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Activation of native TRPC1/C5/C6 channels by endothelin-1 is mediated by both PIP_3 and PIP_2 in rabbit coronary artery myocytes

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We investigate activation mechanisms of native TRPC1/C5/C6 channels (termed TRPC1 channels) by stimulation of endothelin-1 (ET-1) receptor subtypes in freshly dispersed rabbit coronary artery myocytes using single channel recording and immunoprecipitation techniques. ET-1 evoked non-selective cation channel currents with a unitary conductance of 2.6 pS which were not inhibited by either ET_A or ET_B receptor antagonists, respectively BQ-123 and BQ788, when administered separately. However, in the presence of both antagonists, ET-1-evoked channel activity was abolished indicating that both ET_A and ET_B receptor stimulation activate this conductance. Stimulation of both ET_A and ET_B receptors evoked channel activity which was inhibited by the protein kinase C (PKC) inhibitor chelerythrine and by anti-TRPC1 antibodies indicating that activation of both receptor subtypes causes TRPC1 channel activation by a PKC-dependent mechanism. ET_A receptor-mediated TRPC1 channel activity was selectively inhibited by phosphoinositol-3-kinase (PI-3-kinase) inhibitors wortmannin (50 nM) and PI-828 and by antibodies raised against phosphoinositol-3,4,5-trisphosphate (PIP_3), the product of PI-3-kinase-mediated phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP_2). Moreover, exogenous application of diC8-PIP_3 stimulated PKC-dependent TRPC1 channel activity. These results indicate that stimulation of ET_A receptors evokes PKC-dependent TRPC1 channel activity through activation of PI-3-kinase and generation of PIP_3. In contrast, ET_B receptor-mediated TRPC1 channel activity was inhibited by the PI-phospholipase C (PI-PLC) inhibitor U73122. 1-Oleoyl-2-acetyl-*sn*-glycerol (OAG), an analogue of diacylglycerol (DAG), which is a product of PI-PLC, also activated PKC-dependent TRPC1 channel activity. OAG-induced TRPC1 channel activity was inhibited by anti-phosphoinositol-4,5-bisphosphate (PIP_2) antibodies and high concentrations of wortmannin (20 μM) which depleted tissue PIP_2 levels. In addition exogenous application of diC8-PIP_2 activated PKC-dependent TRPC1 channel activity. These data indicate that stimulation of ET_B receptors evokes PKC-dependent TRPC1 activity through PI-PLC-mediated generation of DAG and requires a permissive role of PIP_2. In conclusion, we provide the first evidence that stimulation of ET_A and ET_B receptors activate native PKC-dependent TRPC1 channels through two distinct phospholipids pathways involving a novel action of PIP_3, in addition to PIP_2, in rabbit coronary artery myocytes.

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Abbreviations AgP, antigenic peptide; CPA, cyclopiazonic acid; DAG, diacylglycerol; ET-1, endothelin-1; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol; PI-3-kinase, phosphoinositol-3-kinase; PIP_2, phosphatidylinositol-4,5-bisphosphate; phosphatidylinositol-3,4,5-trisphosphate; PLC, phosphoslipase C; PKC, protein kinase C; TRPC, canonical transient receptor potential.

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Introduction

Endothelin-1 (ET-1) produces vasoconstriction by a direct action on vascular smooth muscle cells through stimulation of predominantly ET_A receptors, although ET_B receptors are involved in some vascular beds (Sumner *et al.* 1992; Davenport & Battistini, 2002). Moreover in the coronary circulation activation of ET-1 receptors has been linked to exaggerated constriction of human coronary artery leading to myocardial ischaemia in coronary artery disease (Schiffrin & Touyz, 1998; Kinlay *et al.* 2001).

ET-1-induced vasoconstriction is mediated almost entirely by influx of Ca^{2+} ions through voltage-independent ion channels (see Miwa *et al.* 2005). These data suggest that ET-1 contracts vascular smooth muscle by opening Ca^{2+}-permeable non-selective cation channels. Consistent with this notion we demonstrated that ET-1 activates two distinct types of canonical transient receptor potential (TRPC) channels in freshly dispersed rabbit coronary myocytes. At low concentrations (1–10 nM) ET-1 activates a non-selective cation channel with four subconductance states of between 16 and 68 pS (Peppiatt-Wildman *et al.* 2007). These responses were mediated mainly by ET_A receptors and were mimicked by the diacylglycerol (DAG) analogue, 1-oleoyl-2-acetyl-*sn*-glycerol (OAG) via a protein kinase C (PKC)-independent mechanism. Evidence indicated that this cation channel protein is a heteromeric structure consisting of TRPC3/TRPC7 subunits (Peppiatt-Wildman *et al.* 2007).

In contrast at higher concentrations (100 nM) ET-1 evokes a PKC-dependent 2.6 pS Ca^{2+}-permeable cation channel which has characteristics of a heteromeric TRPC1/TRPC5/TRPC6 structure (subsequently referred to as TRPC1 channels, Saleh *et al.* 2008). With this concentration of ET-1 the TRPC3/TRPC7 conductance is not observed.

In the present study we have investigated the transduction mechanisms linking ET-1 receptors to native TRPC1 ion channels described above in coronary artery myocytes. The results demonstrate that TRPC1 channels may be activated by stimulation of either ET_A or ET_B receptors using two distinct phosphoinositide signalling pathways involving respectively phosphatidylinositol 3,4,5-trisphosphate (PIP_3) and phosphatidylinositol 4,5-bisphosphate (PIP_2). This is the first demonstration that PIP_3, in addition to PIP_2, activates native TRPC1 channels.

Methods

Cell isolation

New Zealand White rabbits (2–3 kg) were killed using I.V. sodium pentobarbitone (120 mg kg^{-1}, in accordance with the UK Animals (Scientific Procedures Act) 1986). Experimental methods were carried out as specified by St George's animal welfare committee and according to the policies of *The Journal of Physiology* (Drummond, 2009). Right and left anterior descending coronary arteries were dissected free from fat and connective tissue in physiological salt solution containing (mM): NaCl (126), KCl (6), glucose (10), Hepes (11), $MgCl_2$ (1.2) and $CaCl_2$ (1.5), with pH adjusted to 7.2 with 10 M NaOH. An incision was made along the longitudinal axis of the blood vessels and the exposed endothelium was gently removed using a cotton bud. Enzymatic digestion and smooth muscle cell isolation were subsequently carried using methods previously described (Saleh *et al.* 2006).

Electrophysiology

Single channel currents were recorded in voltage-clamp mode using cell-attached and inside-out patch configurations (Hamill *et al.* 1981) with a HEKA EPC 8 patch-clamp amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany) at room temperature (20–23°C). Patch pipettes were manufactured from borosilicate glass to produce pipettes with resistances of 6–10 MΩ for isolated patch recording when filled with patch pipette solution. To reduce 'line' noise the recording chamber (vol. *ca* 150–200 μl) was perfused using two 20 ml syringes, one filled with external solution and the other used to drain the chamber, in a 'push and pull' technique. The external solution could be exchanged twice within 30 s. In cell-attached patch recording, the membrane potential was set to ∼0 mV using a high KCl bathing solution (see below). In both cell-attached and inside-out patch recordings, +70 mV was applied to the patch and held at this level except for measuring current–voltage (I–V) relationships when the applied patch voltage was manually altered between +120 mV and −50 mV. According to convention in the text membrane potential is given with respect to the internal potential and thus, the resting holding potential is referred to as −70 mV.

Single channel currents were initially recorded onto digital audiotape (DAT) using a Sony PCM-R300 digital tape-recorder (BioLogic Science Instruments, Claix, France) at a bandwidth of 5 kHz (HEKA EPC 8 patch-clamp amplifier) and a sample rate of 48 kHz. For off-line analysis, single channel currents were filtered at 100 Hz (see below, −3 db, low pass 8-pole Bessel filter, model LP02, Frequency Devices Inc., Ottawa, IL, USA) and acquired using a Digidata 1322A and pCLAMP 9.0 at a sampling rate of 1 kHz. Data were captured with a Dell Dimension 5150 personal computer.

Single channel current amplitudes were calculated from idealised traces of at least 60 s in duration using the 50% threshold method and analysed using pCLAMP

v.9.0 software with events lasting for <6.664 ms (2 × rise time for a 100 Hz, −3 db, low pass filter) being excluded from analysis. Single channel current amplitude histograms were plotted and fitted with Gaussian curves with the peak of these curves determining the unitary amplitude of the single channel currents. Open probability (NP_o) was calculated automatically using pCLAMP 9. Figure preparation was carried out using Origin 6.0 software (OriginLab Corp., Northampton, MA, USA) where inward single channel openings are shown as downward deflections.

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), pH adjusted 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), Na_2ATP (1) and 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′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) (0.1), niflumic acid (0.1) and nicardipine (0.005), pH adjusted to 7.2 with NaOH. Under these conditions voltage-dependent Ca^{2+} currents, K^+ currents, swell-activated Cl^- currents and Ca^{2+}-activated Cl^- conductances are abolished and non-selective cation currents could be recorded in isolation.

Anti-TRPC1 (which detects TRPC1 proteins with a predicted molecular mass of ∼100 kDa) and anti-PIP$_2$ antibodies (which detect liposome complex of PIP$_2$ molecules with a predicted molecular mass of ∼75 kDa, see manufacturer's data sheet and Fukami *et al.* 1988) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and alomone labs (Israel), anti-PIP$_3$ antibodies were from MBL (Japan) and anti-β-actin antibodies were from Sigma (UK). Pre-incubation of anti-TRPC1 antibodies with its antigenic peptide was carried out in a 1 : 2 ratio for at least 2 h in control experiments. Unless otherwise stated all other drugs were purchased from Calbiochem (UK), Sigma (UK) or Tocris (UK) and agents were dissolved in distilled H_2O or DMSO (0.1%). DMSO alone had no effect on channel activity. The values are the mean of n cells ± S.E.M. Statistical analysis was carried out using paired (comparing effects of agents on the same cell) or unpaired (comparing effects of agents between cells) Students' t test with the level of significance set at $P < 0.05$.

