J Physiol 587.3 (2009) pp 531–540

The Journal of Physiology

Obligatory role for phosphatidylinositol 4,5-bisphosphate in activation of native TRPC1 store-operated channels in vascular myocytes

Sohag N. Saleh, Anthony P. Albert and William A. Large

Ion Channels and Cell Signalling Research Centre, Division of Basic Medical Sciences, St George's, University of London, Cranmer Terrace, London SW17 0RE, UK

In the present study the effect of phosphatidylinositol 4,5-bisphosphate (PIP_2) was studied on a native TRPC1 store-operated channel (SOC) in freshly dispersed rabbit portal vein myocytes. Application of diC8-PIP_2, a water soluble form of PIP_2, to quiescent inside-out patches evoked single channel currents with a unitary conductance of 1.9 pS. DiC8-PIP_2-evoked channel currents were inhibited by anti-TRPC1 antibodies and these characteristics are identical to SOCs evoked by cyclopiazonic acid (CPA) and BAPTA-AM. SOCs stimulated by CPA, BAPTA-AM and the phorbol ester phorbol 12,13-dibutyrate (PDBu) were reduced by anti-PIP_2 antibodies and by depletion of tissue PIP_2 levels by pre-treatment of preparations with wortmannin and LY294002. However, these reagents did not alter the ability of PIP_2 to activate SOCs in inside-out patches. Co-immunoprecipitation techniques demonstrated association between TRPC1 and PIP_2 at rest, which was greatly decreased by wortmannin and LY294002. Pre-treatment of cells with PDBu, which activates protein kinase C (PKC), augmented SOC activation by PIP_2 whereas the PKC inhibitor chelerythrine decreased SOC stimulation by PIP_2. Co-immunoprecipitation experiments provide evidence that PKC-dependent phosphorylation of TRPC1 occurs constitutively and was increased by CPA and PDBu but decreased by chelerythrine. These novel results show that PIP_2 can activate TRPC1 SOCs in native vascular myocytes and plays an important role in SOC activation by CPA, BAPTA-AM and PDBu. Moreover, the permissive role of PIP_2 in SOC activation requires PKC-dependent phosphorylation of TRPC1.

(Resubmitted 18 November 2008; accepted after revision 29 November 2008; first published online 1 December 2008)
Corresponding author A. P. Albert: Ion Channels and Cell Signalling Research Centre, Division of Basic Medical Sciences, St George's, University of London, Cranmer Terrace, London SW17 0RE, UK. Email: aalbert@sgul.ac.uk

In vascular smooth muscle canonical transient receptor potential (TRPC) channels are involved in many physiological responses including contraction, cell growth, proliferation and migration (see Large, 2002; Beech *et al.* 2004; Firth *et al.* 2007). A key question concerns the activation mechanism of TRPC channels, which are frequently described as either receptor-operated or store-operated channels (ROCs and SOCs, respectively). In freshly dispersed vascular myocytes TRPC ROCs are stimulated by G-protein-coupled agonists such as noradrenaline, angiotensin II (Ang II) or endothelin-1 (ET-1) coupled to either phospholipase C (PLC, TRPC6 in rabbit portal vein, Inoue *et al.* 2001; mesenteric artery, Saleh *et al.* 2006; TRPC3/TRPC7 in rabbit coronary artery, Peppiatt-Wildman *et al.* 2007) or phospholipase D (TRPC3 in rabbit ear artery, Albert *et al.* 2005, 2006). In all these cases it seems that diacylglycerol (DAG) which is produced by phospholipase stimulation plays an important role in channel activation and may actually be the gating molecule (Albert & Large, 2006; Albert *et al.* 2008). SOCs are activated by depletion of intracellular Ca^{2+} stores and there is now considerable evidence that TRPC proteins also form SOCs in native vascular smooth muscle with both TRPC1 and TRPC5 as suggested components of SOCs (Xu & Beech, 2000; Xu *et al.* 2006; Saleh *et al.* 2006, 2008). In vascular smooth muscle protein kinase C (PKC) appears to have an important role in activation of TRPC SOCs (Albert & Large, 2002*b*; Albert *et al.* 2007). In addition Ca^{2+}-independent phospholipase A_2 has also been suggested to be involved in activating SOCs (Smani *et al.* 2004).

Phosphatidylinositol 4,5-bisphosphate (PIP_2) is an important signalling molecule, which is cleaved by PLC to inositol 1,4,5-trisphosphate (IP_3) and DAG and both

DOI: 10.1113/jphysiol.2008.166678

these products have well established cellular effects. However, recently there has been much interest in the direct actions of PIP_2 on ion channels, including TRP channels (Suh & Hille, 2005; Hardie, 2007; Rohacs, 2007; Voets & Nilius, 2007; Nilius *et al.* 2008). In HEK293 cells PIP_2 increased activity of expressed TRPC3, TRPC6 and TRPC7 channel activity (Lemonnier *et al.* 2008), decreased TRPC4α activity (Otsuguro *et al.* 2008) and produced complex effects on TRPC5 channels (Trebak *et al.* 2008). In freshly dispersed vascular myocytes we demonstrated that endogenous PIP_2 inhibited native TRPC6 channels (Albert *et al.* 2008). These data indicated that PIP_2 was bound to TRPC6 in unstimulated cells and following receptor stimulation by Ang II, optimal channel stimulation was produced by hydrolysis of this bound PIP_2 and simultaneous activation of TRPC6 channels by DAG, possibly at the same PIP_2-binding site on the channel molecule (Albert *et al.* 2008).

In the present study we investigated the role of PIP_2 in activation of native TRPC1 SOCs in rabbit portal vein myocytes, which have characteristics of a heterotetrameric channel consisting of TRPC1/TRPC5/TRPC7 subunits (Saleh *et al.* 2008). These results show that PIP_2 stimulates this ion channel and that there is an obligatory role for endogenous PIP_2 in TRPC1 SOC activation.

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). Portal vein was dissected free from fat and connective tissue and enzymatically digested into single myocytes using methods previously described (Saleh *et al.* 2006).

Electrophysiology

Single cation currents were recorded with an HEKA EPC8 patch-clamp amplifier (HEKA Instruments Inc., Bellmore, NY, USA) at room temperature (20–23°C) using cell-attached and inside-out patch configurations (Hamill *et al.* 1981) and data acquisition and analysis protocols as previously described (Saleh *et al.* 2006). Briefly, single channel current amplitudes were calculated from idealized traces that were filtered off-line at 100 Hz with an 8-pole Bessel filter (Frequency Devices, Haverhill, MA, USA) and sampled at 1 kHz. Traces of at least 60 s in duration were used to calculate open probability and construct fitted-level amplitude histograms and events lasting for < 6.664 ms ($2 \times$ rise time for a 100 Hz, -3 db, low pass filter) were excluded from analysis using the 50% threshold method. Figure preparation was carried out using Origin 6.0 (OriginLab Corp., Northampton, MA, USA) where inward single channel currents are shown as downward deflections. Open probability (NP_o) was calculated using the equation:

$$NP_o = \frac{\sum (O_n n)}{T}$$

where n is number of simultaneously open channels in the patch, O_n is time spent at the open level for each channel (i.e. $n-1$) and T is total recording time.

