Biochemical and Biophysical Research Communications 383 (2009) 130–134

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

TRPV3, a thermosensitive channel is expressed in mouse distal colon epithelium

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ARTICLE INFO

Article history:
Received 24 March 2009
Available online 29 March 2009

Keywords:
Calcium-permeable channel
Transient receptor potential channels
Thermoreceptor
Calcium imaging
Carvacrol
ATP
Distal colon
Mouse

ABSTRACT

The thermo-transient receptor potential (thermoTRP) subfamily is composed of channels that are important in nociception and thermo-sensing. Here, we show a selective expression of TRPV3 channel in the distal colon throughout the gastrointestinal tract. Expression analyses clearly revealed that TRPV3 mRNA and proteins were expressed in the superficial epithelial cells of the distal colon, but not in those of the stomach, duodenum or proximal colon. In a subset of primary epithelial cells cultured from the distal colon, carvacrol, an agonist for TRPV3, elevated cytosolic Ca^{2+}concentration in a concentration-dependent manner. This response was inhibited by ruthenium red, a TRPV channel antagonist. Organotypic culture supported that the carvacrol-responsive cells were present in superficial epithelial cells. Moreover, application of carvacrol evoked ATP release in primary colonic epithelial cells. We conclude that TRPV3 is present in absorptive cells in the distal colon and may be involved in a variety of cellular functions.

Introduction

The transient receptor potential (TRP) channel family is one of the largest families of ion channels and is composed of six subfamilies: TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystin), TRPL (mucolipin) and TRPA (ankyrin) [1–3]. It contains themoreceptors known as 'thermoTRPs (TRPV1, TRPV2, TRPV3, TRPV4, TRPM8 and TRPA1), which can be activated by increase and decrease in ambient temperature. Among thermoTRPs, TRPV3, whose gene is immediately adjacent to that of TRPV1 in human and mouse genomes can be activated by warm temperatures close to core body temperature [1–4]. It can alternatively be activated by diphenyl-containing compounds or camphor [1,2]. Unlike other temperature-sensitive TRPV channels, TRPV3 is expressed at very low levels in sensory neurons but are prominently expressed within keratinocytes [1,3]. Nevertheless, mice lacking the TRPV3 channel exhibit abnormal behavioral responses to innocuous and noxious heat [6], suggesting that the epithelial TRPV3 channel is indeed involved in cutaneous thermosensation.

The gastrointestinal tract is a sensory organ that responds to a large array of signals originating in the lumen, including nutrients, non-nutritive substances, mechanical factors and microorganisms [7]. The mucosa therefore requires ion channels and receptors in response to various stressors including warm and cold stimuli; for example, TRPV1, TRPV2, TRPV4, TRPM8 and TRPA1 are expressed in vagal afferent neurons projecting to stomach and upper gut [8], whereas TRPA1 plays a role in serotonin secretion in enterochromaffin cells [9]. Since it has been reported that the responses to temperature changes in body core thermoreceptors are similar to those of cutaneous thermoreceptors [10,11], it is possible that a TRP channel may be expressed in the gastrointestinal epithelium. In this study, we examined expression of themoTRPs in the mouse lower gastrointestinal tract and demonstrated that functional TRPV3 channel is specifically expressed in superficial epithelial cells in the mouse distal colon.

Materials and methods

The Center of Experimental Animal Sciences at Nagoya City University approved the following experiments.

Isolation of colonic epithelial cells. Preparation and characterization of colonic epithelial cells was done as previously described [12]. Briefly, distal colons excised from anesthetized C57BL/6J mice were cut open, gently stretched (epithelial side up), and incubated with 2.5 mg/ml dispase (Invitrogen) for 2 h at room temperature. The tissue was then treated with 0.05% trypsin in 0.02% EDTA for 20 min at 37 °C and resuspended in 10% FCS-DMEM (Invitrogen, Carlsbad, CA, USA). The resulting single cell suspension ($10–15 \times 10^4$ cells/ml) was used for the calcium imaging analysis.

