

Transient receptor potential vanilloid 4 (TRPV4)-dependent calcium influx and ATP release in mouse oesophageal keratinocytes

Hiroshi Mihara^{1,2}, Ammar Boudaka¹, Toshiro Sugiyama², Yoshinori Moriyama³ and Makoto Tominaga^{1,4}

¹Division of Cell Signaling, Okazaki Institute for Integrative Bioscience (National Institute for Physiological Sciences), National Institutes of Natural Sciences, Okazaki, Japan

²Department of Gastroenterology, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama, Japan

³Department of Membrane Biochemistry, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan

⁴Department of Physiological Sciences, The Graduate University for Advanced Studies, Okazaki, Japan

Non-technical summary In the oesophagus the ion channel TRPV4 senses multiple stimuli, including heat and mechanical stimulation. TRPV4 activation causes ATP release from oesophageal cells, which could be important in oesophageal disease mechanisms.

Abstract Gastro-oesophageal reflux disease (GERD) is a multi-factorial disease that may involve oesophageal hypersensitivity to mechanical or heat stimulus as well as acids. Intraganglionic laminar endings (IGLEs) are the most prominent terminal structures of oesophageal vagal mechanosensitive afferents and may modulate mechanotransduction via purinergic receptors. Transient receptor potential channel vanilloid 4 (TRPV4) can detect various stimuli such as warm temperature, stretch and some chemicals, including 4 α -phorbol 12,13-didecanoate (4 α -PDD) and GSK1016790A. TRPV4 is expressed in many tissues, including renal epithelium, skin keratinocytes and urinary bladder epithelium, but its expression and function in the oesophagus is poorly understood. Here, we show anatomical and functional TRPV4 expression in mouse oesophagus and its involvement in ATP release. TRPV4 mRNA and protein were detected in oesophageal keratinocytes. Several known TRPV4 activators (chemicals, heat and stretch stimulus) increased cytosolic Ca²⁺ concentrations in cultured WT keratinocytes but not in TRPV4 knockout (KO) cells. Moreover, the TRPV4 agonist GSK1016790A and heat stimulus evoked TRPV4-like current responses in isolated WT keratinocytes, but not in TRPV4KO cells. GSK1016790A and heat stimulus also significantly increased ATP release from WT oesophageal keratinocytes compared to TRPV4KO cells. The vesicle-trafficking inhibitor brefeldin A (BFA) inhibited the ATP release. This ATP release could be mediated by the newly identified vesicle ATP transporter, VNUT, which is expressed by oesophageal keratinocytes at the mRNA and protein levels. In conclusion, in response to heat, chemical and possibly mechanical stimuli, TRPV4 contributes to ATP release in the oesophagus. Thus, TRPV4 could be involved in oesophageal mechano- and heat hypersensitivity.

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Corresponding author M. Tominaga: Division of Cell Signaling, Okazaki Institute for Integrative Bioscience, National Institutes of Natural Sciences, Okazaki, Aichi 444-8787, Japan. Email: tominaga@nips.ac.jp

Abbreviations 4 α -PDD, 4 α -phorbol 12,13-didecanoate; BFA, brefeldin A; CK14, cytokeratin 14; EET, epoxyeicosatrienoic acids; ENRD, endoscopy-negative reflux disease; GERD, gastro-oesophageal reflux disease; GSK, GSK1016790A, a TRPV4 agonist; IGLEs, intraganglionic laminar endings; MHC2, myosin heavy chain 2; NPPB, 5-nitro-2-(3-phenylpropylamino)-benzoate; PPI, proton pump inhibitor; RR, ruthenium red; TRPV4, transient receptor potential vanilloid 4; VNUT, vesicular nucleotide transporter.

H. Mihara and A. Boudaka contributed equally to this work.

Introduction

Gastro-oesophageal reflux disease (GERD) is a multi-factorial disease that often impairs health-related quality of life (Flook & Wiklund, 2007). The cardinal symptoms of GERD are heartburn and regurgitation and affect 10–20% of adults in western countries and about 5% of adults in Asia (Dent *et al.* 2005; Wong & Kinoshita, 2006). Oesophageal acid exposure in GERD patients may be sufficiently severe to cause endoscopically visible mucosal damage (erosive oesophagitis), but many patients have no endoscopically visible oesophageal mucosal damage despite having GERD symptoms and oesophageal acid exposure. This latter condition is termed endoscopy-negative reflux disease (ENRD) and is quite common worldwide (Dent, 2007). Although symptoms for most GERD patients resolve with proton pump inhibitor (PPI) therapy, 19–44% of GERD patients only exhibit partial or no response (van Pinxteren *et al.* 2001; Donnellan *et al.* 2005). Oesophageal hypersensitivity and functional heartburn are the most prevalent causes of such PPI-resistant GERD patients (Mainie *et al.* 2006; Sharma *et al.* 2008). Many studies demonstrated that this patient population also has increased oesophageal sensitivity to mechanical or heat stimuli (Trimble *et al.* 1995; Drewes *et al.* 2006; Reddy *et al.* 2007).

Intraganglionic laminar endings (IGLEs) (Rodrigo *et al.* 1975) are derived from nodose ganglion neurons (Neuhuber, 1987; Berthoud & Powley, 1992) and represent the most prominent terminal structures of vagal mechanosensitive afferent fibres throughout the gastrointestinal tract (Berthoud *et al.* 1997). Immunohistochemical studies identified P2X₂ and P2X₃ purinergic receptors in the IGLEs (Raab & Neuhuber, 2005), and electrophysiological experiments demonstrated that adenosine triphosphate (ATP) may play a modulatory role in IGLE mechanotransduction processes (Zagorodnyuk *et al.* 2003). However, the underlying molecular mechanisms of mechano-ATP transduction are poorly understood.

