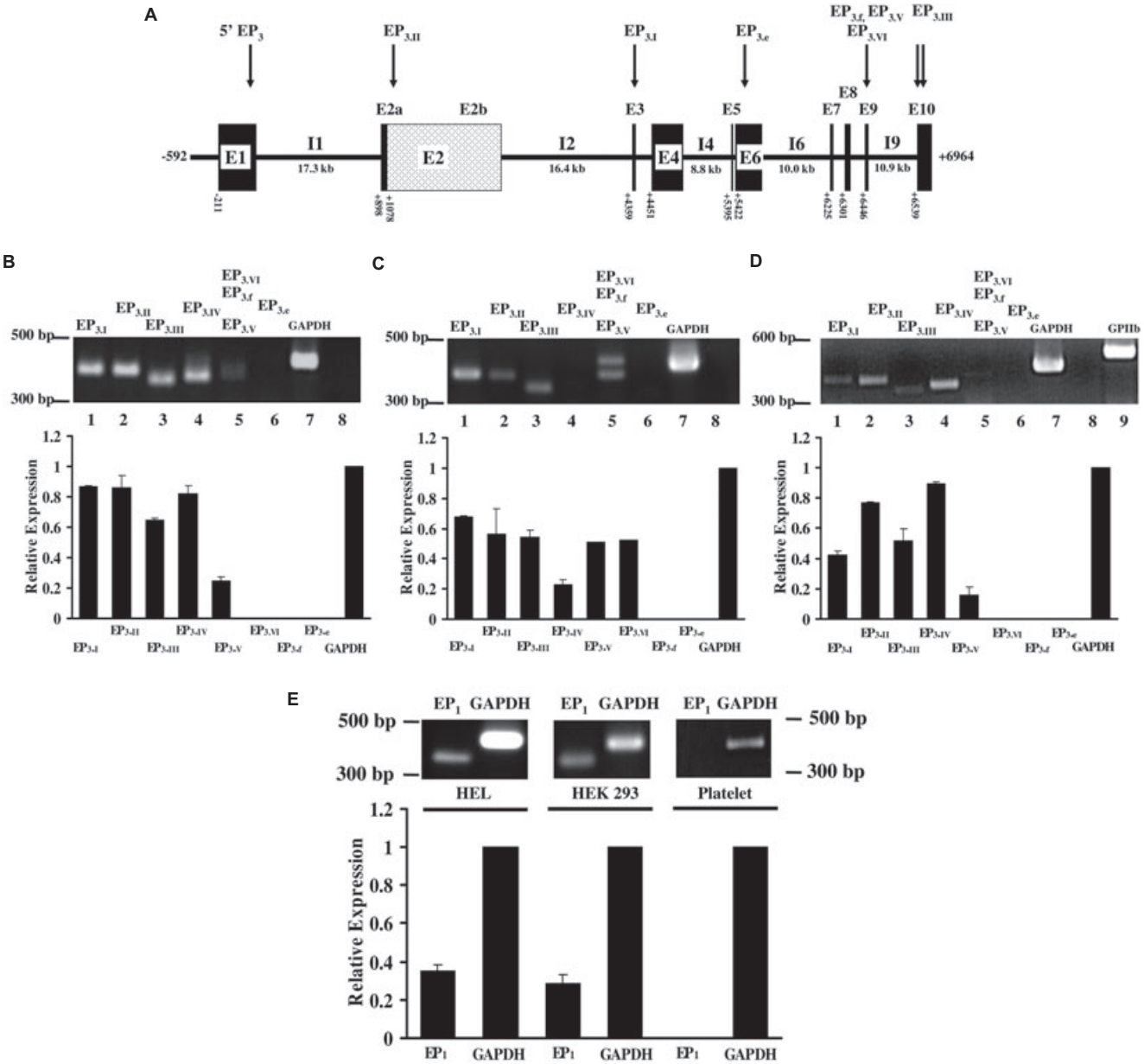

Figure 8 Identification of prostaglandin E_2 receptor $(EP)_3$ isoform(s) in human erythroleukaemic (HEL) 92.1.7 and human embryonic kidney (HEK) 293 cells. (A) Schematic of the human EP_3 gene organization composed of exon (E)1–E10 separated by intron (I)1–I9. The lower numbers indicate the 5′ position of the exon sequences within the EP_3 mRNAs, where nucleotide numbers are assigned relative to the translation initiation codon, ATG designated +1. E1 encodes the 5′-untranslated region (UTR), the translational initiation codon and the EP_3 receptor coding sequence through to sequences within transmembrane (TM) domain 6, with the first intron (I1) occurring at 898 bp downstream of the ATG. E2a encodes the remaining TM6 in addition to TM7 and 10 amino acids of the carboxyl-terminal tail domain. E1 and E2a are common to all eight EP_3 isoforms, with isoform-specific regions encoding the respective C-tail domains and/or 3′-UTRs formed by E2b and/or combinations of E3–E10. The positions of the universal 5′ primer (5′ EP_3) and of each of the isoforms-specific 3′ primers used in RT-PCR analysis are indicated by the upper arrows. (B–D) Agarose gel electrophoresis of RT-PCR products derived from first strand (1°) cDNA from HEL 92.1.7 (B), HEK 293 (C) cells or human platelets (D): Lane 1, $EP_{3.I}$; Lane 