Activation of native TRPC1/C5/C6 channels by endothelin-1 is mediated by both PIP₃ and PIP₂ 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 ETₐ or ETₐ 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 ETₐ and ETₐ receptor stimulation activate this conductance. Stimulation of both ETₐ and ETₐ 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. ETₐ 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 (PIP₃), the product of PI-3-kinase-mediated phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP₂). Moreover, exogenous application of diC₈-PIP₃ stimulated PKC-dependent TRPC1 channel activity. These results indicate that stimulation of ETₐ receptors evokes PKC-dependent TRPC1 channel activity through activation of PI-3-kinase and generation of PIP₃. In contrast, ETₐ 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 (PIP₂) antibodies and high concentrations of wortmannin (20 μM) which depleted tissue PIP₂ levels. In addition exogenous application of diC₈-PIP₃ activated PKC-dependent TRPC1 channel activity. These data indicate that stimulation of ETₐ receptors evokes PKC-dependent TRPC1 activity through PI-PLC-mediated generation of DAG and requires a permissive role of PIP₂. In conclusion, we provide the first evidence that stimulation of ETₐ and ETₐ receptors activate native PKC-dependent TRPC1 channels through two distinct phospholipids pathways involving a novel action of PIP₃, in addition to PIP₂, 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; PIP₂, phosphatidylinositol-4,5-bisphosphate; phosphatidylinositol-3,4,5-trisphosphate; PLC, phospholipase C; PKC, protein kinase C; TRPC, canonical transient receptor potential.
Introduction

Endothelin-1 (ET-1) produces vasoconstriction by a direct action on vascular smooth muscle cells through stimulation of predominantly ETₐ receptors, although ETₐ 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 Ca²⁺ ions through voltage-independent ion channels (see Miwa et al. 2005). These data suggest that ET-1 contracts vascular smooth muscle by opening Ca²⁺-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 ETₐ 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 Ca²⁺-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 ETₐ or ETₐ receptors using two distinct phosphoinositide signalling pathways involving respectively phosphatidylinositol 3,4,5-trisphosphate (PI₃P) and phosphatidylinositol 4,5-bisphosphate (PI₄P). This is the first demonstration that PI₃P, in addition to PI₄P, 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), MgCl₂ (1.2) and CaCl₂ (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 Digitata 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 with the peak of these curves 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), CaCl₂ (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 Ca²⁺ entry through voltage-dependent Ca²⁺ channels. The bathing solution used in inside-out experiments (intracellular solution) contained (mM): CsCl (18), caesium aspartate (108), MgCl₂ (1.2), Hepes (10), glucose (11), BAPTA (1), CaCl₂ (0.48), free internal Ca²⁺ concentration approximately 100 nM as calculated using EQCAL software), Na₂ATP (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), CaCl₂ (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 (0.0002), pH adjusted to 7.2 with NaOH. Under these conditions voltage-dependent Ca²⁺ currents, K⁺ currents, swell-activated Cl⁻ currents and Ca²⁺-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-PIP₂ antibodies (which detect liposome complex of PIP₂ 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-PIP₂ 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 H₂O 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-blotting and immunoprecipitation. Dot-blotting 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\textsubscript{A} and ET\textsubscript{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\textsubscript{A} and ET\textsubscript{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\textsubscript{A} and ET\textsubscript{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 1A, 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\textsubscript{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\textsubscript{r}) of about 0 mV. These responses were seen in approximately 90% of patches tested. Figure 1Ab illustrates that the channel current amplitude histogram of

![Figure 1](image)