TRPV4 was originally reported as an osmo- or mechano-sensor (Liedtke *et al.* 2000; Strotmann *et al.* 2000) that can be activated by diverse chemical stimuli, including the synthetic phorbol ester 4 α -phorbol 12,13-didecanoate (4 α -PDD), GSK1016790A (Watanabe *et al.* 2002a; Willette *et al.* 2008), as well as moderate warmth (>27°C) (Guler *et al.* 2002; Watanabe *et al.* 2002b). TRPV4 is widely expressed throughout the body, including renal epithelia, skin keratinocytes, hippocampal neurons, endothelial cells and urinary bladder epithelia, thereby contributing to numerous physiological processes (Shibasaki *et al.* 2007; Sokabe *et al.* 2010). We recently showed that TRPV4 was highly expressed in the complex urothelium lining the luminal side of the bladder and that isolated urothelial cells release ATP upon mechanical stimulus that induced TRPV4 activation

(Mochizuki *et al.* 2009). Loss of TRPV4 leads to an increased micturition threshold, a larger bladder capacity and disrupted voiding behaviour, suggesting that normal sensitivity to bladder distension is lost in TRPV4-deficient mice (Gevaert *et al.* 2007). In contrast, intravesical instillation of GSK1016790A resulted in a decreased micturition threshold and increased voiding frequency, suggesting that excessive TRPV4 activation can induce bladder overactivity (Thorneloe *et al.* 2008). Furthermore, the TRPV4 antagonist HC-067047 was shown to be a potential treatment for cystitis-induced bladder dysfunction (Everaertsa *et al.* 2010b). In the last three decades, ATP has become widely recognized as a neurotransmitter in the central nervous system and also in peripheral tissues, including the gastrointestinal tract (Burnstock *et al.* 1978; Edwards *et al.* 1992; Galligan & Bertrand, 1994; McConalogue *et al.* 1996). SLC17A9 (vesicular nucleotide transporter (VNUT)) has been recently identified as a novel vesicular nucleotide transporter, and VNUT is reportedly involved in vesicular storage and ATP exocytosis in taste cells and during T-cell activation (Sawada *et al.* 2008; Iwatsuki *et al.* 2009; Tokunaga *et al.* 2010).

In this study, we investigated the mechanism of ATP release from oesophageal keratinocytes in response to mechanical, heat or chemical stimuli. Our results indicate that ATP release from oesophageal keratinocytes is regulated by TRPV4 channel-dependent Ca²⁺ influx and mediated by exocytosis with vesicles expressing VNUT.

Methods

Animals

Male C57BL/6NCR mice (6–8 weeks old; SLC) were used for a control. TRPV4-deficient (TRPV4KO) mice (Mizuno *et al.* 2003) and TRPV3-deficient (TRPV3KO) mice (Moqrich *et al.* 2005) were both backcrossed on a C57BL/6NCR background. Mice were housed in a controlled environment (12 h light–12 h dark cycle; room temperature, 22–24°C; 50–60% relative humidity) with free access to food and water. All procedures involving the care and use of animals were approved by The Institutional Animal Care and Use Committee of the National Institutes of Natural Sciences and carried out in accordance with the NIH *Guide for the care and use of laboratory animals* (NIH publication no. 85–23, revised 1985).

Reverse transcription PCR analysis

To examine TRP channel mRNA expression in the mouse oesophagus, total RNA (1 μ g) isolated from whole oesophagus and cultured oesophageal keratinocytes

Table 1. Characteristics of primary and secondary antisera used for immunochemistry

Tissue antigen	Host	Dilution	Source (Reference)
CK14	Rabbit	1:500	COVANCE
TRPV4	Rabbit	1:500	B. Nilius (Mochizuki <i>et al.</i> 2009)
VNUT	Rabbit	1:500	(Tokunaga <i>et al.</i> 2010)
DAPI		1:1000	Dojin Chemical Corp.
Secondary antibodies used for immunochemistry.			
Antibody label	Dilution	Source	
Goat anti-rabbit IgG-Alexa488	1:1500	Invitrogen, Inc.	
Goat anti-rabbit IgG-Alexa635	1:1500	Invitrogen, Inc.	

was used for reverse transcription using the Superscript III first-strand synthesis system for RT-PCR (Invitrogen). PCR was performed using rTaq DNA polymerase (TaKaRa) in an iCycler (Bio-Rad) with specific primer sets (Supplemental Table S1) for several TRP channels including TRPV2, TRPV3 and TRPV4, the keratinocyte marker cytokeratin 14 (CK14), the skeletal muscle marker myosin heavy chain 2 (MHC2) and vesicular nucleotide transporter (VNUT). PCR conditions used were: 1 cycle at 94°C for 2 min; 40 cycles at 94°C for 10 s; 55°C for 10 s; and 72°C for 30 s; and 1 cycle at 72°C for 2 min.

Immunochemistry

All experiments were repeated on specimens from at least three mice. Antibody information is summarized in Table 1. For section preparation, mice were anaesthetized with diethyl ether and perfused through the heart with 4% paraformaldehyde in PBS. The oesophagi were removed and further fixed at 4°C for 6 h. Tissues were washed in PBS (3 times, 15 min each), placed in PBS–sucrose (PBS containing 20% sucrose) and stored at 4°C overnight. Next, they were embedded in OCT compound (Tissue Tek, Elkhart, IN, USA), and 14- μ m-thick sections were collected onto slides and dried at room temperature for 1 h. Preparations were cleared with PBS plus 0.3% Triton X-100 (PBS–T 0.3%) 3 times for 5 min each. Non-specific antibody binding was reduced by incubation in BlockAce (Yukijirushi, Sapporo, Japan) in PBS–T 0.3% for 1 h at room temperature prior to antibody exposure. Preparations were analysed using a fluorescent microscope and a confocal laser scanning microscope (LSM 510, Carl Zeiss).

For immunocytochemistry, wild-type (WT) keratinocytes cultured on glass coverslips for 3 days were used. The cells were fixed at 4°C for 20 min with the same fixative. Samples were cleared with PBS–T 0.1% 3 times for 5 min each. PBS–T 0.1% with 3% bovine serum albumin (BSA; Sigma) was used as a blocking solution.

Preparations were analysed similarly to the oesophagus sections.

