British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2012.01937.x
www.brjpharmacol.org

RESEARCH PAPER

Pharmacological profile of phosphatidylinositol 3-kinases and related phosphatidylinositols mediating endothelin$_A$ receptor-operated native TRPC channels in rabbit coronary artery myocytes

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Keywords
canonical transient receptor potential; endothelin; phosphatidylinositol 3-kinase; phosphatidylinositol; vascular smooth muscle

Received
13 December 2011
Revised
27 January 2012
Accepted
20 February 2012

BACKGROUND AND PURPOSE

Endothelin$_A$ (ET_A) receptor-operated canonical transient receptor potential (TRPC) channels mediate Ca^{2+} influx pathways, which are important in coronary artery function. Biochemical pathways linking ET_A receptor stimulation to TRPC channel opening are unknown. We investigated the involvement of phosphatidylinositol 3-kinases (PI3K) in ET_A receptor activation of native heteromeric TRPC1/C5/C6 and TRPC3/C7 channels in rabbit coronary artery vascular smooth muscle cells (VSMCs).

EXPERIMENTAL APPROACH

A pharmacological profile of PI3K was created by studying the effect of pan-PI3K, pan-Class I PI3K and Class I PI3K isoform-selective inhibitors on ET_A receptor-evoked single TRPC1/C5/C6 and TRPC3/C7 channel activities in cell-attached patches from rabbit freshly isolated coronary artery VSMCs. The action of phosphatidylinositol 3-phosphate- [PI(3)P], 4-phosphate- [PI(4)P] and 5-phosphate- [PI(5)P] containing molecules involved in PI3K-mediated reactions were studied in inside-out patches. Expression of PI3K family members in coronary artery tissue lysates were analysed using quantitative PCR.

KEY RESULTS

ET_A receptor-operated TRPC1/C5/C6 and TRPC3/C7 channel activities were inhibited by wortmannin. However, ZSTK474 and AS252424 reduced ET_A receptor-evoked TRPC1/C5/C6 channel activity but potentiated TRPC3/C7 channel activity. All the PI(3)P-, PI(4)P- and PI(5)P-containing molecules tested induced TRPC1/C5/C6 channel activation, whereas only PI(3)P stimulated TRPC3/C7 channels.

CONCLUSIONS AND IMPLICATIONS

ET_A receptor-operated native TRPC1/C5/C6 and TRPC3/C7 channel activities are likely to be mediated by Class I PI3Kγ and Class II/III PI3K isoforms, respectively. ET_A receptor-evoked and constitutively active PI3Kγ-mediated pathways inhibit TRPC3/C7 channel activation. PI3K-mediated pathways are novel regulators of native TRPC channels in VSMCs, and these signalling cascades are potential pharmacological targets for coronary artery disease.

Abbreviations

AS252424, 5-[[5-(4-fluro-2-hydroxyphenyl)-2-furanyl]methylene]-2,4-thiazolidinedione; BQ788, N-[(cis-2,6-dimethyl-1-piperidinyl)carbonyl]-4-methyl-L-leucyl-1-(methoxycarbonyl)-D-tryptophyl-D-norleucine; IC87114, 2-[6-amino-9H-purin-9-yl)methyl]-5-methyl-3-(2-methylphenyl)-4(3H)-quinazolinone; PIK75, N-((1E)-(6-bromoimidazo[1,2-a]pyridin-3-yl)methylene)-N′-methyl-N″-(2-methyl-5-nitrobenzene)sulfonohyrazide; PI3K, phosphatidylinositol 3-kinase; PI, phosphatidylinositol; PI(3)P, phosphatidylinositol 3-phosphate; PI(4)P, phosphatidylinositol 4-phosphate; PI(5)P, phosphatidylinositol 5-phosphate; PI(3,4)P2, phosphatidylinositol 3,4-bisphosphate; PI(3,5)P2, phosphatidylinositol 3,5-bisphosphate; PI(4,5)P2, phosphatidylinositol 4,5-bisphosphate; PI(3,4,5)P3, phosphatidylinositol 3,4,5-trisphosphate; TGX221, (+-)-7-methyl-2-(morpholin-4-yl)-9-(1-phenylaminoethyl)-pyrido[1,2-a]-pyrimidin-4-one; TRPC, canonical transient receptor potential; U73122, 1-[6-[[17β0-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1-H-pyrrole-2,5-dione; VSMC, vascular smooth muscle cells; ZSTK474, 2-(2-difluoromethylbenzimidazol-1-yl)-4,6-dimorpholino-1,3,5-triazine

Introduction

Endothelin-1 (ET-1) is one of the most potent endogenous vasoconstrictors identified so far, and also it is associated with vascular remodelling and angiogenesis (Nguyen *et al.*, 2010; Thorin and Webb, 2010). ET-1 acts at two GPCR subtypes, ET_A and ET_B, which are expressed on the plasmalemma of vascular smooth muscle cells (VSMCs). ET_A receptors are thought to be predominantly involved in ET-1-evoked contraction, proliferation and pro-inflammatory effects on VSMCs. Blockade of ET_A receptors increases blood flow and perfusion and reduces fatty streaks and endothelial dysfunction in coronary arteries, and therefore pharmacological agents that reduce ET_A receptor-mediated pathways are candidates for prevention and treatment of coronary artery disease (Nguyen *et al.*, 2010; Thorin and Webb, 2010). The significance of the present work is in further understanding of these ET_A receptor-mediated signalling pathways, which enhances our knowledge of potential therapeutic targets for coronary artery disease.

It is evident that ET_A receptor-mediated vascular effects are correlated with a rise in $[Ca^{2+}]_i$ of VSMCs, which involves a significant contribution by Ca^{2+} influx (Tykocki and Watts, 2010). There is no clear picture on the mechanisms governing ET_A receptor-mediated Ca^{2+} influx in VSMCs, although activation of canonical transient receptor potential (TRPC) channel proteins has been proposed (Bergdahl *et al.*, 2003; Ko *et al.*, 2004; Tykocki and Watts, 2010).

The family of TRPC1-C7 channel subunits form Ca^{2+}-permeable non-selective cation channels, which are important regulators of Ca^{2+} influx in VSMCs (Abramowitz and Birnbaumer, 2009; Dietrich *et al.*, 2010). It is apparent that two TRPC channel subgroups are expressed in VSMCs; TRPC1 channels (channels containing TRPC1 subunits) and channels composed of TRPC3/C6/C7 subunits (Albert *et al.*, 2009; Large *et al.*, 2009; Albert, 2011), with these two TRPC channel subgroups having distinctive activation mechanisms. TRPC1 channels are activated by receptor- and intracellular Ca^{2+} store-dependent pathways, which involve PKC-dependent phosphorylation of TRPC1 proteins, and obligatory roles for the phosphatidylinositols (PIs), PI 4,5-bisphosphate [$PI(4,5)P_2$] and PI 3,4,5-trisphosphate [$PI(3,4,5)P_3$] (Albert and Large, 2002; Saleh *et al.*, 2006; 2008; 2009a,b; Albert *et al.*, 2009; Large *et al.*, 2009; Albert, 2011). TRPC3/C6/C7 channels are activated by a GPCR or constitutively active pathways, which involve phospholipase-mediated generation of DAG that induces channel opening via PKC-independent mechanisms (Helliwell and Large, 1997; Inoue *et al.*, 2001; Albert *et al.*, 2003; 2005; 2006; Peppiatt-Wildman *et al.*, 2007; Large *et al.*, 2009; Albert, 2011). DAG is proposed to activate TRPC3/C6/C7 channels by removal of an inhibitory action of $PI(4,5)P_2$, which is bound to channel proteins at rest (Albert *et al.*, 2008; Large *et al.*, 2009; Ju *et al.*, 2010; Albert, 2011). Moreover, TRPC3/C6/C7 channels are inhibited by PKC stimulation, which is produced by receptor-mediated generation of DAG (Albert and Large, 2004; Saleh *et al.*, 2006) and TRPC1 channel-mediated Ca^{2+} influx (Shi *et al.*, 2010). As such, TRPC3/C6/C7 channels are only activated by lower concentrations of receptor agonists, whereas TRPC1 channels are induced by a wide range of receptor stimuli concentrations (Large *et al.*, 2009; Shi *et al.*, 2010; Albert, 2011).

In coronary artery VSMCs, differences between biophysical properties, blocking actions of anti-TRPC antibodies, pharmacology, and co-immunoprecipitation indicate that stimulation of ET_A receptors by low concentrations of ET-1 (1–10 nM) activate two distinct native TRPC channels composed of TRPC1/C5/C6 with a unitary conductance of about 3 pS (Saleh *et al.*, 2008; 2009b; Albert *et al.*, 2009) and TRPC3/C7 channels with four sub-conductance states between 15 and 70 pS (Peppiatt-Wildman *et al.*, 2007; Albert *et al.*, 2009). In contrast, ET_A receptor activation with higher concentrations of ET-1 (100 nM) only activates TRPC1/C5/C6 channels (Saleh *et al.*, 2009b). ET_A receptor-induced TRPC1/C5/C6 channel activation is governed by phosphatidylinositol 3-kinase (PI3K)-mediated generation of $PI(3,4,5)P_3$ whereas stimulation of these channels by ET_B receptor activation requires $PI(4,5)P_2$ (Saleh *et al.*, 2009b). It is unclear how ET_A receptors activate TRPC3/C7 channels (Peppiatt-Wildman *et al.*, 2007).

The PI3K family consists of three classes of isoforms (Classes I, II and III), which phosphorylate PI, PI 4-phosphate [PI(4)P], PI 5-phosphate [PI(5)P] and $PI(4,5)P_2$ at their D3 hydroxyl groups to generate, respectively, PI(3)P, $PI(3,4)P_2$, $PI(3,5)P_2$ and $PI(3,4,5)P_3$ (Hawkins *et al.*, 2006; Vanaesebroeck *et al.*, 2010). PI3K-dependent pathways are considered to be important in vascular physiology and pathology (Oudit *et al.*, 2004; Morello *et al.*, 2009) and are proposed to mediate ET-1-evoked Ca^{2+} influx pathways (Kawanabe *et al.*, 2004; Miwa *et al.*, 2005; Ivey *et al.*, 2008). The present work investigates the role of PI3K isoforms using selective pharmacological inhibitors, and associated PI substrates and products, in ET_A receptor stimulation of native TRPC1/C5/C6 and TRPC3/C7 channels in freshly isolated coronary artery VSMCs.

