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ORIGINAL PAPER

Potential role of transient receptor potential (TRP) channels in bladder cancer cells

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Abstract Transient receptor potential (TRP) channels play important roles in thermal, chemical, and mechanical sensation in various tissues. In this study, we investigated the differences in urothelial TRP channels between normal urothelial cells and bladder cancer cells. TRPV2 and TRPM7 expression levels and TRPV2 activator-induced intracellular Ca^{2+} increases were significantly higher, whereas TRPV4 expression and TRPV4 activator-induced intracellular Ca^{2+} increases were significantly lower in mouse bladder cancer (MBT-2) cells compared to normal mouse urothelial cells. The proliferation rate of MBT-2 cells overexpressing dominant-negative TRPV2 was significantly increased. In contrast, treatment with TRPV2 activators significantly decreased the proliferation rate. TRPM7-overexpressing MBT-2 cells proliferated more slowly, as compared to mock-transfected cells. Moreover, expression of dominant-negative TRPV2 significantly decreased plasma membrane Ca^{2+} permeability of MBT-2 cells as compared to that in mock-transfected cells. Increases in the expression of *TRPV2* mRNA, immunoreactivity, and TRPV2 activator-induced intracellular Ca^{2+} were also observed in T24 human bladder cancer cells. These results suggested that TRPV2 and TRPM7 were functionally expressed in bladder cancer cells and served as negative regulators of bladder cancer cell proliferation, most likely to prevent excess mechanical stresses.

Keywords Bladder cancer · TRP channel · Urothelial cells · Mechanosensation

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Introduction

The transient receptor potential (TRP) superfamily of cation channels plays critical roles in sensory physiology including thermal, chemical, and mechanical responses, in many types of cells. Recent studies have suggested that environmental factors, such as temperature, chemical exposure, pH, and mechanical stress, are sensed by specific TRP channels, which may play a role in the regulation of proliferation, differentiation, apoptosis, and oncogenesis [1–4]. Changes in the expression of TRPM1 in melanoma cells and TRPV6 and TRPM8 in prostate cancer cells have been reported [5–8]. However, the physiological roles of these TRP channels in cancer cells are still poorly understood.

Bladder cancer is the fourth most common cancer in men and has the eighth highest cancer-related mortality rate in adult men [9]. Approximately 70–90 % of new cases are superficial tumors (stage Ta/T1/in situ). The standard treatment for these patients is transurethral resection. However,

the tumor recurrence rate following transurethral resection of bladder tumors is relatively high (30–60 %). Postoperative intravesical chemotherapy and immunotherapy have been used as adjuvant treatments in patients with a high risk of recurrence. However, these treatments only provide an 8 % reduction in recurrence in this patient population [9].

Everaerts et al. [10] demonstrated the functional expression of TRPV4, TRPV2, and TRPM7 in mouse urothelial cells. TRPV4, which is thought to be a sensor for hypotonicity [11, 12], senses distension of the bladder urothelium; this signal is converted to an ATP signal in the micturition reflex pathway during urine storage [13]. A progressive decrease in the expression of the short splice-variant (s-TRPV2) accompanied by a marked increase in the expression of full-length TRPV2 (f-TRPV2) was found in high-grade and advanced-stage urothelial carcinoma tissues [14]. In addition, TRPV2 has been reported to induce apoptotic cell death in human T24 bladder cancer cells [15]. However, the expression patterns and functions of TRP channels in cancer are still not completely understood.

In this study, we sought to investigate the physiological roles of TRP channels in bladder cancer cells by comparing the expression of TRP channels in normal mouse urothelial cells and MBT-2 bladder cancer cells. To elucidate the physiological implications of TRP channel expression, we focused on the correlation between TRP channels and intracellular Ca^{2+} homeostasis, which is one of the key determinants of cancer cell proliferation.

Materials and methods

Chemicals

2-Aminoethoxydiphenyl borate (2-APB), lysophosphatidylcholine (LPC), GSK1016790A (GSK), and ionomycin (Iono) were obtained from Sigma.

Animals

All animal experiments were performed using 8-week-old male wild-type (WT) C3H/HeN mice (CLEA Japan Inc., Tokyo, Japan). All procedures were conducted in accordance with the policies of the Institutional Animal Care and Use Committee, National Institute for Physiological Sciences.

Preparation of primary mouse urothelial cells

Whole bladders were taken from anesthetized mice, and urothelial cells were prepared using previously reported procedures [13], with slight modification. Briefly, the bladder was everted by pushing the dome downward through the bladder neck with a blunt 18-gauge needle. The "everted ball bladder" was incubated with 0.05 % trypsin–EDTA (Life Technologies, Carlsbad, CA, USA) for 30 min on a shaker at 37 °C, and urothelial cells were harvested in supplemented keratinocyte serum-free medium (KSFM; Life Technologies) at 1×10^6 cells/mL. Drops of the cell suspension were seeded on fibronectin (50 μg/mL)-coated 60-mm dishes or cover slips (diameter 12 mm; Warner Instruments Inc., Hamden, CT, USA). All experiments with urothelial cells were performed after they had formed clusters, approximately 72 h after the start of cultivation.

