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RESEARCH ARTICLE

Mitochondria regulate TRPV4-mediated release of ATP

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Background and Purpose: Ca^{2+} influx via TRPV4 channels triggers Ca^{2+} release from the IP_3-sensitive internal store to generate repetitive oscillations. Although 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 PLC blocker U73122 and the inositol trisphosphate receptor 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.

KEYWORDS
endothelium, inositol 1,4,5-trisphosphate (IP_3), intercellular Ca^{2+} waves, intercellular communication, mitochondria, pannexin, purinergic receptors, TRPV4, vascular

Abbreviations: ALS, asymmetric least squares; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazine; CPA, cyclopiazonic acid; *F*, fluorescence intensity; F_0, baseline fluorescence intensity; GSK, *N*-[(1*S*)-1-[[4-[(2*S*)-2-[[(2,4-dichlorophenyl)sulfonyl]amino]-3-hydroxy-1-oxopropyl]-1-piperazinyl]carbonyl]-3-methylbutyl]benzo[*b*]thiophene-2-carboxamide; HC067047, 2-methyl-1-[3-(4-morpholinyl)propyl]-5-phenyl-*N*-[3-(trifluoromethyl)phenyl]-1*H*-pyrrole-3-carboxamide; IP_3, inositol 1,4,5-trisphosphate; MOPS, 3-(*N*-morpholino)propanesulfonic acid sodium salt; PIP_2, phosphatidylinositol 4,5-bisphosphate; PSS, physiological saline solution; U73122, 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione; $\Delta\Psi_m$, mitochondrial membrane potential; CICR, Ca^{2+}-induced Ca^{2+} release; ER, endoplasmic reticulum; PLC, phospholipase C; SD, standard deviation.

1 | INTRODUCTION

The endothelium is a monolayer of cells that lines all blood vessels, and it plays a 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 & 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; Giorgi et al., 2018; Marcu et al., 2014). Mitochondrial Ca^{2+} uptake is controlled by the potential ($\Delta\Psi_m \sim -180$ mV) across the inner mitochondrial membrane that is generated via the proton gradient (Katakam et al., 2013). Depolarisation 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-trisphosphate** (IP_3) receptor activity on the internal Ca^{2+} store to modulate cell activity (Augustin et al., 1994; Correa et al., 2011; Csordás et al., 2006; Duchen, 2000; Narayanan et al., 2010; Olson et al., 2010; Rizzuto et al., 1993; Swärd et al., 2002; Szado et al., 2003). However, much less is known of the contribution of mitochondria in regulating Ca^{2+} influx in the endothelium.

The **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; Patel et al., 2021; Shigematsu et al., 2010; Strotmann et al., 2000). TRPV4 is a Ca^{2+} permeant channel that responds to thermal and mechanical stimuli, biochemical activators, GPCRs 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; Greenstein et al., 2020; Heathcote et al., 2019) 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 mitochondria 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 that Ca^{2+} influx is required for TRPV4-mediated Ca^{2+} release from internal stores. However, when $\Delta\Psi_m$ is depolarised, TRPV4 activation generates large-scale, multicellular 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.

What is already known

- TRPV4-mediated Ca^{2+} influx triggers Ca^{2+} release from the IP_3-sensitive store and vasodilation.

What does this study add

- When mitochondria are depolarised, TRPV4 activity triggers Ca^{2+} release in the absence of Ca^{2+} influx.
- With depolarised mitochondria, TRPV4 activation triggers ATP release via pannexins to activate purinergic cell-surface receptors.

What is the clinical significance

- In pathological conditions, depolarised mitochondria reconfigure TRPV4-mediated signalling, promoting intercellular communication and coordinated cell activity.

2 | METHODS

2.1 | Animals

All animal husbandry and experimental procedures were carried out in accordance with the relevant UK Home Office Regulations (Schedule 1 of the Animals [Scientific Procedures] Act 1986, UK) and were prior approved by the University of Strathclyde Animal Welfare and Ethical Review Body. Strathclyde BPU is a conventional unit that undertakes FELASA quarterly health monitoring. Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020).

Male Sprague–Dawley rats (10–12 weeks old; 250–300 g), from an in-house colony, were used for the study. The animals were housed three 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% and 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 killed by cervical dislocation, and the mesenteric bed was 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 were not used.

2.2 | 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 microscissors and pinned flat on a customised 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 (5 μM) (Abcam, UK) with 0.02% pluronic F-127 (Sigma, USA) in PSS, and incubated at 37°C for 30 min. After loading, the artery was washed gently with PSS.

2.3 | Imaging

Endothelial cell Ca^{2+} activity was acquired at 10 Hz on an upright fluorescence microscope (FN-1, Nikon, Japan) equipped with a 40× objective lens (0.8 numerical aperture; Nikon, Japan) and a back-illuminated electron-multiplying charge-coupled device (EMCCD) camera (1024 × 1024 13-μm 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. Microscope control and Ca^{2+} imaging recordings (10-min duration) were achieved using the open-source software, Micro-Manager (Edelstein et al., 2010, 2014).

2.4 | Experimental protocol

In experiments with GSK1016790A (GSK; 20 nM), the drug was perfused (2.5 ml min^{-1}) into the bath, and Ca^{2+} activity was recorded for a 10-min period. Drugs took 2 min to reach the imaging bath. Thus, each Ca^{2+} recording contained a 2-min period of baseline activity and 8 min of GSK-evoked activity. At the end of the recording period, the preparations were washed for 10 min with PSS and allowed to re-equilibrate for a further 10 min. 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 to GSK in Ca^{2+}-free PSS was obtained. The tissue was then perfused for 10 min with PSS to replenish the internal Ca^{2+} store and allowed to re-equilibrate for 10 min before subsequent recordings in the absence of extracellular Ca^{2+} (5 min before recording). The effect of various pharmacological interventions (CCCP/HC067047/caffeine/U73122) on GSK-evoked endothelial Ca^{2+} responses was studied in paired or unpaired experiments, as described in the text.

2.5 | Data analysis

Single-cell Ca^{2+} activity (200–300 cells per preparation) was analysed using custom-written Python software (Wilson et al., 2020). First, imaging datasets were grouped by experiment (e.g., control and treatment datasets). Circular regions of interest (ROIs; 10-μm diameter) were then generated for each cell within the control dataset of each imaging group, and these ROIs were projected across all other datasets in the group. Ca^{2+} responses for each cell were then extracted by averaging the fluorescence intensity (F) across all pixels in each ROI for each image frame. Ca^{2+} traces were smoothed using a Savitzky–Golay (21-point, third-order) filter and expressed as fractional changes in fluorescence (F/F_0) from baseline (F_0). The baseline was automatically determined by averaging the fluorescence intensity of the 10-s (100-frame) portion of the signal that exhibited the least noise.