Evidence was obtained showing that PI3K-mediated signalling pathways are pivotal in ET_A receptor-evoked TRPC1/C5/C6 and TRPC3/C7 channel activation. The Class I PI3Kγ isoform are likely to couple ET_A receptors to TRPC1/C5/C6 channel opening, whereas Class II and/or Class III PI3K isoforms are likely to mediate ET_A receptor stimulation of TRPC3/C7 channels. Moreover, we showed for the first time that PI(3)P-, PI(4)P- and PI(5)P-containing molecules are novel gating ligands of native TRPC1 and TRPC3/C6/C7 channel subtypes.

Methods

All terms used for receptors, ion channels and enzymes are in accordance with the nomenclature of Alexander *et al.* (2011).

Cell isolation

All animal care and procedures were approved by St. George's, University of London animal welfare committee. Animals were allowed free access to food and water and kept on a 12–12 h light/dark cycle. New Zealand White rabbits (2–3 kg) were killed using i.v. sodium pentobarbitone (120 $mg \cdot kg^{-1}$) in accordance with the UK Animals Scientific Procedures Act, 1986. Right and left anterior descending coronary arteries were dissected free from fat and connective tissue, and the endothelium was removed with a cotton bud. Physiological salt solution contained (mM): NaCl (126), KCl (6), glucose (10), HEPES (11), $MgCl_2$ (1.2), $CaCl_2$ (1.5) and pH to 7.2 with 10 M NaOH. Enzymatic isolation of VSMCs was carried out using methods previously described (Peppiatt-Wildman *et al.*, 2007; Saleh *et al.*, 2008; 2009b).

Electrophysiology

Single channel cation currents using cell-attached and inside-out patches were made with an AXOpatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA) at room temperature (20–23°C) as previously described (Peppiatt-Wildman *et al.*, 2007; Saleh *et al.*, 2008; 2009b). Single channel *I/V* relationships were obtained by manually altering the holding potential of –70 mV between –120 mV and +120 mV. Single TRPC1 and TRPC3/C6/C7 channel subgroups were analysed according to Shi *et al.* (2010). Single channel currents records were filtered at 0.1–0.5 kHz (-3 dB, low pass 8-pole Bessel filter, Frequency Devices, model LP02, Scensys Ltd., Aylesbury, UK) and acquired at 1–5 kHz [Digidata 1322A (Axon Instruments Inc., Union City, CA, USA) and pCLAMP v9.0 software (Molecular Devices, LLC, Sunnyvale, CA, USA)]. Single channel current amplitudes were calculated from idealized traces of ≥30 s in duration using the 50% threshold method and analysed using pCLAMP v9.0 software. Events lasting between <1.32 and <6.64 ms (×2 rise time of filtering used, see above) were excluded during creation of idealized traces to maximize the number of channel openings reaching their full current amplitude. Open probability (NP_o) was used as a measure of channel activity and was calculated automatically by pCLAMP v9.0 software. Single channel current amplitude histograms were plotted from the event data of the idealized traces, using bin widths appropriate for the unitary amplitudes. Amplitude histograms were fitted using Gaussian curves, with peak values corresponding to channel open levels. Mean channel amplitudes at different membrane potential were plotted, and current–voltage relationships curves were fitted by linear regression with the gradient-determining conductances values. Figure preparation was carried out using MicroCal Origin software 6.0 (Originlab Corporation, Northampton, MA, USA) where inward single cation channel openings are shown as downward deflections.

Dot-blots

Dot-blots were performed using techniques described previously (Saleh *et al.*, 2009b). Rabbit coronary arteries were mixed with radioimmunoprecipitation assay buffer (Sigma-Aldrich Company Ltd., Gillingham, UK) containing protease inhibitor (Roche Diagnostics Ltd., West Sussex, UK) and sonicated at 4°C for 3 h. Tissue lysates were centrifuged at 6200× *g* at 4°C for 10 min, and 10 μL of tissue lysate supernatant was added to a PVDF membrane. Membranes were dried and placed into 5% blocking buffer and left on a rocker at room temperature for 1 h. Membranes were incubated with appropriate primary antibodies overnight at 4°C. Following removal of primary antibodies, PVDF membranes were washed for 1 h with PBS and incubated for 1 h with horseradish peroxidase-conjugated secondary antibody diluted 1:5000. After three washes in PBS containing 0.1% Tween, PVDT membranes were treated with ECL chemiluminescence reagents (Pierce Biotechnology Inc., Rockford, IL, USA) for 1 min and exposed to photographic films.

RNA extraction and cDNA synthesis

Total RNA was extracted from rabbit fresh enzymatically-dispersed coronary arteries using the RNAqueous Small Phenol-Free Total RNA Isolation Kit (Life Technologies, Paisley, UK) according to the manufacturer's instruction. RNA quality was measured using Nanodrop ND1000 spectrophotometer (Thermo Scientific, Loughborough, UK) and RNA reverse-transcribed to cDNA using High Capacity RNA-to-cDNA Kit (Life Technologies). Negative controls were performed in the absence of reverse transcriptase (-RT) to check for genomic contamination.

End-point PCR

End-point PCR was performed using GoTag® DNA Polymerase (Promega, Southampton, UK) and under the following conditions: initial denaturation at 94°C for 2 min; PCR cycles: 94°C for 30 s, 55°C for 30 s and 72°C for 30 s; repeated for 40 cycles; final extension for 10 min. PCR product was checked on 1% agarose gel electrophoresis. If there were no visible bands, 5 μL of the PCR product was used as a template to perform a second round PCR with 20 cycles of 94°C for 30 s and 55°C for 30 s and 72°C for 30 s using the same pair of primers and same initial denaturation and final extension times. Negative control with no template was performed to check for contamination. PCR product amplified was confirmed by sequence analysis (Beckman Coulter Genomics, High Wycombe, UK) and checked for human analogues using the National Center for Biotechnology Information Basic Local Alignment Search Tool programme.

qPCR

qPCR was performed with The QuantiFast SYBR Green PCR Kit (Qiagen, Crawley, UK) and using a CFX96™ Real-Time

PCR Detection System (Bio-Rad, Hemal Hempstead, UK). Duplicate reactions were carried out in 20 μL volumes including 1 μL of cDNA, 10 μL of SYBR Green Master Mix (Qiagen), 2 μL of sense primer and 2 μL of anti-sense primer. The cycling conditions were as follows: initial denaturation at 95°C for 5 min followed by 50 cycles of 95°C for 10 s, combined annealing and extension at 65°C for 30 s. Melt curve analysis was performed to ensure that each primer set amplified a single product that shows a single peak in the melt curve. No template controls were applied to check for contamination. Cycle threshold (Ct) values were calculated using CFX65™ Manager Software (Bio-Rad). Standard curves were plotted using fourfold serial dilution of cDNA to determine the efficiency of amplification and R^2 values. The expression of RNA relative to β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was calculated using equation $2^{-\Delta Ct}$.

Primers

The housekeeping genes β-actin and GAPDH were used as references. Primers (see Tables S1 and S2 in supplementary data) were designed using Primer 3 software. All primers designed flank a region that contains at least one intron to avoid genomic DNA contamination. All primers were synthesized by Invitrogen (Life Technologies).

Solutions and drugs

In cell-attached patch experiments, the membrane potential was set to approximately 0 mV by perfusing cells in a KCl external solution containing (mM): KCl (126), $CaCl_2$ (1.5), HEPES (10) and glucose (11), and pH to 7.2 with 10 M KOH. Nicardipine (5 μM) was also included to prevent smooth muscle cell contraction by blocking Ca^{2+} entry through voltage-dependent Ca^{2+} channels. The bathing solution used in inside-out experiments (intracellular solution) contained (mM): CsCl (18), caesium aspartate (108), $MgCl_2$ (1.2), HEPES (10), glucose (11), BAPTA (1), $CaCl_2$ (0.48, free internal Ca^{2+} concentration approximately 100 nM as calculated using EQCAL software; Biosoft, Cambridge, UK), Na_2ATP (1), NaGTP (0.2); pH 7.2 with Tris. The patch pipette solution used for both cell-attached and inside-out patch recording (extracellular solution) was K^+ free and contained (mM): NaCl (126), $CaCl_2$ (1.5), HEPES (10), glucose (11), TEA (10), 4-AP (5), iberiotoxin (0.0002), 4,4′-Diisothiocyano-2,2′-stilbenedisulfonic acid (DIDS) (0.1), niflumic acid (0.1) and nicardipine (0.005); pH to 7.2 with NaOH. The use of blockers of voltage dependent calcium channels (nicardipine), K^+ currents (TEA, 4-AP, iberiotoxin), swell-activated Cl^- currents (DIDS) and Ca^{2+}-activated Cl^- conductances (niflumic acid) allowed non-selective cation currents to be recorded in isolation.

All PI molecules were ordered in a water soluble diC8 form from Echelon Biosciences (Salt Lake City, UT, USA). Unless otherwise stated, all other drugs were purchased from Calbiochem (Nottingham, UK), Sigma Aldrich or Tocris (Bristol, UK), and agents were dissolved in distilled H_2O or dimethyl sulfoxide (DMSO) (0.1%). DMSO alone had no effect on channel activity.

Statistical analysis

The values are presented as the mean of *n* cells ± SEM. Statistical analysis was carried out using Student's paired (comparing effects of agents on the same cell) or unpaired (comparing effects of agents between cells) *t*-test with the level of significance set at $P < 0.05$.

Results

Effect of wortmannin on two distinct ET_A receptor-operated native TRPC channels in coronary artery VSMCs

In 46/80 cell-attached patches, stimulation of ET_A receptors by bath application of 10 nM ET-1 (in the presence of the ET_B receptor antagonist BQ788, 100 nM) evoked two distinct native cation channel currents in freshly isolated coronary artery VSMCs (Figure 1A). One channel had a unitary conductance of about 3 pS, whereas the second had four subconductance levels between 15 and 70 pS (data not shown). Both 3 pS and 15–70 pS channels had reversal potentials (E_r) of about 0 mV. We have previously described the properties of these two channels and shown that the 3 pS channel is composed of heteromeric TRPC1/C5/C6 subunits (Saleh *et al.*, 2008; 2009b), whereas the 15–70 pS channel consists of a heteromeric TRPC3/C7 channel structure (Peppiatt-Wildman *et al.*, 2007). In the remaining 34/80 cell-attached patches, stimulation of ET_A receptors with 10 nM ET-1 (also in the presence of 100 nM of BQ788) only evoked 3 pS channel currents (see Figure 2B). Stimulation of ET_A receptors either activated TRPC1/C5/C6 and TRPC3/C7 channels or only TRPC1/C5/C6 channels is likely to be due to differences in expression levels of these two channel subtypes; not all patches contain TRPC3/C7 channels. Also, ET_A receptor-mediated pathways may inhibit TRPC3/C7 channel activity as previously described for angiotensin II in mesenteric artery VSMCs (Saleh *et al.*, 2006; Shi *et al.*, 2010).