Immunoprecipitation and Western blotting

Dissected tissues were flash frozen and stored in 10 mM TRIS-HCl (pH 7.4) at −80°C for subsequent use. Tissues were defrosted and mechanically disrupted with an Ultraturrax homogeniser and further disrupted by sonication on ice for at least 2 h. Tissues were subsequently centrifuged at 25 000 g for 30 min at 4°C and the supernatant was discarded. The total cell lysate (TCL) was then collected by centrifugation at 11 200 g for 10 min in 10 mg ml^{-1} RIPA lysis buffer (Santa Cruz Biotechnology), supplemented with protease inhibitors. Protein content was quantified using the Bio-Rad protein dye reagent (Bradford method). TCL was retained on ice for subsequent experimental procedures including dot-blots and immunoprecipitation. Dot-blots were carried out by 'spotting' 2–5 μl of TCL on prepared immobilon-p polyvinylidene difluoride (PVDF) membranes. Membranes were allowed to dry prior to detection using conventional Western blotting techniques (see later). The immunoprecipitation protocol was carried out using the Millipore Catch and Release® kit, where spin columns were loaded with 500 μg of TCL and 2–6 μg of antibody and immunoprecipitated for 2 h at room temperature.

Immunoprecipitated samples were eluted with Laemmli sample buffer and incubated at 60°C for 5 min. One-dimensional protein gel electrophoresis was performed in 4–12% Bis-Tris Gels in a Novex mini-gel system (Invitrogen) with 10–20 μg of total protein loaded in each lane. Separated proteins were transferred onto PVDF membranes using the Invitrogen iBlot apparatus. Western blotting was subsequently carried out on membranes which were incubated with the appropriate primary antibody for 2 h at room temperature. Where possible, alternative antibodies raised against different epitopes were used for immunoprecipitation and Western blot analysis. Following antibody removal membranes were washed for 2 h with milk/phosphate-buffered saline with Tween 20 (PBST) and were subsequently incubated with horseradish peroxidase-conjugated secondary antibody diluted 1 : 1000–5000 in milk/PBST. Membranes were then washed 3 times for 15 min in PBST, followed by a final wash in PBS before being treated with ECL chemiluminescence reagents (Pierce Biotechnology, Inc., Rockford, IL, USA) for 1 min and exposed to photographic films. Data shown represents n values of at least three separate experiments.

Results

Stimulation of ET_A and ET_B receptors activate 2–3 pS cation channel currents in rabbit coronary artery myocytes

In initial experiments we investigated the identity of the ET-1 receptor subtype involved in activating native 2–3 pS cation channel currents. For these experiments 100 nM ET-1 was used since at these concentrations ET-1 does not activate the TRPC3/C7 conductance expressed in this preparation (see Peppiatt-Wildman *et al.* 2007 and Introduction) and the 2–3 pS conductance is recorded in isolation. Both ET_A and ET_B G-protein-coupled receptors are expressed in vascular smooth muscle (see Miwa *et al.* 2005) and therefore we studied the effect of selective concentrations of ET_A and ET_B receptor antagonists, respectively BQ-123 and BQ-788 (Davenport, 2002), on ET-1-induced native 2–3 pS channel activity in cell-attached patches from freshly dispersed coronary artery myocytes.

Figure 1*A*, *E* and *F* shows that bath application of 100 nM ET-1 activated cation channel activity at −70 mV which had a mean peak open probability (NP_o) of 0.25 ± 0.07 ($n = 10$) and was composed of channel openings with a unitary conductance of 2.6 pS and a reversal potential (E_r) of about 0 mV. These responses were seen in approximately 90% of patches tested. Figure 1*Ab* illustrates that the channel current amplitude histogram of

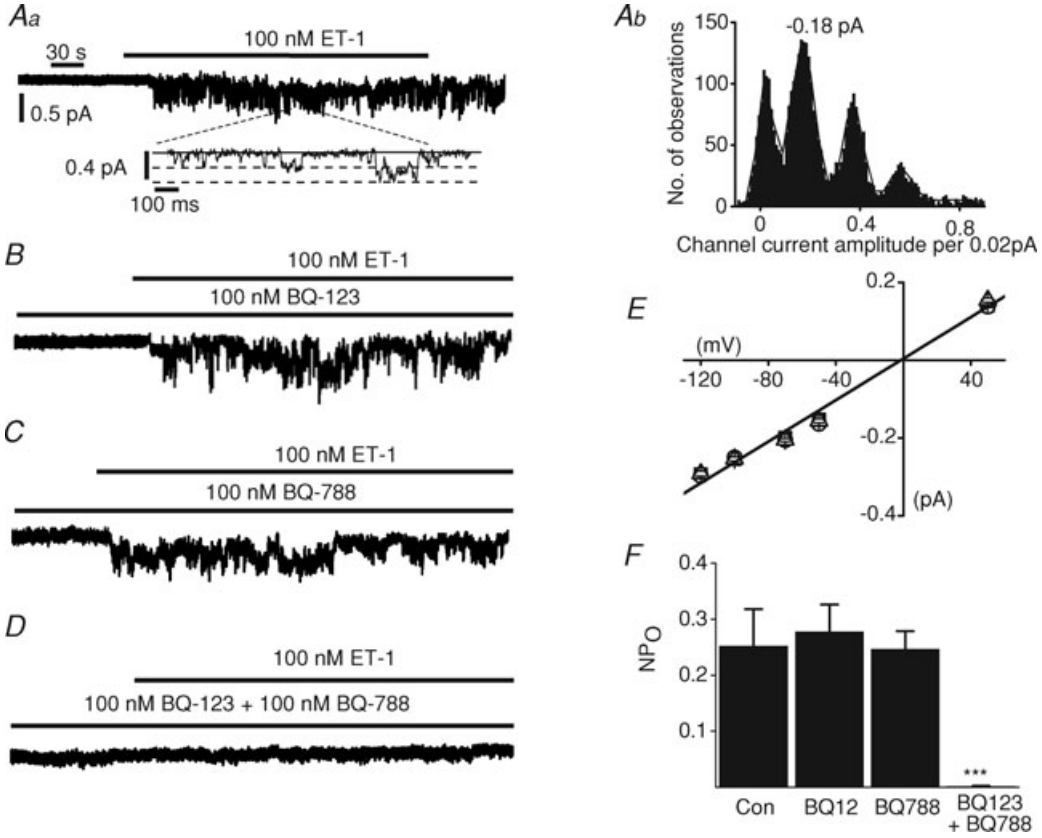

Figure 1. Stimulation of ET_A and ET_B receptors activates 2–3 pS cation channel currents in cell-attached patches from freshly dispersed coronary artery myocytes
Aa, bath application of 100 nM ET-1 induced cation channel activity at an applied patch voltage of +70 mV. According to convention we will refer to this as −70 mV membrane potential throughout the text (see Methods). *Ab*, amplitude histogram of channel currents shown in *Aa* could be fitted with the sum of four Gaussian curves indicating 1 closed and 3 multiple open levels inferring that the patch contained at least 3 channels. *B* and *C*, ET-1 evoked cation channel activity in the presence of respectively either the ET_A receptor antagonist 100 nM BQ-123 or the ET_B receptor antagonist 100 nM BQ-788. *D* and *F*, ET-1-induced cation channel activity was blocked in the presence of a mixture of 100 nM BQ-123 and 100 nv BQ-788 at −70 mV. *E*, *I–V* relationship of cation channel currents evoked by ET-1 (open circles), ET-1 in the presence of BQ-123 (open squares) and ET-1 in the presence of BQ-788 (filled squares) showing that they all had a unitary conductance of 2.6 pS and E_r of about 0 mV. Each point represents at least $n = 6$. *F*, mean data showing neither BQ-123 nor BQ-788 inhibited ET-1-evoked channel activity when applied separately. However when the antagonists were added together ET-1-evoked cation channel activity was abolished. Each value is the mean of 10 patches.

ET-1-evoked channel activity shown in Fig. 1*Aa* could be fitted by the sum of four Gaussian curves representing one closed and three open levels of the same conductance, i.e. there were at least three channels in the patch. Figure 1*B* and *F* shows that pre-treatment with the ET_A receptor antagonist 100 nM BQ-123 for 5 min had no effect on ET-1-induced channel activity ($n = 10$). In addition, Fig. 1*C* and *F* shows that pre-treatment with the ET_B receptor antagonist 100 nM BQ-788 for 5 min also had no effect on ET-1-evoked channel activity ($n = 10$). Figure 1*E* shows that ET-1 activated the same 2.6 pS channel currents in the presence of either BQ-123 or BQ-788 and in the absence of receptor antagonists. However, Fig. 1*D* and *F* illustrates that that pre-treatment with co-application of both 100 nM BQ-123 and 100 nM BQ-788 for 5 min almost completely abolished ET-1-induced channel activity ($n = 8$, $P < 0.001$).

These data showing that both ET_A and ET_B receptor antagonists must be present to block channel activity by ET-1 indicate that stimulation of both ET_A and ET_B receptors can lead to channel opening.

