Mitochondria regulate TRPV4 mediated release of ATP

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Abstract

Background and Purpose Ca^{2+} influx via TRPV4 triggers Ca^{2+} release from the IP_3-sensitive internal store to generate repetitive oscillations. While mitochondria are acknowledged regulators of IP_3-mediated Ca^{2+} release, how TRPV4-mediated Ca^{2+} signals are regulated by mitochondria is unknown. We show that depolarised mitochondria switch TRPV4 signalling from relying on Ca^{2+}-induced Ca^{2+} release at IP_3 receptors, to being independent of Ca^{2+} influx and instead mediated by ATP release via pannexins.

Experimental Approach TRPV4 evoked Ca^{2+} signals were individually examined in hundreds of cells in the endothelium of rat mesenteric resistance arteries using the indicator Cal520.

Key Results TRPV4 activation with GSK1016790A(GSK) generated repetitive Ca^{2+} oscillations that required Ca^{2+} influx. However, when the mitochondrial membrane potential was depolarised, by the uncoupler CCCP or complex I inhibitor rotenone, TRPV4 activation generated large propagating, multicellular, Ca^{2+} waves in the absence of external Ca^{2+}. The ATP synthase inhibitor oligomycin did not potentiate TRPV4 mediated Ca^{2+} signals. GSK-evoked Ca^{2+} waves, when mitochondria were depolarised, were blocked by the TRPV4 channel blocker HC067047, the SERCA inhibitor cyclopiazonic acid, the phospholipase C (PLC) blocker U73122 and the inositol triphosphate receptor (IP_3R) blocker caffeine. The Ca^{2+} waves were also inhibited by the extracellular ATP blockers suramin and apyrase and the pannexin blocker probenecid.

Conclusion and Implications These results highlight a previously unknown role of mitochondria in shaping TRPV4 mediated Ca^{2+} signalling by facilitating ATP release. When mitochondria are depolarised, TRPV4-mediated release of ATP via pannexin channels activates plasma membrane purinergic receptors to trigger IP_3 evoked Ca^{2+} release.

Abbreviations: IP_3, inositol 1,4,5-trisphosphate; IP_3R, inositol 1,4,5-trisphosphate receptor; TRPV transient receptor potential vanilloid; ACh, acetylcholine; CPA, cyclopiazonic acid; PLC, phospholipase C; ATP, Adenosine triphosphate; MOPS, 3-(N-Morpholino)propanesulfonic acid sodium salt, 4-Morpholinepropanesulfonic acid sodium salt; EDTA, Ethylenediaminetetraacetic acid; U73122, 1-[6-[[(17β)-3-Methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione; HC067047, 2-Methyl-1-[3-(4-morpholinyl)propyl]-5-phenyl-N-[3-(trifluoromethyl)phenyl]-1H-pyrrole-3-carboxamide ; CCCP, carbonyl cyanide m-chlorophenyl hydrazine; GSK, N-[(1S)-1-[[4-[(2S)-2-[[(2,4-Dichlorophenyl)sulfonyl]amino]-3-hydroxy-1-oxopropyl]-1-piperazinyl]carbonyl]-3-methylbutyl]benzo[b]thiophene-2-carboxamide; $\Delta\Psi_m$, mitochondrial membrane potential; F, fluorescence intensity; F_0 baseline fluorescence intensity; PIP_2, phosphatidylinositol 4,5-bisphosphate. PSS, physiological saline solution; ALS, asymmetric least squares; DMSO, Dimethyl sulfoxide.

What is already known

- Ca^{2+} influx via TRPV4 triggers Ca^{2+} release from the IP_3-sensitive internal store to generate repetitive oscillations and vasodilation.

What this study adds

- When mitochondria are depolarised, Ca^{2+} influx is not required for TRPV4 to trigger Ca^{2+} release from the IP_3-sensitive internal store.
- TRPV4 activation triggers ATP release via pannexins to activate purinergic cell surface receptors when mitochondria are depolarised.

Clinical significance

- In pathological conditions, depolarised mitochondria will reconfigure TRPV4 mediated Ca^{2+} signalling to promote intercellular communication and coordinated cell activity.

Introduction

The endothelium is a monolayer of cells that lines all blood vessels and it plays critical role in regulating angiogenesis, vascular tone, permeability and leukocyte trafficking (Augustin et al., 1994; McCarron et al., 2017). Ca^{2+} signalling links extracellular activators to endothelial control of vascular function and also regulates intracellular processes involved in cell proliferation, protein expression and apoptosis (Clapham, 2007; Hill-Eubanks et al., 2011; Ottolini and Sonkusare, 2021).

Intracellular Ca^{2+} signalling is tightly controlled by processes that regulate release and uptake of the ion from the internal store, and influx and efflux of Ca^{2+} across the plasma membrane. Mitochondria are important regulators of intracellular Ca^{2+} signalling. Mitochondria regulate Ca^{2+} signalling by uptake of the ion into the organelle to alter local and global Ca^{2+} concentrations (Csordás et al., 2006; Marcu et al., 2014; Giorgi et al., 2018). Mitochondrial Ca^{2+} uptake is controlled by the potential ($\Delta\Psi_m$~-180mV) across the inner mitochondrial membrane that is generated via the proton gradient (Katakam et al., 2013). Depolarization of $\Delta\Psi_m$ decreases mitochondrial Ca^{2+} uptake (Chikando et al., 2013; Zhang et al., 2019). By regulating Ca^{2+} in small subcellular regions, mitochondria exert control of inositol 1,4,5-triphosphate (IP_3) receptor activity on the internal Ca^{2+} store to modulate cell activity (Rizzuto et al., 1993; Augustin et al., 1994; Duchen, 2000; Swärd et al., 2002; Szado et al., 2003; Csordás et al., 2006; Narayanan et al., 2010; Olson et al., 2010; Correa et al., 2011). However, much less is known of the contribution of mitochondria in regulating Ca^{2+} influx in the endothelium.

The transient receptor potential type 4 (TRPV4) ion channel is a member of TRP superfamily of non-selective cation channels that is expressed on the plasma membrane of various cell types (Liedtke et al., 2000; Strotmann et al., 2000; Shigematsu et al., 2010; Patel et al., 2021). TRPV4 is a Ca^{2+} permeant channel that responds to thermal and mechanical stimuli, biochemical activators, G-protein coupled receptor and pharmacological agonists such as GSK1016790A (GSK) (Harraz et al., 2018). TRPV4 may mediate physiological responses by generating Ca^{2+} induced Ca^{2+} release (CICR) (Dunn et al., 2013; Heathcote et al., 2019; Greenstein et al., 2020) at IP_3 receptors to regulate vascular tone (Heathcote et al., 2019).

We sought to determine if mitochondria regulate TRPV4 mediated Ca^{2+} signalling. Previous studies have shown that TRPV4 interacts with mitochondrial proteins including Hsp60 and

Mfn1/2 to regulate mitochondrial structure and function (Kumar et al., 2018). This observation highlights a potential interaction between mitochondrial and TRPV4 in regulating intracellular signalling. However, precisely how mitochondria regulate TRPV4-mediated Ca^{2+} signalling is unclear. Here, the effect of mitochondria in regulating TRPV4-mediated intracellular Ca^{2+} responses has been examined in the endothelium of intact resistance arteries. As previously reported (Heathcote et al., 2019), we find Ca^{2+} influx is required for TRPV4-mediated Ca^{2+} release from the internal store. However, when $\Delta\Psi_m$ is depolarised, TRPV4 activation generates large-scale, multi-cellular propagating Ca^{2+} waves that do not require Ca^{2+} influx. These waves arise from ATP release via pannexin channels that activate plasma membrane purinergic receptors to evoke Ca^{2+} release.

