Title: Obligatory role for PKCδ in PIP_2-mediated activation of store-operated TRPC1 channels in vascular smooth muscle cells

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Key points

- In vascular smooth muscle cells (VSMCs), activation of Ca^{2+}-permeable store-operated channels (SOCs) composed of canonical transient receptor potential channel 1 (TRPC1) subunits mediate Ca^{2+} entry pathways which regulate contraction, proliferation and migration that are processes associated with vascular disease.

- Activation of TRPC1-based SOCs requires protein kinase C (PKC) activity, which is proposed to phosphorylate TRPC1 proteins to promote channel opening by phosphatidylinositol 4,5-bisphosphate (PIP_2). We investigated the identity of the PKC isoform involved in activating TRPC1-based SOCs in rat mesenteric artery VSMCs.

- TRPC1-based SOCs were reduced by PKCδ inhibitors and knockdown of PKCδ expression. Store depletion induced interactions between TRPC1 and PKCδ and PKCδ-dependent phosphorylation of TRPC1. Furthermore, generation of store-operated interactions between PIP_2 and TRPC1 and activation of TRPC1-based SOCs by PIP_2 required PKCδ.

- These findings reveal that PKCδ activity has an obligatory role in activating TRPC1-based SOCs, through regulating PIP_2-mediated channel opening.

Abstract

In vascular smooth muscle cells (VMSCs), stimulation of Ca^{2+}-permeable canonical transient receptor potential channel 1 (TRPC1)-based store-operated channels (SOCs) mediate Ca^{2+} entry pathways which regulate cell contraction, proliferation and migration that are processes associated with vascular disease. It is therefore important to understand how TRPC1-based SOCs are activated. Stimulation of TRPC1-based SOCs requires protein kinase C (PKC) activity, with store-operated PKC-dependent phosphorylation of TRPC1 essential for channel opening by phosphatidylinositol 4,5-bisphosphate (PIP_2). Experimental protocols used to activate TRPC1-based SOCs suggest that the PKC isoform involved requires diacylglycerol (DAG) but is Ca^{2+}-insensitive, which are characteristics of the novel group of PKC isoforms (δ, ε, η, θ). Hence the present study examines if a novel PKC isoform(s) is involved in activating TRPC1-based SOCs in contractile rat mesenteric artery VSMCs. Store-operated whole-cell cation currents were blocked by Pico145, a highly selective and potent TRPC1/4/5 channel blocker and T1E3, a TRPC1 blocking antibody. PKCδ was expressed in VSMCs, and selective PKCδ inhibitory peptides and knockdown of PKCδ expression with morpholinos oligomers inhibited TRPC1-based SOCs. TRPC1 and PKCδ interactions and phosphorylation of TRPC1 induced by store depletion were both reduced by pharmacological inhibition and PKCδ knockdown. In addition, store-operated PIP_2 and TRPC1 interactions were blocked by PKCδ inhibition, and PKCδ was required for PIP_2-mediated activation of TRPC1 currents. These results identify involvement of PKCδ in stimulation of TRPC1-based SOCs and highlights that store-operated PKCδ activity is obligatory for channel opening by PIP_2, the likely activating ligand.

Miguel Martín-Aragón Baudel obtained his PhD in Edinburgh Napier University under the supervision of Mark Darlison and Amy Poole before moving to St George's University of London to work as a postdoctoral research assistant with Anthony Albert. He is currently working at the University of California, Davis with Manuel Navedo continuing his research interests in ion channel-mediated mechanisms driving vascular contraction and role in diseases such as hypertension and diabetes.

Introduction

Store-operated channels (SOCs) are Ca^{2+}-permeable plasmalemmal ion channels activated by depletion of cytosolic endo/sarcoplasmic (ER/SR) Ca^{2+} stores (Martín-Aragón Baudel et al, 2020). In vascular smooth muscle cells (VSMCs), SOCs are stimulated by vasoconstrictors that activate the $G\alpha q$ protein-coupled receptor signaling pathway leading to phospholipase C (PLC) activity, phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis, inositol 1,4,5-trisphosphate (IP_3) generation and IP_3-mediated depletion of SR Ca^{2+} stores. As such SOCs induce Ca^{2+} entry pathways that regulate vasoconstrictor-mediated contraction, proliferation, and migration and are considered drug targets for treatment of vascular diseases such as hypertension and atherosclerosis.

There is substantial evidence that SOCs represent a diverse family of ion channels with differing molecular composition, activation mechanisms and functions (Albert & Large, 2003; Albert et al, 2007; 2009; Cheng et al, 2013; Prakriya & Lewis, 2015; Ong et al, 2016). In native contractile VSMCs, SOCs have relatively low Ca^{2+} permeability, linear current-voltage rectification properties, a single channel conductance of 2-3 pS, and are composed of canonical transient receptor potential (TRPC) channels (Trepakova et al, 2001; Albert & Large, 2002a; Bergdahl et al, 2005; Xu & Beech, 2001; Xu et al, 2005a; Liu et al, 2005a; 2005b; Albert et al, 2006; Saleh et al, 2006; 2008; 2009a; Shi et al, 2012a; 2014; 2016; 2017a). It is proposed that molecular composition involves a heteromeric TRPC1/C5 template with TRPC1 the critical subunit for conferring activation by store depletion, hence the term TRPC1-based SOCs (Xu & Beech, 2001; Xu et al, 2005a; Saleh et al, 2008; Shi et al, 2012a). In comparison, synthetic VSMCs that are involved in cell proliferation, migration and growth and are associated with more pathological functions exhibit TRPC1-based SOCs and also SOCs with properties similar to Orai1-based calcium release-activated channels (CRACs) such as high Ca^{2+} permeability, pronounced inward rectification, single channel conductance in the fS range, and composition of Orai1 proteins (Berra-Romani et al, 2008; Beech, 2012; Trebak, 2012, Prakriya & Lewis, 2015). It is important to highlight that in this study, we examined activation mechanisms of TRPC1-based SOCs in freshly isolated and primary cultured single VSMCs and tissue lysates that have a native contractile phenotype (Shi et al, 2016; 2017a) and are unlikely to involve Orai1 proteins (Shi et al, 2017b). These TRPC1-based SOCs are likely to be important in regulating contractility and switching of VSMCs from contractile to synthetic phenotypes (Berra-Romani et al, 2008; Matchkov et al, 2013).

How store depletion stimulates TRPC-based SOCs is controversial, especially compared to Orai1-based CRACs where it is well-established that store depletion induces the ER/SR store Ca^{2+} sensor stromal interaction molecule 1 (STIM1) to oligomerise and translocate to the plasma membrane where it interacts with Orai1 to induce channel assembly and gating (Prakriya & Lewis, 2015). However, there is growing evidence that store depletion also activates TRPC-based SOCs through STIM1-mediated

processes, potentially involving direct interactions between TRPC and Orai1 proteins (Liao et al, 2014), activation of Orai1-based CRACs as a prerequisite for TRPC1 channel opening (Ambudkar et al, 2017), and direct interactions between TRPC and STIM1 proteins (Worley et al, 2007; Yuan et al, 2009; Lee et al, 2014; Asanov et al, 2015). In native contractile VSMCs, our recent work indicates that store depletion stimulates TRPC1-based SOCs through a novel STIM1-mediated Gαq/PLCβ1 pathway which is likely to induce channel opening by regulating interactions between protein kinase C and PIP_2 (Shi et al, 2016; 2017a).

There is considerable evidence that protein kinase C (PKC) activity is critical for activation of TRPC1-based SOCs in native contractile VSMCs (Large et al, 2009; Albert, 2011). PKC inhibitors prevent activation of TRPC1-based SOCs and reduce store-operated phosphorylation of TRPC1, and PKC activators stimulate TRPC1-based SOCs (Saleh et al, 2008; 2009a; Shi et al, 2012a; Shi et al, 2016). Activation of TRPC1-based SOCs and phosphorylation of TRPC1 by physiological vasoconstrictors are also reduced by PKC inhibitors (Albert & Large, 2002b; Saleh et al, 2006; 2009b; Shi et al, 2012b; 2014, 2016). It is proposed that PKC activity stimulates opening of TRPC1-based SOCs through phosphorylation of TRPC1 proteins to promote PIP_2 binding that acts as the activating ligand (Shi et al, 2014; 2016; 2017a). As such, activation of TRPC1-based SOCs by store depletion or PKC activators is inhibited by anti-PIP_2 antibodies and pre-treatment with PI4-kinase inhibitors which deplete PIP_2 levels (Saleh et al, 2009a; Shi et al, 2012b). In addition, activation of TRPC1-based SOCs by the water soluble PIP_2 analogue diC8-PIP_2 is prevented by PKC inhibitors (Saleh et al, 2009a; Shi et al, 2012b). These results indicate that interactions between PKC activity and PIP_2 have obligatory roles in activation of TRPC1-based SOCs; PKC cannot activate TRPC1-based SOCs without PIP_2 and vice versa (Martín-Aragón Baudel et al, 2020). The present study further examines the importance of PKC activity in activating TRPC1-based SOCs by investigating the PKC isoform(s) involved.

The PKC family comprises of at least 11 homologous serine/threonine kinases divided into three groups according to their basic structure and activation requirements: conventional PKC isoforms (α, βI, βII and γ) require both Ca^{2+} and diacylglyerol (DAG), novel PKC isoforms (δ, ε, η and θ) require DAG but are Ca^{2+}-insensitive, and atypical PKC isoforms (ζ, ι and λ) are activated by lipid mediators such as phosphatidylserine and do not require Ca^{2+} or DAG (Salamanca & Khalil, 2005; Ringvold & Khalil, 2017). Many of these PKC isoforms are expressed in VSMCs and are proposed to regulate several physiological and pathological processes, including those reported to involve a role for TRPC1-based SOCs (Salamanca & Khalil, 2005; Ding et al, 2011; Fan et al, 2014; Ringvold & Khalil, 2017). In native contractile VSMCs, stimulation of TRPC1-based SOCs and PKC-dependent phosphorylation of TRPC1 by store-depletion requires PLCβ1 activity (Shi et al, 2016), DAG activates TRPC1-based SOCs through

a PKC-dependent mechanism (Saleh et al, 2006; 2008; Large et al, 2009; Albert, 2011), and TRPC1-based SOCs are activated by store depleting agents which are likely to increase (e.g. the SR Ca^{2+}-ATPase inhibitor cyclopiazonic acid (CPA)), decrease (e.g. the cell-impermeable and -permeable high affinity Ca^{2+} chelators BAPTA and BAPTA-AM), or produce little change in $[Ca^{2+}]_i$ (e.g. the cell-permeable low affinity Ca^{2+} chelator TPEN). These results suggest that the PKC isoform involved requires DAG but it is likely to be Ca^{2+}-insensitive; these are characteristics of the novel group of PKC isoforms. These ideas on the identity of the PKC isoform involved form the basis of the present work, and our findings indicate that the PKCδ novel isoform is the likely candidate involved in activation of TRPC1-based SOCs in native contractile rat mesenteric artery VSMCs.