The relationship between NP_o and PIP_2 concentration ($[PIP_2]$) for inside out patches was fitted with the Hill equation: $y = \frac{y_{max}}{(1+K_d/[PIP_2])^{n_H}}$ where y_{max} = maximum NP_o, K_d is the apparent dissociation constant and n_H is the Hill coefficient.

Immunoprecipitation and Western blotting

Total cell lysate (TCL) was extracted and quantified as previously described (Saleh *et al.* 2008). The immunoprecipitation protocol was carried out using the Catch and Release® kit (Upstate Biotechnology, Lake Placid, NY, USA), where spin columns were loaded with 300–600 μg of cell lysates, 2–4 μg of antibody, 2 μg of β-actin antibody, and immunoprecipitated on an end-over-end stirrer for 2 h at room temperature.

Protein samples were eluted with Laemmli sample buffer and incubated at 60°C for 5 min. One-dimensional protein gel electrophoresis, semi-dry transfer and Western blot procedures were performed as previously described (Saleh *et al.* 2008). Whenever possible, alternative antibodies raised against different epitopes were used for immunoprecipitation and Western blot analysis. Secondary horseradish peroxidase-conjugated goat anti-rabbit, goat anti-mouse (Sigma) and donkey anti-goat (Millipore) antibodies were added according to the host species of the primary antibody used for the Western blot and subsequently detected with ECL reagents (Saleh *et al.* 2008). In loading control experiments anti-β-actin antibodies (mouse monoclonal, Sigma, UK) were also added to the immunoprecipitate and immunoblot procedures to show that expression levels of β-actin did not change during the experimental conditions. Moreover immunoblot control data showed that anti-PIP_2 and anti-TRPC1 antibodies did not recognize β-actin following immunoprecipitation with only anti-β-actin antibodies. Data shown represent n-values of at least three separate experiments.

Anti-TRPC1, anti-PIP_2 and anti-phosphoserine/threonine antibodies

Polyclonal antibodies for TRPC1 raised against different intracellular epitopes were purchased from Santa Cruz

Biotechnology (Santa Cruz, CA, USA) and Alomone Laboratories (Jerusalem, Israel). The specificity of these antibodies for their target proteins have been previously confirmed (Liu *et al.* 2005*b*; Sours *et al.* 2006). Mouse monoclonal PIP$_2$ antibody generated against liposomes of human origin constituted with the phospholipid (Osborne *et al.* 2001) was purchased from Assay Designs (Ann Arbor, MI, USA) and Santa Cruz Biotechnology and has a predicted molecular mass of 74 kDa according to the manufacturer's specifications. This anti-PIP$_2$ antibody has previously been used in electrophysiological (Liou *et al.* 1999; Bian *et al.* 2001; Ma *et al.* 2002; Pian *et al.* 2006; Xie *et al.* 2008) and immunoprecipitation experiments (Asteggiano *et al.* 2001; Beauge *et al.* 2002; Yue *et al.* 2002) to investigate the role of PIP$_2$ in regulating ion channels and exchangers. Anti-phosphoserine and anti-phosphothreonine antibodies were purchased from Santa Cruz Biotechnology and both were used together in immunoprecipitation and Western blotting experiments.

Solutions and drugs

In cell-attached patch experiments the membrane potential was set to 0 mV by perfusing cells in a KCl external solution containing (mM): KCl (126), $CaCl_2$ (1.5), Hepes (10) and glucose (11), pH to 7.2 with 10 M KOH. Nicardipine (5 μM) was also included to prevent smooth muscle cell contraction by blocking Ca^{2+} entry through voltage-dependent Ca^{2+} channels. Immediately prior to patch excision for inside-out recordings and within a 30 s period the bathing solution was exchanged for an intracellular solution containing (mM): CsCl (18), caesium aspartate (108), $MgCl_2$ (1.2), Hepes (10), glucose (11), BAPTA (1), $CaCl_2$ (0.48, free internal Ca^{2+} concentration approximately 100 nM as calculated using EqCal software; Biosoft, Great Shelford, UK), Na_2ATP (1), NaGTP (0.2) pH 7.2 with Tris. U73122 at 2 μM and 50 nM wortmannin were also included for all experiments with diC8-PIP$_2$ to prevent conversion of PIP$_2$ into IP$_3$, DAG and phosphatidylinositol 3,4,5-trisphosphate (PIP$_3$).

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

DiC8-PIP$_2$ was from Cayman Chemical Co. (Ann Arbor, MI, USA) whilst all other drugs were purchased from Calbiochem (UK), Sigma (UK) or Tocris Bioscience (Bristol, UK). Reagents were dissolved in distilled H_2O or DMSO (0.1%) and DMSO alone had no effect on SOC activity. The values are the mean of n cells $\pm$ S.E.M. Statistical analysis was carried out using Students' t test for paired (comparing effects of agents on the same cell) or unpaired data (comparing effects of agents between cells) with the level of significance set at $P < 0.05$.

Results

DiC8-PIP$_2$ activates TRPC1 SOCs in rabbit portal vein myocytes

In the first series of experiments we investigated the effect of exogenous diC8-PIP$_2$, a water-soluble form of PIP$_2$, on cyclopiazonic acid (CPA)-evoked SOC activity in inside-out patches. In these experiments we initially evoked SOC activity by bath applying the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase inhibitor CPA (10 μM) to cell-attached patches and when channel activity reached plateau levels patches were excised into the inside-out configuration. SOC activity was maintained in excised patches for periods of up to 45 min and did not run down in the presence of ATP and GTP. This configuration allowed diC8-PIP$_2$ to be bath applied to the cytosolic surface of the patches. Figure 1*A* shows that bath application of 10 μM diC8-PIP$_2$ to inside-out patches significantly increased mean open probability (NP_o) of CPA-evoked SOC activity from 0.18 ± 0.05 to 0.37 ± 0.07 at -80 mV ($n = 5$, $P < 0.05$) and this effect was readily reversed following the removal of diC8-PIP$_2$ (not shown). The bathing solution also contained 2 μM U73122 and 50 nM wortmannin (see Methods) to ensure that this was an effect of PIP$_2$ itself and not a metabolite. Channel current amplitude histograms shown in Fig. 1*B* illustrate that CPA-evoked channel currents had similar peak amplitudes in the absence (Fig. 1*Ba*) and presence (Fig. 1*Bb*) of diC8-PIP$_2$, which suggests that diC8-PIP$_2$ increased activity of the same CPA-induced channels.