Methods

Animals

All animal husbandry and euthanasia were carried out in accordance with the prior approval of the University of Strathclyde Animal Welfare and Ethical Review Body and under relevant UK Home Office Regulations, [Schedule 1 of the Animals (Scientific Procedures) Act 1986, UK]. Studies are reported in compliance with the ARRIVE guidelines (McGrath and Lilley, 2015). Strathclyde BPU is a conventional unit which undertakes FELASA quarterly health monitoring. Male Sprague-Dawley rats (10-12 week old; 250 - 300 g), from an in-house colony, were used for the study. The animals were housed 3 per cage in North Kent Plastic cages (model RC2F) with 'Sizzle Nest' nesting material. A 12:12 light dark cycle was used with a temperature range of 19 – 23°C (set point 21°C) and humidity levels between 45-65%. Animals had free access to fresh water and standard chow (Special Diet Services RM1). The enrichment in the cages was aspen wood chew sticks and hanging huts. Animals were euthanized by cervical dislocation and the mesenteric bed removed and transferred immediately to a physiological saline solution (PSS) buffer (composition below). In most experiments, controls and treatments were carried out in the same tissue, so blinding and randomisation was not used.

Tissue preparation

All experiments were performed using second- or third-order mesenteric arteries as previously described (Zhang et al., 2019). Arteries were cut longitudinally with micro-scissors and pinned flat on a customized Sylgard-coated bath chamber, with the endothelium facing upwards (*en face* preparation). The endothelium was then loaded with acetoxymethyl ester form of the Ca^{2+} indicator, Cal520-AM (5μM) (Abcam, UK) with 0.02% pluronic F-127 (Sigma, USA) in PSS and incubated at 37^{o}C for 30 min. After loading, the artery was washed gently with PSS.

Imaging

Ca^{2+} responses were acquired at 10 Hz on an upright fluorescence microscope (FN-1, Nikon, Japan) equipped with a 40X objective lens (0.8 numerical aperture; Nikon, Japan) and a back-illuminated electron-multiplying charge-coupled device (EMCCD) camera (1024×1024

13μM pixels; iXon 888, Andor, UK)(Glitsch et al., 2002). Fluorescence excitation (488 nM wavelength for Cal520) was provided by an illumination system CoolLED pE-4000. The system was controlled, and Ca^{2+} images recorded using the open source software, Micro-Manager (Edelstein et al., 2010, 2014).

Experiment protocol:

In experiments with GSK (20 nM), the drug was perfused into the bath followed by a 10-minute wash with PSS and 10-minute re-equilibration. In experiments in the absence of external Ca^{2+}, a Ca^{2+}-free PSS was perfused into the bath for 5 min to eliminate extracellular Ca^{2+} before the control response (10 min recording) to GSK in Ca^{2+}-free PSS was obtained. The tissue was then perfused for 10 min with PSS to replenish the internal store followed by equilibration for 10 minutes before subsequent recordings in the absence of extracellular Ca^{2+} (5 minutes before recording). The effect of various pharmacological interventions (CCCP/HC067047/caffeine/U73122) on GSK-evoked endothelial Ca^{2+} responses were studied in paired or unpaired experiments as described in the text.

Data analysis

The Ca^{2+} response in 200-300 cells in each preparation were analysed using custom-written Python software (Wilson et al., 2020). Within each cell, a region of interest (ROI) was generated (10 μm diameter circle) and the ROI was assigned an identification number. This enabled the responses in the same cells to be compared before and after treatment. Cells were aligned between recordings to compensate for subtle shifts in position of ROIs before processing analysis as previously described. The Ca^{2+} response from each ROI were extracted and expressed as ratios (F/F_0) of fluorescence counts (F) against baseline values (F_0). The baseline was identified automatically as portion of the signal exhibiting the lowest background noise during 100 consecutive frames (10 seconds) prior to the introduction of any agonists or antagonists. Ca^{2+} activity was identified using a zero-crossing peak-detection algorithm as fluctuations that exceed 5 times the standard deviation of baseline noise (Wilson et al., 2016; Lee et al., 2018). The amplitude and number of oscillations from each cell were extracted automatically.

The Ca^{2+} response to TRPV4 activation contained two main components: a ‘slow’ persistent Ca^{2+} elevation in baseline Ca^{2+} levels and a fast intra-cellular Ca^{2+} wave. The proportion of each component of the response changed after mitochondrial depolarisation. To determine the

underlying mechanisms, we isolated the two components from each F/F_0 trace. The slow persistent Ca^{2+} elevation (baseline) component of each signal was extracted by applying an asymmetric least squares (ALS) smoothing function (Heathcote et al., 2019). Signalling metrics describing this slow persistent Ca^{2+} elevation then calculated from a fitted sigmoidal curve. Cells were considered to exhibit a persistent Ca^{2+} elevation if the slow F/F_0 component rose by more than ten-fold the standard deviation of baseline noise. The fast component of each signal was extracted by dividing each F/F_0 trace by the ALS-smoothed signal. This procedure effectively flattens the baseline and removes any slow drift from the signal (Heathcote et al., 2019).

Statistical Analysis

The data and statistical analysis in this study comply with the recommendations of the British Journal of Pharmacology on experimental design and analysis in pharmacology (Curtis et al., 2018). The n numbers shown are the numbers of biological replicates (number of animals). For each replicate, a single field of endothelial cells was studied. Summary data are presented graphically as paired mean responses, or as the grand mean ± SEM of n biological replicates. Data were assessed for variance homogeneity (F-test) before statistical tests were performed. Raw peak F/F_0 responses were analysed statistically using either a paired/unpaired Student's t-test or a one-way ANOVA with Tukey's multiple comparisons test using GraphPad Prism 8.0 (GraphPad, La Jolla, CA, USA). $p < 0.05$ was considered statistically significant.

Reagents and chemicals

The PSS consisted of (in mM): 145.0 NaCl, 2.0 MOPS (3-(N-Morpholino) propanesulfonic acid, 4-Morpholinepropanesulfonic acid), 4.7 KCl, 1.2 NaH_2PO_4, 5.0 Glucose, 0.02 EDTA (Ethylenediaminetetraacetic acid), 1.17 $MgCl_2$, 2.0 $CaCl_2$ (pH adjusted to 7.4 with NaOH). In experiments using Ca^{2+}-free PSS, $CaCl_2$ was replaced with $MgCl_2$ on an equimolar basis and Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA, 1 mM) was included. Cal520/AM was obtained from Abcam UK, Pluronic F-127 from Invitrogen UK, HC 067047 from TOCRIS UK. GSK1016790A(GSK), Carbonyl cyanide m-chlorophenyl hydrazine (CCCP), cyclopiazonic acid (CPA), caffeine, PLC inhibitor (U73122), Oligomycin and Rotenone were obtained from Sigma UK. The stock solution of caffeine was prepared in PSS solution. All other drugs and chemicals were dissolved in DMSO (Dimethyl sulfoxide, 100%) and diluted using PSS to the desired concentration.

Data availability

All data underpinning this study is available from the authors upon reasonable request.

Results

The TRPV4 agonist GSK (20 nM) generated rapid and reproducible Ca^{2+} oscillations in the endothelium (~200 endothelial cells) of mesenteric resistance arteries (Figure 1A, B). GSK-evoked Ca^{2+} responses were reproducible in amplitude and the percentage of cells responding to each activation (Figure 1C-E). Previous findings show that TRPV4-mediated Ca^{2+}-influx induces Ca^{2+} release at IP_3 receptors to cause repetitive Ca^{2+} oscillations (Glitsch et al., 2002; Dunn et al., 2013; Heathcote et al., 2019).

Mitochondria are key regulators of IP_3-evoked Ca^{2+} release (Rizzuto et al., 1993; Ivanova et al., 2019). Mitochondria may also regulate TRPV4 activity. The mitochondria membrane potential is the primary driver for Ca^{2+} uptake to mitochondria (Miller, 1998). Therefore, the uncoupler carbonyl cyanide m-chlorophenyl hydrazine (CCCP) and the complex I inhibitor, rotenone, were each used to depolarise the mitochondrial membrane potential (Wilson et al., 2019; Zhang et al., 2019) and explore the effect of compromised mitochondria on TRPV4-mediated Ca^{2+} signalling (Chalmers and McCarron, 2008).