It is generally considered that stimulation of TRPC channels in VSMCs is mediated by activation of GPCRs linked to phospholipase-dependent signalling pathways and generation of DAG, which activates channels via PKC-independent and -dependent mechanisms (see Introduction). However, PI3K-mediated processes have been proposed to mediate ET-1-evoked Ca^{2+} influx (Kawanabe *et al.*, 2004; Miwa *et al.*, 2005; Ivey *et al.*, 2008) and ET_A receptor-evoked TRPC1/C5/C6 channels in VSMCs (Saleh *et al.*, 2009b). We compared the effect of PLC and PI3K inhibitors on ET_A receptor-evoked TRPC1/C5/C6 and TRPC3/7 channel activities in cell-attached patches. ET_A receptor-evoked TRPC1/C5/C5 and TRPC3/C7 channel activities were unaffected by co-application of the PLC inhibitor U73122 (2 μM) (Yule and Williams, 1992), whereas the pan-PI3K inhibitor wortmannin (50 nM) (Powis *et al.*, 1994) reduced both TRPC1/C5/C6 and TRPC3/C7 channel activities by over 95% (Figure 1B and C).

Differential actions of ZSTK474, a pan-Class I PI3K inhibitor, on ET_A receptor-operated native TRPC1/C5/C6 and TRPC3/C7 channels

We studied the role of Class I PI3K isoforms in ET_A receptor stimulation of TRPC1/C5/C6 and TRPC3/C7 channels using the pan-Class I PI3K inhibitor ZSTK474 (Kong and Yamori,

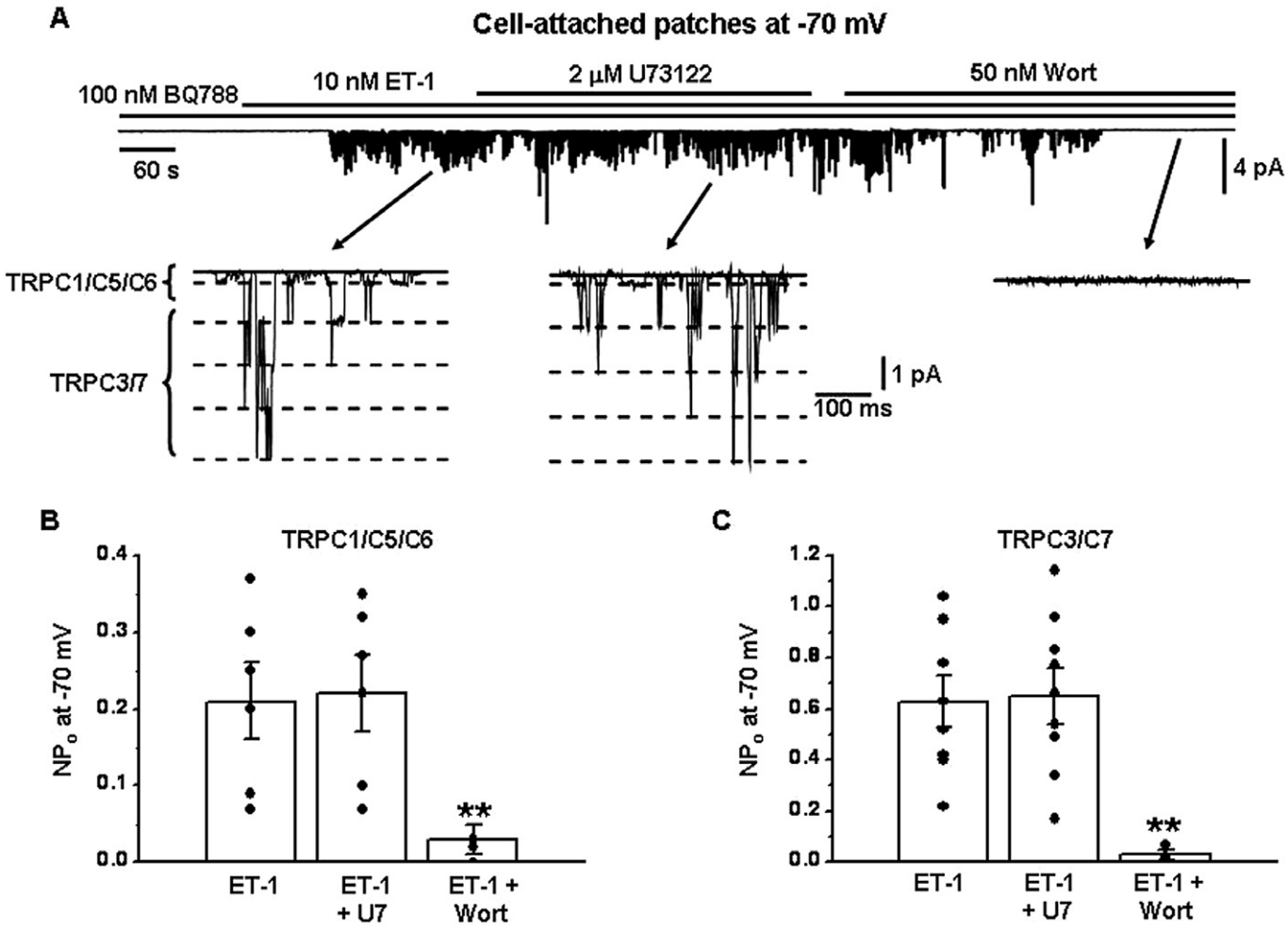

Figure 1

Effect of wortmannin on ET_A receptor-evoked native TRPC1/C5/C6 and TRPC3/C7 channel activities in rabbit coronary artery VSMCs. (A) Bath application of 10 nM ET-1, in the presence of the ET_B receptor antagonist 100 nM BQ788, activated TRPC1/C5/C6 and TRPC3/C7 channels in cell-attached patches held at –70 mV, which were unaffected by U73122 but blocked by wortmannin (Wort). Bold and dashed lines in insets show, respectively, closed and open levels. (B) and (C) Mean data showing that wortmannin significantly inhibited both ET_A receptor-mediated TRPC1/C5/C6 and TRPC3/C7 channel activities. $n \geq 6$, $^{**}P < 0.01$.

2007). Co-application of 100 nM ZSTK474 had differential actions on ET_A receptor-evoked TRPC1/C5/C6 and TRPC3/C7 channel activities; TRPC1/C5/C6 channel activity was inhibited by over 95% (Figure 2), whereas TRPC3/C7 channel activation was potentiated by over twofold (Figure 2). ZSTK474-induced augmentation of ET_A receptor-evoked TRPC3/C7 was blocked by 50 nM wortmannin by 96 ± 2% ($n = 4$, $P < 0.01$, Figure 2A and C). ZSTK474-mediated inhibition of ET_A receptor-evoked TRPC1/C5/C6 was similar in patches containing both TRPC1/C5/C6 and TRPC3/C7 channel activities (Figure 2A) or in patches containing only TRPC1/C5/C6 channel activity (Figure 2B).

Pharmacological profile of isoform-selective PI3K inhibitors on ET_A receptor-induced native TRPC1/C5/C6 and TRPC3/C7 channels

The family of Class I PI3K consists of four isoforms of catalytic subunits (PI3Kα, β, δ and γ) (Hawkins *et al.*, 2006; Vanaesebroeck *et al.*, 2010). We investigated the effect of Class I PI3K isoforms in mediating ET_A receptor-evoked TRPC1/C5/C6 and TRPC3/C7 channel activities using established isoform-selective inhibitors, which we used at ≤5 times the concentration of IC_{50} values for other PI3K isoforms (Knight *et al.*, 2006; Chaussade *et al.*, 2007; Hayakawa *et al.*, 2007). To clearly distinguish the effect of PI3K inhibitors on TRPC1/C5/C7 and TRPC3/C7 channel opening, we studied the effects of these inhibitors on cell-attached patches in which ET_A receptor stimulation evoked either TRPC1/C5/C6 and TRPC3/C7 channel activities (Figure 3) or only TRPC1/C5/C6 channel activity (Figure 4). Figures 3 and 4 show that patches containing ET_A receptor-evoked TRPC1/C5/C6 and TRPC3/C7 channel activities or only TRPC1/C5/C6 channel activity were unaffected by TGX211 (100 nM), PIK75 (100 nM) and IC87114 (600 nM), which are selective inhibitors of PI3Kα, β and δ isoforms respectively. In contrast, AS252424, a PI3Kγ isoform inhibitor, had differential actions on TRPC1/C5/C6 and TRPC3/C7 channels; Figure 3(iv) and B show that