To investigate whether diC8-PIP$_2$-induced increases in SOC activity were due to either potentiation of CPA-evoked responses or direct activation of SOCs, we studied the effect of diC8-PIP$_2$ on quiescent inside-out patches in the presence of 2 μM U73122 and 50 nM wortmannin. Figure 1*C* and *D* shows that bath application of 10 μM diC8-PIP$_2$ to an inside-out patch activated channel currents which had a mean NP_o of 0.27 ± 0.04 ($n = 16$) at -80 mV and a slope conductance between -50 mV and -120 mV of 1.9 pS. These data indicate that diC8-PIP$_2$ activates channel currents with a similar conductance to that of native TRPC SOCs previously described in portal vein myocytes (Albert & Large, 2002*a*; Saleh *et al.* 2008). TRPC1 has been previously proposed to be a component of SOCs in portal vein myocytes (Saleh *et al.* 2008) and Fig. 1*C* shows that bath application of an anti-TRPC1 antibody (1 : 200 dilution) significantly

inhibited diC8-PIP_2-induced channel activity by 76 ± 6% at –80 mV ($n = 5$, $P < 0.01$). This inhibition was abolished when the antigenic peptide (also 1 : 200 dilution) was preincubated with the antibody ($n = 5$, data not shown).

Effect of an anti-PIP_2 antibody on SOC activity

The above results indicate that exogenous diC8-PIP_2 activates ion channels with properties similar to TRPC1 SOCs previously described in portal vein myocytes (cf. Albert & Large, 2002*a*; Saleh *et al.* 2008). Therefore we investigated the role of endogenous PIP_2 in activating these SOCs by studying the effect of an anti-PIP_2 antibody on SOCs in inside-out patches.

In the first experiments SOC activity was induced in cell-attached patches by CPA or BAPTA-AM and then patches were excised and anti-PIP_2 antibodies were applied to the internal membrane surface of the patches. Figure 2*Aa* and *b* and *B* shows that mean NP_o of SOC activity, initially induced by 10 μM CPA or 50 μM BAPTA-AM, was significantly inhibited by anti-PIP_2 antibodies (1 : 200 dilution) by 91 ± 3% ($n = 7$) and 82 ± 9% ($n = 7$), respectively. This effect was not seen when the anti-PIP_2 antibody was denatured prior to application by boiling for a period of 30 min ($n = 4$, data not shown). In addition Fig. 2*Ac* and *B* show that anti-PIP_2 antibodies also produced marked inhibition of SOC activity induced by the PKC-activating phorbol ester phorbol 12,13-dibutyrate (PDBu, 1 μM), which has been previously shown to activate SOCs in this preparation (Albert & Large, 2002*b*; Saleh *et al.* 2008). In these experiments inside-out patches were prepared from quiescent cells before application of PDBu and anti-PIP_2 antibodies, which significantly reduced activity by 92 ± 2% ($n = 5$). Figure 2*C* shows that tissue lysates from portal vein immunoprecipitated with anti-TRPC1

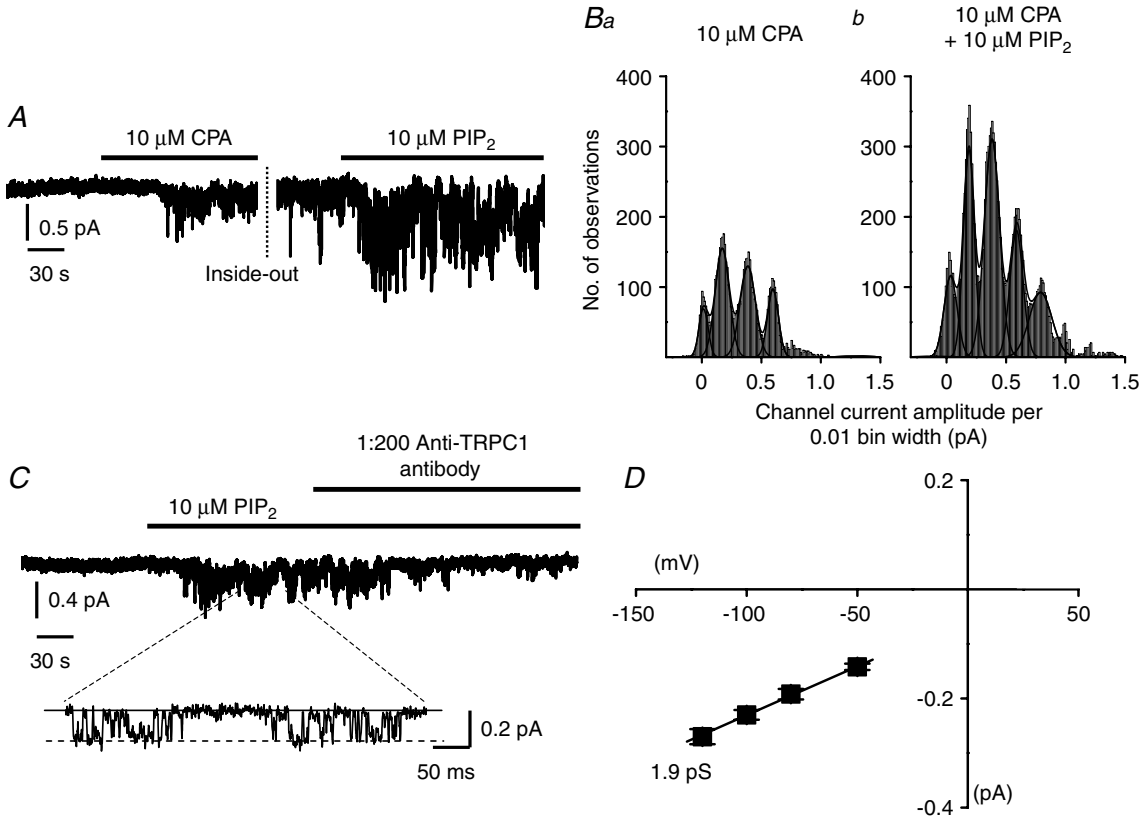

Figure 1. diC8-PIP_2 activates TRPC1 SOCs in rabbit portal vein myocytes
A, bath application of 10 μM CPA evoked SOC activity in a cell-attached patch held at −80 mV. After excision into the inside-out configuration, SOC activity was potentiated by bath application of 10 μM diC8-PIP_2. *B*, fixed level channel current amplitude histograms of SOC activity shown in *A*. Histograms could be fitted by the sum of several Gaussian curves showing the presence of more than one channel in the patch. Note that the peaks of the Gaussian curves have similar values in the absence (*a*) and presence of diC8-PIP_2 (*b*) indicating that this phospholipid increased the activity of the same channel. *C*, bath application of 10 μM diC8-PIP_2 activated channel currents in a quiescent inside-out patch held at −80 mV which were inhibited by anti-TRPC1 antibodies (Santa Cruz, 1 : 200 dilution). *D*, diC8-PIP_2-induced channel currents had a slope conductance of 1.9 pS between −120 and −50 mV.

antibodies then Western blotted with anti-PIP$_2$ antibodies detected a band of ∼70 kDa, which is the predicted band for the PIP$_2$ complex with this antibody (see Methods). Moreover Fig. 2*C* shows that preincubation of anti-TRPC1 antibodies with its antigenic peptide (AgP) reduced detection of the band with anti-PIP$_2$ antibodies without affecting expression levels of β-actin proteins.