In the first series of experiments, endothelial cells responses to GSK (20nM) were recorded in the same preparation in the absence and then presence of CCCP (1μM) (Figure 2A). CCCP by itself did not alter baseline Ca^{2+} (not shown). GSK-evoked Ca^{2+} signals were changed substantially in the presence of CCCP. Rather than repetitive oscillations, GSK-evoked large sustained increases in Ca^{2+} that propagated across cells (slow Ca^{2+} signals) (Figure 2B, C; Supplementary Video 1).

These signals appeared to be coordinated in clusters of cells that activated their neighbours (Supplementary Video 1). When extracellular Ca^{2+} was removed, GSK by itself failed to generate a Ca^{2+} response (Figure 2). However, when the mitochondrial membrane was depolarised with CCCP in the absence of external Ca^{2+}, GSK evoked a large-scale Ca^{2+} response that propagated across cells (Figure 2B, E, F Supplementary Video 1). These results suggest that when mitochondria are depolarised, TRPV4 triggers Ca^{2+} release from the internal Ca^{2+} store without a requirement for Ca^{2+}-induced Ca^{2+} release. Since mitochondrial depolarization abolished the fast component of GSK-evoked Ca^{2+} signals, only slow waves were investigated further. All subsequent experiments were performed in Ca^{2+}-free PSS.

When mitochondria were depolarised using the complex I inhibitor, rotenone (Figure 3), similar results to those of CCCP were obtained – GSK evoked an endothelial Ca^{2+} response that did not require the influx of external Ca^{2+}. The magnitude of the response was lower in the presence of rotenone (versus CCCP) which is consistent with the slower rate of mitochondrial depolarisation achieved with this drug (Zhang et al., 2019). Conversely, GSK did not generate large-scale Ca^{2+} waves in the presence of the ATP synthase inhibitor, oligomycin, (Figure 3). These findings suggest that the mitochondria membrane potential rather than ATP production is important in TRPV4 coupling to mitochondria.

To confirm that the Ca^{2+} release evoked by GSK arose due to TRPV4 channel activation, the specific TRPV4 antagonist HC067047 (HC067; 10μM; 50 min incubation) was used. HC067 prevented GSK-evoked Ca^{2+} signals when mitochondria were depolarised (Figure 4A-D).

To determine the source of the Ca^{2+} generating the TRPV4-mediated Ca^{2+} waves uncovered after mitochondrial depolarisation, the SERCA ATPase inhibitor, cyclopiazonic acid (CPA, 10 μM) was used to deplete the endoplasmic reticulum (ER) Ca^{2+} store. After store depletion with CPA, TRPV4 activation failed to generate a Ca^{2+} response in the presence of depolarised mitochondria (Figure 5A). The amplitude (F/F_0) and percentage of active cells each were significantly reduced in the CPA treated group (Figure 5B, C, D). Together, these results confirm that TRPV4 activation is required for compromised mitochondria-regulated internal Ca^{2+} release from the ER.

IP_3Rs are the main intracellular ligand-gated Ca^{2+} channel expressed on the ER membrane of endothelial cells (Supattapone et al., 1988; Yoshida and Imai, 1997; Bosanac et al., 2002). Ryanodine receptors play little role in Ca^{2+} signalling in endothelial cells (Santulli et al., 2017; Buckley et al., 2020). Caffeine is a potent IP_3R inhibitor (Brown et al., 1992; Sei et al., 2001) and was used to determine if IP_3R activation contributed to GSK-evoked Ca^{2+} release that is uncovered following mitochondrial depolarisation. TRPV4-mediated Ca^{2+} responses, after mitochondrial depolarisation with CCCP, were inhibited by pre-treatment with caffeine (10mM; 5 min incubation) (Figure 6A, B, C). After the washout of caffeine, the Ca^{2+} response to GSK (in CCCP) recovered (Figure 6A, B, C, D). These data indicate that TRPV4 activation, following mitochondrial depolarization, evokes IP_3R-mediated Ca^{2+} release from the ER.

Phospholipase C (PLC), an important component of the IP_3 signalling system, converts phosphatidylinositol 4,5-bisphosphate (PIP_2) to IP_3 and diacylglycerol (Essen et al., 1997; Kadamur and Ross, 2013). To examine if PLC was involved in TRPV4-mediated Ca^{2+} release when mitochondria are depolarised the PLC inhibitor, U73122, was used (Figure 7). After incubation with U73122 (2μM; 15min), GSK (in CCCP) failed to generate a Ca^{2+} response (Figure 7A, B). The amplitude of the Ca^{2+} response and the percentage of active cells remained near baseline levels. (Figure 7C, D).

The question arises as to how TRPV4 activation generates IP_3-mediated waves that move from neighbour to neighbour when mitochondria are depolarised (Supplementary Video 1). The progression of the wave appears to be consistent with a self-propagating event (Supplementary Video 1). This type of wave has been shown to occur in other tissue via release of ATP (Daneva et al.; Anselmi et al., 2008; Isakson and Thompson, 2014). To examine the role of purinergic transmission in TRPV4-mediated Ca^{2+} release following mitochondrial depolarization (in the presence of CCCP), the effects of the ATPase, apyrase (5 units/ml; 30 minutes), the purinergic receptor blocker, suramin (100 μM, 10 minutes), and the pannexin-1 blocker, probenecid (1 mM; 30 min), were each examined. TRPV4-mediated Ca^{2+} waves were significantly reduced in the presence versus absence of apyrase, suramin, or probenecid (Figure 7). Collectively, these results suggest that when mitochondria are depolarised TRPV4 activation triggers ATP release via pannexin-1 channels. Extracellular ATP then acts on plasma membrane purinergic receptors to stimulate PLC activation and Ca^{2+} release from the ER.

Discussion

TRPV4 is a plasma membrane expressed, non-selective cation channel that is permeable to Ca^{2+} and is activated by mechanical, osmotic, and chemical stimuli (White et al., 2016). Ca^{2+} influx via TRPV4 triggers Ca^{2+} release from the IP_3-sensitive internal store to generate repetitive oscillations that cause many of the physiological responses derived from the channel's activity (Heathcote et al., 2019; Shen et al., 2019). Under normal conditions, when mitochondria are polarised, TRPV4-mediated release of Ca^{2+} from the IP_3-sensitive store is completely dependent on Ca^{2+} influx and signals appear as relatively fast intracellular oscillations (Dunn et al., 2013; Heathcote et al., 2019). In the absence of external Ca^{2+}, TRPV4 activation does not cause a significant intracellular Ca^{2+} response (Heathcote et al., 2019). The present study demonstrates that when the mitochondrial membrane potential is depolarised, Ca^{2+} signals evoked by TRPV4 activation change to large sustained Ca^{2+} release events and slow propagating waves that propagate within and between cells in the absence of Ca^{2+} influx. These Ca^{2+} responses occur via a TRPV4-mediated release of ATP through pannexin channels. ATP then activates cell surface purinergic receptors to trigger IP_3 evoked Ca^{2+} release from the ER.

TRPV4 activation has previously been shown to stimulate the release of autocrine/paracrine regulators, including ATP from various cells (Gevaert et al., 2007; Gradilone et al., 2007; Silva and Garvin, 2008). For example, in human pulmonary fibroblasts TRPV4 activation resulted in ATP being release into the extracellular space (Rahman et al., 2018). In cholangiocyte cilia, TRPV4 activation induces bicarbonate secretion via apical ATP release (Gradilone et al., 2007). In thick ascending limbs of the renal medulla, decreases in osmolality stimulate ATP release via TRPV4 activation (Silva and Garvin, 2008). In the present study, when mitochondria are depolarised, Ca^{2+} waves evoked by TRPV4 activation are blocked by the purinergic receptor antagonist suramin, and the ATPase, apyrase. These findings suggest an involvement of ATP in the TRPV4-mediated Ca^{2+} response when mitochondria are compromised.

