Regulation of Extracellular Signal-Regulated Kinase Cascades by α- and β-Isoforms of the Human Thromboxane A₂ Receptor

SINEAD M. MIGGIN and B. THERESE KINSELLA

Department of Biochemistry, Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Dublin, Ireland

Received August 13, 2001; accepted December 22, 2001

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Thromboxane A₂ (TXA₂) stimulates mitogenic growth of vascular smooth muscle. In humans, TXA₂ signals through two TXA₂ receptor (TP) isoforms, termed TPα and TPβ. To investigate the mechanism of TXA₂-mediated mitogenesis, regulation of extracellular signal-regulated kinase (ERK) signaling was examined in human embryonic kidney 293 cells stably overexpressing the individual TP isoforms. The TXA₂ mimetic 9,11-dideoxy-9,11-c-methano epoxy prostaglandin F₂α (U46619) elicited concentration- and time-dependent activation of ERK1 and -2 through both TPs with maximal TPα- and TPβ-mediated ERK activation observed after 10 and 5 min, respectively. U46619-mediated ERK activation was inhibited by the TP antagonist [1S-[1α,2β]-5Z,9β,11α,13α,14α,15β,15-[2-(phenylamino)carbonyl]hydrazine methyl]-7-oxabicyclo-[2,2,1]-hept-2-yl]-5-heptenoic acid (SQ29,548), and by the mitogen-activated protein kinase (ERK) signaling was examined through similar mechanisms, although the time frame for maximal ERK activation and PKC dependence differs.

The prostanoid thromboxane A₂ (TXA₂) mediates a number of cellular responses, including platelet aggregation and contraction of vascular smooth muscle (Narumiya et al., 1999). TXA₂ may stimulate mitogenic and/or hypertrophic growth of vascular smooth muscle (Morinelli et al., 1994), and a number of studies have shown that the TXA₂ mimetic U46619 elicits extracellular signal-regulated kinase (ERK) activation in porcine, rat, bovine, and human smooth muscle cells, respectively (Morinelli et al., 1994; Jones et al., 1995; Grosser et al., 1997; Miggin and Kinsella, 2001). The TXA₂ receptor (TP), a member of the G protein-coupled receptor (GPCR) superfamily, is primarily coupled to Gq-dependent activation of phospholipase (PL) C (Narumiya et al., 1999). Previous studies have shown that down-regulation of diacyl glycerol (DAG)-regulated protein kinase (PK) C isoforms inhibits the ability of the Gq-coupled GPCRs to activate ERK signaling (Bogoyevitch et al., 1994). This effect is mediated through the ubiquitously expressed serine/threonine kinase c-Raf (Hagemann and Rapp, 1999). c-Raf, the most studied member of the Raf family, which also includes A-Raf and B-Raf, interacts directly with GTP-bound Ras, activating the ERK signaling cascade (Hagemann and Rapp, 1999). The Raf isoforms may be differentially regulated in response to diverse stimuli (Hagemann and Rapp, 1999); for example, although c-Raf is inhibited by cAMP-dependent

ABBREVIATIONS: TXA₂, thromboxane A₂; TP, thromboxane A₂ receptor; GPCR, G protein-coupled receptor; PL, phospholipase; DAG, diacyl glycerol; PK, protein kinase; PI3K, phosphoinositide 3-kinase; SH, src-homology; PG, prostaglandin; PD, prostaglandin D₂; ppERK, phosphorylated extracellular signal-regulated kinase; ppPKB, phosphorylated protein kinase B; PAGE, polyacrylamide gel electrophoresis; PTx, pertussis toxin; SMC, smooth muscle cell.
PKA, A-Raf is insensitive (Hageman and Rapp, 1999; Suttor et al., 1999). On the other hand, PKA not only blocks c-Raf activation but also may phosphorylate the low-molecular-mass guanine nucleotide binding protein Rap 1, which in turn recruits and causes sustained activation of B-Raf (Schmitt and Stork, 2000).

Phosphoinositide 3-kinase (PI3K) has been identified as one of the main mediators of growth factor-regulated cell proliferation and cell survival, transmitting antiangiogenic signals. The PI3K class Ia family members consist of a p110α, β, or δ catalytic subunit and a p85α or β adapter subunit. Growth factor stimulation of cells is mediated in part by interaction of the src-homology (SH)2 domains of the p85 adaptor subunits with tyrosine-phosphorylated proteins, such as receptor tyrosine kinases and growth factor receptor binding protein-2 (Wang et al., 1995; Sugden and Clerk, 1997). The PI3K class Iβ family, consisting of a p110γ catalytic subunit, is stimulated by G protein βγ subunits and does not interact with SH2 domain-containing adapters (Lopez-Ilasaca et al., 1998). Both class Iα and Iβ PI3Ks interact with GTP-bound Ras, which can act as both an effector and regulator of PI3K (Sugden and Clerk, 1997). Several studies suggest that PKB/Akt activation, by complex formation with the phosphatidylinositol-3,4,5-trisphosphate lipid products of PI3K, is a key event in the realization of the antiangiogenic effect of PI3K (Datta et al., 1999).

In humans, molecular cloning has identified two receptors for TXA₂, termed TPα and TPβ, which arise by differential splicing and which differ exclusively in their carboxyl terminal (C) tails (Kinsella, 2001). Although both TPα and TPβ exhibit identical G protein-dependent coupling to PLC, the main effector of TP receptor activation, they display differential regulation of their secondary effector adenylyl cyclase and the novel, high-molecular-weight G protein Gh (Walsh et al., 1998; Walsh and Kinsella, 2000). TPα and TPβ exhibit different patterns of expression (Miggin and Kinsella, 1998); and they are subject to differential homologous desensitization (Kinsella, 2001) and heterologous desensitization by a number of other prostanoids, including prostaglandin (PG)I₂, PGE₂, and PGD₂ (Walsh et al., 2000; Walsh and Kinsella, 2000; Kinsella, 2001). In view of these findings highlighting critical differences between the TP isoforms, the aim of the current study was to define the key regulatory elements involved in TXA₂-mediated activation of the ERK signaling cascade and to investigate whether the individual TP receptors may regulate this essential mitogenic cascade. These studies provide the first in-depth study defining the mechanisms of TXA₂-mediated ERK activation through the human TP receptors.