**Figure 1.** Stimulation of ET\textsubscript{A} and ET\textsubscript{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\textsubscript{A} receptor antagonist 100 nM BQ-123 or the ET\textsubscript{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 nM 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\textsubscript{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. 1A 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 1B 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. 1C 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 1E 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. 1D 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 2A shows that the mean $N_P$ 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 $0.37 \pm 0.04$ to $0.06 \pm 0.01$ (83 ± 5% inhibition, n = 6, P < 0.01) by the PKC inhibitor chelerythrine (3 μM). Figure 2B shows that the mean $N_P$ of ET_B receptor-mediated channel activity, activated by 100 nM ET-1 in the presence of 100 nM BQ-123, was significantly reduced from $0.38 \pm 0.04$ to $0.07 \pm 0.01$ (81 ± 6% inhibition, n = 6, P < 0.01) by the PKC inhibitor chelerythrine (3 μM). Figure 2C shows that the mean $N_P$ 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 $0.37 \pm 0.04$ to $0.06 \pm 0.01$ (83 ± 5% inhibition, n = 6, P < 0.01) by the PKC inhibitor chelerythrine (3 μM). Figure 2D shows that the mean $N_P$ of ET_B receptor-mediated channel activity, activated by 100 nM ET-1 in the presence of 100 nM BQ-123, was significantly reduced from $0.38 \pm 0.04$ to $0.07 \pm 0.01$ (81 ± 6% inhibition, n = 6, P < 0.01) by the PKC inhibitor chelerythrine (3 μM). Figure 2E shows that the mean $N_P$ 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 $0.37 \pm 0.04$ to $0.06 \pm 0.01$ (83 ± 5% inhibition, n = 6, P < 0.01) by the PKC inhibitor chelerythrine (3 μM). Figure 2F shows that the mean $N_P$ of ET_B receptor-mediated channel activity, activated by 100 nM ET-1 in the presence of 100 nM BQ-123, was significantly reduced from $0.38 \pm 0.04$ to $0.07 \pm 0.01$ (81 ± 6% inhibition, n = 6, P < 0.01) by the PKC inhibitor chelerythrine (3 μM).
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 ± 3% inhibition, n = 7, P < 0.01) by 3 μM chelerythrine.

Figure 2C 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 ± 5% inhibition, n = 5, P < 0.01). Moreover Fig. 2D 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 ± 8% inhibition, n = 5, P < 0.01). In control experiments, Fig. 2E 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 3A 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 ± 4% inhibition, n = 7, P < 0.01) whereas Fig. 3B 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 activity.

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**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 ETₐ receptors. Therefore, we investigated the effects of several established inhibitors of other phospholipases that may be involved in ETₐ receptor-mediated activation of TRPC1 channel currents. Figure 3C shows that pharmacological inhibitors of phosphatidylcholine-PLC (PC-PLC, 100 μM D-609, n = 5), cytosolic and Ca²⁺-dependent and-independent forms of phospholipase A₂ (PLA₂, 100 μM AACOCF₃, n = 4 and 100 μM PACOCF₃, n = 4) and phospholipase D (PLD, 100 μM C₂-ceramide, n = 6) had no effect on ETₐ receptor-mediated TRPC1 channel activity. Stimulation of ETₐ receptors has also been shown to activate Rho kinase (Ivey et al. 2008) but Fig. 3C shows that the Rho kinase inhibitors HA-110 (5 μM, n = 5) and Y27632 (1 μM, n = 5) had no effect on ETₐ receptor-mediated TRPC1 channel activity. These data suggest that PC-PLC, PLA₂, PLD and Rho kinase are also not involved in TRPC1 channel activation initiated by ETₐ receptor stimulation.

Previous studies have shown that stimulation of ETₐ receptors can activate phosphoinositol-3-kinase (PI-3-kinase), which phosphorylates PIP₂ to form PIP₃, 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 ETₐ and ET₉ 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 4A shows that 50 nM wortmannin significantly reduced mean NP₀ of ETₐ receptor-mediated TRPC1 channel activity from 0.23 ± 0.06 to 0.02 ± 0.01 (94 ± 4% inhibition, n = 6, P < 0.01). In addition, Fig. 4B shows that 3 μM PI-828 also significantly attenuated mean NP₀ of ETₐ receptor-mediated TRPC1 channel activity from 0.14 ± 0.04 to 0.02 ± 0.01 (85 ± 5% inhibition, n = 5, P < 0.01). Importantly, Fig. 4C and D illustrates that 50 nM wortmannin (control mean NP₀ of 0.18 ± 0.06 and 0.18 ± 0.07 in wortmannin, n = 6) and 3 μM PI-828 (control mean NP₀ of 0.31 ± 0.11 and 0.24 ± 0.08 in PI-828, n = 6) had no effect on ET₉ 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 ETₐ and ET₉ receptors evokes TRPC1 channel activity via different signal transduction mechanisms. Stimulation of ETₐ receptors is coupled to TRPC1 channels via a PI-3-kinase-dependent pathway whereas a PI-PLC-dependent pathway links ET₉ 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 ETₐ receptors activates TRPC1 channel currents via a PI-3-kinase-mediated pathway in cell-attached patches
A and B show that ETₐ 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 ET₉ receptor-mediated TRPC1 channel activity.
Involvement of PIP₃ in ETA receptor-mediated activation of TRPC1 channel currents

The above results suggest that generation of PIP₃ produced from the action of PI-3-kinase on PIP₂ is required for ETₐ receptor-mediated TRPC1 channel stimulation. Consequently we investigated if exogenous PIP₃ directly activates TRPC1 channel currents in coronary artery myocytes.