TRPV4-mediated release of ATP appears to occur via pannexin channels. Pannexins are broadly expressed (including in endothelial cells) large-pore ion channels that function by releasing large signalling molecules (Lohman et al., 2015; Sharma et al., 2018; Yang et al., 2020). Pannexin channels (in addition to connexons) are widely reported to be a principal

conduit for ATP release in multiple cell types (Dahl and Locovei, 2006; Locovei et al., 2006; Iglesias et al., 2009; MacVicar and Thompson, 2010; D'hondt et al., 2011) including endothelial cells (Good et al., 2021; Maier-Begandt et al., 2021). The present findings show the pannexin blocker probenecid inhibited the TRPV4-mediated Ca^{2+} response that occurs when mitochondria are depolarised, suggesting that ATP release is inhibited. The other major pannexin blocker often used in intact tissue studies is carbenoxolone. However, carbenoxolone is a potent IP_3R blocker, prohibiting its use in the present experiments (Buckley et al., 2021).

The mechanism that links TRPV4 to ATP release via pannexins is not clear. In other systems Ca^{2+} influx via TRPV4 activates pannexins (Rahman et al., 2018). However, in the present experiments, TRPV4-mediated activation of pannexins occurred in a Ca^{2+}-free external solution. Ca^{2+} influx therefore cannot provide the pannexin activation mechanism. Pannexin gating is regulated by membrane potential (Marrelli et al., 2007; Ma et al., 2009, 2012) and the open probability of single pannexin channel increases in a voltage-dependent manner (Romanov et al., 2012). Pannexin channel gating is also regulated by the metabolic status of the cell and increases with reactive oxygen species production (Romanov et al., 2012). It is tempting to speculate that when mitochondria are depolarised the change in redox status of the cell will sensitize pannexins to membrane potentials changes. TRPV4 generates an inward current which may act to depolarise the plasma membrane potential resulting in pannexin channel opening and ATP release. TRPV4 channels are also reported to interact with mitochondria proteins including Hsp60 and, Mfn1/2 to regulate mitochondrial structure and function (Kumar et al., 2018). Perhaps this forms a link allowing mitochondria to regulate TRPV4 activity.

The progression of TRPV4-evoked Ca^{2+} waves through neighbouring cells when mitochondria are depolarised appears consistent with a self-propagating event, i.e., the wave appears to be responsible for its own propagation (Video 1). In other cells, ATP-induced increases in Ca^{2+} activate connexin hemichannels to trigger ATP release and regenerative Ca^{2+} waves (Anselmi et al., 2008). Ca^{2+} increases trigger ATP release to generate Ca^{2+} responses in neighbouring cells. The Ca^{2+} response, in these neighbouring cells, in turn generates ATP release (Anselmi et al., 2008). The release of ATP may also explain the regenerative propagation of multicellular Ca^{2+} waves on TRPV4 activation in the present study. ATP released from one cell may trigger Ca^{2+} release from neighbouring cells and the

Ca^{2+} increase itself will further activate TRPV4 to promote further ATP release. TRPV4 is activated by intracellular Ca^{2+} (Strotmann et al., 2003) and IP_3 may sensitize the channel to activation (Fernandes et al., 2008).

A distinguishing feature of the TRPV4-mediated Ca^{2+} response when mitochondria are depolarised is that the Ca^{2+} signal remains elevated rather than undergoing repetitive oscillations, as occurs when mitochondria are polarised. ATP depletion following the release of the purine from the cell, may result in attenuation of Ca^{2+} pump activity normally responsible for removing Ca^{2+} from the cytoplasm. The depletion of ATP may occur rapidly when mitochondria are depolarised since mitochondrial ATP synthesis is blocked by the organelles depolarisation. Cytosolic cytochrome c release from depolarised mitochondria may also interact with the IP_3 receptors to block a negative feedback system in which Ca^{2+} limits its own release (Wozniak et al., 2006) resulting in a more sustained increase in Ca^{2+}.

Mitochondria are widely acknowledged to regulate IP_3-mediated Ca^{2+} signalling. Ca^{2+} uptake by mitochondria controls the local concentration of the ion near IP_3Rs to generate a feedback effect of Ca^{2+} on IP_3R activity (Olson et al., 2010; Decuypere et al., 2011). Mitochondria also evoke long-distance control of IP_3R activity mediated by ATP production (Rizzuto et al., 1993; Carafoli, 2003; Csordás et al., 2006; Szabadkai et al., 2006; Wilson et al., 2019). In each case, depolarisation of the mitochondrial membrane potential is a critical parameter in the control of IP_3-evoked Ca^{2+} signalling (Abramov and Duchen, 2008; Chikando et al., 2013; Storey and Lambert, 2017). When the mitochondrial membrane potential is depolarised, mitochondrial Ca^{2+} uptake and ATP production are inhibited. As a result, mitochondrial control of IP_3R-mediated Ca^{2+} release is disabled and Ca^{2+} released is suppressed in endothelial cells and smooth muscle cells (Chalmers and McCarron, 2009; Olson et al., 2010; Wilson et al., 2019). This mitochondrial control of Ca^{2+} release is exerted at IP_3Rs rather than the production of IP_3 via PLC. Evidence for this is found in the observation that when PLC is bypassed by evoking Ca^{2+} release by photo release of caged IP_3, Ca^{2+} release is inhibited when the mitochondrial membrane potential is depolarised (McCarron and Muir, 1999; Chalmers and McCarron, 2008, 2009; Olson et al., 2010; Decuypere et al., 2011; Wilson et al., 2019). These previous reports emphasise the unexpected nature of the present findings. Depolarisation of mitochondria normally inhibits Ca^{2+} release – here TRPV4 mediated Ca^{2+} release is facilitated when mitochondria are depolarised. The mechanisms underlying the differences are unclear but it appears that TRPV4 activation alters the way in which

mitochondria normally regulate the IP_3R. TRPV4 channels are reported to interact with mitochondrial proteins including Hsp60 and, Mfn1/2 to regulate mitochondrial structure and function. It is tempting to speculate that this type of link may provide a route for activated TRPV4 to modulate mitochondrial control of IP_3R.

Pannexins form plasma membrane channels that are closed under physiological conditions. Pannexins may open during pathological conditions (e.g., ischemia and hypoxia) to release signalling molecules (Pelegrin and Surprenant, 2006; Sridharan et al., 2010). The present study demonstrates that pannexins contribute to TRPV4 mediated Ca^{2+} responses when mitochondria are compromised. Pannexin channels do not contribute to the TRPV4-mediated Ca^{2+} response under normal physiological conditions (i.e. when mitochondria a polarised). However, our results suggest that when the mitochondrial membrane potential is depolarised, TRPV4 triggers release of ATP via a pannexin channel and this released ATP activates membrane bound P2Y receptors to elicit PLC and IP_3-mediated Ca^{2+} release from internal stores and resulting in propagating Ca^{2+} waves (Figure 8). This mechanism requires PLC and IP_3R activation, indicating that P2X receptors are not required. Additionally, the Ca^{2+} response occurred when extracellular Ca^{2+} was removed. The mitochondrial membrane potential is normally highly polarised (~-180 mV) but is critically regulated by physiological and pathological status. The mitochondrial membrane potential will depolarise in hypoxic or anoxic conditions. These conditions may provide the trigger to initiate this process.

These present findings reveal a new link between mitochondria and TRPV4 activation and highlight the plasticity of TRPV4 channels in modulating endothelial Ca^{2+} responses. Changes in metabolic status of cells, by altering mitochondrial function, will reconfigure Ca^{2+} signalling pathways. In these circumstances, TRPV4 activation is likely to be a major regulator of Ca^{2+} signalling generating large sustained increases in Ca^{2+} concentration.

Declaration of conflict of interest

The authors do not have conflicts of interest to declare.

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Author contributions:

ZX & JGM developed the concept. XZ performed the experiments. XZ analysed the data. XZ, MDL, CB, CW & JGM interpreted the data. XZ & JGM drafted the manuscript. XZ, MDL, CB CW & JGM edited the manuscript. CW, CB & JGM sourced funding. All authors approved the final version of the manuscript.