**Experimental Procedures**

**Materials**

PD 98059, GF 109203X, Tyrphostin AG1478, PP2, and H-89 were purchased from Calbiochem-Novabiochem (Nottingham, UK). 5-Heptenoic acid, 7,6,9-H3-9-epoxy-1-oxecynil-2-oxacyclobutylic[2.2.1]hept-5-yl]-(1R)-1-oxo-4,5-β-2), 6β(1E,3S*)-9,11-dideoxy-9α,11β-methano epoxy prostaglandin F₂α. U46619) and [1S-[1α,2β-[52]-3,4α-][3-[3-[(2-phenylamino)[carboxylsubunit(l)]hydroxyl]methyl]-1,2,4-oxacyclobutylic[2.2.1]hept-2-yl]-5-oxo heptanoic acid (SQ29,548) were purchased from Cayman Chemical (Ann Arbor, MI). [γ²³P]ATP (6000 Ci/mmol; 10 mCi/ml) was purchased from PerkinElmer Life Sciences (Boston, MA). Anti-ACTIVE mitogen-activated protein kinase rabbit polyclonal antibody was purchased from Promega (Madison, WI). Chemiluminescence blotting substrate (peroxidase), rat monoclonal 3F10 anti-hemagglutinin (HA)-peroxidase, and β-galactosidase staining kit (###28-673) were purchased from Roche Applied Science (Sussex, UK). Affinity-purified rabbit polyclonal anti-ERK1 (691); anti-Ha-Ras 259 rat monoclonal antibody (Sc 35); anti-GRK2/β-ARK1 (C15, Sc-562); anti-Rap1B (C-17, Sc 1481); anti-Gβδ(C-16, Sc 379); anti-Gy2 (A-16, Sc 374); anti-PI3K (H-199; Sc 7177); and horseradish peroxidase-conjugated anti-rabbit, -mouse, or -rat IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Aprotinin, benzamidine, leupeptin, myelin basic protein (MBP), phorbol-12-myristate-13-acetate (PMA), protein A-Sepharose CL-4B, protein G-agarose, phosphatidylinositol-3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002), epidermal growth factor, and wortmannin were purchased from Sigma Chemical (St. Louis, MO). Anti-HA 101I was purchased from BABCO (Berkeley, CA). I-Block was purchased from Tropix (Bedford, MA). Anti-phosphorylation-specific (pS473) PKB antibodies to detect PKB were purchased from BABCO (Berkeley, CA). Anti-PI3K antibodies to detect ERK1/2 were purchased from Tropix (Bedford, MA).

**Fig. 1.** Concentration-dependent effect of U46619 on ERK1 and ERK2 activation in HEK.TPα10 and HEK.TPβ3 cells. HEK.TPα10 cells (A) and HEK.TPβ3 cells (B) were stimulated for 10 min with 0 to 1000 nM U46619. A and B, blots (top) were screened with anti-ACTIVE-ERK to detect the phosphorylated, active forms of ERK (ppERK1/2), whereas blots (bottom) were screened with anti-ERK antibodies to detect ERK1/2 immunoreactive protein. Results are representative of three independent experiments. C, fold increases in ERK (ppERK1/2) phosphorylation in HEK.TPα10 cells (A) and HEK.TPβ3 cells (B), respectively, are presented as mean fold increases of basal ERK phosphorylation ± S.E.M. (n = 3), where the levels of basal ERK phosphorylation in vehicle-treated cells are assigned a value of 1.0. *, p < 0.05, **, p < 0.01 indicate that the levels of U46619-mediated ERK activation (ppERK) were significantly greater compared with basal levels.
[\textsuperscript{3}H]SQ29,548 (50.4 Ci/mmol) was obtained from PerkinElmer Life Sciences. All other chemicals and reagents were of AnalaR grade or molecular biology grade and were used without further purification.

**Methods**

**Plasmids.** The plasmid pCMV5:Ha-ras has been described previously (Kinsella et al., 1991). The plasmid pCMV5 Ha-ras\textsuperscript{N17} encoding the dominant negative (DN) Ha-Ras\textsuperscript{N17}, whereby Ser\textsuperscript{17} of Ha-Ras was mutated to Asn\textsuperscript{17}, was constructed by site-directed mutagenesis with the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The DN [or kinase dead (KD)] plasmids pEF-BOS-c-raf\textsuperscript{KD}-HA\textsuperscript{2} (K375W), pEF-BOS-A-raf\textsuperscript{KD}-HA\textsuperscript{2} (K336W), pEF-BOS\DeltaRfI:p85-HA\textsuperscript{2} (p85 DN), and the wild-type pEF-BOS\DeltaRfI:p85-HA\textsuperscript{2}, described previously (Sutor et al., 1999), were kindly donated by Dr. Larry M. Karnitz (Division of Radiation Oncology, Mayo Clinic, Rochester, MN). The plasmid pcDNA3:PI3K\textsuperscript{KD}-HA\textsuperscript{2} (K832R) was a generous gift from Dr. Reinhard Wetzker (Max-Planck Research Unit, Jena, Germany). The plasmid pRK5:ARK1(495-689), as described previously (Koch et al., 1994), was a generous gift from Prof. Robert Lefkowitz (Howard Hughes Medical Institute, Duke University Medical Center, Durham, NC). The plasmid pCMVrap1b\textsuperscript{N17}, as described previously (Schmitt and Stork, 2000), was a generous gift from Dr. Philip Stork (Vollum Institute, Oregon Health Sciences University, Portland, OR). The full-length rat G\textsubscript{11} and bovine G\textsubscript{12} were amplified by reverse transcriptase-polymerase chain reaction followed by subcloning into pcDNA3 (Invitrogen, Carlsbad, CA); the cloned cDNAs were verified by nucleotide sequence analysis and were confirmed to be identical to the previously published sequences for the rat G\textsubscript{11} (GenBank accession no. U34958) and bovine G\textsubscript{12} (GenBank accession no. M37183) sequences, respectively.

**Cell Culture and Transfection.** Human embryonic kidney (HEK) 293 cells were obtained from the American Type Culture Collection (Manassas, VA) and were routinely grown in minimal essential medium (MEM), 10% fetal bovine serum (FBS), unless otherwise indicated. The previously described recombinant
HEK.TPα10 and HEK.TP3 cell lines, stably overexpressing TPα and TPβ3, respectively (Walsh et al., 1998, 2000), were routinely grown in MEM, 10% FBS unless otherwise indicated. For transient transfections, HEK.TPα10 and HEK.TP3 cells were plated in 10-cm culture dishes 24 h before transfection at a density of 2 × 10^6 cells/10-cm dish in MEM, 10% FBS. Cells were cotransfected with 25 μg of pCMV5-, pcDNA3-, or pEF-BOS-based vectors, or as negative controls, with 25 μg of the appropriate empty vector, either pCMV5 or pcDNA3, plus 10 μg of pAdV by using the calcium phosphate/DNA coprecipitation technique (Walsh et al., 1998). HEK 293 cells transiently transfected in this way were routinely harvested 48 h post-transfection. However, for the measurement of mitogenic responses, 48 h post-transfection the complete medium (MEM, 10% FBS) was replaced with serum-free medium (MEM, 0% FBS) and the cells were incubated for a further 48 h to induce quiescence. In all cases, transient expression of proteins was confirmed by Western blot analysis at 48 to 96 h post-transfection. Specifically, expression of Ha-Ras and Ha-RasN17 was confirmed by immunoblot analysis using anti-Ha-Ras antibody (259; Santa Cruz Biotechnology) to confirm expression of Ha-Ras (lane 2) and Ha-Ras N17 (lane 3) with HEK 293 cells serving as a reference (lane 1).