Primary culture of oesophageal keratinocytes

Male C57BL/6NCR mice (6–8 weeks old; SLC), TRPV4KO and TRPV3KO mice were killed by cervical dislocation following light diethyl ether anaesthesia. The entire lengths of oesophagi were placed in cold (4°C) PBS without Ca²⁺ and Mg²⁺. The muscle layer was removed using forceps and the remaining inner tissue containing the keratinous layer was incubated at 4°C in 1 ml trypsin solution (Invitrogen) for 5–8 h. The gelatinized submucosal layer and lamina propria mucosae were peeled away and the keratinocytes harvested with a cell scraper (Greiner Bio-one). The resulting cells were plated on glass coverslips, dishes or fibronectin-coated silicone chambers after filtering with a cell strainer (BD Falcon). The cells were incubated in MCDB 153 medium (modified from that described in Sokabe *et al.* 2010) containing 5 μ g ml⁻¹ insulin, 0.4 μ g ml⁻¹ hydrocortisone, 14.1 μ g ml⁻¹ phosphorylethanolamine, 10 ng ml⁻¹ epidermal growth factor (all from Sigma), 10 μ g ml⁻¹ transferrin (Funakoshi, Japan), 40 μ g ml⁻¹ bovine pituitary gland extract (Kyokuto, Japan), 25 μ g ml⁻¹ gentamicin, 50 U ml⁻¹ penicillin, 50 μ g ml⁻¹ streptomycin and 10% fetal bovine serum (FBS). After incubating at 37°C for 24 h, the medium was changed to a medium lacking FBS. Cells were then maintained at 37°C in a humidified atmosphere of 5% CO₂ in air. Half of the medium was changed every 2 days.

Ca²⁺ imaging

Fura-2 fluorescence was measured in primary oesophageal keratinocytes cultured for 2–3 days with a standard bath solution containing 140 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂, 10 mM Hepes and 10 mM glucose at pH 7.4 (adjusted with NaOH). The ratios of fluorescence intensities obtained with fura-2 emission at 340 nm and

380 nm are shown. 4α -PDD and GSK1016790A were used as TRPV4 agonists, and a broad TRP channel blocker, ruthenium red (RR), was used as an antagonist (both from Sigma). Ionomycin (Sigma) was used to confirm cell viability. Temperature changes were achieved using an inline-heater (SH-27B and TC-344B, Warner Instruments) with a thermocouple (TA-30, Warner Instruments) used to measure bath temperature (Mandadi *et al.* 2009). Stretch stimulus was applied with an elastic silicone chamber (STB-CH-04, STREX, Osaka, Japan) and an extension device (modified version of STB150, STREX), which were established for use with primary urothelial cells (Mochizuki *et al.* 2009). All experiments were performed at room temperature (25°C). F_{340}/F_{380} was calculated and acquired with an imaging processing system (IP-Lab, Scanalytics Inc., Rockville, MD, USA) and ImageJ software (<http://rsb.info.nih.gov/ij/>). Changes in ratio (Δ) were calculated by subtracting mean basal from peak values.

Electrophysiology

The standard bath solution for the patch-clamp experiments was the same as that used for the Ca^{2+} imaging experiments. Pipette solutions for whole-cell recordings contained 140 mM KCl, 5 mM EGTA and 10 mM Hepes, pH 7.4. Primary oesophageal keratinocytes were used within 3 h of isolation. Heat stimulus was applied in the same system used for Ca^{2+} imaging experiments. Whole-cell recording data were sampled at 10 kHz and filtered at 5 kHz for analysis (Axon 200B amplifier with pClamp software, Molecular Devices, Foster City, CA, USA). Voltage ramp pulses from -100 mV to $+100$ mV (500 ms) were applied every 6–10 s to generate an I - V curve.

Measurement of ATP release (luciferin–luciferase assay)

The concentration of ATP released from oesophageal keratinocytes cultured in 24-well plates was measured by a luciferin–luciferase assay using an ATP analyser (AF-100, DKK-TOA Co., Tokyo, Japan) and a method slightly modified from that previously described (Dutta *et al.* 2004). Primary oesophageal keratinocytes cultured in 24-well plates for 4–5 days to full confluence were incubated in Tyrode solution for 1 h at 37°C to measure basal ATP release. The superfusate was collected for measurements of released ATP and replaced with Tyrode solution containing the TRPV4 agonist GSK or an exocytic stimulator, 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB; Sigma) (Dolovcak *et al.* 2009). The superfusate was collected after 15 min and the ratio of released ATP (15 min stimulation/control condition) was calculated.

For measurement of heat-induced ATP release, the solution was first maintained at room temperature (25°C) for 1 h in Tyrode solution, after which the solution was heated to 38.5°C by addition of preheated solution and incubated for an additional 15 min at 38.5°C. To block ATP vesicle trafficking, cells were pretreated with brefeldin A (10 μ M, BFA; Wako, Japan) for 1 h (Tokunaga *et al.* 2010). An aliquot (500 μ l) of superfusate was mixed with 50 μ l luciferin–luciferase assay mixture for luminometric ATP measurements.

Data analysis and statistics

Values in Ca^{2+} imaging, patch-clamp experiments and ATP measurements are presented as means \pm SEM from three or more independent experiments. Student's t test or non-parametric Bonferroni-type multiple comparison were used. $P < 0.05$ was considered statistically significant.