MBT-2 cells

MBT-2 cells were derived from carcinogen (*N*-[4-(5-nitro-2-furyl)-2-thiazolyl]-formamide)-induced bladder tumors in C3H/He mice. This cell line has been used as an animal model to evaluate the efficacy of antitumor drugs in bladder cancer [16, 17]. MBT-2 cells (provided by Dr. Yamazaki, Kyoto University, Japan) were maintained in RPMI-1640 medium supplemented with 10 % heat-inactivated fetal bovine serum, 2 mM L-glutamine, nonessential amino acids (1×), and 1 mM sodium pyruvate at 37 °C in the presence of 5 % CO_2. Cells were passaged twice per week using 0.25 % trypsin and 0.02 % EDTA (Life Technologies). For MTS assays, 0.5 μg of plasmid DNA containing dominant-negative TRPV2 [18, 19], rat TRPV2 (provided by Dr. David Julius, University of California San Francisco, CA, USA), or human TRPM7 (provided by Dr. Yasuo Mori, Kyoto University, Japan) in OPTI-MEM (Life Technologies) were transfected into MBT-2 cells (2×10^4 cells/well in 24-well plates, Falcon) using Lipofectamine Plus Reagent (Life Technologies). After a 3.5-h incubation, the medium was changed to normal MBT-2 medium, and cells were again incubated for 27 h at 37 °C in the presence of 5 % CO_2 before use.

T24 cells

The T24 cell line is a poorly differentiated human bladder urothelial carcinoma cell line. T24 cells (obtained from the Human Science Research Resources Bank, Tokyo, Japan) were cultured in Eagle's Minimum Essential Medium (EMEM; Wako, Osaka, Japan), containing 10 % heat-inactivated fetal bovine serum, 1× nonessential amino acids (Life Technologies), and 1 mM sodium pyruvate (Life Technologies) at 37 °C in the presence of 5 % CO_2. Cells were passaged twice per week using 0.25 % trypsin and 0.02 % EDTA (Life Technologies).

Real-time polymerase chain reaction (PCR) of TRP channels

Total RNA was isolated using a Sepasol G kit (Nakarai, Kyoto, Japan), and first-strand cDNA was synthesized

using Superscript III reverse transcriptase (Life Technologies). Real-time PCR was performed with 10 μL SYBR Master Mix Reagent (Takara, Otsu, Japan), 0.25 μM specific primer sets (for all TRPMs, TRPVs, TRPCs, and TRPA1; see Table S1), and 1 μL of the prepared cDNA. Real-time PCR analysis was performed using a StepOne analyzer (Life Technologies). The temperature profile consisted of 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 1 min. To discriminate specific amplification from non-specific amplification, melting curve analysis was performed at the end of each PCR assay. To determine the starting cDNA amount, purified PCR products with known concentrations were serially diluted and used as standards.

Immunostaining

For immunocytochemistry, primary mouse urothelial cells, MBT-2 cells, and T24 cells were treated with phosphate-buffered saline with 0.1 % Tween-20 (PBST) and fixed with 4 % paraformaldehyde (PFA). Cells were incubated with primary antibodies [rabbit anti-TRPV2 antibodies (1:300, Transgenic Inc., Kumamoto, Japan) or rabbit anti-TRPV4 antibodies (1:200, Abcam, Tokyo, Japan)] overnight at 4 °C, followed by second antibodies (Alexa Rb 488, 1:500, Life Technologies) for 1 h at room temperature. The images were captured with a fluorescent microscope (Olympus, Tokyo, Japan).

Fluorescence measurement

Primary cultures of mouse urothelial cells, MBT-2 cells, and T24 cells were loaded with a fluorescent Ca^{2+} indicator (5 μM fura-2-acetoxymethyl ester, Life Technologies) in KSFM at 37 °C for 60 min. The extracellular solution contained 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose, with the pH adjusted to 7.4 using NaOH. Measurements of cytosolic Ca^{2+} concentrations were performed by ratiometric imaging with fura-2 at 340 and 380 nm, and the emitted light signal was read at 510 nm with a CCD camera (CoolSnap ES, Roper Scientific/Photometrics, Fairfax, VA, USA). The F 340/F 380 ratio was calculated and acquired with an imaging processing system (IP-Lab, Scanalytics Inc., Rockville, MD, USA) and ImageJ (http://rsbweb.nih.gov/ij/). Changes in the delta ratio were calculated by subtracting basal values from peak values and then normalized to that of ionomycin (5 μM, Sigma), which was used to examine cell viability.

