The TRPV4 channel is a novel regulator of intracellular Ca$^{2+}$ in human esophageal epithelial cells

Takashi Ueda,1 Michiko Shikano,1,2 Takeshi Kamiya,2 Takashi Joh,2 and Shinya Ugawa1

Departments of 1Neurobiology and Anatomy, and 2Gastroenterology and Metabolism, Graduate School of Medical Sciences, Nagoya City University, Nagoya, Japan

Submitted 16 November 2010; accepted in final form 11 April 2011

Ueda T, Shikano M, Kamiya T, Joh T, Ugawa S. The TRPV4 channel is a novel regulator of intracellular Ca$^{2+}$ in human esophageal epithelial cells. Am J Physiol Gastrointest Liver Physiol 301: G138–G147, 2011. First published April 14, 2011; doi:10.1152/ajpgi.00511.2010.—The esophageal epithelium has sensory properties that enable it to sustain normal barrier function. Transient receptor potential vanilloid 4 (TRPV4) is a Ca$^{2+}$-permeable channel that is activated by extracellular hypotonicity, polymaturated fatty acids, phorbol esters, and elevated temperature. We found that TRPV4 is expressed in both human esophageal tissue and in HET-1A cells, a human esophageal epithelial cell line. Specific activation of TRPV4 by the phorbol ester 4x-phorbol 12,13-didecanoate (4x-PDD) increased intracellular Ca$^{2+}$ in a subset of HET-1A cells. Elevated temperature strongly potentiated this effect at low concentrations of 4x-PDD, and all of the responses were inhibited by the TRPV antagonist ruthenium red. TRPV4 activation differentially affected cell proliferation and cell viability; HET-1A cell proliferation was increased by 1 μM 4x-PDD, whereas higher concentrations (10 μM and 30 μM) significantly decreased cell viability. Transient TRPV4 activation triggered ATP release in a concentration-dependent manner via gap-junction hemichannels, including pannexin 1 and connexin 43. Furthermore, TRPV4 activation for 24 h did not increase the production of interleukin 8 (IL-8) but reduced IL-1β-induced IL-8 production. Small interference RNA targeted to TRPV4 significantly attenuated all of the 4x-PDD-induced responses in HET-1A cells. Collectively, these findings suggest that TRPV4 is a novel regulator of Ca$^{2+}$-dependent signaling pathways linked to cell proliferation, cell survival, ATP release, and IL-8 production in human esophageal epithelial cells.

The TRPV4 channel is present in some stratified squamous epithelia. In the skin, TRPV4 is expressed in differentiated keratinocytes and at very low levels in sensory neurons. Nevertheless, there are significant differences in thermal preference between TRPV4-null mice and wild-type mice, suggesting that epidermal TRPV4 channels may participate in the detection of warm (that is, comfortable) skin temperatures (18). Moreover, a recent study of TRPV4-null mice found that TRPV4 channels associate with AJs between the differentiated cells located beneath the cornified layer of the epidermis, where they promote cell-to-cell junction formation (33). TRPV4 is also present in the superficial layer of mouse corneal epithelium, and its activation contributes to a regulatory volume decrease triggered by hypotonic exposure (25). In contrast, TRPV4 is mainly expressed in the undifferentiated basal cells of the urothelium and is involved in stretch-induced ATP release from these cells (6). Because TRPV4-null mice exhibit abnormal voiding behavior and the amplitude of spontaneous contractions in explanted TRPV4-null bladder strips is significantly reduced, it has been suggested that TRPV4 channels in basal cells play a critical role in urothelium-mediated signal transduction via ATP release in response to intravesical mechanical stress. However, TRPV4 expression in the esophageal epithelium has not been previously investigated.

The goal of this study was to determine whether TRPV4 is expressed in the human esophagus and in the established

Address for reprint requests and other correspondence: T. Ueda, Dept. of Neurobiology and Anatomy, Graduate School of Medical Sciences, Nagoya City Univ., I Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya, Aichi 467-8601, Japan (e-mail: ueda@med.nagoya-cu.ac.jp).
nontumorigenic esophageal epithelial cell line HET-1A (15, 28, 36). We used HET-1A cells to test whether TRPV4 activation leads to elevated $[\text{Ca}^{2+}]_{i}$, ATP release, cell proliferation/cell death, and the secretion of the multifunctional cytokine IL-8. In addition, we examined the effect of TRPV4 activation on IL-8 production induced by IL-1β stimulation, as recent studies suggest that increasing $[\text{Ca}^{2+}]_{i}$ by activating TRPV4 channels may represent a potential therapeutic approach to treating polycystic kidney disease (7) and rheumatoid arthritis (13).

**MATERIALS AND METHODS**

**Reagents.** 4α-PDD, ionomycin, GdCl₃, carbenoxolone (CBX), flufenamic acid (FFA), bafilomycin A (BFA), bisindolylmaleimide XI hydrochloride (BIM) and DMEM were purchased from Sigma-Aldrich (St. Louis, MO). Ruthenium red (RuR) was obtained from Wako Pure Chemical (Osaka, Japan). Keratinocyte medium (KM) and fetal calf serum were purchased from ScienCell Research Laboratories (Carlsbad, CA) and Biological Industries (Beit HaEmek, Israel), respectively.