2, $EP_{3.II}$; Lane 3, $EP_{3.III}$; Lane 4, $EP_{3.IV}$; Lane 5 $EP_{3.V}$; $EP_{3.VI}$; $EP_{3.f}$; Lane 6, $EP_{3.e}$; Lane 7, glyceraldehyde-3-phosphate dehydrogenase (GAPDH); Lane 8, negative control PCRs carried out with primary cDNA template in the absence of primers; Lane 9 (Panel D only), GPIIb. (E) Agarose gel electrophoresis of RT-PCR for products for EP_1 and GAPDH derived from primary cDNA from HEL 92.1.7 (left-hand panel), HEK 293 (middle-hand panel) cells or human platelets (right-hand panel), as indicated. Densitiometric analysis of $EP_{3.I}$, $EP_{3.II}$, $EP_{3.III}$, $EP_{3.IV}$, $EP_{3.V}$, $EP_{3.VI}$, $EP_{3.f}$ and $EP_{3.e}$ mRNA relative to GAPDH mRNA expression in HEL (B), HEK 293 (C) cells or human platelets (D) and of EP_1 mRNA relative to GAPDH mRNA expression in HEL cells, HEK 293 cells or human platelets (E) is shown in graphical form below the respective panels.

Identification of EP_3 receptor isoforms expressed in HEL and HEK 293 cells

In humans, there are eight distinct isoforms of the EP_3 receptor subtype that arise by alternate splicing of a primary RNA transcript [Figure 8A (Kotani *et al.*, 1997)]. In an attempt to ascertain the basis of the differential EP_1/EP_3 receptor-mediated signalling observed in HEL versus HEK 293 cells responsible for mediating PLCβ-dependent $[Ca^{2+}]_i$ mobiliza-