Methods

Ethical Approval

All animal procedures were carried out in accordance with guidelines laid down by St George's, University of London Animal Welfare Committee and conform with the principles and regulations described by the Service Project Licence: 70/8512, and also to the principles and regulations of *The Journal of Physiology* as described by Grundy (2015). Male Wistar rats (8-12 weeks old) were used for the purpose of this study. Rats were supplied from Charles River, UK and housed and maintained in standard sized plastic cages at the Biological Research Facility at St George's, University of London, with a 12 h light-dark cycle, ambient room temperature of 18-20^{o}C, relative humidity of approximately 50%, and water and lab rodent diet (Specialist Dietary Services, UK) available *ad libitum*. Animals were culled by cervical dislocation in accordance with the UK Animals Scientific Procedures Act of 1986 and as revised by European Directive 2010/63/EU.

Cell Isolation and tissue lysates

Mesenteric arteries were dissected and cleaned of adherent fat in physiological salt solution containing (mmol/L): 126 NaCl, 6 KCl, 10 Glucose, 11 HEPES, 1.2 $MgCl_2$, and 1.5 $CaCl_2$, with pH adjusted to 7.2 with 10 mol/L NaOH. Single VSMCs were enzymatically dispersed and tissue lysates prepared as previously described (Shi et al, 2016; 2017a).

PKCδ knockdown

Knockdown of PKCδ was performed by transfection of vessel segments with morpholino antisense oligonucleotides as described previously (Stott et al, 2018). PKCδ morpholino oligomers and a control oligomer containing five mismatched nucleotides (10 μM, Genetools) were mixed with lipofectamine 2000 (Life Technologies) in Opti-MEM and left at room temperature for 2 h. Mesenteric artery segments

were cultured with this mix in DMEM F-12 with 1% penicillin/streptomycin at 37°C for 48 h. Vessel segments were then enzymatically dispersed into single VSMCs or used as tissue lysates as required.

Electrophysiology

Whole-cell and cell-attached patch clamp techniques were used to record TRPC1-based SOCs with an Axopatch 200B amplifier (Axon Instruments, Union City, CA, USA) at room temperature (20–23°C) using bath and patch pipette solutions, data analysis and experimental protocols as described previously (Shi et al, 2017a). In experiments investigating the effects of phorbol 12,13-dibutyrate (PBDu), a phorbol ester and PKC activator, and diC8-PIP_2 on whole-cell cation currents 750 ms duration voltage ramps from +100 mV to - 150 mV were applied every 30 s from a holding potential of 0 mV. The patch pipette solution contained (mM): CsCl (126), $MgCl_2$ (1.2), Hepes (10), glucose (11), BAPTA (10), $CaCl_2$ (4.8, free internal Ca^{2+} concentration approximately 100 nM as calculated using MaxChelator (maxchelator.stanford.edu)), Na_2ATP (1), NaGTP (0.2) pH 7.2 with Tris. The external solution contained (mM): NaCl (126), $CaCl_2$ (1.5), Hepes (10), glucose (11), DIDS (0.1), niflumic acid (0.1), and nicardipine (0.005) pH 7.2 with NaOH.

Immunoprecipitation and western blotting

Freshly isolated or cultured vessel segments were prepared for immunoprecipitation, one-dimensional protein gel electrophoresis and immunoblotting as previously described (Shi et al, 2017a). Primary antibodies used: mouse anti-PKCε (Santa Cruz, sc-1681, 1:500), mouse anti-PKCθ (Santa Cruz, sc-1680, 1:500), mouse anti-PKCη (Santa Cruz, sc-136036, 1:500), rabbit anti-PKCδ (Abcam, ab182126, 1:1000). Rabbit anti-TRPC1 antibody (1 µg/mL) was generated by GenScript (Piscataway, NJ, USA) using peptide sequences from a previously characterized putative extracellular region (Xu & Beech, 2001). Visualization were performed using anti-rabbit and anti-mouse secondary antibodies conjugated to IRDye 800RD or IRDye 680CW (1:10,000; Li-Cor Biosciences, Cambridge, United Kingdom) as appropriate and the Odyssey Infrared Imaging System (Li-Cor Biosciences,). Protein band intensities were measured using Image Studio software (Li-Cor Biosciences) and normalized to smooth muscle actin (Abcam, #ab5694, 1:2000) when quantified.

Immunofluorescence

Single VSMCs were fixed with 4% (w/v) paraformaldehyde for 15 min, cells were treated with 0.1 mol/L glycine for 5 minutes and permeabilized with phosphate-buffered saline (PBS) containing 0.1% (v/v) Triton X-100 (PBS-T) for 15 min at room temperature. Cells were then incubated with PBS-T containing 1% (w/v) bovine serum albumin (BSA) for 1 h at room temperature to block non-specific binding of antibodies. Immunostaining was performed with mouse anti-PKCε (Santa Cruz, sc-1681, 1:500), mouse anti-PKCθ (Santa Cruz, sc-1680, 1:200), rabbit anti-PKCη (Abcam, ab4134, 1:200), rabbit anti-PKCδ

(Abcam, ab182126, 1:1000) overnight at 4°C. Cells were then washed and incubated with a 488 fluorophore-conjugated donkey anti-goat secondary antibody (1:1000; A-11055, Alexa Fluor, UK) for 1 h at room temperature. Unbound secondary antibodies were removed by washing with PBS, and nuclei were labelled with 4, 6-diamidino-2-phenylindole (DAPI) mounting medium (Sigma, UK). Control experiments were performed by omitting either primary or secondary antibodies. Cells were imaged using a Nikon A1R confocal microscope. PIP_2 immunofluorescence experiments were performed in the presence of saponin with rabbit anti-PIP_2 (Santa Cruz, sc-53412, 1:50) according to (Edimo et al, 2016).

Proximity Ligation Assay

Interactions between different proteins were studied with Duolink® in situ detection kit (Sigma, UK) as previously described (Shi et al, 2017a). Single VSMCs were fixed and permeabilized as per immunofluorescence and blocking was performed with Duolink blocking buffer for 1 h at 37°C. Cells were then incubated with appropriate antibodies overnight at 4°C as per immunofluorescence experiments plus mouse anti-P-Ser (Santa Cruz, sc-16B4, 1:100), mouse anti-P-Thr (Santa Cruz, sc-5267, 1:100), mouse anti-TRPC1 (Santa Cruz, sc-133076, 1:100) and rabbit anti-TRPC5 (T5E3) which was generated by GenScript (Piscataway, NJ, USA) using peptide sequences from a previously characterized putative extracellular region (1 µg/mL, Xu et al, 2001; 2005a; 2005b). The rest of the protocol continued as per manufacturer's instructions. Fluorescent puncta were visualized with Nikon A1R confocal microscope and images analyzed with ImageJ Fiji. The mean number of puncta per cell was calculated by counting the number of particles across a z-stack of the cell.

Reagents

Pico145 was a generous gift provided by Robin S Bon and David J Beech (University of Leeds, UK). The cell permeable PKCδ inhibitor, δV1-TAT peptide (RRRQRRKKRGY-SFNSYELGSL) was synthesized by Mimotopes (Wirral, UK). PKCδ non-permeable $\delta PKC_{8\text{-}17}$ peptide inhibitor (SFNSYELGSL) was purchased from AnaSpec (AnaSpec, EGT Corporate Headquarters, USA). PKCε peptide inhibitor was purchased from Santa Cruz. All other drugs were purchased from Sigma-Aldrich or Tocris (Abingdon, UK). Agents were dissolved in distilled H_2O or 0.1% DMSO. DMSO alone had no effect on whole-cell or single channel currents.

Statistical analysis

All statistical analysis was performed using GraphPad Prism 8 (La Jolla, CA). Data was calculated as mean ± SD, with n=number of data points. Data points were generated from at least three different isolated VSMCs or tissue lysate preparations. To compare between two or more current-voltage relationships, two-way ANOVA with Tukey multiple comparisons test was used, and differences in

means at -80 mV are shown in text. To compare between two data sets, paired or unpaired t-tests were used. The level of significance for all statistical tests was set at $p < 0.05$.

Results

PKC-dependent store-operated currents are composed of TRPC1 subunits in rat mesenteric artery VSMCs

In our first series of experiments, we confirmed that PKC-dependent TRPC1-based SOCs are functionally expressed in native contractile VSMCs from freshly isolated rat mesenteric arteries using a highly selective and potent TRPC1/C4/C5 channel blocker Pico145 (Rubaiy et al, 2017), an externally-acting TRPC1 blocking antibody T1E3 (Xu & Beech, 2001, Xu et al, 2005b) and the pan-PKC isoform selective inhibitor GF109203X. Figure 1A shows that passive depletion of internal Ca^{2+} stores, following cell dialysis with a patch pipette solution containing high concentrations of BAPTA and no added Ca^{2+}, induced whole-cell cation currents which had a relatively linear current-voltage (I/V) relationship, an E_{rev} between 0 mV and +20 mV, with a mean control amplitude of -1.15 $\pm$ 0.62 pA/pF at -80 mV which increased to a mean peak amplitude of -2.74 $\pm$ 0.88 pA/pF (n=6, p=0.0015) and reduced to -0.89 $\pm$ 0.35 pA/pF by bath application of Pico145 (n=6, p=0.0002, two-way ANOVA, Tukey's multiple comparisons test). Figures 1B & C show that bath application of T1E3 and G109203X also inhibited store-operated whole-cell currents, with mean peak amplitudes at -80 mV reduced respectively from -2.96 $\pm$ 0.92 pA/pF to -0.84 $\pm$ 0.43 (n=6, p=0.0001) and from -2.14 $\pm$ 0.92 pA/pF to -0.91 $\pm$ 0.45 (n=7, p=0.0051, two-way ANOVA, Tukey's multiple comparisons test).