In experiments examining the repeat activation of endothelial cells with GSK under control conditions (presence of external Ca^{2+}, no pharmacological interventions), the amplitude of individual endothelial cell Ca^{2+} activity was calculated by averaging the amplitude of F/F_0 traces over the 8-min activation period. In these experiments, cells were considered to be responsive to GSK if the average F/F_0 signal exceeded baseline values by more than 10-fold the standard deviation (SD) of baseline noise.

We previously demonstrated that the Ca^{2+} response to TRPV4 activation contains two components: a 'slow' persistent elevation in F/F_0, consistent with an elevation in baseline Ca^{2+} levels, and fast spikes in F/F_0, consistent with intracellular Ca^{2+} waves (Heathcote et al., 2019). The contribution of each component to the overall response changes after pharmacological intervention. Thus, in experiments to determine the mechanisms responsible for GSK-evoked Ca^{2+} activity, we isolated and compared the two components of each F/F_0 trace. The slow persistent Ca^{2+} elevation (baseline) component was extracted by applying an asymmetric least squares (ALS) smoothing function. 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. Unless otherwise noted, the reported amplitude of the slow component was calculated by averaging the slow F/F_0 signal over the 8-min period following the introduction of GSK to the imaging chamber. Cells were considered to be responsive to GSK if this average amplitude exceeded baseline values by more than 10-fold the SD of baseline noise. Fast Ca^{2+} activity was identified using a zero-crossing peak detection algorithm on the fast F/F_0 signal (Lee et al., 2018; Wilson et al., 2016), which extracts the amplitude of all peaks exceeding five times the SD of baseline noise. Cells were considered to be responsive to GSK in the fast domain if they exhibited at least one such peak. The reported amplitude of the fast component was calculated by averaging the amplitude of all detected peaks in the 8-min period following the introduction of GSK to the imaging chamber unless otherwise indicated (as in Figure 4).

2.6 | Statistical analysis

The statistical analyses 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 ± SD 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.

2.7 | Materials

The PSS consisted of (in mM): 145.0 NaCl, 2.0 MOPS (3-(*N*-morpholino)propanesulfonic acid sodium salt, 4-morpholinepropanesulfonic acid sodium salt), 4.7 KCl, 1.2 NaH_2PO_4, 5.0 glucose, 0.02 EDTA, 1.17 $MgCl_2$ and 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 EGTA (1 mM) was included. Cal520/AM was obtained from Abcam, UK; Pluronic F-127 from Invitrogen, UK; and HC067047 from Tocris (Abingdon, UK). GSK1016790A (GSK), carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP), **cyclopiazonic acid** (CPA), caffeine, the phospholipase C (PLC) inhibitor U73122, oligomycin and rotenone were obtained from Sigma (Dorset, UK). The stock solution of caffeine was prepared in PSS solution. All other drugs and chemicals were dissolved in DMSO (100%) and diluted using PSS to the desired concentration.

2.8 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22 (Alexander, Christopoulos et al., 2021; Alexander, Kelly et al., 2021; Alexander, Mathie et al., 2021).

3 | 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 in 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 (Dunn et al., 2013; Glitsch et al., 2002; Heathcote et al., 2019).

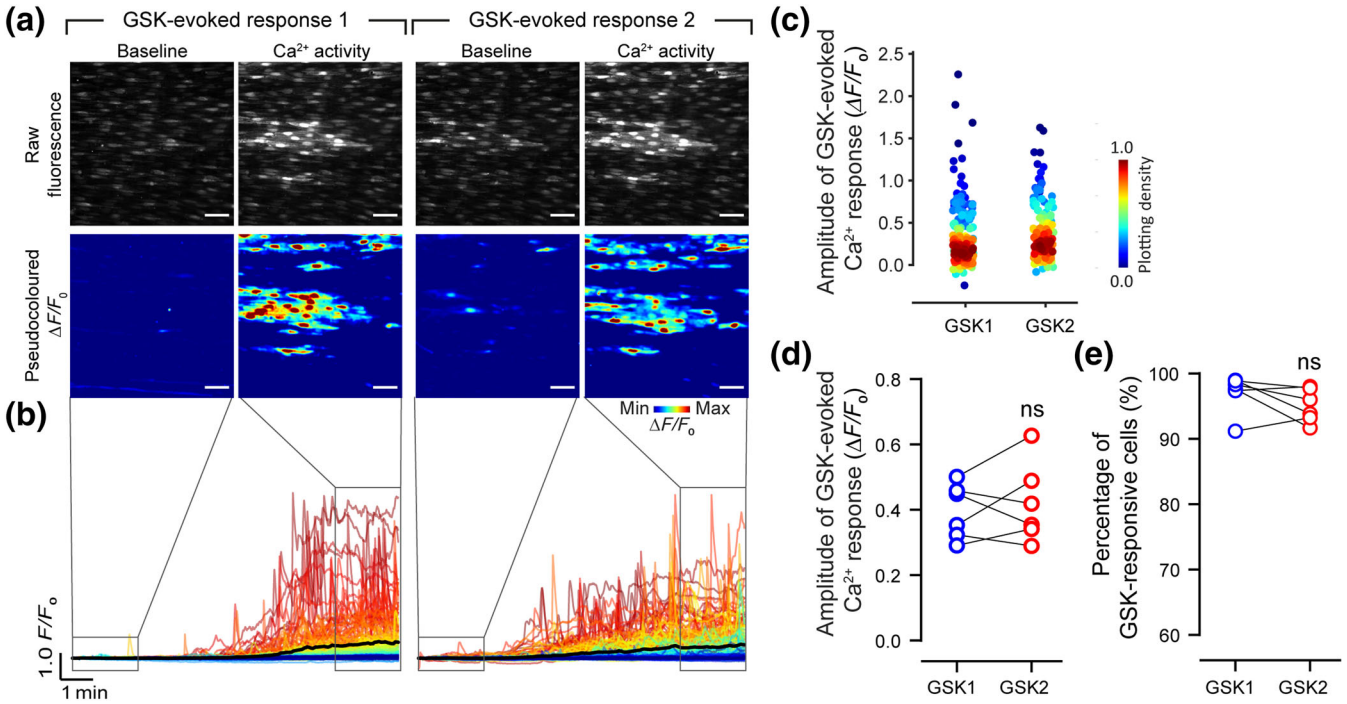

FIGURE 1 Activation of TRPV4 channels evokes reproducible endothelial cell Ca^{2+} responses. Representative mesenteric artery endothelial cell Ca^{2+} images (a) and corresponding single-cell Ca^{2+} traces (b), showing TRPV4-mediated (GSK1016790A, GSK, 20 nM; 1.5 min) Ca^{2+} signalling on two consecutive occasions with a wash (10 min) and rest (10 min) between GSK applications. 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-min 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) and (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. ns, paired *t* test

Mitochondria are key regulators of IP_3-evoked Ca^{2+} release (Ivanova et al., 2019; Rizzuto et al., 1993). Mitochondria may also regulate TRPV4 activity. The mitochondrial membrane potential is the primary driver for Ca^{2+} uptake to mitochondria (Miller, 1998). Therefore, the uncoupler 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 & McCarron, 2008).