Results

TRPV4 and VNUT mRNAs are expressed in oesophageal keratinocytes

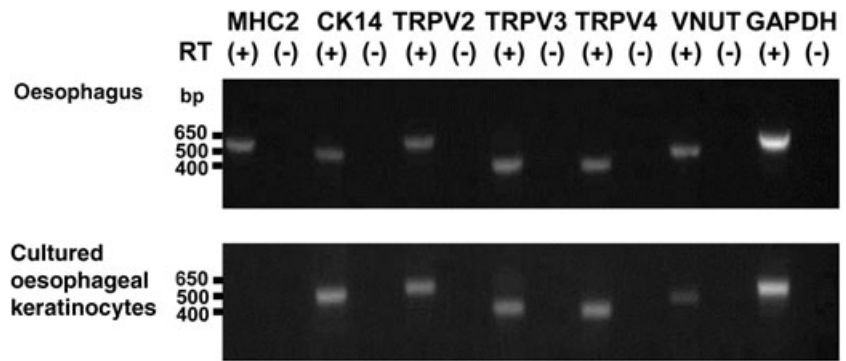
Given the previous findings of TRPV3 and TRPV4 expression in mouse keratinocytes (Mandadi *et al.* 2009; Sokabe *et al.* 2010), the mechanosensitivity of TRPV2 (Muraki *et al.* 2003; Mihara *et al.* 2010; Shibasaki *et al.* 2010) and the contribution of VNUT to ATP exocytosis (Sawada *et al.* 2008), we wanted to first confirm mRNA expression of TRPV2, TRPV3, TRPV4 and VNUT in the oesophagus and cultured oesophageal keratinocytes. mRNA for TRP channels and VNUT together with the control CK14 (a keratinocyte marker) was detected in whole oesophagus and in cultured oesophageal keratinocytes (Fig. 1).

Expression of TRPV4 protein in oesophageal keratinocytes

To examine TRPV4 expression at a protein level, immunohistochemistry of oesophageal tissue from mice was performed. TRPV4-like immunoreactive (IR) cells were observed in WT oesophageal keratinous layers with the CK14 keratinocyte marker, while TRPV4-like IR cells were not observed in TRPV4KO oesophagus (Fig. 2). TRPV4-like immunoreactivity appeared homogeneous in keratinous layers.

Figure 1. Expression of TRPV4 and VNUT mRNAs in whole oesophagus and cultured oesophageal keratinocytes

Expression of mRNA for MHC2, CK14, TRPV2, TRPV3, TRPV4, VNUT (vesicular nucleotide transporter) and GAPDH was examined with (+) and without (-) RT reaction. Expected sizes of the amplified fragments were 562, 523, 552, 421, 404, 466 and 545 bp, respectively. TRPV2, TRPV3, TRPV4 and VNUT mRNAs were also detected in cultured oesophageal keratinocytes. MHC2, skeletal muscle marker; CK14, keratinocyte marker.



TRPV4-mediated cytosolic Ca^{2+} increase in primary oesophageal keratinocytes

To confirm functional TRPV4 expression in primary oesophageal keratinocytes, we used a fluorescent Ca^{2+} imaging system ($10 \mu M$ fura-2 AM) to examine the response to TRPV4 agonists (4α -PDD and GSK1016790A) and physiological stimuli (heat and mechanical stimuli) in primary oesophageal keratinocytes. We first confirmed the cellular purity of cultured oesophageal keratinocytes using CK14 immunoreactivity (Supplemental Fig. S1), which indicated that almost all cells were CK14-IR keratinocytes. Response traces for 4α -PDD with WT and TRPV4KO keratinocytes are shown in Fig. 3A. 4α -PDD and the TRPV4-specific agonist, GSK1016790A, significantly increased intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) in WT keratinocytes, but not in TRPV4KO cells. These increases were also abolished by pretreatment with a broad TRP channel blocker, ruthenium red (RR), or in the absence of extracellular Ca^{2+} ($Ca^{2+}(-)$) (Fig. 3A and B). Heat stimulus (25 to $40^\circ C$) also increased $[Ca^{2+}]_i$ in oesophageal keratinocytes from WT, TRPV4KO and TRPV3KO mice, although the

$[Ca^{2+}]_i$ increases were significantly smaller for TRPV4KO keratinocytes (Fig. 3C and D). Omitting Ca^{2+} from the bath solution or pretreating with RR significantly reduced the $[Ca^{2+}]_i$ increases, suggesting that heat-induced $[Ca^{2+}]_i$ increases were mediated by Ca^{2+} influx mainly through plasma membrane TRPV4 channels (Fig. 3C and D). On the other hand, heat-induced $[Ca^{2+}]_i$ increases were not significantly affected by the loss of TRPV3, another heat-sensitive channel (Fig. 3D) while camphor, a TRPV3 agonist, induced $[Ca^{2+}]_i$ increases that were significantly larger in WT oesophageal keratinocytes compared to TRPV3KO cells (Supplemental Fig. S2), suggesting that the contribution of TRPV3 to heat-evoked $[Ca^{2+}]_i$ increases could be modest. Another physiological stimulus, mechanical stretch, increased $[Ca^{2+}]_i$ in WT keratinocytes, but not in TRPV4KO cells but only in the presence of extracellular Ca^{2+} (Fig. 3E and F). These data indicate that TRPV4 is functionally expressed in oesophageal keratinocytes and senses physiological stimuli such as heat and mechanical stretch. In addition, TRPV4 seems to be more predominantly involved in heat-evoked responses than is TRPV3.

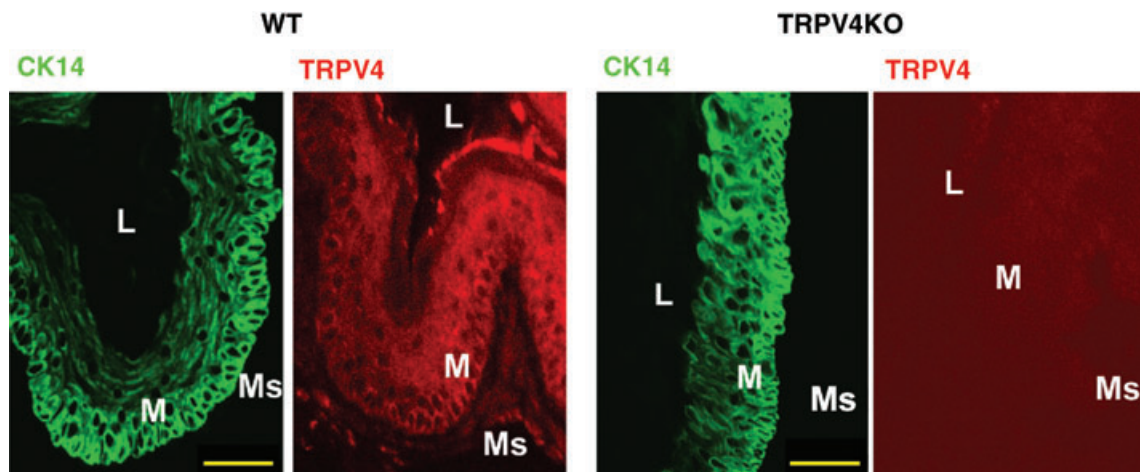


Figure 2. TRPV4 immunoreactivity in WT and TRPV4KO mouse oesophagus

TRPV4 immunoreactivity was observed in WT but not TRPV4KO oesophageal mucosal layers. Scale bars indicate $50 \mu m$. CK14, keratinocyte marker; L, lumen; M, mucosa; Ms, muscle.