Organotypic culture. Distal colons excised from anesthetized C57BL/6J mice were cut open, gently stretched (epithelial side up), and incubated with 2.5 mg/ml dispase (Invitrogen) for 2 h at room temperature. The tissues were then plated and fixed on cov-

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doi:10.1016/j.bbrc.2009.03.143

erglasses with Cell Tak medium (BD Biosciences, Bedford, MA, USA).

Reverse transcription-polymerase chain reaction (RT-PCR) analysis. Mouse RNA was isolated from the stomach, duodenum, proximal (proximal third) and distal (distal third) colons and skin (positive control). Then, 3 μg of RNA was subjected to random-primer reverse transcription using SuperScript II (Invitrogen). Next, 1/40 of the sample was amplified by PCR for 30 cycles with the following primers: TRPV1 (GenBank Accession No. NM_001001445), sense 5′-ctatgatcgcaggagcatcttcga-3′ (336–359) and antisense 5′-gaacttcacaatggccagctggtt-3′ (778–801) (product = 466 base pairs (bp)), TRPV2 (GenBank Accession No. NM_011706), sense 5′- ctcca ctggaaagacgtgcctgat-3′ (699–722) and antisense 5′- atcaggc acgtctttccagtggag-3′ (1099–1122) (product = 424 bp), TRPV3 (GenBank Accession No. NM_145099), sense 5′-cgctggcctcactgattgagaa-3′ (1875–1896) and antisense 5′-cccatgcggaatctgcttctca-3′ (2196–2217) (product = 343 bp), TRPV4 (GenBank Accession No. NM_022017), sense 5′-caaccagccgcacatcgtc aactaa-3′ (962–984) and antisense 5′-agggaggagaggtcgtagagaga-3′ (1326–1348) (product = 387 bp), TRPM8 (GenBank Accession No. NM_134252), sense 5′-ggtatggagagatttcccgagaca-3′ (2222–2245) and antisense 5′-cttcatcacagaagaggacgaaga-3′ (2501–2524) (product = 303 bp), TRPA1 (GenBank Accession No. NM_177781), sense 5′-gactaccctgtgcaccaggcagcat-3′ (625–649) and antisense 5′-cagtggc tccctgggttgcagcaaa-3′ (862–886) (product = 262 bp), β-actin (GenBank Accession No. NM_007393), sense 5′-gatcctgaccgagcgtggcta ca-3′ (652–674) and antisense 5′-acggatgtcaacgtcacacttca-3′ (927–949) (product = 298 bp). As a negative control, the reverse transcription step was omitted and the isolated RNA was analyzed in the same way. The PCR products obtained were separated by 1% agarose gel electrophoresis. The molecular identity and homogeneity of the resulting PCR products were checked by DNA sequencing.

In situ hybridization. C57BL/6J mice at 8 weeks of age (8 wk) were decapitated under deep anesthesia (Nembutal, intraperitoneal injection, 50 mg/kg), and their distal colons were quickly removed. Histological preparation of 10 μm sections and *in situ* hybridization were performed as previously described [13]. A [^{35}S]UTP-labeled complementary RNA probe for mouse TRPV3 (mTRPV3) was generated from nucleotide sequences (nucleotides 1725–2437) (GenBank Accession No. NM_145099).

Immunoblotting. Total protein extracts were obtained from HEK293T cells transfected with the empty pcDNA 3.1 expression vector (Invitrogen) or mTRPV3-containing pcDNA 3.1 and mouse distal colon. Equal amounts of protein (25 μg) were resolved by sodium dodecyl sulfate–polyacrylamide (10%) gel electrophoresis. TRPV3 was detected by an anti-TRPV3 antibody (C-12) (Santa Cruz, Santa Cruz, CA, USA), followed by horseradish peroxidase-labeled anti-goat IgG secondary antibody and then visualized with the ECL Western blotting Detection kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Immunohistochemistry. Fresh frozen sections were obtained from C57BL/6J mouse distal colon using a cryostat. After sections were air-dried, they were fixed in 4% paraformaldehyde/0.1 M phosphate buffer (pH 7.4) (4% PFA) for 20 min at 4 °C and incubated with the anti-TRPV3 antibody (1:1000) in phosphate-buffered saline containing 0.3% Triton X-100 overnight at 4 °C, followed by the Alexa 488-conjugated donkey anti-goat IgG secondary antibody (Invitrogen). Specificity was confirmed by signal ablation with its antigenic peptide [TRPV3 (C-12) p, Santa Cruz] according to the manufacturer's protocol.