tion and the differential cross-talk with the TP receptor isoforms, RT-PCR analysis was employed to identify the profile of EP_1 and EP_3 receptor isoform expressed therein (Table 1 & Figure 8). The predominant EP_3 receptor isoforms found in HEL cells were $EP_{3.I}$, $EP_{3.II}$, $EP_{3.III}$ and $EP_{3.IV}$, with $EP_{3.I}$, $EP_{3.II}$ and $EP_{3.IV}$ exhibiting the greatest and almost equivalent levels of expression (Figure 8B) while expression of $EP_{3.III}$ was estimated to be approximately 80% of the latter isoforms. RT-PCR also demonstrated that $EP_{3.V}$ was expressed in HEL cells at approximately 30% of $EP_{3.I}$; however, the EP_3 receptor isoforms $EP_{3.VI}$, $EP_{3.e}$ and $EP_{3.f}$ were not detected. While multiple EP_3 receptor isoforms were also expressed in HEK 293 cells, the profile of expression between the HEL and HEK cell types was found to be substantially different (Figure 8C). $EP_{3.I}$, $EP_{3.II}$, $EP_{3.III}$ and, to a lesser extent, $EP_{3.IV}$ were expressed in HEK 293 cells, albeit at varied and lower levels of expression than in HEL cells (Figure 8C). In particular, in contrast to HEL cells, the expression of $EP_{3.IV}$ in HEK 293 cells was less than 50% of the other isoforms detected. In addition, HEK 293 cells also expressed $EP_{3.V}$ and $EP_{3.VI}$, at levels similar to $EP_{3.III}$, but each of those receptor isoforms were absent from HEL cells. Moreover, as in HEL cells, receptor isoforms $EP_{3.e}$ and $EP_{3.f}$ were not expressed in HEK 293 cells. In view of the differences in the profile of expression between the EP_3 receptor isoforms in the HEK 293 relative to the megakaryocytic HEL cell lines, we also investigated their expression in human platelets. RT-PCR analysis confirmed that the profile of the EP_3 receptor isoform expression in human platelets was almost identical to that in HEL cells, most noteworthy with the abundant expression of $EP_{3.IV}$, relative to that in HEK 293 cells (Figure 8D). Additionally, while $EP_{3.V}$ and $EP_{3.VI}$ isoforms were expressed in the latter cells, neither isoform was expressed in HEL cells or in platelets (Figure 8D). Moreover, as in HEL and HEK 293 cell lines, $EP_{3.e}$ and $EP_{3.f}$ receptor isoforms were not expressed in platelets.

The EP_1 receptor subtype (Figure 8E), which was originally cloned from HEL cells (Funk *et al.*, 1993), was also expressed but at levels significantly less than that of the EP_3 receptor isoforms $EP_{3.I–IV}$ isoforms (Figure 8B) but notably was absent from human platelets (Figure 8E). Consistent with previous reports (Kelley-Hickie and Kinsella, 2004), EP_1 receptors were also expressed in HEK 293 cells albeit at equivalent levels relative to that in HEL cells (Figure 8C).

Taken together, data herein establish that signalling through the TP receptor isoforms expressed in the erythroleukaemic cell line, HEL 92.1.7, are subject to cross-talk and augmentation of signalling through the activation of PTX-sensitive isoforms of the EP_3 subtype of the EPs. Furthermore, selective activation of the different EP receptor subtypes may lead to opposing intermolecular cross-talk between TPα/TPβ receptor signalling where EP_1 receptor-induced signalling mediates desensitization, while that through EP_3 receptors augments TPα/TPβ receptor signalling.

Discussion and conclusions

As stated, it has been established that 17-phenyl trinor PGE_2 acting through the EP_1 subtype of the EP family desensitized signalling by the TPα and TPβ isoforms of the human TP endogenously expressed in human renal mesangial cells and by the individual the TP receptor isoforms stably expressed in HEK 293 cells (Walsh and Kinsella, 2000; Kelley-Hickie and Kinsella, 2004). However, in human platelets, 17-phenyl trinor PGE_2 did not induce $[Ca^{2+}]_i$ mobilization *per se* nor desensitize subsequent TP receptor-mediated signalling (Walsh and Kinsella, 2000). Here, we have sought to investigate the possible cross-talk between EP_1 and TP receptor signalling in HEL 92.1.7 cells, a widely used model cell line possessing megakaryocytic characteristics and known to endogenously express TPα, TPβ and EP_1 receptors (Long *et al.*, 1990; Funk *et al.*, 1993; Schwaner *et al.*, 1995; Coyle and Kinsella, 2005; 2006).