**Saturation Radioligand Binding.** Radioligand binding was performed essentially as described previously (Miggin and Kinsella, 1998). Briefly, HEK 293 cells transiently transfected with pCMV5:TPα were harvested by centrifugation at 500g at 4°C for 5 min and then washed three times in phosphate-buffered saline (PBS). Protein concentrations were determined using the Bradford assay (Miggin and Kinsella, 1998). Cells were then harvested and resuspended in modified Ca^2+/-Mg^2+/-free Hanks' buffered salt solution, containing 10 mM HEPES, pH 7.67, 0.1% bovine serum albumin at a final concentration of 1 mg/ml. Saturation radioligand binding experiments with the TP antagonist [3H]SQ29,548 (20 nM; 50.4 Ci/mmol) were carried out at 30°C for 30 min in 100-μl reactions by using approximately 100 μg of total cellular protein per assay. Nonspecific binding was determined in the presence of excess nonlabeled 10 μM SQ29,548. Reactions were terminated by the addition of ice-cold 10 mM Tris, pH 7.4, followed by filtration through Whatman GF/C glass filters (Whatman, Maidstone, UK). After washing of the filters three times with 4 ml of 10 mM Tris, pH 7.4, liquid scintillation counting of the filters in 5 ml of scintillation cocktail was performed. Results are expressed as picomoles of [3H]SQ29,548 incorporated per milligram of cell protein ± S.E.M., where n = 3.

**β-Galactosidase Staining.** Briefly, HEK 293 cells were transiently transfected with pHM6lacZ (Roche Applied Science) by using the calcium phosphate/DNA coprecipitation technique (Walsh et al., 1998). After 48 and 96 h, β-galactosidase activity was assessed using the β-galactosidase staining kit, essentially as described by the manufacturer (Roche Applied Science). Briefly, cells were washed once with PBS. After removal of the PBS, the cells were fixed with PBS containing 2% formaldehyde (3 ml/10-cm dish) for 15 min at room temperature. Cells were washed three times with PBS followed by analysis of β-galactosidase activity with a staining solution (1 part X-gal:19 parts iron buffer). After incubation of the cells for 60 min at 37°C, 5% CO_2, stained and nonstained cells were counted in a num-

---

**Fig. 3.** Effect of coexpression of Ha-Ras and Ha-RasN17 on U46619-induced activation of ERK in HEK.TPα10 and HEK.TPβ3 cells. HEK.TPα10 (A) and HEK.TPβ3 (B) were transiently transfected with either pCMV5:Ha-Ras, pCMV5:Ha-RasN17, or as a control, with the vector pCMV5. Cells were stimulated with U46619 for 10 min (+), with cells exposed to vehicle alone (−) serving as references. A and B, blots were screened with anti-ACTIVE-ERK to detect ppERK1/2 (A and B, top) or with anti-ERK antibodies to detect ERK1/2 immunoreactive protein (A and B, bottom). Results are representative of four independent experiments. (C) fold increases of basal ERK phosphorylation ± S.E.M. (n = 4), where the levels of basal ERK phosphorylation in vehicle-treated cells (−) are assigned a value of 1.0. *, p < 0.05 and **, p < 0.01 indicate that the levels of U46619-mediated ppERK activation were significantly enhanced/reduced in the presence of Ha-ras and Ha-rasN17, respectively. (D) immunoblot analysis of total cellular protein (100 μg) by using anti-Ha-Ras antibody (259; Santa Cruz Biotechnology) to confirm expression of Ha-Ras (lane 2) and Ha-RasN17 (lane 3) with HEK 293 cells serving as a reference (lane 1).
Assay. Induced ppERK activation were significantly reduced in the presence of a value of 1.0.

**Determination of ERK Activity by Using an in Vitro Kinase Assay.** HEK.TPα10 and HEK.TPβ3 cells were seeded at 4 x 10^5 cells/60-mm dish in MEM, 10% FBS. After 24 h, cells were exposed to MEM, 0.5% FBS to induce quiescence. After a further 48 h, the cells were exposed to test agents for the appropriate times, as indicated in the respective figure legends. ERK activity was determined by monitoring ERK-mediated phosphorylation of its substrate myelin basic protein as described previously

**Determination of ERK/PKB Activation by Immunoblot Analysis.** HEK.TPα10 and HEK.TPβ3 cells were seeded at 1.5 x 10^6 cells/10-cm dish in MEM, 10% FBS. After 24 h, cells were exposed to MEM, 0% FBS to induce quiescence. After 48 h, the cells were exposed to test compounds, as indicated in the respective figure legends. Cellular lysates were prepared as described previously

**Fig. 4.** Effect of GF 109203X on U46619-induced activation of ERK1 and ERK2 in HEK.TPα10 and HEK.TPβ3 cells. HEK.TPα10 (A) and HEK.TPβ3 (B) cells were transiently transfected with pEG-BOSAR1Δs85-HA and pADVA by using the Effectene transfection reagent essentially as described by the manufacturer (QIAGEN, Valencia, CA). After 48 h, cells were stimulated with U46619 (100 nM; 10 min) with nonstimulated cells serving as a reference. After harvesting, aliquots (80 µg) of whole cell protein were resolved by SDS-PAGE and electroblotted onto PVDF membranes. For immunoprecipitations, whole cell protein (500 µg) from each transfection was immunoprecipitated using the anti-TPβ (1/100) antisera directed to peptide sequences unique to TPα (amino acid residues SLSLQPQLTQRSGLQ; a peptide) and TPβ (amino acid residues LPFEPPGTAKLRKD; β peptide) C-tail sequences, as described previously (Miggin and Kinsella, 2001). Alternatively, as negative controls, whole cell protein (500 µg) from each transfection was subjected to immunoprecipitation by using the control preimmune TPα (1/100) and TPβ (1/100) sera. Immunoprecipitations were carried out essentially as described previously (Walsh et al., 2000; Miggin and Kinsella, 2001). Briefly, cells were washed once with ice-cold phosphate-buffered saline (3 ml/dish) and were then lysed with 0.6 ml of radioimmunoprecipitation buffer [50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40 (w/v), 0.5% sodium deoxycholate (w/v), 0.1% SDS (w/v) containing 10 mM sodium fluoride, 25 mM sodium pyrophosphate, 1 µg/ml leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml antipain, and 1 mM sodium orthovanadate]. After incubation on ice for 15 min, cells were harvested and disrupted by sequentially passing through hypodermic needles of decreasing bore size (gauge 20, 23, and 26), and soluble lysates were harvested by centrifugation at 13,000g at room temperature for 5 min. Immunoprecipitations of TPα and TPβ receptors from the cell lysates was performed using either anti-TPα (1/100) or anti-TPβ (1/100) antisera as appropri-
Fig. 5. Effect of H-89 on U46619-induced activation of ERK1 and ERK2 in HEK.TPx10 and HEK.TPβ3 cells. HEK.TPx10 (A) and HEK.TPβ3 (B) cells were preincubated for 5 min in the absence or presence of 10 μM H-89 before stimulation for 10 min with 100 nM U46619, 10 ng/ml EGF with cells exposed to vehicle alone serving as references. A and B, blots were screened with anti-ACTIVE-ERK to detect ppERK1/2 (A and B, top) or with anti-ERK antibodies to detect ERK1/2 immunoreactive protein (A and B, bottom). Results are representative of four independent experiments. C, fold increases in ERK phosphorylation (ppERK1/2) in A and B are presented as mean fold increases of basal ERK phosphorylation ± S.E.M. (n = 4), where the levels of basal ERK phosphorylation in vehicle-treated cells (–) are assigned a value of 1.0. ** indicates that the levels of U46619-mediated ppERK activation were significantly reduced in the presence (p ≤ 0.01) of H-89.