Stimulation of ET_A and ET_B receptors activates TRPC1 channel currents through a PKC-dependent mechanism

Previously we have shown that ET-1 and agents that deplete internal Ca^{2+} stores, cyclopiazonic acid (CPA) and BAPTA-AM, evoke native 2.6 pS TRPC1 channel currents in coronary artery myocytes which are inhibited by PKC inhibitors (Saleh *et al.* 2008; Albert *et al.* 2009). Therefore we investigated the role of PKC and TRPC1 subunits in mediating both ET_A and ET_B receptor-mediated channel activity. In these experiments we bath applied ET-1 in the presence of either BQ-788 or BQ-123 to evoke respectively ET_A or ET_B receptor-coupled pathways in cell-attached patches.

Figure 2*A* shows that the mean NP_o of ET_A receptor-mediated channel activity, activated by 100 nM ET-1 in the presence of 100 nM BQ-788, was significantly reduced from 0.37 ± 0.04 to 0.06 ± 0.01 ($83 \pm 5\%$ inhibition, $n = 6$, $P < 0.01$) by the PKC inhibitor chelerythrine (3 μM). Figure 2*B* shows that the mean

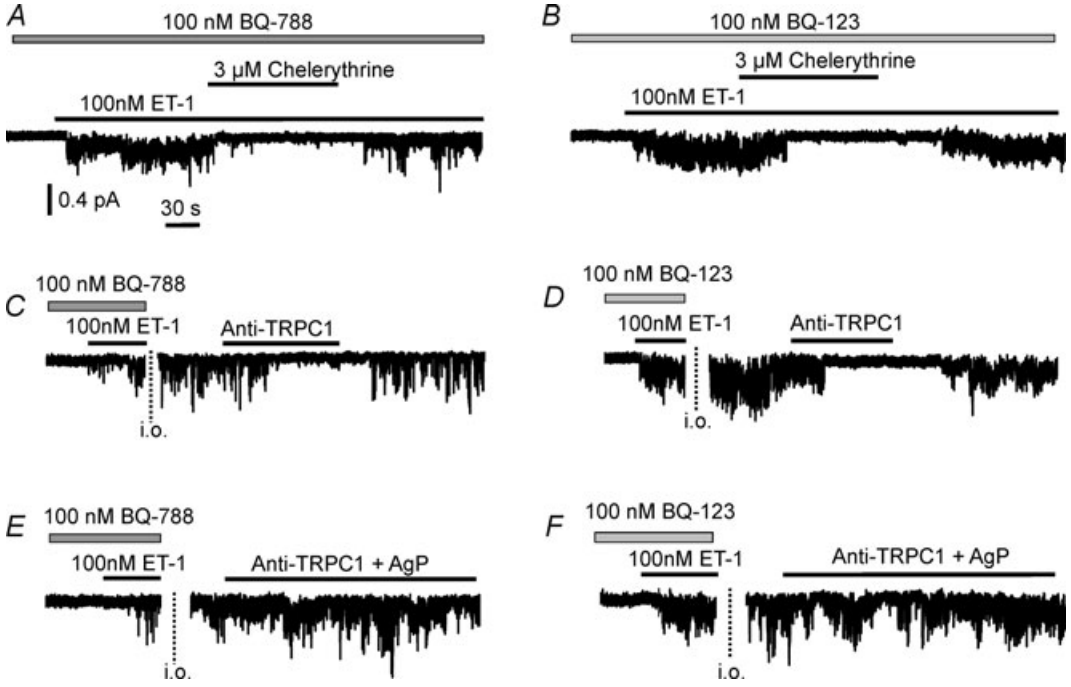

Figure 2. Stimulation of ET_A and ET_B receptors activates native TRPC1 channel currents via a PKC-dependent mechanism
A and *B*, stimulation of ET_A (100 nM BQ-788 present) or ET_B receptors (100 nM BQ-123 present) with 100 nM ET-1 evoked channel activity that was inhibited by co-application of 3 μM chelerythrine in cell-attached patches held at −70 mV. *C* and *D*, stimulation of respectively ET_A and ET_B receptors evoked channel activity initially induced in cell-attached patches which was inhibited by application of 1 : 200 dilution of anti-TRPC1 antibodies following excision of patches into the inside-out configuration (i/o). *E* and *F*, following pre-incubation of anti-TRPC1 antibodies (1 : 200) with its antigenic peptide (1 : 100, AgP) bath application of the complex had no effect on ET_A receptor-mediated or ET_B receptor-mediated channel activity.

NP_o of ET_B receptor-mediated channel activity, activated by 100 nM ET-1 in the presence of 100 nM BQ-123, was also significantly inhibited from 0.31 ± 0.08 to 0.05 ± 0.02 ($84 \pm 3\%$ inhibition, $n = 7$, $P < 0.01$) by 3 μM chelerythrine.

Figure 2*C* illustrates that ET_A receptor-mediated channel activity in cell-attached patches was maintained following excision into the inside-out configuration and that bath application of anti-TRPC1 antibodies to the cytosolic surface of these inside-out patches significantly reduced mean NP_o of ET_A receptor-mediated activity from 0.19 ± 0.03 to 0.01 ± 0.01 ($95 \pm 5\%$ inhibition, $n = 5$, $P < 0.01$). Moreover Fig. 2*D* shows that anti-TRPC1 antibodies also significantly inhibited the mean NP_o of ET_B receptor-mediated channel activity from 0.22 ± 0.08 to 0.03 ± 0.02 ($87 \pm 8\%$ inhibition, $n = 5$, $P < 0.01$). In control experiments, Fig. 2*E* and *F* show that following pre-incubation with their antigenic peptide, anti-TRPC1 antibodies had no effect on ET_A receptor-mediated or ET_B receptor-mediated channel activity ($n = 4$ for each). Channel activity often recovered, at least partially, following washout of anti-TRPC1 antibodies indicating some degree of reversibility in the conditions used.

These data show that stimulation of both ET_A and ET_B receptors activates TRPC1 channel currents through a PKC-dependent mechanism in coronary artery myocytes.

Distinct signalling pathways mediate ET_A and ET_B receptor stimulation of TRPC1 channel activity

In the next series of experiments we investigated the signalling pathways linking ET_A and ET_B receptors to PKC-mediated opening of TRPC1 channels. ET_A and ET_B G-protein-coupled receptors can be linked to different phospholipases that generate the endogenous PKC activator diacylglycerol (DAG, Ivey *et al.* 2008). Therefore we investigated the effect of biochemically characterised pharmacological inhibitors of endogenous phospholipases on ET_A and ET_B receptor-mediated TRPC1 channel activity in cell-attached patches.

Figure 3*A* shows that the phosphoinositol-phospholipase C (PI-PLC) inhibitor U73122 (2 μM) significantly inhibited the mean NP_o of ET_B receptor-mediated TRPC1 channel activity from 0.26 ± 0.05 to 0.04 ± 0.02 ($89 \pm 4\%$ inhibition, $n = 7$, $P < 0.01$) whereas Fig. 3*B* demonstrates that this PI-PLC inhibitor had no effect on ET_A receptor-mediated TRPC1 channel activity (control mean NP_o was 0.21 ± 0.06 and 0.18 ± 0.05 in U73122, $n = 7$). In addition, 2 μM U73343, an inactive analogue of U73122, had no effect on ET_B receptor-mediated TRPC1 channel activity ($n = 4$, data not shown).

The above studies indicate that a PI-PLC-mediated mechanism couples ET_B receptors to TRPC1 channel

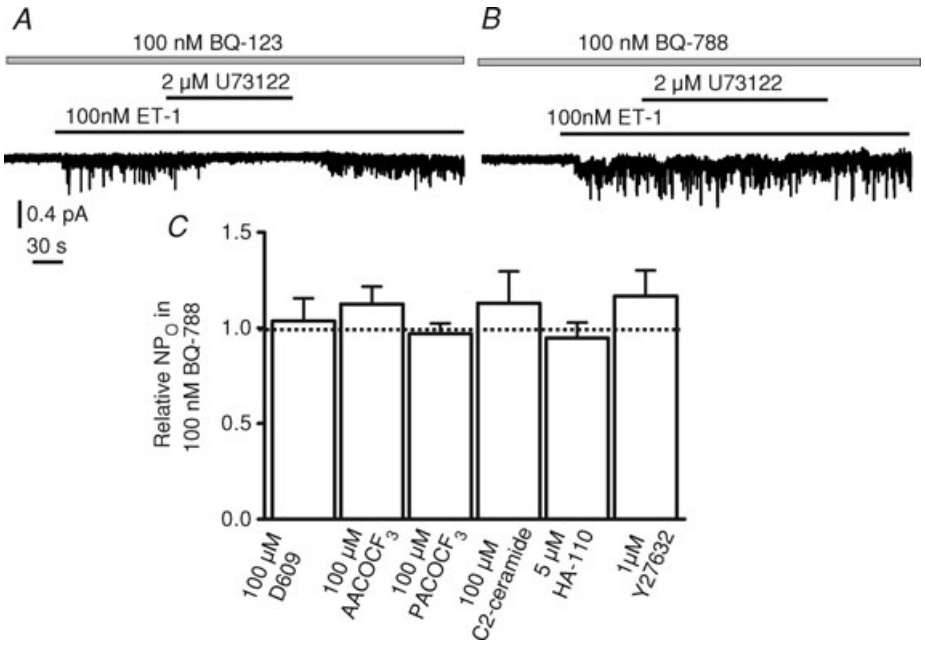

Figure 3. Stimulation of ET_B, but not ET_A, receptors activates TRPC1 channel currents via a PLC-mediated transduction pathway in cell-attached patches
A, ET_B receptor-mediated TRPC1 channel activity was inhibited by co-application of 2 μM U73122 at −70 mV. *B*, ET_A receptor-mediated TRPC1 channel activity was unaffected by co-application of 2 μM U73122. *C*, mean data showing that ET_A receptor-mediated TRPC1 channel activity (ET-1-evoked NP_o in the presence of 100 nM BQ-788) at −70 mV was also unaffected by co-application of inhibitors against different phospholipases and Rho kinase (see text for details).