In contrast to its effects in platelets (Walsh and Kinsella, 2000), 17-phenyl trinor PGE_2 induced IP_3 generation and $[Ca^{2+}]_i$ mobilization in HEL cells and did not desensitize but rather augmented, signalling in response to the TXA_2 mimetic U46619. Furthermore, the augmentation was reciprocal, with TP receptor signalling increasing 17-phenyl trinor PGE_2-induced signalling. The specificity of the augmentation was confirmed in HEL cells whereby the α_2-adrenoceptor agonist UK14304 did not augment but, rather, desensitized EP receptor signalling and vice versa while additional EP (sulprostone and PGE_2) and TP (I-BOP) receptor agonists also showed reciprocal augmentations between TP and EP receptor signalling. 17-phenyl trinor PGE_2 signalling and augmentation of TP receptor signalling was insensitive to both the EP/DP receptor antagonist AH6809 and the more selective EP_1 receptor antagonist SC19220 in HEL cells. In contrast to its effects in HEK.TPα and HEK.TPβ cells, where EP_1 receptor-dependent desensitization occurs through direct PKC phosphorylation of Thr^{337} and Thr^{399} within the unique C-tail domains of TPα and TPβ receptors, respectively (Kelley-Hickie and Kinsella, 2004), 17-phenyl trinor PGE_2-mediated $[Ca^{2+}]_i$ mobilization and augmentation of TP receptor signalling were unaffected by either PKC, or indeed PKA, inhibition. While EP_1 receptor-mediated signalling leads to $[Ca^{2+}]_i$ mobilization through G_q/PLCβ coupling (Narumiya *et al.*, 1999; Bos *et al.*, 2004; Alfranca *et al.*, 2006), signalling by EP_3 receptors occurs through PTX-sensitive $G_i/G_{\beta\gamma}$-dependent activation of PLCβ (Coleman *et al.*, 1994; Narumiya *et al.*, 1999). Here, 17-phenyl trinor PGE_2-mediated $[Ca^{2+}]_i$ mobilization was sensitive to both the PLCβ inhibitor U73122 and to PTX, suggesting that it was signalling through the EP_3, as opposed to the EP_1 receptor subtype in HEL cells.

Further clarification of the nature of the EP receptor-mediated responses in HEL cells was obtained by the use of the more recently described highly specific EP receptor agonists ONO-D1-004 (EP_1), ONO-AE1-259 (EP_2), ONO-AE1-248 (EP_3) and ONO-AE1-329 (EP_4 (Yamamoto *et al.*, 1999; Narumiya and FitzGerald, 2001; Kubo *et al.*, 2004; Norel *et al.*, 2004; Takahashi *et al.*, 2005). The EP_3 receptor agonist ONO-AE1-248, but not the EP_1, EP_2 or EP_4 receptor agonists, induced $[Ca^{2+}]_i$ mobilization and IP_3 generation in HEL cells, which was insensitive to the selective EP_1 (ONO-8713) and EP_4 (ONO-AE-208) but not to the EP_3 (ONO-AE3-240) receptor antagonists. While ONO-AE1-248-mediated signalling occurred through PTX-sensitive, $G_i/G_{\beta\gamma}$-dependent activation of PLCβ, unlike that in mesangial cells or in the clonal HEK 293 cell lines (Walsh and Kinsella, 2000; Kelley-Hickie and Kinsella, 2004) neither AH6809 nor SC19220 antagonized its