Fig. 6. Effect of coexpression of DN A-Raf and DN c-Raf on U46619-induced ERK activation in HEK.TPx10 and HEK.TPβ3 cells. HEK.TPx10 (A) and HEK.TPβ3 (B) were transiently transfected with either pEF-BOS:A-Raf.HA2 DN (DN A-Raf) or pEF-BOS:c-Raf.HA2 DN (DN c-Raf) or the empty vector (Control). Cells were stimulated with U46619 for 10 min (+), with cells exposed to vehicle alone (–) serving as references. A and B, blots were screened with anti-ACTIVE-ERK to detect ppERK1/2 (A and B, top) or with anti-ERK antibodies to detect ERK1/2 immunoreactive protein (A and B, bottom). Results are representative of four independent experiments. C, fold increases in ERK phosphorylation (ppERK1/2) in A and B are presented as mean fold increases of basal ERK phosphorylation ± S.E.M. (n = 4), where the levels of basal ERK phosphorylation in vehicle-treated cells (–) are assigned a value of 1.0. *, p ≤ 0.05 and **, p ≤ 0.01 indicate that the levels of U46619-mediated ppERK activation were significantly reduced in the presence of DN A-Raf and c-Raf. D, immunoblot analysis of total cellular lysate (30 μg) to confirm expression of c-Raf (lane 2) and A-Raf (lane 3) with nontransfected HEK 293 cells serving as a reference (lane 1).
ate. Immunoprecipitates were resolved by SDS-PAGE followed by electroblotting onto PVDF membranes. Thereafter, membranes were screened by immunoblot analysis by using either the anti-HA 3F10 peroxidase conjugate (to detect coimmunoprecipitation of HA-tagged p85 with TPα and TPβ) or by using the anti-HA-101r (BABCO) followed by peroxidase-conjugated anti-mouse IgG (to detect HA-tagged p85 cellular protein). To confirm the specificity of the anti-TPα and anti-TPβ antisera to immunoprecipitate TPα and TPβ, the previously described cell lines HEK.HATPα and HEK.HATPβ were used (Walsh et al., 2000). Both HA-tagged TPα and HA-tagged TPβ were subjected to immunoprecipitation by using the anti-TPα antisera (1/100), anti-TPβ (1/100), or anti-HA 101r (1/300; BABCO) antisera as described previously (Walsh et al., 2000) with HEK 293 cells serving as a control. Immunoprecipitates were resolved by SDS-PAGE followed by electroblotting onto PVDF membranes. Thereafter, membranes were screened by immunoblot analysis by using the anti-HA 3F10 peroxidase conjugate (to detect immunoprecipitation of HA-tagged TPα and TPβ).

**Results**

**U46619-Mediated ERK Activation in HEK.TPα10 and HEK.TPβ3 Cells.** Maximal concentration-dependent activation of ERK1 and ERK2 occurred in HEK.TPα10 cells after their exposure to 100 nM U46619 (Fig. 1, A and C). In HEK.TPβ3 cells, maximal ERK1/2 activation occurred after their exposure to 100 to 300 nM U46619 (Fig. 1, B and C).

Exposure of HEK.TPα10 (Fig. 2, A and E) and HEK.TPβ3 (Fig. 2, C and E) cells to U46619 induced time-dependent ERK1/2 activation with maximal responses observed at 10 min in HEK.TPα10 cells and at 5 min in HEK.TPβ3 cells, respectively. ERK activation was also determined using an in vitro kinase reaction with MBP serving as an ERK phosphorylation substrate. U46619 induced a time-dependent activation of ERK in HEK.TPα10 cells and in HEK.TPβ3 cells with maximal MBP phosphorylation observed after 10 min (Fig. 2, B and F) and after 5 min (Fig. 2, D and F), respectively.

**Fig. 7.** Effect of coexpression of Rap1bN17 on U46619-induced ERK activation in HEK.TPα10 and HEK.TPβ3 cells. HEK.TPα10 (A) and HEK.TPβ3 (B) were transiently transfected with pCMV:Rap1bN17 (RapN17) or with pCMV (Control). Cells were stimulated with U46619 for 10 min (+), with cells exposed to vehicle alone (−) serving as references. A and B, blots were screened with anti-ACTIVE-ERK to detect ppERK1/2 (A and B, top) or with anti-ERK antibodies to detect ERK1/2 immunoreactive protein (A and B, bottom). Results are representative of four independent experiments. C, fold increases in ERK phosphorylation (ppERK1/2) in A and B are presented as mean fold increases of basal ERK phosphorylation ± S.E.M. (n = 4), where the levels of basal ERK phosphorylation in vehicle-treated cells (−) are assigned a value of 1.0. **, p ≤ 0.01 indicates that the levels of U46619-mediated ppERK activation were significantly reduced in the presence of Rap1bN17.
Fig. 8. Effect of wortmannin and LY294002 on U46619-induced activation of ERK1 and ERK2 in HEK.TPα10 and HEK.TPβ3 cells. HEK.TPα10 (A) and HEK.TPβ3 (B) cells were preincubated with either wortmannin (wort; 400 nM; 30 min) or LY294002 (LY; 50 μM; 30 min). Subsequently, the medium was supplemented with U46619 (U4; 100 nM; 10 min), with cells exposed exclusively to U46619 or vehicle alone (Control) serving as references. A and B, blots were screened with anti-ACTIVE-ERK to detect ppERK1/2 (A and B, top) or with anti-ERK antibodies to detect ERK1/2 immunoreactive protein (A and B, bottom). Results are representative of four independent experiments. C, fold increases in ERK phosphorylation (ppERK1/2) in A and B are presented as mean fold increases of basal ERK phosphorylation ± S.E.M. (n = 4), where the levels of basal ERK phosphorylation in vehicle-treated cells (−) are assigned a value of 1.0. *, p ≤ 0.05 and **, p ≤ 0.01 indicate that the levels of U46619-mediated ppERK activation were significantly reduced in the presence of wortmannin and LY294002.