**HET-1A cell culture.** HET-1A cells (15, 28, 36) were obtained from the American Type Culture Collection and grown in T-75 flasks that were precoated with collagen (AteloCell, IPC-30; KOKEN, Tokyo, Japan). The cells were maintained in KM supplemented with penicillin and streptomycin in a humidified incubator with 5% CO₂-95% air at 37°C. The cells were passaged weekly and were used for experiments between passages 4 and 18. For calcium imaging, HET-1A cells were grown on glass coverslips that were precoated with collagen. The cells were allowed to recover for at least 48 h after plating before the experiments were performed.

**RT-PCR.** Total RNA was isolated from HET-1A cells, and 3 μg of RNA was used as a template for random-primer reverse transcription using SuperScript III (Invitrogen, Carlsbad, CA). Next, 1/40 of the sample was amplified by PCR for 30–35 cycles with the following primers: TRPV4 (GenBank Accession No. NM_021625), 5′-TACCTGTGTGCCATGTTCATCT-3′ and 5′-TGCTATAGGTCCCCGT-CAGCTT-3′ (position 1,521–1,899, yielding a 379-bp amplicon); TRPV1 (NM_080704) 5′-CTGCGGACCCACTCCAAAAGGA-3′ and 5′-AGAGCAAGCAGGTCTTCAGATC-3′ (310 – 681, 372 bp); pannexin 1 (Panx1; NM_015368), 5′-CTGTTCTGCGGTTCGCAGCT-3′ and 5′-AGATGCCAAGCCAATGAGT-3′ (759 –1,202, 444 bp); pannexin 2 (Panx2; NM_052839), 5′-CTCGGCACCAAGAAGGCCAA-3′ and 5′-CAGCGTGTTGATGTGCAGCA-3′ (1,564 –1,893, 330 bp); pannexin 3 (Panx3; NM_052959), 5′-CTGGCCTTGTCTCATGTCACT-3′ and 5′-CGAGGTGAAGATGAGCAAGA-3′ (349 – 664, 316 bp); connexin 32 (Cx32;NM_001097642), 5′-ATGAACTGGA-CAGGTTTGTAC-3′ and 5′-ATGTGTTGCTGGTGAGCCA-3′ (96 – 397, 302 bp); connexin 43 (Cx43; NM_000165), 5′-ACTTGGCGTGACTTCACT-3′ and 5′-CATCAGTTGGCAACTTGA-3′.
(201–608, 408 bp); vesicular nucleotide transporter (VNUT, also referred to as SLC17A9; NM_022082), 5'-TTCTATCCTCTCTCCTGGCT-3' and 5'-GCTGATGATGGCCACAAGGT-3'(950–1,417, 468 bp); P2X7 receptor (NM_002562), 5'-CTCCAGTAACTGCTGTCGCT-3' and 5'-AGCTCTGAGGTGGTGATGCA-3' (1,170–1,631, 462 bp); H9252-actin (NM_001101), 5'-GATCCTCACCGAGCGCGGCTACA-3' and 5'-GCGGATGTCCACGTCACACTTCA-3' (657–954, 298 bp). As a negative control, the reverse transcription step was omitted, and the isolated RNA was processed and analyzed in the same way. The PCR products were separated in 1% agarose gels by electrophoresis. The molecular identity and homogeneity of the PCR products were confirmed by DNA sequencing.

**Plasmid construction and transfection.** The coding sequence of mouse TRPV4 cDNA was subcloned into the pcDNA3.1 vector (Invitrogen), yielding the construct mTRPV4-pcDNA. The empty pcDNA3.1 vector (empty-pcDNA) was used as a negative control. Human embryonic kidney 293T (HEK293T) cells were cultured with DMEM supplemented with 10% FCS (vol/vol) at 37°C in humidified air with 5% CO2-95% air. The cells were seeded onto 60-mm dishes and incubated at 37°C. After 24 h, the cells were washed with DMEM, transiently transfected with mTRPV4-pcDNA or empty-pcDNA using Lipofectamine 2000 (Invitrogen), and incubated for 48 h.