Figure 1

There is considerable evidence that heteromeric TRPC1/C5 molecular structures compose TRPC1-based SOCs in VSMCs from different rabbit, mouse, and human vascular beds (see Introduction). In proximity ligation assay (PLA) studies, Figure 2 shows that fluorescent puncta with a mean number per cell of 51.5 $\pm$ 12.6 (n=14) produced using anti-TRPC1 and anti-TRPC5 antibodies were present at, or close to, the plasma membrane in freshly isolated rat mesenteric artery VSMCs.

Figure 2

These findings clearly confirm that PKC-dependent TRPC1-based SOCs are functionally expressed in native contractile rat mesenteric artery VSMCs, similar to previous studies in VSMCs from different rabbit, mouse and human vascular preparations (see Introduction).

PKCδ is the dominant novel PKC isoform in VSMCs

The PKC isoform involved in activating TRPC1-based SOCs in VSMCs requires DAG but is Ca^{2+} insensitive and is likely to be a member of the novel PKC isoform family (see Introduction), we therefore investigated the expression of novel PKC isoforms in tissue lysates from freshly isolated rat mesenteric arteries. We used brain lysates as positive controls where novel PKC isoforms have been previously identified (Callender & Newton, 2017; Fleegal et al, 2006; Popp et al 2006; Wang et al, 2019; Yang et al, 2019).

Figure 3A shows PKCδ expression with relatively low levels or little expression of PKCε, PKCη and PKCθ were found in tissue lysates from rat mesenteric arteries. Using the same anti-PKC novel isoform antibodies, expression of PKCδ, PKCε, PKCη and PKCθ were all present in brain lysates. In addition, Figure 3B shows that immunocytochemical studies revealed PKCδ staining at, or close to, the plasma membrane of VSMCs with little staining recorded for PKCε, PKCη and PKCθ isoforms.

Figure 3

PKCδ activity is essential for activation of TRPC1-based SOCs

In our next series of experiments, we examined if pharmacological inhibition of PKCδ and knockdown of PKCδ expression using morpholino technology resulted in an anticipated decrease in activation of TRPC1-based SOCs. We focused on the PKCδ isoform based on the expression studies detailed above and previous data indicating that expression and function of PKCδ in vascular smooth muscle (Salamanca & Khalil, 2005; Ringvold & Khalil, 2017).

Figure 4A reveals that store-operated whole-cell TRPC1-based currents were inhibited by bath application of the cell-permeable PKCδ inhibitor δV1-TAT, with mean peak amplitude reduced from -6.18 ± 2.34 pA/pF to -1.26 ± 0.57 pA/pF at -80 mV (n=6, p=0.0001, two-way ANOVA, Tukey's multiple comparisons test). Figure 4B also shows that following inclusion of the cell-impermeable PKCδ peptide inhibitor δPKC_{8-17} in the patch pipette solution store depletion induced a small whole-cell TRPC1-based current with mean control amplitude increasing from -0.97 ± 0.27 pA/pF to a mean peak amplitude of -2.01 ± 1.1 pA/pF at -80 mV (n=6, p=0.0902, two-way ANOVA, Tukey's multiple comparisons test).

Since expression of PKCε was also observed in VSMCs (see Figure 3) and that other studies have indicated expression of PKCε in the vasculature (Salamanca & Khalil, 2005; Ringvold & Khalil, 2017), we studied the potential contribution of this PKC isoform in activating store-operated TRPC1-based SOCs. Figure 4C shows following inclusion of a PKCε-specific peptide inhibitor in the patch pipette

solution store depletion activated whole-cell TRPC1 currents with a mean control amplitude increasing from -0.84 ± 0.21 pA/pF to -3.39 ± 1.42 pA/pF at -80 mV (n=5, p=0.0006) which was subsequently inhibited by bath application of δV1-TAT to -0.99 ± 0.56 pA/pF (n=5, p=0.0012, two-way ANOVA, Tukey's multiple comparisons test).

Figure 4

Figure 5A shows that incubation of freshly isolated rat mesenteric artery segments with antisense morpholino oligomers against PKCδ for 48 h significantly decreased protein expression of PKCδ by about 50% compared to a mismatched oligomer (n=3, p=0.0202, unpaired t-test). Figure 5B shows that in VSMCs isolated from vessel segments expressing mismatched (scrambled) oligomers store depletion induced whole-cell TRPC1 currents with relative linear rectification and a mean control amplitude at -80 mV which increased from -1.07 ± 0.68 pA/pF to a mean peak amplitude of -2.57 ± 0.89 pA/pF (n=9, p=0.0003, two-way ANOVA, Tukey's multiple comparisons test), which were similar to currents recorded from freshly isolated VSMCs (see Figures 1 and 4). In contrast, Figure 5B shows that in VSMCs expressing specific PKCδ oligomers store depletion activated small whole-cell TRPC1 currents, with control mean amplitude at -80 mV increasing from -1.05 ± 0.59 pA/pF to a mean peak amplitude of -1.36 ± 0.69 pA/pF (n=10, p=0.8240, two-way ANOVA, Tukey's comparisons test). As such, mean peak amplitudes of store-operated TRPC1 current at -80 mV were greatly reduced in VSMCs expressing specific PKCδ compared to scrambled oligomers (p=0.0051, two-way ANOVA, Tukey's comparisons test).

Figure 5

Store depletion induces TRPC1 and PKCδ interactions and PKCδ-dependent phosphorylation of TRPC1

The above pharmacological and molecular evidence indicate that PKCδ activity is essential for activation of TRPC1-based SOCs in native contractile VSMCs. Therefore, we investigated if store depletion induced PKCδ and TRPC1 interactions and PKCδ-dependent phosphorylation of TRPC1 in freshly isolated rat mesenteric artery tissue lysates and VSMCs.

Figure 6A shows that immunoprecipitation with anti-TRPC1 antibodies followed by immunoblot with an anti-PKCδ antibody revealed that interactions between these two proteins are absent in unstimulated tissue but occur following incubation with BAPTA-AM or TPEN. Furthermore, PLA studies shown in Figure 6B confirmed that interactions between TRPC1 and PKCδ were absent in unstimulated VSMCs,

but that robust puncta at, or close to, the plasma membrane were induced between TRPC1 and PKCδ following pre-treatment with BAPTA-AM and TPEN.

Figure 6

PLA experiments performed with a mixture of anti-phosphorylated serine/threonine and anti-TRPC1 antibodies revealed a low number of fluorescence puncta in unstimulated freshly isolated VSMCs, which is indicative of a low level of basal TRPC1 phosphorylation. Figure 7A shows that the mean number of puncta per cell was increased following pre-treatment with BAPTA-AM from 6.1 ± 3.9 (n=19) to 20.5 ± 9.9 (n=30, p=0.0001) and inhibited by co-application of δV1-TAT to 8.3 ± 4.2 (n=25, p=0.0001, unpaired t-test). In addition, Figure 7B shows that transfection of vessel segments with specific PKCδ morpholino oligomers also reduced BAPTA-AM-induced increase in mean puncta number compared to scrambled oligomers from 22.4 ± 10.4 (n=30) to 7.2 ± 4.9 (n=25, p=0.0001, unpaired t-test). Importantly, Figures 7A and B show a similar mean number of puncta using mismatched oligomers compared to non-transfected freshly isolated VSMCs under unstimulated (8.7 ± 4.3, n=26 vs 6.1 ± 3.9, n=19, p=0.049) or BAPTA-AM-treated conditions (22.4 ± 10.4, n=30 vs 20.5 ± 9.9, n=30, p=0.4653, unpaired t-test), indicating that the transfection process had little effect on this mechanism.

Taken together, these results indicate that store depletion induces interactions between TRPC1 and PKCδ in VSMCs, and that these interactions cause PKCδ-dependent phosphorylation of TRPC1.

Figure 7

PKCδ mediates store-operated interactions between PIP_2 and TRPC1, and PIP_2-evoked TRPC1 currents

In our previous work, we identified that PIP_2 has an obligatory role in the activation of TRPC1-based SOCs in VSMCs, potentially as the activating ligand, and that this process requires PKC-dependent phosphorylation of TRPC1 (see Introduction). Therefore, we investigated if store-operated interactions between PIP_2 and TRPC1 require PKCδ activity using immunocytochemistry and PLA, and if PKCδ is essential for activation of store-operated whole-cell TRPC1 currents by PIP_2.

In freshly isolated rat mesenteric artery VSMCs, Figure 8A shows that PIP_2 immunostaining was highly expressed at, or close to, the plasma membrane in unstimulated cells and BAPTA-AM and δV1-TAT had no obvious effect on this distribution. To provide evidence that the staining produced with anti-PIP_2 antibodies was related to endogenous PIP_2, Figure 8A shows that pre-treatment with high

concentrations of wortmannin (20 μM), which inhibits PI-4/PI-5 kinases to reduce PIP_2 recycling and promote PIP_2 depletion, produced a considerable reduction in the PIP_2 signal (Suh & Hille, 2005; Saleh et al, 2009; Shi et al, 2014). In control experiments, TRPC1 immunostaining was unaffected by BAPTA-AM, δV1-TAT or wortmannin treatment (Fig. 8A). We next adapted this immunostaining protocol for PLA experiments, and Figure 8B reveals that there was a low level of interactions between PIP_2 and TRPC1 in unstimulated VSMCs which were increased by BAPTA-AM from a mean puncta number per cell of 7.6 ± 5.2 (n=23) to 34.6 ± 14.1 (n=24, p=0.0001) which were inhibited in the presence of δV1-TAT or following pre-treatment of wortmannin to respectively 6.5 ± 2.9 (n=20, p=0.0001) to 6.1 ± 3.7 (n=19, p=0.0001, unpaired t-test).