Measurements of [Ca^{2+}]i concentration. Both single cell suspension and organotypic cultures of mouse distal colonic epithelial cells were plated and fixed on coverglasses with Cell Tak medium. The fixed cells were incubated with the fluorescent Ca^{2+}indicator, fura-2 acetoxymethyl ester (10 μM) (Invitrogen), in assay buffer (10 mM Hepes, 130 mM NaCl, 10 mM glucose, 5 mM KCl, 2 mM $CaCl_2$ and 1.2 mM $MgCl_2$, pH 7.4, 290 mOsm) for 30 min at room temperature. The loading solution was washed out and the cells were incubated in 500 μl of assay buffer and stimulated with carvacrol (300 μM and 1 mM) (Sigma–Aldrich, St. Louis, MO, USA), allyl isothiocyanate (AITC) (100 μM) (Wako, Osaka, Japan), ruthenium red (10 μM) (Wako, Osaka, Japan), or ionomycin (3 μM) (Sigma–Aldrich) using a bath perfusion system at a flow rate of 1–2 ml/min. We recorded [Ca^{2+}]i changes using an Olympus IX-70 microscope equipped with the ARGUS/HisCa system (Hamamatsu Photonics, Hamamatsu, Japan) [14]. Acquisition and analysis of the fluorescence images were done using ARGUS/HisCa software (v 1.65).

ATP release assay. Following a period of stabilization during which baseline samples were collected, a single cell suspension (10,000–15,000 cells, 100 μl) isolated from distal colon was treated with 1 mM carvacrol or 3 μM ionomycin for 2 min. These supernatants (100 μl) were assayed for ATP released from the cells using the ATP Bioluminescent Assay Kit (FL-AA, Sigma–Aldrich) and a luminometer.

Statistical analysis. Pooled data are shown as means ± SE. Groups were compared with the Student's *t*-test. A value of $P < 0.05$ was considered significant.

Results

Expression of TRPV3 mRNA in mouse distal colonic epithelium

We carefully isolated the total RNA of mouse tissues and performed RT-PCR using specific primer sets. As shown in Fig. 1A, thermoTRP transcripts except for TRPM8 could be amplified from colonic tissues. TRPV3 transcript was specifically observed in the