**Small-interference RNA.** Oligonucleotides targeting human TRPV4 (siRNA1: Silencer predesigned siRNA ID s34001, 5'-UUCUCUAUGAUUGCUGUC-3'; and siRNA2: Silencer predesigned siRNA ID s34002, 5'-AUCUUGUAAACAAACUUGG-3'), Panx1 (ID s24448, 5'-UAAUAGCCCAGGUAGAUAC-3'), and scrambled control (Silencer negative control #1 siRNA; AM4611) were purchased from Applied Biosystems (Foster City, CA). HET-1A cells were transfected with 30 pmol of either the scrambled control, siRNA-TRPV4, or siRNA-Panx1 using Lipofectamine 2000. At 48 h posttransfection, the cells were replated, incubated an additional 48 h, and then used in assays. Because the TRPV4 siRNA1 failed to inhibit expression (as

![Fig. 2. 4α-PDB induces a transient increase in intracellular Ca²⁺ concentration ([Ca²⁺]) in HET-1A cells. A: representative trace showing that HET-1A cells respond to 4α-PDB, a specific TRPV4 agonist. The responses were blocked by ruthenium red (RuR), a TRPV channel blocker. The effect was reproducible although repetitive stimulation at 10 μM desensitized channel activity. B: RuR significantly blocked the 4α-PDB-induced increase in [Ca²⁺]. In this and subsequent figures, the numbers in parentheses indicate the total number of cells measured. The data represent the means ± SE of at least 3 independent experiments. Significant differences between the control group and the respective groups are indicated (***P < 0.001). C: concentration-response relationship of a 4α-PDB-induced increase in [Ca²⁺]. In this and subsequent figures, the numbers in parentheses indicate the total number of cells measured. The data represent the means ± SE of at least 3 independent experiments. Significant differences between the control group and the respective groups are indicated (***P < 0.001). D: at 37°C, the cells were robustly activated by 4α-PDB even at 1 μM. 4α-PDB at 3 μM evoked a greater calcium response. E: 4α-PDB at 1 μM failed to stimulate HET-1A cells at room temperature (20–25°C) but caused a significant increase of [Ca²⁺] at 37°C. The data represent the means ± SE of at least 3 independent experiments.
determined at both the mRNA and protein level), siRNA2 was used for these experiments.

Western blot analysis. HET-1A cells or HEK293T cells transfected with mTRPV4-pcDNA or empty-pcDNA were washed three times with PBS and then lysed in 100 μl of lysis buffer [20 mM Tris-HCl (pH 7.4), 0.1% SDS, 1% Triton X-100, and 1% sodium deoxycholate]. The lysates were centrifuged at 17,300 g for 30 min at 4°C, and the protein concentration in the supernatant was measured with the DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). Equal amounts of total protein (20 μg) were resolved by SDS-polyacrylamide (7.5%) gel electrophoresis. TRPV4 was detected with an anti-TRPV4 antibody raised against the synthetic peptide CDGHQQ-GYAPKWARADEALP (corresponding to amino acid residues 853–871 of rat TRPV4) (1:1000) (ACC-034; Alomone Laboratories, Jerusalem, Israel), followed by a horseradish peroxidase-labeled anti-rabbit IgG secondary antibody (1:1000) (Promega, Madison, WI), which was then visualized with the ECL Western Blotting Detection kit (GE Healthcare, Buckinghamshire, UK). β-Actin was detected with a monoclonal anti-actin antibody (clone AC-40, Sigma) followed by a horseradish peroxidase-labeled anti-mouse IgG secondary antibody (Promega), which was then visualized with the ECL detection kit.

Immunohistochemistry. Frozen sections of human normal adult esophagus (from a 66-yr-old male, T1234106, lot. A804297) were obtained from BioChain Institute (Hayward, CA). After being air-dried, the HET-1A cells and human frozen sections were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 20 min at 4°C, then incubated overnight at 4°C with the anti-TRPV4 antibody (1:1000) in phosphate-buffered saline containing 0.3% Triton X-100. After being washed, the samples were incubated with Alexa 488-conjugated donkey anti-rabbit IgG secondary antibody (1:1000) (Invitrogen). The specificity of the primary antibody was confirmed by signal ablation using the antigenic peptide according to the manufacturer’s specifications.

Measurement of [Ca2+]. HET-1A cells that were fixed on collagen-coated coverslips were moved to a chamber for Ca2+ imaging and incubated at room temperature for 30 min with the ratiometric fluorescent Ca2+ indicator fura-2 AM (acetoxyethyl ester) at 10 μM final concentration (Invitrogen) in assay buffer (containing 1 mM HEPES, 130 mM NaCl, 10 mM glucose, 5 mM KCl, 2 mM CaCl2, and 1.2 mM MgCl2, pH 7.4, 290 mOsm). The loading solution was washed out, and the cells were incubated in 500 μl of assay buffer and stimulated with 4α-PDD (0.01, 0.1, 0.3, 1.0, 10.0 or 20.0 μM), RuR (10 μM) or iomycin (3 μM) using a bath perfusion system with a flow rate of 2–3 ml/min. All calcium influx experiments were performed at room temperature (20–25°C), except when examining the effect of elevated temperature. In the experiments, the bath solution was maintained at 37°C with an electronic thermometer (PTC-201; Unique Medical, Tokyo, Japan). We recorded changes in [Ca2+], using an IX-70 microscope (Olympus, Tokyo, Japan) equipped with the ARGUS/HisCa system (Hamamatsu Photonics, Hamamatsu, Japan) (40). Fluorescent images were acquired and analyzed with ARGUS/HisCa software, version 1.65 (Hamamatsu).