Figure 8

In freshly isolated VSMCs, Figure 9A shows that bath application of the phorbol ester 12,13-dibutyrate (PDBu) increased whole-cell non-selective cation currents from a mean control amplitude of -0.71 ± 0.13 pA/pF to a mean peak amplitude of -2.12 ± 0.8 pA/pF at -80 mV (n=8, p=0.0001, two-way ANOVA , Tukey's comparisons test). In contrast, inclusion of diC8-PIP_2 in the patch pipette solution had no effect on whole-cell cation currents (-80 mV, -0.75 ± 0.18 vs -1.0 ± 0.08, n=5, p=0.6151, two-way ANOVA, Tukey's comparisons test). Figure 9B shows that dialyzing cells with diC8-PIP_2 in the patch pipette solution increased PDBu-activated whole-cell cation currents, with mean peak amplitude at -80 mV increased from -2.12 ± 0.8 pA/pF (n=8, Fig. 9A) to -2.88 ± 0.78 pA/pF at -80 mV (n=7, p=0.0291, unpaired t-test). Figure 9B also shows that PDBu-evoked whole-cell cation currents induced in the presence of diC8-PIP_2 were inhibited by bath applications of T1E3 and δV1-TAT to respectively -1.13 ± 0.24 pA/pF (n=5, p=0.0001) and -0.84 ± 0.38 pA/pF at -80 mV (n=7, p=0.0001 two-way ANOVA, Tukey's comparisons test) indicating that these currents were mediated by TRPC1 and required PKCδ activity.

Figure 9

Noradrenaline-evoked TRPC1 channels require PKCδ

The present study clearly demonstrates that the PKCδ isoform is important for activation of TRPC1 channels by agents that deplete internal Ca^{2+} stores. Therefore, in the final series of experiments we investigated the physiological significance of these findings by examining whether PKCδ is involved in activation of TRPC1 channels by the vasoconstrictor and α_1-adrenoceptor agonist methoxamine (MO). Figure 10 shows that in unstimulated VSMCs there was a low level of spontaneous 2 pS TRPC1-based channel activity in cell-attached patches held at -80 mV, and that mean peak open probability (NP_o) was increased following bath application of MO from 0.14 ± 0.07 to 0.51 ± 0.2 (n=6, p=0.0035) and was

significantly reduced by subsequent co-application of δV1-TAT to 0.11 ± 0.08 (n=6, p=0.0029, paired t-test).

Figure 10

Discussion

The present work reveals that activity of PKCδ, a member of the novel PKC isoform subgroup, is essential for activation of TRPC1-based channels evoked by store depletion and α_1-adrenoceptor stimulation in native contractile rat mesenteric artery VSMCs. It is proposed that PKCδ-dependent phosphorylation of TRPC1 is required for TRPC1 channel activation by PIP_2, the likely activating ligand. These results provide further evidence that PKC activity and PIP_2 are obligatory for activation of TRPC1-based SOCs in VSMCs, ion channels implicated in regulating contraction, proliferation and migration that are processes linked to hypertension and atherosclerosis. As such, regulating PKCδ activity and PIP_2 levels may be therapeutic targets for these and other vascular diseases.

TRPC1-based SOCs require PKC activity

Our results show that the well-established protocol for inducing store depletion, inclusion of a high concentration of BAPTA in the patch pipette solution, evoked a whole-cell non-selective cation current with a relatively linear I/V relationship with E_{rev} of between 0 mV and +20 mV in freshly isolated rat mesenteric artery VSMCs which is similar to currents previously recorded in rabbit and mouse VSMCs (Shi et al 2012a; 2014; 2016; 2017a, 2017b). There is considerable evidence using pharmacological agents, antibodies as blocking agents and TRPC1 knockout mice that these store-operated currents are mediated by a heteromeric TRPC1/TRPC5 molecular template (Xu & Beech, 2001; Xu et al, 2005a; Saleh et al, 2008; Shi et al, 2012a). In further support of this proposal, the present study shows that store-operated whole-cell currents were inhibited by Pico145, a highly selective and potent TRPC1/C4/C5 channel blocker (Rubaiy et al, 2017) and T1E3, a TRPC1 antibody raised against an extracellular pore region and known to act as a blocking agent (Xu & Beech, 2001; Xu et al, 2005b). In addition, using PLA, the current work reveals that TRPC1 and TRPC5 co-localize within <40 nm of each other at, or close to, the plasma membrane of VSMCs. It should be noted that the electrophysiological and pharmacological properties of these store-operated currents in freshly isolated VSMCs exhibiting a contractile phenotype are considerably different to those of Orai1-based CRACs (Prakriya & Lewis, 2015) indicating that Orai1 proteins are unlikely to be involved as previously shown (Shi et al, 2017b).

Previously studies show that PKC activity and PKC-mediated phosphorylation of TRPC1 is essential for activation of TRPC1-based SOCs in native contractile VSMCs (Albert & Large, 2002b; Saleh et al 2006;

2008; Large et al, 2009; Albert, 2011; Shi et al 2012a; 2014; 2016). The present study provides further support for an excitatory role for PKC, with the pan-PKC isoform selective inhibitor GF109203X inhibiting store-operated whole-cell TRPC1 currents in freshly isolated rat mesenteric artery VSMCs. Identifying the specific PKC isoform responsible for activating TRPC1-based SOCs was therefore the main aim of the present work.

PKCδ is the PKC isoform responsible for activation of TRPC1-based SOCs and store-operated TRPC1 phosphorylation

The PKC family are subdivided into conventional PKC (α, β, and γ), novel PKC (δ, ϵ, η, and θ), and atypical PKC (ζ and ι/κ) isoforms (Salamanca & Khalil, 2005; Ringvold & Khalil, 2017). Given that DAG analogues activate TRPC1 channels through a PKC-dependent mechanism in VSMCs (Saleh et al, 2006; 2008; Large et al 2009; Albert, 2011), and that the Ca^{2+} store depletion protocols used to activate TRPC1-based SOCs are likely to increase, decrease, or not alter $[Ca^{2+}]_i$, the PKC isoform(s) responsible is likely to belong to the novel subfamily (see Introduction).

Western blotting and immunocytochemical analysis demonstrated that PKCδ is expressed in native contractile rat mesenteric artery VSMCs and was distributed at, or close to, the plasma membrane. There was greater expression of PKCδ than other novel PKC isoforms in VSMCs and tissue lysates, however there should be caution in suggesting that PKCδ is the dominant novel PKC isoform in VSMCs as expression variations may be linked to potential differences between the affinity and specificity of the anti-novel PKC isoform antibodies used.

Inhibition of PKCδ activity via well-characterized selective PKCδ peptide inhibitors and knockdown of PKCδ expression using morpholino oligomers resulted in a significant reduction in the development and peak amplitude of store-operated whole-cell TRPC1 currents. Moreover, immunoprecipitation and PLA studies revealed that store depletion induced interactions between TRPC1 and PKCδ. Importantly, PLA experiments showed that store depletion induced puncta formation between anti-TRPC1 and anti-phosphorylated serine and threonine antibodies that were greatly reduced by PKCδ peptide inhibitors and PKCδ selective morpholino oligomers, which indicates that store depletion induces PKCδ-dependent phosphorylation of TRPC1. Taken together, these findings provide strong evidence that the PKCδ isoform is essential for the activation of TRPC1-based SOCs in native contractile VSMCs.

PKCδ has also been reported to participate in store-operated Ca^{2+} entry in airway smooth muscle (Gao et al 2012), proliferation and migration of VSMCs (Ding et al 2011; Fan et al 2014), development of the myogenic response (Kashihara et al 2008), and membrane insertion of TRPM4 channels and associated

vasoconstriction (Garcia et al 2010; Crnich et al 2010). Interestingly, TRPC1 has also been associated with many of these vascular functions (Earley & Brayden, 2015), and therefore in the future it may be useful to investigate the functional association between TRPC1 and PKCδ activities in the vasculature. In contrast to the results above, PKC activity has an inhibitory action on Orai1-based CRACs with PKC inhibitors including GF109203X and knockdown of PKCβ isoform increasing Orai1 activity (Kawasaki et al 2010), which further indicates that Orai1 proteins or Orai1-based CRACs are not involved in activation of TRPC1-based SOCs in native contractile VSMCs.

PKCδ activity is required for PIP_2-mediated activation of TRPC1 channels

We have previous described the obligatory requirement of PIP_2 in the activation of TRPC1-based SOCs, and proposed that for this phospholipid to act as the activating ligand it requires PKC-dependent phosphorylation of TRPC1 (Large et al, 2009; Saleh et al, 2009a; 2009b; Albert, 2011; Shi et al, 2012a; 2012b; 2014). In the present study, we show using PLA that store depletion induces interactions between PIP_2 and TRPC1 at, or close to, the plasma membrane of VSMCs which are reduced a PKCδ peptide inhibitor. To provide evidence that endogenous PIP_2 is involved in these results, we showed that a high concentration of wortmannin (20 μM) which depletes PIP_2 levels through inhibiting PI-4/PI-5 kinase-mediated PIP_2 synthesis (Suh & Hille, 2005) reduced PIP_2 immunostaining and store-operated associations between TRPC1 and PIP_2. It should be noted that high concentration of wortmannin also inhibit myosin light chain kinase (MLCK) and PI(3) kinase, but previous findings have shown that lower concentrations (<1 μM) which inhibit MLCK and PI-3 kinase but do not deplete PIP_2 levels had no effect on activation of TRPC1 channel activity in VSMCs (Saleh et al, 2009; Shi et al, 2014). The PKC activator PDBu activated whole-cell TRPC1 currents which were increased in amplitude when the water soluble PIP_2 analogue diC8-PIP_2 was included in the patch pipette solution and were inhibited by a PKCδ peptide inhibitor. Cell dialysis with diC8-PIP_2 failed to activate any whole-cell currents on its own as previously indicated (Albert et al, 2008), which indicates that the effects of PDBu and PIP_2 are not merely additive but are likely to represent synergism between the two molecules. Taken together, these results re-enforce the idea that PIP_2 is the activating ligand of TRPC1-based SOCs, and that this requires PKCδ-dependent phosphorylation of TRPC1.

The proposed excitatory roles of PKC and PIP_2 on TRPC1-based SOCs are opposite to the action of these molecules in the activation of non-TRPC1-containing channels, e.g. TRPC3/C6/C7 channels, in native contractile VSMCs (Venkatachalan et al, 2003; Albert & Large, 2004; Large et al, 2009; Shi et al, 2010; Albert, 2011). This subgroup of TRPC channels, known as receptor-operated channels (ROCs), are activated by receptor-mediated generation of DAG which leads to channel opening via PKC-independent mechanisms, with subsequent PKC activity induced by DAG producing channel inhibition.