Agents that deplete PIP$_2$ levels inhibit SOC activity

These data suggest that endogenous PIP$_2$ associated with TRPC1 proteins may have an important role in activating SOCs in portal vein myocytes. Therefore we investigated the effect of depleting endogenous PIP$_2$ levels on SOC activity in cell-attached patches. To deplete PIP$_2$ we used 20 μM wortmannin and 100 μM LY294002 which at these concentrations inhibit phosphoinositol (PI)-4-kinases leading to a reduction in generation of PIP$_2$ and consequently a depletion of tissue PIP$_2$ levels (Suh & Hille, 2005; Albert *et al.* 2008).

Figure 3*Aa* and *b* shows that pretreatment of portal vein myocytes with 20 μM wortmannin for 15 min almost obliterated the ability of CPA and PDBu to induce SOC activity, and mean NP_o was decreased by 98 ± 1% ($n = 8$) and 97 ± 2% ($n = 9$), respectively, compared to untreated control cells ($n = 6$ and $n = 5$). Moreover Fig. 3*Ac* shows that pretreatment of myocytes with 100 μM LY294002 for 15 min also reduced NP_o of CPA-induced SOC activity by a mean value of 99 ± 1% ($n = 6$) compared to control cells ($n = 6$). However Fig. 3*Aa–c* shows that in cells pretreated with wortmannin or LY294002, bath application of 10 μM diC8-PIP$_2$ in the inside-out configuration was still able to induce SOC activity. In the presence of wortmannin mean NP_o of CPA- and PDBu-induced SOC activity was significantly increased by diC8-PIP$_2$ from, respectively, 0.02 ± 0.01 to 0.29 ± 0.07 ($n = 4$, $P < 0.05$) and 0.02 ± 0.01 to 0.51 ± 0.09 ($n = 5$,

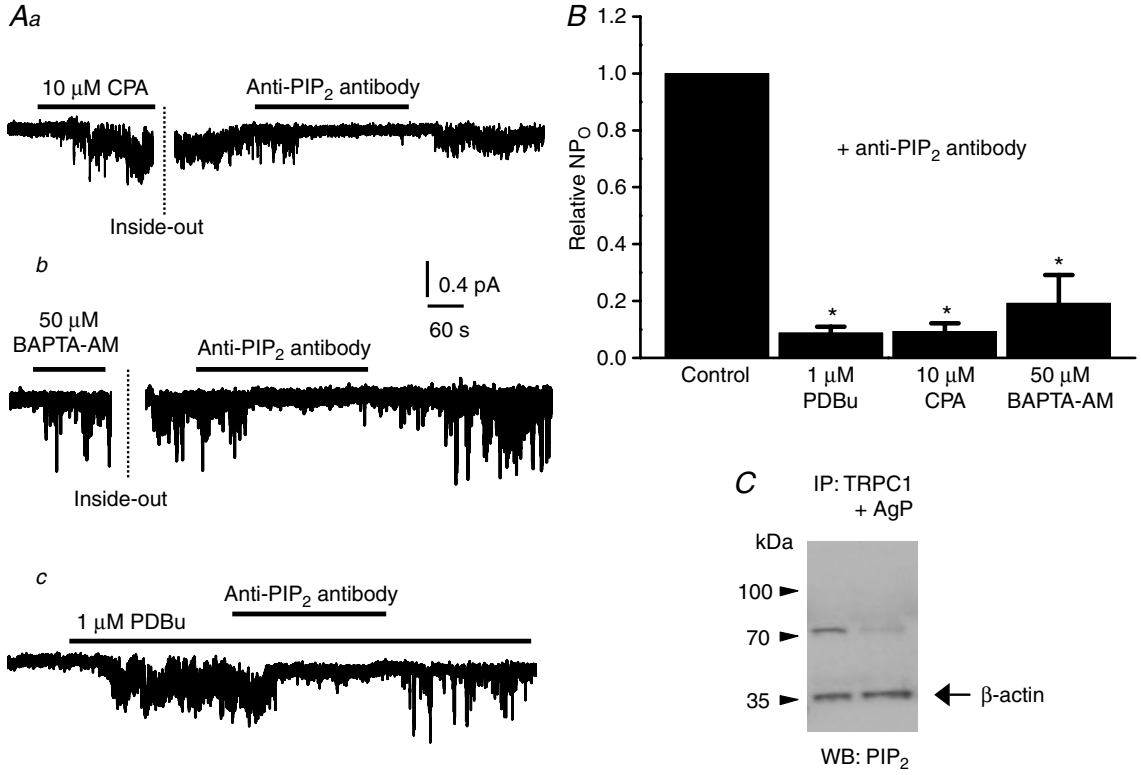

Figure 2. Anti-PIP$_2$ antibodies inhibit TRPC1 SOC activity
A, bath application of anti-PIP$_2$ antibodies (1 : 200 dilution) to the cytosolic surface of inside-out patches held at –80 mV markedly inhibited SOC activity initially induced by 10 μM CPA (*a*) or 50 μM BAPTA-AM (*b*) in cell-attached patches and 1 μM PDBu (*c*) applied to a quiescent inside-out patch. *B*, mean data showing that anti-PIP$_2$ antibodies significantly reduced SOC activity evoked by CPA, BAPTA-AM and PDBu (*$P < 0.05$). *C*, co-immunoprecipitation experiments where tissue lysates from portal vein were immunoprecipitated (IP) with an anti-TRPC1 antibody (Santa Cruz) and then Western blotted (WB) with an anti-PIP$_2$ antibody. In control conditions a band of ∼70 kDa was observed (see Methods), which was absent after pre-treatment of the anti-TRPC1 antibody with its antigenic peptide (AgP). Note that bands detected with an anti-β-actin antibody were unaffected by pretreatment with the antigenic peptide.