Figure 5A and B shows that bath application of 3 μM diC8-PIP₃, a water soluble form of PIP₃, 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-PIP₃ was approximately 1 μM and maximum channel activation was obtained with 10–20 μM diC8-PIP₃ (data not shown). Figure 5C and D also illustrates that mean $NP_o$ of diC8-PIP₃-evoked channel activity in inside-out patches was significantly inhibited from 0.21 ± 0.09 to 0.02 ± 0.02 (94 ± 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 ± 7% inhibition, $n = 5$, $P < 0.01$) with anti-TRPC1 antibodies.

The role of endogenous PIP₃ in ETₐ receptor-mediated stimulation of TRPC1 channel activity was investigated using an anti-PIP₃ antibody. Figure 5E shows that the mean $NP_o$ of ETₐ receptor-mediated TRPC1 channel stimulation, initially activated in cell-attached patches in the presence of the ET₃ receptor antagonist BQ-788, was significantly reduced from 0.25 ± 0.07 to 0.01 ± 0.01 (97 ± 1% inhibition, $n = 6$, $P < 0.01$) by bath application of an anti-PIP₃ antibody to the cytosolic surface of the patches. In contrast, Fig. 5F illustrates that an anti-PIP₃ antibody had no effect on ET₃ receptor-mediated TRPC1 channel activity (control mean $NP_o$ from 0.48 ± 0.08 to 0.51 ± 0.11 in anti-PIP₃ antibody, $n = 6$).

These data clearly show that exogenous PIP₃ and ET-1 activate the same PKC-dependent TRPC1 channel currents and also indicate that endogenous PIP₃ mediates activation of TRPC1 channel currents by ETₐ receptor stimulation.

Involvement of PIP₂ in ET₆ receptor-mediated activation of TRPC1 channel currents

The above results indicate that ET₆ 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 PIP₂...
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 PIP2 in PKC-dependent activation of TRPC1 channels in portal vein smooth muscle cells (Saleh et al. 2009) and therefore we investigated the effects of PIP2 in coronary artery myocytes.

Bath application of 10 μM diC8-PIP2 to inside-out patches induced cation channel activity which had a mean NPo of 0.34 ± 0.11 at −70 mV (n = 11) and a unitary conductance of 2.6 pS and an Er of about 0 mV (Fig. 6Aa and b). In addition, the mean NPo of diC8-PIP2-evoked channel activity was significantly reduced by co-application of anti-TRPC1 antibodies (from 0.24 ± 0.08 to 0.02 ± 0.01, 88 ± 6% inhibition, n = 5, P < 0.01, Fig. 6B) and by 3 μM chelerythrine (from 0.31 ± 0.08 to 0.05 ± 0.03, 97 ± 6% inhibition, n = 6, P < 0.01, Fig. 6C). These data provide evidence that exogenous PIP2 activates TRPC1 channel currents via a PKC-dependent mechanism.

OAG-evoked channel activity was significantly inhibited by anti-TRPC1 antibodies (Fig. 6D, mean NPo from 0.23 ± 0.08 to 0.03 ± 0.01, 87 ± 1% inhibition, n = 4, P < 0.01) and by anti-PIP2 antibodies (Fig. 6E, mean NPo from 0.32 ± 0.06 to 0.01 ± 0.01, 97 ± 1% inhibition, n = 6, P < 0.01) in inside-out patches. Figure 6F also shows that when tissues were pre-treated with 20 μM wortmannin for 30 min to deplete tissue PIP2 levels (see Fig. 7) OAG did not evoke TRPC1 channel activity in cell-attached patches (mean NPo of 0.01 ± 0.01, n = 6). These results suggest that endogenous PIP2 has an obligatory role for OAG-evoked TRPC1 channel activation.

These studies demonstrate that stimulation of ETB 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 PIP2.