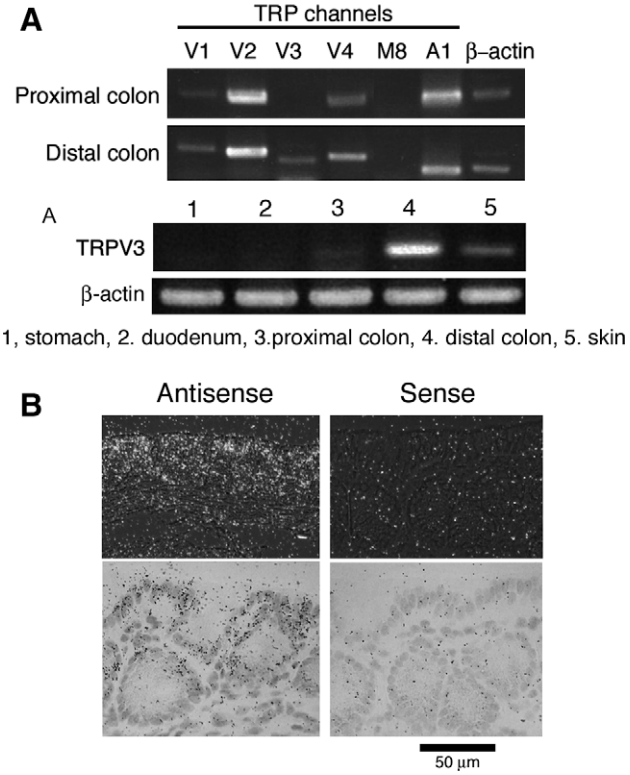

Fig. 1. (A) RT-PCR analysis of thermoTRP transcripts in mouse tissues. Control β-actin fragments are shown. (B) Expression of TRPV3 transcripts in the mouse distal colon by *in situ* hybridization. Strong expression of TRPV3 transcripts was observed in the superficial epithelial cells in the mouse distal colon when the TRPV3 antisense probe was used, whereas no signal was observed when the TRPV3 sense probe was employed. Scale bars: 50 μm.

distal part of colon. It was not amplified from the stomach, duodenum or proximal colon. We next performed *in situ* hybridization using [^{35}S]UTP-labeled riboprobes. TRPV3 transcripts were expressed intensely in the superficial epithelial cells in the distal colon (Fig. 1B). Labeling was not detected when the corresponding sense probe was used (Fig. 1B). In contrast, no apparent RNA signal for TRPV1, TRPV2, TRPV4 or TRPM8 channel was observed in the epithelium (data not shown).

Expression of TRPV3 proteins in mouse distal colonic epithelium

To confirm the expression of TRPV3 in the mouse distal colon at the protein level, we performed an immunoblot analysis using a rabbit polyclonal antibody against peptides mapping near the C-terminus of TRPV3. This antibody bound to a prominent band of ~80 kDa in total extracts of cultured HEK293T cells transfected with the mouse TRPV3 complementary DNA (Fig. 2A, lane 1), but not in those transfected with the corresponding empty vector (Fig. 2A, lane 2), indicating that this antibody specifically recognizes mouse TRPV3 proteins. Next, immunoblot analysis of the lysates derived from mouse distal colon performed with the same antibody detected the ~80 kDa protein band (Fig. 2A, lane 3), indicating that TRPV3 protein is expressed here.

We subsequently performed fluorescent immunohistochemical analyses on mouse distal colon to precisely localize TRPV3 within the colonic mucosa. The analyses revealed that the TRPV3 channel proteins were located mainly in the superficial epithelial cells of the colonic mucosa (Fig. 2B). This antibody stained keratinocytes in the mouse skin (Fig. 2C), in concordance with a previous study. In contrast, no signal was detected in the proximal colon (Fig. 2D). Finally, preabsorption with a specific antigenic peptide for TRPV3 blocked fluorescence in the tissues (Fig. 2E), indicating that TRPV3 protein is expressed in the superficial epithelial cells of the mouse distal colon.

Functional expression of TRPV3 in mouse distal colonic epithelium

To identify the functional activity of TRPV3 in mouse colonic epithelial cells, we examined the effects of carvacrol, a TRPV3 agonist, on cytosolic Ca^{2+}regulation using a Ca^{2+}imaging system and a Ca^{2+} fluorescent probe (10 μM fura-2/AM) in primary colonic epithelial cells. In 15% of cells (30 of 200), the application of 300 μM carvacrol caused a slight increase in intracellular ionic calcium ($[Ca^{2+}]i$) and the sequential addition of 1000 μM carvacrol markedly increased $[Ca^{2+}]i$ from a resting level of 0.51 ± 0.01 to 0.66 ± 0.03 ($n = 30$, $P < 0.01$) (Fig. 3A). Because carvacrol also activates the TRPA1 channel, allyl isothiocyanate (AITC), a TRPA1 ligand, was applied to the isolated cells. However, the colonic epithelial cells did not respond to 100 μM AITC (Fig. 3B). Repetitive application of 1 mM carvacrol also evoked an increase in $[Ca^{2+}]i$, and this response was blocked by 10 μM ruthenium red (RuR), a non-selective antagonist of TRPV channels (Fig. 3B). In organotypic cell culture, moreover, we found that the carvacrol-responsive cells were specifically located at the luminal surface and upper portion of colonic crypt, but not in the middle or deep portions of the crypt (Fig. 3C).