Interestingly, stimulation of TRPC1-based SOCs are proposed to inhibit TRPC6-based SOCs through inducing Ca^{2+} influx and PKC activity, which suggests the involvement of a conventional PKC isoform (Shi et al, 2010). A distinct role of PIP_2 on TRPC3/C6/C7-based ROCs is less clear with both inhibitory and excitatory actions on channel activity proposed (Albert et al, 2008; Imai et al, 2012). These findings further indicate that TRPC1-based SOCs and TRPC3/C6/C7-based ROCs form distinct channel structures with differing activation mechanisms involving differing PKC isoforms, and likely distinct functions in VSMCs.

Computation predication of potential PKCδ-dependent phosphorylation sites within the TRPC1 sequence using GPS 3.0 (Xue et al, 2008) reveals five intracellular serine residues, with Ser619 and Ser752 at the C-terminal domain of significance as both these sites are close to a known PIP_2-binding domain (Kwon et al, 2007). Hence it is possible that PKCδ-dependent phosphorylation of one or both sites increases PIP_2 binding to TRPC1 leading to channel opening. Other studies have demonstrated that protein kinase A (PKA), protein kinase G (PKG), and calmodulin kinase II (CaMKII) have inhibitory actions on TRPC1-based SOCs in VSMCs (Liu et al, 2005a; Albert et al, 2006; Chen et al, 2011), and therefore perhaps TRPC1 phosphorylation by these kinases inhibit TRPC1-based SOCs by reducing TRPC1 and PIP_2 interactions. Similar roles for kinase activities in modulating lipid-protein interactions are well-established in the regulation of K^+ channel subtypes (Logothetis et al, 2015).

An important question is how PKCδ activity is stimulated by store depletion? Our proposed hypothesis based on previous work indicates that store depletion induces STIM1-TRPC1 interactions, which stimulate a Gq-PLCβ1 pathway that generates DAG and PKC activity leading to PKC-dependent phosphorylation of TRPC1 (Martín-Aragón Baudel, 2020). PKC-dependent phosphorylation of TRPC1 leads to dissociation of interactions between TRPC1 and the PIP_2 binding protein myristoylated alanine-rich C-kinase substrate (MARCKS), with the latter molecule then releasing PIP_2 into the local environment allowing it to act as the channel activating ligand. The present study highlights that the PKCδ isoform is likely to be an essential PKC isoform in this process; this will be important to investigate in detail in future studies.

Physiological relevance of PKCδ-mediated TRPC1 channels

TRPC1 channel activity induced by the α_1-adrenoceptor agonist and vasoconstrictor methoxamine was reduced by a PKCδ peptide inhibitor, which suggests that a similar store-operated activation process involving PKCδ may be used by physiological stimulants of TRPC1 channels. This is further indicated by findings showing that PLCβ1 and STIM1 knockdown both significantly inhibit noradrenaline-evoked TRPC1 channel activity (Shi et al, 2016; 2017b), and that noradrenaline induces an increase in PKC-

dependent phosphorylation of TRPC1 and stimulates MARCKS to release PIP_2 which is then available to interacts with TRPC1 (Shi et al, 2014). It is previously proposed that vasoconstrictor agents may also activate TRPC1-based SOCs independently of store deletion (Albert & Large, 2002b; Saleh et al, 2006; Large et al, 2009; Saleh et al, 2009b, Shi et al, 2010; 2012b; Albert, 2011), which suggests that TRPC1 channels may also act as ROCs. The current and previous findings certainly suggest that a significant contribution to physiological stimulation of TRPC1 channels is likely via a store-operated pathway.

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Additional information

Competing Interests

The authors declare that they have no competing interests.

Author contributions

MMAB, JS, WL and AA all contributed to the conception or design of the work, analysis of data or interpretation of data for the work and were involved in drafting the work or revising it critically for important intellectual content. MMAB and JS were involved in the acquisition of data. All authors approved final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Statistical summary document

Excel template with detail of statistical tests performs on data is submitted with this manuscript.

Figure Legends

Figure 1. TRPC1 compose PKC-dependent SOCs in native contractile VSMCs

A, Representative trace and mean I/V relationships showing that development of store-operated whole-cell currents from control to peak levels in freshly isolated rat mesenteric artery VSMCs following obtaining whole-cell configuration (wc) was inhibited by bath application of Pico145. Vertical deflections represent currents evoked by voltage ramps from +100 mV to −150 mV (750 ms duration) every 30 s from a holding potential of 0 mV. *B* and *C*, Mean data showing that bath applications of T1E3 (1 μg/mL) and GF109203X (3 μM) inhibited store-operated whole-cell currents.

Figure 2. Expression of TRPC1-TRPC5 interactions in native contractile VSMCs

Representative PLA images from two different freshly isolated rat mesenteric artery VSMCs showing association between TRPC1 and TRPC5. These images, and other PLA figures shown, represent puncta observed across a z-stack of each cell. Scale bar represents 10 μm.

Figure 3. Expression of novel PKC isoforms in native contractile VSMCs

A, Representative western blots showing expression of the novel PKC isoforms PKCε, PKCθ, PKCη and PKCδ in rat mesenteric artery VSMCs and brain (B) protein lysates. *B*, Representative immunocytochemical images of rat mesenteric VSMCs immunolabelled with the same anti-novel PKC isoform antibodies used in *A*. Scale bar presents 10 μm.

Figure 4. Pharmacological PKCδ inhibitors reduce TRPC1-based SOCs

A, Representative trace and mean I/V relationship showing that peak amplitude of store-operated whole-cell TRPC1 currents in freshly isolated rat mesenteric artery VSMCs were reduced by bath application of δV1-TAT. *B*, A small store-operated whole-cell TRPC1 current was induced following inclusion of $\delta PKC_{8\text{-}17}$ peptide inhibitor in the patch pipette solution. *C*, Inclusion of εPKC peptide inhibitor in the patch pipette appeared to have little effect on development of store-operated whole-cell TRPC1 which were subsequently inhibited by bath application of δV1-TAT.

Figure 5. Knock-down of PKCδ expression reduces activation of TRPC1-based SOCs

A, Representative western blots and mean data showing that PKCδ-specific morpholino oligomers reduced PKCδ expression in rat mesenteric artery protein lysates compared to scrambled /mismatched oligomers (Scram). Band intensity was normalized to β-GAPDH to calculate % expression (vs scrambled, n=3, p=0.0202, unpaired t-test). *B,* Mean I/V relationships showing that mean peak amplitude of store-operated whole-cell TRPC1 currents were reduced in the presence of PKCδ-specific (n=10) compared to scrambled (n=9) morpholino oligomers.

Figure 6. Store depletion induces associations between TRPC1 and PKCδ in native contractile VSMCs

A, Representative western blots with anti-TRPC1 and anti-PKCδ antibodies following immunoprecipitation of freshly isolated rat mesenteric artery tissue lysates with an anti-TRPC1 antibody shows that pre-treatment with the BAPTA-AM and TPEN induced associations between TRPC1 and PKCδ but did not alter TRPC1 expression. *B*, Representative images of proximity ligation assays from freshly isolated rat mesenteric artery VSMCs showing low level of association between TRPC1 and PKCδ in unstimulated VSMCs which was greatly increased following pre-treatment with TPEN and BAPTA-AM for 10 min. Scale bars represents 10 μm.

Figure 7. Store depletion induces PKCδ-dependent phosphorylation of TRPC1

A, Representative and mean data from proximity ligation assays showing pre-treatment with BAPTA-AM (n=30) for 10 min greatly increased number of puncta per cell representing associations between TRPC1 and phosphorylated serine and threonine residues compared to control values (n=19, p=0.0001), which were reduced in the presence of δV1-TAT (n=25, p=0.0001, unpaired t-test). *B*, Representative and mean data from proximity ligation assays showing BAPTA-AM-induced associations between TRPC1 and phosphorylated serine and threonine residues were reduced in VSMCs from vessels transfected with PKCδ-specific (n=24, p=0.0001) compared to scrambled morpholino oligomers (n=30, p=0.0001, unpaired t-test). Scale bars represents 10 μm. ****p=0.0001.

Figure 8. Store-operated interactions between TRPC1 and PIP_2 require PKCδ

A, Representative images from freshly isolated rat mesenteric VSMCs showing expression of PIP_2 (green) and TRPC1 (red) following pretreatment with BAPTA-AM for 10 min, wortmannin (Wort) for 20 min, and δV1-TAT for 10 min. *B*, Representative images and mean data from PLA experiments showing that control interactions between TRPC1 and PIP_2 (n=23) were greatly increased by pre-treatment with BAPTA-AM for 10 min (n=23, p=0.0001), which were reduced by co-application with δV1-TAT (n=20, p=0.0001) and wortmannin (n=19, p=0.0001, unpaired t-test). Scale bars represents 10 μm.

Figure 9. PIP_2 increases PDBu-induced whole-cell TRPC1 currents

A, Mean I/V relationships from freshly isolated rat mesenteric artery VSMCs showing that bath application of PDBu but not inclusion of diC8-PIP_2 in the patch pipette solution induced a whole-cell current. *B*, Mean I/V relationships showing that following cell dialysis with diC8-PIP_2, PDBu-evoked whole-cell currents had greatly increased peak amplitude compared to currents induced by PDBu alone shown in *A* and were inhibited by T1E3 and δV1-TAT.

Figure 10. Methoxamine-evoked TRPC1 channel activity is mediated by PKCδ

A and *B,* Representative trace and mean open probability (NP_o) data showing that bath application of methoxamine evoked TRPC1 channel activity in cell-attached patches held at -80 mV (n=6, p=0.0035), which was reduced by co-application of δV1-TAT (n=6, p=0.0029, paired t-test).