Measurement of plasma membrane Ca^{2+} permeability

To measure plasma membrane Ca^{2+} permeability, we performed experiments using fura-2, as described previously [20]. Dominant-negative TRPV2 was transfected into MBT-2 cells together with Ds-Red plasmid (1/10 dilution), and Ca^{2+} imaging was performed 24 h later using fura-2. The initial Ca^{2+} levels in the cells were measured, and cells were then treated with 2 mM EDTA solution without Ca^{2+}. After the treatment, basal Ca^{2+} levels in the cells were determined. Next, 2 mM Ca^{2+} solution was added again to measure the peak ratio of the intracellular Ca^{2+} increase in Ds-Red-positive cells to measure plasma membrane Ca^{2+} permeability. All data were normalized to the value of ionomycin.

Proliferation assay

MBT-2 cells overexpressing dominant-negative TRPV2 [18, 19], which was reported to inhibit endogenous mouse TRPV2 activity, were used for proliferation assays. Rat TRPV2 and human TRPM7 were used for overexpression of TRPV2 and TRPM7, respectively. A TRPV2 activator, i.e., LPC (30 μM) or 2-APB (100 μM), was added 24 h before the proliferation assay. First, the medium was changed to 300 μL of normal MBT-2 medium with 60 μL of CellTiter96 Aqueous One Solution Cell Proliferation Assay (Promega, WI, USA), and cells were incubated for 3.5 h at 37 °C in the presence of 5 % CO_2. Absorbance was analyzed with a Multiskan Spectrum plate reader (Thermo Scientific, MA, USA) at a wavelength of 490 nm. The baseline value (wells without cells) was subtracted from each reading.

Statistical analysis

Data are represented as the means ± SEMs. Statistical analysis was performed using a Student's t test. Differences with p values of <0.05 were considered significant.

Results

Expression of TRP channel mRNA in mouse urothelial cells

Several members of the TRP family have been shown to exhibit altered expression patterns in cancer cells, and *Trpv1*, *Trpv2*, *Trpv4*, *Trpm7*, *Trpm8*, and *Trpa1* mRNAs have been reported to be expressed in mouse urothelial cells [10, 21]. We examined the expression of 22 genes (Table S1) belonging to four subfamilies of TRP channels

