Biochemistry and Biophysics Reports 20 (2019) 100672

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

Differential modulation of transendothelial electrical resistance by TRPV4 agonists is mediated by apoptosis and/or necrosis

N. Pairet[c,d,*], S. Mang[a,b], T. Kiechle[a], N. Laufhäger[a], P. Dietl[c], D.J. Lamb[a]

[a] Immunology & Respiratory Diseases Research, Boehringer Ingelheim Pharma GmbH & Co. KG, D-88397, Biberach an der Riß, Germany
[b] Institute of Immunology, Hannover Medical School, D-30625, Hannover, Germany
[c] Institute of General Physiology, University of Ulm, Ulm, Germany
[d] Drug Discovery Sciences, Boehringer Ingelheim Pharma GmbH & Co. KG, D-88397, Biberach an der Riß, Germany

A B S T R A C T

Transient receptor potential vanilloid 4 (TRPV4) has been implicated in many disease conditions also in the lung. Its activation leads to an increase endothelial permeability in an intracellular calcium-influx dependent manner. We investigated its function *in vitro* on primary human endothelial cells using two TRPV4 agonists, GSK1016790A and 4α-Phorbol 12,13-didecanoate (4α-PDD) and a selective TRPV4 blocker GSK2193874. Both TRPV4 agonists leaded to a reduction in transendothelial electrical resistance (TER) which was mediated however by differential cytotoxic effects. 4α-PDD induced apoptosis that could not be blocked by TRPV4 inhibition in HUVECs, whereas GSK1016790A selectively activated TRPV4 and reduced TER as a consequence of cellular necrosis. TRPV4 mediated cytotoxicity is poorly described and may provide significant context to the role of TRPV4 in barrier-function.

1. Introduction

Transient receptor potential vanilloid 4 (TRPV4) is a voltage-gated calcium permeable channel that belongs to the Transient Receptor Potential (TRP) superfamily of cation channels [1]. It is expressed in numerous cell types including endothelial cells [2] and is activated by a variety of chemical and physical stimuli such as mechanical stress [3–5]. Previously it has been implicated in many disease conditions, including the pulmonary disorders chronic obstructive pulmonary disease (COPD), acute lung injury, acute respiratory distress syndrome (ARDS) and pulmonary edema formation [6,7]. TRPV4 regulates vascular permeability [8] and its activation, whether via physical stimuli such as mechanical ventilation, pulmonary venous hypertension or with pharmacological tools leads to an increase endothelial permeability in an intracellular calcium-influx dependent manner [9,10]. TRPV4 regulates the integrity of the alveolar barrier and its activation has been showed to causes endothelial detachment from the basement membrane, leading to disruption of the pulmonary endothelial barrier, resulting in pulmonary edema formation and alveolar flooding [10,11]. These properties make TRPV4 an exciting target in disease research.

Many of these functional observations have been obtained using small molecule TRPV4 agonists in both cellular and animal models. However, we questioned the link between pharmacological activation of the channel by agonists and the corresponding functional changes in barrier function after we observed different Ca^{2+} influx profiles with two different TRPV4 agonists resulting in the same functional changes within the cell, particularly when there is no affirmed signal transduction pathway that can be followed to substantiate such a link. For better understanding of TRPV4 biology and its role in regulating the endothelial membrane integrity, we investigated its activation and inhibition in an *in vitro* model on Human umbilical vein endothelial cells (HUVECs) using two reported selective activators of TRPV4, GSK1016790A and 4α-Phorbol 12,13-didecanoate (4α-PDD) [8,12] and an orally active, potent and selective TRPV4 blocker GSK2193874 that has been promoted as an excellent tool for further understanding of TRPV4 biology *in vitro* and *in vivo* [6].

2. Methods

2.1. TER measurement

The CellZscope Automated Cell Monitoring System (nanoAnalytics GmbH; Münster, Germany) was used for continuous measurement of transepithelial/transendothelial electrical resistance (TER). Human umbilical vein endothelial cells (HUVECs, EndoGRO™, SCCE001, Merck Millipore, USA) were seeded at a density of 3×10^4 cells per transwell filters (Corning #3470; 0.4 µM Poren; Polystyrene; 24 wp) in 100 µl EndoGRO-LS Complete Culture Media Kit (SCME001, Millipore, Billerica, MA, USA) and incubated at 37 °C in humidified air for 24 h and then in humidified air for another 24 h in an Invivo$_2$ 300 Hypoxia Chamber (Ruskinn Technology, Pencoed, UK) at 1% O_2, 5% CO_2 at 37 °C. Afterwards cells on transwell filters were transferred in the

* Corresponding author. Drug Discovery Sciences, Boehringer Ingelheim Pharma GmbH & Co. KG, Birkendorfer Straße 65, D-88397, Biberach an der Riß, Germany.
E-mail address: nicolas.pairet@boehringer-ingelheim.com (N. Pairet).

https://doi.org/10.1016/j.bbrep.2019.100672
Received 25 August 2018; Received in revised form 15 February 2019; Accepted 30 July 2019
Available online 19 September 2019

machine and another 160 µl of medium was given on the apical side of the transwell filters. To maintain optimal culture conditions, the CellZscope was placed in a tissue culture incubator (37 °C, 5% CO_2) and TER measurement was initiated. Cells were preincubated in the presence or absence of different concentrations of the TRPV4 antagonist GSK2193874 for 1 h or more and afterwards different concentrations of the TRPV4 agonists 4α-PDD or GSK1016790A were added from a 10-fold concentrate in medium on the apical side of the transwell filters and TER was measured continually for up to 15 h. It is possible that this procedure induced a degree of cellular stress with cell manipulations being performed under hypoxia and the experiment being performed under normoxia. However, we achieved similar results when cells were cultured and treated entirely under normoxic conditions, albeit with a lower assay window.

For supplement part: TER measurement, without preincubation at hypoxia, was also performed in small airway epithelial cells (SAECs) differentiated on transwells and cultured in air-liquid interface (ALI). Small airway epithelial cells (ATCC #PCS-301-010 Lot: 61913333) were cultured and differentiated following the Lonza Clonetics™ S-ALI^{TM} air-liquid interface medium protocol. Briefly SAECs were seeded into cell culture flask (T175 NUNC flask, 178883, Thermo Fischer) on day - 8 in Clonetics S-ALI growth medium. On day - 4 cells were trypsinised and seeded with a density of 22×10^3 cells/well on Corning Transwell filters (Corning #3470; 0.4 µm Poren; Polystyrene; 24 wp). On day 0 airlift of the cells was performed by removing the apical medium and substituting the basolateral growth medium with S-ALI Differentiation Medium (Clonetics S-ALI differentiation medium). On the apical side cells were washed to remove growth factors. SAECs were then differentiated in air-liquid interface (ALI) for at least 4 weeks with basolateral medium changes 3 times a week with apical washing step ones a week. Afterwards ALI cultures on transwell filters were placed in the cellZscope and medium was added basolaterally and apically to enable impedance measurement as previously described. The cellZscope was placed in an incubator at 37 °C, 5% CO_2 in humidified air and TER measurement was initiated. Cells were preincubated in presence or absence of different concentrations of the TRPV4 antagonist GSK2193874 for 1 h or more and afterwards were treated with the TRPV4 agonist GSK1016790A and TER measurement was performed continuously for up to 24 h.