Cell viability and proliferation assay. The viability and proliferation of HET-1A cells were evaluated using the Cell Counting Kit-8 (Dojin, Kumamoto, Japan), which is based on the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were plated on 96-well plates (at 5 × 10^3 cells/well), treated with 4α-PDD (3 or 10 μM) without changing the medium, and incubated at 37°C. After 5 min, the contents of the wells were centrifuged at 600 g for 2 min, and 100 μl of the supernatant was collected for ATP measurements. ATP concentration was determined by adding 100 μl of luciferin-luciferase reagent from the ATP Bio-luminescent Assay Kit (FL-AA, Sigma-Aldrich) to the 100-μl sample. The resulting chemiluminescence was measured with a Lumat LB 9507 tube luminometer (Berthold Technologies, Bad Wildbad, Germany) and is expressed in RLU. In some experiments, the cells were pretreated with a channel blocker [GdCl3 (100 μM), CBX (50 μM), FFA (50 μM), or BFA (50 nM)] for 30 min at 37°C.

ELISA for IL-8 production. The cells were plated in 96-well plates (2 × 10^4 cells/well) and stimulated with 1 μM 4α-PDD in the absence or presence of 5 ng/ml IL-1β for 24 h at 37°C. For control groups, vehicle solution (DMSO) without 4α-PDD was applied to cells that had been pretreated with or without IL-1β. IL-8 in all samples was measured in triplicate using the Quantikine Human IL-8 Immunoassay Kit (R&D Systems, Minneapolis, MN). The numbers in parentheses indicate the number of experiments.
CXCL8/IL-8 ELISA kit, D8000C (R&D Systems, Minneapolis, MN) according to the manufacturer’s specifications. Briefly, 100-μl assay diluents and 50-μl standards or samples from each well were incubated for 2 h at room temperature. After being washed with wash buffer, the samples were incubated for 1 h with an horseradish peroxidase-conjugated anti-IL-8 antibody against IL-8 and then with a substrate reagent (Molecular Devices). The values were compared against a standard curve that was generated using known concentrations of IL-8 to calculate IL-8 concentration in the samples (in pg/ml). In some experiments, the HET-1A cells were treated for 24 h at 37°C with the selective PKC inhibitor BIM (2 μM), together with 5 ng/ml IL-1β and 1 μM 4α-PDD.

Statistical analysis. Pooled data are shown as the means ± SE. The differences between groups were analyzed by an unpaired Student’s t-test and were considered significant when \( P < 0.05 \).

RESULTS

TRPV4 expression in HET-1A cells and human esophageal tissue. Total RNA was isolated from both early- and late-passage HET-1A esophageal epithelial cells and human esophageal tissue. RT-PCR was then performed using intron-spanning TRPV4-specific primers (see MATERIALS AND METHODS). Products of the expected size (379 bp) and nucleotide sequence for TRPV4-derived mRNA were amplified from the HET-1A cells and human esophageal tissue (Fig. 1A). No product was observed when the reverse transcriptase was replaced with water during the RT reaction (data not shown).

To confirm the presence of TRPV4 protein in HET-1A cells, we next performed Western blot analysis using a rabbit polyclonal antibody raised against a peptide near the COOH terminus of the TRPV4 protein (see MATERIALS AND METHODS). As shown in Fig. 1B, this anti-TRPV4 antibody was bound tightly to a prominent band of about 100 kDa in total extracts from cultured HEK293T cells transfected with mouse TRPV4 cDNA but not in extracts from cells transfected with the empty vector, indicating that this antibody recognizes TRPV4 protein. This is consistent with a previous study in which an immunoreactive protein band of ~100 kDa was detected in HeLa cells that expressed TRPV4 protein cloned from human tracheal epithelial cells (1). In HET-1A cells, this antibody detected two bands of ~75 and 100 kDa; these bands were not detected when the antibody was preabsorbed with the synthetic peptide. This indicates that TRPV4 protein is endogenously expressed in HET-1A cells. The 75-kDa band may represent a TRPV4 variant, as reported previously (1).

We next performed fluorescent immunohistochemistry on human esophagus tissue to determine the precise location of the TRPV4 protein within the esophageal mucosa. We found strong positive TRPV4 immunoreactivity in the basal cells of the esophageal epithelium, and this immunoreactivity grew weaker as we examined cells closer to the luminal surface (Fig. 1C, a). This antibody also stained HET-1A cells (Fig. 1C, c). Immunoreactivity was markedly reduced by preabsorbing the antibody with the antigenic TRPV4 peptide (Fig. 1C, b and d).