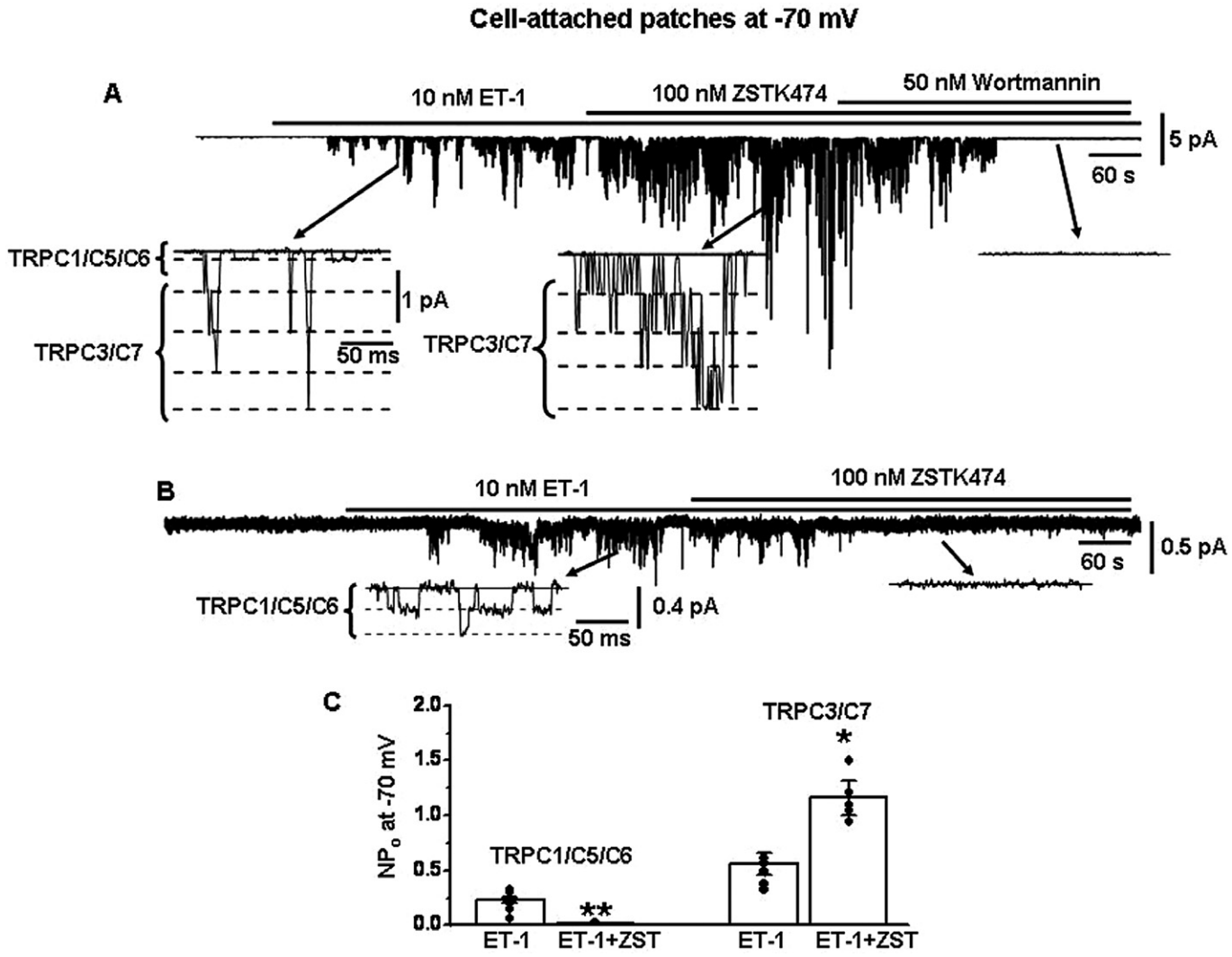

Figure 2

Differential effects of the selective pan-Class I PI3K inhibitor ZSTK474 on ET_A receptor-evoked TRPC1/C5/C6 and TRPC3/C7 channel activities. (A) ZSTK474 inhibited TRPC1/C5/C6 and potentiated TRPC3/C7 channel activities evoked by ET_A receptor stimulation in cell-attached patches held at –70 mV. Moreover, ET_A receptor-mediated TRPC3/C7 channel activity in the presence of ZSTK474 was inhibited by wortmannin. Note that the middle inset shows that TRPC1/C5/C6 channel openings are absent in the presence of ZSTK474. (B) Illustration of a cell-attached patch in which ET_A receptor stimulation only induced TRPC1/C5/C6 channel activity, which was inhibited by ZSTK474. (C) Mean data showing that ZSTK474 significantly inhibited TRPC1/C5/C6 but potentiated TRPC3/C7 channel activities evoked by ET_A receptor stimulation. $n \geq 5$, $^*P < 0.05$, $^{**}P < 0.01$.

300 nM AS252424 potentiated TRPC3/C7 channel activity by over twofold, whereas Figure 4(iv) and B illustrate that 300 nM AS252424 reduced ET_A receptor-evoked TRPC1/C5/C6 channel activity by over 95%. It should be noted that ZSTK474- and AS252424-mediated inhibition of ET_A receptor-evoked TRPC1/C5/C6 was similar in patches containing both TRPC1/C5/C6 and TRPC3/C7 channel activities [Figures 2A and 3A(iv)] to that in patches containing only TRPC1/C5/C6 channel activity [Figures 2B and 4A(iv)].

Effect of ZSTK474 and AS252424 on quiescent cell-attached patches

It is possible that the potentiating actions of ZSTK474 and AS252424 on ET_A receptor-evoked TRPC3/C7 channel activity reflect channel inhibition by constitutive Class I PI3K isoform activity. We studied this hypothesis by investigating the effect of ZSTK474 and AS252424 on unstimulated cell-attached patches. Bath application of 100 nM ZSTK474 (Figure 5A) and 300 nM AS252424 (Figure 5B) activated channel currents with a mean NP_O of, respectively, 0.86 ± 0.11 ($n = 9$) and 0.89 ± 0.09 ($n = 9$) in quiescent cell-attached patches held at –70 mV; both had four amplitude levels between –1 pA and –4.5 pA [Figure 5A(ii) (iii) and B(ii) (iii)] that related to sub-conductance states between 15 and 70 pS, which all had E_r of about 0 mV, similar to ET_A receptor-evoked TRPC3/C7 channel currents (Peppiatt-Wildman *et al.*, 2007). In addition, ZSTK474- and AS252424-evoked TRPC3/C7 channel activities were both inhibited by co-application of 50 nM

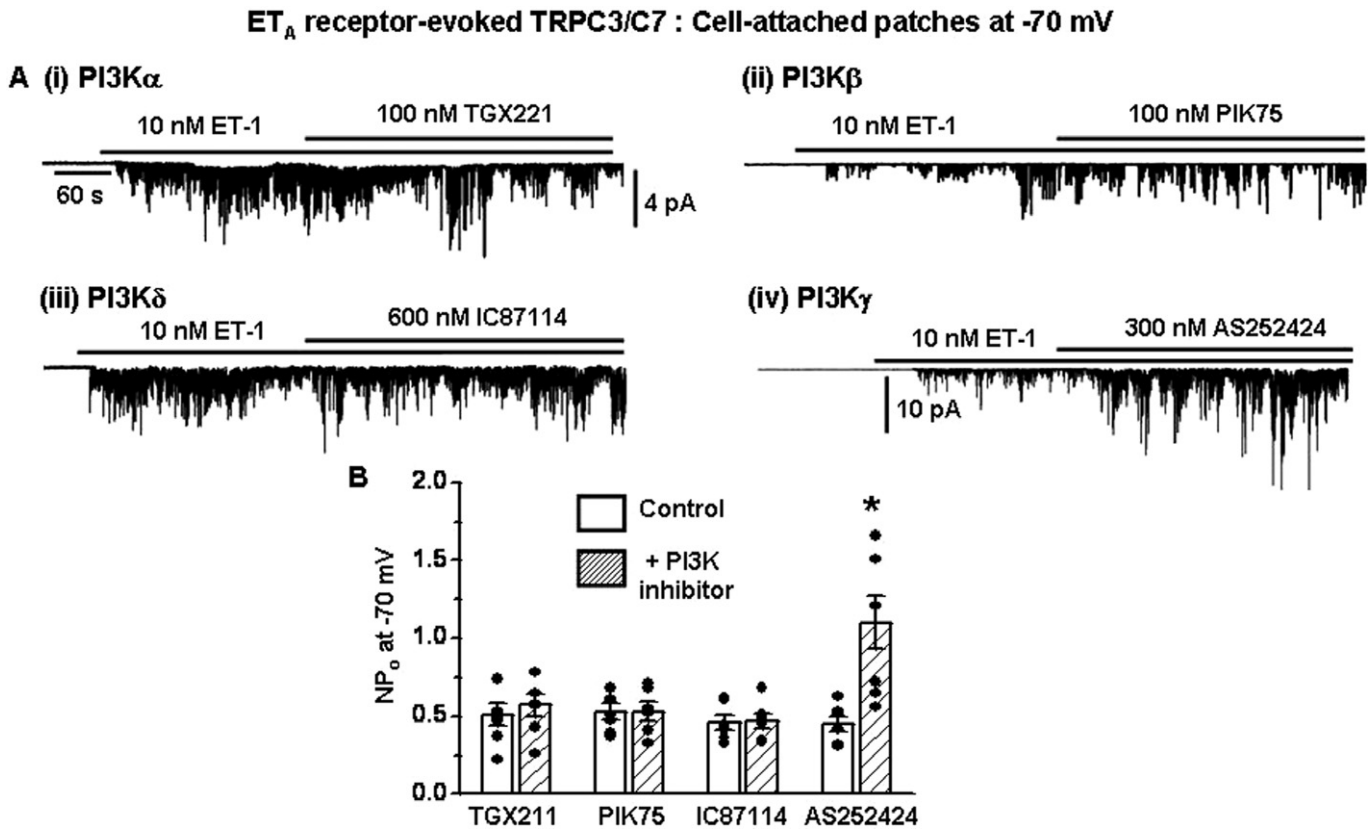

Figure 3

Class I PI3Kγ isoform inhibitor potentiates ET$_A$ receptor-evoked TRPC3/C7 channel activity. (A) Effect of selective Class I PI3K isoform inhibitors (i) TGX221, (ii) PIK75, (iii) IC87114, and (iv) AS252424 on cell-attached patches held at −70 mV, which contained both ET$_A$ receptor-induced TRPC1/C5/C6 and TRPC3/C7 channel activities. (B) Mean data showing the effect of Class I PI3K isoform inhibitors on TRPC3/7 channel activity. $n \geq 5$, $^*P < 0.05$. Note that only AS252424 potentiated ET$_A$ receptor-evoked TRPC3/C7 channel activity.

wortmannin by 97 ± 2% [$n = 9$, $P < 0.01$, Figure 5A(i)] and 95 ± 3% [$n = 5$, $P < 0.05$, Figure 5B(i)], respectively.