2.2. Calcium 6 assay on the FLIPR^{TETRA}

Pharmacological activation and inhibition of TRPV4 was analyzed using the FLIPR Calcium 6 Assay kit (molecular devices #R8191 bulk kit) and was performed according to the manufacturer's instructions. Briefly HUVECs were seeded at 1×10^4 cells/well (SCME001, Millipore, Billerica, MA, USA) on assay plates (384 well Poly-D-Lysin black/clear bottom, Biocoat #4663) and incubated for 24 h. Cells were incubated for 2 h with the calcium 6 dye (in assay buffer (HBSS [+ $CaCl_2$/$MgCl_2$] + 20 mM Hepes + 0.1% BSA; pH 7,4) at 37 °C in 5% CO_2, humidified air. Compounds were preincubated for 15 min or 1 h before stimulation (FLIPR^{TETRA}, Molecular Devices, excitation 470–495 nm, emission 515–575 nm) and the concentration-dependent inhibition or activation of calcium influx was determined.

2.3. TRPV4 agonism effect on LDH release

Cells were seeded (25×10^3 cells/well for HUVECs) in appropriate medium on 96 well culture plates (NunclonTM Delta Surface, Thermo scientific) and incubated for 24 h. Afterwards cells were preincubated for 1 h in the presence or absence of the TRPV4 antagonist GSK2193874 in 100 µL medium. Medium was removed one more time and cells were incubated at 37 °C in 5% CO_2, humidified air for up to 12 h in 100 µL medium in presence or absence of different concentrations of the TRPV4 agonist GSK1016790A or 4α-Phorbol 12, 13-didecanoate (4α-PDD). Then supernatant was collected at different time points and lactate dehydrogenase (LDH) release was detected using a CytoTox96® Non-Radioactive Cytotoxicity Assay kit (Promega, Madison, WI) following manufacturers instruction and using a SpectrMax M5 plate reader (Molecular Devices, Sunnyvale, CA). Percent cytotoxicity is calculated by the following formula: 100 x Experimental LDH release divided by the maximum LDH release (= lysate).

Experiments were also performed with Hank's Balanced Salt Solution (HBSS, Gibco, Life technologies, Grand Island, NY) in the presence or absence of $CaCl_2$ and $MgCl_2$.

2.4. RealTime-Glo™ annexin V Apoptosis and Necrosis Assay

The RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay (Promega, Madison, USA) is a live-cell real-time assay that measures the exposure of phosphatidylserine (PS) on the outer leaflet of the cell membrane during the apoptotic process and is detected by annexin V binding with a simple luminescence signal. The assay also includes a cell-impermeant, profluorescent DNA dye, which detects necrosis. In the assay, time-dependent increases in luminescence that occur before increases in fluorescence reflect the apoptotic process. A significant time delay between the emergences of PS, indicated by Annexin V binding, leading to a luminescence signal and the loss of membrane integrity visualized by fluorescence signal, indicate an apoptotic phenotype leading to secondary necrosis. Increases in fluorescence or increase in both luminescence and fluorescence concurrently consist with necrosis or other non-apoptotic mechanisms.

The RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay were performed as prescribed by the manufacturer. Briefly HUVECs were seeded with a density of 25×10^3 cells/well on 96 well white plates (96F Nunclon™ Delta White Microwell SI, Thermo Fisher Scientific, Roskilde, Denmark) in 50 µl medium (SCME001, Millipore, Billerica, MA, USA) and incubated at 37 °C in 5% CO_2, humidified air for 24 h. Afterwards cells were preincubated for 1 h in the presence or absence of the TRPV4 antagonist GSK2193874. Cells were then treated with different concentrations of the TRPV4 agonists GSK1016790A or 4α-PDD and directly equal volume of detection reagent was added, the plate was covered with an imaging seal (4titude 4ti-0516/96, LabSource, Switzerland) and a kinetic mode (1 read every 30 s for up to 20 h) using a multimode instrument with temperature control was initiated for assay signal detection.

2.5. Cell-IQ®

HUVECs were seeded in medium (SCME001, Millipore, Billerica, MA, USA) on 96 well culture plates (NunclonTM Delta Surface, Thermo scientific) and incubated at 37 °C in 5% CO_2, humidified air for 24 h. Afterwards cells were preincubated for 1 h in the presence or absence of the TRPV4 antagonist GSK2193874. Cells were then incubated at 37 °C in 5% CO_2, humidified air for up to 4 h in 100 µL medium in presence or absence of different concentrations of the TRPV4 agonist GSK1016790A and live cell imaging was recorded in a Cell-IQ® (Chip-Man Technologies, Tampere, Finland).

2.6. Calculations & statistics

For statistical analyses Graph Pad Prism Software for Windows version 7 was used. Significance levels are shown as * $p \leq 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ or "ns" for not significant ($p > 0.05$); ANOVAs were corrected for multiple comparisons with a Tuckey correction.

2.7. Ethics statement

Human cells sourced from commercial vendors were verified to have associated, signed informed consents in place. This process was endorsed by a panel of senior company scientists and physicians.

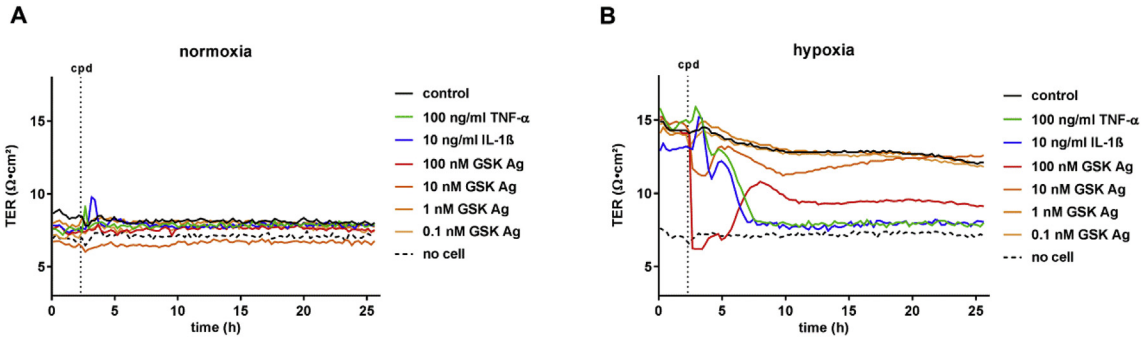

Fig. 1. Effect of hypoxia on TER in HUVECs. (A) TER measurement in the cellZscope before and after addition of different concentrations of the TRPV4 agonist GSK1016790A (Ag), TNF-α or IL-1ß in cells preincubated at normoxia. (B) TER measurement after addition of different concentrations of the TRPV4 agonist GSK1016790A (Ag), TNF-α or IL-1β in cells preincubated for 24 h at hypoxia (1% O_2, 5% CO_2 at 37 °C) prior to TER measurement. Grid lines labelled with cpd showing time points of compound addition in the graphs.