stimulation but is unlikely to be involved in activating native TRPC1 channels through stimulation of ET_A receptors. Therefore we investigated the effects of several established inhibitors of other phospholipases that may be involved in ET_A receptor-mediated activation of TRPC1 channel currents. Figure 3*C* shows that pharmacological inhibitors of phosphatidylcholine-PLC (PC-PLC, 100 μM D-609, $n = 5$), cytosolic and Ca^{2+}-dependent and -independent forms of phospholipase A_2 (PLA_2, 100 μM $AACOCF_3$, $n = 4$ and 100 μM $PACOCF_3$, $n = 4$) and phospholipase D (PLD, 100 μM C2-ceramide, $n = 6$) had no effect on ET_A receptor-mediated TRPC1 channel activity. Stimulation of ET_A receptors has also been shown to activate Rho kinase (Ivey *et al.* 2008) but Fig. 3*C* shows that the Rho kinase inhibitors HA-110 (5 μM, $n = 5$) and Y27632 (1 μM, $n = 5$) had no effect on ET_A receptor-mediated TRPC1 channel activity. These data suggest that PC-PLC, PLA_2, PLD and Rho kinase are also not involved in TRPC1 channel activation initiated by ET_A receptor stimulation.

Previous studies have shown that stimulation of ET_A receptors can activate phosphoinositol-3-kinase (PI-3-kinase), which phosphorylates PIP_2 to form PIP_3, with the latter phospholipid capable of stimulating PKC activity (see review by Ivey *et al.* 2008). Therefore we investigated the role of a PI-3-kinase-mediated mechanism on ET_A and ET_B receptor-mediated TRPC1 channel activity in cell-attached patches using selective concentrations of wortmannin and a structurally different compound, PI-828, which both inhibit PI-3-kinase. Figure 4*A* shows that 50 nM wortmannin significantly reduced mean NP_o of ET_A receptor-mediated TRPC1 channel activity from 0.23 ± 0.06 to 0.02 ± 0.01 ($94 \pm 4\%$ inhibition, $n = 6$, $P < 0.01$). In addition Fig. 4*B* shows that 3 μM PI-828 also significantly attenuated mean NP_o of ET_A receptor-mediated TRPC1 channel activity from 0.14 ± 0.04 to 0.02 ± 0.01 ($85 \pm 5\%$ inhibition, $n = 5$, $P < 0.01$). Importantly, Fig. 4*C* and *D* illustrates that 50 nM wortmannin (control mean NP_o of 0.18 ± 0.06 and 0.18 ± 0.07 in wortmannin, $n = 6$) and 3 μM PI-828 (control mean NP_o of 0.31 ± 0.11 and 0.24 ± 0.08 in PI-828, $n = 6$) had no effect on ET_B receptor-mediated TRPC1 channel activity indicating that these reagents do not have direct non-specific effects on TRPC1 channel currents.

These results provide evidence that stimulation of ET_A and ET_B receptors evokes TRPC1 channel activity via different signal transduction mechanisms. Stimulation of ET_A receptors is coupled to TRPC1 channels via a PI-3-kinase-dependent pathway whereas a PI-PLC-dependent pathway links ET_B receptor-mediated TRPC1 channel opening, and both these pathways are likely to induce TRPC1 channel activity through a PKC-dependent mechanism.

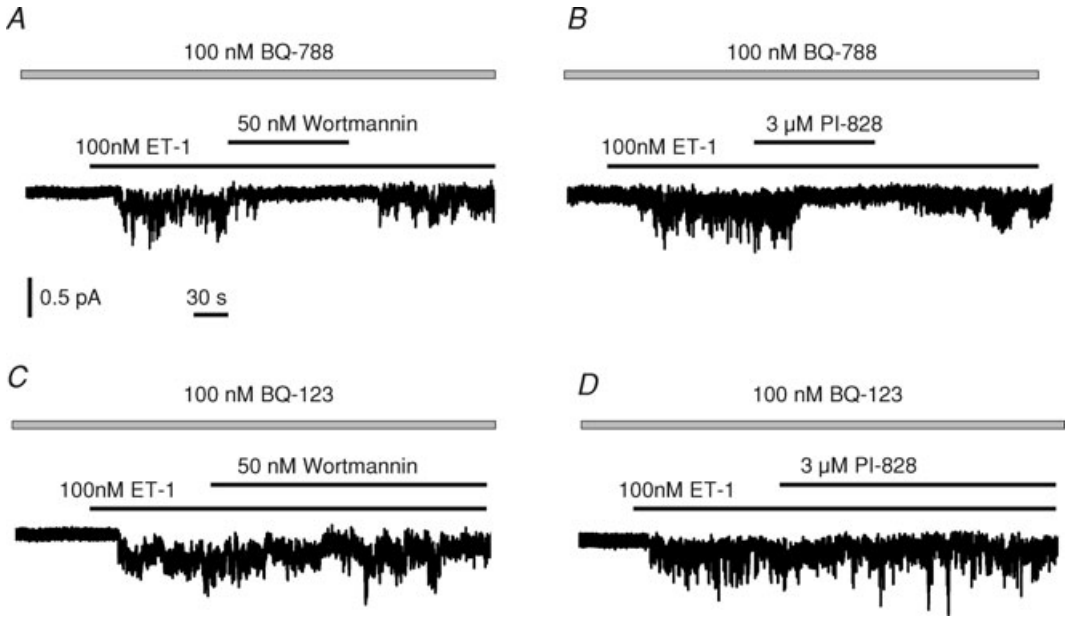

Figure 4. Stimulation of ET_A receptors activates TRPC1 channel currents via a PI-3-kinase-mediated pathway in cell-attached patches
A and *B* show that ET_A receptor-mediated TRPC1 channel activity was inhibited by co-application of 50 nM wortmannin or 3 μM PI-828, whereas *C* and *D* show that these compounds had no effect on ET_B receptor-mediated TRPC1 channel activity.

Involvement of PIP_3 in ET_A receptor-mediated activation of TRPC1 channel currents

The above results suggest that generation of PIP_3 produced from the action of PI-3-kinase on PIP_2 is required for ET_A receptor-mediated TRPC1 channel stimulation. Consequently we investigated if exogenous PIP_3 directly activates TRPC1 channel currents in coronary artery myocytes.

Figure 5*A* and *B* shows that bath application of 3 μM diC8-PIP_3, a water soluble form of PIP_3, to the cytosolic surface of inside-out patches activated cation channel activity with a mean NP_o of 0.32 ± 0.06 ($n = 11$) and a unitary conductance of 2.6 pS with an E_r of about 0 mV. The threshold concentration of diC8-PIP_3 was approximately 1 μM and maximum channel activation was obtained with 10–20 μM diC8-PIP_3 (data not shown). Figure 5*C* and *D* also illustrates that mean NP_o of diC8-PIP_3-evoked channel activity in inside-out patches was significantly inhibited from 0.21 ± 0.09 to 0.02 ± 0.02 ($94 \pm 4\%$ inhibition, $n = 5$, $P < 0.01$) by co-application with 3 μM chelerythrine and from 0.28 ± 0.05 to 0.03 ± 0.02 ($84 \pm 7\%$ inhibition, $n = 5$, $P < 0.01$) with anti-TRPC1 antibodies.

The role of endogenous PIP_3 in ET_A receptor-mediated stimulation of TRPC1 channel activity was investigated using an anti-PIP_3 antibody. Figure 5*E* shows that the mean NP_o of ET_A receptor-mediated TRPC1 channel stimulation, initially activated in cell-attached patches in the presence of the ET_B receptor antagonist BQ-788, was significantly reduced from 0.25 ± 0.07 to 0.01 ± 0.01 ($97 \pm 1\%$ inhibition, $n = 6$, $P < 0.01$) by bath application of an anti-PIP_3 antibody to the cytosolic surface of the patches. In contrast, Fig. 5*F* illustrates that an anti-PIP_3 antibody had no effect on ET_B receptor-mediated TRPC1 channel activity (control mean NP_o from 0.48 ± 0.08 to 0.51 ± 0.11 in anti-PIP_3 antibody, $n = 6$).

These data clearly show that exogenous PIP_3 and ET-1 activate the same PKC-dependent TRPC1 channel currents and also indicate that endogenous PIP_3 mediates activation of TRPC1 channel currents by ET_A receptor stimulation.