Competing Interests

None

Reference

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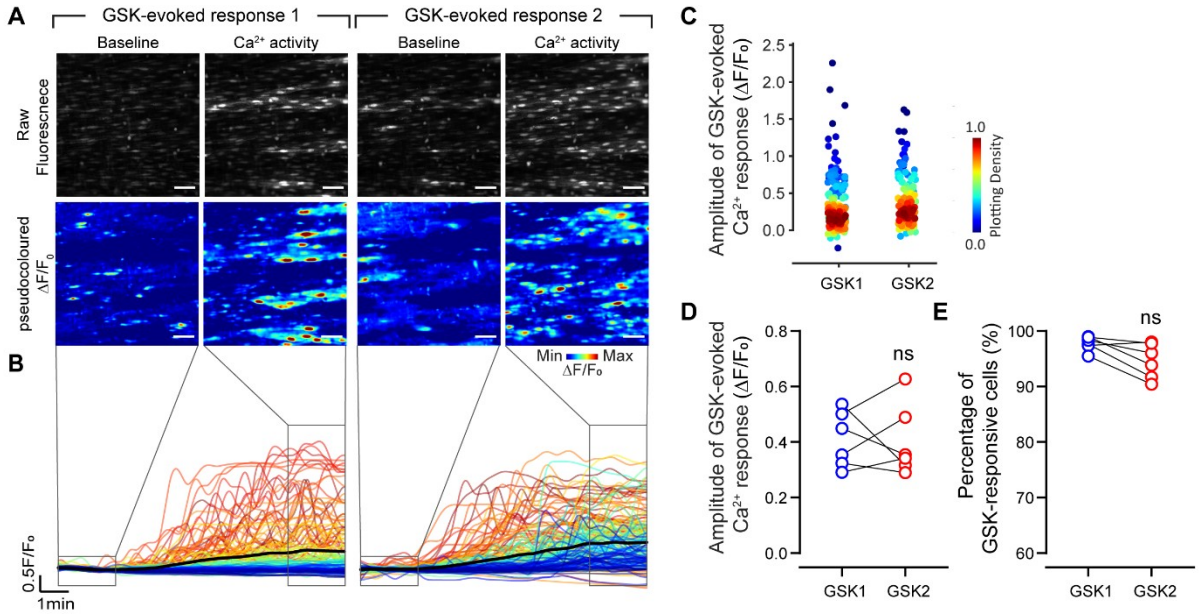

Figure 1. Activation of TRPV4 channels evokes reproducible endothelial cell Ca^{2+} responses. (A-B) Representative mesenteric artery endothelial cell Ca^{2+} images (A), and corresponding single-cell Ca^{2+} traces (B), showing TRPV4-mediated (GSK1016790A, GSK, 20 nM) Ca^{2+} signalling on two consecutive occasions. In A, the upper panels are raw fluorescence images, and the lower panels are pseudocoloured $\Delta F/F_0$ maximum intensity projections showing total Ca^{2+} activity over the indicated 2-minute period shown in B. Scale bars = 50 µm. In B, single-cell Ca^{2+} traces are coloured (from blue to red) according to the intensity of the Ca^{2+} response. The average response is overlaid in black. (C) Single-cell Ca^{2+} measurements (mean $\Delta F/F_0$) from the data shown in A-B. Individual data points have been coloured (from blue, low to red, high) according to the density of individual values. (D-E) Paired summary data showing the reproducibility of TRPV4-mediated endothelial Ca^{2+} responses. * $p < 0.05$, paired t test.

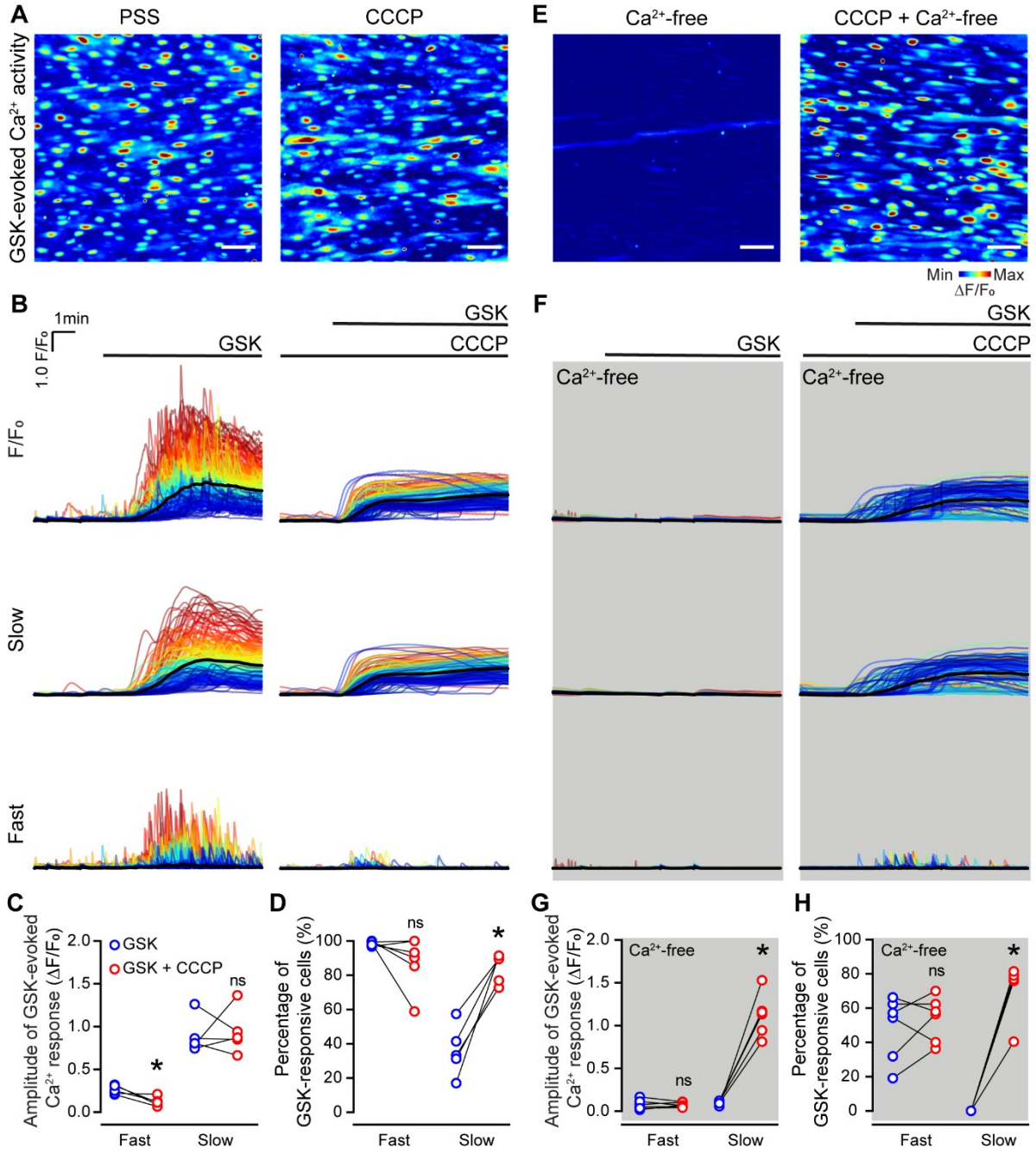

Figure 2. Mitochondrial depolarisation enables TRPV4-mediated endothelial cell Ca^{2+} signalling in the absence of extracellular Ca^{2+}. Ca^{2+} activity images, corresponding single-cell Ca^{2+} traces, and paired summary data showing the effect of the mitochondrial uncoupler, CCCP (1 μM), on TRPV4-mediated (GSK1016790A, GSK, 20 nM) endothelial cell Ca^{2+} signalling in the presence (A-D) and absence (E-H) of extracellular Ca^{2+}. In B & F, F/F_0 Ca^{2+} signals (top) were decomposed into fast (middle) and slow (bottom) components. Extracellular Ca^{2+} is required for TRPV4-mediated endothelial Ca^{2+} activity. However, this requirement is lost when mitochondria are depolarised with the uncoupler CCCP. * $p < 0.05$, paired t test.