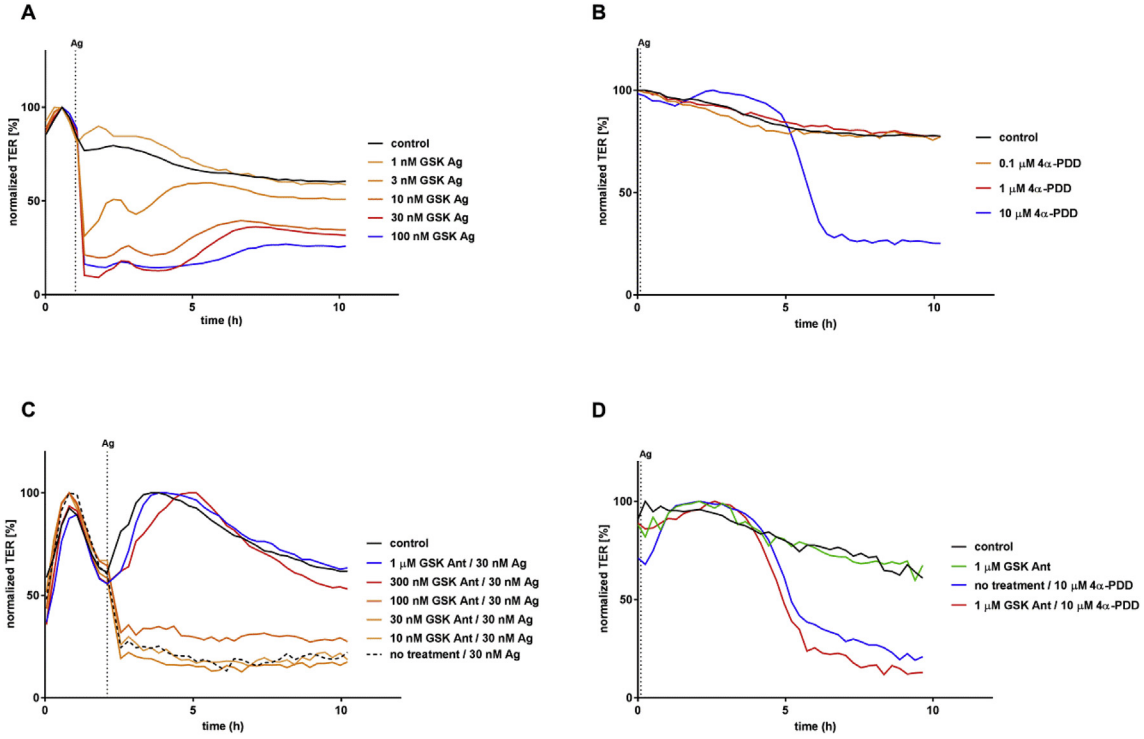

Fig. 2. Effect of TRPV4 agonism on TER in HUVECs. (A) TER measurement in the cellZscope before and after addition of different concentrations of the TRPV4 agonist (Ag) GSK1016790A. (B) TER measurement after addition of different concentrations of the TRPV4 agonist (Ag) 4α-PDD. (C) TER measurement with cells preincubated for 1 h in presence or absence of different concentrations of the TRPV4 antagonist GSK2193874 (Ant) and treated afterwards with the TRPV4 agonist (Ag) GSK1016790A (30 nM). (D) TER measurement with cells preincubated in presence or absence of the TRPV4 antagonist GSK2193874 (Ant) and treated afterwards with the TRPV4 agonist 4α-PDD (10 μM). Grid lines labelled with Ag showing time points of TRPV4 agonist addition in the graphs. Data are shown as mean only n = 3–5 performed statistical test: ordinary one-way ANOVA * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$.

3. Results

3.1. TER measurement in HUVECs

Transient receptor potential vanilloid 4 (TRPV4) has been suggested to be a critical regulator of endothelial barrier integrity. Pharmacological activation of TRPV4 was therefore studied in a cellZscope allowing continuous measurement of transepithelial/transendothelial electrical resistance (TER). During method establishment a TER improvement was observed when endothelial cells were cultured for 24 h under the more physiological hypoxic conditions (1% O_2, 5% CO_2 at 37 °C) prior to TER measurement. Under these conditions it was possible to establish a model with an assay window high enough to test compound effect on TER in HUVECs. An initial TER improvement was observed when cells were cultured 24 h under hypoxic conditions (Fig. 1, B) compared to cells cultured under normoxia (Fig. 1, A). With cells cultured under hypoxia the assay window was improved and compound effect on TER could be investigated. A dose-dependent decrease in TER was observed after addition of the TRPV4 agonist GSK1016790A. Interestingly, the cytokines TNF-α [100 ng/ml] and IL-1β [10 ng/ml] also induced a reduction in TER after compound addition.

3.2. Effect of TRPV4 agonism on TER

To investigate the effect of TRPV4 on the vascular-barrier integrity, HUVECs were seeded on transwell filters for TER measurement. Addition of GSK1016790A resulted in a concentration-dependent

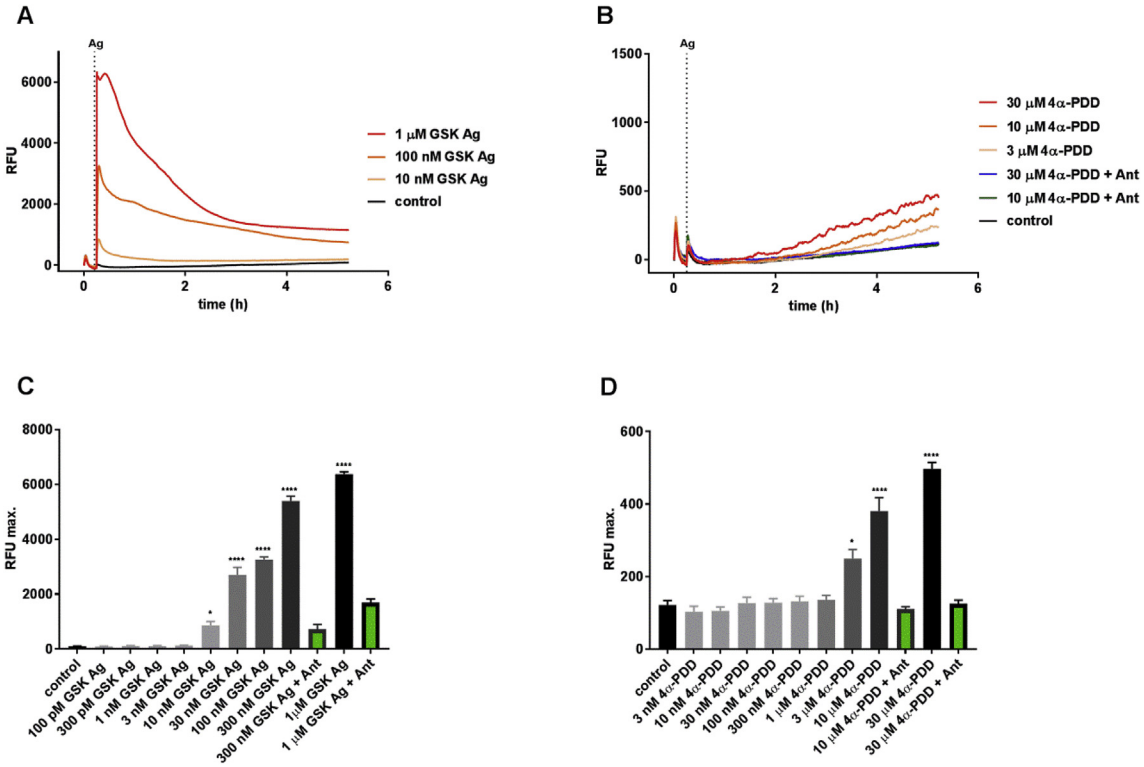

Fig. 3. TRPV4 mediated Calcium influx with FLIPR Calcium 6 Assay in HUVECs. (A) Calcium influx measured in the FLIPR^{TETRA} before and after addition of different concentrations of the TRPV4 agonist (Ag) GSK1016790A. (B) Calcium influx measurement after addition of different concentrations of the TRPV4 agonist (Ag) 4α-PDD. (C) Calcium influx maxima in cells preincubated for 15 min in presence or absence of the TRPV4 antagonist GSK2193874 (Ant, 1 μM) and treated afterwards with different concentrations of the TRPV4 agonist (Ag) GSK1016790A. (D) Calcium influx in cells preincubated for 15 min in presence or absence of the TRPV4 antagonist GSK2193874 (Ant, 1 μM) and treated afterwards with different concentrations of the TRPV4 agonist 4α-PDD. Data are shown as mean ± SEM; (n = 3; *p < 0.05; ****p < 0.0001 one-way ANOVA Tukey's multiple comparisons test). Grid lines labelled with Ag showing time points of TRPV4 agonist addition in the graphs.