ATP release by carvacrol in mouse distal colonic epithelium

Multiple stressors evoke ATP release for immune defense, cell volume regulation, cell proliferation and mitogenesis, apoptosis and epithelial ion and water transport in surrounding cells [15]. To examine whether TRPV3 activity is involved in functional signaling in response to a stress stimulus, an ATP release assay was performed in mouse distal colon mucosa. Carvacrol (1 mM) stimulation caused increased ATP release from the cells (212 ± 18 fmol per 10^5 cells, $n = 10$, $P < 0.01$ vs. a resting level of 145 ± 15 fmol, $n = 10$) (Fig. 4). Ionomycin, a calcium ionophore, induced greater ATP release from the colonic cells (314 ± 37 fmol, $n = 10$, $P < 0.01$

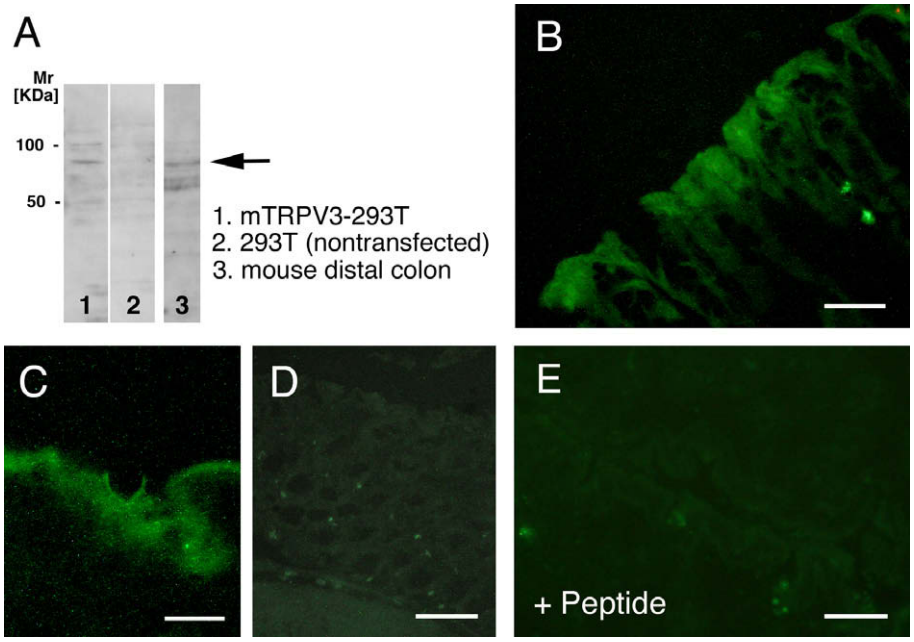

Fig. 2. Immunoblotting and immunohistochemical analysis of TRPV3 in the mouse distal colon. (A) Detection of TRPV3 protein (arrow) in total protein extracts of TRPV3-expressing or -non-expressing HEK293T cells (lane 1 and lane 2, respectively) and detection of TRPV3 protein in the protein extracts from mouse distal colon (lane 3). Mr: molecular mass standards. (B) Cellular localization of TRPV3 protein in the mouse distal colonic mucosa. (C) The TRPV3-immunoreactivity was also found in skin keratinocyte, as previously described. (D) No immunoreactivity was observed in the proximal colonic mucosa. (E) TRPV3-immunoreactivity in the distal colonic mucosa after preabsorption treatment with the antigenic peptide. Scale bars: 50 μm.