In the first series of experiments, endothelial cell responses to GSK (20 nM) were recorded in the same preparation in the absence and then presence of CCCP (1 μM) (Figure 2a). GSK-evoked Ca^{2+}

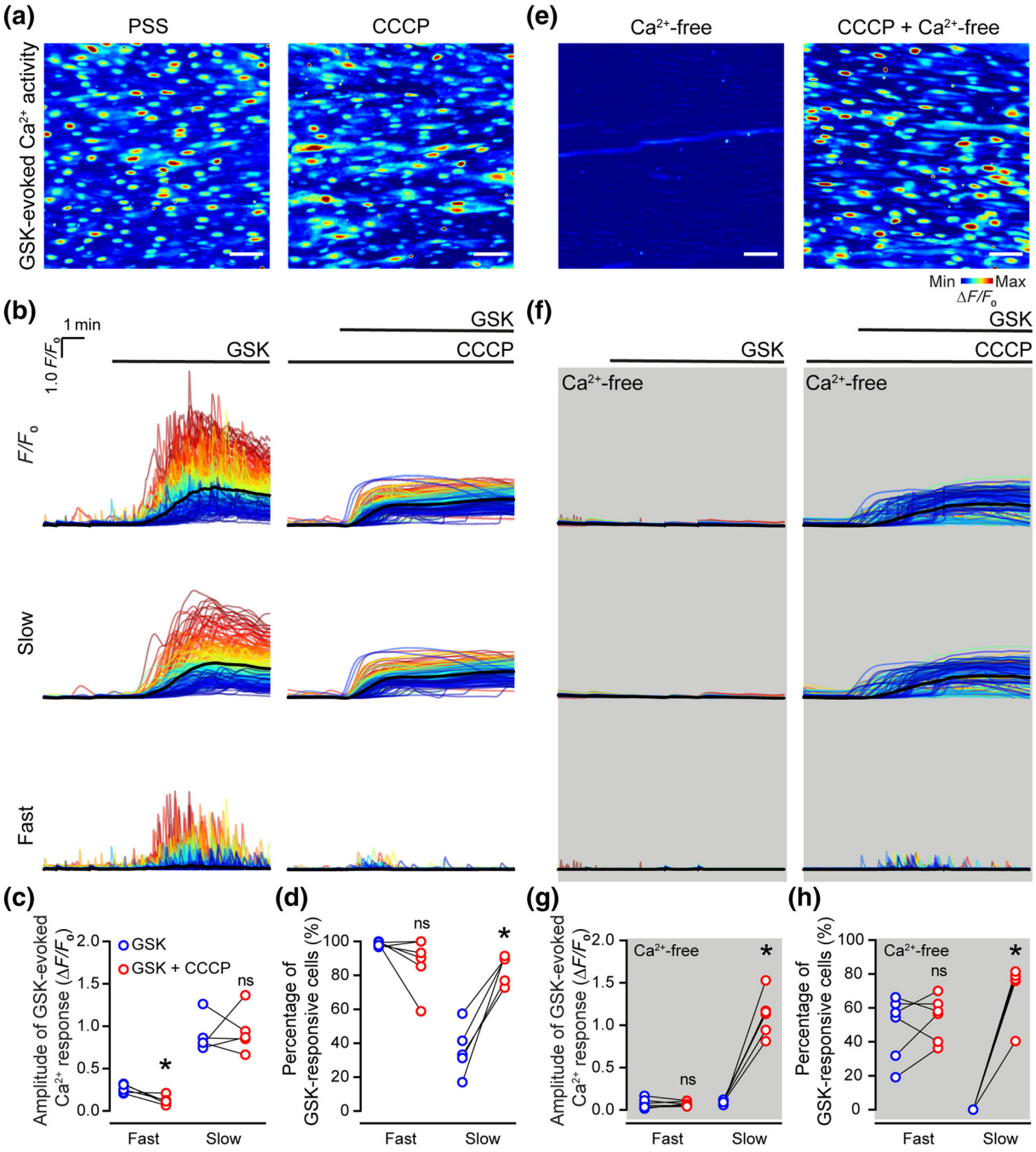

FIGURE 2 Mitochondrial depolarisation enables TRPV4-mediated endothelial cell Ca^{2+} signalling in the absence of extracellular Ca^{2+}. Ca^{2+} activity images, corresponding single-cell Ca^{2+} traces, and paired summary data showing the effect of the mitochondrial uncoupler, CCCP (1 μM), on TRPV4-mediated (GSK1016790A, GSK, 20 nM) endothelial cell Ca^{2+} signalling in the presence (a–d) and absence (e–h) of extracellular Ca^{2+}. In (b) and (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. *$P < 0.05$, significant effect of CCCP; paired *t* test

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 and Video S1).

These signals appeared to be coordinated in clusters of cells that activated their neighbours (Video S1). When extracellular Ca^{2+} was removed, GSK by itself failed to generate a Ca^{2+} response (Figure 2). However, when the mitochondrial membrane potential was depolarised with CCCP in the absence of external Ca^{2+}, GSK still evoked a large-scale Ca^{2+} response that propagated across cells (Figure 2b,e,f and Video S1). These results suggest that when mitochondria are depolarised, TRPV4 triggers Ca^{2+} release from the internal Ca^{2+} store without a requirement for CICR in contrast to the findings when mitochondria are polarised (Figure 2). Because mitochondrial depolarisation abolished the fast component of GSK-evoked Ca^{2+} signals, only slow waves were investigated further. To determine the mechanisms underlying TRPV4-mediated Ca^{2+} release when mitochondria are depolarised, all subsequent experiments were performed in Ca^{2+}-free PSS.