decrease in TER and the effect occurred directly after agonist addition (Fig. 2, A). In contrast, when endothelial cells were stimulated with 4α-Phorbol 12,13-didecanoate (4α-PDD), the TER decrease began only about 5 h after agonist addition and only with a high concentration of agonist (10 μM; Fig. 2, B). One hour preincubation with different doses of the TRPV4 antagonist GSK2193874 resulted in a concentration-dependent inhibition of GSK1016790A agonism effect on TER (Fig. 2, C) but in contrast was not able to inhibit the effect of 4α-PDD (Fig. 2, D).

3.3. TRPV4 mediated calcium influx

TRPV4 activation with GSK1016790A resulted in a direct and strong increase in intracellular calcium concentration in a dose-dependent manner (Fig. 3, A) that could be nearly completely blocked by preincubation for 15 min with 1 μM of the TRPV4 antagonist GSK2193874 (Fig. 3, C). Activation of TRPV4 with 4α-PDD led to a very small increase of intracellular calcium concentration, that occurred hours after agonist addition (Fig. 3, B) and in contrast to the effects on TER, agonism by both agonists could be blocked by inhibition with GSK2193874 (Fig. 3, D).

3.4. TRPV4 antagonist reverse the effect of TRPV4 agonism

To investigate whether the effect of TRPV4 activation can not only be prevented but also reversed by TRPV4 inhibition, HUVECs were treated firstly with the TRPV4 agonist GSK1016790A and afterwards with the TRPV4 antagonist GSK2193874 in TER and intracellular calcium concentration measurement. HUVECs that were first stimulated with 15 nM of the TRPV4 agonist GSK1016790A and that were subsequently left untreated for 30 min after agonist exposure, showed a drop in TER that remained until the end of the experiment. In contrast HUVECs treated within the first 30 min of agonism with 1 μM of the TRPV4 antagonist GSK2193874 showed a recovery in TER to that of the control group treated only with 0.1% DMSO (Figs. S1 and A) similar result was observed in the intracellular calcium influx measurement (Figs. S1 and B). In the calcium measurement a too low concentration of the TRPV4 agonist GSK1016790A resulted in a assay window not high enough to be reversed and the effect of a too high concentration of GSK1016790A could not be reversed by TRPV4 antagonism with GSK2193874 (Figs. S1 and C).

3.5. TRPV4 mediated cytotoxicity

To investigate whether cell viability is impacted by TRPV4 activation, HUVECs were exposed to different concentrations of TRPV4 agonists with or without preincubation with 1 μM of the TRPV4 antagonist GSK2193874 for 1 h. Cells exposed up to 3 μM 4α-PDD showed no significant LDH release compared to the control groups. Cells exposed to 10 μM 4α-PDD showed no increase in cytotoxicity after 3 h but cytotoxicity began to increase after 8 h and reached a maximum after 12 h. This effect could not be blocked when cells were preincubated with the TRPV4 antagonist (Fig. 4A and B). In contrast activation of TRPV4 with the agonist GSK1016790A led to a rapid concentration-dependent increase in cytotoxicity (Fig. 4, C), even with low concentration of the agonist (Fig. 4, D). In contrast to 4α-PDD, the effect of TRPV4 activation with GSK1016790A could completely be blocked by preincubation with the TRPV4 antagonist GSK2193874 (Fig. 4, E).