Involvement of PIP_2 in ET_B receptor-mediated activation of TRPC1 channel currents

The above results indicate that ET_B receptor-mediated TRPC1 channel activity is coupled to a PI-PLC pathway and to stimulation of PKC (see Figs 2 and 3). Previous work suggests that this biochemical cascade is likely to involve generation of DAG, through hydrolysis of PIP_2

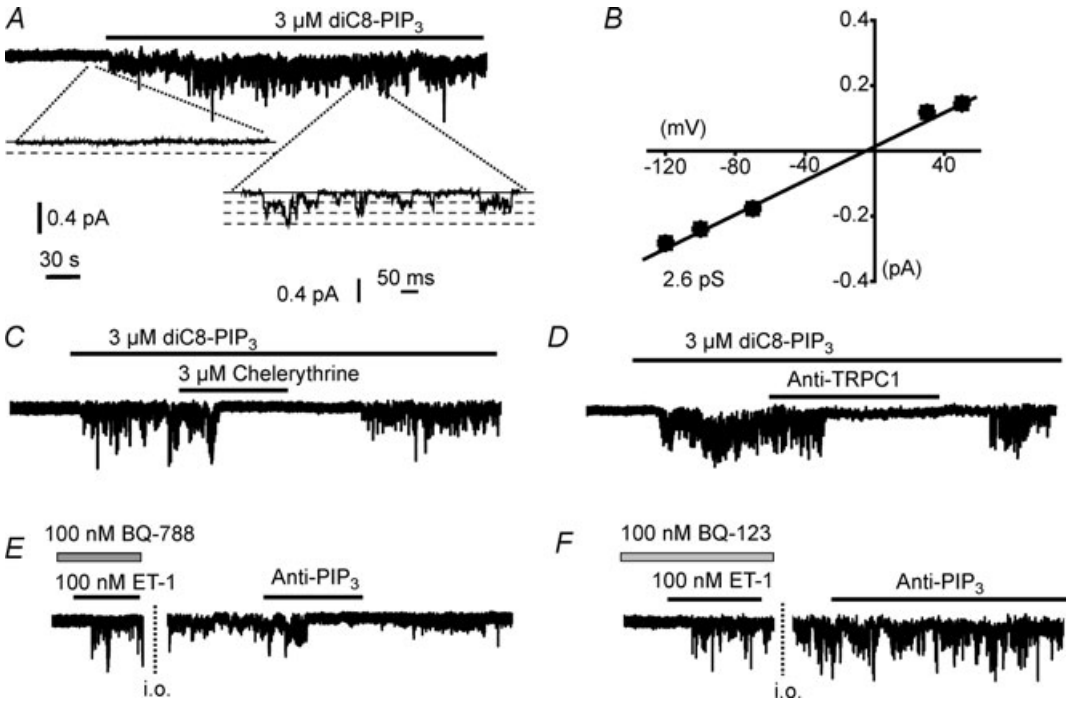

Figure 5. PIP_3 activates TRPC1 channel currents via a PKC-dependent mechanism
A, bath application of 3 μM diC8-PIP_3 activates cation channel activity in an inside-out patch at −70 mV. *B*, current–voltage relation of diC8-PIP_3-evoked cation channel activity yielded a unitary conductance of 2.6 pS and an E_r of about 0 mV. Each point was at least $n = 4$. *C* and *D*, respectively 3 μM chelerythrine and 1 : 200 dilution of anti-TRPC1 antibodies inhibited diC8-PIP_3-induced channel activity in inside-out patches at −70 mV. *E* and *F*, co-application of 1 : 200 dilution of anti-PIP_3 antibodies reduced ET_A receptor-mediated TRPC1 channel activity (*E*) but had no effect on ET_B receptor-mediated TRPC1 channel activity (*F*).

by PI-PLC, since 1-oleoyl-2-acetyl-*sn*-glycerol (OAG), a cell-permeant DAG analogue, activates TRPC1 channel activity through a PKC-dependent mechanism in rabbit mesenteric artery, portal vein and also coronary artery (see Albert & Large, 2002; Saleh *et al.* 2006, 2008). Moreover, our recent findings extended this hypothesis by proposing an obligatory role for PIP_2 in PKC-dependent activation of TRPC1 channels in portal vein smooth muscle cells (Saleh *et al.* 2009) and therefore we investigated the effects of PIP_2 in coronary artery myocytes.

Bath application of 10 μM diC8-PIP_2 to inside-out patches induced cation channel activity which had a mean NP_o of 0.34 ± 0.11 at -70 mV ($n = 11$) and a unitary conductance of 2.6 pS and an E_r of about 0 mV (Fig. 6*Aa* and *b*). In addition, the mean NP_o of diC8-PIP_2-evoked channel activity was significantly reduced by co-application of anti-TRPC1 antibodies (from 0.24 ± 0.08 to 0.02 ± 0.01, $88 \pm 6\%$ inhibition, $n = 5$, $P < 0.01$, Fig. 6*B*) and by 3 μM chelerythrine (from 0.31 ± 0.08 to 0.05 ± 0.03, $97 \pm 6\%$ inhibition, $n = 6$, $P < 0.01$, Fig. 6*C*). These data provide evidence that exogenous PIP_2 activates TRPC1 channel currents via a PKC-dependent mechanism.

OAG-evoked channel activity was significantly inhibited by anti-TRPC1 antibodies (Fig. 6*D*, mean NP_o from 0.23 ± 0.08 to 0.03 ± 0.01, $87 \pm 1\%$ inhibition, $n = 4$, $P < 0.01$) and by anti-PIP_2 antibodies (Fig. 6*E*, mean NP_o from 0.32 ± 0.06 to 0.01 ± 0.01, $97 \pm 1\%$ inhibition, $n = 6$, $P < 0.01$) in inside-out patches. Figure 6*F* also shows that when tissues were pre-treated with 20 μM wortmannin for 30 min to deplete tissue PIP_2 levels (see Fig. 7) OAG did not evoke TRPC1 channel activity in cell-attached patches (mean NP_o of 0.01 ± 0.01, $n = 6$). These results suggest that endogenous PIP_2 has an obligatory role for OAG-evoked TRPC1 channel activation.

These studies demonstrate that stimulation of ET_B receptors induces TRPC1 channel activation through stimulation of PI-PLC to generate DAG which activates PKC leading to channel opening through a mechanism involving endogenous PIP_2.

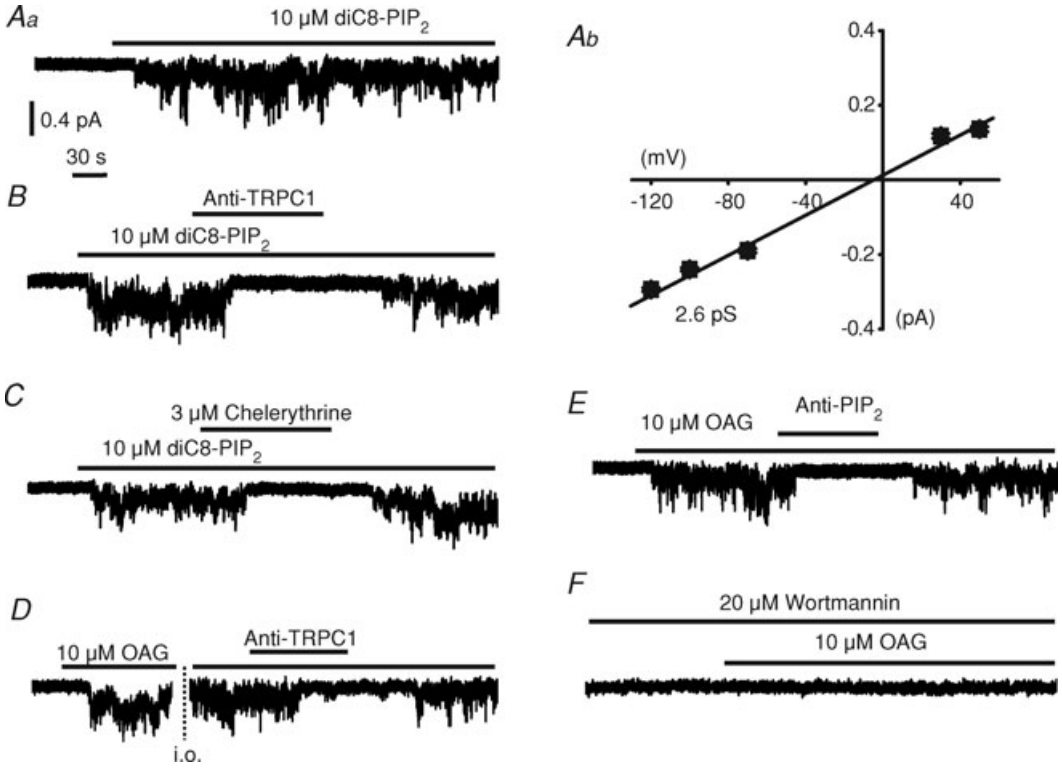

Figure 6. Obligatory role of PIP_2 in mediating OAG-induced TRPC1 channel activity via a PKC-dependent mechanism

Aa and *b*, bath application of 10 μM diC8-PIP_2 activates cation channel activity in inside-out patches at -70 mV which has a unitary conductance of 2.6 pS and a E_r of about 0 mV. *B* and *C*, diC8-PIP_2-evoked channel activity is inhibited by 1 : 200 anti-TRPC1 antibodies (*B*) and also by 3 μM chelerythrine (*C*). *D* and *E*, OAG-induced channel activity is inhibited by 1 : 200 anti-TRPC1 antibodies (*D*) and also by 1 : 200 anti-PIP_2 antibodies (*E*) in inside-out patches held at -70 mV. *F*, pre-treatment with 20 μM wortmannin for 30 min prevented activation of TRPC1 activity by OAG in a cell-attached patches at -70 mV.

PIP_3 evokes TRPC1 channel activity independently of PIP_2

The present work shows that stimulation of ET_A receptors activates TRPC1 activity through a PI-3-kinase-mediated pathway involving PIP_3 (see Figs 4 and 5). Moreover we demonstrate that exogenous diC8-PIP_3 evokes PKC-dependent TRPC1 channel activity (see Fig. 5). In contrast, our data indicate that activation of TRPC1 channels by stimulation of ET_B receptors involves a permissive role for PIP_2. In a previous report we stated that PIP_2 had an obligatory role for TRPC1 channel activation in rabbit portal vein myocytes (Saleh *et al.* 2009). Therefore we investigated whether endogenous PIP_2 was necessary for activation of TRPC1 channels by PIP_3 in coronary artery smooth muscle cells.