Fig. 4. siRNA-TRPV4 reduces 4α-PDD-induced calcium responses in HET-1A cells. A: in siRNA2-TRPV4-transfected HET-1A cells (siRNA2TRPV4), 10 μM 4α-PDD did not elicit a significant calcium response. B: siRNA-TRPV4 reduced the number of the cells showing a 4α-PDD-induced calcium response. The numbers in parentheses indicate the number of cells measured. C: responses obtained from individual siRNA2-TRPV4-transfected HET-1A cells were significantly reduced as well. The numbers in parentheses indicate the number of cells measured. The data represent the means ± SE, and significant differences between the control group (scramble) and the respective groups are indicated (***\( P < 0.001 \)).
The effects of 4α-PDD on [Ca$^{2+}$], in HET-1A cells. To identify the functional activity of TRPV4 channels, we examined the effects of 4α-PDD, a selective TRPV4 agonist, on cytosolic Ca$^{2+}$ levels using a Ca$^{2+}$ imaging system and a Ca$^{2+}$-fluorescent probe (10 μM fura-2 AM) in HET-1A cells. Single applications of 4α-PDD produced highly variable responses in HET-1A cells from different coverslips. In 24.6% of HET-1A cells examined (55 of the 224 cells), the application of 3 μM 4α-PDD caused a slight increase in [Ca$^{2+}$], and the sequential addition of 10 μM 4α-PDD markedly increased [Ca$^{2+}$], from a resting level of 0.2 ± 0.01 to 0.3 ± 0.03 (n = 20, P < 0.01) at room temperature (Fig. 2A). Increasing the concentration of 4α-PDD to 20 μM increased [Ca$^{2+}$] in 127 (56.7%) of the 224 cells. All 4α-PDD responses were blocked by the presence of the TRPV channel blocker RuR (10 μM) (Fig. 2, A and B). Figure 2C summarizes the concentration-response relationships of the 4α-PDD-induced [Ca$^{2+}$], elevations in HET-1A cells.

Because moderate temperatures (25–34°C) activate TRPV4 channel and sensitize the channel activities induced by a wide range of microenvironmental chemical and physical signals (5, 18, 23), we examined the effect of elevated temperature on the 4α-PDD response in HET-1A cells. For this experiment, 4α-PDD was applied to the cells at 37°C, which is the temperature at which the cells are cultured and the physiological temperature of human esophageal tissue. At this temperature, 27 (27%) of the 105 HET-1A cells tested responded robustly to only 1 μM 4α-PDD (Fig. 2, D and E). This response to 1 μM 4α-PDD at 37°C was comparable to the response to 10 μM at room temperature, indicating that elevating temperature to physiological levels greatly facilitated the ability of the channels to activate. However, because RuR is not a specific TRPV4 channel blocker and 4α-PDD may activate other TRP channels at higher concentrations, we examined the ability of 4α-PDD to elevate [Ca$^{2+}$], in HET-1A cells transfected with either siRNA-TRPV4 (siRNA2, to selectively knock down TRPV4 expression) or a scrambled (nonsilencing) siRNA. Transfection with siRNA2 effectively blocked the expression of TRPV4 at the mRNA (Fig. 3A) and protein (Fig. 3, B and C) levels by about 30% after 72–96 h. In addition, the response to 10 μM 4α-PDD at room temperature was significantly decreased in siRNA2-transfected HET-1A cells (Fig. 4A). Specifically, only 6.3% (5/80) siRNA2-transfected HET-1A cells responded to 4α-PDD, compared with 18.8% (21/112) of scrambled-siRNA-transfected cells (Fig. 4B). Furthermore, the absolute responses of individual 4α-PDD-responding siRNA2-transfected cells were significantly smaller than the responses elicited in scrambled-siRNA-transfected cells (Fig. 4C). Thus the intracellular calcium elevations induced by 4α-PDD at 10 μM are likely exclusively mediated by TRPV4 channels in HET-1A cells.

The effect of TRPV4 activation on cell viability. Recent studies have suggested that several Ca$^{2+}$-permeable TRPV channels are involved in the migration and proliferation of certain epithelial cells (31, 46). We examined the influence of TRPV4-mediated Ca$^{2+}$ influx on HET-1A cell viability using a quantitative colorimetric assay kit based on the MTT test. Surprisingly, 4α-PDD had the opposite effect on cell viability at different concentrations. Stimulation for 24 h at 37°C with 1 μM 4α-PDD slightly but significantly increased cell viability to 115 ± 4% (n = 30 wells, P < 0.05 vs. control), whereas stimulation with 10 and 30 μM 4α-PDD dramatically decreased cell viability to 77 ± 6% (n = 30 wells, P < 0.01 vs. control) and 39 ± 3% (n = 30 wells, P < 0.001 vs. control), respectively (Fig. 3A). To confirm that the increased cell viability was of the result of modified cell proliferation, we