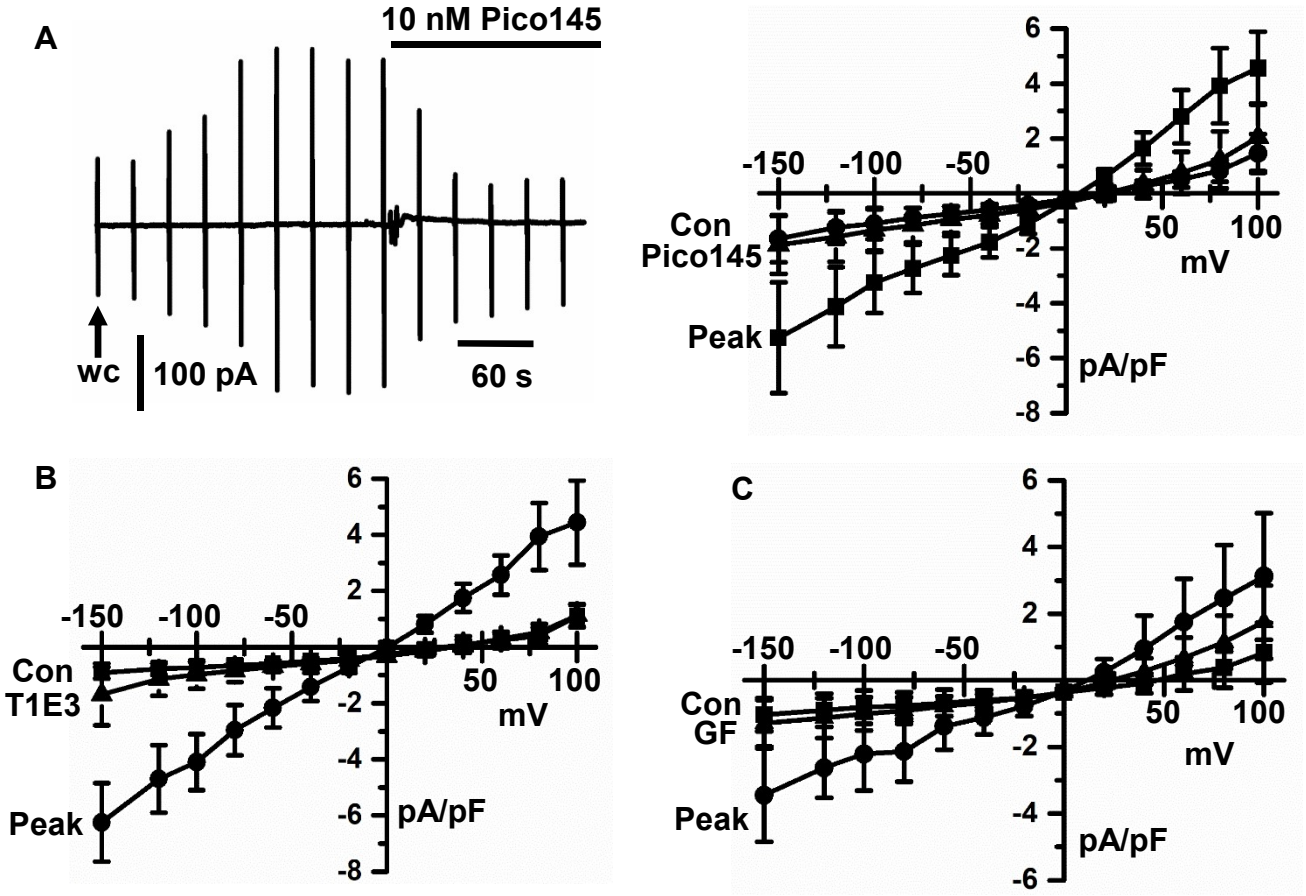
A
10 nM Pico145
wc
100 pA
60 s
Con
Pico145
Peak
-150
-100
-50
50
100
mV
pA/pF
6
4
2
-2
-4
-6
-8
B
Con
T1E3
Peak
C
Con
GF
Peak

Figure 1

Anti-TRPC1 + anti-TRPC5

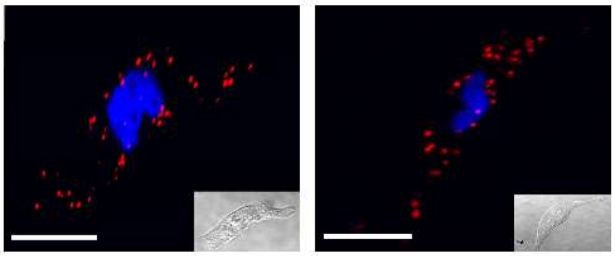

Figure 2

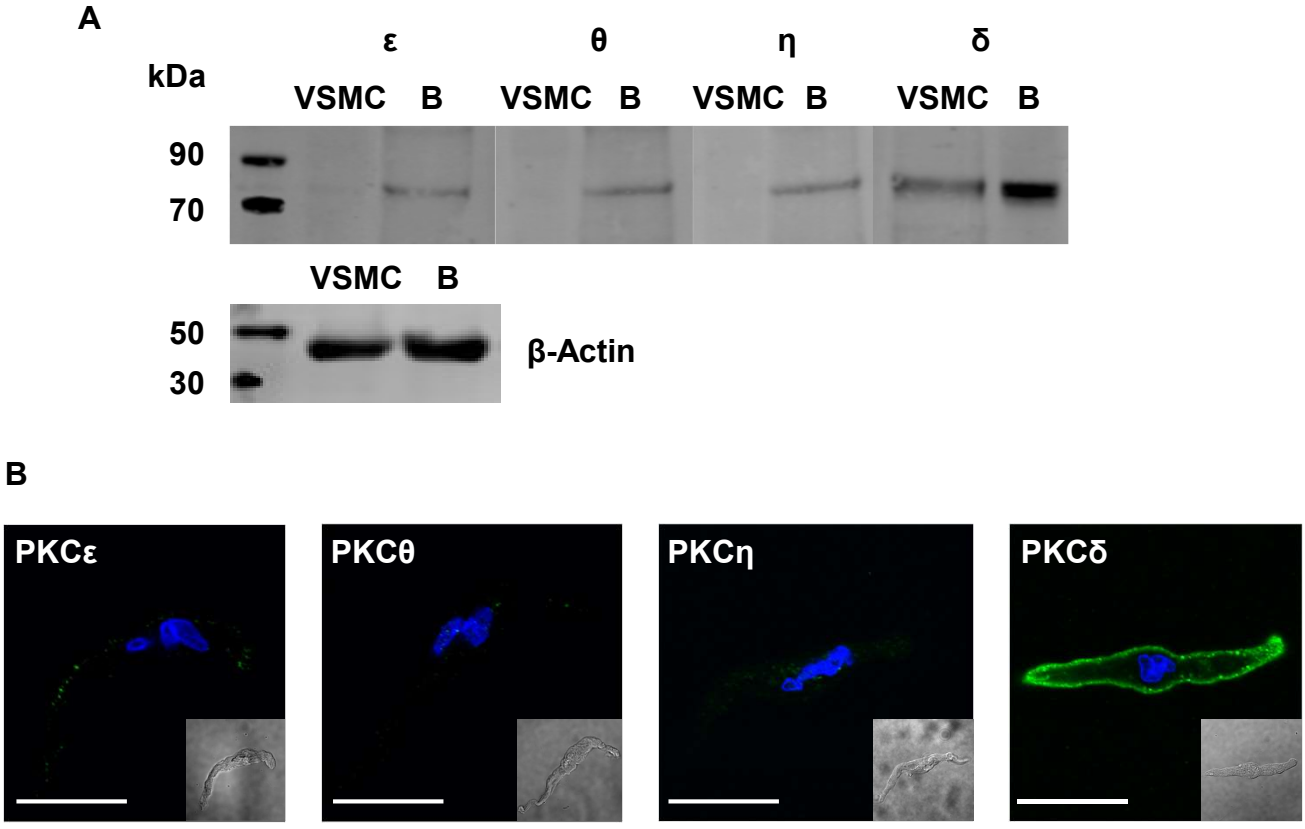
A
ε
θ
η
δ
kDa
VSMC B
VSMC B
VSMC B
VSMC B
90
70
VSMC B
50
30
β-Actin
B
PKCε
PKCθ
PKCη
PKCδ

Figure 3

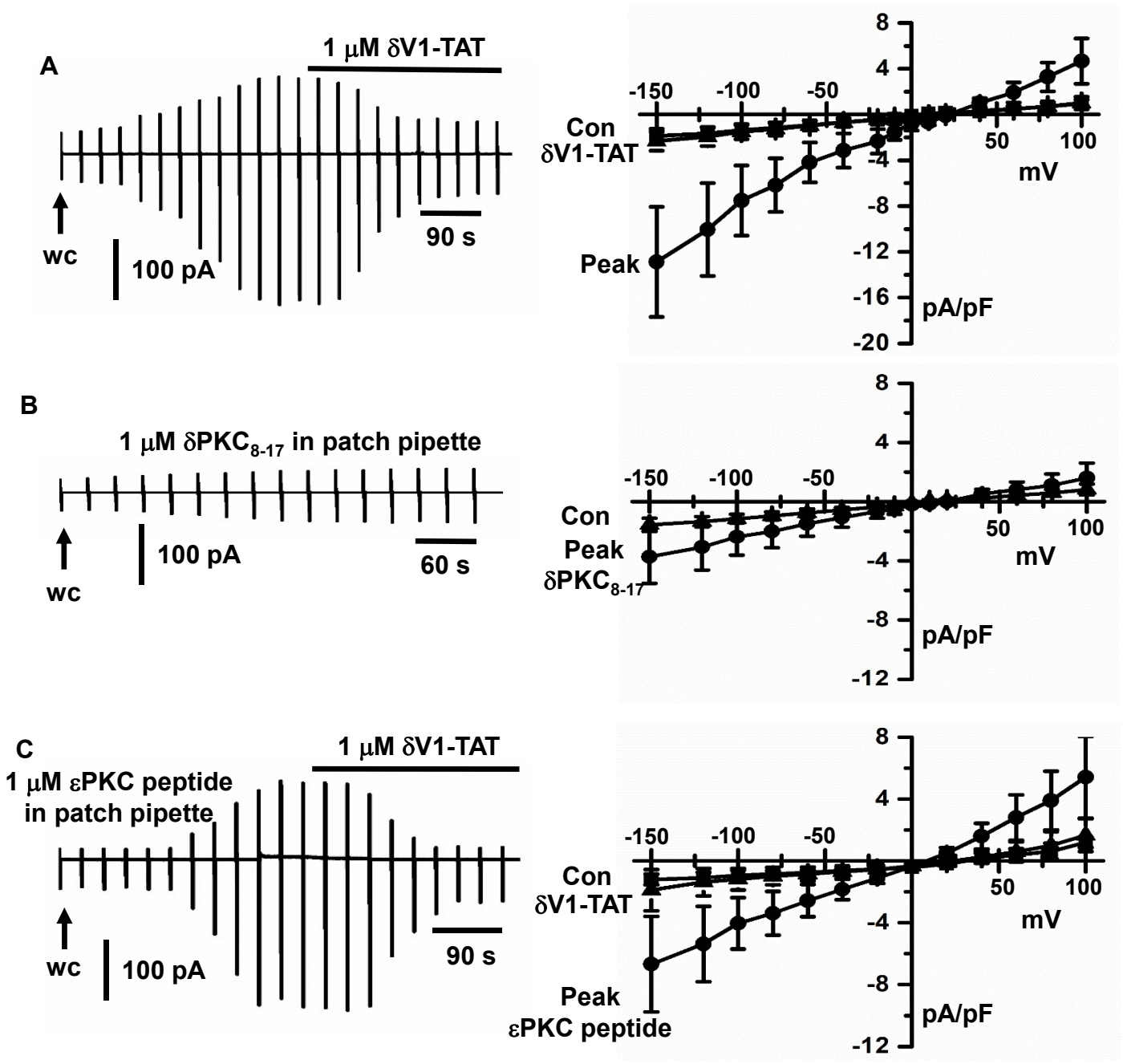
A
1 μM δV1-TAT
wc
100 pA
90 s
Con
δV1-TAT
Peak
mV
pA/pF
B
1 μM δPKC8-17 in patch pipette
wc
100 pA
60 s
Con
Peak
δPKC8-17
mV
pA/pF
C
1 μM εPKC peptide
in patch pipette
1 μM δV1-TAT
wc
100 pA
90 s
Con
δV1-TAT
Peak
εPKC peptide
mV
pA/pF