Effect of PI3K-generated PIs on quiescent inside-out patches

The differential effects of PI3K-mediated pathways on TRPC1/C5/C6 and TRPC3/C7 channels shown above imply that downstream products (Hawkins *et al.*, 2006; Vanaesebroeck *et al.*, 2010) are likely to have important actions on activation mechanisms of these two native TRPC channels. Bath application of the water soluble diC8-PI(3)P (10 μM) to the cytosolic surface of unstimulated inside-out patches held at −70 mV activated two distinct channels; one channel had an unitary conductance of 2.8 pS, whereas the other channel had four sub-conductance states of 16, 32, 49 and 68 pS, and both channel types had E_r of about 0 mV (data not shown, Figure 6A). In contrast, PI(3,4)P_2, PI(3,5)P_2, and PI(3,4,5)P_3 (all at 10 μM) only activated channel currents with unitary conductances of about 3 pS (Figure 6B). Interestingly, substrates of PI3K-mediated pathways, PI(4)P, PI(5)P and PI(4,5)P_2 (all at 10 μM) (Hawkins *et al.*, 2006; Vanaesebroeck *et al.*, 2010), also activated channels with conductances of about 3 pS (Figure 6C). Non-phosphorylated PI had no effect on quiescent inside-out patches (Figure 6D and E). All PIs were applied in the presence of 50 nM wortmannin and 2 μM U73122 to ensure that these were effects of the PI itself and not a metabolite of PI3K and PLC pathways, respectively (Saleh *et al.*, 2009a,b).

To test the hypothesis that ET$_A$ receptor stimulation evokes TRPC1/C5/C6 and TRPC3/C7 channel activities through generation of PI(3)P, we carried out dot-blots studies that showed that ET$_A$ receptor stimulation leads to production of PI(3)P levels [Figure 6F(i)] through a wortmannin-sensitive process [Figure 6F(ii)].

Effect of PIs on PI(3)P-induced TRPC3/C7 channel activity

Earlier studies proposed that PI(4,5)P_2, a substrate of PLC- and also PI3K-mediated pathways, has a pronounced inhibitory action on the TRPC3/C6/C7 channel subgroup in VSMCs (Albert *et al.*, 2008; Ju *et al.*, 2010), and therefore we examined if the excitatory actions of PI(3)P on TRPC3/C7 channel activity is regulated by equimolar concentrations of PI(4,5)P_2. TRPC3/C7 channel activity induced by 10 μM diC8-PI(3)P in inside-out patches was almost abolished by co-application of 10 μM diC8-PI(4,5)P_2 (Figure 7A and D). Moreover, other substrates of PI3K-mediated pathways, PI(4)P and PI(5)P, and also PI3K-generated products, PI(3,4)P_2, PI(3,5)P_2 and PI(3,4,5)P_3

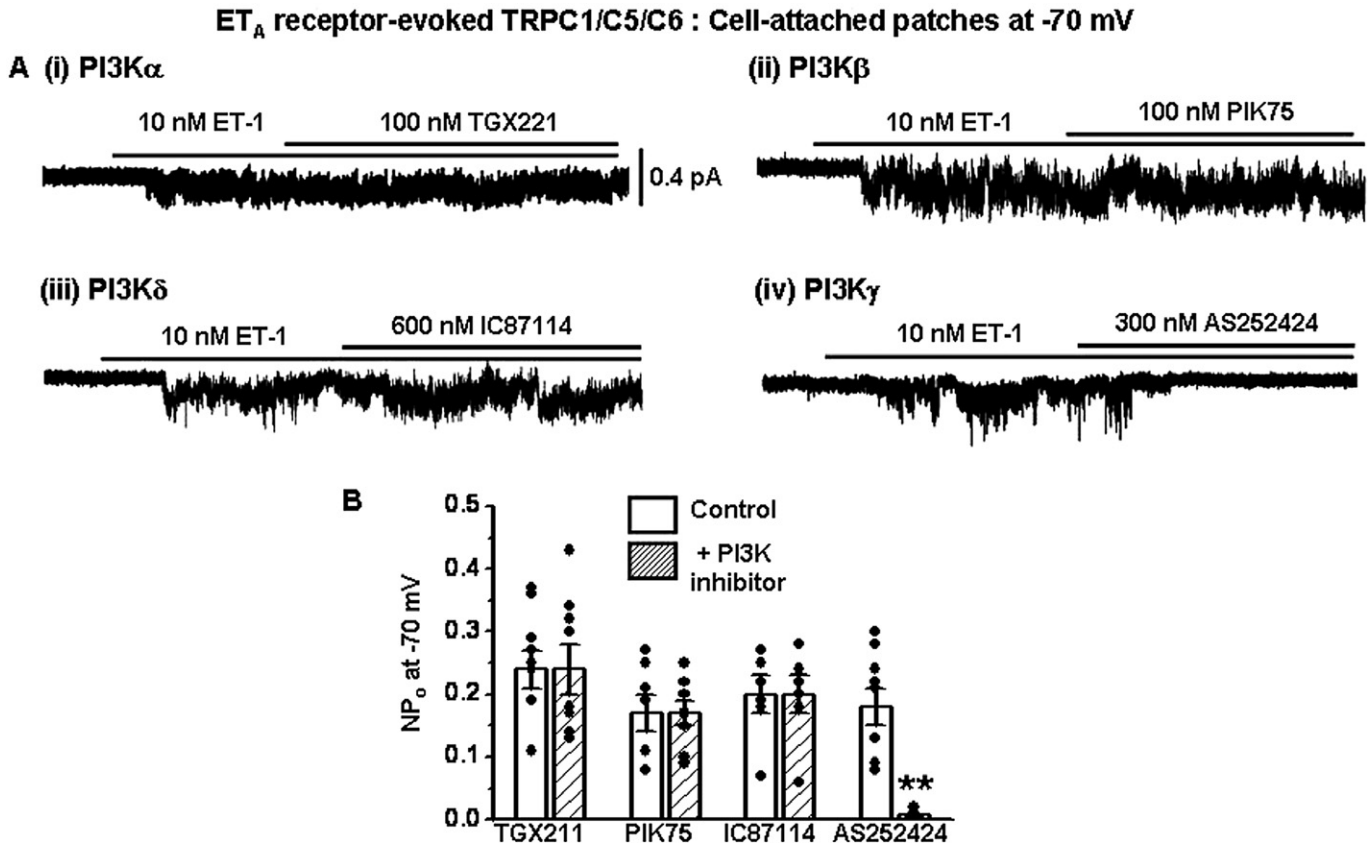

Figure 4

Class I PI3Kγ isoform inhibitor reduces ET$_A$ receptor-evoked TRPC1/C5/C6 channel activity. (A) Effect of selective Class I PI3K isoform inhibitors (i) TGX221, (ii) PIK75, (iii) IC87114, and (iv) AS252424 on cell-attached patches held at –70 mV, which contained only ET$_A$ receptor-induced TRPC1/C5/C6 activity. (B) Mean data showing the effect of Class I PI3K isoform inhibitors on TRPC1/C5/C6 channel activity. $n \geq 5$, $^{**}P < 0.01$. Note that only AS252424 inhibited ET$_A$ receptor-evoked TRPC1/C5/C6 channel activity.

(all at 10 μM), produced marked inhibition of PI(3)P-induced TRPC3/C7 channel activity (Figure 7B–D). Unphosphorylated PI (10 μM) had no effect on PI(3)P-evoked TRPC3/C7 channel activity (Figure 7B and D).

Effects of the G-protein βγ subunit inhibitor, gallein, on ET$_A$ receptor-operated native TRPC1/C5/C6 and TRPC3/C7 channels

The present work proposes that ET$_A$ GPCR stimulation induces native TRPC1/C5/C6 and TRPC3/C7 channels via, respectively, Class I PI3Kγ and Class II and/or III PI3K isoforms in coronary artery VSMCs. We therefore examined if the established pathway of G-protein βγ subunits coupled to PI3K is responsible for linking ET$_A$ receptor to channel activation (Stephens *et al.*, 1994; Clapham and Neer, 1997; Leopolt *et al.*, 1998). ET$_A$ receptor stimulation of TRPC1/C5/C6 and TRPC3/C7 channel activities was inhibited by co-application of 20 μM gallein, a G-protein βγ subunit inhibitor (Seneviatne *et al.*, 2011), by over 90% in cell-attached patches (Figure 8). In control experiments, gallein did not inhibit TRPC1/C5/C6 and TRPC3/C7 channel activities induced by 10 μM diC8-PI(3)P in inside-out patches (Figure 8B and C).

Expression of Class I, II and III PI3K isoforms in coronary artery tissue lysates

We carried out PCR studies to identify the expression of Class I, II and III PI3K isoforms in freshly dispersed coronary arteries. Coronary artery tissue lysates expressed transcripts for all known Class I, II and III PI3K isoforms after two sequential end-point PCR cycle steps (Figure 9A). Quantitative PCR using SYBR Green technology was used to assess the relative abundance of Class I, II and III PI3K isoforms in coronary artery tissue lysates. Melt-curve analysis was used to confirm specificity of primers (Figure 9B), and the efficiency of primer sets was established by the generation of standard curves from serial dilutions of cDNA from coronary artery tissue lysates (Figure 9C). The relative abundance of mRNA encoding PI3K isoforms was quantified on the basis of the expression ratio of target genes against two reference genes, β-actin [Figure 9D(i) and E] and GAPDH [Figure 9D(ii) and F]. Amplification plots of target genes against β-actin and GAPDH show that all known Class I, II, III PI3K isoforms are expressed in coronary artery tissue lysates, with PI3Kα expression levels considerably higher than levels of other PI3K isoforms (determined using two different primer sets, Figure 9G and H). Importantly, PI3Kγ