When mitochondria were depolarised using the complex I inhibitor, rotenone (Figure 3), results similar to those of CCCP were obtained; GSK-evoked an endothelial Ca^{2+} response that did not

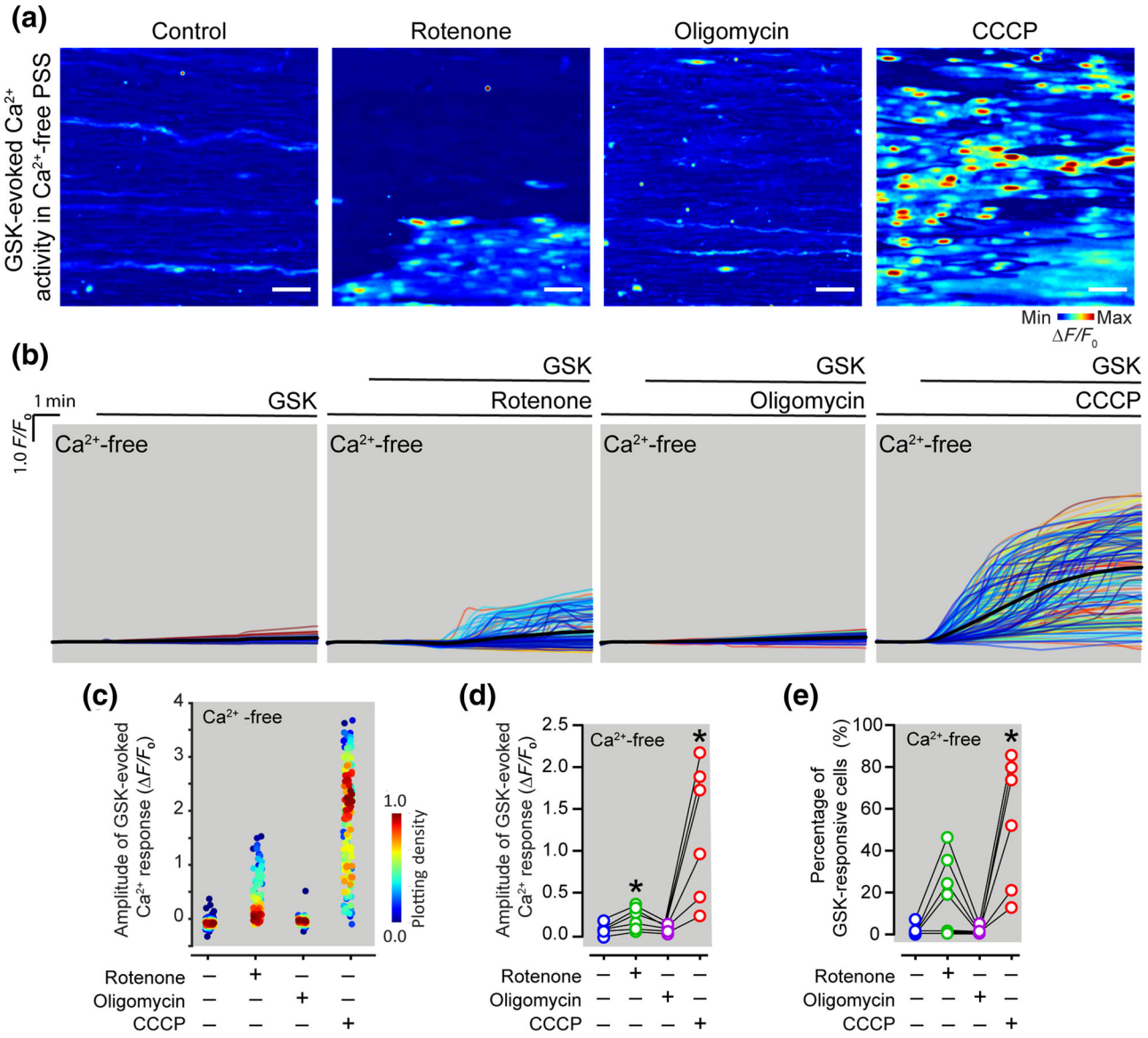

FIGURE 3 Mitochondria restrain TRPV4-mediated endothelial cell Ca^{2+} release. Ca^{2+} activity images (a) and corresponding single-cell Ca^{2+} traces (b) from a single field of cells showing the effects of various mitochondrial inhibitors on TRPV4-mediated (GSK1016790A, GSK, 20 nM) endothelial Ca^{2+} 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) and (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, significant effect of rotenone or CCCP; one-way ANOVA with paired multiple comparisons