Figure 4

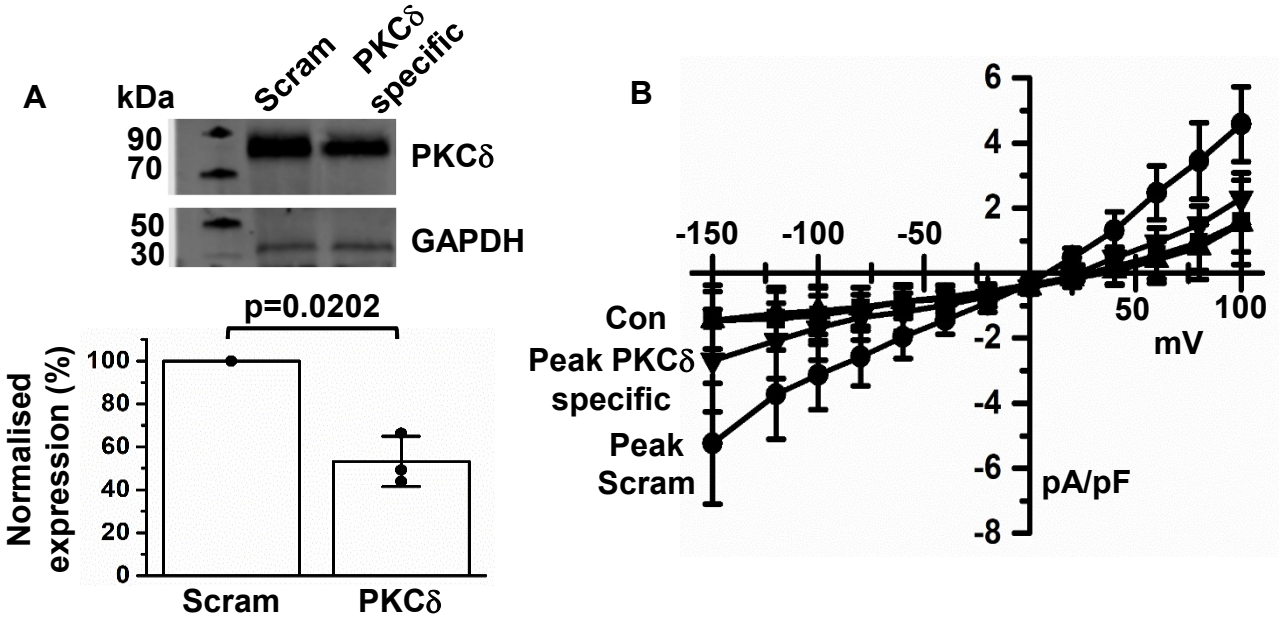
A
kDa
Scram
PKCδ specific
90
70
PKCδ
50
30
GAPDH
p=0.0202
Normalised expression (%)
100
80
60
40
20
0
Scram
PKCδ
B
6
4
2
-150
-100
-50
50
100
mV
Con
Peak PKCδ specific
Peak Scram
-2
-4
-6
-8
pA/pF

Figure 5

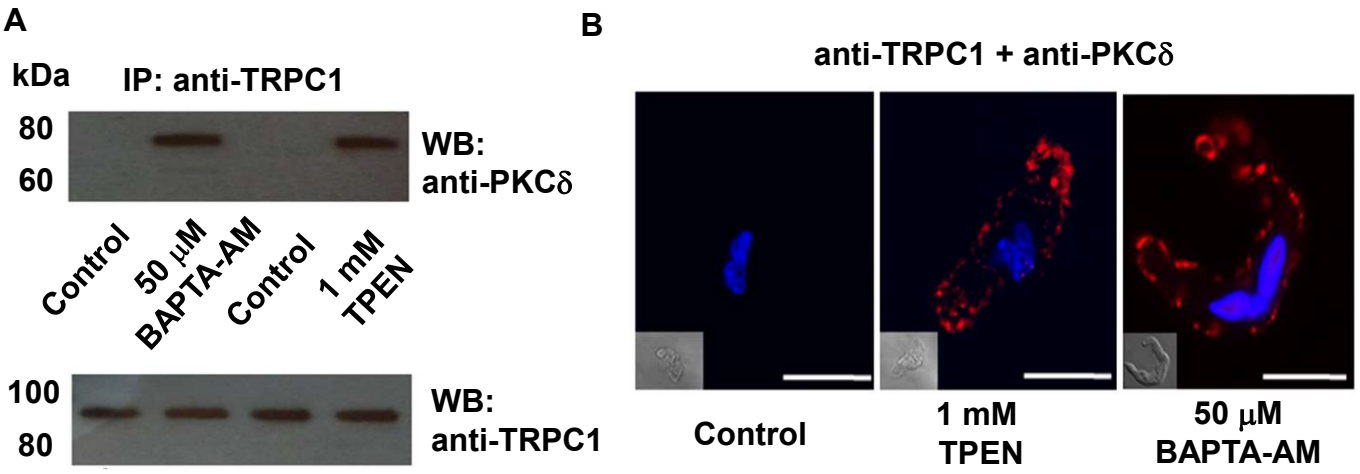
A
kDa
IP: anti-TRPC1
80
60
WB:
anti-PKCδ
Control
50 μM
BAPTA-AM
Control
1 mM
TPEN
100
80
WB:
anti-TRPC1
B
anti-TRPC1 + anti-PKCδ
Control
1 mM
TPEN
50 μM
BAPTA-AM

Figure 6

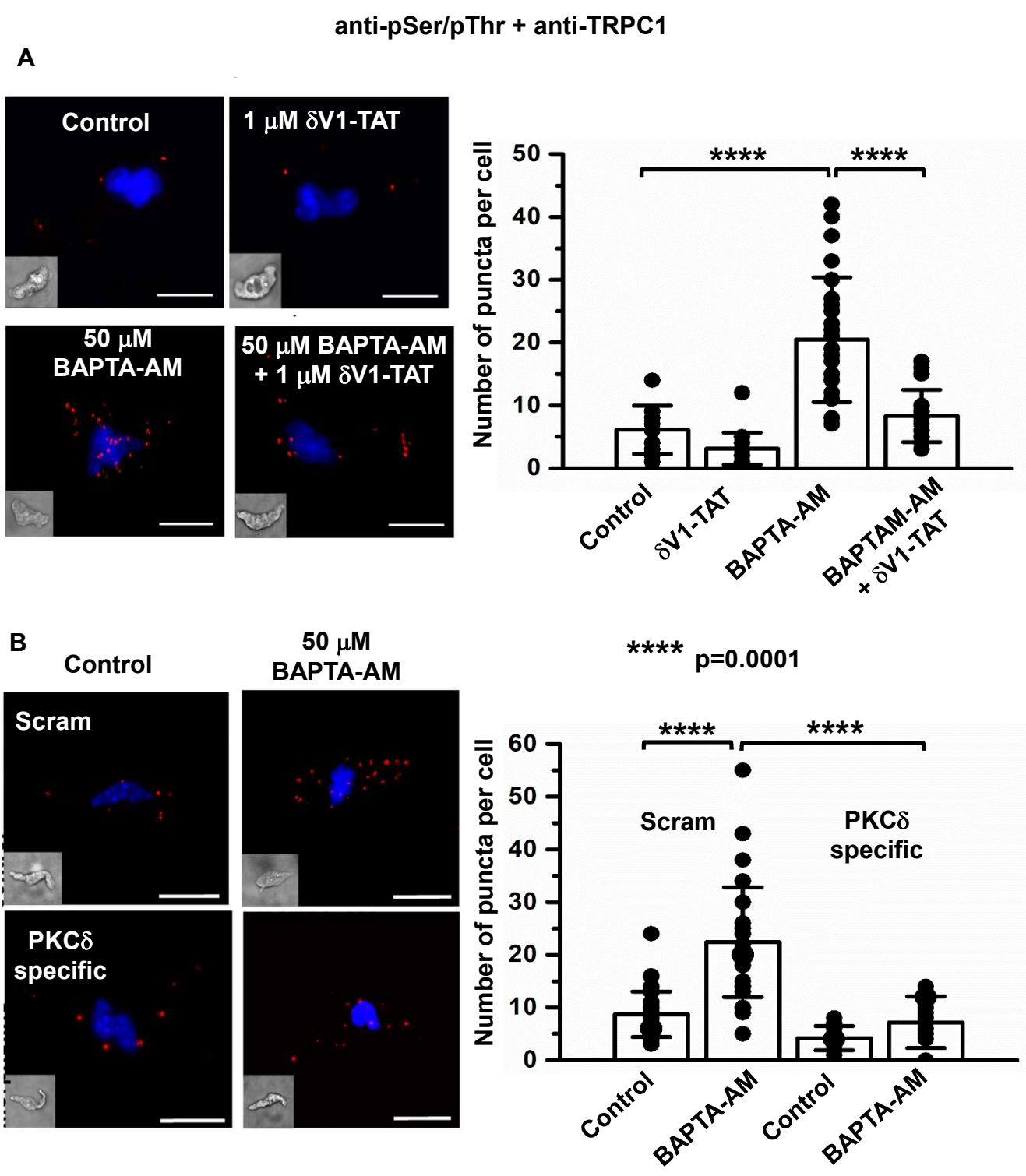
anti-pSer/pThr + anti-TRPC1
A
Control
1 μM δV1-TAT
50 μM BAPTA-AM
50 μM BAPTA-AM + 1 μM δV1-TAT
Number of puncta per cell
****
****
Control
δV1-TAT
BAPTA-AM
BAPTAM-AM + δV1-TAT
B
Control
50 μM BAPTA-AM
Scram
PKCδ specific
**** p=0.0001
Scram
PKCδ specific
Control
BAPTA-AM
Control
BAPTA-AM