$P < 0.05$). In addition, in the presence of LY294002 the mean NP_o of CPA-evoked SOC activity was significantly enhanced from 0.01 ± 0.01 to 0.12 ± 0.05 ($n = 3$, $P < 0.05$) by diC8-PIP_2.

To confirm that wortmannin and LY294002 lowered PIP_2 levels we carried out co-immunoprecipitation and Western blotting studies. Figure 3*Ba* shows that pretreatment of portal vein tissue with 20 μM wortmannin reduced total PIP_2 levels measured with Western blotting (Fig. 3*Ba* left panel) and following immunoprecipitation (Fig. 3*Ba* right panel). In addition Fig. 3*Bb* illustrates that pre-treatment of portal vein tissue with 20 μM wortmannin did not alter expression levels of TRPC1 proteins, which were detected as bands at a molecular mass of ~90 kDa (see Methods). Moreover Fig. 3*Bc* shows that pretreatment of portal vein tissue with either 20 μM wortmannin or 100 μM LY294002 predictably reduced association of PIP_2 with TRPC1 proteins.

These results provide powerful evidence that endogenous PIP_2 is obligatory for SOC activation by CPA and PDBu in portal vein myocytes. Moreover these data indicate that interactions between TRPC1 proteins and PIP_2 have an important role in stimulating SOC activity.

Protein kinase C regulates diC8-PIP_2-induced activation of SOCs

We have previously shown that PKC inhibitors chelerythrine and Ro 31-8220 inhibit SOCs activated by CPA, PDBu and BAPTA-AM in rabbit portal vein myocytes (Albert & Large, 2002*b*; Liu *et al.* 2005*a*) and in other vascular preparations (Saleh *et al.* 2006, 2008). As the present work indicates that PIP_2 is also obligatory for SOC activation we investigated whether these two pathways were dependent on one another or could activate SOCs independently.

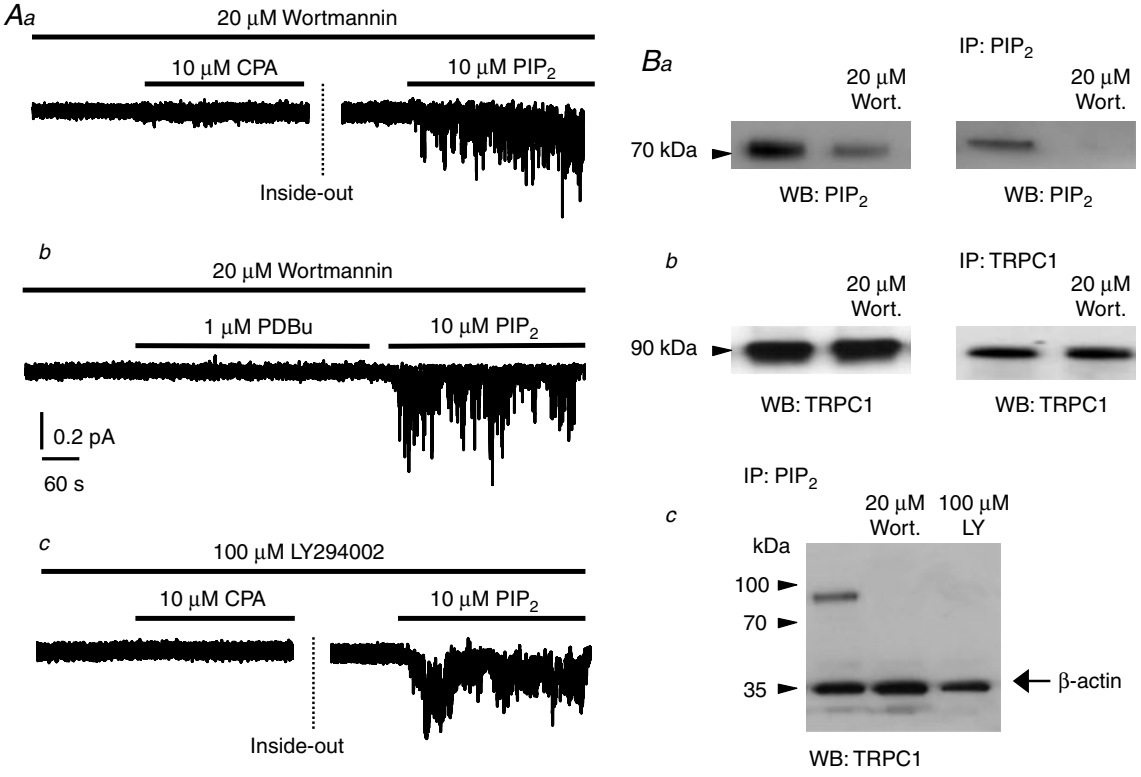

Figure 3. Depletion of endogenous PIP_2 inhibits SOC activity
A, pre-treatment of portal vein tissue with 20 μM wortmannin for 15 min prevented SOC activation by 10 μM CPA (*a*) in cell-attached patches and 1 μM PDBu (*b*) in inside-out patches held at –80 mV. Pre-treatment with 100 μM LY294002 also inhibited SOC activity induced by CPA in a cell-attached patch held at –80 mV (*c*). Note that in inside-out patches diC8-PIP_2 activated SOC activity in the presence of wortmannin (*a* and *b*) and LY294002 (*c*). *Ba*, pre-treatment of tissue lysates from portal vein with 20 μM wortmannin for 15 min reduced total PIP_2 levels detected with WB (left panel) and following IP and WB (right panel) using anti-PIP_2 antibodies. *Bb*, pre-treatment with 20 μM wortmannin had no effect on expression levels of TRPC1 detected with WB and following IP and WB using anti-TRPC1 antibodies (Santa Cruz). *Bc*, co-immunoprecipitation experiment showing that pre-treatment of portal vein tissue with 20 μM wortmannin and 100 μM LY294002 inhibited association of PIP_2 with TRPC1 proteins following IP with anti-PIP_2 and WB with anti-TRPC1 antibodies.