Fig. 9. Role of class I PI3Ks on U46619-induced activation of ERK in HEK.TPα10 and HEK.TPβ3 cells. HEK. TPα10 (A) and HEK.TPβ3 (B) cells were transiently transfected with either pcDNA3:Gβδ1 plus pcDNA3:Gγ2 (Gβγ), pRK5:βARK1(495-689) minigene (β-ARK), pcDNA3:PI3KγDN (PI3KγDN), or with the vector pcDNA3 (Control). In addition, cells were preincubated with PTX (50 ng/ml; 16 h). Subsequently, cells were stimulated for 10 min with 100 nM U46619 (+), with cells exposed exclusively to U46619 or vehicle alone (−) serving as a reference. A and B, blots were screened with anti-ACTIVE-ERK to detect ppERK1/2 (A and B, top) or with anti-ERK antibodies to detect ERK1/2 immunoreactive protein (A and B, bottom). Results are representative of four independent experiments. C and D, fold increases in ERK phosphorylation (ppERK1/2) in A and B, respectively, are presented as mean fold increases of basal ERK phosphorylation ± S.E.M. (n = 4), where the levels of basal ERK phosphorylation in vehicle-treated cells (−) are assigned a value of 1.0. The levels of U46619-mediated ppERK activation were not significantly altered in the presence of PTX, Gβγ, β-ARK, or PI3KγDN.
specificity of U46619-induced ERK1/2 activation was confirmed whereby both the selective TP antagonist SQ29,548 and the mitogen-activated protein kinase kinase 1/2 inhibitor PD 98059 abolished ERK activation (ppERK1/2; Fig. 2, A and C) and MBP phosphorylation (data not shown) in both HEK.TPα10 and HEK.TPβ3 cells. In all cases, equal protein loading and ERK1 and ERK2 expression was confirmed using an anti-ERK antibody to detect total ERK protein expression (Figs. 1, A and B; 2, A and C, bottom).

Thereafter, to determine the role of various protein mediators, such as Ras or PI3K isozymes, in U46619/TXA2-induced ERK signaling, HEK.TPα10 cells and HEK.TPβ3 cells were transiently cotransfected with cDNAs encoding either wild-type or dominant negative forms of those mediators. To establish the efficiency of transfection and to confirm sustained protein expression at 48 to 96 h post-transfection, for each independent experiment, HEK 293 cells were transfected with the vector pCMV5:TPα10 and TP expression was determined by saturation radioligand binding analysis with [3H]SQ29,548 at 48 and 96 h post-transfection. Routinely, TPα expression was 1.98 ± 0.14 and 1.57 ± 0.12 pmol of [3H]SQ29,548/mg of cell protein after 48 and 96 h post-transfection, respectively. Thus, no significant reduction in TPα protein expression occurred at 96 h post-transfection compared with TPα expression at 48 h (p = 0.1259). As an additional control, to determine the efficiency of transfection, HEK 293 cells were transfected with the expression vector pHM6: lacZ. At 48 and 96 h post-transfection, β-galactosidase activity was quantified. Routinely, by using the calcium phosphate/DNA coprecipitation technique, 35 to 40% and 30 to 35% cells expressed β-galactosidase activity at 48 and 96 h post-transfection, respectively.

To investigate the role of Ha-Ras in TP-mediated ERK activation, the effect of overexpression of Ha-Ras and its dominant negative Ha-RasN17 (Schmitt and Stork, 2000) were investigated. Overexpression of Ha-Ras and Ha-RasN17 was confirmed by Western blot analyses (Fig. 3D). Coexpression of Ha-Ras augmented U46619-induced ERK1/2 activation in both HEK.TPα10 and HEK.TPβ3 cells compared with control (pCMV5-transfected) cells (Fig. 3, A–C), whereas Ha-RasN17 significantly reduced U46619-induced ERK1/2 activation in both cell types (Fig. 3, A–C). Furthermore, overexpression of Ha-Ras or Ha-RasN17 in HEK.TPα10 and HEK.TPβ3 cells neither elicited ERK activation in the absence of U46619 (Fig. 3, A–C) nor affected the overall level of TP expression compared with nontransfected cells (data not shown).

Preincubation of HEK.TPα10 and HEK.TPβ3 cells with the EGF receptor inhibitor AG1478 (125 nM; 30 min) or the src inhibitor PP2 (100 nM; 15 min) significantly inhibited U46619-mediated ERK1/2 activation. Specifically, in HEK.TPα10 cells, 7.57 ± 0.45-, 4.22 ± 0.06- (p < 0.01), and 3.61 ± 0.15 (p < 0.01)-fold increases in U46619-mediated ERK activation over basal levels were observed in the ab-

**Fig. 10.** Effect of dominant negative p85 adaptor subunit of PI3K on TPα- and TPβ-mediated ERK activation. HEK.TPα10 (A) and HEK.TPβ3 (B) cells were transiently transfected with pEF-BOSRI:p85Δ. HA2(p85DN) or with the empty vector (Control). Cells were stimulated with 100 nM U46619 for 10 min (+), with cells exposed to vehicle alone (−) serving as references. A and B, blots were screened with anti-ACTIVE-ERK to detect ppERK1/2 (A and B, top) or with anti-ERK antibodies to detect ERK1/2 immunoreactive protein (A and B, bottom). Results are representative of four independent experiments. C, fold increases in ERK phosphorylation (ppERK1/2) in A (HEK.TPα10) and B (HEK.TPβ3), respectively, are presented as mean fold increases of basal ERK phosphorylation ± S.E.M. (n = 4), where the levels of basal ERK phosphorylation in vehicle-treated cells (−) are assigned a value of 1.0.
sence and presence of either AG1478 or PP2, respectively. In HEK.TPβ3 cells, 5.25 ± 0.36- to 2.23 ± 0.28- (p < 0.005), and 2.91 ± 0.63 (p < 0.05)-fold increases in U46619-mediated ERK activation over basal levels were observed in the absence and presence of either AG1478 or PP2, respectively.

**Effect of GF 109203X and H-89 on TPα and TPβ-Mediated ERK Activation.** Preincubation of HEK.TPα10 cells with the PKC inhibitor GF 109203X, at a previously established PKC-selective inhibitory concentration (Martiny-Baron et al., 1993), led to near complete inhibition of U46619-induced ERK1/2 activation (Fig. 4A). In HEK.TPβ3 cells, GF 109203X only partially inhibited U46619-mediated ERK1/2 activation (Fig. 4B). Specifically, GF 109203X pretreatment reduced U46619-mediated ERK activation in HEK.TPα10 cells from a 7.57 ± 0.45- to 2.15 ± 0.42-fold increase over basal levels (Fig. 4C; n = 8). However, in HEK.TPβ3 cells, U46619-mediated ERK activation was reduced from a 5.25 ± 0.36- to 3.11 ± 0.22-fold increase over basal levels (Fig. 4C; n = 8). Moreover, the effects of GF 109203X on U46619-induced ERK activation in HEK.TPα10 and HEK.TPβ3 cells were concentration-dependent (data not shown). Specifically, IC50 values of 206 and 840 nM GF 109203X were determined in HEK.TPα10 and HEK.TPβ3 cells, respectively. As a control, GF 109203X inhibited PMA-induced ERK activation, indicating that it was used at an effective, inhibitory concentration (Fig. 4, A and B).