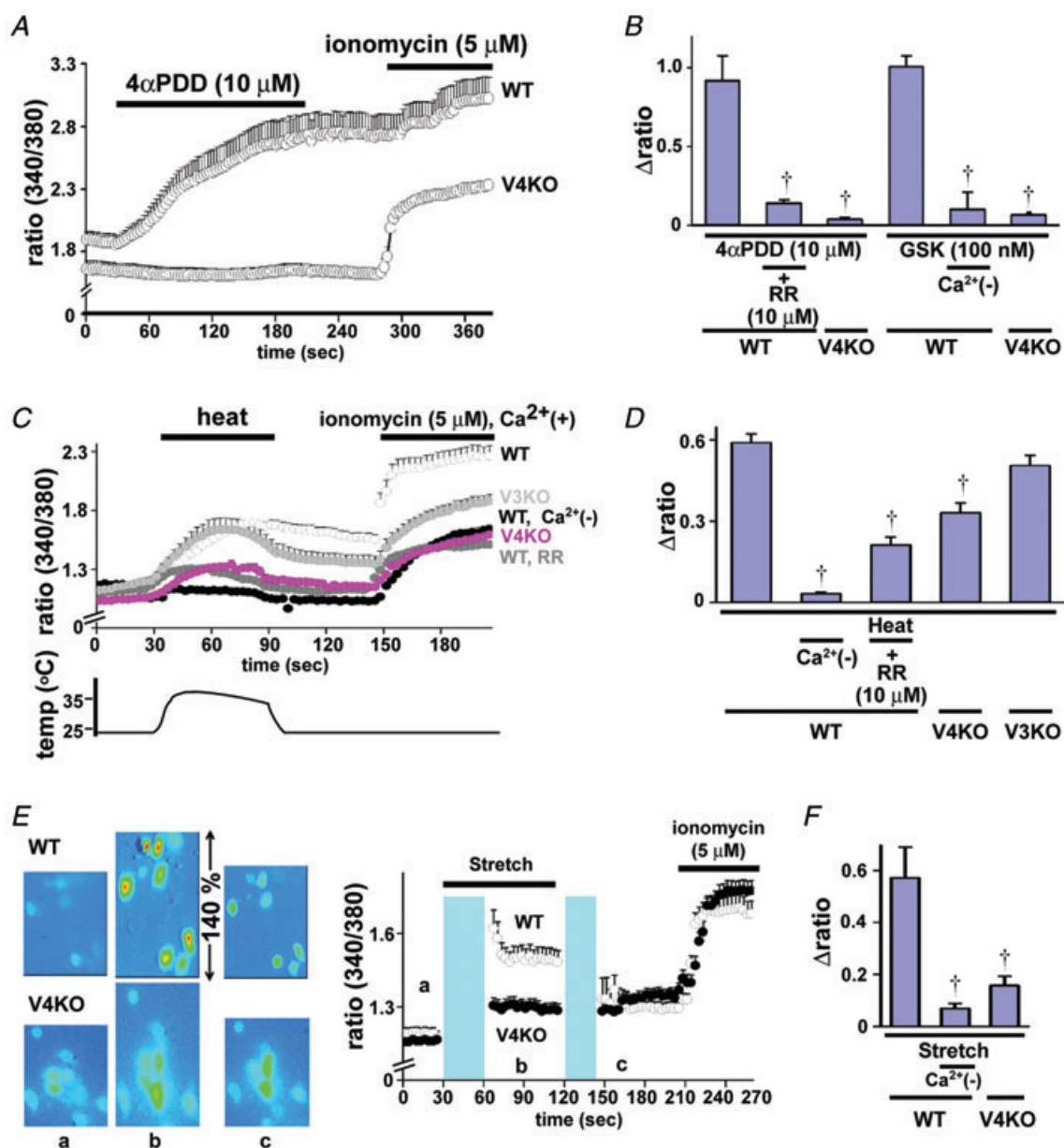


Figure 3. TRPV4-mediated cytosolic Ca^{2+} increase in primary oesophageal keratinocytes

A, traces for $[\text{Ca}^{2+}]_i$ changes (340/380 nm ratio) in the response to the TRPV4-specific agonist 4α -PDD, in WT and TRPV4KO (V4KO) keratinocytes (mean \pm SEM). Bars indicate the duration of chemical application. **B**, 4α -PDD and another TRPV4-specific agonist GSK1016790A (GSK), increased $[\text{Ca}^{2+}]_i$ in WT oesophageal keratinocytes, but not in TRPV4KO cells. The increases were significantly inhibited by a broad TRP channel blocker, ruthenium red (RR), or in the absence of extracellular Ca^{2+} ($\text{Ca}^{2+}(-)$) (\dagger , * $P < 0.05$ vs. WT). Changes in ratio (Δ) were calculated by subtracting mean basal from peak values. **C**, traces for $[\text{Ca}^{2+}]_i$ changes in response to heat stimuli (25 to 40°C) in WT (in the presence or absence of extracellular Ca^{2+}) or TRPV4KO keratinocytes (mean \pm SEM). Bars indicate the duration of heat stimuli or a chemical application. **D**, $[\text{Ca}^{2+}]_i$ increase was larger in TRPV4KO keratinocytes and inhibited by RR or in the $\text{Ca}^{2+}(-)$ condition ($\dagger P < 0.05$ vs. WT). $[\text{Ca}^{2+}]_i$ increase in TRPV3KO keratinocytes was similar to that for WT. **E**, representative pseudocolour images and traces for $[\text{Ca}^{2+}]_i$ changes in response to mechanical stimuli (140%) in WT and TRPV4KO keratinocytes cultured on silicon chambers (mean \pm SEM). Images were obtained at points a, b and c indicated on the traces. A mechanical stimulus was applied between the blue boxes (black bar). **F**, $[\text{Ca}^{2+}]_i$ increase was significantly smaller in TRPV4KO than in WT keratinocytes and was inhibited significantly in the absence of Ca^{2+} ($\text{Ca}^{2+}(-)$, $\dagger P < 0.05$ vs. WT).