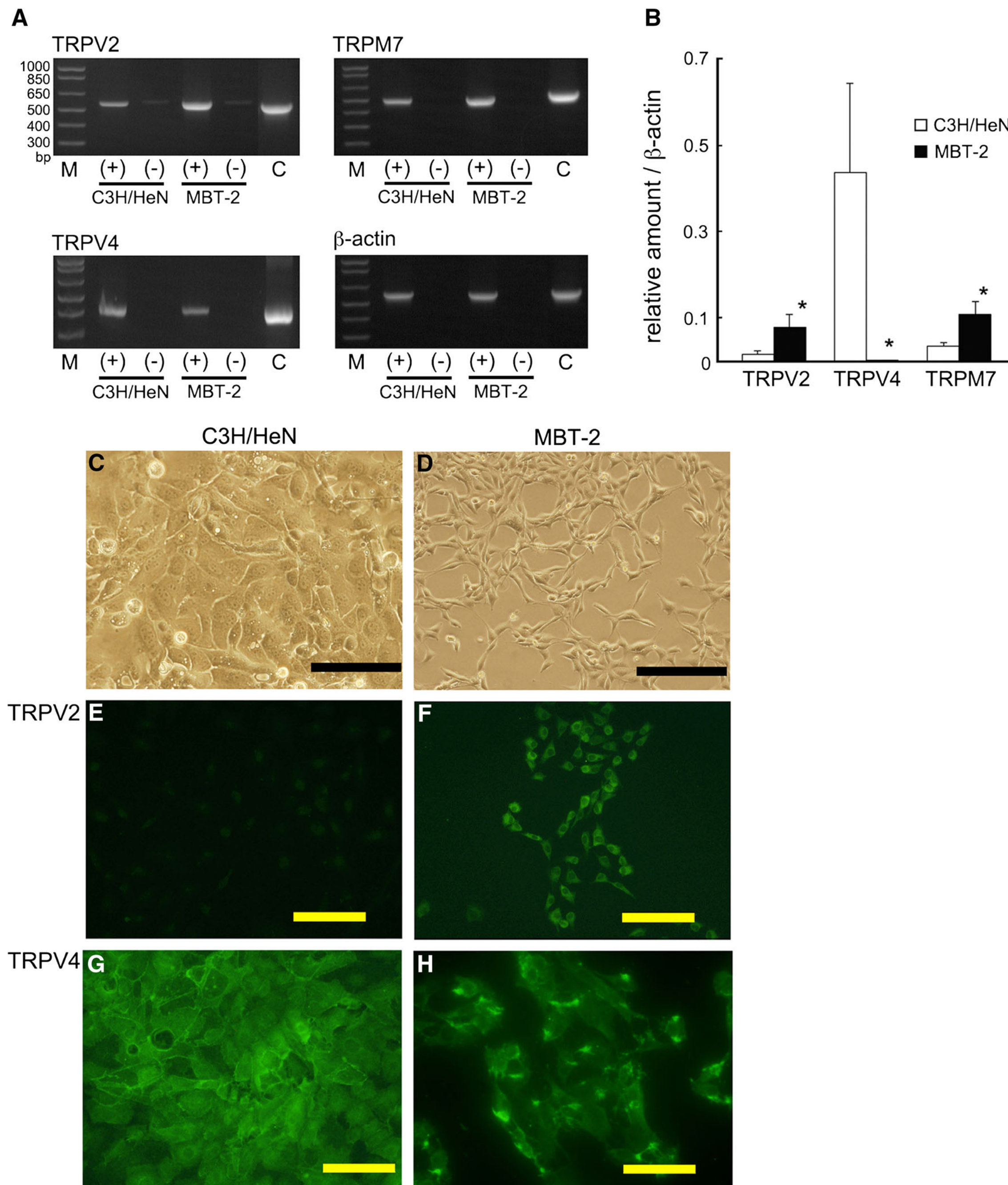

Fig. 1 Expression of TRPV2, TRPV4, and TRPM7 in primary urothelial cells from C3H/HeN mice and in MBT-2 cells. **a** Amplified fragments of *Trpv2*, *Trpv4*, *Trpm7*, and *β-actin* mRNAs of primary urothelial cells from C3H/HeN mice and MBT-2 cells. *C* (control) and *M* indicate the PCR using plasmid DNA and markers, respectively. (+) and (−) indicate with and without the RT reaction, respectively. Expected sizes for the amplified fragments for *Trpv2*, *Trpm7*, *Trpv4*, and *β-actin* are 552, 495, 404, and 572 bp, respectively. **b** Quantitative analysis of mRNA expression of *Trpv2*, *Trpm7*, and *Trpv4* in C3H/HeN cells and MBT-2 cells using quantitative real-time RT-PCR. *$p < 0.05$ vs. C3H/HeN cells ($n = 3$). **c**, **d** Phase-contrast images of urothelial cells from C3H/HeN mice (72 h culture, **c**) and MBT-2 cells (**d**) under light microscopy. *Scale bars* 500 μm. **e**–**h** Immunocytochemical analysis of urothelial cells from C3H/HeN mice and MBT-2 cells using anti-TRPV2 antibodies (**e**, **f**) or anti-TRPV4 antibodies (**g**, **h**). *Scale bars* 200 μm (**e**, **f**) and 100 μm (**g**, **h**)

(TRPV, TRPM, TRPC, and TRPA) in primary mouse urothelial cell cultures obtained from C3H/HeN mice and in MBT-2 cells using real-time reverse transcription (RT)-PCR. Among the genes examined, significant expression of *Trpv2*, *Trpv4*, *Trpm4*, and *Trpm7* was observed in urothelial cells from C3H/HeN mice and in MBT-2 cells (data not

shown). Because no significant changes in the expression of *Trpm4* mRNA were observed between primary urothelial cells and cancer cells, we decided not to focus on TRPM4. As shown in Fig. 1a, we confirmed the expression of *Trpv2*, *Trpv4*, and *Trpm7* both in primary urothelial cells from C3H/HeN mice and in MBT-2 cells. The expression levels of *Trpv2* and *Trpm7* mRNAs were increased in MBT-2 cells compared with primary urothelial cells from C3H/HeN mice, while that of *Trpv4* mRNA was decreased in MBT-2 cells (Fig. 1a); these differences were statistically significant (Fig. 1b).

TRP protein expression in mouse urothelial cells

Next, we compared the cell shapes of primary mouse urothelial cells and MBT-2 cells. Primary urothelial cells showed a relatively large cell size, while MBT-2 cells appeared slightly longer and thinner (Fig. 1c, d). We performed a TRPV2 immunofluorescence study for primary mouse urothelial cell cultures and MBT-2 cells. In this analysis, MBT-2 cells showed clear immunoreactivity with anti-TRPV2 antibodies, whereas normal urothelial cells from C3H/HeN mice displayed almost negligible TRPV2 expression (Fig. 1e, f), consistent with the differences in their mRNA expression levels (Fig. 1a, b). TRPV4-like immunoreactivity was observed both in normal urothelial cells from C3H/HeN mice and in MBT-2 cells, while the signals appeared weaker in MBT-2 cells (Fig. 1g, h).

TRP channel function in mouse urothelial cells

To determine whether the TRPV2 protein expressed in MBT-2 cells was functional, we performed a Ca^{2+}-imaging experiment by examining the effects of the TRPV2 agonists, 2-APB and LPC, on primary mouse urothelial cell cultures and MBT-2 cells. While LPC has been reported to activate TRPC5 as well as TRPV2, TRPC5 was expressed only at very low, negligible levels compared to TRPV2, TRPV4, and TRPM7, as measured by real-time PCR. 2-APB and LPC evoked significantly larger increases in intracellular Ca^{2+} concentrations in MBT-2 cells than in WT urothelial cells (Fig. 2a–d, g). On the other hand, the TRPV4-mediated intracellular Ca^{2+} increase induced by GSK1016790A (GSK) was significantly lower in MBT-2 cells than in normal urothelial cells from C3H/HeN mice (Fig. 2e–g). These results were consistent with the gene expression patterns of TRPV2 and TRPV4 at both mRNA and protein levels (Fig. 1). The ionomycin responses of MBT-2 cells were lower than those in normal urothelial cells. We hypothesized that this may be because the loading efficiency of fura-2 was different between cell types. This difference was corrected by normalizing data as the Δratio of interest/Δratio of ionomycin.