Preincubation of quiescent HEK.TPα10 and HEK.TPβ3 cells with the potent PKA inhibitor H-89 (K_i = 48 nM), at a previously established inhibitory concentration (Daaka et al., 1997), significantly reduced U46619-mediated ERK1/2 activation but, as a control, had no effect on EGF-mediated ERK activation (Fig. 5, A–C). At higher concentrations, H-89 may inhibit other serine/threonine kinases, for example, PKC (K_i = 31.7 μM). However, we routinely use H-89 at a concentration below the K_i for PKC and have previously demonstrated that at the concentration used, PKA is inhibited, without effect on PKC (Walsh et al., 2000; Walsh and Kinsella, 2000). Additionally, the potent PKA inhibitor KT 5720 (1 μM; 45 min) significantly reduced U46619-mediated ERK activation in HEK.TPα10 and HEK.TPβ3 cells (data not shown). In all cases, equal protein loading and equal ERK1 and ERK2 expression were confirmed for each sample by using an anti-ERK antibody (Figs. 4, A and B; 5, A and B, bottom). It has recently been reported that H-89 may as an antagonist of certain GPCRs, thereby calling into question its utility as a selective PKA inhibitor (Penn et al., 1999). To rule out the possibility that H-89 may act as an antagonist of the hTPs, we investigated the effect of H-89 on ligand binding by both the TPα and TPβ isoforms by using [3H]SQ29,548 as selective TP radioligand. In keeping with our previous reports (Walsh et al., 2000), H-89 had no affect on ligand binding by either TPα or TPβ isoforms. Specifically, HEK.TPα10 cells exhibited 1.97 ± 0.42 and 2.25 ± 0.42 pmol of [3H]SQ29,548 bound/mg of protein in the absence and presence of 10 μM H-89, respectively. HEK.TPβ3 cells exhibited 1.93 ± 0.13 and 2.09 ± 0.1 pmol of [3H]SQ29,548 bound/mg of protein in the absence and presence of H-89, respectively.

**Role of A-Raf, c-Raf, and Rap1b in TPα- and TPβ-Mediated ERK Activation.** Coexpression of the DN forms of either A-Raf (A-RafDN) or c-Raf (c-RafDN) decreased U46619-mediated ERK activation in both HEK.TPα10 and HEK.TPβ3 cells but did not affect the level of basal ERK activation in either cell type (Fig. 6, A and B). Overexpression of both A-RafDN and c-RafDN were confirmed by western blot analyses (Fig. 6D). Additionally, HEK.TPα10 and HEK.TPβ3 cells transiently transfected with either A-RafDN or c-RafDN neither elicited ERK activation in the absence of U46619.
Coexpression of Rap1bN17 resulted in a partial inhibition of U46619-mediated ERK activation in both HEK.TPα10 and HEK.TPβ3 cells (Fig. 7, A–C). For each independent experiment, overexpression of Rap1bN17 was confirmed by Western blot analysis (data not shown). Additionally, HEK.TPα10 and HEK.TPβ3 cells transiently transfected with Rap1bN17 neither elicited ERK activation in the absence of U46619 (Fig. 7, A and B) nor exhibited altered levels of TP expression compared with control nontransfected cells (data not shown).

**Role of Class IB PI3K in TPα- and TPβ-Mediated ERK Activation.** Pretreatment of quiescent HEK.TPα10 and HEK.TPβ3 cells with the PI3K inhibitor wortmannin, at an established PI3K-selective inhibitory concentration (Leopoldt et al., 1998), led to near complete inhibition of U46619-mediated ERK activation (Fig. 8, A and C). Moreover, pretreatment of HEK.TPα10 and HEK.TPβ3 cells with the more selective PI3K inhibitor LY294002, at an established inhibitory concentration (Vlahos et al., 1994), also significantly inhibited U46619-mediated ERK activation compared with cells exposed exclusively to U46619 under similar conditions (Fig. 8, A–C).

Coexpression of PI3KγDN (with/without the adaptor subunit p101; data not shown) did not affect U46619-induced activation of ERK in either HEK.TPα10 or HEK.TPβ3 cells compared with vehicle-treated cells (Fig. 9, A–D). Moreover, transient overexpression of the β-ARK1 (495–689) minigene, to sequester Gβγ subunits (Koch et al., 1994), or the Gβ1γ2 subunit did not affect U46619-mediated ERK activation in HEK.TPα10 or HEK.TPβ3 cells (Fig. 9, A–D). Pretreatment of quiescent HEK.TPα10 and HEK.TPβ3 cells with pertussis toxin (PTx), at a concentration established to inhibit Gαi signaling (Lawler et al., 2001; data not shown), did not affect U46619-induced ERK1/2 activation compared with cells treated with U46619 alone (Fig. 9, A–D). Additionally, HEK.TPα10 and HEK.TPβ3 cells transiently transfected with either the PI3KγDN or the β-ARK1DN (495–689) minigene or the Gβ1γ2 subunit did not elicit ERK activation in the absence (−) of U46619 (Fig. 9, A–D). In all cases, equal protein loading and equal ERK1/2 expression was confirmed using an anti-ERK antibody (Fig. 9, A and B, bottom). For each independent experiment, transient overexpression of Gβγ, β-ARK and PI3KγDN was confirmed by Western blot analyses (data not shown).

**Role of Class IA PI3K in TPα- and TPβ-Mediated ERK Activation.** Because the Gβγ-regulated class 1α subfamily of

![Fig. 12. Time-dependent effect of U46619 on PKB activation in HEK.TPα10 and HEK.TPβ3 cells. HEK.TPα10 (A) and HEK.TPβ3 (B) cells were stimulated for 0 to 60 min with 100 nM U46619. Additionally, in A (HEK.TPα10 cells) and B (HEK.TPβ3 cells), cells were preincubated with wortmannin (wort; 400 nM; 30 min) before stimulation of cells with U46619 (100 nM; 5 min). A and B, blots were screened with anti-phosphorylation-specific PKB to detect the phosphorylated, active forms of PKB (ppPKB). Results are representative of three independent experiments. C, fold increases in PKB (ppPKB) phosphorylation in A (HEK.TPα10 cells) and B (HEK.TPβ3 cells), respectively, are presented as mean fold increases of basal PKB phosphorylation ± S.E.M. (n = 3), where the levels of basal PKB phosphorylation in vehicle-treated cells are assigned a value of 1.0. ∗, p ≤ 0.05 and ∗∗, p ≤ 0.01 indicate that the levels of U46619-mediated PKB activation (ppPKB) were significantly greater compared with basal levels.
PI3K does not seem to be involved in TP-mediated ERK activation, the role of PI3K class 1x was investigated. Coexpression of a dominant negative form of the class 1a adaptor subunit (p85\textsuperscript{DN}; Sutor et al., 1999) resulted in a marginal decrease in U46619-mediated ERK activation in both HEK.TP\textalpha 10 and HEK.TP\beta3 cells (Fig. 10, A–C). In addition, overexpression of the wild-type p85 in both HEK.TP\textalpha 10 and HEK.TP\beta3 cells augmented U46619-mediated ERK activation (data not shown).