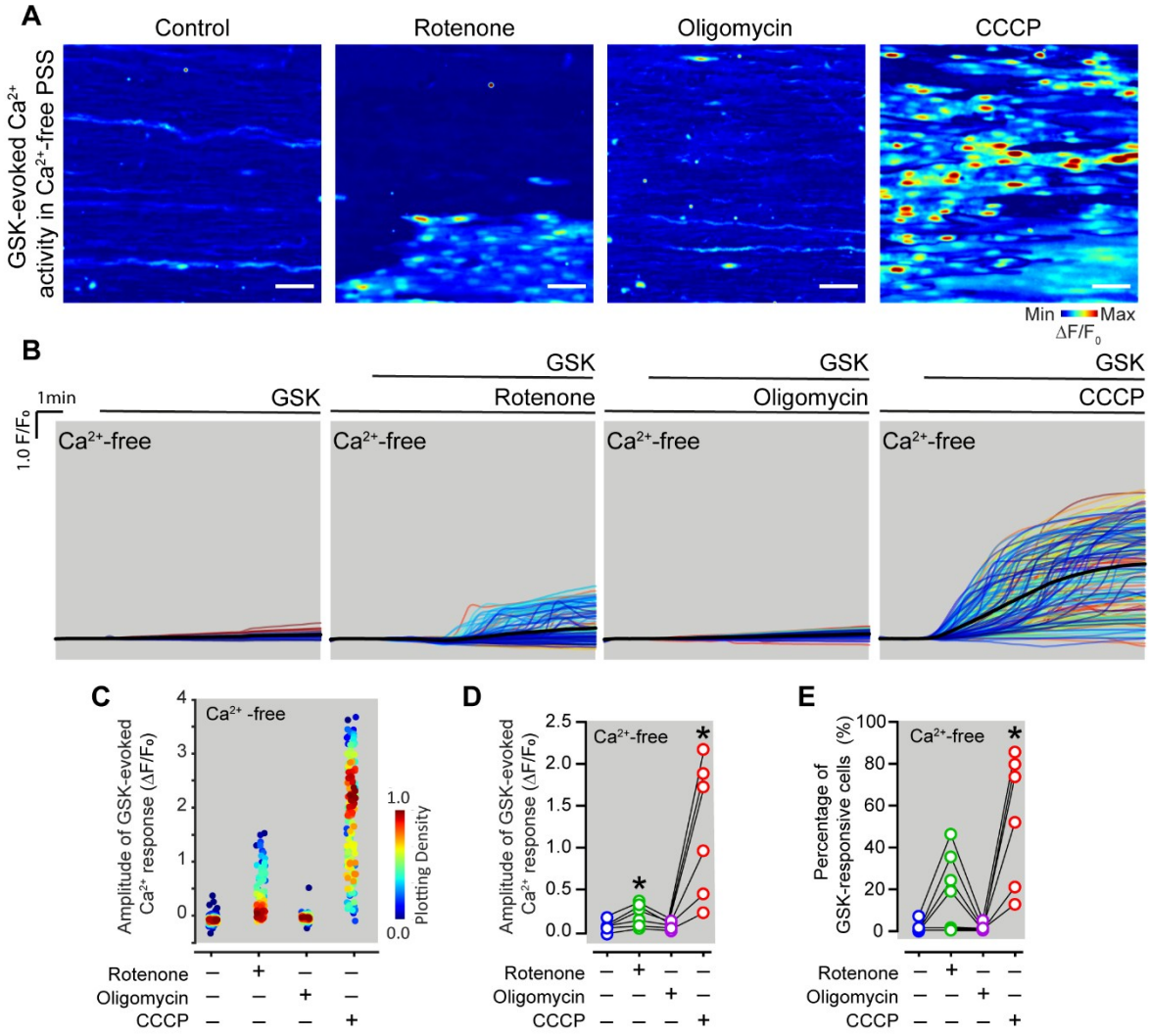

Figure 3. Mitochondria restrain TRPV4-mediated endothelial cell Ca^{2+} release. (A-B) Ca^{2+} activity images (A) and corresponding single-cell Ca^{2+} traces (B) from a single field of cells showing the effects of various mitochondrial inhibitors on TRPV4-mediated (GSK1016790A, GSK, 20 nM) endothelial Ca^{2+} release. Rotenone (1 μM) was used to inhibit mitochondrial complex I, oligomycin (1 μM) was used to inhibit mitochondrial complex V, and CCCP (1 μM) was used to uncouple mitochondria. All experiments were performed in a Ca^{2+}-free bath solution to eliminate Ca^{2+} influx. C) Single-cell Ca^{2+} measurements (mean $\Delta F/F_0$) from the data shown in A-B. (D-E) Paired summary data showing the effect of the various mitochondrial inhibitors on the amplitude on TRPV4-mediated endothelial Ca^{2+} release. * $p < 0.05$, one-way ANOVA with paired multiple comparisons.

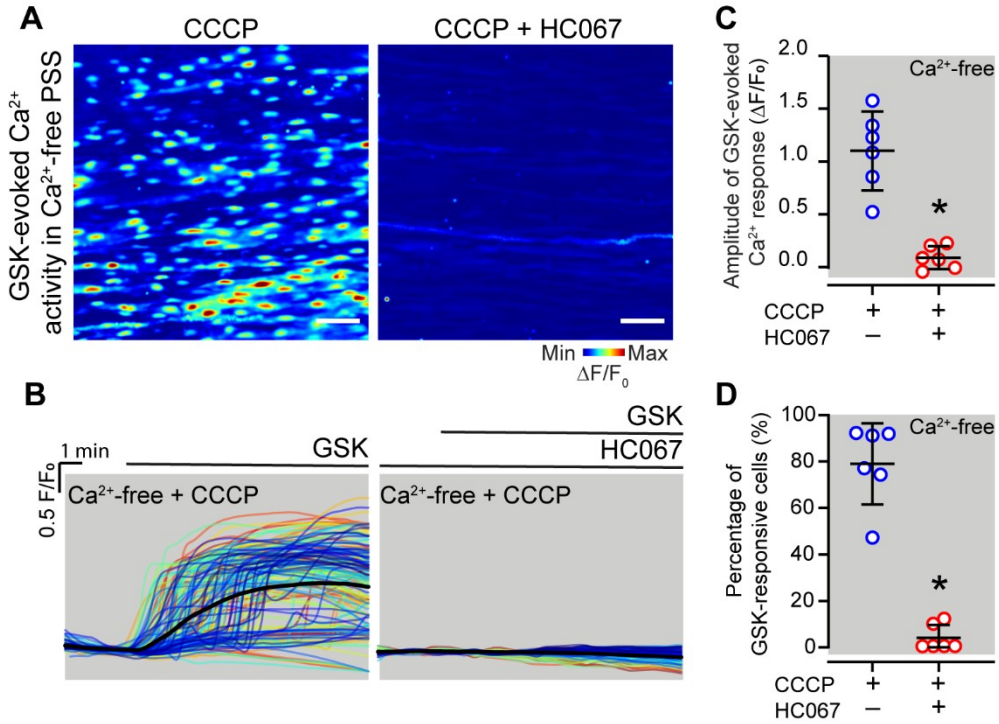

Figure 4. Ca^{2+} release evoked by the GSK1016790A arises via TRPV4 channel activation. (A-B) Ca^{2+} activity images (A) and corresponding single-cell Ca^{2+} traces (B) showing the effects of the specific TRPV4 channel antagonist, HC067047 (10μM), on endothelial cell Ca^{2+} responses evoked by GSK1016790A (GSK, 20 nM). All experiments were performed in a Ca^{2+}-free solution, to eliminate Ca^{2+} influx, and in the presence of the mitochondrial uncoupler, CCCP (1 μM), to inhibit mitochondrial function. (C-D) Summary data showing the effect of TRPV4 channel blockade on GSK-evoked endothelial Ca^{2+} release. * $p < 0.05$, unpaired t test.