Figure 7*A* shows that following pre-treatment of myocytes with 20 μM wortmannin for 30 min to deplete PIP_2 levels (see Fig. 5*Ca*) bath application of 3 μM diC8-PIP_3 activated TRPC1 channel activity with a mean peak NP_o value of 0.31 ± 0.05 ($n = 6$) in inside-out patches, which is similar to control values of channel activity induced by 3 μM PIP_3 in the absence of wortmannin (see above and Fig. 7*Aa*). Figure 7*B* shows that anti-PIP_2 antibodies had no effect on PIP_3-induced TRPC1 channel activity in inside-out patches (control mean NP_o of 0.23 ± 0.06 and 0.24 ± 0.08 in the presence of anti-PIP_2 antibodies, $n = 6$). Both of these procedures blocked OAG-evoked TRPC1 channel activity (cf. Fig. 6*E* and *F*). In other experiments the anti-PIP_2 antibody reduced TRPC1 channel activation by both ET_A and ET_B receptor stimulation (data not shown). This is predictable since PIP_2 acts as a substrate for PIP_3 generated by PI-3-kinase (ET_A pathway) and DAG produced by PI-PLC (ET_B pathway).

Therefore with regard to direct TRPC1 channel activation endogenous PIP_2 is not obligatory for TRPC1 channel activation by PIP_3 (ET_A receptor pathway) but

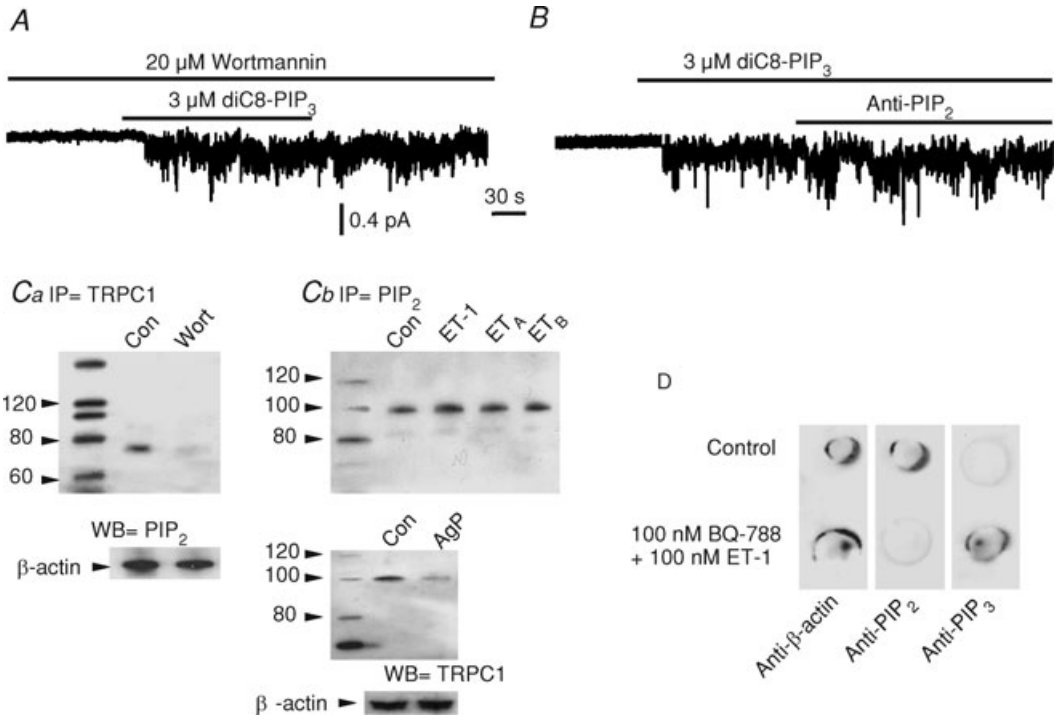

Figure 7. PIP_3 activation of TRPC1 channel activity is independent of PIP_2
A, diC8-PIP_3-evoked TRPC1 channel activity in inside-out patches held at −70 mV was not inhibited by pre-treatment with 20 μM wortmannin for 30 min. *B*, 1 : 200 anti-PIP_2 antibodies did not inhibit diC8-PIP_3-mediated channel activation. *Ca*, co-immunoprecipitation experiment showing association between PIP_2 and TRPC1 proteins at rest (Con) after immunoprecipitation with anti-TRPC1 antibodies and blotting with anti-PIP_2 antibodies which was reduced following pre-treatment with 20 μM wortmannin (Wort). *Cb*, upper panel shows co-immunoprecipitation experiment following immunoprecipitation with anti-PIP_2 antibodies and blotting with anti-TRPC1 antibodies illustrating that PIP_2 association with TRPC1 proteins is unaltered following pre-treatment with 100 nM ET-1 and stimulation of ET_A (100 nM ET-1 + 100 nM BQ-788) or ET_B (100 nM ET-1 + 100 nM BQ-123) receptors. Lower panel shows a Western blot in which following preincubation with its antigenic peptide (AgP), detection of TRPC1 protein with anti-TRPC1 antibodies was reduced. *Ca* and *b* also show that wortmannin and antigenic peptide had no effect on expression of β-actin proteins. *D*, dot-blot showing the presence of PIP_2 but not PIP_3 levels in tissue lysates at rest and the reduction of PIP_2 levels and increase of PIP_3 levels following stimulation of ET_A receptors.

is necessary for OAG (DAG)-induced (ET_B receptor pathway) TRPC1 channel stimulation.

To further investigate the role of PIP_2 and PIP_3 in mediating ET-1-induced TRPC1 channel activation we carried out co-immunoprecipitation and dot-blot studies. Figure 7*Ca* illustrates a co-immunoprecipitation experiment which shows that at rest PIP_2 is associated with TRPC1 proteins in coronary artery when tissue lysates were immunoprecipitated with anti-TRPC1 antibodies and then blotted with anti-PIP_2 antibodies to detect a predicted band of ∼75 kDa (see Methods). In addition Fig. 7*Ca* shows that pre-treatment of coronary arteries with 20 μM wortmannin for 30 min reduced PIP_2 association with TRPC1 proteins whereas total β-actin levels were not altered. The upper panel in Fig. 7*Cb* shows that stimulation of ET_A or ET_B receptors did not alter PIP_2 association with TRPC1 proteins following immunoprecipitation with anti-PIP_2 antibodies and blotting with anti-TRPC1 antibodies to detect a predicted band of ∼100 kDa (see Methods). The middle panel shows a control experiment in which pre-incubation of the anti-TRPC1 antibody with its antigenic peptide (AgP) reduced the detection of the predicted band for TRPC1 proteins on a Western blot. The lower panel shows that the antigenic peptide had no effect on the expression of β-actin.

It was not possible to detect total PIP_3 levels using Western blotting or association between PIP_3 and TRPC1 proteins using co-immunoprecipitation at rest or after stimulation of ET_A and ET_B receptors. This is probably due to resting and receptor-mediated generation of PIP_3 levels being too small to resolve with the limited amounts of available coronary artery tissue. Therefore we measured PIP_3 and PIP_2 levels using tissue lysate and dot-blot techniques with their respective antibodies. Figure 7*D* illustrates that at rest total cell lysates from coronary arteries contained detectable PIP_2 but not PIP_3 whereas upon stimulation of ET_A receptors (ET-1 in the presence of BQ-788) the levels of PIP_2 were reduced and generation of PIP_3 was detected whereas levels of β-actin were unaffected.

These data provide novel evidence that PIP_3 can activate TRPC1 channels independently of PIP_2 in coronary artery myocytes.

Discussion

The present work provides the first evidence that stimulation of ET_A and ET_B receptors by ET-1 activates native TRPC1 channel currents in freshly dispersed coronary artery myocytes by two distinct parallel phosphoinositide signalling pathways. Evidence is provided to show that stimulation of ET_A receptors evokes TRPC1 channel currents through PI-3-kinase-mediated generation of PIP_3 which leads to opening of TRPC1 channels, possibly by a direct action. In contrast ET_B receptors are coupled to PI-PLC and production of DAG leading to PIP_2-mediated TRPC1 channel activation. Moreover it appears that PKC is involved in activation of TRPC1 channel currents by both PIP_3 and PIP_2. Previously we have shown a permissive role for PIP_2 in activating TRPC1 channels in rabbit portal vein myocytes (Saleh *et al.* 2009) but this is the first demonstration that PIP_3 also activates native TRPC1 channel currents. Furthermore, to our knowledge, this is the first evidence that PI-3-kinase may be involved in activation of TRPC1 channel. Importantly, this pathway involving PI-3-kinase-mediated generation of PIP_3 represents a novel activation mechanism of TRPC channels.

ET_A receptor transduction mechanism and activation of TRPC1 channel currents

ET_A receptor-mediated stimulation of TRPC1 channel activity is blocked by PI-3-kinase inhibitors and by an anti-PIP_3 antibody which did not inhibit TRPC1 channel activation induced by ET_B receptor stimulation. Moreover exogenous PIP_3 applied to inside-out patches evoked cation channel currents with identical properties to those stimulated by ET-1, i.e. native TRPC1 channels. Importantly, PIP_3-induced TRPC1 channel activation did not require endogenous PIP_2 since PIP_3 readily activated TRPC1 channel currents in tissues pre-treated with high concentrations of wortmannin, which reduced association of PIP_2 with TRPC1. Moreover an anti-PIP_2 antibody which blocked responses to OAG did not inhibit PIP_3-evoked TRPC1 channel activity. Thus generation of PIP_3 by stimulation of ET_A receptors activates TRPC1 channels with PIP_3 possibly being the activating ligand, which represents a novel mechanism of ion channel activation.