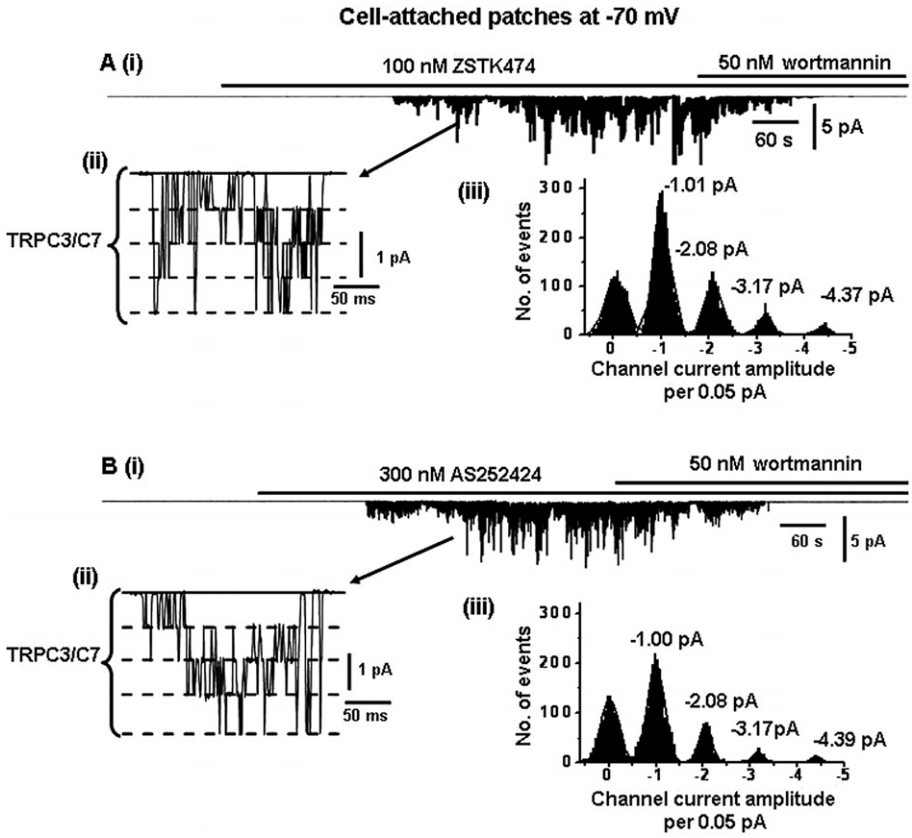

Figure 5

ZSTK474 and AS252424 activate TRPC3/C7 channel activity in unstimulated VSMCs. (A) (i) Bath application of ZSTK474 activated cation channel activity in quiescent cell-attached patches held at −70 mV, which was inhibited by wortmannin. (ii) Inset showing ZSTK474-evoked channel activity on a faster time scale. (iii) Amplitude histogram showing that ZSTK474-evoked channel currents illustrated in (i) had four sub-conductance levels between −1 and −4.5 pA at −70 mV. (B) Similar results were obtained with AS252424.

and Class II and III PI3K isoforms are expressed in coronary artery tissue lysates.

Discussion

PI3K mediates ET_A receptor stimulation of native heteromeric TRPC1/C5/C6 and TRPC3/C7 channels

The present study shows that low concentrations of wortmannin, a pan-PI3K inhibitor (Powis *et al.*, 1994), almost abolished ET_A receptor-operated native TRPC1/C5/C6 and TRPC3/C7 channel activities, which were unaffected by the PLC inhibitor U73122 (Yule and Williams, 1992). The G-protein βγ subunit inhibitor gallein (Seneviatne *et al.*, 2011) also markedly reduced ET_A receptor-evoked TRPC1/C5/C6 and TRPC3/C7 channel activities, but gallein did not inhibit activation of TRPC1/C5/C6 and TRPC3/C7 channels by PI(3)P, a downstream product of PI3K. Figure 10 highlights our proposal that stimulation of ET_A receptors coupled to G-protein βγ subunits leading to activation of different PI3K isoforms produces opening of two distinct native TRPC channels in freshly isolated rabbit coronary artery VSMCs. G-protein βγ-mediated activation of PI3K isoforms is a well-characterized biochemical pathway in many cell types (Stephens *et al.*, 1994; Clapham and Neer, 1997; Leopolt *et al.*, 1998).

In VSMCs, receptor/$G_{\alpha q/11}$-evoked PLC and constitutively active $G_{\alpha i/o}$-evoked phospholipase D activities are currently thought to be the major signalling pathways involved in activating native TRPC channels (Abramowitz and Birnbaumer, 2009; Albert *et al.*, 2009; Large *et al.*, 2009; Dietrich *et al.*, 2010; Albert, 2011). The present study, in conjunction with our earlier work (Saleh *et al.*, 2009b), indicates that $G_{\beta\gamma}$-PI3K-mediated pathways must also be considered important in native TRPC channel activation.

PI3Kγ mediates ET_A receptor-induced TRPC1/C5/C6 channel activation

ET_A receptor stimulation leads to PI3K-mediated generation of $PI(3,4,5)P_3$ and opening of TRPC1/C5/C6 channels in coronary artery VSMCs (Saleh *et al.*, 2008; 2009b). The present work extends these ideas to suggest that the Class I PI3Kγ isoform mediates ET_A receptor stimulation of TRPC1/C5/C6 channels (Figure 10). These conclusions are based upon marked inhibition of TRPC1/C5/C6 channel activity by the pan-Class I PI3K inhibitor, ZSTK474 (Kong and Yamori, 2007)

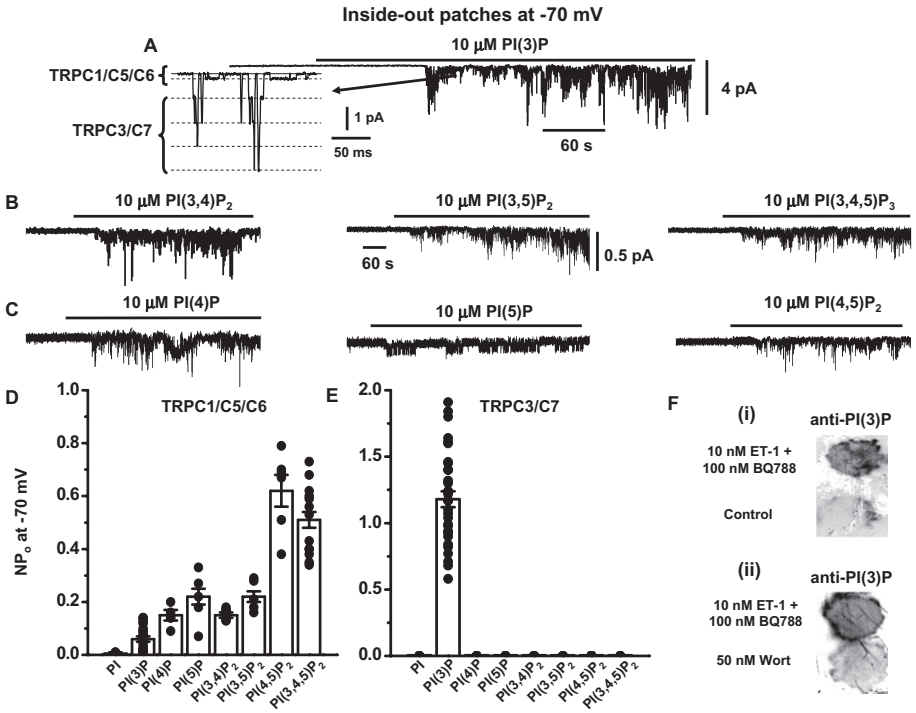

Figure 6

Selective excitatory actions of PI(3)P and other PIs on TRPC1/C5/C6 and TRPC3/C7 channel activities. (A) Bath application of PI(3)P activated both TRPC1/C5/C6 and TRPC3/C7 channel activities in inside-out patches held at −70 mV. (B) and (C) Bath application of other PI(3)-containing molecules only activated TRPC1/C5/C6 channel activity in inside-out patches. (D) and (E) Mean data showing the effect of PI molecules on TRPC1/C5/C6 and TRPC3/C7 channel activities, respectively. Note that PI failed to activate either TRPC1/C5/C6 or TRPC3/C7 channels. All PIs used were diC8 molecules. (F) Dot-blots showing (i) the absence of PI(3)P in tissue lysates at rest and an increase in PI(3)P levels following ET_A receptor stimulation, and (ii) ET_A receptor-mediated increase in PI(3)P levels was prevented by pre-incubation with wortmannin (Wort).

and the selective Class I PI3Kγ blocker, AS252424 (Knight *et al.*, 2006; Chaussade *et al.*, 2007; Hayakawa *et al.*, 2007). Inhibitors of Class I PI3Kα, β and δ isoforms had no effect on channel activity.

PI3K has been suggested to mediate the ET-1-evoked cation conductances involved in Ca^{2+} influx and vasoconstriction of rabbit basilar artery (Kawanabe *et al.*, 2004; Miwa *et al.*, 2005). Angiotensin II-evoked stimulation of L-type Ca^{2+} channels in rat portal vein is proposed to involve G-protein βγ subunits coupled to PI3Kγ and production of $PI(3,4,5)P_3$ (Viard *et al.*, 1999; Quignard *et al.*, 2001; Le Blanc *et al.*, 2004), and PI3Kγ is reported to be important for regulating Ca^{2+} oscillations and contraction of murine airway smooth muscle (Jiang *et al.*, 2010).

Class II and/or Class III PI3K isoforms mediate ET_A receptor-evoked TRPC3/C7 channel activity

In contrast to the inhibitory actions of ZSTK474 and AS252424 on TRPC1/C5/C6 channel activity, both these agents had excitatory actions on ET_A receptor-stimulated TRPC3/C7 channel activity indicating that PI3Kγ-mediated pathways are involved in inhibiting these channels. Moreover, ZSTK474 and AS252424 also activated channels with similar conductance values as TRPC3/C7 channels in unstimulated cells. These results indicate that ET_A receptor-mediated and constitutively active PI3Kγ exerts a powerful inhibitory action on native TRPC3/C7 channels in coronary artery VSMCs, which maintain these channels in a closed state (Figure 10).

ZSTK474- and AS252424-induced potentiation of ET_A receptor-evoked TRPC3/C7 channel activity and activation of TRPC3/C7 channels in quiescent patches were both inhibited by low concentrations of wortmannin. These results indicate that non-Class I PI3K isoforms, most likely Class II and/or III PI3K isoforms, mediate activation of TRPC3/C7 channels. Moreover, they suggest that these Class II and/or Class III PI3K isoforms can be constitutively active. The qPCR studies confirmed that all known catalytic subunits of Class I, II and III PI3K isoforms are expressed in coronary artery tissue lysates. There are few established inhibitors of Class II and III PI3K isoforms, and so future experiments using protein knock-down approaches will be required to identify PI3K isoforms mediating ET_A receptor-evoked TRPC3/C7 channel activation.