Figure 4*Aa* and *b* compares control responses to diC8-PIP$_2$ to responses in cells pretreated with 1 μM PDBu, a PKC activator, for 15 min in inside-out patches held at –80 mV. These data show that mean NP_o of SOC activity induced by 10 μM diC8-PIP$_2$ was significantly increased from 0.27 ± 0.04 ($n = 16$) to 0.72 ± 0.15 ($n = 5$, $P < 0.01$) in the presence of PDBu. Figure 4*Ac* shows that the same experiment done in reverse, i.e. PDBu applied after PIP$_2$, also enhanced PIP$_2$-evoked SOC activity from 0.33 ± 0.06 to 0.86 ± 0.14 ($n = 6$, $P < 0.01$). In contrast pre-application of the PKC inhibitor chelerythrine greatly reduced the ability of 10 μM diC8-PIP$_2$ to activate SOCs (Fig. 4*Ad* and *B*. Figure 4*B* shows mean concentration–response curves of diC8-PIP$_2$-activated SOCs in control conditions and following pre-treatment with either PDBu or chelerythrine, and clearly illustrates that PDBu increased mean NP_o of diC8-PIP$_2$-induced SOC activity at all concentrations tested and produced a larger maximum response. Interestingly, the concentration–response curves of diC8-PIP$_2$-induced SOC activity had similar n_H values of 1.7 and 1.6 in the absence and presence of PDBu, respectively, whilst the EC_{50} values were also similar in all conditions, around 3 μM. Thus the major effect of PDBu appears to be an increase in the number of available channels in the normal concentration range of PIP$_2$.

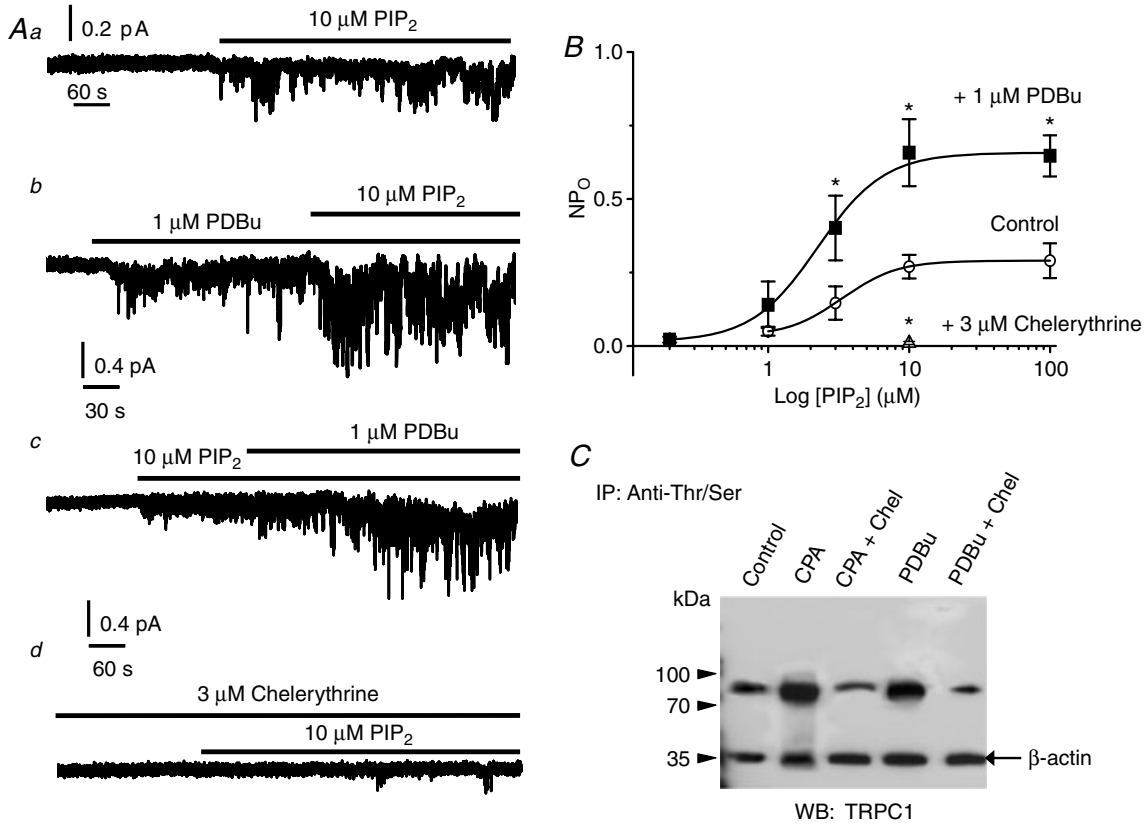

Figure 4. PKC-dependent phosphorylation associated with TRPC1 proteins is pivotal for activation of SOC activity by diC8-PIP$_2$

Aa, control response of 10 μM diC8-PIP$_2$ applied to the internal surface of an inside-out patch. *b*, 1 μM PDBu greatly enhanced responses to diC8-PIP$_2$ in patches held at –80 mV. *c*, a representative trace where 10 μM diC8-PIP$_2$ was applied to an inside-out patch first and subsequent addition of PDBu potentiated channel activity. *d*, pre-incubation with 3 μM chelerythrine almost completely abolished the ability of diC8-PIP$_2$ to activate SOCs. *B*, mean concentration–response curves of diC8-PIP$_2$-induced SOC activity in inside-out patches held at –80 mV fitted with Hill plots (see Methods) in control conditions and in the presence of PDBu and chelerythrine. In the presence of 1 μM PDBu the diC8-PIP$_2$-evoked increase in NP_o was obtained by subtracting the value in PDBu alone from the NP_o in PDBu plus diC8-PIP$_2$. Note that the EC_{50} values of the concentration–response curves in control conditions and in the presence of PDBu are similar ~3 μM. Each point is from at least $n = 4$, $^*P < 0.05$. *C*, co-immunoprecipitation experiments showing that pre-treatment of portal vein tissue with 10 μM CPA and 1 μM PDBu increases phosphorylated serine and threonine residues on TRPC1 proteins detected on Western blots. Note that CPA- and PDBu-evoked increases in TRPC1 phosphorylation are inhibited by pre-incubation with 3 μM chelerythrine.

Protein kinase C-dependent phosphorylation of TRPC1

These data with chelerythrine indicate that constitutive PKC activity is required for activation of SOCs by diC8-PIP_2 and that increased PKC stimulation augments diC8-PIP_2 induced activation of SOCs. Therefore we investigated the effect of agents that activate SOC activity on PKC-dependent phosphorylation of TRPC1 proteins and also their effect on PIP_2 interactions with TRPC1 proteins using immunoprecipitation methods.

Figure 4*C* shows that unstimulated tissue lysates from portal vein immunoprecipitated with both anti-phosphoserine and -threonine antibodies then blotted with anti-TRPC1 detected a band of ∼90 kDa. In addition Fig. 4*C* shows that pre-treatment of tissue lysates with 10 μM CPA and 1 μM PDBu increased intensity of the TRPC1 protein bands, which were inhibited by pre-incubation with 3 μM chelerythrine. Pre-treatment with 20 μM wortmannin had no effect on unstimulated, CPA- or PDBu-induced phosphorylation of TRPC1 proteins (data not shown). Interestingly pre-treatment of tissue lysates from portal vein with 10 μM CPA or 1 μM PDBu for 15 min did not alter the level of association between TRPC1 proteins and PIP_2 (data not shown). These data indicate that TRPC1 proteins are constitutively phosphorylated and that this phosphorylation can be increased by CPA and PDBu.