TRP channel expression in human urothelial cancer cells

In order to confirm the expression of TRP channels in human urothelial cells, we used T24 human bladder carcinoma cells [14]; however, we failed to obtain human urothelial cell samples. We found obvious expression of *Trpv2*, *Trpv4*, and *Trpm7* mRNAs in T24 cells (Fig. 3a). In contrast to mouse urothelial cells, T24 cells seemed to express considerable amounts of *Trpv4* mRNA. TRPV2-like and TRPV4-like immunoreactivity was observed in T24 cells (Fig. 3b, c), consistent with the results of mRNA expression analysis. Because human TRPV2 does not respond to 2-APB [22], we used LPC as an agonist for human TRPV2. LPC (100 μM) caused a large increase in intracellular Ca^{2+} concentrations in T24 cells (Fig. 3d), while GSK caused only a modest increase in intracellular Ca^{2+} concentrations (Fig. 3e); these results were slightly different from the results of protein expression analysis. The LPC-induced increase in intracellular Ca^{2+} concentrations in T24 cells was observed in the presence of extracellular Ca^{2+}, but was smaller in the absence of extracellular Ca^{2+} (Fig. 3f), suggesting that the increase in intracellular Ca^{2+} concentration was due to the influx of Ca^{2+}, likely through TRPV2.

TRPV2 and TRPM7 negatively regulated MBT-2 cell proliferation

To clarify the physiological roles of TRPV2 and TRPM7 in bladder cancer cells, we performed proliferation assays using MBT-2 cells overexpressing TRPV2 or TRPM7. Our results indicated that TRPM7 suppressed MBT-2 cell proliferation ($p = 0.02$, $n = 6$, Fig. 4a). However, TRPV2 did not change MBT-2 cell proliferation, probably due to the strong endogenous TRPV2 expression observed in MBT-2 cells ($p = 0.527$, $n = 6$, Fig. 4a). Indeed, dominant-negative TRPV2 significantly enhanced MBT-2 cell proliferation ($p < 0.001$, $n = 6$, Fig. 4a). Moreover, TRPV2 activators (LPC and 2-APB) significantly suppressed MBT-2 cell proliferation ($p < 0.005$, $n = 6$, Fig. 4b). These results suggested that both TRPV2 and TRPM7 negatively regulated MBT-2 cell proliferation.

Plasma membrane Ca^{2+} permeability

To investigate whether the plasma membrane Ca^{2+} permeability was altered by the introduction of the dominant-negative TRPV2, we evaluated the relative Ca^{2+} permeability of MBT-2 cells using a Ca^{2+} imaging technique [20]. Figure 5a, b show time-dependent changes in the intracellular Ca^{2+} concentration, before, during, and after treatment with 2 mM EDTA with or without the dominant-

A C3H/HeN
Iono.
2-APB
Ratio (340/380 nm)
4.5
3.5
2.5
1.5
0
0 1 2 3 4 min
B MBT-2
Iono.
2-APB
Ratio (340/380 nm)
4.5
3.5
2.5
1.5
0
0 1 2 3 4 min
C C3H/HeN
Iono.
LPC
Ratio (340/380 nm)
4.5
3.5
2.5
1.5
0
0 1 2 3 4 5 min
D MBT-2
Iono.
LPC
Ratio (340/380 nm)
4.5
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0
0 1 2 3 4 5 min
E C3H/HeN
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GSK
Ratio (340/380 nm)
5.5
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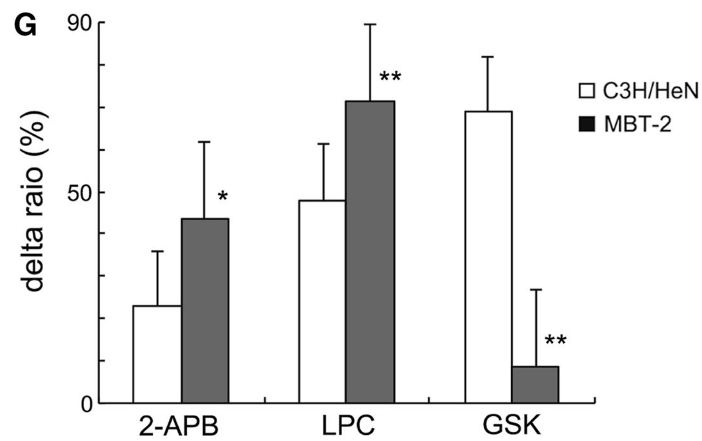
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50
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C3H/HeN
MBT-2
2-APB
LPC
GSK