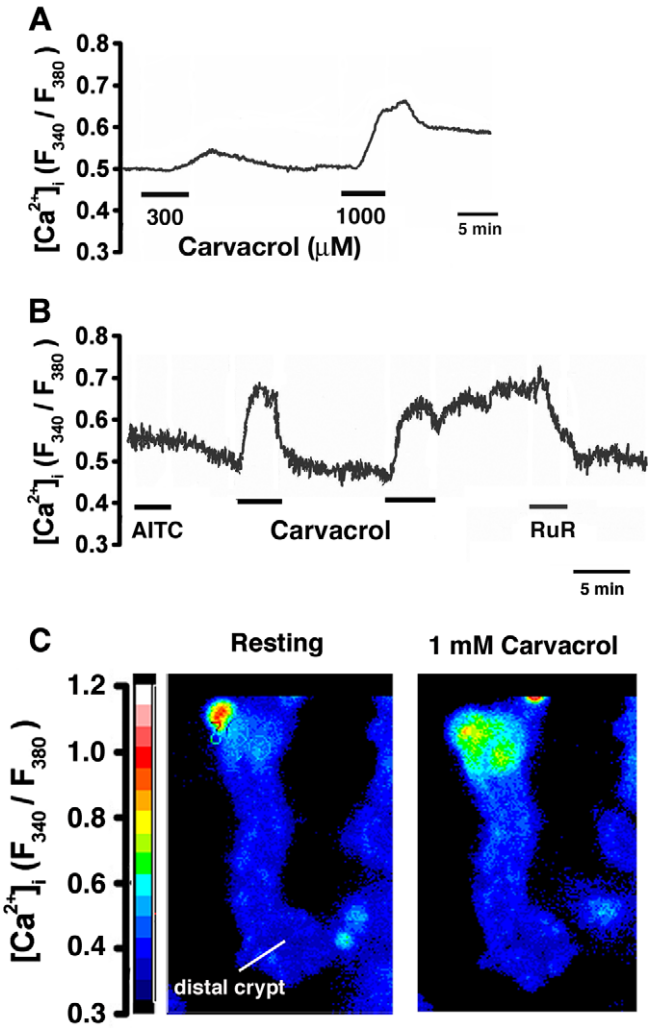

Fig. 3. Intracellular Ca^{2+} ($[Ca^{2+}]i$) response in primary cultured mouse distal colonic epithelial cells. (A) A subset of the cells responded to carvacrol, a TRPV3 agonist, in a dose-dependent manner. (B) Allyl isothiocyanate (AITC) did not evoke $[Ca^{2+}]i$ increases in carvacrol-reactive cells. Repetitive carvacrol stimulation (1 mM) caused responses without desensitization. The responses were abolished by extracellular ruthenium red (RuR, 10 μM), a nonselective TRPV channel blocker. (C) Organotypic calcium imaging analysis in primary cultured mouse distal colonic epithelium. Carvacrol (1 mM) evoked $[Ca^{2+}]i$ increases in the epithelial cells at the upper part of crypt, but not in cells at the middle and distal crypt.

vs. resting level), indicating a significant effect of intracellular calcium on ATP release (Fig. 4).

Discussion

ThermoTRPs are critical contributors to normal pain and temperature sensation [1–3]. When these channels were first discovered, attention was primarily focused on their potential contributions to direct thermal activation of peripheral sensory neurons. In these neurons, TRPV1 is both a receptor for capsaicin and other related pungent vanilloid compounds and a "heat receptor" capable of directly depolarizing neurons in response to temperatures >42 °C. TRPV2 exhibits a threshold for activation of 52 °C, 10 °C higher than that of TRPV1. However, recent anatomical, physiological, and behavioral studies have provided evidence that TRPV3 and TRPV4 expressed in skin epithelial cells can also contribute to thermosensation *in vitro* and *in vivo* [1,2,4,6]. Heat-evoked responses in mammalian cells expressing TRPV4 were detected once temperature reached 27–34 °C, whereas TRPV3 is activated by warm temperature with a threshold between 34 and 39 °C close to core body temperature.

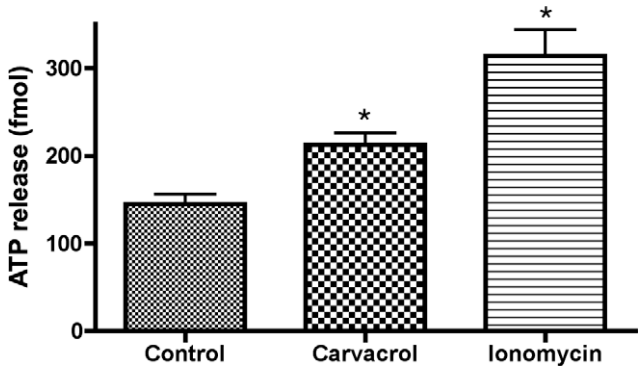

Fig. 4. ATP release after carvacrol stimulus in the mouse distal colon. Ionomycin, a calcium ionophore, was used to determine the involvement of $[Ca^{2+}]i$ increase in ATP release. Experimental data were obtained from 10 separate assays. $^{*}P < 0.01$ vs. control.