Stimulation of ET_A receptors expressed in Chinese hamster ovary cells has been shown to increase PI-3-kinase activity and PIP_3 formation which was inhibited by low concentrations of wortmannin (Sugawara *et al.* 1996). Our data also show that ET_A receptor stimulation increases PIP_3 production. In vascular smooth muscle ET-1 receptor stimulation leads to activation of several signalling pathways including PI-3-kinase (see review by Boualleque *et al.* 2007) and this mechanism is involved in vasoconstriction (Kawanabe *et al.* 2004). Previously PIP_3 has been shown to bind to expressed TRPC1 proteins (Kwon *et al.* 2007) although another study suggested that PIP_3 did not activate expressed TRPC1 channels (Tseng *et al.* 2004). However in the same work it was shown that PIP_3 produces marked stimulation of TRPC6 channel activity (Tseng *et al.* 2004). Previously we indicated that the 2.6 pS ET-1-induced conductance in coronary artery

myocytes may be a heteromeric channel consisting of TRPC1, TRPC5 and TRPC6 subunits (Saleh *et al.* 2008). Therefore it is possible that the heteromeric structure of native TRPC1 channels is more sensitive to PIP_3 then heterologously expressed TRPC1 proteins or that PIP_3 binds to proposed TRPC5 or TRPC6 subunits of the native conductance in coronary artery myocytes.

The present work does not reveal how ET_A receptors are linked to PI-3-kinase in coronary arteries but in other systems it has been shown, and is generally accepted, that $G_{\beta\gamma}$ subunits activate PI-3-kinase (see Clapham & Neer, 1997; Vanhaesebroeck *et al.* 1997).

ET_B receptor transduction mechanism and activation of TRPC1 channel currents

The present work shows that ET_B receptor-induced stimulation of TRPC1 channel activity was markedly inhibited by the PI-PLC inhibitor U73122, which did not effect ET_A receptor-mediated activation of TRPC1 channel activity. In addition OAG, an analogue of DAG which is a product of PI-PLC stimulation, induced TRPC1 channel activity which was also inhibited by an anti-PIP_2 antibody. Moreover OAG did not evoke TRPC1 channel activity in cells pre-treated with high concentrations of wortmannin, which depleted tissue PIP_2 levels. These electrophysiological data are consistent with a pathway in which ET_B receptors are coupled to PI-PLC, which generates DAG and subsequently induces PIP_2-mediated activation of TRPC1 channels.

Application of exogenous PIP_2 evoked TRPC1 channel currents and co-immunoprecipitation studies showed that PIP_2 co-associated with TRPC1 proteins in resting and ET-1-stimulated tissues. This finding is similar to a previous study in rabbit portal vein myocytes in which it was concluded that PIP_2 is tethered to TRPC1 proteins at rest but PKC-mediated phosphorylation of TRPC1 proteins was necessary to cause channel opening (Saleh *et al.* 2009, see Large *et al.* 2009 for more detail). We propose that a similar mechanism may be important for ET_B receptor stimulation in coronary artery myocytes.

Therefore the present work shows that both PIP_2 and PIP_3 can activate TRPC1 channels in coronary artery myocytes and our evidence is that PIP_3 is obligatory for ET_A receptor-mediated stimulation of TRPC1 channels whereas PIP_2 is necessary for ET_B receptor-mediated activation of the same ion channel.

An interesting observation is that ET_A and ET_B receptor-mediated TRPC1 channel activity is not additive and that antagonism of both ET_A and ET_B receptors is required to block ET-1-induced activation of TRPC1 channels. This suggests that both pathways were equally effective in activating TRPC1 channels with the conditions used in our experiments and may indicate a safeguard mechanism for channel activation. Moreover these data indicate how two receptor subtypes converge onto the same TRPC1 channel utilising different transduction pathways.

Role of PKC in activation mechanism of native TRPC1 channels by ET_A and ET_B receptor stimulation

Stimulation of TRPC1 activity by both ET_A and ET_B receptors in coronary artery myocytes was almost abolished by the PKC inhibitor chelerythrine. In addition, the responses of PIP_3 and PIP_2, the proposed mediators of respectively ET_A and ET_B receptors stimulation, were also blocked by chelerythrine. Therefore it is evident that PKC plays a central role in the activation mechanism of TRPC1 channels by ET-1. Previously we demonstrated in rabbit portal vein myocytes that TRPC1 channel activation by the sarcoplasmic reticulum Ca^{2+}-ATPase inhibitor cyclopiazonic acid (CPA), phorbol 12,13-dibutyrate (PDBu), a PKC stimulant, and PIP_2 was associated with phosphorylation of TRPC1 proteins which was inhibited by chelerythrine (Saleh *et al.* 2009). Ahmmed *et al.* (2004) also demonstrated that PKC-evoked phosphorylation of expressed TRPC1 channels regulated store-operated Ca^{2+} entry in cultured endothelial cells. Importantly, the present work adds significant support for the postulated activation mechanism of TRPC1 channels (see Large *et al.* 2009 for fully explanation) by showing that PIP_3, another notable endogenous phospholipid, also acts as a stimulatory ligand of TRPC1 channels and requires a PKC-dependent process which is likely to involve phosphorylation of TRPC1 subunits. In future experiments it will be interesting to investigate the molecular basis of PIP_3/PIP_2-mediated activation mechanisms of native TRPC1 channels using expressed heterotetrameric channels involving TRPC1 subunits.

It has been shown that PIP_3 also activates some PKC isoforms *in vitro* (Nakanishi *et al.* 1993). Therefore on ET_A receptor stimulation production of PIP_3 is likely both to activate PKC and also to activate TRPC1 channels, which leads to opening of channels through a positive feedback process in which increased PKC-dependent phosphorylation of TRPC1 proteins results in greater PIP_3-mediated channel activity. A similar transduction mechanism has been proposed to link expressed M_2 muscarinic receptors to an endogenous chloride channel in *Xenopus* oocytes (Wang *et al.* 1999).

The observation that bath application of ET-1 evoked channel activity recorded in a cell-attached patch suggests that important signalling molecule(s) outlined above translocate from receptors stimulated outside the patch to ion channels underneath the pipette tip. A characteristic of native TRPC channels is that once these signalling pathways are activated by bath applied agonists in

the cell-attached configuration channel activity persists after excision into the inside-out configuration. In this configuration there is no agonist present and it is possible that processes that normally inhibit channel activity are lost (e.g. cytosolic factors) when the membrane patch is excised.

Multiple transduction mechanisms and TRPC channels in vascular smooth muscle

In cell lines, receptor-mediated activation of expressed TRPC channels is generally shown to be via stimulation of $G_{\alpha q/11}$ and activation of PI-PLC (e.g. see Hardie, 2007), but in vascular smooth muscle more diverse signalling pathways are involved. Therefore α_1-adrenoceptors and angiotensin II (Ang II) receptors are coupled to TRPC6 channels via PI-PLC in respectively rabbit portal vein and mesenteric artery myocytes (Helliwell & Large, 1997; Inoue *et al.* 2001; Saleh *et al.* 2006). In contrast constitutive TRPC3 channels in rabbit ear artery myocytes are coupled to $G_{\alpha i/o}$ proteins linked PC-PLD-induced production of DAG (Albert & Large, 2004; Albert *et al.* 2005, 2006). The present work adds yet another signalling cascade for TRPC channels in which ET_A receptor stimulation causes PI-3-kinase-mediated production of PIP_3 to activate TRPC1 channels.

Agents that deplete intracellular Ca^{2+} stores also stimulate TRPC1 channel activity and therefore these channels are often termed store-operated channels (SOCs). The present results with ET-1 and previous work with noradrenaline in portal vein (Albert & Large, 2002) and Ang II in mesenteric artery (Saleh *et al.* 2006) indicate that membrane-delimited lipid pathways induce TRPC1 channel activity in isolated patches. Consequently TRPC1 channels behave more as receptor-operated channels than as SOCs according to their strict definition.

Phospholipids and TRPC channels

There is increasing evidence that phospholipids regulate transient receptor potential channels including TRPC channel subtypes in native vascular myocytes and in expression systems (Hardie 2007; Rohacs, 2007; Voets & Nilius, 2007; Nilius *et al.* 2008; Large *et al.* 2009). Endogenous PIP_2 inhibits the excitatory effects of DAG on TRPC6 in mesenteric artery myocytes (Albert *et al.* 2008) and also inositol 1,4,5-trisphosphate potentiates the excitatory effects of DAG on both native TRPC6 and TRPC1 channels in rabbit portal vein myocytes (Albert & Large, 2003; Liu *et al.* 2005; Saleh *et al.* 2008). PIP_2 has also been shown to have complex actions on expressed TRPC conductances with this phospholipid increasing TRPC3, TRPC6 and TRPC7 channel activity (Lemonnier *et al.* 2007), inhibiting TRPC4 whole-cell currents (Otsuguro *et al.* 2008) and having both excitatory and inhibitory effects on TRPC5 channel activity (Trebak *et al.* 2008). There is little information on the action on PIP_3 on TRPC channels although this phospholipid has been shown to increase expressed TRPC6-mediated Ca^{2+} entry in HEK293 cells recorded with a Ca^{2+}-sensitive dye (Tseng *et al.* 2004). However the present data provide the first direct evidence that PIP_3 activates native TRPC channels in any cell type.