These co-immunoprecipitation data together with functional studies suggest that CPA and PDBu increase PKC-dependent phosphorylation of TRPC1 proteins, which promotes PIP_2-mediated SOC activity.

Discussion

The results from the present study show that PIP_2 activates a TRPC1 SOC in freshly dispersed rabbit portal vein smooth muscle cells. In addition, evidence is presented which indicates that endogenous PIP_2 is required for SOC activation in response to agents that deplete intracellular Ca^{2+} stores. To our knowledge these findings are the first to report that PIP_2 activates a native TRPC1 SOC.

We initially found that PIP_2 augmented SOC activity when applied to the cytosolic surface of patches where activity had been first induced by the Ca^{2+}-ATPase inhibitor CPA. Subsequently we also found that PIP_2 alone applied to the cytosolic surface of a quiescent inside-out patch readily activated the same 2 pS non-selective cation channel. Moreover anti-TRPC1 antibodies inhibited the PIP_2-evoked channel activity as seen in previous studies where the channel was activated by CPA and BAPTA-AM (Saleh *et al.* 2008). These characteristics of the PIP_2-evoked channel are similar to the properties of channel currents evoked by store-depletors previously described in this preparation (Albert & Large, 2002*a*; Liu *et al.* 2005*a*; Saleh *et al.* 2008). PIP_2-evoked channel currents were observed in the presence of the PLC inhibitor U73122 and 50 nM wortmannin, which selectively inhibits phosphoinositol-3-kinase. Consequently PIP_2, and not a metabolite, activates the ion channel, which has properties of a TRPC1/TRPC5/TRPC7 heterotetramer (Saleh *et al.* 2008).

Role of endogenous PIP_2 in SOC stimulation

Single channel activity evoked by CPA, BAPTA-AM or PDBu was markedly inhibited by an anti-PIP_2 antibody and this inhibitory effect was abolished when the antibody was denatured. Moreover, the same anti-PIP_2 antibody increased TRPC6 channel activity in rabbit mesenteric artery myocytes where PIP_2 had an inhibitory role (Albert *et al.* 2008), which suggests that this is not a non-selective effect of this antibody. Treatment with high concentrations of wortmannin and LY294002, which depleted total tissue PIP_2 levels, almost obliterated SOC channel activation by CPA and PDBu. However, in the same cells, in the inside-out configuration PIP_2 always evoked SOC activity when applied to the internal surface of the membrane indicating that the channels functioned normally. Co-immunoprecipitation studies illustrated that TRPC1 was associated with PIP_2 in portal vein tissue, whilst treatment with wortmannin or LY294002 depleted both total tissue PIP_2 levels and reduced co-association between TRPC1 and PIP_2. Together these results strongly indicate an obligatory role for endogenous PIP_2 in activation of the TRPC1 SOC by store-depleting reagents in portal vein myocytes.

Interaction between PIP_2 and PKC on TRPC1 SOC activation

We have previously shown that CPA-evoked SOCs in the portal vein are dependent on PKC for activation (Albert & Large, 2002*b*; Saleh *et al.* 2008). The present work demonstrates that agents which affect PKC activity had a profound effect on the ability of PIP_2 to activate SOCs. PIP_2 produced a much larger increase in channel NP_o in cells pre-treated with a PKC activator compared to control cells. Interestingly there was little change in the concentration range at which PIP_2 evoked channel activity. Instead in the presence of PDBu effective PIP_2 concentrations produced greater channel activity with an increased maximum response. These effects were most likely to be due to an increase in the number of active channels within the patch and not to channel insertion since the experiments were carried out in the inside-out configuration. Furthermore in cells pre-treated with the PKC inhibitor chelerythrine there was marked suppression of PIP_2-evoked channel activity. These data

indicate that PKC-dependent phosphorylation of the SOC regulates the ability of PIP_2 to activate the channel. Co-immunoprecipitation experiments provided evidence that TRPC1 is phosphorylated constitutively and that CPA and PDBu increased the levels of TRPC1 phosphorylation. Previously we have reported that the phosphatase inhibitor calyculin A induced SOC activity also suggesting constitutive phosphorylation of SOCs (Albert & Large, 2002*b*). Therefore our data suggest that the ability of PIP_2 to evoke the SOC depends on PKC-dependent phosphorylation of TRPC1 and previously it has been shown that PKCα-dependent phosphorylation of TRPC1 regulates store-operated Ca^{2+} entry in cultured endothelial cells (Ahmmed *et al.* 2004). Interestingly TRPC1 co-associated with PIP_2 in unstimulated cells and this interaction did not appear to be increased by CPA or PDBu. Therefore it is possible that PIP_2 is tethered to TRPC1 at rest but only activates the channel when TRPC1 is phosphorylated by PKC.

Recently several studies have implicated a role for stromal interaction molecule-1 (STIM1) in the activation of SOCs composed of TRPC1 and Orai1 complexes in heterologous expression systems (Huang *et al.* 2006; Ong *et al.* 2007; Yuan *et al.* 2007) and cultured smooth muscle cells (Li *et al.* 2008). The roles of STIM-1 or Orai1 have not been assessed in native vascular preparations but it will be of future interest to see if portal vein SOCs are composed of STIM1/TRPC1/Orai1 ternary complexes and whether STIM1 can activate these channels in the absence of PIP_2 or PKC phosphorylation.

Conclusions

This study shows that PIP_2 activates a native SOC with TRPC1 properties in vascular myocytes. Previously we postulated that this SOC may possess a TRPC1/TRPC5/TRPC7 heteromeric structure. Moreover it appears that endogenous PIP_2 is obligatory for channel activation by agents such as CPA, BAPTA-AM and PDBu. Finally PIP_2 stimulation of the SOC appears to be regulated by PKC-dependent phosphorylation of TRPC1 induced by procedures that deplete internal Ca^{2+} stores. In physiological conditions SOCs are activated by G-protein-coupled receptor stimulation that often utilizes PLC-mediated cleavage of PIP_2 to produce IP_3 and DAG. It seems curious that an agonist that decreases the concentration of PIP_2, which is essential for SOC activation, is able to activate these channels (Albert & Large, 2002*b*). It is possible that there are separate microdomains of PIP_2; one pool of PIP_2 may be in a complex with the pharmacological receptor and PLC while a second pool of PIP_2, which is not accessible to PLC, may be linked to the TRPC1 SOC complex. Further experiments are needed to test this hypothesis. It is clear from the present work that endogenous PIP_2 is required for activation of TRPC1 SOC, in contrast to its effect on TRPC6 ROC where PIP_2 is an endogenous inhibitor of channel activation by DAG (Albert *et al.* 2008).