Figure 6. Obligatory role of PIP2 in mediating OAG-induced TRPC1 channel activity via a PKC-dependent mechanism

Aa and b, bath application of 10 μM diC8-PIP2 activates cation channel activity in inside-out patches at −70 mV which has a unitary conductance of 2.6 pS and an Er of about 0 mV. B and C, diC8-PIP2-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-PIP2 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.
PIP3 evokes TRPC1 channel activity independently of PIP2

The present work shows that stimulation of ETA receptors activates TRPC1 activity through a PI-3-kinase-mediated pathway involving PIP3 (see Figs 4 and 5). Moreover we demonstrate that exogenous diC8-PIP3 evokes PKC-dependent TRPC1 channel activity (see Fig. 5). In contrast, our data indicate that activation of TRPC1 channels by stimulation of ETB receptors involves a permissive role for PIP2. In a previous report we stated that PIP2 had an obligatory role for TRPC1 channel activation in rabbit portal vein myocytes (Saleh et al. 2009). Therefore we investigated whether endogenous PIP2 was necessary for activation of TRPC1 channels by PIP3 in coronary artery smooth muscle cells.

Figure 7A shows that following pre-treatment of myocytes with 20 μM wortmannin for 30 min to deplete PIP2 levels (see Fig. 5Ca) bath application of 3 μM diC8-PIP3 activated TRPC1 channel activity with a mean peak \( N_P \) 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 PIP3 in the absence of wortmannin (see above and Fig. 7Aa). Figure 7B shows that anti-PIP2 antibodies had no effect on PIP3-induced TRPC1 channel activity in inside-out patches (control mean \( N_P \) of 0.23 ± 0.06 and 0.24 ± 0.08 in the presence of anti-PIP2 antibodies, n = 6). Both of these procedures blocked OAG-evoked TRPC1 channel activity (cf. Fig. 6E and F). In other experiments the anti-PIP2 antibody reduced TRPC1 channel activation by both ETA and ETB receptor stimulation (data not shown). This is predictable since PIP2 acts as a substrate for PIP3 generated by PI-3-kinase (ETA pathway) and DAG produced by PI-PLC (ETB pathway).

Therefore with regard to direct TRPC1 channel activation endogenous PIP2 is not obligatory for TRPC1 channel activation by PIP3 (ETA receptor pathway) but...
is necessary for OAG (DAG)-induced (ET_\text{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 7Ca 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. 7Ca 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. 7Cb shows that stimulation of ET_\text{A} or ET_\text{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_\text{A} and ET_\text{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 7D illustrates that at rest total cell lysates from coronary arteries contained detectable PIP_2 but not PIP_3 whereas upon stimulation of ET_\text{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_\text{A} and ET_\text{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_\text{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_\text{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_3 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 TRPC1 channels.

**ET_\text{A} receptor transduction mechanism and activation of TRPC1 channel currents**

ET_\text{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_\text{A} 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_\text{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_\text{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_\text{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 heteromorphic structure of native TRPC1 channels is more sensitive to PIP3 then heterologously expressed TRPC1 proteins or that PIP3 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_{b/γ} 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-PIP2 antibody. Moreover OAG did not evoke TRPC1 channel activity in cells pre-treated with high concentrations of wortmannin, which depleted tissue PIP2 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 PIP2-mediated activation of TRPC1 channels.

Application of exogenous PIP2 evoked TRPC1 channel currents and co-immunoprecipitation studies showed that PIP2 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 PIP2 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 PIP2 and PIP3 can activate TRPC1 channels in coronary artery myocytes and our evidence is that PIP3 is obligatory for ET_A receptor-mediated stimulation of TRPC1 channels whereas PIP2 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 PIP3 and PIP2, 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 PIP2 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 PIP3, 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 PIP3/IP2-mediated activation mechanisms of native TRPC1 channels using expressed heterotetrameric channels involving TRPC1 subunits.

It has been shown that PIP3 also activates some PKC isoforms in vitro (Nakanishi et al. 1993). Therefore on ET_A receptor stimulation production of PIP3 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 PIP3-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 \(\alpha_1\)-adrenoceptors and angiotensin II (Ang II) receptors are coupled to TRPC6 channels via PI-PLC in respectively rabbit portal vein and mesenteric artery myocytes (Hellwell & 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\(\alpha\) 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 (Otsguero 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\(\alpha\) and ET\(\beta\) 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.

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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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