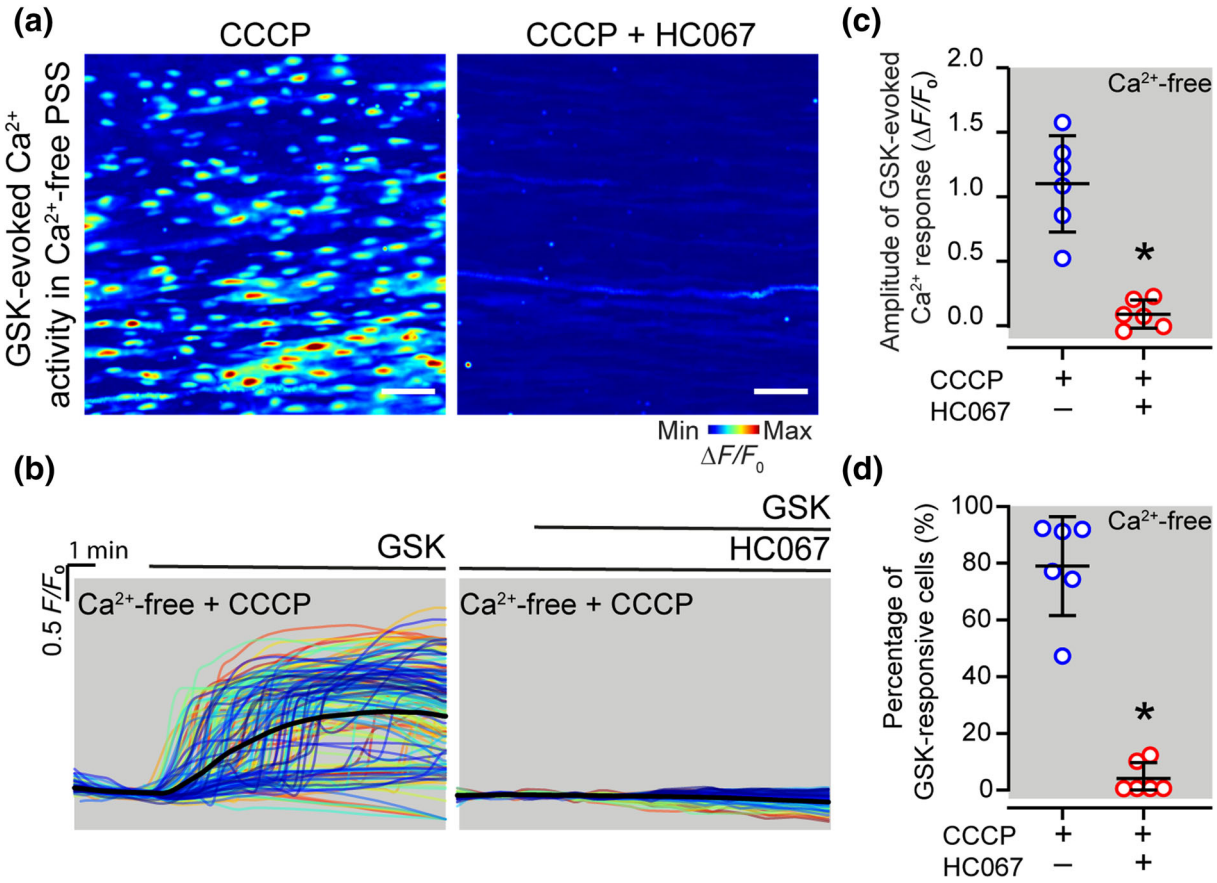

FIGURE 4 Ca^{2+} release evoked by the GSK1016790A arises via TRPV4 channel activation. Ca^{2+} activity images (a) and corresponding single-cell Ca^{2+} traces (b) showing the effects of the specific TRPV4 channel antagonist, HC067047 (10 μM), on endothelial cell Ca^{2+} responses evoked by GSK1016790A (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 (individual values with means ± SD) showing the effect of TRPV4 channel blockade on GSK-evoked endothelial Ca^{2+} release. *$P < 0.05$, significant effect of HC067; unpaired *t* test

require the influx of external Ca^{2+}. The magnitude of the response was lower in the presence of rotenone (compared with 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; 10 μM; 50-min incubation) was used. HC067 prevented GSK-evoked Ca^{2+} signals when mitochondria were depolarised (Figure 4a–d).

To determine the source of Ca^{2+} that generated the TRPV4-mediated Ca^{2+} waves after mitochondrial depolarisation, the **SERCA ATPase** inhibitor, CPA (20 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 when mitochondria are depolarised (Figure 5a). The amplitude (F/F_0) and percentage of active cells each were significantly reduced in the CPA-treated group (Figure 5b–d). Together, these results confirm that TRPV4 activation is required for compromised mitochondria-regulated internal Ca^{2+} release from the ER.

IP_3 receptors are the main intracellular ligand-gated Ca^{2+} channel expressed on the ER membrane of endothelial cells (Bosanac et al., 2002; Supattapone et al., 1988; Yoshida & Imai, 1997). **Ryanodine receptors** play little role in Ca^{2+} signalling in endothelial cells (Buckley et al., 2020). Caffeine is a potent inhibitor of IP_3 receptors (Bezprozvanny et al., 1994; Brown et al., 1992; Ehrlich et al., 1994; Hirose et al., 1993; Parker & Ivorra, 1991) and was used to determine if activation of IP_3 receptors contributed to GSK-evoked Ca^{2+} release that is uncovered after mitochondrial depolarisation. Caffeine blocks IP_3 receptors ($K_D = 1.6$ mM) over the same concentration range that activates ryanodine receptors, and an almost complete inhibition occurs at a concentration of 10 mM (Bezprozvanny et al., 1994; Ehrlich et al., 1994). Because there does not appear to be a significant contribution of ryanodine receptors to Ca^{2+} signalling in the endothelium (Buckley et al., 2020; Mumtaz et al., 2011), caffeine is a very useful inhibitor of IP_3 receptors.

TRPV4-mediated Ca^{2+} responses, after mitochondrial depolarisation with CCCP, were inhibited by pre-treatment with caffeine (10 mM; 5-min incubation) (Figure 6a–c). After the washout of