◀**Fig. 2** Changes in cytosolic Ca^{2+} concentrations in response to agonist application in urothelial cells from C3H/HeN mice and MBT-2 cells. **a**–**f** Changes in cytosolic Ca^{2+} concentrations (indicated by the 340/380 nm excitation ratio, with SEM) in response to TRPV2 agonists (2-APB and LPC; **a**–**d**) or a TRPV4 agonist [GSK1016790A (GSK); **e**, **f**] in urothelial cells from C3H/HeN mice (*left*) and in MBT-2 cells (*right*). *Bars* indicate the time of agonist application. **g** Differences in changes of cytosolic Ca^{2+} concentrations (delta ratio %: difference between the basal values and peak values normalized to that of ionomycin) between urothelial cells from C3H/HeN mice and MBT-2 cells. $n = 100–130$ in three independent experiments. *, **$p < 0.05$, $p < 0.01$ vs. C3H/HeN. *Iono* ionomycin

negative TRPV2. These results suggested that dominant-negative TRPV2 actually decreased plasma membrane Ca^{2+} permeability (Fig. 5c).

Discussion

In the present study, we found increases in *Trpv2* and *Trpm7* mRNA expression and a decrease in *Trpv4* mRNA expression in MBT-2 cells compared to normal urothelial cells. Furthermore, we confirmed the expression of *Trpv2*, *Trpv4*, and *Trpm7* mRNAs in T24 human bladder cancer cells and observed TRPV2-like and TRPV4-like immunoreactivity in T24 cells. In addition, we found evidence of TRPV2 activity using a Ca^{2+}-imaging experiment with TPRV2 agonists in mouse and human bladder cancer cell lines, although we could not exclude the possibility that TRPV2 activators had other targets, such as TRPC5. We observed reduced cell proliferation in MBT-2 cells treated with TRPV2 activators, enhanced proliferation in MBT-2 cells expressing dominant-negative TRPV2, and decreased plasma membrane Ca^{2+} permeability in MBT-2 cells expressing dominant-negative TRPV2, suggesting the importance of TRPV2 for intracellular Ca^{2+} homeostasis, likely acting as a negative regulator of proliferation in bladder cancer.

The expression of TRPV2 has been reported to be increased in human bladder cancer and to increase with the progression of cancer stage [14], suggesting the functional involvement of TRPV2 in bladder cancer. TRPV2 is a highly permeable Ca^{2+} channel and is activated by mechanical stimulus. Although we still do not know the actual stimulant for TRPV2 in these situations, we speculate that TRPV2 could be activated by rapid cell growth or migration, during which the cell membrane could be distended or unknown stimulants for TRPV2 (e.g., LPC or other lipids) could be released by membrane distension in cancer cells. Indeed, other reports have also shown that TRPV2 can be activated under steady state conditions during cancer cell migration [23]. This could in turn cause Ca^{2+} influx, leading to the suppression of cell growth and migration, which will prevent excess mechanical stress