Fig. 5. 4α-PDD differentially affects cell viability and proliferation in HET-1A cells depending on the degree of TRPV4 activation. A: 4α-PDD at 1 μM increased cell viability, whereas higher concentrations of 10 and 30 μM decreased cell viability. B: increased proliferation by 1 μM 4α-PDD was confirmed by the bromodeoxyuridine (BrdU) incorporation ELISA assay. C: this effect of 4α-PDD disappeared in HET-1A cells transfected with siRNA2-TRPV4 (siRNA2[TRPV4]). The data represent the means ± SE of at least 3 independent experiments. Significant differences between the control group and the respective groups are indicated (*P < 0.05; **P < 0.01; ***P < 0.001; n.s., not significant).
channel activation. Cells in a concentration-dependent manner (3/8251-PDD increased ATP release from the brief stimulation with 4
stimulus using an ATP release assay in HET-1A cells (Fig. 6). Is involved in functional signaling in response to a stress
immune defense, cell volume regulation, cell proliferation, and epithelial ion and water transport
mitogenesis, apoptosis, and epithelial ion and water transport

The data represent the means ± SE of at least 3 independent experiments. Significant differences between the control group and the respective groups are indicated (*P < 0.05; **P < 0.001).

**A** 4α-PDD-induced TRPV4 activation in HET-1A cells. Extracellular ATP concentrations were measured 5 min after stimulation. A: 4α-PDD induced ATP release in a concentration-dependent manner. The responses were inhibited by simultaneous administration of RuR. The data represent the means ± SE of at least 3 independent experiments. Significant differences between the control group and the respective groups are indicated (***P < 0.001; **P < 0.001). B: role of TRPV4 in ATP release was examined using siRNA. 4α-PDD significantly increased extracellular ATP concentration in HET-1A cells transfected with scrambled siRNA but failed to increase extracellular ATP in cells treated with siRNA against TRPV4 (siRNA2TRPV4). The data represent the means ± SE of at least 3 independent experiments. Significant differences between the control group and the respective groups are indicated (*P < 0.05; n.s., not significant).

**B** Relative ATP release (RLU)

---

investigated the effect of 4α-PDD on BrdU incorporation. Figure 5B shows that 0.1, 1.0 and 10.0 μM 4α-PDD significantly increased BrdU incorporation, indicating that 4α-PDD modulates the proliferation of HET-1A cells. Knockdown of TRPV4 with siRNA2-TRPV4 transfection completely blocked the increased proliferation induced by stimulating cells with 1 μM 4α-PDD for 24 h at 37°C (Fig. 5C).

**ATP release from HET-1A cells by 4α-PDD-induced TRPV4 channel activation.** Many stressors evoke ATP release for immune defense, cell volume regulation, cell proliferation, mitogenesis, apoptosis, and epithelial ion and water transport in surrounding cells (2). We examined whether TRPV4 activity is involved in functional signaling in response to a stress stimulus using an ATP release assay in HET-1A cells (Fig. 6). Brief stimulation with 4α-PDD increased ATP release from the cells in a concentration-dependent manner (3 μM: 2.3 × 10^6 ± 3.4 × 10^5 RLU; 10 μM: 2.7 × 10^6 ± 1.8 × 10^5 RLU) (Fig. 6A). Both of these levels were significantly greater (P < 0.01 and P < 0.001, respectively) than resting release (which was 1.6 × 10^6 ± 1.7 × 10^5 RLU, n = 10). RuR significantly reduced ATP release induced by 10 μM 4α-PDD to 2.0 × 10^6 ± 1.4 × 10^5 RLU (n = 10, P < 0.01 vs. 10 μM 4α-PDD without RuR) (Fig. 6A). Moreover, transfection with siRNA2-TRPV4 completely suppressed the 4α-PDD-induced ATP release from HET-1A cells (Fig. 6B).

ATP is released from cells via several different efflux mechanisms, including maxianion channels (30), volume-sensitive outwardly rectifying chloride channels (10), members of the ATP-binding cassette protein family (35), gap junction hemichannels (37), P2X7 receptor channels/pores (27), and vesicle exocytosis (26). To determine which pathway mediates ATP efflux in HET-1A cells, we first performed a RT-PCR analysis. In these cells, putative transcripts coding for Panx1 and Cx43 were amplified, whereas those coding for the Panx2, Panx3, Cx32, VNUT, and P2X7 channels were barely detectable (Fig. 7A). We next examined the effect of several inhibitors on TRPV4-induced ATP release in HET-1A cells. Figure 7B shows that pretreatment with CBX and FFA (inhibitors of gap junction hemichannels, such as Panx and Cx) significantly attenuated ATP release, whereas GdCl3 (a maxianion channel