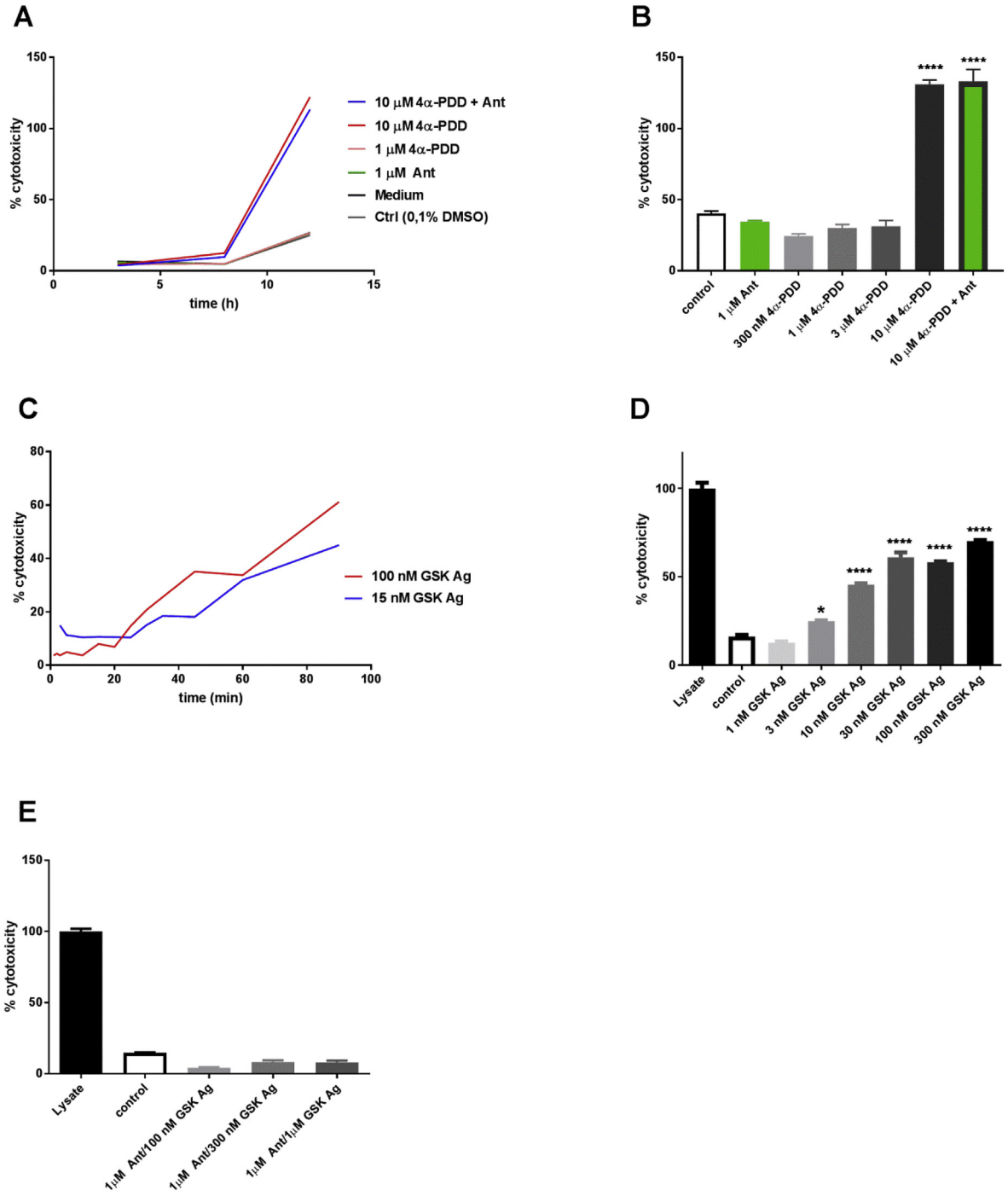

Fig. 4. TRPV4 mediated lactate dehydrogenase (LDH) release in HUVECs. (A) Kinetic of HUVECs preincubated for 1 h in the presence or absence of the TRPV4-Antagonist GSK2193874 (1 μM, Ant) and afterwards exposed to different concentration of the TRPV4 agonist 4α-PDD (time points 3, 8, and 12 h). (B) Dose-response of cells preincubated in the presence or absence of the TRPV4-Antagonist GSK2193874 (1 μM) and afterwards incubated with different concentration of the TRPV4 agonist 4α-PDD for 12 h. (C) Kinetic of cells preincubated for 1 h in the presence or absence of the TRPV4-Antagonist GSK2193874 (1 μM) and afterwards exposed to the TRPV4 agonist GSK1016790A (15 or 100 nM) for up to 90 min. (D) Dose-response of cells incubated with different concentration of the TRPV4 agonist GSK1016790A for 4 h. (E) Cells preincubated in the presence or absence of the TRPV4-Antagonist GSK2193874 (1 μM) and afterwards incubated with different concentration of the TRPV4 agonist GSK1016790A for 3.5 h. Data are shown as mean only or mean ± SEM; (n = 6; *p < 0.05; ****p < 0.0001 one-way ANOVA Tukey's multiple comparisons test).

3.6. Dependence on calcium for TRPV4 induced LDH release

After having shown that the TRPV4 agonist GSK1016790A induces a large increase in intracellular calcium concentration in HUVECs and also induces cytotoxicity, further investigations were made on the question whether the cytotoxic effect induced by the TRPV4 activator GSK1016790A is dependent on extracellular calcium influx. Therefor HUVECs were incubated for 1 h in absence or presence of calcium with 100 nM GSK1016790A in HBSS and LDH release was recorded. The TRPV4 agonist GSK1016790A showed, in cells incubated in HBSS with calcium, the same cytotoxic effect as in cells treated with the agonist in medium. 100 nM of the agonist induced a strong and significant

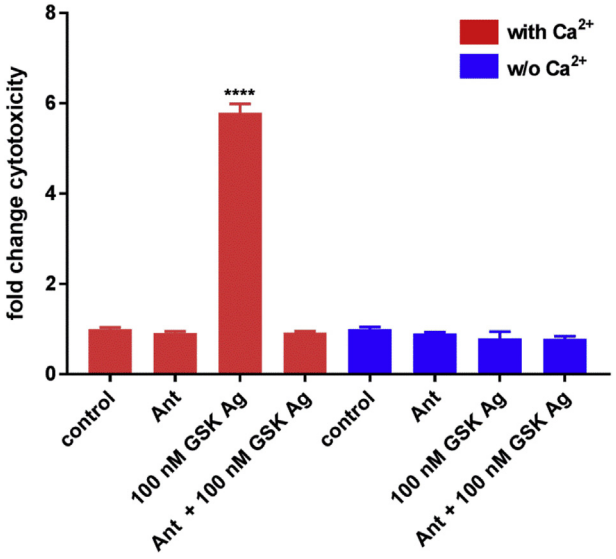

Fig. 5. Calcium dependent TRPV4 induced LDH release. Fold change cytotoxicity in HUVECs preincubated in presence or absence of the TRPV4 antagonist GSK2193874 (Ant, 1 μM) and afterwards exposed to 100 nM of the TRPV4 agonist GSK1016790A in HBSS with or HBSS without calcium for 1 h. Data are shown as mean ± SEM; (n = 6; ****p < 0.0001 one-way ANOVA Tukey's multiple comparisons test).

increase in LDH release, that could be blocked by preincubation with the TRPV4 antagonist. In contrast the TRPV4 agonist GSK1016790A [100 nM] caused no cytotoxic effect compared to control, when incubated in HBSS without calcium (Fig. 5).

3.7. TRPV4 activation in the RealTime-Glo™ annexin V Apoptosis and Necrosis Assay

To investigate whether the TRPV4 induced cytotoxicity is an apoptotic or necrotic process, HUVECs were exposed to different concentrations of the TRPV4 agonists GSK1016790A and 4α-PDD after being preincubated in presence or absence of the TRPV4 antagonist GSK2193874 (1 μM). The TRPV4 agonist GSK1016790A showed a direct, strong and concentration-dependent increase in the fluorescence signal from the DNA-intercalating dye, similar to the necrosis inducer Digitonin, that could be significantly blocked when pretreated with the TRPV4 antagonist GSK2193874 (Fig. 6A and B). In contrast the agonist 4α-PDD (10 μM) leads to an increase in the fluorescence signal that began only after 8 h and reached his maximum signal after more than 15 h and the agonist effect could not be blocked by the TRPV4 antagonist GSK2193874 (Fig. 6A and B).

Luminescence detection of phosphatidylserine (PS) on the outer leaflet of the cell was not increased by the TRPV4 agonist GSK1016790A compared with the control group. The agonist 4α-PDD (10 μM) in contrast showed an increase in the luminescence signal beginning at 5 h and reaching is maximum signal at 8 h. The effect of 4α-PDD could not be blocked by pretreatment with the TRPV4 antagonist GSK2193874 (Fig. 6C and D).

The necrotic signal observed by TRPV4 agonism with GSK1016790A was also captured using live cell imaging (Fig. S2). Cells were observed to first swell and then burst, an effect also blocked by antagonism with GSK2193874.

4. Discussion

To investigate TRPV4 biology and its role in regulating the endothelial membrane integrity, we used two reported selective activators of TRPV4, GSK1016790A and 4α-Phorbol 12,13-didecanoate (4α-PDD) [8,12] and a potent and selective TRPV4 blocker GSK2193874[6]. We use a primary human cell to avoid any potential artefacts that may be attributable to a cancer cell line. HUVECs were initially selected because of the reported TRPV4 mediated effects on vascular leakage [9,11].

During method establishment we found that HUVECs cultured under normoxia exhibited relatively low baseline TER levels, but under the physiologically-relevant hypoxia for these cells (oxygen tension in the umbilical cord is reported to be approximately 25 mmHg compared to normal adult arterial values of > 80 mmHg) the TER level increased to a level suitable to test these compounds. It is reported that hypoxia can induce a large variety of biological active agents in endothelial cells, e.g. vascular endothelial growth factor (VEGF) [13], that has been further implicated to mediate loss of transepithelial resistance (TER) [14]. Furthermore Hypoxia-induced hyperpermeability of rat glomerular endothelial cells has been reported, involving hypoxia-inducible factor-2α (HIF-2α), that mediated changes in the expression of occludin and ZO-1 inducing permeability [15].