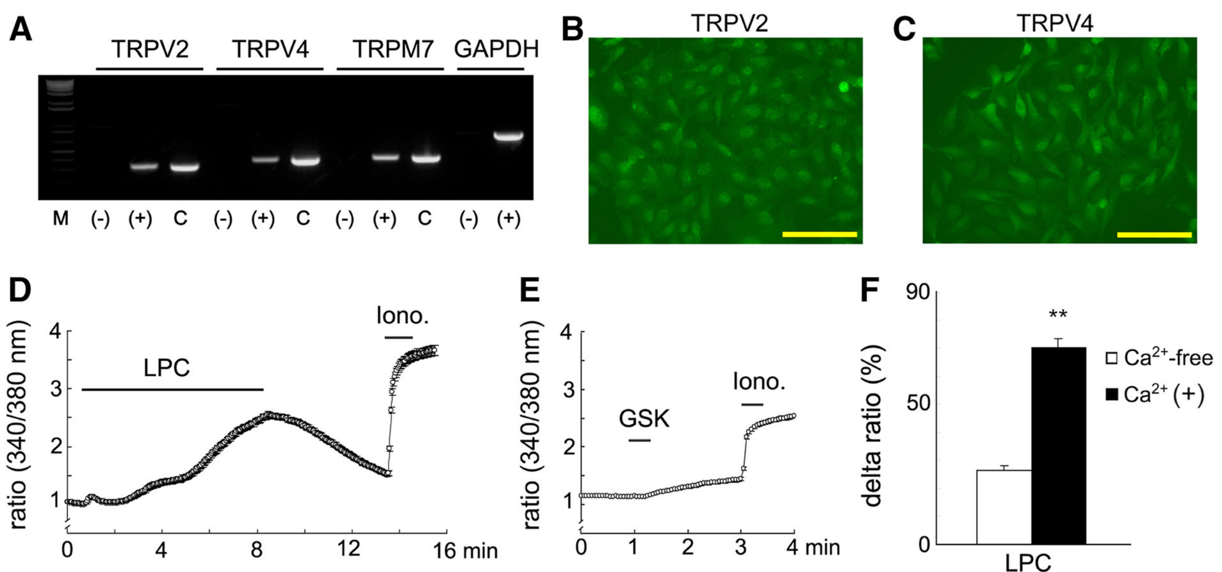

Fig. 3 Expression of TRPV2 and TRPV4 in T24 cells. **a** Amplified fragments of *TRPV2*, *TRPV4*, *TRPM7*, and *GAPDH* mRNAs in T24 cells. *C* (control) and *M* indicate the PCR result using plasmid DNA and markers, respectively. (+) and (−) indicate with and without the RT reaction, respectively. Expected sizes of the amplified *TRPV2*, *TRPM7*, *TRPV4*, and *GAPDH* fragments are 326, 378, 398, and 742 bp, respectively. **b**, **c** Immunocytochemical analysis of T24 cells using anti-TRPV2 antibodies (**b**) or anti-TRPV4 antibodies (**c**). *Scale bars* 200 μm. **d**, **e** Changes in cytosolic Ca^{2+} concentrations (indicated by the 340/380 nm excitation ratio, with SEM) in response to a TRPV2 agonist (LPC; D) or a TRPV4 agonist (GSK; E) in T24 cells. **f** Comparison of LPC-induced increases in cytosolic Ca^{2+} concentrations in the presence and absence of extracellular Ca^{2+}, which was normalized to the response of ionomycin. *$p < 0.01$ vs. Ca^{2+}-free. $n = 208$ and 109 for Ca^{2+} (+) and Ca^{2+}-free, respectively. *Iono* ionomycin

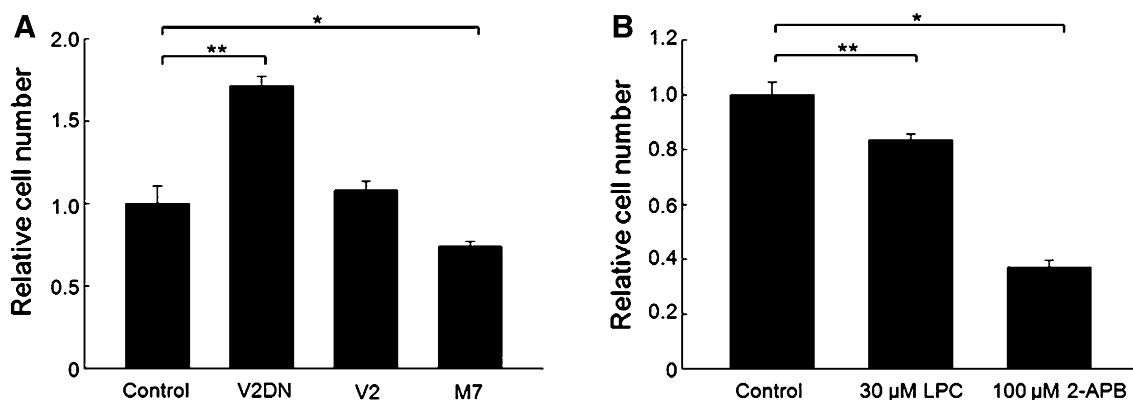

Fig. 4 Proliferation of MBT-2 cells expressing TRPV2 and TRPM7. **a** Effects of dominant-negative TRPV2, full-length TRPV2, and full-length TRPM7 on the proliferation of MBT-2 cells, as assessed with MTS assays. Cell numbers were normalized to values in the control (mock-transfected) cells. V2DN, cells expressing dominant-negative TRPV2; V2, cells expressing full-length TRPV2; M7, cells expressing full-length TRPM7. Bars ± SEM ($n = 6$), $*p = 0.02$, $**p < 0.001$. **b** Effects of TRPV2 activators on MBT-2 cell proliferation. LPC (30 μM) or 2-APB (100 μM) was added 24 h before MTS assay ($*p < 0.001$, $n = 6$; $**p = 0.007$, $n = 6$). Bars ± SEM