---

Panx1 and Cx43 are involved in 4α-PDD-induced ATP release in HET-1A cells. A: RT-PCR analysis revealed that Panx1 and Cx43 are candidate molecules that are permeable to ATP in HET-1A cells. TRPV4 and β-actin were used as positive controls for mRNA isolated from HET-1A cells. Right: primer sets for vesicular nucleotide transporter (VNUT) and the P2X7 receptor correctly amplified the target genes in the foliate papillae (Fp) and the cerebral cortex (Cc), respectively. B: HET-1A cells were pretreated with carbexonolone (CBX; 50 μM), flufenamic acid (FFA; 50 μM), GdCl3 (Gd; 100 μM), or batrilocycin A (BFA; 50 nM) for 30 min at 37°C. Five minutes after stimulation with 4α-PDD, the supernatants were collected, and ATP content was measured (n = 10). Each value represents the mean ± SE. Significant differences between the control group (4α-PDD) and the respective groups are indicated (*P < 0.05; ***P < 0.001). RLU, relative luminescence units.
inhibitor) and BFA (an inhibitor of the vesicular H+/ATPase) were without effect. We examined the effect of GdCl₃ at several concentrations (50, 100, and 200 µM), all of which failed to inhibit ATP release in HET-1A cells (data not shown). To confirm the involvement of gap junction hemichannels in TRPV4-mediated ATP release, we further examined the effect of 4α-PDD on ATP release using HET-1A cells transfected with either siRNA-Panx1A or scrambled siRNA (Fig. 8). Transfection with siRNA-Panx1, but not the scrambled siRNA, suppressed 4α-PDD-induced ATP release from HET-1A cells (Fig. 8B).

The inhibitory effect of TRPV4 activation on IL-8 release induced by IL-1β. In recent years, many factors that induce IL-8 production in esophageal epithelial cells, including PAR2 (48) and CaSR (15), have been studied. This is because increased IL-8 expression has been found in esophageal biopsy specimens from patients with reflux esophagitis (12, 34). Moreover, extracellular ATP stimulates cytokine production in C6 glioma cells (14) and keratinocytes (47). Thus we addressed the effect of TRPV4 activation on IL-8 production in HET-1A cells treated with or without IL-1β (5 ng/ml). Stimulation with 1 µM 4α-PDD for 24 h at 37°C did not increase IL-8 production (Fig. 9A). In contrast, treatment with IL-1β for 24 h robustly enhanced IL-8 production to 1,212 ± 61 pg/ml, compared with 2.5 ± 3 pg/ml in HET-1A cells that were not treated with IL-1β. Furthermore, the IL-1β-induced increase in IL-8 production was significantly reduced by treatment with 1 µM 4α-PDD (to 1,000 ± 39.8 pg/ml, *P < 0.001 vs. IL-1β without 4α-PDD, *n = 10 wells). As shown in Fig. 9B, silencing TRPV4 with siRNA2-TRPV4 attenuated the inhibitory effect of 4α-PDD on IL-8 production, suggesting that TRPV4 negatively regulates the robust production of IL-8 in HET-1A cells. It has been reported that phorbol esters activated human TRPV4 channels via PKC-dependent and -independent pathways (45). Thus we studied the effect of BIM, a selective PKC inhibitor, on 4α-PDD-mediated inhibition of
IL-1β-induced IL-8 production. We compared the effect of 4α-PDD in the presence or absence of BIM. The presence of BIM did not affect IL-1β-induced IL-8 production but did influence the inhibitory effect of 4α-PDD (Fig. 9C).

**DISCUSSION**

Our study demonstrates the presence of TRPV4 transcript and protein in basal cells of the human esophageal epithelium. Furthermore, we provide evidence that TRPV4 is a molecular component of stimulus-induced Ca\(^{2+}\) influx in the HET-1A human esophageal epithelial cell line, which retains the characteristics of basal epithelial cells (15). First, 4α-PDD, a potent activator of TRPV4 channels (41), increased [Ca\(^{2+}\)]\(_i\) in a subset of HET-1A cells. Second, elevated temperature (37°C), a modifier of TRPV4 activation (5), strongly potentiated the effect of low 4α-PDD levels on TRPV4. Third, all of these responses were inhibited by RuR, a TRPV channel antagonist, and by transfection of siRNA to silence TRPV4 expression. Finally, TRPV4-specific immunoreactivity was detected in HET-1A cells. Moreover, 43% of HET-1A cells were resistant to 4α-PDD at the submaximum concentration of 20 μM, indicating heterogeneous expression of TRPV4 throughout the cell population.

The physiological role of TRPV4 channels in basal esophageal epithelial cells is presently unknown. Our Western blot analysis showed a prominent band of about 100 kDa, suggesting that the TRPV4 protein complement in HET-1A cells is composed primarily of full-length TRPV4 (TRPV4-A) (1). TRPV4-A can respond to a wide variety of endogenous physical and chemical stimuli, including mechanical and osmotic stressors, AA, the endocannabinoids anandamide and 2-AG, and cytochrome P450 components in the inhibitory signaling cascade. TRPV4 channels (41), increased [Ca\(^{2+}\)]\(_i\) in the HET-1A human esophageal cell line, which retains the characteristics of basal esophageal epithelial cells (15). First, 4α-PDD, a potent activator of TRPV4 channels (41), increased [Ca\(^{2+}\)]\(_i\) in a subset of HET-1A cells. Second, elevated temperature (37°C), a modifier of TRPV4 activation (5), strongly potentiated the effect of low 4α-PDD levels on TRPV4. Third, all of these responses were inhibited by RuR, a TRPV channel antagonist, and by transfection of siRNA to silence TRPV4 expression. Finally, TRPV4-specific immunoreactivity was detected in HET-1A cells. Moreover, 43% of HET-1A cells were resistant to 4α-PDD at the submaximum concentration of 20 μM, indicating heterogeneous expression of TRPV4 throughout the cell population.