Gating of native TRPC1/C5/C6 channels by PI3K-mediated PI molecules

$PI(4,5)P_2$ is obligatory for activation of native TRPC1/C5/C7 channels in portal vein VSMCs, through a process requiring PKC-dependent phosphorylation of TRPC1 proteins (Saleh *et al.*, 2009a). In coronary artery VSMCs, ET_A receptor-evoked

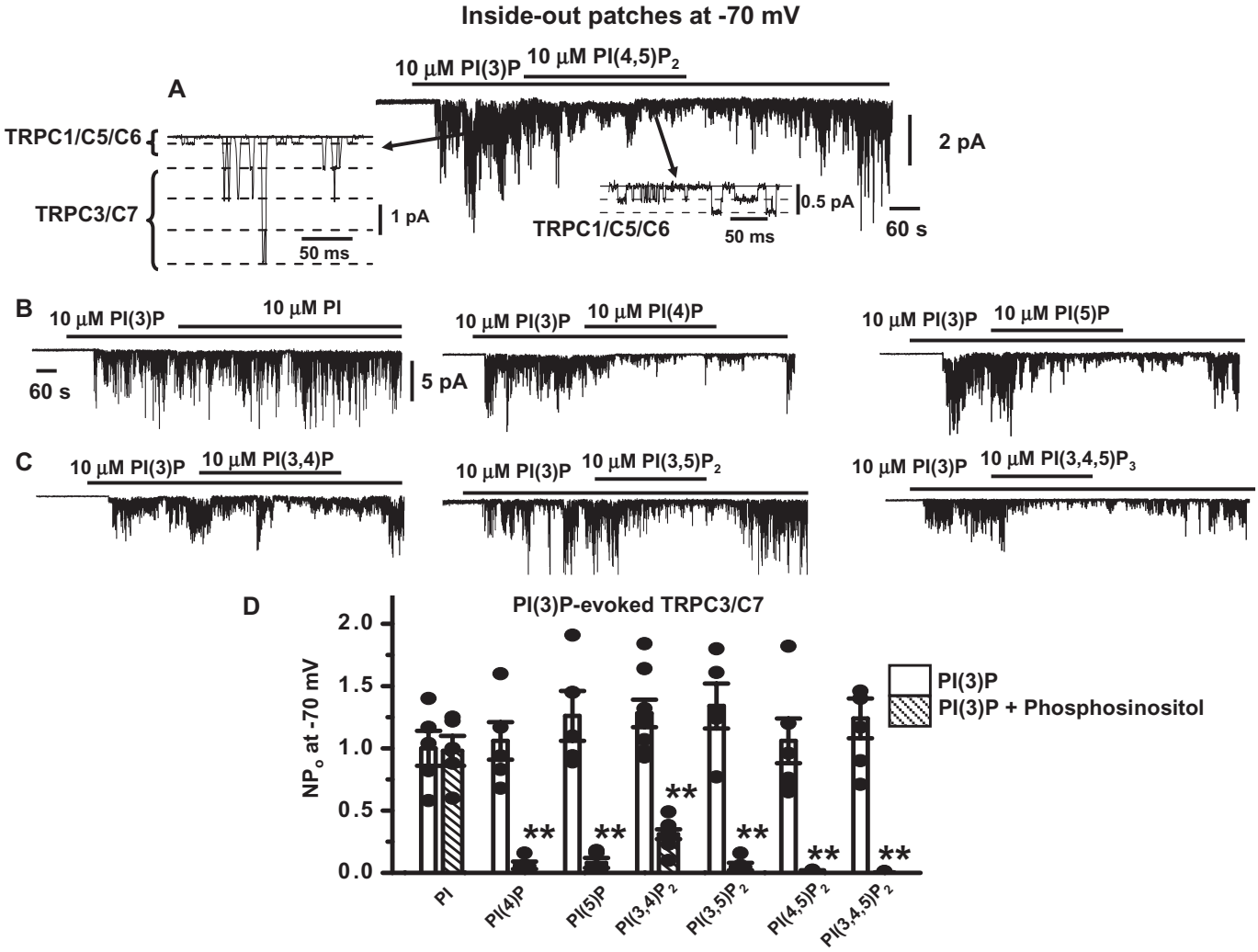

Figure 7

PI(3)P-evoked TRPC3/C7 channel activity is inhibited by other PIs. (A) PI(3)P-activated TRPC3/C7 channel activity is inhibited by co-application of equimolar PI(4,5)P_2 concentrations in inside-out patches held at –70 mV. Note that the insets show that TRPC1/C5/C6 channels are present with PI(3)P and also with PI(3)P + PI(4,5)P_2 conditions. (B) Substrates of PI3K-mediated reactions, PI(4)P and PI(5)P, and (C) products of PI3K-mediated reactions inhibited PI(3)P-evoked TRPC3/C7 channel activities. (C) Mean data showing that PI molecules significantly inhibited PI(3)P-evoked TRPC3/C7 channel activity. $n \geq 5$, $^{**}P < 0.01$. Note that PI had no effect on PI(3)P-induced TRPC3/C7 channel activity.

activation of TRPC1/C5/C6 channels is proposed to be mediated by PI3K through generation of PI(3,4,5)P_3, which acts as a gating ligand via a PKC-dependent mechanism (Saleh *et al.*, 2008; 2009b).

The present work builds upon these earlier findings with PI(3,4,5)P_3 to reveal that other signalling molecules generated by PI3K, PI(3)P, PI(3,4)P_2, PI(3,5)P_2 and PI(3,4,5)P_3 activate TRPC1/C5/C6 channels in inside-out patches of coronary artery VSMCs, which indicates that these ligands are also gating molecules. Future studies will need to determine if TRPC1/C5/C6 channel opening by these PI3K-generated molecules requires PKC-dependent phosphorylation of TRPC1, and/or TRPC5/C6 subunits, and also to examine which TRPC subunit(s) confers activation by PI. Recent work has indicated that TRPC1 confers activation by PI(4,5)P_2 and PI(3,4,5)P_3 to a native heteromeric TRPC1/C5 channel in murine mesenteric artery (Shi *et al.*, 2011).

PI(3)P is novel activator of TRPC3/C7 channels

The established view is that TRPC channels mediated by TRPC3/C6/C7 subunits are activated by phospholipase-generated DAG through a PKC-independent mechanism (Abramowitz and Birnbaumer, 2009; Dietrich *et al.*, 2010). In VSMCs, DAG is proposed to gate channels composed of TRPC3/C6/C7 subunits by competing with PI(4,5)P_2, which is bound to channel proteins at rest and acts as a physiological antagonist (Albert *et al.*, 2008; Large *et al.*, 2009; Ju *et al.*, 2010; Albert, 2011). In agreement with these studies, previous evidence indicates that TRPC3/C7 channels in coronary

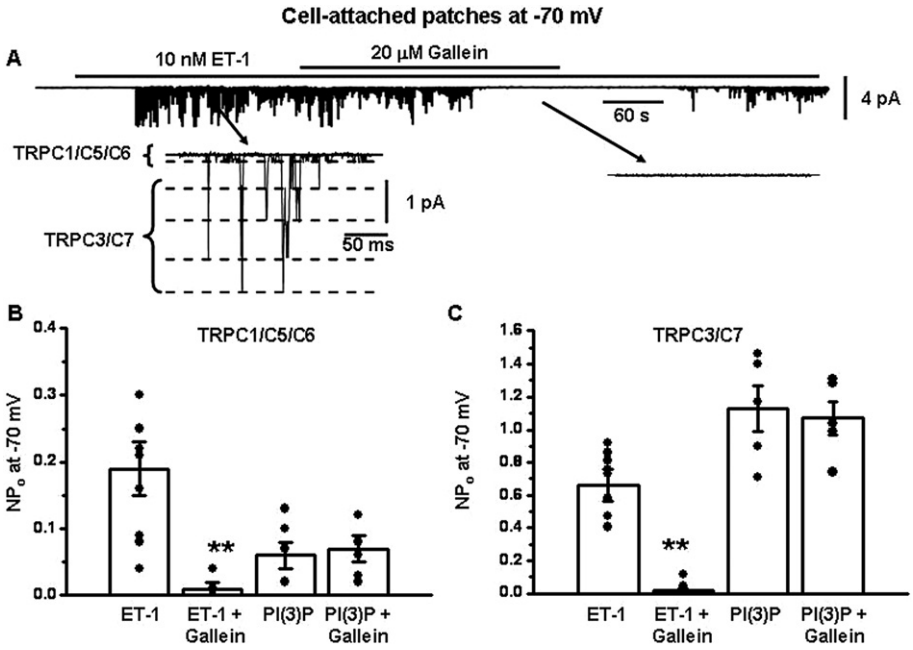

Figure 8

Effect of the G-protein βγ subunit inhibitor gallein on ET_A receptor-evoked TRPC1/C5/C6 and TRPC3/C7 channel activities. (A) Co-application of gallein inhibited both ET_A receptor-evoked TRPC1/C5/C6 and TRPC3/C7 channel activities in cell-attached patches held at –70 mV. (B) and (C) Mean data showing that gallein significantly inhibited ET_A receptor-evoked TRPC1/C5/C6 and TRPC3/C7 channel activities in cell-attached patches, respectively. In addition, gallein had no effect on PI(3)-evoked TRPC1/C5/C6 or TRPC3/C7 channel activities in inside-out patches. $n \geq 5$, $^{**}P < 0.01$.

artery VSMCs are activated by DAG via a PKC-independent mechanism (Peppiatt-Wildman *et al.*, 2007). However, the present results indicate that PI3K, and not PLC, signalling pathways govern ET_A receptor stimulation of TRPC3/C7 channels. The important conclusion from these findings is that although DAG can activate TRPC3/C7 channels (Peppiatt-Wildman *et al.*, 2007), PI(3)P and not this triglyceride is involved in ET_A receptor-mediated activation of native TRPC3/C7 channels in coronary artery VSMCs. It will be interesting to investigate whether PI(3)P is an activating ligand at other TRPC3/C6/C7 channel subgroups in different cell types.

We obtained compelling evidence that PI(3)P is the only PI3K-generated molecule that activates TRPC3/C7 channels. Furthermore, $PI(3,4)PI_2$, $PI(3,5)P_2$, $PI(4,5)P_2$ and $PI(3,4,5)P_3$ induced a pronounced inhibition of PI(3)P-evoked TRPC3/C7 channel activity. As PI(3)P is the only PI3K-generated molecule that activates TRPC3/C7, it is most likely that this PI mediates ET_A receptor stimulation of TRPC3/C7 channel activity. In agreement with this hypothesis, dot-blot analysis showed that ET_A receptor stimulation generates PI(3)P through a wortmannin-sensitive process in coronary artery tissue lysates. Moreover, PI(3)P is considered to be produced by the action of Class II/III PI3K isoforms (Hawkins *et al.*, 2006; Vanaesebroeck *et al.*, 2010). These results represent a significant advance in our understanding of the native TRPC3/C6/C7 channel subgroup; PI(3)P is a novel gating ligand (Figure 10). It will be important to further investigate interactions between PI(3)P (and other PIs) and DAG in activating the TRPC3/C6/C7 channel subgroup.