signalling in HEL cells. Moreover, ONO-AE1-248 augmented U46619-induced signalling and in a reciprocal manner. Consistent with the hypothesis that the 17-phenyl trinor PGE_2-mediated effect may be occurring through an EP_3, as opposed to EP_1 receptor mechanism, the EP_3 (ONO-AE3-240) but not the EP_1 (ONO-8713) receptor antagonist inhibited 17-phenyl trinor PGE_2-mediated $[Ca^{2+}]_i$ mobilization in HEL cells. Conversely, the EP_1 antagonist ONO-8713, but not the EP_3 receptor antagonist ONO-AE3-240, inhibited 17-phenyl trinor PGE_2-induced $[Ca^{2+}]_i$ mobilization in the clonal HEK 293 cell lines stably overexpressing TPα and TPβ receptors, respectively. Hence, taken together, these data confirm that there are fundamental differences in EP-mediated PLCβ activation/$[Ca^{2+}]_i$ signalling between HEL cells and that previously identified in primary human mesangial cells and in the HEK.TPα or HEK.TPβ cell lines, whereby it mainly occurs in the former through a PTX-sensitive, EP_3 receptor-dependent mechanism while in the latter cell types, it occurs through an EP_1 receptor-dependent mechanism (Kelley-Hickie and Kinsella, 2004). Moreover, ligands, such as 17-phenyl trinor PGE_2, displaying overlapping specificity between the EP_1/EP_3 receptor subtypes signal through activation of the EP_3 subtype in HEL cells but signal through the EP_1 receptor subtype in human mesangial cells and HEK 293 cells to mediate augmentation or desensitization of TP receptor signalling respectively. It is arguable that the observed differences in the clonal HEK.TPα and HEK.TPβ cells, relative to that in HEL cells, may be due to the effect of ectopic overexpression of the TP receptor isoforms and/or of the coupling G protein (Gαq). However, owing to the fact that such differences in signalling and desensitization/augmentation also occurs in primary human mesangial cells makes it less likely that those differences are due to artefacts of overexpression of the TP receptor isoforms or of $G\alpha_q$ in the respective HEK 293 cell lines.

In order to address the nature of the cell-specific differences in EP receptor-mediated signalling, the profile of expression of the EP_1 and EP_3 receptor isoforms was examined by RT-PCR. HEL and HEK 293 cells were found to express equivalent levels of the EP_1 subtype while both cell lines expressed multiple EP_3 receptor isoforms, albeit with different and distinct profiles. While $EP_{3.I–III}$ isoforms were abundantly expressed in both cell lines, differences were seen for other isoforms. In particular, while HEL cells expressed high levels of $EP_{3.IV}$ receptor isoforms, HEK 293 cells showed minimal expression of the $EP_{3.IV}$ isoform. In addition, expression of the $EP_{3.V}$ and $EP_{3.VI}$ receptor isoforms was restricted to HEK 293 cells. The latter data are consistent with previous reports where RT-PCR analysis of various human tissues revealed that $EP_{3.V}$ and $EP_{3.VI}$ isoforms have a more limited expression than other EP_3 receptor isoforms, being confined primarily to uterine tissue with lower, but modest, levels detected in the lung and kidney (Kotani *et al.*, 2000). Our present data also established that the $EP_{3.e}$ and $EP_{3.f}$ receptor isoforms were not detected in either HEL or HEK 293 cell lines. Hence, owing to the near equivalent level of expression of the EP_1 receptor subtype in HEL and HEK 293 cells, it is evident that differential expression of that subtype *per se* cannot account for the observed differences in signalling or intermolecular cross-talk between the two cell types. On the other hand, it is indeed evident that there are significant differences in the profile of EP_3 receptor isoforms expressed between the two cell lines. Moreover, RT-PCR analysis confirmed that the profile of EP_3 receptor isoform expression in human platelets was almost identical to that in the HEL cell line with the notable exception that the EP_1 receptor subtype was not detectable in the former. The absence of EP_1 receptors in human platelets is entirely consistent with previous findings of Ma *et al.* (2001) who also reported an absence of its expression in mouse platelets .