However, in our model hypoxia improved initial TER in HUVECs and we concluded that this assay procedure (1% O_2, 5% CO_2 at 37 °C) represented more physiological conditions for Human umbilical vein endothelial cells rather than hypoxic conditions, leading to an improvement of initial TER, that remained constant during electrical resistance measurement in the cellZscope. A thought shared in the literature, evaluating the consequences of physioxia on cells and importantly emphasizing the discrepancy between *in vivo* and *in vitro* tissue and cells oxygen status, which can have significant impact on experimental outcome. Pointing out, that the values corresponding to the physioxia of different tissues are ranging between 11% and 1% O_2, whereas current *in vitro* experimentations are usually performed in 19.95% O_2, an artificial context as far as oxygen balance is concerned, concluding that most of the experiments performed in so-called normoxia might be misleading [16]. In this review the pO_2 in umbilical vein blood, that is in direct contact to Human umbilical vein endothelial cells, is also mentioned to be normally between 20 and 30 mmHg [16,17], indicating that 1% O_2 should represent more physiological levels of oxygen (physioxia) in HUVECs than the so-called normoxia (19.5% O_2) in the incubator.

TRPV4 agonism with GSK1016790A on the endothelial cell layer resulted in a strong decrease in TER in a dose-dependent manner directly after agonist addition, and could be completely abolished by the TRPV4 antagonist. In contrast, 4α-Phorbol 12,13-didecanoate (4α-PDD) reduced TER only approximately 5 h after agonism and only with a high concentration of 4α-PDD which could not be blocked by the TRPV4 antagonist. The effects on TER correlated with the intracellular calcium influx measurement over time. These data suggest that GSK1016790A induced TER reduction is mediated via TRPV4 in an intracellular calcium-influx dependent manner, whereas 4α-PDD-mediated TER reduction may be independent of TRPV4 activation, although we cannot exclude the possibility that GSK2193874 and 4α-PDD do not share a competitive binding site. Interestingly, the effect of the TRPV4 agonist GSK1016790A on TER and intracellular calcium influx could not only be blocked by preincubation with the TRPV4 antagonist but that it was also possible to reverse the TRPV4 activation on both calcium influx and TER reduction by administration of the antagonist after agonist addition within in a certain time range. These data suggest that not only GSK2193871 is capable of displacing GSK1016790A from its binding site, but that reduction in endothelial barrier permeability can be reversed if the intracellular calcium concentrations fall within a certain time frame (approximately 30 min in these experiments). However, beyond this limited time window, the loss of barrier permeability appears more permanent and that subsequent non-calcium-dependent processes are responsible.

In these studies, we used HUVECs as a cellular test system. However, we also replicated selected findings in primary human epithelial cells and cell lines (Fig. S3). Furthermore, these data are consistent with the *ex in vivo* findings in which TRPV4 in murine isolated

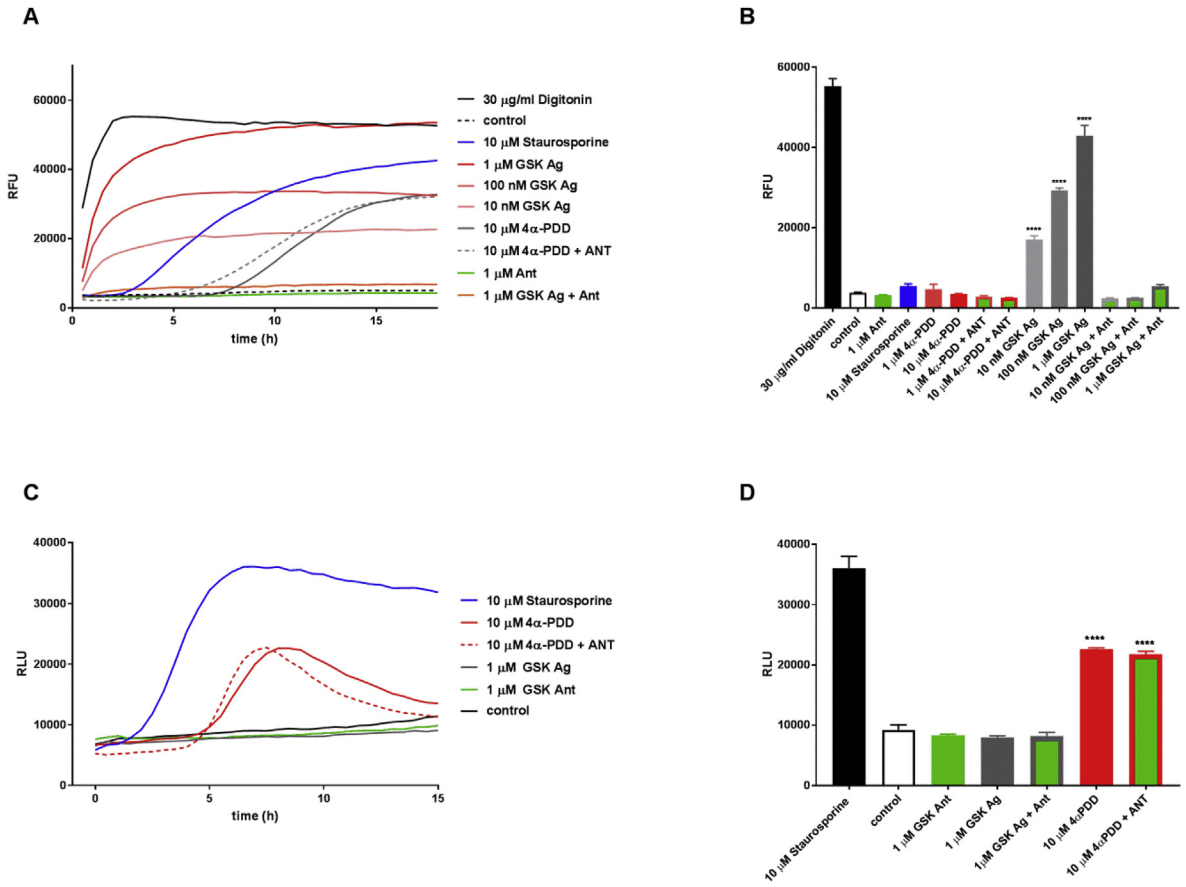

Fig. 6. TRPV4 activation in the RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay. (A) Fluorescence measurement (in relative fluorescence units RFU) over time (indicating necrosis) beginning 30 min after agonist addition in HUVECs preincubated in presence or absence of the TRPV4 antagonist GSK2193874 (Ant, 1 µM) and afterwards treated with different concentrations of the TRPV4 agonist GSK1016790A, 4α-PDD or staurosporine (Apoptosis inducer) or digitonin (necrosis inducer). (B) Fluorescence measurement in HUVECs preincubated in presence or absence of the TRPV4 antagonist GSK2193874 (Ant, 1 µM) and afterwards treated with different concentrations of the TRPV4 agonist GSK1016790A and 4α-PDD after 3 h. (C) Luminescence measurement (in relative light units RLU) over time (indicating apoptosis) beginning after agonist addition in HUVECs preincubated in presence or absence of the TRPV4 antagonist GSK2193874 (Ant, 1 µM) and afterwards treated with different concentrations of the TRPV4 agonist GSK1016790A, 4α-PDD or staurosporine as an apoptosis inducer. (D) Luminescence measurement in HUVECs preincubated in presence or absence of the TRPV4 antagonist GSK2193874 (Ant, 1 µM) and afterwards treated with different concentrations of the TRPV4 agonist GSK1016790A, 4α-PDD or staurosporine after 8 h. Data are shown as mean only or mean ± SEM; (n = 4; ****p < 0.0001 one-way ANOVA Tukey's multiple comparisons test).

lungs regulates vascular permeability and its activation, whether via physical stimuli such as mechanical stress or with pharmacological tools leads to an increase endothelial and epithelial permeability in an intracellular calcium-influx dependent manner [9–11,18]. It has also been reported that 4α-PDD activity on Ca^{2+} influx and whole-cell currents in human embryonic kidney (HEK) cells is approximately 300 fold less potent than GSK1016790A and had only a weak ability to contract bladder strips compared to GSK1016790A. Furthermore 4α-PDD has been reported to be less selective compared to GSK1016790A [19,20] that is consistent with our experimental observations. Additionally the exclusivity of 4α-PDD for TRPV4 has been put in question, by the fact that it can activates mouse DRG neurons independently of TRPV4, by the fact that it stimulated a dose-dependent increase in $[Ca^{2+}]_i$ in neurons from WT and TRPV4-KO mice, with the proportion of responding neurons and magnitude of increase unaffected by the genotype [21].