Substrates of PI3K-mediated pathways modulate TRPC1/C5/C6 and TRPC3/C7 channel activity

Our data indicate that substrates of PI3K-mediated pathways [PI(4)P, PI(5)P and $PI(4,5)P_2$] activate TRPC1/C5/C6 channels but inhibit PI(3)P-evoked TRPC3/C7 channels in coronary artery VSMCs. These results suggest that specific PI(3) molecule-acting phosphatases (e.g. phosphatase and tensin homologue deleted on chromosome 10) acting at PI3K-mediated products to generated these substrates (Hawkins *et al.*, 2006; Vanaesebroeck *et al.*, 2010) are also likely to have important roles in regulating native TRPC channel regulation in VSMCs. PI molecules shown in the present study to activate TRPC1/C5/C6 and TRPC3/C7 channels and also inhibit PI(3)P-evoked TRPC3/C7 channel activity bind to expressed TRPC1, C5 C6 and C7 proteins (Kwon *et al.*, 2007). Interestingly, the non-phosphorylated PI molecule, PI, which we showed has no effect on TRPC1/C5/C6 and TRPC3/C7 channel activities (see Figures 5 and 6), does not bind to expressed TRPC proteins (Kwon *et al.*, 2007).

Importance of results

TRPC channels and PI3K have been independently reported to contribute to vascular tone, cell growth, proliferation, migration and survival of VSMCs and have both been implicated in vascular diseases such as hypertension and neointima hyperplasia (Huang and Kontos, 2002; Oudit *et al.*, 2004; Huang *et al.*, 2005; Abramowitz and Birnbaumer, 2009; Morello *et al.*, 2009; Dietrich *et al.*, 2010). To our

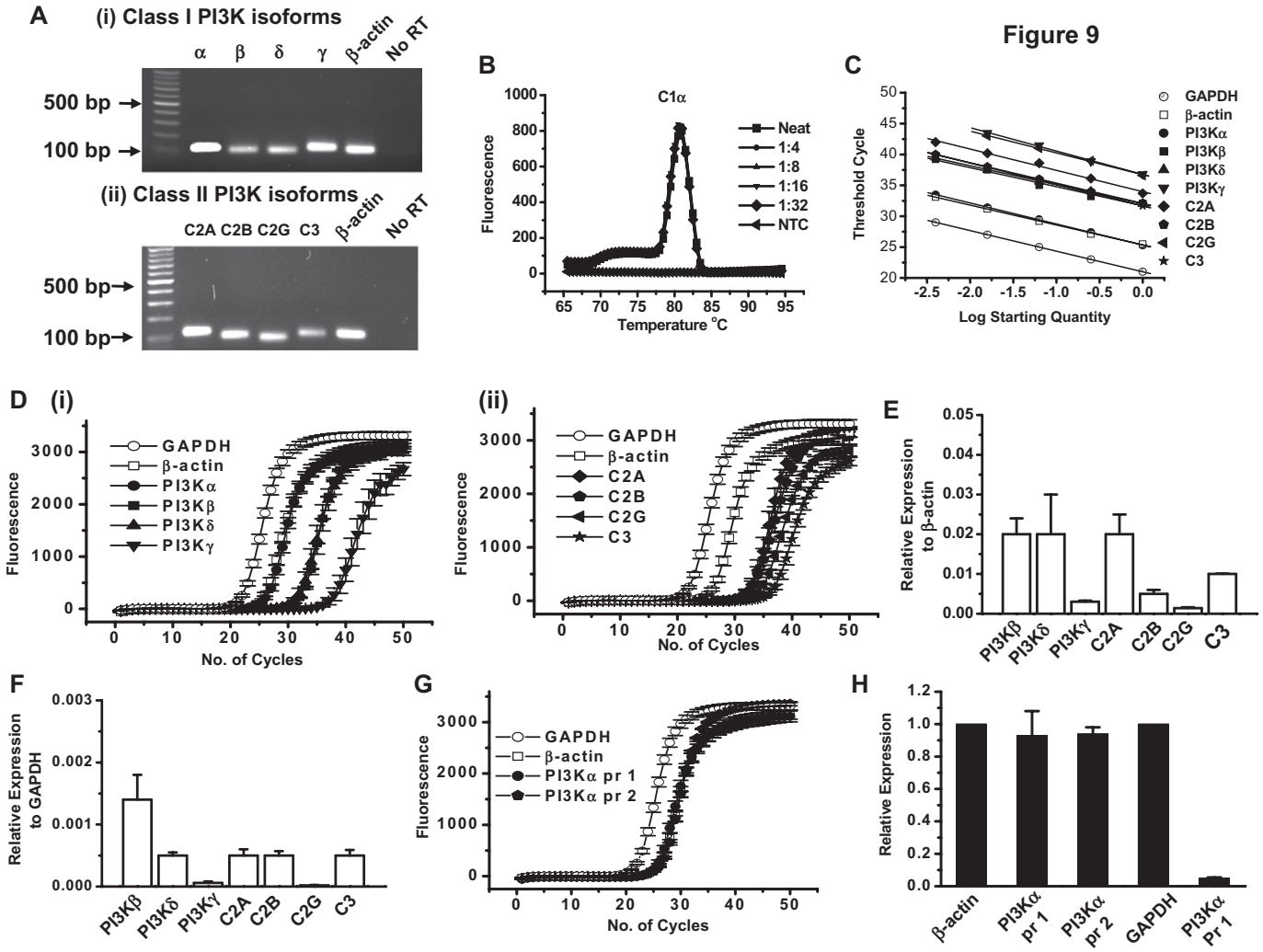

Figure 9

Expression of PI3K isoforms in freshly dispersed coronary arteries. (A) End-point RT-PCR of (i) Class I and (ii) Class II/III (C2/3) PI3K isoforms following one 40-cycle plus one 20-cycle protocols. (B) Representative dissociation profile for the Class I PI3Kα pr 1 in serially diluted concentrations of rabbit coronary artery cDNA. (C) Standard curves for all PI3K, β-actin and GAPDH primers used. (D) Amplification plots for (i) Class I and (ii) Class II/III PI3K primer sets. (E) Relative expression of primer sets for Class I (excluding Class I PI3Kα), II and III PI3K isoforms, which are normalized to β-actin and (F) to GAPDH. (G) Amplification plots and (H) relative expression of two different Class I PI3Kα primer sets, using β-actin and GAPDH as controls. All values shown were determined from at least three different animals.

knowledge, our results demonstrate for the first time that PI3Kγ and other Class II/III PI3K isoforms are likely to be important in regulating distinct native TRPC channels in VSMCs. Our findings provide an important rational for investigating the role of ET_A receptor-PI3K-TRPC channel pathways in coronary artery function, for example, contraction, membrane potential regulation, cell migration and proliferation. Moreover, our data indicate that pharmacological manipulation of specific PI3K isoforms and TRPC channels involved in these ET_A receptor-PI3K-TRPC pathways may be beneficial for the treatment and prevention of cardiovascular disease.

Of further value, our results highlight the novel roles of PI molecules in the regulation of native TRPC channels. PI molecules containing a phosphorylated D3, D4 or D5 hydroxyl group activate a native TRPC1 channel, whereas only PI(3)P activates a TRPC3/C6/C7 channel subtype. These novel findings provide an intriguing proposition for gating of the TRPC3/C6/C7 channel subgroup; PI containing D3 phosphorylation produces channel activation, whereas PI containing D4/D5 phosphorylations produces potent, and dominant, channel inhibition.

Acknowledgements

This work was supported by the British Heart Foundation Project grant, PG/07/079/23568 and PG/08/042/25066, to Anthony P. Albert and William A. Large. We would like to thank Ken Laing and Alison Davis for technical advice in qPCR.

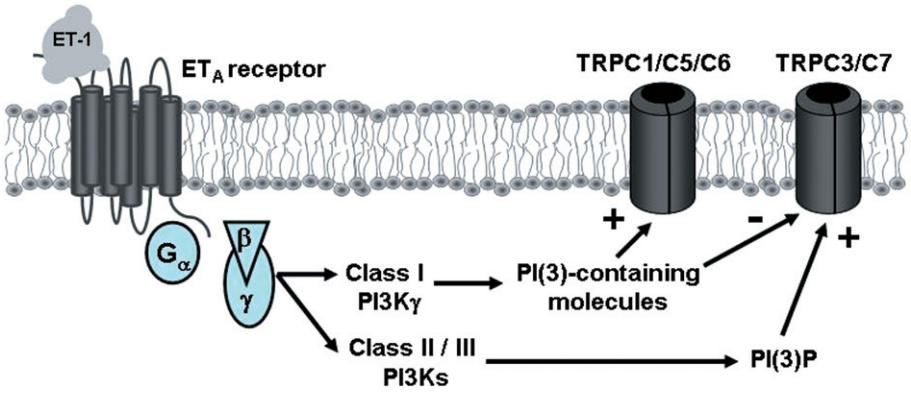

Figure 10

Schematic representation of proposed activation mechanisms of ET_A receptor-evoked TRPC1/C5/C6 and TRPC3/C7 channels. Stimulation of ET_A GPCRs leads to Gβγ subunits activation of different PI3K isoforms. It is proposed that the PI3Kγ-mediated generation of PI(3)-containing molecules [PI(3)P, PI(3,4)P_2, PI(3,5)P_2, PI(3,4,5)P_3] produce gating of TRPC1/C5/C6 channels and TRPC3/C7 channel inhibition. In contrast, Class II and /or III PI3K isoform-mediated generation of PI(3)P induced TRPC3/C7 channel gating. It is important to note that PI(3)P is represented has a direct activating ligand at TRPC3/C7 channels.

Conflicts of interest

None.

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Supporting information

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

Table S1 Conventional PCR primers used for end-point PCR
Table S2 qPCR primers

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