While the various EP_3 receptor isoforms are reported to exhibit identical PGE_2 binding and coupling to G_i-mediated inhibition of adenylyl cyclase, they also display numerous functional differences (Dey *et al.*, 2006; Sugimoto and Narumiya, 2007). In particular they exhibit differential abilities to couple to PLCβ-mediated $[Ca^{2+}]_i$ mobilization (An *et al.*, 1994; Schmid *et al.*, 1995; Dey *et al.*, 2006; Sugimoto and Narumiya, 2007). For example, the $EP_{3.I–III}$ isoforms can couple to PLCβ when expressed in COS-7, CHO and BHK cell lines (An *et al.*, 1994; Yang *et al.*, 1994; Kotani *et al.*, 1995; Schmid *et al.*, 1995). In contrast, the remaining $EP_{3.IV}$, $EP_{3.V}$, $EP_{3.VI}$ $EP_{3.e}$ and $EP_{3.f}$ receptor isoforms were unable to couple to IP_3 generation or to $[Ca^{2+}]_i$ mobilization (Schmid *et al.*, 1995; Kotani *et al.*, 2000). The abundant expression of the $EP_{3.I–III}$ isoforms detected herein in both HEL and HEK 293 cells suggests that the ability of the EP_3 receptor agonists to induce PLCβ-mediated $[Ca^{2+}]_i$ mobilization in the former but not in the latter cell line may not due to differences in the profile of EP_3 receptor isoform expression between the cell types. Consistent with this, attempts were made here to ascertain which EP_3 receptor isoform(s) have the capacity to couple to PLCβ activation by overexpressing each of the individual EP_3 isoforms in HEK 293 cells and examining their ability to induce IP_3 generation and $[Ca^{2+}]_i$ mobilization in response to the EP_3 receptor antagonist ONO-AE3-240. However, in that heterologous system, none of the EP_3 receptor isoforms induced agonist-dependent IP_3 generation or $[Ca^{2+}]_i$ mobilization, suggesting that other cell type-specific factors, such as differences in the profiles of heterotrimeric G protein and/or PLCβ isoform expression in combination with the profile of expression of EP_3 receptor isoforms may account for the cell-specific differences in signalling.

During megakaryocyte maturation, platelets evolve with changes occurring not only in the profile of receptors but also of the various heterotrimeric G proteins expressed (Sasaki *et al.*, 1997; van der Vuurst *et al.*, 1997; 1998). For example, van der Vuurst *et al.* (1997) established that while various megakaryocyte cell lines, representing different stages of differentiation/maturation, and platelets express similar levels of TP receptors, the TXA_2 mimetic U46619 does not induce $[Ca^{2+}]_i$ mobilization during the early stages of maturation while in platelets there is a substantial $[Ca^{2+}]_i$ response. They suggested that the appearance of U46619-mediated $[Ca^{2+}]_i$ response in cells representing the later stages of megakaryocyte maturation and in platelets was due to the appearance of G_{16}, a member of the G_q family that is expressed primarily in haemapoietic cells and exhibits promiscuity in G protein-coupled receptor (GPCR) coupling (Baltensperger and Porzig, 1997; van der Vuurst *et al.*, 1997). In platelets, there are several members of the G_i family present, suggesting a degree of redundancy (Yang *et al.*, 2002). However, these authors argue that GPCRs have preferred G_i partners but that, in the

absence of the preferred partner, other G_i family members can substitute in a somewhat promiscuous, if less efficient, manner (Yang *et al.*, 2002). It is possible that while HEL cells express multiple G_i proteins to which EP_3 receptor isoforms may preferentially couple, a suitable G_i partner may not be present in platelets or in HEK 293 cells to enable efficient EP_3 isoform coupling to $G_i/G_{\beta\gamma}$-mediated PLCβ activation in those cell types. Clarification of this point will require further investigation.

While the various underlying mechanisms of intermolecular cross-talk involving desensitization of signalling have been studied extensively (Lefkowitz, 1998; Bunemann *et al.*, 1999), the actual mechanisms leading to augmentation between signalling systems are numerous and less clearly defined requiring investigation of each individual occurrence. Augmentation of signalling has been observed between other members of the prostanoid subfamily of GPCRs. Van der Vuurst *et al.* (1997) demonstrated that cAMP elevation in response to the prostacyclin receptor (IP) agonist iloprost was enhanced by the TXA_2 mimetic U46619 in mature megakaryoblastic cell lines. Likewise, Wilson *et al.* (2004) demonstrated augmentation of signalling between TPα and the human IP receptors in HEK 293 cells. Co-expression of each receptor led to enhanced cicaprost- and/or U46619-induced cAMP generation and U46619-mediated $[Ca^{2+}]_i$ mobilization compared with that which occurred in cells expressing the individual TPα or IP receptors (Wilson *et al.*, 2004). In the latter case, enhancement of signalling was attributed to oligomerization between TPα receptors and the human IP receptors (Wilson *et al.*, 2004). Here, attempts were made to address the possibility that TPα and/or TPβ receptors may actually oligomerize with EP_3 receptor isoforms through co-immunoprecipitation experiments in HEK 293 cells stably overexpressing FLAG-tagged TPα and TPβ receptors and transiently transfected with individual haemagglutinin-tagged EP_3 receptor isoforms. However, while no evidence was found to support oligomerization between the various EP_3 and TP receptor isoforms in HEK 293 cells, the possibility that it may occur in HEL cells, or indeed in other cell types, and that it may also play a role in the reciprocal augmentation between EP_3 and TP receptor isoforms cannot be excluded.