Thereafter, to investigate whether the p85 subunit may directly associate with the TP isoforms, coimmunoprecipitation of TP\alpha and TP\beta with p85 was investigated using TP isoform-specific antisera directed to residues within the unique C-tails of TP\alpha and TP\beta (Miggin and Kinsella, 2001). Association of the wild-type p85 (data not shown) and p85\textsuperscript{DN} with both TP\alpha and TP\beta was confirmed by the coimmunoprecipitation of the HA-tagged p85 with the anti-TP antisera (Fig. 11, A and C, lane 2). However, coimmunoprecipitation of the HA-tagged p85 with the anti-TP antisera confirmed the specificity of the coimmunoprecipitation of both TP\alpha or TP\beta, as detected using their respective isoform specific antisera (data not shown).

To demonstrate the specificity of the TP\alpha and TP\beta antisera, HA-tagged TPs were immunoprecipitated with anti-TP\alpha antisera, anti-TP\beta antisera, and, as a control, with the anti-HA 101r antibody directed to the HA-epitope tag. Immunoprecipitations of HA-tagged TP\alpha and TP\beta were confirmed by back blotting with the anti-HA 3F10-POD-conjugated antibody (Fig. 11, B and D, lane 5). Although the anti-TP\alpha antisera resulted in the immunoprecipitation of a broad protein band corresponding to the glycosylated and nonglycosylated forms of TP\alpha, anti-TP\beta antisera did not immunoprecipitate TP\alpha (Fig. 11B; compare lane 2 to 3, respectively). Similarly, although the anti-TP\beta antisera resulted in the immunoprecipitation of a broad protein band corresponding to the glycosylated and nonglycosylated forms of TP\beta, the anti-TP\alpha antisera did not immunoprecipitate TP\beta (Fig. 11D; compare lane 3 to 2, respectively). Taken together, these data confirm the specificity of the anti-TP antisera and suggest that PI3K class 1x can interact with both TP\alpha and TP\beta to mediate ERK activation.

As an extension of these studies, to establish whether TP\alpha and TP\beta may, in turn, stimulate PKB/Akt activation in response to PI3K activation, the effect of U46619 on PKB activation was examined. Exposure of HEK.TP\textalpha 10 and HEK.TP\beta3 cells to U46619-induced time-dependent activations of PKB with maximal phosphorylation observed at 5 min (Fig. 12, A and C) and at 10 to 20 min (Fig. 12, B and C), respectively. In addition, U46619-induced activation of PKB in HEK.TP\textalpha 10 and HEK.TP\beta3 cells was attenuated when cells were preincubated with the PI3K inhibitors wortmannin (Fig. 12, A and B) and LY294002 (data not shown).

Fig. 13. Proposed model for TP-mediated mitogen-activated protein kinase activation. A, ligand activation of the TX\textalpha  receptor TP\alpha and TP\beta isoforms leads to Gq-mediated activation of PLC, leading to increases in intracellular concentrations of IP\textbeta and DAG, and concomitant activation of Ca\textsuperscript{2+}–sensitive, DAG-regulated PKC. A key target of PKC is its positive regulation of c-Raf and, to a lesser extent, A-Raf leading to mitogen-activated protein kinase kinase 1 and 2 phosphorylation and activation of ERK1 and -2. B, in addition, ligand activation of TP\alpha and TP\beta may lead to Gs-dependent activation of adenylyl cyclase (AC) resulting in increases in intracellular concentrations of cAMP and, in turn, activation of H-89-sensitive PKA. PKA may, in turn, phosphorylate c-Raf to inhibit its actions but may also phosphorylate Rap1b, which, in turn, sequesters B-Raf leading to sustained activation of B-Raf, leading to ERK activation. C, ligand activation of TP\alpha and TP\beta may also signal through activation of wortmannin and LY294002-sensitive PI3K class 1x members, through unknown mechanisms but which may involve direct interaction of TPs and TP\beta with the p85 adaptor subunit, leading to Ras signaling and ERK activation (a) and leading to PKB activation (d). Ligand activation of TP\alpha and TP\beta may also signal toward transactivation of AG1478-sensitive epidermal growth factor receptor (EGF-R) through an unknown mechanisms involving PP2-sensitive src, leading to activation of ERK1 and -2. Although the TP antagonist SQ29,548 completely inhibits TP-mediated mitogenesis, PTx, G\textalpha subunits, and bARK1(495-689) minigene are without an effect on this pathway. Solid arrows and/or (+) indicate positive effects; broken lines and/or (−) indicate inhibitory effects. ? indicates whether PI3K acts as upstream or downstream of ras.
**Discussion**

TXA$_2$ has been implicated as a positive mediator of mitogenic/hypertrophic responses in vascular smooth muscle (Morinelli et al., 1994). Both the TXA$_2$ mimetics [1S-[1α,2α(Z), 3β(1E,3S*)]-7-[[[3-hydroxy-4-(4-iodophenox)I]-1-butenyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid and U46619 activate ERK1/2 in porcine, rat, and bovine aortic SMCs, respectively (Morinelli et al., 1994; Jones et al., 1995; Grosser et al., 1997), although the mechanisms of ERK activation remain poorly defined. Recently, Gao et al. (2001) reported that TP-mediated ERK activation in human endothelial ECV304 cells involved transactivation of the EGF receptor. They proposed that TP-mediated mitogenesis occurred in a PTx-sensitive, G$_{i/o}$-src-EGF receptor-dependent mechanism (Gao et al., 2001). In another study in cultured human uterine SMCs, we have established that TP-mediated ERK activation in response to U46619 and the F2 isoprostane 8-epi PGF$_2$α also involves transactivation of the EGF receptor; however, ERK activation was insensitive to PTx but occurred in a PKA-, PKC-dependent manner in a pathway that also required the participation of PI3K (Miggin and Kinsella, 2001). Thus, it seems that there are differences in TP-mediated ERK activation in human endothelial and in human uterine SMCs, readily distinguishable on the basis of their apparent sensitivity to PTx and the requirement for other signaling elements in human SMCs. Thus, in the current study, we first sought to fully define the mechanisms leading to TXA$_2$-mediated ERK activation. Second, we sought to establish whether the individual TP$_{α}$ and TP$_{β}$ receptor isoforms regulate ERK signaling and to define the key elements of those cascade(s).

In the present study, the previously described HEK 293 cell lines stably overexpressing either TP$_{α}$ (HEK.TP$_{α}$10) or TP$_{β}$ (HEK.TP$_{β}$3) (Walsh et al., 1998, 2000) were used to investigate the mechanisms of TP$_{α}$- and TP$_{β}$-mediated ERK signaling. U46619 elicited concentration- and time-dependent activations of ERK1/2 through both TP$_{α}$ and TP$_{β}$. Maximal U46619-induced ERK activations in HEK.TP$_{α}$10 and in HEK.TP$_{β}$3 cells were observed at 10 and 5 min, respectively. The relevance of differential rates of ERK activation through the TP isoforms remains to be determined, but may be related to a requirement for rapid ERK activation through TP$_{β}$ in certain tissues that express higher levels of this isoform, for example, fetal vascular smooth muscle (Miggin and Kinsella, 1998).