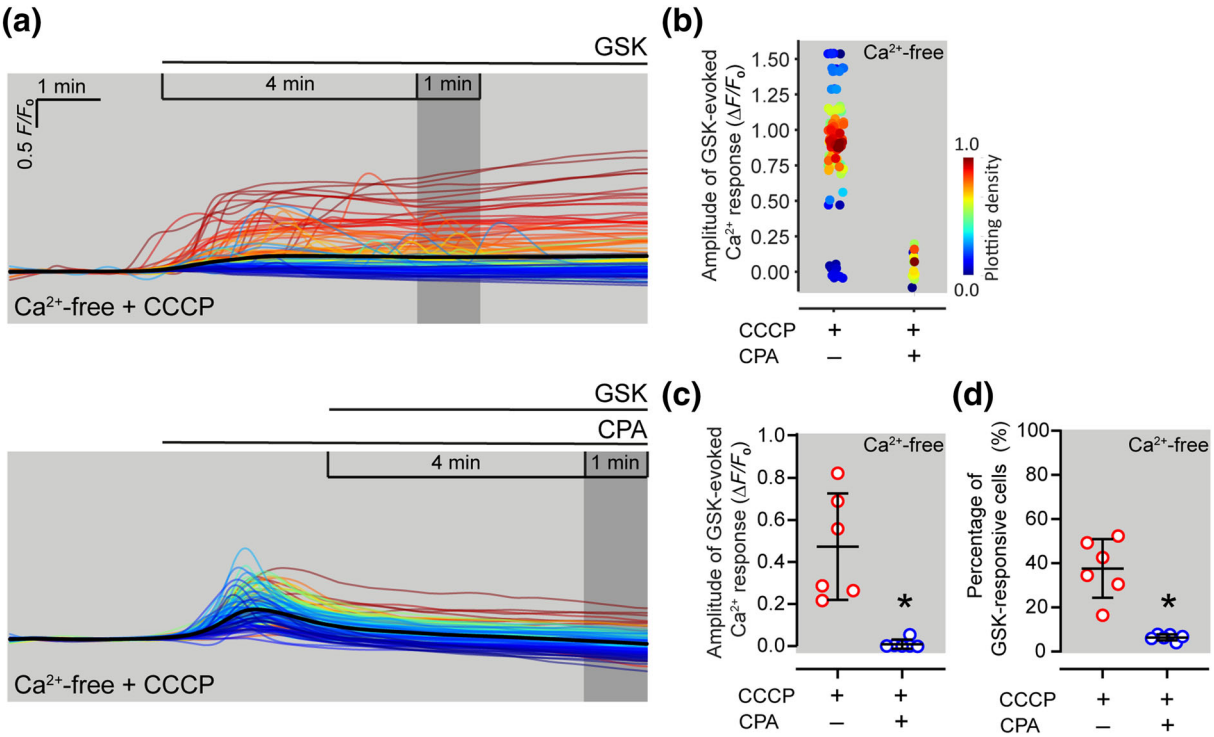

FIGURE 5 Depletion of internal Ca^{2+} stores prevents TRPV4 Ca^{2+} release. After incubation in a Ca^{2+}-free bath and CCCP (1 μM), the endothelium was activated by GSK1016790A (20 nM). A different preparation was perfused by the sarcoendoplamic reticulum ATPase (SERCA) inhibitor, cyclopiazonic acid (CPA, 20 μM), in a Ca^{2+}-free bath solution then CCCP (1 μM). GSK (20 nM) was induced when the internal store was depleted. (a) Representative single-cell endothelial Ca^{2+} traces showing the effect of CPA on TRPV4-mediated Ca^{2+} release. (b) Single-cell Ca^{2+} measurements (mean $\Delta F/F_0$) from the data shown in (a) and (b). $\Delta F/F_0$ values are mean Ca^{2+} levels during the fifth minute following activation of TRPV4 channels with GSK. Thus, the 4-min bar above the traces indicates a time period of GSK incubation and the 1-min bar (with shading) the period of measurement. Summary data (individual values with means ± SD) showing the amplitude of Ca^{2+} signals (c) and percentage of active cells (d) in the fifth minute after GSK was induced. *P < 0.05, significant effect of CPA; unpaired t test

caffeine, the Ca^{2+} response to GSK (in CCCP) recovered (Figure 6a–d). Caffeine inhibited basal activity in the absence and presence of CCCP, consistent with the involvement of IP_3 receptors in spontaneous activity in endothelial cells (Figure S1). Taken together, our results showed that TRPV4 activation, following mitochondrial depolarisation, evoked IP_3 receptor-mediated Ca^{2+} release from the ER.

PLC, an important component of the IP_3 signalling system, converts phosphatidylinositol 4,5-bisphosphate (**PIP_2**) to IP_3 and diacyl glycerol (Essen et al., 1997; Kadamur & 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 (2 μM; 15 min), 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 (Video S1). The progression of the wave appears to be consistent with a self-propagating event (Video S1). This type of wave has been shown to occur in other tissues via a propagated release of ATP (Anselmi et al., 2008; Daneva et al., 2021; Isakson & Thompson, 2014). To examine the role of purinergic transmission in TRPV4-mediated Ca^{2+} release following mitochondrial depolarisation (in the presence of CCCP), the effects of the ATPase, apyrase (5 $U \cdot ml^{-1}$; 30 min), the purinergic receptor blocker, **suramin** (100 μM, 10 min), 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). Pannexin channels do not appear to contribute to the TRPV4-mediated Ca^{2+} response under normal physiological conditions (i.e., when mitochondria are polarised; Figure S2). 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.

4 | 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

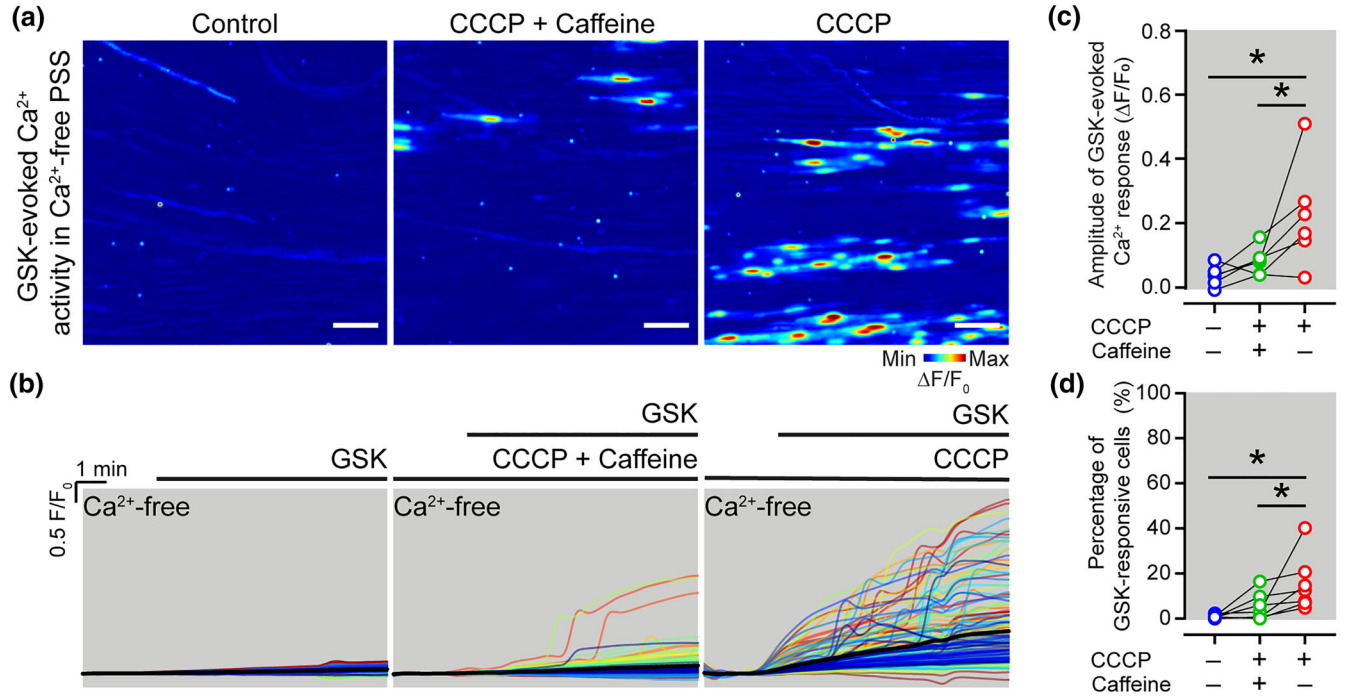

FIGURE 6 IP_3 receptor inhibition prevents TRPV4-mediated Ca^{2+}release. Pseudocoloured $\Delta F/F_0$ maximum intensity projections (a) and corresponding single-cell Ca^{2+} traces (b) showing the effect of the IP_3 receptor inhibitor, caffeine (10 mM), on endothelial cell Ca^{2+} release evoked by 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 IP_3 receptor blockade on mitochondrial-restrained TRPV4-mediated endothelial Ca^{2+} responses. *$P < 0.05$, significantly different as indicated; one-way ANOVA with paired multiple comparisons