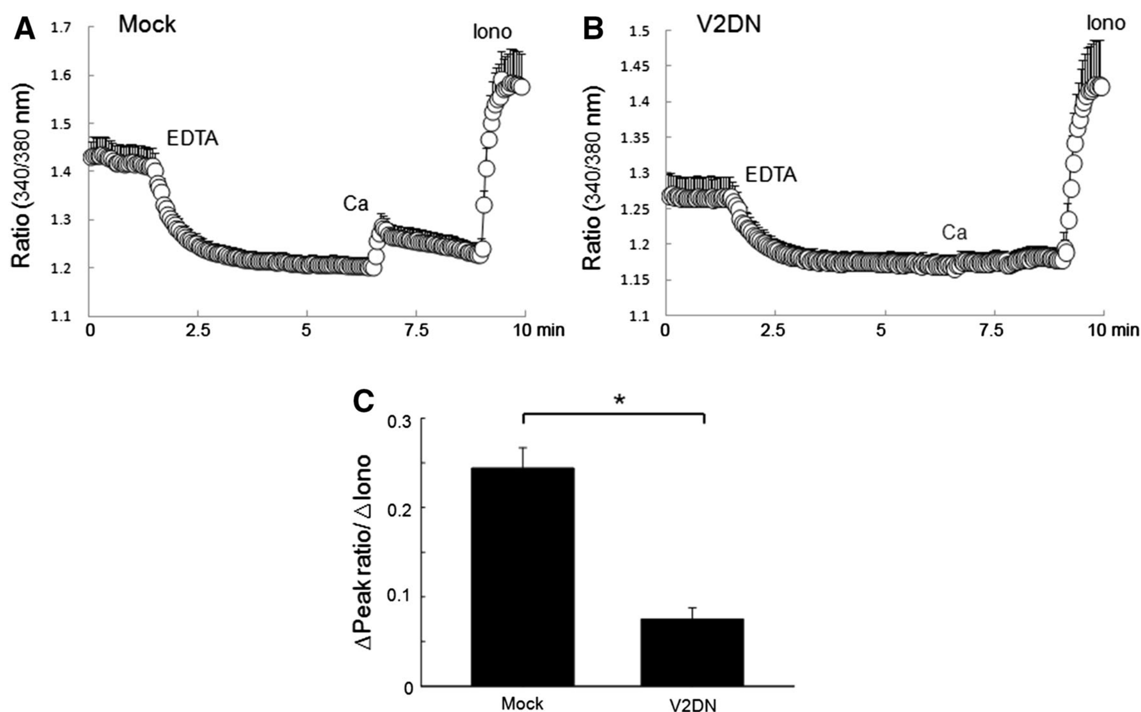

Fig. 5 Permeability of the plasma membrane to Ca^{2+} in MBT-2 cells. **a**, **b** Time-dependent changes in intracellular Ca^{2+} concentrations before, during, and after treatment of cells with 2 mM EDTA. In cells expressing dominant-negative TRPV2 (**b**), the peak value was smaller than that in mock-transfected cells (**a**). **c** Statistical analysis of the peak value normalized to ionomycin. $*p < 0.001$, $n = 11$

resulting from cell proliferation. Yamada et al. [15] reported that TRPV2 activation leads to cell death rather than cell growth or migration in bladder cancer cells, indicating that TRPV2 negatively regulates bladder cancer cell proliferation. It is possible that excess Ca^{2+} influx through TRPV2 could cause Ca^{2+} overload in certain circumstances, leading to cell death. Another possibility is a live-cell extrusion mechanism to prevent the accumulation of excess epithelial cells [24]. This phenomenon was observed in Madin-Darby canine kidney (MDCK) cells and zebrafish cells, and another candidate mechanosensor, PIEZO1, was shown to be involved in the detection of this excess cell density, most likely through direct mechanosensation [25, 26]. In most cases, excess mechanical stress

is avoided in the remaining cells, allowing the cells to be maintained in a healthier state. In renal tubules, polycystin-2 (TRPP2) is thought to be important for fluid-shear stress sensation [27]. Recently, TRPP2 has been reported to act as a negative regulator of proliferation [28]. These data suggest the importance of the sensation of mechanical stress in renal tubule and bladder cells.

Although some studies have shown that TRPM7 is involved in various cancers, such as breast cancer and gastric cancer [29, 30], we found, for the first time, that *Trpm7* expression was significantly increased in MBT-2 cells compared to control primary urothelial cells from C3H/HeN mice. Since TRPM7 has also been reported to be activated by mechanical stimuli [31, 32], these data suggested that TRPM7 played a role similar to that of TRPV2 in bladder cancer cells. Future studies are needed to elucidate the function of TRPM7 in urothelial cells.

We previously reported that TRPV4 detects bladder distension and that this information is transmitted to the sensory neurons via ATP [13]. It is not known why the expression of *Trpv4* mRNA was almost completely abolished in MBT-2 cells, while *Trpv4* mRNA was expressed at normal levels in primary urothelial cells from C3H/HeN mice. Modest responses to GSK were also observed in MBT-2 cells and T24 cells. Interestingly, loss of TRPV4 can lead to a reduced sensation of urine accumulation in the bladder, as has been shown in TRPV4-deficient mice [33]. Therefore, TRPV4 may be less important in cancer cells than in normal urothelium, which has to sense urine volume via TRPV4 activation. One possibility is that ATP secretion may require the expenditure of large amounts of cell energy in cancer cells. Therefore, we speculate that MBT-2 cells decreased the activity of the TRPV4-ATP system to escape energy loss.

In conclusion, in MBT-2 mouse bladder cancer cells, we observed increased expression of TRPV2 and TRPM7 compared with primary normal urothelial cells. In particular, TRPV2 seemed to be important for intracellular Ca^{2+} homeostasis and was found to negatively regulate MBT-2 cell proliferation. High expression and activity of TRPV2 was also observed in T24 human bladder cells, suggesting a critical role for TRPV2 in cancer physiology. Although both TRPV2 is known to be ubiquitously expressed in many tissues and cells, the fact that bladder epithelium can be treated locally indicates that manipulation of TRPV2 activity could interfere with the proliferation of bladder cancer.

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Conflict of interest The authors declare that they have no conflicts of interest.

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