We also questioned the link between pharmacological activation of TRPV4 and the corresponding functional observations on barrier integrity when there is no affirmed signal transduction pathway that can be followed to substantiate such a link. We hypothesized that such effects may also be caused by cytotoxicity. Interestingly, we observed differential cytotoxic effects in endothelial cells induced by the two TRPV4 agonists at concentrations within the pharmacological range. HUVECs exposed to the agonist 4α-PDD (10 µM) showed a time-dependent release of lactate dehydrogenase, a cytotoxicity marker released by damaged cells, beginning after 8 h and reaching a maximum after 12 h. Similar to the TER observations, this could not be blocked with the TRPV4 antagonist GSK2193874. Necrosis was confirmed with a DNA-intercalating dye, but was preceded by an increase in phosphatidylserine on the outer leaflet of the cell membrane, indicating an apoptotic process followed by secondary necrosis, which was apparently independent of TRPV4, again suggesting a possible off-target mechanism in HUVECs. In contrast activation of TRPV4 with the agonist GSK1016790A lead to a rapid concentration-dependent increase in both LDH release and DNA dye intercalation within the first hours, even with a low concentration of the agonist that could completely be blocked with the TRPV4 antagonist. Furthermore this effect was dependent upon extracellular calcium. No cytotoxic effect occurred, when cells were incubated with GSK1016790A [100 nM] in HBSS in the absence of calcium. Live cell imaging showed that within the first few minutes after TRPV4 activation with GSK1016790A cellular swelling and blebbing occurred, followed by apparent bursting of the plasma membrane. Cellular swelling and blebbing has been reported in the literature [11], following lung exposure to TRPV4 agonists resulting in a loss of barrier function. Because of the chronological relationship between GSK1016790A-mediated increases in intracellular calcium

concentrations, the cellular swelling and cytotoxicity, we speculate that this effect maybe a consequence of rapid water entry into the cell following the rapid and high concentrations of intracellular calcium.

The physiological Ca^{2+} concentration in extracellular biologic fluids (and media mimicking these conditions) ranges from 1.6 to 2 mM, in contrast the cytosolic free Ca^{2+} concentration is kept by cells around 100 nM producing an extremely large electrochemical gradient between extracellular and intracellular Ca^{2+} concentrations, meaning that for a cell at rest the [Ca^{2+}] is ~20.000 times lower in the cytoplasm than outside the cell [22–24]. TRP channels modulate the cations flux through plasma membranes down their electrochemical gradients, thereby playing an important role in raising the free intracellular Ca^{2+} concentration [23,25]. TRPV4 has been implicated in the control of regulatory volume decrease (RVD), a regulatory response to cell swell exposed to hypotonic solution that is normally associated with changes in intracellular calcium concentration [26]. TRPV4 has been shown to provide the Ca^{2+} signal, required to activate further Ca^{2+} potassium channel and the subsequent RVD in epithelial cells and also interacts with aquaporins to control RVD in astrocytes [26–28]. This is an important observation that suggests a disruption of cell volume regulation may have crucial consequences for cell signalling, barrier integrity and cell viability [27]. Finally TRPV4–AQP4 interactions have been promoted to constitute a molecular system that fine-tunes astroglial volume regulation by integrating osmosensing, calcium signalling, and water transport and, when over-activated, triggers pathological swelling [28]. These prior finding support our speculation that pharmacological activation of TRPV4, leads to a permanent opening of TRPV4 channels in HUVECs, that we do not expect to mimic the situation in real life when activated by a physiological trigger, and led to an extreme calcium-influx followed by water entry, disturbing cell volume regulation and leading to the observed excessive cell swelling and the followed disruption of the cell membrane.

Alternatively, it has been suggested that intracellular calcium mediates expression of ligands that bind to and activate death receptors such as CD95[29] although within the time frame of these experiments, it seems unlikely that transcriptional changes could occur. Another possibility is that mitochondria may respond to an apoptotic Ca^{2+} signal by the selective release of cytochrome *c* or through enhanced production of reactive oxygen species and opening of an inner mitochondrial membrane pore [29]. Our findings are supported by the observations of Olivan et al. (2018), who showed in parallel similar TRPV4 mediated cytotoxic effects on melanoma cells and keratinocytes [30].

In summary we explained the functional effects of TRPV4 activation on TER with differential cytotoxic effects induced by two widely-published TRPV4 agonists in HUVECs. We conclude that in this test system, 4α-PDD may not be a selective activator of TRPV4 and mediates TER reduction via apoptosis. In contrast GSK1016790A selectively activates TRPV4 even with low nanomolar concentrations, but that TER reduction is also a consequence of cellular necrosis, during which the cells swell leading to membrane disruption and collapse of the cells. Cell death plays an important role in regulating barrier integrity and TRPV4 mediated cytotoxicity in endothelial cells, but also in epithelial cells, is poorly described in the literature and we believe that these findings add significant context to many reported and further studies concerning the role of TRPV4 in endothelial and epithelial barrier-function.

Acknowledgements

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2019.100672.

Transparency document

Pairet, Nicolas (2019): The role of TRPV4 in membrane barrier integrity and inhibition in stretch-induced pathological lung cellular responses during mechanical ventilation. Open Access Repositorium der Universität Ulm. Dissertation. http://dx.doi.org/10.18725/OPARU-12279.

Transparency document related to this article can be found online at https://doi.org/10.1016/j.bbrep.2019.100672.