Augmentation of signalling is a well documented phenomenon in platelets with numerous agonists such as adrenaline, 5-HT, ADP, platelet-activating factor and thrombin shown to act synergistically in platelet activation and aggregation (Rasheed and Saeed, 2004). For example, cross-talk between the two purinergic receptors $P2Y_1$ and $P2Y_{12}$ is a means of subtly modulating the response of human platelets to ADP, where $P2Y_{12}$ receptors potentiate the $P2Y_1$ receptor-induced $[Ca^{2+}]_i$ response by inhibition of adenylyl cyclase and activation of PI3K (Hardy *et al.*, 2004). On the other hand, $P2Y_1$ receptors do not augment $P2Y_{12}$-induced $[Ca^{2+}]_i$ signalling but, rather, activation of $P2Y_1$ receptors inhibits PI3K-dependent signalling through a Src tyrosine kinase-dependent manner to inhibit $P2Y_{12}$-mediated $[Ca^{2+}]_i$ signalling (Hardy *et al.*, 2004). Furthermore, it has also been established that $P2Y_{12}$-induced ADP secretion augments TP receptor-mediated platelet aggregation through a mechanism involving PI3K activation to regulate the second wave of platelet secretion and aggregation (Li *et al.*, 2003). Hence, it is possible that the augmentation between TP- and EP_3 receptor-mediated signalling observed here in HEL cells may also involve PI3K activation. In support of this, TP receptor signalling has been shown to lead to PI3Kγ-dependent Akt phosphorylation (Li *et al.*, 2003) while the PI3K inhibitors wortmannin and LY294002 inhibited U46619-mediated ERK activation in HEK.TPα and HEK.TPβ cells (Miggin and Kinsella, 2002b). Moreover, in preliminary studies, we have found that both wortmannin and LY294002 inhibited 17-phenyl trinor PGE_2-induced augmentation of U46619-mediated signalling in HEL cells suggesting that the EP_3 receptor-mediated augmentation of TP receptor signalling may indeed involve PI3K (Figure S1). While, to date, there is no direct evidence in the literature that EP_3 receptor-mediated signalling leads to PI3K activation or to Akt phosphorylation *per se*, because activation of members of the class 1B subfamily of PI3K is generally known to occur through a PTX-sensitive Gβγ-dependent mechanism (Stephens *et al.*, 1997), it is indeed formally possible and may, in turn, provide a mechanism for the observed augmentation between EP_3- and TP receptor-mediated signalling. Consistent with the latter, through additional preliminary findings, we have confirmed that 17-phenyl trinor PGE_2 induces Akt phosphorylation in HEL cells that is sensitive to inhibition by both wortmannin and LY294002 (Figure S1). Hence, it is possible that the EP_3 receptor-mediated augmentation of TP signalling occurs through EP_3 receptor-regulated PI3K/AKT signalling. A full assessment of this possibility will require further investigation.