The role of Ras in GPCR-mediated activation of the ERK signaling cascade is well documented. Indeed, U46619-induced rapid Ras activation platelets (Shock et al., 1997). In this study, overexpression of Ha-Ras augmented U46619-induced ERK activation in both HEK.TP$_{α}$10 and HEK.TP$_{β}$3 cells, whereas overexpression of dominant negative Ha-Ras$^{N17}$ partially inhibited U46619-induced ERK activation in both cell lines. Concurring with findings in uterine SMCs and ECV304 cells (Gao et al., 2001; Miggin and Kinsella, 2001), we have also established that both TP$_{α}$- and TP$_{β}$-mediated ERK activation involves transactivation of the EGF receptor, and is src-dependent.

Previous studies have demonstrated that PKC activation was necessary for TXA$_2$-stimulated hypertrophic growth in vascular smooth muscle (Craven et al., 1996) and that U46619-induced activation of Ras in platelets was PKC-dependent (Shock et al., 1997). Furthermore, inhibition of GF 109203X-sensitive PKCs led to a decrease in U46619-induced ERK activation in cultured human SMCs (Miggin and Kinsella, 2001). Thus, the role of PKC in U46619-mediated activation of ERK through both TP$_{α}$ and TP$_{β}$ was investigated. In contrast to the strong PKC-dependent activation of ERK in HEK.TP$_{α}$10 cells, U46619-induced ERK activation in HEK.TP$_{β}$3 cells was only partially dependent on GF 109203X-sensitive PKCs. The precise mechanism of the differential PKC dependence of TXA$_2$-induced ERK activation through either TP$_{α}$ or TP$_{β}$ remains to be fully investigated; however, it is possible that the TP isoforms may represent differential targets of PKC isoform activation. In keeping with this, we have previously established that the TP$_{α}$ and TP$_{β}$ isoforms are subject to differential PGE$_2$-induced desensitization in a mechanism most probably involving direct PKC phosphorylation of the TP isoforms within their unique C-tail domains (Walsh and Kinsella, 2000).

Typically, increased levels of intracellular cAMP were thought to attenuate the ERK signaling cascade (Burgering et al., 1993). Recent evidence now suggests that, in certain situations, elevations of cAMP may actually activate the ERK signaling cascade (Frödin et al., 1994), through both the G$_{α}$ subunit and the G$_{βγ}$ subunits of the activated G$_{s}$ (Faure et al., 1994). Interestingly, correlating with findings in human uterine SMCs and in COS-7 cells (Faure et al., 1994; Miggin and Kinsella, 2001), we found that U46619-induced ERK activation was partially dependent on PKA, because H-89 significantly decreased ERK activation through both TP$_{α}$ and TP$_{β}$.

Taken together, our studies have shown that both PKC and PPA are involved in ERK activation through the TP isoforms. It is postulated that the positive effects of PKC may be mediated through any one of the PKC-sensitive Raf isozymes, such as c-Raf (Anton and Wengnolle, 1998). However, because c-Raf is inhibited by PPA, it is also postulated that other Raf isoforms may be involved in TXA$_2$-mediated ERK activation. To address this question, the direct role of A-Raf and c-Raf in TP-mediated ERK activation was examined. Both the PPA-insensitive A-Raf and the PPA-sensitive c-Raf were demonstrated to regulate U46619-induced ERK activation through TP$_{α}$ and TP$_{β}$. Additionally, overexpression of Rap1b$^{N17}$ significantly reduced ERK activation through TP$_{α}$ and TP$_{β}$, suggesting that the somewhat novel PPA-modulated Rap1/B-Raf mechanism of ERK regulation (Schmitt and Stork, 2000) may also be involved in TP-mediated ERK activation. The availability of dominant negative forms of B-Raf would add further clarification to this point.

GPCRs have been shown to regulate the class I family of PI3Ks (Vanhaesebroeck et al., 1997), although a mechanism has not yet been clearly defined. In the current study, using the PI3K inhibitors wortmannin and LY294002, we have shown that ERK activation via TP$_{α}$ and TP$_{β}$ is mediated through PI3K, correlating with findings in human uterine SMCs (Miggin and Kinsella, 2001). Additionally, the dominant negative PI3K$^{K832P}$ (Lopez-Ilasaca et al., 1998) did not affect U46619-mediated ERK activation in either cell type. Moreover, overexpression of G$βγ$-a or the $Gβγ$ antagonist $β$-ARK1(495-689) minigene (Koch et al., 1994) did not affect ERK activation through TP$_{α}$ and TP$_{β}$. Additionally, PTx did not affect ERK activation through TP$_{α}$ and TP$_{β}$. These data
suggest that PI3K class 1β is not involved in TP-mediated ERK activation.

In previous studies, Morinelli et al. (1997) demonstrated that exposure of A7r5 cells overexpressing TPα to I-BOP led to tyrosine phosphorylation of both the TPα itself and the p85 adaptor subunit of class IA PI3Ks. In view of these findings and our studies indicating a specific role for wortmannin- and LY294002-sensitive PI3Ks, distinct from the class 1β family, we sought to investigate a possible role of class IA PI3Ks in TPα- and TPβ-mediated ERK activation. Overexpression of a dominant negative form of PI3K class 1A adaptor subunit of class I A PI3Ks. In view of these findings and our studies indicating a specific role for wortmannin- and LY294002-sensitive PI3Ks, distinct from the class 1β family, we sought to investigate a possible role of class IA PI3Ks in TPα- and TPβ-mediated ERK activation.

Through these studies, we provide novel evidence suggesting that class IA, not class 1β, PI3K mediates ERK activation through TPα and TPβ. Several studies have indicated that PKB/Akt activation is a key event in the realization of the apoptotic effect of PI3K (Datta et al., 1999). Through follow-up studies, we established that TPα and TPβ induced activation of PKB/Akt through wortmannin- and LY294002-sensitive phosphorylation of PKB (S473). Thus, in keeping with its mitogenic actions, it seems that TXA2 and its mimetics may not only promote mitogenesis through activation through PKB/Akt and its downstream signaling. These findings contrast those of Gao et al. (2000) in that the regulation of the ERK signaling cascades through TPα and TPβ is multifaceted with multiple intermediates participating in this highly regulated signaling network, and studies presented herein have identified the key components involved in the regulation of TXA2-mediated ERK activation.

References


Address correspondence to: B. Therese Kinsella, Department of Biochemistry, Conway Institute of Biomolecular and Biomedical Research, Merville House, University College Dublin, Belfield, Dublin 4, Ireland. E-mail: therese.kinsella@ucd.ie