TRPV4-mediated current responses in primary oesophageal keratinocytes

We next performed patch-clamp experiments using acute isolated oesophageal keratinocytes. GSK1016790A (300 nM) evoked current responses with a strongly outwardly rectifying current–voltage (I – V) relationship (Fig. 4A–C) in WT keratinocytes, but not in TRPV4KO cells. This I – V relationship is consistent with a previous report (Willette *et al.* 2008). In addition, GSK1016790A-induced currents could be blocked by RR (10 μ M). Currents at -100 mV were significantly larger in WT keratinocytes than in TRPV4KO cells (Fig. 4C). Heat stimulus (25 to 45°C) also evoked inward currents at -60 mV with an outwardly rectifying I – V relationship

(Fig. 4D and E), but the currents were significantly smaller in TRPV4KO keratinocytes compared to WT and TRPV3KO cells (Fig. 4F), which is consistent with the Ca^{2+} imaging data (Fig. 3C and D).

VNUT immunoreactivity in oesophageal keratinocytes

We previously showed that urinary bladder epithelium senses mechanical stretch via TRPV4 and releases ATP (Mochizuki *et al.* 2009). On the other hand, it was reported that T-cells can translate the Ca^{2+} signals into ATP release via VNUT (Tokunaga *et al.* 2010) and that ATP acts as a neuromodulator for mechanotransduction in IGLs via a (P2X₂)–P2X₃ receptor-mediated pathway, especially in

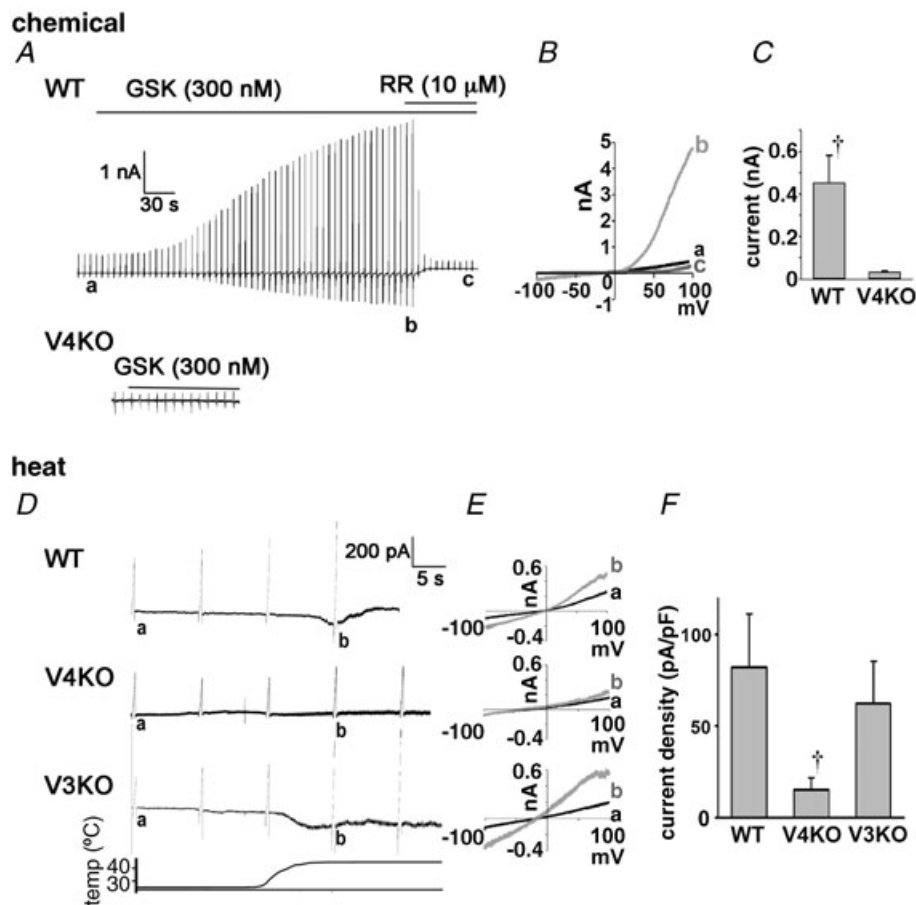


Figure 4. TRPV4 agonist- and heat-induced current responses in primary oesophageal keratinocytes. A, GSK1016790A (GSK, 300 nM) evoked current responses in WT, but not in TRPV4KO cells. The currents were blocked by ruthenium red (RR; 10 μ M). B, currents in response to ramp pulses at points a, b and c shown in A show a strongly outwardly rectifying I – V relationship. C, significantly larger inward currents at -100 mV were obtained from WT keratinocytes compared with TRPV4KO cells ($\dagger P < 0.05$). D, heat stimuli (25 to 45°C) evoked inward currents at -60 mV in WT and TRPV3KO (V3KO) keratinocytes, but not in TRPV4KO cells. E, currents in response to ramp pulses at points a and b shown in D show outwardly rectifying I – V relationship in WT and TRPV3KO cells. F, significantly larger inward currents at -60 mV were obtained from WT keratinocytes compared with TRPV4KO ($\dagger P < 0.05$ vs. WT or TRPV3KO).

the abdominal portion of the mouse oesophagus (Raab & Neuhuber, 2005; Kestler *et al.* 2009). These results led us to hypothesize that mechanical stimulus activates TRPV4 expressed in oesophageal keratinocytes, leading to VNUT-dependent exocytotic ATP release, followed by transmission of signals to the CNS via activation of purinergic receptors expressed in IGLs. To verify this hypothesis, we examined oesophageal VNUT expression. VNUT-like immunoreactivity was observed in both the WT oesophageal keratinous layer (Fig. 5A) and cultured keratinocytes (Fig. 5B). The punctate expression pattern observed in cultured keratinocytes might indicate vesicle clusters.