Thus in the current study, it was established that contrary to that in primary human mesangial cells and transfected HEK 293 cell lines, 17-phenyl trinor PGE_2 mediates $[Ca^{2+}]_i$ mobilization through the activation of PTX-sensitive isoforms of the EP_3 receptor subtype of the PGE_2 receptors in HEL cells. Moreover, 17-phenyl trinor PGE_2 selectively activates EP_1 and EP_3 subtypes of the EP receptor family to differentially regulate TP receptor-mediated signalling through distinct intermolecular cross-talk mechanisms whereby activation of the EP_1 receptor subtype impairs/desensitizes while the EP_3 receptor subtype augments TPα/TPβ receptor signalling in a cell type-specific manner. These differential effects are not predictable from the expression of the EP_1 subtype and/or the profile of the EP_3 receptor isoforms. While distinct functional differences between the human TPα and TPβ receptor isoforms have been extensively investigated (Hirata *et al.*, 1996; Walsh and Kinsella, 2000; Walsh *et al.*, 2000b; Kinsella, 2001; Reid and Kinsella, 2003; Kelley-Hickie and Kinsella, 2004), the functional differences in the multiple human EP_3 receptor isoforms are still poorly defined. The significance of the existence of eight human EP_3 receptor isoforms and expression of multiple isoforms in any given cell and or/tissue type is unclear and requires further investigation. Taken together these data highlight the difficulty of defining EP receptor subtype-specific effects due to overlapping specificities of the many available agonists/antagonists. Furthermore, there is a requirement for the development and careful assessment of pharmacological agents to specifically target individual EP receptor subtypes or isoforms such that functional differences and intermolecular cross-talk with other receptors may be clearly defined.

Acknowledgements

This research was supported by grants to BTK from the Wellcome Trust, Science Foundation Ireland and The Health Research Board (Ireland). We are grateful to Dr James McRedmond for his advice in preparing RNA from purified human platelets and to N. Shigeta, ONO Pharmaceutical Co. Ltd., Osaka, Japan for the gift of the ONO-series of EP ligands.

conflict of interest

None.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Effect of wortmannin and LY294002 on EP receptor signalling in HEL cells. HEL cells, preloaded with FURA2/AM, were incubated for 30 min with either 400 nM wortmannin (A–D) or 50 μM LY294002 (E–H) prior to stimulation with 1 μM U46619 or 1 μM 17-phenyl trinor PGE_2 either alone or in sequential combination, where the agonists were added at times indicated by the arrows. Results presented are representative of at least four independent experiments, where actual mean changes in intracellular Ca^{2+} mobilized ($\Delta[Ca^{2+}]_i$, nM) are: (A) $\Delta[Ca^{2+}]_i = 37.2 \pm 6.4$; (B) $\Delta[Ca^{2+}]_i = 177 \pm 15.4$; (C) 17-phenyl trinor PGE_2, $\Delta[Ca^{2+}]_i = 177 \pm 15.4$, U46619, $\Delta[Ca^{2+}]_i = 72.4 \pm 3.8$; (D) U46619, $\Delta[Ca^{2+}]_i = 37.2 \pm 6.4$, 17-phenyl trinor PGE_2, $\Delta[Ca^{2+}]_i = 312 \pm 13.5$; (E) $\Delta[Ca^{2+}]_i = 30.1 \pm 3.2$; (F) $\Delta[Ca^{2+}]_i = 181.3 \pm 10.2$; (G) U46619, $\Delta[Ca^{2+}]_i = 30.1 \pm 3.2$, 17-phenyl trinor PGE_2, $\Delta[Ca^{2+}]_i = 243 \pm 6.5$; (H) 17-phenyl trinor PGE_2, $\Delta[Ca^{2+}]_i = 181.3 \pm 10.2$, U46619, $\Delta[Ca^{2+}]_i = 78.5 \pm 3.2$. (I) HEL cells were incubated for 30 min with either vehicle (-), 400 nM wortmannin or 50 μM LY294002 followed by stimulation for 10 min with either 1 μM U46619 or 1 μM 17-phenyl trinor PGE_2. Thereafter, samples (50 μg protein per lane) were resolved by SDS-PAGE and initially immunoblotted (IB) with *anti*-phospho-Akt (Ser^{473}) antibody (upper panel); followed by *anti*-Akt antibody (lower panel). Results presented are representative of three independent experiments. The relative position of the 58 kDa molecular size marker is indicated to the left of the panels. EP, PGE_2 receptor; HEL, human erythroleukaemic; PG, prostaglandin.

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