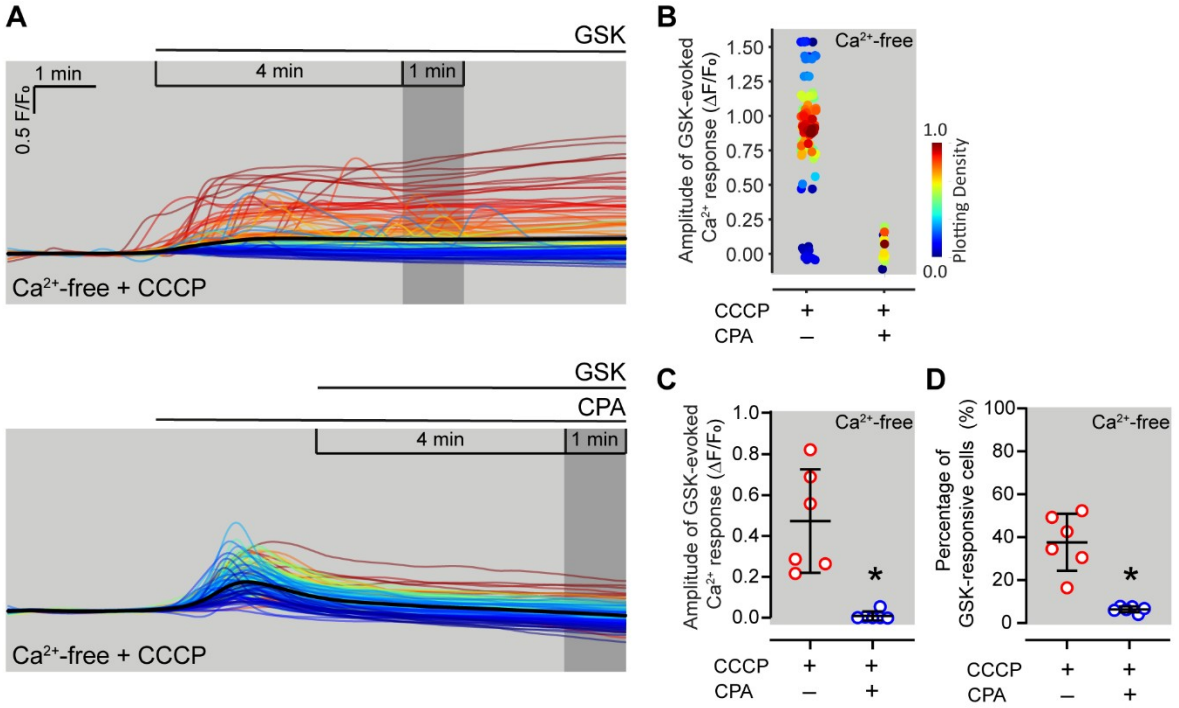

Figure 5. Depletion of internal Ca^{2+} stores prevents TRPV4 Ca^{2+} release. (A) Representative single-cell endothelial Ca^{2+} traces showing the effect of the sarcoendoplasmic reticulum ATPase (SERCA) inhibitor, cyclopiazonic acid (CPA, 50 µM), on TRPV4-mediated (GSK1016790A, GSK, 20 nM) Ca^{2+} release. All experiments were performed in a Ca^{2+}-free solution, to eliminate Ca^{2+} influx, and in the presence of the mitochondrial uncoupler, CCCP (1 µM), to inhibit mitochondrial function. (B) Single-cell Ca^{2+} measurements (mean $\Delta F/F_0$) from the data shown in A-B. $\Delta F/F_0$ values are mean Ca^{2+} levels during the 5th minute following activation of TRPV4 channels with GSK. (C-D) Summary data showing the effect of SERCA inhibition on GSK-evoked endothelial Ca^{2+} release. * $p <$ 0.05, unpaired t test.

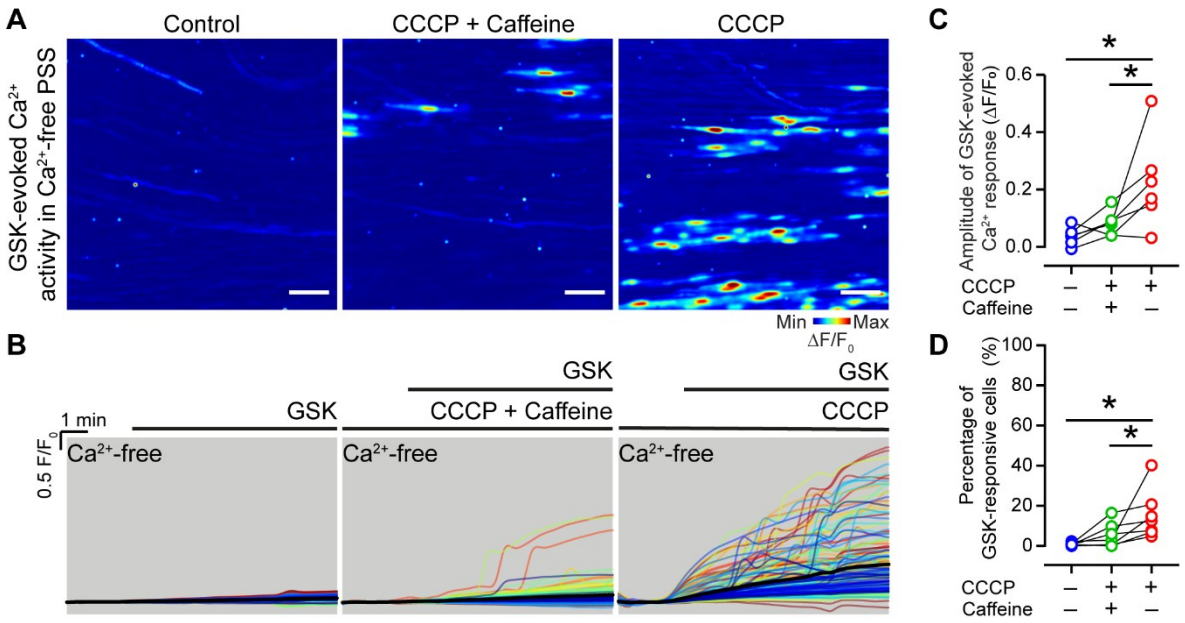

Figure 6. IP_3 receptor inhibition prevents TRPV4-mediated Ca^{2+}release. (A-B) Pseudocolored $\Delta F/F_0$ maximum intensity projections (A) and corresponding single-cell Ca^{2+} traces (B) showing the effect of the IP_3 receptor inhibitor, caffeine (10 mM), on endothelial cell Ca^{2+} release evoked by repeat activation of TRPV4 channels (GSK1016790A, GSK, 20 nM). All experiments were performed in a Ca^{2+}-free solution, to eliminate Ca^{2+} influx, and CCCP (1 μM) was introduced to inhibit mitochondrial function. (C-D) Paired summary data showing the effect of the IP_3 receptor blockade on mitochondrial-restrained TRPV4-mediated endothelial Ca^{2+} responses. * $p < 0.05$, one-way ANOVA with paired multiple comparisons.