References

[1] T. Voets, et al., Molecular determinants of permeation through the cation channel TRPV4, J. Biol. Chem. 277 (2002) 33704–33710, https://doi.org/10.1074/jbc.M204828200.
[2] S. Wu, et al., Ca2+ entry via α1G and TRPV4 channels differentially regulates surface expression of P-selectin and barrier integrity in pulmonary capillary endothelium, Am. J. Physiol. Lung Cell Mol. Physiol. 297 (2009) L650–L657, https://doi.org/10.1152/ajplung.00015.2009.
[3] J. Vriens, et al., Cell swelling, heat, and chemical agonists use distinct pathways for the activation of the cation channel TRPV4, Proc. Natl. Acad. Sci. 101 (2004) 396–401, https://doi.org/10.1073/pnas.0303329101.
[4] A.E. Loot, et al., Role of cytochrome P450-dependent transient receptor potential V4 activation in flow-induced vasodilatation, Cardiovasc. Res. 80 (2008) 445–452, https://doi.org/10.1093/cvr/cvn207.
[5] S.A. Mendoza, et al., TRPV4-mediated endothelial Ca2+ influx and vasodilation in response to shear stress, Am. J. Physiol. Heart Circ. Physiol. 298 (2010) H466–H476, https://doi.org/10.1152/ajpheart.00854.2009.
[6] K.S. Thorneloe, et al., An orally active TRPV4 channel blocker prevents and resolves pulmonary edema induced by heart failure, Sci. Transl. Med. 4 (2012) 159ra148.
[7] S. Balakrishna, et al., TRPV4 inhibition counteracts edema and inflammation and improves pulmonary function and oxygen saturation in chemically induced acute lung injury, Am. J. Physiol. Lung Cell Mol. Physiol. 307 (2014) L158–L172, https://doi.org/10.1152/ajplung.00065.2014.
[8] R.N. Willette, et al., Systemic activation of the transient receptor potential vanilloid subtype 4 channel causes endothelial failure and circulatory collapse: Part 2, J. Pharmacol. Exp. Ther. 326 (2008) 443–452, https://doi.org/10.1124/jpet.107.134551.
[9] K. Hamanaka, et al., TRPV4 initiates the acute calcium-dependent permeability increase during ventilator-induced lung injury in isolated mouse lungs, Am. J. Physiol. Lung Cell Mol. Physiol. 293 (2007) L923–L932, https://doi.org/10.1152/ajplung.00221.2007.
[10] M.-Y. Jian, J.A. King, A.-B. Al-Mehdi, W. Liedtke, M.I. Townsley, High vascular pressure–induced lung injury requires P450 epoxygenase–dependent activation of TRPV4, Am. J. Respir. Cell Mol. Biol. 38 (2008) 386–392, https://doi.org/10.1165/rcmb.2007-0192OC.
[11] D.F. Alvarez, et al., Transient receptor potential vanilloid 4-mediated disruption of the alveolar septal barrier: a novel mechanism of acute lung injury, Circ. Res. 99 (2006) 988–995, https://doi.org/10.1161/01.res.0000247065.11756.19.
[12] K.S. Thorneloe, et al., N-((1S)-1-{[4-((2S)-2-{[(2,4-dichlorophenyl)sulfonyl]amino}-3-hydroxypropanoyl)-1 -piperazinyl]carbonyl}-3-methylbutyl)-1-benzothiophene-2-carboxamide (GSK1016790A), a novel and potent transient receptor potential vanilloid 4 channel agonist induces urinary bladder contraction and hyperactivity: Part I, J. Pharmacol. Exp. Ther. 326 (2008) 432–442, https://doi.org/10.1124/jpet.108.139295.
[13] A. Namiki, et al., Hypoxia induces vascular endothelial growth factor in cultured human endothelial cells, J. Biol. Chem. 270 (1995) 31189–31195.
[14] R. Ghassemifar, C.M. Lai, P.E. Rakoczy, VEGF differentially regulates transcription and translation of ZO-1alpha+ and ZO-1alpha- and mediates trans-epithelial resistance in cultured endothelial and epithelial cells, Cell Tissue Res. 323 (2006) 117–125, https://doi.org/10.1007/s00441-005-0046-7.
[15] P.-L. Luo, Y.-J. Wang, Y.-Y. Yang, J.-J. Yang, Hypoxia-induced hyperpermeability of rat glomerular endothelial cells involves HIF-2α mediated changes in the expression of occludin and, Braz. J. Med. Biol. Res. 51 (2018) e6201, , https://doi.org/10.1590/1414-431X20186201.
[16] A. Carreau, B.E. Hafny-Rahbi, A. Matejuk, C. Grillon, C. Kieda, Why is the partial oxygen pressure of human tissues a crucial parameter? Small molecules and hypoxia, J. Cell Mol. Med. 15 (2011) 1239–1253, https://doi.org/10.1111/j.1582-4934.2011.01258.x.
[17] E. Gluckman, et al., Hematopoietic reconstitution in a patient with Fanconi's anemia by means of umbilical-cord blood from an HLA-identical sibling, N. Engl. J. Med. 321 (1989) 1174–1178, https://doi.org/10.1056/nejm198910263211707.
[18] J. Yin, et al., Negative-feedback loop attenuates hydrostatic lung edema via a cGMP-dependent regulation of transient receptor potential vanilloid 4, Circ. Res. 102 (2008) 966–974, https://doi.org/10.1161/circresaha.107.168724.
[19] K.S. Thorneloe, et al., < em > N < /em > -((1 < em > S < /em >)-1-{[4-((2 < em > S < /em >)-2-{[(2,4-Dichlorophenyl)sulfonyl]amino}-3-hydroxypropanoyl)-1-piperazinyl]carbonyl}-3-methylbutyl)-1-benzothiophene-2-carboxamide (GSK1016790A), a novel and potent transient receptor potential vanilloid 4 channel agonist induces urinary bladder contraction and hyperactivity: Part I, J. Pharmacol. Exp. Ther. 326 (2008) 432–442, https://doi.org/10.1124/jpet.108.139295.

[20] K.S. Thorneloe, M. Cheung, D.A. Holt, R.N. Willette, PROPERTIES OF the TRPV4 agonist GSK1016790A and the TRPV4 antagonist GSK2193874, Physiol. Rev. 97 (2017) 1231–1232, https://doi.org/10.1152/physrev.00019.2017.
[21] R. Alexander, et al., 4alpha-phorbol 12,13-didecanoate activates cultured mouse dorsal root ganglia neurons independently of TRPV4, Br. J. Pharmacol. 168 (2013) 761–772, https://doi.org/10.1111/j.1476-5381.2012.02186.x.
[22] M.J. Berridge, M.D. Bootman, H.L. Roderick, Calcium signalling: dynamics, homeostasis and remodelling, Nat. Rev. Mol. Cell Biol. 4 (2003) 517–529, https://doi.org/10.1038/nrm1155.
[23] M.D. Bootman, Calcium signaling, Cold Spring Harbor Perspect. Biol. 4 (2012), https://doi.org/10.1101/cshperspect.a011171.
[24] D.E. Clapham, TRP channels as cellular sensors, Nature 426 (2003) 517, https://doi.org/10.1038/nature02196.
[25] S.F. Pedersen, G. Owsianik, B. Nilius, TRP channels: an overview, Cell Calcium 38 (2005) 233–252 https://doi.org/10.1016/j.ceca.2005.06.028.
[26] M. Arniges, E. Vazquez, J.M. Fernandez-Fernandez, M.A. Valverde, Swelling-activated Ca2+ entry via TRPV4 channel is defective in cystic fibrosis airway epithelia, J. Biol. Chem. 279 (2004) 54062–54068, https://doi.org/10.1074/jbc.M409708200.
[27] V. Benfenati, et al., An aquaporin-4/transient receptor potential vanilloid 4 (AQP4/TRPV4) complex is essential for cell-volume control in astrocytes, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 2563–2568, https://doi.org/10.1073/pnas.1012867108.
[28] A.O. Jo, et al., TRPV4 and AQP4 channels synergistically regulate cell volume and calcium homeostasis in retinal Müller glia, J. Neurosci. 35 (2015) 13525–13537, https://doi.org/10.1523/JNEUROSCI.1987-15.2015.
[29] G.E. Kass, S. Orrenius, Calcium signaling and cytotoxicity, Environ. Health Perspect. 107 (1999) 25–35.
[30] A. Olivan-Viguera, et al., Pharmacological activation of TRPV4 produces immediate cell damage and induction of apoptosis in human melanoma cells and HaCaT keratinocytes, PLoS One 13 (2018) e0190307, , https://doi.org/10.1371/journal.pone.0190307.