Figure 7

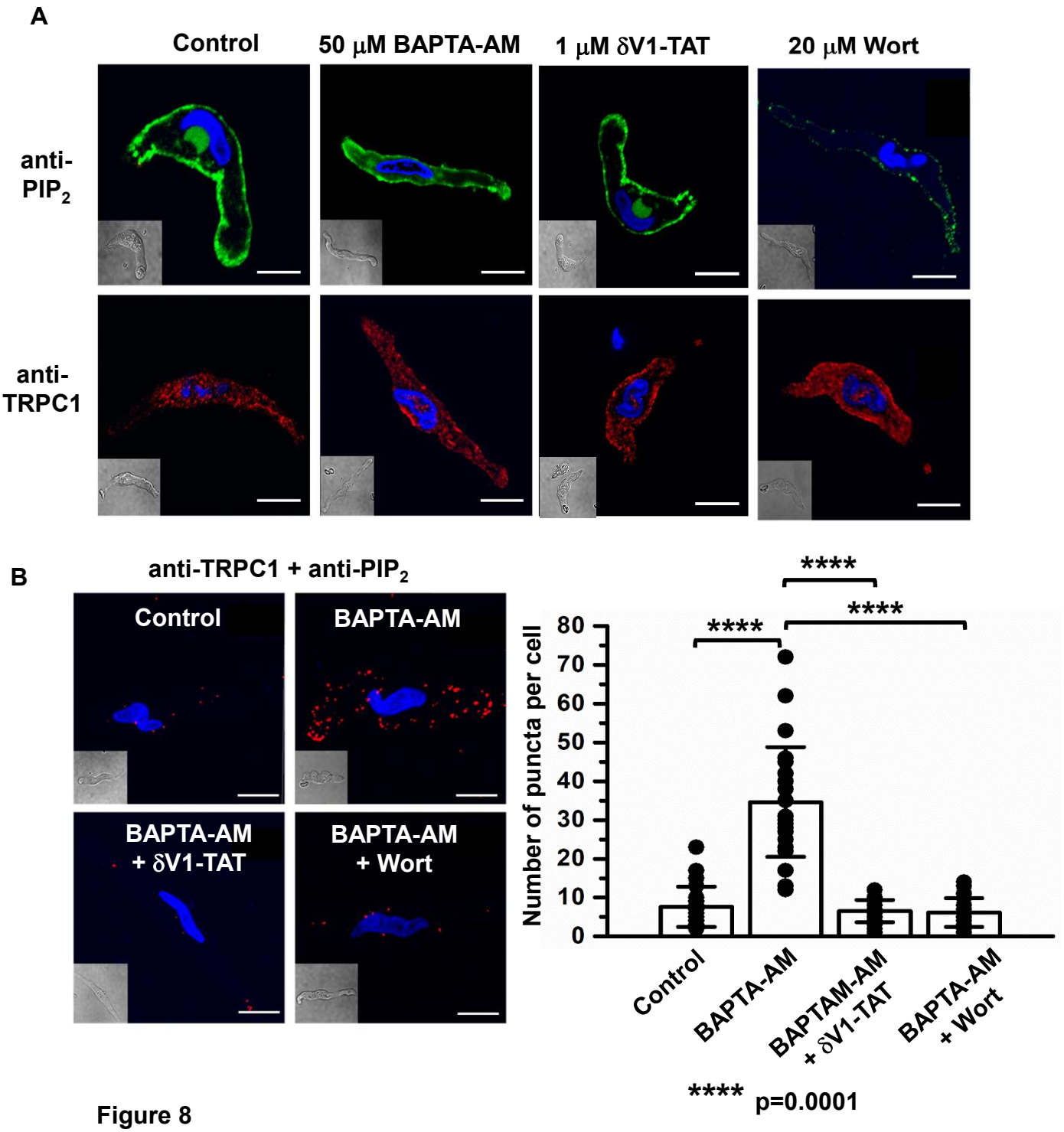
A
Control
50 μM BAPTA-AM
1 μM δV1-TAT
20 μM Wort
anti-PIP2
anti-TRPC1
B
anti-TRPC1 + anti-PIP2
Control
BAPTA-AM
BAPTA-AM + δV1-TAT
BAPTA-AM + Wort
Number of puncta per cell
0
10
20
30
40
50
60
70
80
****
****
****
Control
BAPTA-AM
BAPTAM-AM + δV1-TAT
BAPTA-AM + Wort
**** p=0.0001

Figure 8

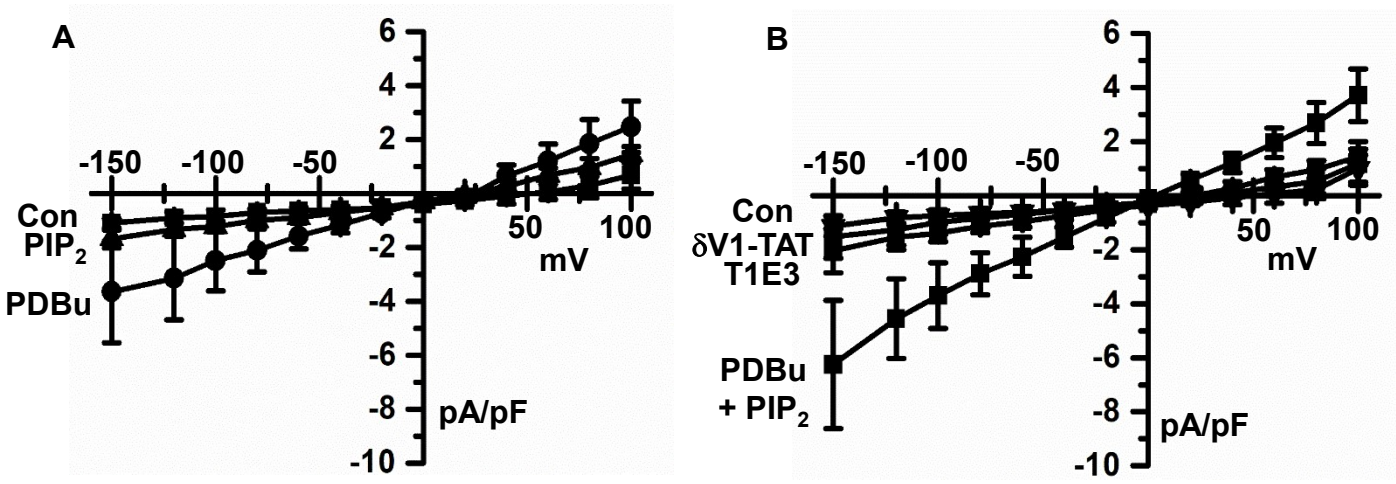
A
6
4
2
-150
-100
-50
50
100
mV
Con
PIP2
PDBu
-2
-4
-6
-8
-10
pA/pF
B
6
4
2
-150
-100
-50
50
100
mV
Con
δV1-TAT
T1E3
PDBu
+ PIP2
-2
-4
-6
-8
-10
pA/pF

Figure 9

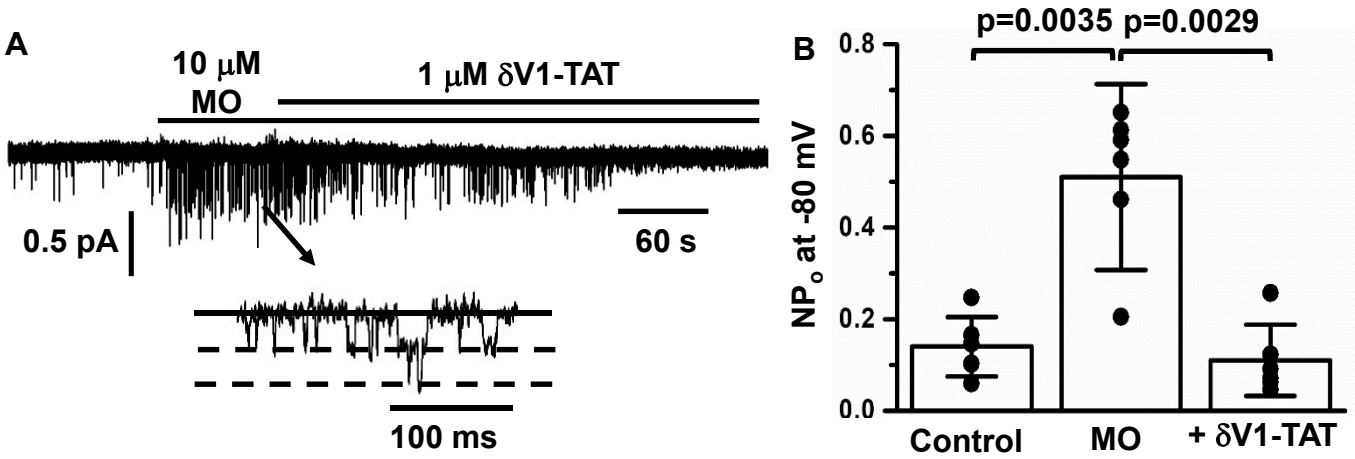
A
10 μM
MO
1 μM δV1-TAT
0.5 pA
60 s
100 ms
B
p=0.0035 p=0.0029
NPo at -80 mV
0.0
0.2
0.4
0.6
0.8
Control
MO
+ δV1-TAT

Figure 10