Conclusion

This study demonstrates that ET-1 activates native TRPC1 channels in rabbit coronary artery myocytes using two distinct phospholipid signalling pathways. The data show that PIP_3 and PIP_2 mediate the responses to respectively ET_A and ET_B receptor stimulation and facilitate opening of native TRPC1 channels. This is the first demonstration that PIP_3 activates native TRPC1 channels in vascular smooth muscle.

References

Ahmmed GU, Mehta D, Vogel S, Holinstat M, Paria BC, Tiruppathu C & Malik AB (2004). Protein kinase Cα phosphorylates the TRPC1 channel and regulates store-operated Ca^{2+} entry in endothelial cells. *J Biol Chem* **279**, 20941–20949.

Albert AP & Large WA (2002). Activation of store-operated channels by noradrenaline via protein kinase C in rabbit portal vein myocytes. *J Physiol* **544**, 113–125.

Albert AP & Large WA (2003). Synergism between inositol phosphates and diacylglycerol on native TRPC6-like channels in rabbit portal vein myocytes. *J Physiol* **552**, 789–795.

Albert AP & Large WA (2004). Inhibitory regulation of constitutive transient receptor potential-like cation channels in rabbit ear artery myocytes. *J Physiol* **560**, 169–180.

Albert AP, Piper AS & Large WA (2005). Role of phospholipase D and diacylglycerol in activating constitutive TRPC-like cation channels in rabbit ear artery myocytes. *J Physiol* **566**, 769–780.

Albert AP, Pucovsky V, Prestwich SA & Large WA (2006). TRPC3 properties of a native constitutively active Ca^{2+}-permeable cation channel in rabbit ear artery myocytes. *J Physiol* **571**, 361–369.

Albert AP, Saleh SN & Large WA (2008). Inhibition of native TRPC6 channel activity by phosphatidylinositol-4,5-bisphosphate in mesenteric artery myocytes. *J Physiol* **586**, 3087–3095.

Albert AP, Saleh SN & Large WA (2009). Identification of canonical transient receptor potential (TRPC) channel proteins in native vascular smooth muscle cells. *Curr Med Chem* **16**, 1158–1165.

Boualleque A, Daou GB & Srivastava AK (2007). Endothelin-1-induced signalling pathways in vascular smooth muscle cells. *Curr Vasc Pharmacol* **5**, 45–52.

Clapham DE & Neer EJ (1997). G proteins $\beta\gamma$ subunits. *Annu Rev Pharmacol Toxicol* **37**, 167–203.

Davenport AP (2002). International Union of Pharmacology. XXIX. Update on endothelin receptor nomenclature. *Pharmacol Rev* **54**, 219–226.

Davenport AP & Battistini B (2002). Classification of endothelin receptors and antagonists in clinical development. *Clinical Sci* **103**, 15–35.

Drummond GB (2009). Reporting ethical matters in *The Journal of Physiology*: standards and advice. *J Physiol* **587**, 713–719.

Fukami K, Matsuoka K, Nakanishi O, Yamakawa A, Kawai S & Takenawa T (1988). Antibody to phospyhatidylinositol 4,5-bisphosphate inhibits oncogene-induced mitogenesis. *Proc Natl Acad Sci U S A* **85**, 9057–9061.

Hamill OP, Marty A, Neher E, Sakmann B & Sigworth FJ (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Arch* **391**, 85–100.

Hardie RC (2007). TRP channels and lipids: from *Drosophila* to mammalian physiology. *J Physiol* **578**, 9–24.

Helliwell RM & Large WA (1997). α_1-Adrenoceptor activation of a non-selective cation current in rabbit portal vein by 1,2-diacyl-*sn*-glycerol. *J Physiol* **499**, 417–428.

Inoue R, Okada T, Onoue H, Harea Y, Shimizu S, Naitoh S, Ito Y & Mori Y (2001). The transient receptor potential protein homologue TRP6 is the essential component of vascular α-adrenoceptor-activated Ca^{2+}-permeable cation channel. *Circ Res* **88**, 325–337.

Ivey ME, Osman N & Little PJ (2008). Endothelin-1 signalling in vascular smooth muscle: pathways controlling cellular functions associated with atherosclerosis. *Atherosclerosis* **199**, 237–147.

Kawanabe Y, Hashimoto N & Masaki T (2004). Effects of phosphoinositide 3-kinase on endothelin-1-induced activation of voltage-independent Ca^{2+} channels and vasoconstriction. *Biochem Pharmacol* **68**, 215–221.

Kinlay S, Behrendt D, Wainstein M, Beltrame, Fang JC, Creager MA, Selwyn AP & Ganz P (2001). Role of endothelin-1 in the active constriction of human atherosclerotic coronary arteries. *Circulation* **104**, 1114–1118.

Kwon Y, Hofmann T & Montell C (2007). Integration of phosphoinositide- and calmodulin-mediated regulation of TRPC6. *Mol Cell* **25**, 491–503.

Large WA, Saleh SN & Albert AP (2009). Role of phosphoinositol 4,5-bisphosphate and diacylglycerol in regulating native TRPC channel proteins in vascular smooth muscle. *Cell Calcium* **45**, 574–582.

Lemonnier L, Trebak M & Putney JW Jr (2007). Complex regulation of the TRPC3, 6 and 7 channel subfamily by diacylglycerol and phosphatidylinositol-4,5-bisphosphate. *Cell Calcium* **43**, 506–514.

Liu M, Albert AP & Large WA (2005). Facilitatory effect of $Ins(1,4,5)P_3$ on store-operated Ca^{2+}-permeable cation channels in rabbit portal vein myocytes. *J Physiol* **566**, 161–171.

Miwa S, Kawanabe Y, Okamoto Y & Masaki T (2005). Ca^{2+} entry involved in endothelin-1-induced contractions of vascular smooth muscle cells. *J Smooth Muscle Res* **41**, 61–75.

Nakanishi H, Brewer KA & Exton JH (1993). Activation of the zeta isozyme of protein kinase C by phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem* **268**, 13–16.

Nilius B, Owsianik G & Voets T (2008). Transient receptor potential channels meet phosphoinositides. *EMBO J* **27**, 2809–2816.

Otsuguro K, Tang J, Tang Y, Xiao R, Freichel M, Tsvilovskyy, Ito S, Flockerzi V, Zhu M & Zholos AV (2008). Isoform-specific inhibition of TRPC4 channel by phosphatidylinositol 4,5-bisphosphate. *J Biol Chem* **283**, 10026–10036.

Peppiatt-Wildman CM, Albert AP, Saleh SN & Large WA (2007). Endothelin-1 activates a Ca^{2+}-permeable cation channel with TRPC3 and TRPC7 properties in rabbit coronary artery myocytes. *J Physiol* **580**, 755–764.

Rohacs T (2007). Regulation of TRP channels by PIP_2. *Pflugers Arch* **453**, 753–762.

Saleh SN, Albert AP, Peppiatt CM & Large WA (2006). Angiotensin II activates two cation conductances with distinct TRPC1 and TRPC6 channel properties in rabbit mesenteric artery myocytes. *J Physiol* **577**, 479–495.

Saleh SN, Albert AP, Peppiatt-Wildman CM & Large WA (2008). Diverse properties of store-operated TRPC channels activated by protein kinase C in vascular myocytes. *J Physiol* **586**, 2463–2476.

Saleh SN, Albert AP & Large WA (2009). Obligatory role for phosphatidylinositol 4,5-bisphosphate in activation of native TRPC1 store-operated channels in vascular myocytes. *J Physiol* **587**, 531–540.

Schiffrin EL & Touyz RM (1998). Vascular biology of endothelin. *J Cardiovasc Pharmacol* **32**, S2–S13.

Sumner MJ, Cannon TR, Mundin JW, White DG & Watts IS. (1992). Endothelin ET_A and ET_B receptors mediate vascular smooth muscle contraction. *Br J Pharmacol* **107**, 858–860.

Sugawara F, Ninomiya H, Okamoto Y, Miwa S, Mazda O, Katsura Y & Masaki T (1996). Endothelin-1-induced mitogenic responses of Chinese hamster ovary cells expressing human endothelinA: the role of a wortmannin-sensitive signalling pathway. *Mol Pharmacol* **49**, 447–457.

Tseng PH, Lin HP, Hu H, Wang C, Zhu MX & Chen CS (2004). The canonical transient receptor potential 6 channel as a putative phosphatidylinositol-3,4,5- trisphosphate-sensitive calcium entry system. *Biochemistry* **43**, 11701–11708.

Trebak M, Lemonnier L, Dehaven WI, Wedel BJ, Bird GS & Putney JW Jr (2008). Complex functions of phosphatidylinositol-4,5-bisphosphate on regulation of TRPC5 cation channels. *Pflugers Arch* **457**, 757–769.

Vanhaesebroeck B, Leevers SJ, Panayotou G & Waterfield MD (1997). Phosphoinositide-3-kinases: a conserved family of signal transducers. *Trends Biochem Sci* **22**, 267–272.

Voets T & Nilius B (2007). Modulation of TRPs by PIPs. *J Physiol* **582**, 939–944.

Wang YX, Dhulipala PD, Li L, Benovic JL & Kotlikoff MI (1999). Coupling of M_2 muscarinic receptors to membrane ion channels via phosphoinositide-3-kinase γ and atypical protein kinase C. *J Biol Chem* **274**, 13859–13864.

Author contributions

S.N.S. carried out the experimental work and figure preparation. A.P.A. and W.A.L. were involved in the conception and design of the study, interpretation of data, and drafting of the manuscript. All authors were involved in revising the manuscript and approved the final version.

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