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 & Garvin, 2008). For example, in human pulmonary fibroblasts, TRPV4 activation resulted in ATP being released 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 & 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 **connexins**) are widely reported to be a principal conduit for ATP release in a wide range of cell types (Dahl & Locovei, 2006; D'hondt et al., 2011; Iglesias et al., 2009; Locovei et al., 2006; MacVicar & Thompson, 2010) including endothelial cells (Good et al., 2021; Maier-Begandt et al., 2021). The present findings show that 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 blocker of IP_3 receptors, 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

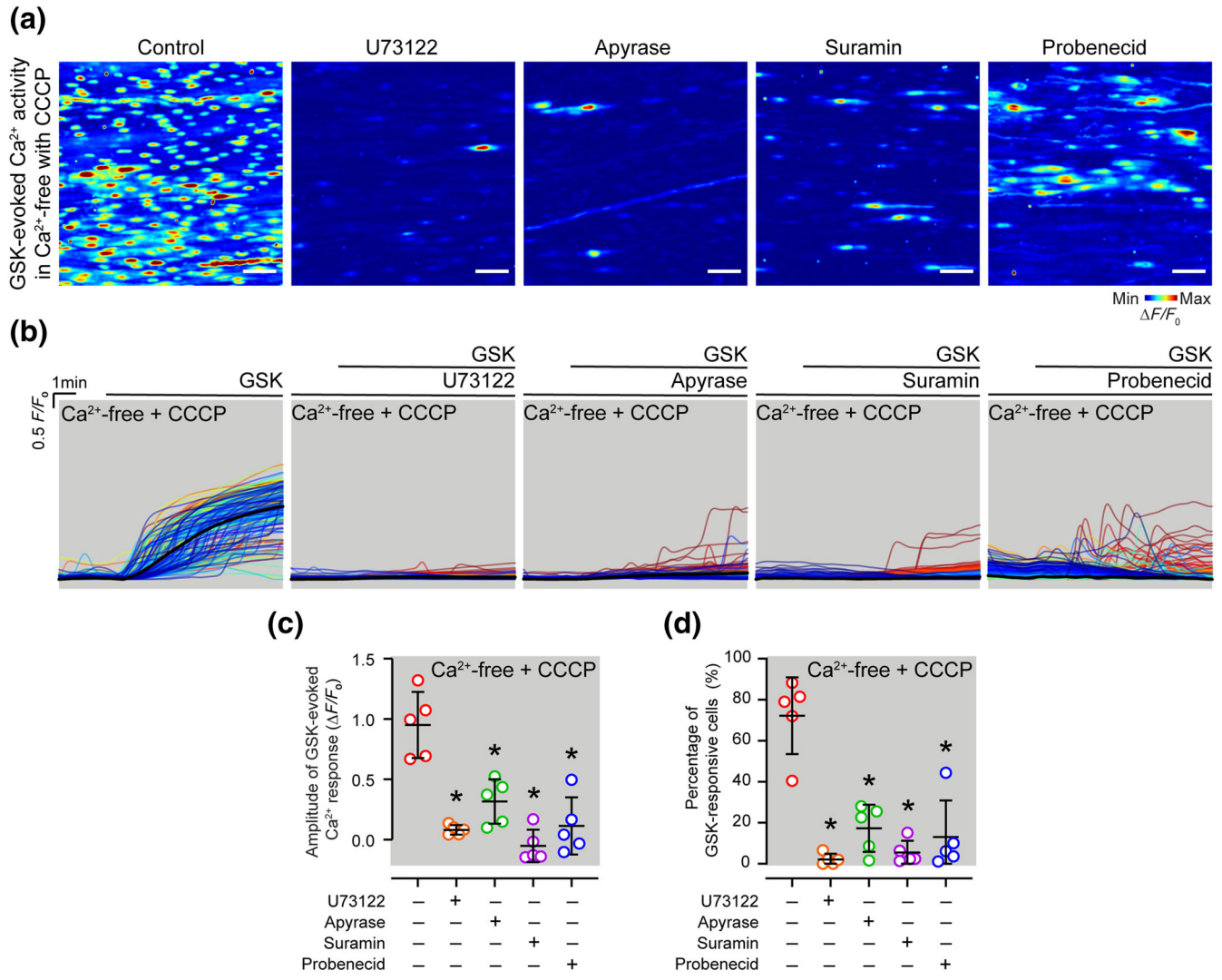

FIGURE 7 TRPV4 channels trigger endothelial cell Ca^{2+} release via pannexin-mediated release of ATP. Pseudocoloured $\Delta F/F_0$ maximum intensity projections (a) and corresponding single-cell Ca^{2+} traces (b) showing TRPV4-mediated (GSK1016790A, GSK, 20 nM) Ca^{2+} release in the absence or presence of the PLC inhibitor, U73122 (2 μM), the ATPase, apyrase (5 $U \cdot ml^{-1}$), 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 (individual values with means ± SD) showing the effect of the various pharmacological agents on the amplitude on mitochondrial-restrained TRPV4-mediated endothelial Ca^{2+} responses. *$P < 0.05$, significantly different from GSK alone; one-way ANOVA with paired multiple comparisons

external solution. Ca^{2+} influx therefore cannot provide the pannexin activation mechanism. Pannexin gating is regulated by membrane potential (Ma et al., 2009, 2012; Marrelli et al., 2007), and the open probability of single pannexin channels 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 sensitise pannexins to membrane potential changes. TRPV4 generates an inward (Na^+ and Ca^{2+}) 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; that is, the wave appears to be responsible for its own propagation (Video S1). 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 sensitise 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 because mitochondrial ATP synthesis is prevented by depolarisation of the organelle. 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_3 receptors to generate a feedback effect of Ca^{2+} on the activity of these receptors (Decuypere et al., 2011; Olson et al., 2010). Mitochondria also evoke long-distance control of IP_3 receptor activity mediated by ATP production (Carafoli, 2003; Csordás et al., 2006; Rizzuto et al., 1993; Szabadkai et al., 2006; Wilson et al., 2019). In each case, depolarisation of the mitochondrial membrane potential is a critical step in the control of IP_3-evoked Ca^{2+} signalling (Abramov & Duchen, 2008; Chikando et al., 2013; Storey & 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_3 receptor-mediated Ca^{2+} release is disabled and Ca^{2+} released is suppressed in endothelial cells and smooth muscle cells (Chalmers & McCarron, 2009; Olson et al., 2010; Wilson et al., 2019). This mitochondrial control of Ca^{2+} release is exerted at IP_3 receptors 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 (Chalmers & McCarron, 2008, 2009; Decuypere et al., 2011; McCarron & Muir, 1999; Olson et al., 2010; Wilson et al., 2019). These previous reports emphasise the unexpected nature of the present findings. Depolarisation of mitochondria normally inhibits Ca^{2+} release — but 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 IP_3 receptors. 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_3 receptors.