TRPV4 activators increased ATP release from oesophageal keratinocytes via exocytosis

To examine whether TRPV4-mediated $[Ca^{2+}]_i$ increases are involved in ATP release from keratinocytes, we measured ATP release in chemical- or heat-stimulated cells from WT, TRPV4KO and TRPV3KO mice using a luciferin–luciferase assay. As the amount of released ATP varied among the agonists/antagonist used, we compared the ratio of stimulated responses to control values (data from WT or TRPV4KO cells kept at 37°C for 15 min). The TRPV4 agonist GSK1016790A robustly increased ATP release from cultured WT oesophageal keratinocytes, but not from TRPV4KO cells (Fig. 6A). The $[Ca^{2+}]_i$ increases were abolished in the absence of extracellular Ca^{2+} or by RR treatment (Fig. 6A), indicating that TRPV4 is essential for GSK1016790A-evoked ATP release. We next examined the effects of heat on ATP release in oesophageal keratinocytes. For this assay, we expressed the results as the actual released ATP amounts

because control values were obtained at 25°C instead of 37°C (control). Heat stimulus (from 25 to 38.5°C and kept at 38.5°C for 15 min) also induced ATP release, although the responses were significantly larger in WT keratinocytes than in TRPV4KO cells (Fig. 6B). The heat-evoked ATP releases were indistinguishable between WT and TRPV3KO cells, further confirming the specific involvement of TRPV4 in the heat-evoked response. To confirm that the ATP-releasing ability is not affected by the loss of TRPV4, ATP release was examined in the presence of 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB), a specific exocytosis activator (Dolovcak *et al.* 2009). The presence of NPPB caused similar ATP release in both WT and TRPV4KO keratinocytes (Fig. 6C), indicating that TRPV4 keratinocytes have a similar ability to release ATP through an exocytotic mechanism. To demonstrate that ATP release involving TRPV4 is mediated by exocytosis, we examined the effect of a vesicle-trafficking inhibitor, brefeldin A (BFA). Pretreatment with BFA (10 μ M) significantly reduced GSK1016790A-induced ATP release from WT keratinocytes (Fig. 6D), supporting the concept that TRPV4 activation induces ATP exocytosis.

Discussion

We identified anatomical and functional TRPV4 expression in mouse oesophageal keratinocytes (Figs 1–4) and showed that TRPV4 activation by GSK1016790A and heat induced ATP release (Figs 5 and 6). ATP released from keratinocytes may be readily metabolized by ectonucleotidases, which could prevent the released ATP from reaching the IGLs located in tunica muscularis. However, because even a keratinocyte monolayer released

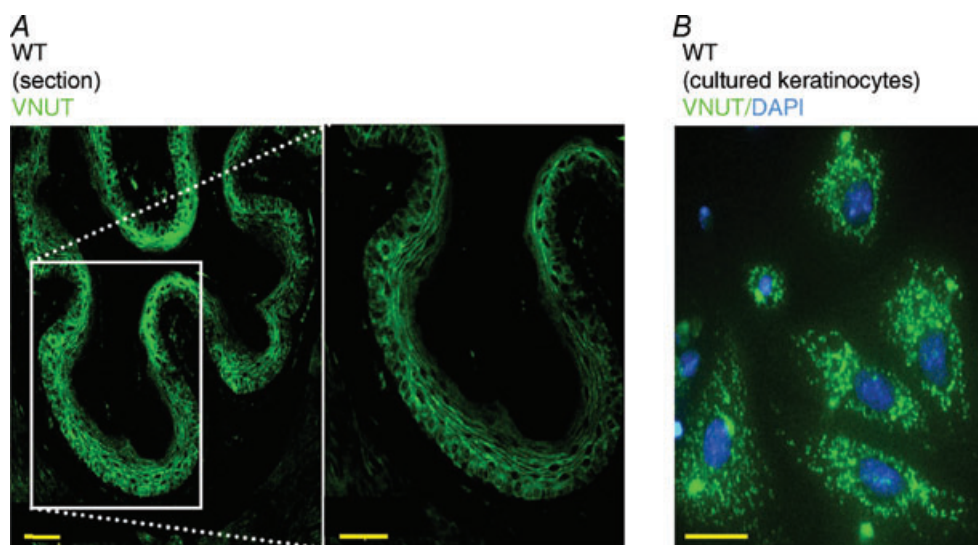


Figure 5. VNUT immunoreactivity in the oesophagus and cultured keratinocytes

A, VNUT immunoreactivity in WT oesophagus mucosal layer. B, a punctate pattern of VNUT immunoreactivity was detected in cultured keratinocytes. Scale bars indicate 50 μ m.