We have described here the isolation and characterization of TRPV3, which is abundantly and selectively expressed in superficial absorptive epithelial cells (enterocytes) in the mouse distal colon throughout the gastrointestinal tract. The full sequence of TRPV3 isolated from the mouse distal colon was identical to that of skin-type TRPV3, indicating that colonic TRPV3 is capable of responding to warm temperatures as well. Because the normal colorectal temperature generally ranges from 36 to 38 °C in the mouse [16] and TRPV3 only responds robustly to small perturbations around the core body temperature, TRPV3 in the distal colonic mucosa may function as an epithelial thermoreceptor involved in body core thermosensation.

Other physiological functions of TRPV3, apart from thermosensation, have been suggested. TRPV3 is activated by a large number of chemical ligands from exogenous and endogenous sources [1,2]. Among TRPV3 agonists, carvacrol, eugenol and thymol are major components of various plants such as oregano, savoy, clove and thyme. Xu et al. reported that TRPV3 is expressed in tongue and nasal epithelium and may be a molecular target of plant-derived skin irritants [5]. They also demonstrated that in mouse 308 keratinocytes, eugenol increased the release of interleukin (IL)-1a, suggesting that TRPV3 activation influences local immune responses. In this context, constitutively active mutations of TRPV3 have been linked to hair loss and atopic dermatitis-like skin lesions in rodents [17]. Transgenic mice with $TRPV3^{Gly573Ser}$, which led to increased ion channel activity, exhibited higher serum levels of CCL11, CCL17, IL-13, IL-17 and MCP-1 and spontaneously developed dermatitis [18]. Further studies are needed to determine whether TRPV3 plays a role in local immune responses in the colonic mucosa as well as in the skin.

In the present study, we demonstrated that mouse distal colonic mucosa released ATP in response to carvacrol, a TRPV3 agonist. Extracellular ATP is known to regulate a variety of functions in epithelial tissues by activating the membrane P2 receptor [15]. In rat distal colonic mucosa, luminal ATP induces K^+ secretion via the P2Y2 receptor [19]. ATP released into the lumen exerted an inhibitory effect on electrogenic Na^+ absorption via the P2Y2 receptor in the distal colon [20,21]. In addition, ATP has been shown to be an afferent transmitter in certain epithelial cells. In mouse taste buds, gustatory stimuli cause receptor cells to secrete ATP through pannexin 1 hemichannels and ATP further stimulates other taste cells to release a second transmitter, serotonin [22]. Wynn et al. have shown that exogenous ATP activates pelvic nerve afferents in the rat colorectum, suggesting that ATP can function as an extracellular signaling molecule in sensory transduction in the gastrointesti-

nal tract [23]. Since TRPV3 is activated by a number of chemical substances and strongly potentiated by phospholipase C, arachidonic acid and other unsaturated fatty acids [1–3], TRPV3 channel may function as a sensor to trigger ATP release from the superficial cells in the distal colon.

In conclusion, we found that transcript and protein encoding TRPV3 were abundantly expressed in the superficial absorptive cells of the mouse distal colon. Thus, we speculate that TRPV3 may play a role in thermo-sensing in the gastrointestinal tract, as originally described in skin. Furthermore, TRPV3 may participate in a variety of cellular functions in the distal colon, including immune defense, cell volume regulation, cell proliferation and mitogenesis, apoptosis and epithelial ion and water transport in surrounding cells.

Acknowledgments

We thank Katsuyuki Tanaka, Kenji Kajita and Masashi Iwata for technical assistance. This investigation was supported by a grant-in-aid for scientific research from the Japan Society for the Promotion of Sciences (to S.U. and S.S.).

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