Pannexins form plasma membrane channels that are closed under physiological conditions. Pannexins may open during pathological conditions (e.g., ischaemia and hypoxia) to release signalling molecules (Pelegrin & 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 activation of IP_3 receptors, 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 (approximately −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.

Although TRPV4 is one of the most studied Ca^{2+} influx pathways in the intact endothelium, the physiological roles of endothelial TRPV4 channels are not fully understood. Early studies proposed a mechanosensory role of endothelial TRPV4 channels, however, more recently, these channels have been shown not to be direct mechanosensors (Nikolaev et al., 2019). Although global $TRPV4^{-/-}$ mice showed unaltered resting BP (Hong et al., 2018; Zhang et al., 2009), a tamoxifen-inducible, endothelium-specific $TRPV4^{-/-}$ had higher resting BP (Ottolini et al., 2020). The latter finding raises the possibility of endothelial TRPV4 being important in BP regulation. TRPV4 may also contribute to pathological responses. Although the physiological roles of pulmonary endothelial TRPV4 channels are unresolved and global $TRPV4^{-/-}$ mice showed unaltered mean pulmonary arterial pressure (Xia et al., 2013), detrimental effects of excessive TRPV4 channel activity in pulmonary endothelium are acknowledged (Alvarez et al., 2006; Suresh et al., 2015; Thorneloe et al., 2012). Excessive endothelial TRPV4 channel activity may also contribute to the hyperinflammatory response and mortality that accompanies sepsis that is mediated in part by Ca^{2+} signalling to induce endothelium-dependent vasodilation (Dalsgaard et al., 2016). On the other hand, reduced endothelial microvascular TRPV4 activity may contribute to diabetic retinopathy (Monaghan et al., 2015). These studies create a complicated picture of the role of endothelial TRPV4 in control of endothelial function and the dysfunction of the channel accompanying disease. Our results suggest that when the mitochondrial membrane potential is depolarised, the Ca^{2+} signals arising from the TRPV4 pathway will be reconfigured from independent responses in cell to coordinated multicellular Ca^{2+} wave that will generate a propagated tissue response. Because these signals occur when the mitochondrial membrane potential is compromised, it is tempting to speculate that the response may form a component of a pathological response.

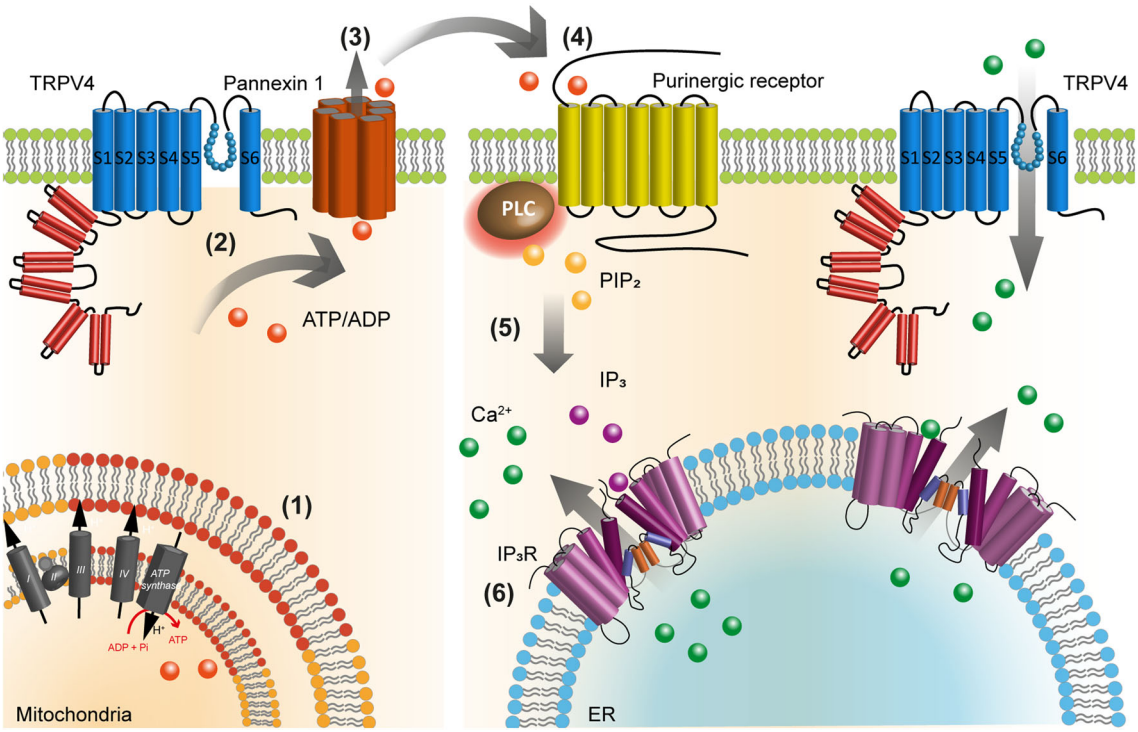

FIGURE 8 Diagram of mitochondrial control of TRPV4-mediated Ca^{2+} signalling. Ca^{2+} influx via TRPV4 channels normally triggers CICR at IP_3 receptors (IP_3R) (right side of diagram). However, when mitochondrial membrane potential is compromised (1), the activation of TRPV4 channels (2) triggers the release of ATP through pannexin hemichannels (3). ATP subsequently activates plasma membrane-bound purinergic receptors (4). The activation of purinergic receptors (P2Y) stimulates PLC to generate IP_3 (5), which subsequently activates IP_3 receptors to evoke Ca^{2+} release from the endoplasmic reticulum (ER) (6). PIP_2, phosphatidylinositol 4,5-bisphosphate

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.

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AUTHOR CONTRIBUTIONS

X.Z. and J.G.M. developed the concept. X.Z. performed the experiments. X.Z. analysed the data. X.Z., M.D.L., C.B., C.W. and J.G.M. interpreted the data. X.Z. and J.G.M. drafted the manuscript. X.Z., M.D.L., C.B., C.W. and J.G.M. edited the manuscript. C.W., C.B. and J.G.M. sourced the funding. All authors approved the final version of the manuscript.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis and Animal Experimentation and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

DATA AVAILABILITY STATEMENT

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

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