The physiological role of TRPV4 channels in basal esophageal epithelial cells is presently unknown. Our Western blot analysis showed a prominent band of about 100 kDa, suggesting that the TRPV4 protein complement in HET-1A cells is composed primarily of full-length TRPV4 (TRPV4-A) (1). TRPV4-A can respond to a wide variety of endogenous physical and chemical stimuli, including mechanical and osmotic stressors, AA, the endocannabinoids anandamide and 2-AG, and cytochrome P450 metabolites of AA, such as epoxyeicosatrienoic acids (18, 23, 42). It is therefore possible that TRPV4 channels function as a multimodal sensor in the basal epithelial cells of the human esophagus. In addition, we speculate that the Ca\(^{2+}\)-permeable TRPV4 channels might participate in other Ca\(^{2+}\)-mediated cellular processes, such as proliferation/differentiation, viability and/or inflammation. Indeed, 4α-PDD at 1 μM increased cell viability and BrdU incorporation in HET-1A cells, whereas higher concentrations (e.g., 30 μM) significantly decreased cell viability. Thus TRPV4 may be a novel regulator of [Ca\(^{2+}\)]\(_i\), that is linked to both cell proliferation and cell death in the basal cells of the human esophagus.

Classic mediators of inflammation, including cytokines and chemokines, have been implicated in the pathophysiology of esophageal inflammation (28, 29). It was recently reported that functional TRPV4 is present in intestinal epithelial cells, and its activation caused a dose-dependent increase in [Ca\(^{2+}\)]\(_i\) and chemokine release, as well as colitis (3). In HET-1A cells, however, stimulating TRPV4 with 1 μM for 24 h at 37°C 4α-PDD was without effect on IL-8 production (Fig. 9). In contrast, TRPV4 activation suppressed IL-1β-induced IL-8 production. This same effect of TRPV4 activation was also observed in the human synoviocytes from patients with rheumatoid arthritis (13). As the inhibitory effect of 4α-PDD on IL-1β-induced IL-8 production was suppressed by a selective PKC inhibitor and TRPV4 knockdown, both TRPV4 and PKC activity may be essential components in the inhibitory signaling cascade. TRPV4 channels may play a role in the immunological function of basal epithelial cells of the human esophagus.

We also found that TRPV4 activation gives rise to ATP release from resting HET-1A cells via gap junction hemichannels, such as Panx1 and Cx43. Extracellular ATP is known to regulate proliferation, differentiation, and the death of keratinocytes by activating the membrane P2 receptor (2, 8). In addition, ATP is an afferent transmitter in certain tissues. Exogenous ATP activates pelvic nerve afferents in the rat colorectum (44), and Ca\(^{2+}\) waves in human epidermal keratinocytes are transmitted to sensory neurons via P2Y2 receptor activation (17). Mouse gustatory stimuli cause taste receptor cells to secrete ATP through Panx1, and ATP further stimulates other taste cells to release a second transmitter, serotonin (11). In the porcine lower esophageal sphincter, relaxation after stimulation of intrinsic inhibitory motor neurons is mediated by nonnitrergic, apamin-sensitive neurotransmission mainly mediated by ATP, ADP, or a related purine acting on P2Y1 receptors (4). A recent study has shown that deletion of P2X3 receptors blunts gastroesophageal sensation in mice (20). Altogether, ATP released from basal esophageal epithelial cells may function as an extracellular signaling molecule in the intraepithelial system controlling proliferation and differentiation as well as in neurosensory transduction in the human esophagus.

In summary, our study is the first to demonstrate the expression of TRPV4 channels in human basal esophageal epithelial cells and HET-1A cells. TRPV4 activation causes increased [Ca\(^{2+}\)]\(_i\) and the release of ATP via Panx1 and Cx43. In addition, TRPV4 activation negatively regulates the production of IL-8. TRPV4 is therefore likely to act as a regulator of multiple Ca\(^{2+}\)-dependent cellular functions in human esophageal epithelial cells.

**ACKNOWLEDGMENTS**

We thank Katsuyuki Tanaka and Kenji Kajita for technical assistance.

**GRANTS**

This investigation was supported by a Grant-In-Aid for scientific research from the Japan Society for the Promotion of Sciences (to T. Ueda, T. Kamiya, and S. Ugawa).

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**REFERENCES**

7. Gradilone SA, Masyuk TV, Huang BQ, Banales JM, Lehmann GL, Radtke BN, Stroope A, Masyuk AI, Splinter PL, LaRusso NF. Acti-
TRPV IN HUMAN ESOPHAGEAL EPITHELIAL CELLS

G147


15. Kalabis J, Oyama K, Okawa T, Nakagawa H, Michaylira CZ, Stairs N, Roper SD.