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*a*). A Ca^{2+}-permeable non-selective cation channel activated by depletion of internal Ca^{2+} stores in single rabbit portal vein myocytes. *J Physiol* **538**, 717–728.

Albert AP & Large WA (2002*b*). 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 (2006). Signal transduction pathways and gating mechanisms of native TRP-like cation channels in vascular myocytes. *J Physiol* **570**, 45–51.

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**, 361–373.

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, Peppiatt-Wildman CM & Large WA (2007). Multiple activation mechanisms of store-operated TRPC channels in smooth muscle cells. *J Physiol* **583**, 25–36.

Asteggiano C, Berberian G & Beauge L (2001). Phosphatidylinositol- 4,5-bisphosphate bound to bovine cardiac Na^+/Ca^{2+} exchanger displays a MgATP regulation similar to that of the exchange fluxes. *Eur J Biochem* **268**, 437–442.

Beauge L, Asteggiano C & Berberian G (2002). Regulation of phosphatidylinositol-4,5-bisphosphate bound to the bovine cardiac Na^+/Ca^{2+} exchanger. *Ann N Y Acad Sci* **976**, 288–299.

Beech DJ, Muraki K & Flemming R (2004). Non-selective cationic channels of smooth muscle and the mammalian homologues of *Drosophila* TRP. *J Physiol* **559**, 685–706.

Bian J, Cui J & McDonaild TV (2001). HERG K^+ channel activity is regulated by changes in phosphatidylinositol-4,5-bisphosphate. *Circ Res* **89**, 1168–1176.

Firth AL, Remillard CV & Yuan JX (2007). TRP channels in hypertension. *Biochim Biophys Acta* **1772**, 895–906.

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.

Huang GN, Zeng W, Kim JY, Yuan JP, Han L, Muallem S & Worley PF (2006). STIM1 carboxyl-terminus activates native SOC, I_{crac} and TRPC1 channels. *Nat Cell Biol* **8**, 1003–1010.

Inoue R, Okada T, Onoue H, Hara Y, Shimizu S, Naitoh S, Ito Y & Mori Y (2001). The transient receptor potential protein homologue TRP6 is the essential component of vascular a1-adrenoceptor-activated Ca^{2+}-permeable cation channel. *Circ Res* **88**, 325–332.

Large WA (2002). Receptor-operated Ca^{2+}-permeable non-selective cation channels in vascular smooth muscle: a physiologic perspective. *J Cardiovasc Electrophysiol* **13**, 493–501.

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.

Li J, Sukumar P, Milligan CJ, Kumar B, Ma ZY, Munsch CM, Jiang LH, Porter KE & Beech DJ (2008). Interactions, functions, and independence of plasma membrane STIM1 and TRPC1 in vascular smooth muscle cells. *Circ Res* **103**, 97–104.

Liou HH, Zhou SS & Huang CL (1999). Regulation of ROMK1 channel by protein kinase A via a phosphatidylinositol-4, 5-bisphosphate-dependent mechanism. *Proc Natl Acad Sci U S A* **96**, 5820–5825.

Liu M, Albert AP & Large WA (2005*a*). 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.

Liu X, Bandyopadhyay BC, Singh BB, Groscher K & Ambudkar IS (2005*b*). Molecular analysis of a store-operated and 2-acetyl-sn-glycerol-sensitive non-selective cation channel. Heteromeric assembly of TRPC1-TRPC3. *J Biol Chem* **280**, 21600–21606.

Ma HP, Saxena S & Warnock DG (2002). Anionic phospholipids regulate native and expressed epithelial sodium channel (ENaC). *J Biol Chem* **277**, 7641–7644.

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

Ong HL, Cheng KT, Liu X, Bandyopadhyay BC, Paria BC, Soboloff J, Pani B, Gwack Y, Srikanth S, Singh BB, Gill DL & Ambudkar IS (2007). Dynamic assembly of TRPC1-STIM1-Orai1 ternary complex is involved in store-operated calcium influx. Evidence for similarities in store-operated and calcium release-activated calcium channel components. *J Biol Chem* **282**, 9105–9116.

Osborne SL, Thomas CL, Gschmeissner S & Schiavo G (2001). Nuclear PtdIns(4,5)P_2 assembles in a mitotically regulated particle involved in pre-mRNA splicing. *J Cell Sci* **114**, 2501–2511.

Otsuguro K, Tang J, Tang Y, Xiao R, Freichel M, Tsvilovskyy V, 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.

Pian P, Bucchi A, Robinson RB & Siegelbaum SA (2006). Regulation of gating and rundown of HCN hyperpolarization-activated channels by exogenous and endogenous PIP_2. *J Gen Physiol* **128**, 593–604.

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.

Smani T, Zakharov SI, Csutora P, Leno E, Trepakova ES & Bolotina VM (2004). A novel mechanism for the store-operated calcium influx pathway. *Nat Cell Biol* **2**, 113–120.

Sours S, Du J, Chu S, Ding M, Zhou XJ & Ma R (2006). Expression of canonical transient receptor potential (TRPC) proteins in human glomerular mesangial cells. *Am J Physiol Renal Physiol* **290**, F1507–F1515.

Suh B-S & Hille B (2005). Regulation of ion channels by phosphatidylinositol-4,5-bisphosphate. *Curr Opin Neurobiol* **15**, 370–378.

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* (in press).

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

Xie LH, Johm SA, Ribalet B & Weiss JN (2008). Phosphatidylinositol- 4,5-bisphosphate (PIP_2) regulation of strong inward rectifier Kir2.1 channels: multilevel positive cooperativity. *J Physiol* **586**, 18833–11848.

Xu SZ & Beech DJ (2000). TrpC1 is a membrane-spanning subunit of store-operated Ca^{2+} channels in native vascular smooth muscle cells. *Circ Res* **88**, 84–87.

Xu SZ, Boulay G, Flemming R & Beech DJ (2006). E3-targeted anti-TRPC5 antibody inhibits store-operated calcium entry in freshly isolated pial arterioles. *Am J Physiol Heart Circ Physiol* **291**, H2653–H2659.

Yuan JP, Zeng W, Huang GN, Worley PF & Muallem S (2007). STIM1 heteromultimerizes TRPC channels to determine their function as store-operated channels. *Nat Cell Biol* **9**, 636–645.

Yue G, Malik B, Yue G & Eaton DC (2002). Phosphatidylinositol 4, 5- bisphosphate (PIP_2) stimulates epithelial sodium channel activity in A6 cells. *J Biol Chem* **277**, 119–1969.

Acknowledgements

This work was supported by The Wellcome Trust.