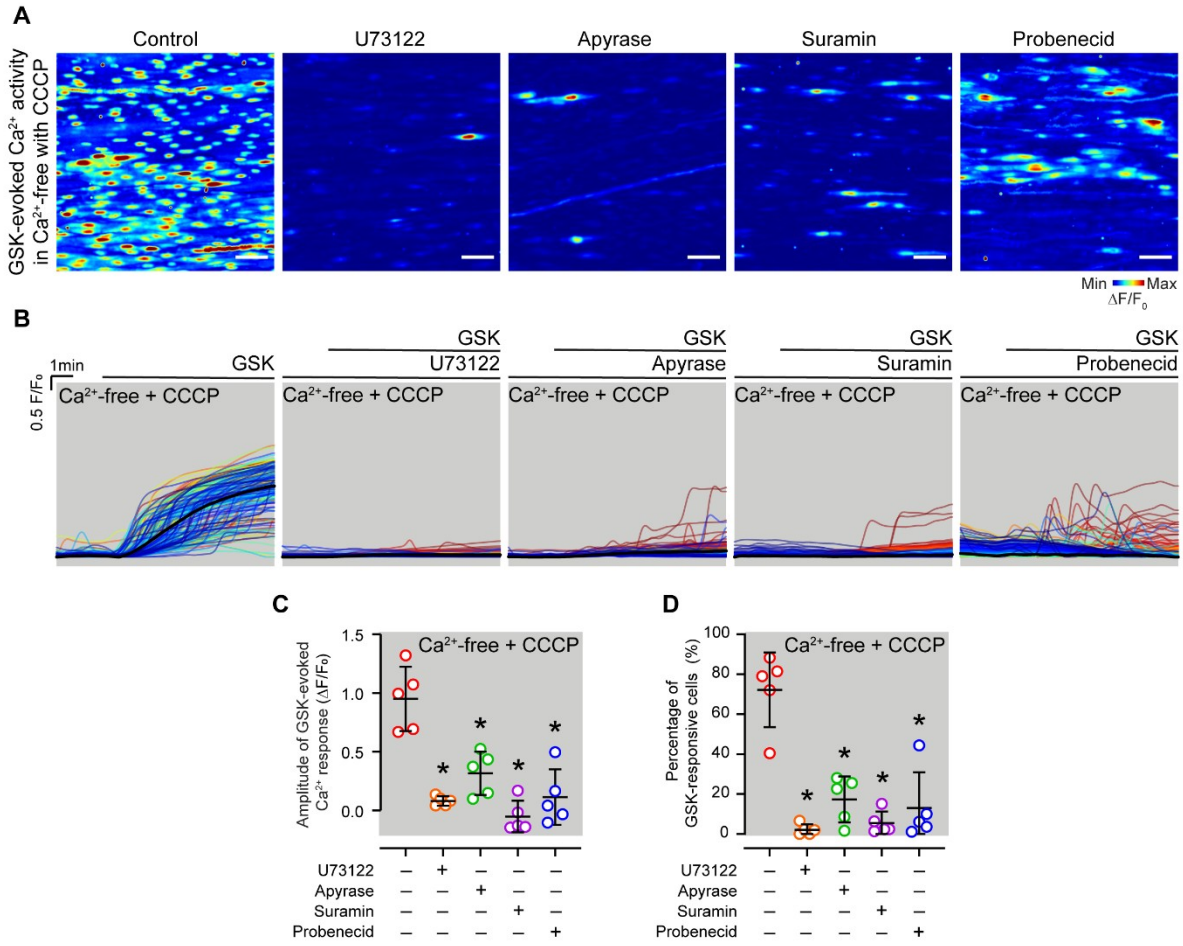

Figure 7. TRPV4 channels trigger endothelial cell Ca^{2+} release via pannexin-mediated release of ATP. (A-B) Pseudocolored $\Delta F/F_0$ maximum intensity projections (A) and corresponding single-cell Ca^{2+} traces (B) showing TRPV4-mediated (GSK1016790A, GSK, 20 nM) Ca^{2+} release in the absence or presence of the phospholipase C inhibitor, U73122 (2 μM), the ATPase, apyrase (5 U/ml), the purinergic receptor antagonist, suramin (100 μM), and the pannexin-1 inhibitor, probenecid (1 mM). All experiments were performed in a Ca^{2+}-free solution, to eliminate Ca^{2+} influx, and in the presence of the mitochondrial uncoupler, CCCP (1 μM), to inhibit mitochondrial function. Image scale bars = 50 μM. (C-D) Summary data showing the effect of the various pharmacological agents on the amplitude on mitochondrial-restrained TRPV4-mediated endothelial Ca^{2+} responses. * $p < 0.05$, one-way ANOVA with paired multiple comparisons.

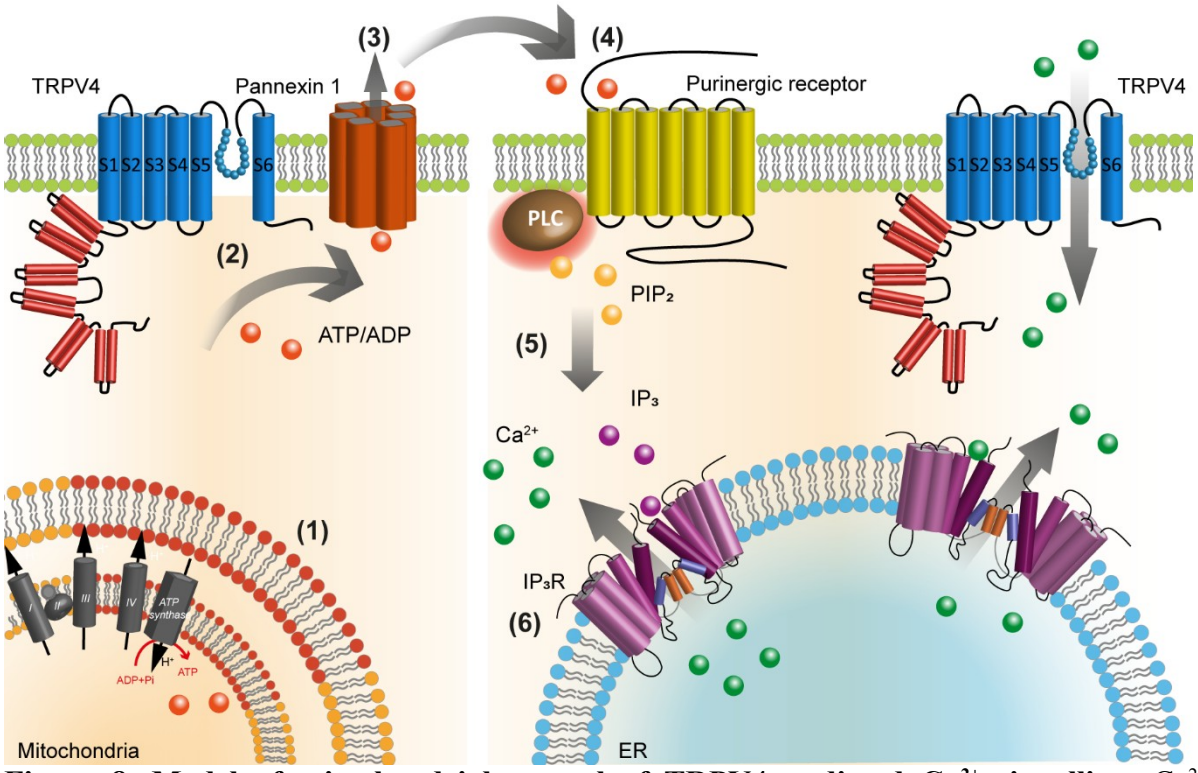

Figure 8. Model of mitochondrial control of TRPV4-mediated Ca^{2+} signalling. Ca^{2+} influx viaTRPV4 normally triggers CICR at IP_3R (right-side). However, when mitochondrial membrane potential is compromised (1), the activation of TRPV4 channels (2) triggers the release of ATP through pannexin hemichannels (3). ATP subsequently activates plasma membrane-bound purinergic receptors (4). The activation of purinergic receptors (P2Y) stimulates phospholipase C (PLC) to generate inositol trisphosphate (IP_3) (5) which subsequently activates IP_3 receptors to evoke Ca^{2+} release from the endoplasmic reticulum (6). IP_3R: inositol triphosphate receptor; ER: endoplasmic reticulum; IP_3: inositol triphosphate; PIP2: phosphatidylinositol 4,5-bisphosphate, PLC: phospholipase C.