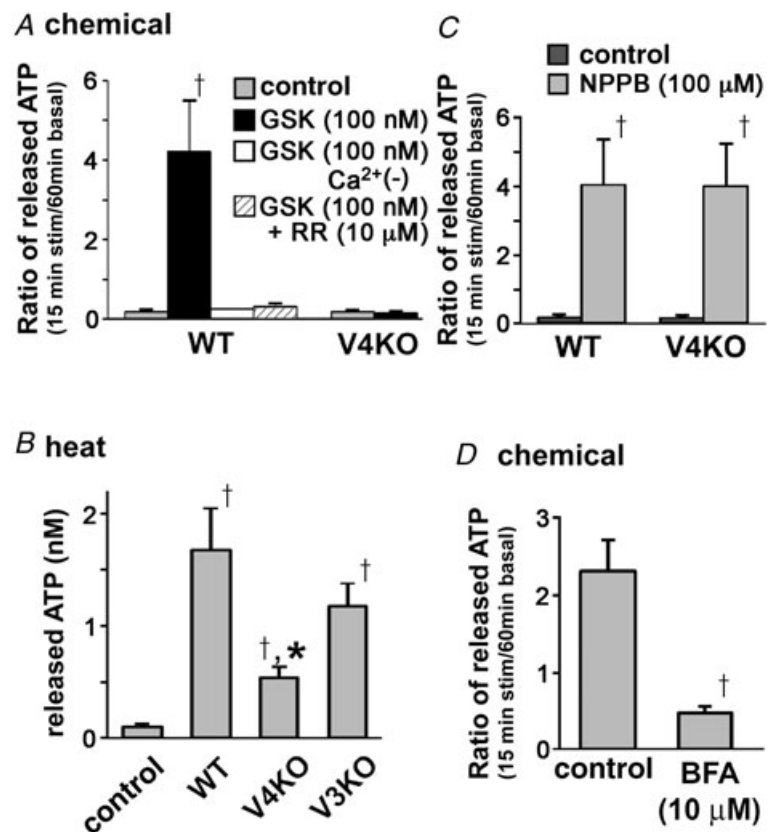
nanomolar levels of ATP (Fig. 6B) and because micromolar ATP release upon heating was estimated for the cultured skin keratinocytes (Mandadi *et al.* 2009), it follows that the oesophageal keratinous layers could release up to micromolar amounts of ATP, which could reach and activate P2X receptors expressed in IGLs as they are in intestine (Bertrand & Bornstein, 2002). Alternatively, vagal and spinal mucosal afferents could detect released ATP because they are reported to abut on and even penetrate the epithelium with small branches (Dutsch *et al.* 1998; Wank & Neuhuber, 2001). Thus, ATP released from oesophageal keratinocytes could act on nerve endings that innervate the oesophagus in different ways. Indeed, P2X₃-like immunoreactivity was observed within nerve fibre arborization in rat pharynx mucosa (Wang & Neuhuber, 2003). Other P2X receptors could also be expressed in mucosal afferents and act as receptors for released ATP. Therefore, upon detecting various stimuli, oesophageal keratinocyte TRPV4 could transmit the information to different kinds of neurons via released ATP.

In a human study, slow heat stimulation of the oesophagus evoked vague perceptions of mild hot and burning sensations, which is indicative of C-fibre activation, even below the threshold temperatures for TRPV1 activation ($\sim 43^\circ\text{C}$) (Olesen *et al.* 2009), suggesting the existence of thermosensors besides TRPV1. Nodose 'nociceptive-like' fibres are exclusively C-fibres that are

sensitive to P2X receptor analogues, whereas jugular 'nociceptive-like' fibres contain both C- and A δ -fibres that are insensitive to P2X receptor analogues in guinea-pigs (Yu *et al.* 2005). These reports suggest that ATP released from keratinocytes might activate P2X receptors expressed in C-fibres that innervate from the nodose ganglion (or dorsal root ganglion). Taken together, TRPV4-mediated ATP release might be involved in vague slow heat-evoked sensations.

Endogenous candidate stimuli for TRPV4 activation are mechanical stimulus, temperature and chemicals. Since TRPV4 is thought to be constitutively active at body temperature, basal TRPV4 activation by body temperature could enhance TRPV4 activity induced by mechanical and chemical stimuli (Everaerts *et al.* 2010a). This concept could be supported by the result showing that GSK1016790A increased ATP release from keratinocytes kept at 37°C (Fig. 6A). TRPV4 can be activated by an endogenously produced 5,6-epoxyeicosatrienoic (5,6-EET) acid, an arachidonic acid (AA) metabolite in endothelial cells (Watanabe *et al.* 2003). Concentrations of 5,6-EET could be elevated in some conditions in the oesophagus. Moreover, ATP-mediated activation of phospholipase C (PLC) is known to modulate TRPV4 activity by sensitizing TRPV4 to EETs (Fernandes *et al.* 2008). Thus, ATP released from damaged tissues could enhance TRPV4 activity, which in turn further enhances ATP release. Such positive

Figure 6. TRPV4 activators increase ATP release from oesophageal keratinocytes via exocytosis. **A**, the TRPV4 agonist GSK1016790A (GSK) increased ATP release from WT cultured oesophageal keratinocytes, but not from TRPV4KO cells. The increase was abolished in the absence of extracellular Ca^{2+} or by ruthenium red (RR) treatment in WT cells ($\dagger P < 0.05$ vs. control). **B**, heat stimulus (from 25 to 38.5°C and kept at 38.5°C for 15 min) significantly increased ATP release from WT cultured keratinocytes compared with WT cells incubated at 37°C for 15 min (control) ($\dagger P < 0.05$ vs. control). The increase was significantly smaller in TRPV4KO cells compared with WT or TRPV3KO cells ($*P < 0.05$). **C**, an exocytic stimulator, 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB), induced ATP release from TRPV4KO keratinocytes similar to that from WT cells ($\dagger, *P < 0.05$ vs. control). **D**, the vesicle-trafficking inhibitor brefeldin A (BFA, $10 \mu\text{M}$) significantly inhibited GSK-induced ATP release ($\dagger P < 0.05$).



feedback mechanisms might explain the mechano- and heat hypersensitivity seen in GERD patients that do not have histological changes.

In conclusion, TRPV4 is functionally expressed in mouse oesophageal keratinocytes and functions as a transducer of heat, chemicals and possibly mechanical stimuli, leading to ATP release via exocytosis. Our results suggest that TRPV4 could be a promising novel therapeutic target for oesophageal hypersensitivity in PPI-resistant GERD patients.

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Author contributions

Conception and design of the experiments: H.M., A.B. and M.T.; data collection: H.M and A.B.; data analysis and interpretation: all authors; drafting manuscript: H.M. and M.T. All authors approved the final version.

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