Transient receptor potential vanilloid 4 (TRPV4) channel is a physiological sensor for hypo-osmolarity, mechanical deformation, and warm temperature. The channel activation leads to various cellular effects involving Ca\(^{2+}\) dynamics. We found that TRPV4 interacts with \(\beta\)-catenin, a crucial component linking adherens junctions and the actin cytoskeleton, thereby enhancing cell-cell junction development and formation of the tight barrier between skin keratinocytes. TRPV4-deficient mice displayed impairment of the intercellular junction-dependent barrier function in the skin. In TRPV4-deficient keratinocytes, extracellular Ca\(^{2+}\)-induced actin rearrangement and stratification were delayed following significant reduction in cytosolic Ca\(^{2+}\) increase and small GTPase Rho activation. TRPV4 protein located where the cell-cell junctions are formed, and the channel deficiency caused abnormal cell-cell junction structures, resulting in higher intercellular permeability in vitro. Our results suggest a novel role for TRPV4 in the development and maturation of cell-cell junctions in epithelia of the skin.

Transient receptor potential vanilloid 4 (TRPV4), a member of the TRP superfamily of cation channels, is a Ca\(^{2+}\)-permeable channel expressed in both neuronal and non-neuronal cells. Channel activation allows cation influx into cells, leading to various Ca\(^{2+}\)-dependent processes. TRPV4 can be activated by a variety of chemical and physical stimuli such as synthetic phorbol ester 4\(\alpha\)-phorbol 12,13-didecanoate (4\(\alpha\)-PDD) (3), a botanical agent (bisandrographolide A) (4), anandamide (5), and it has been proposed that the channel is involved in the detection of warm temperature (12). Skin keratinocytes express another warm temperature-sensitive TRP channel, TRPV3 (activated by temperature above 32°C), which is also implicated in temperature sensation in mice (13). Because both TRPV3 and TRPV4 are expressed in keratinocytes and are activated by similar ranges of temperatures, these channels likely have distinct functions in the skin. Consistent with this idea, a recent report provided evidence that TRPV3, rather than TRPV4, mainly participates in transmission of warm temperature information from keratinocytes to adjacent nerve endings through ATP release (14). It has also been reported that mutation of TRPV3 is linked to defective hair growth and dermatitis in rodents (15, 16), although the involvement of TRPV4 has not been confirmed.

The skin constitutes an interface between the external environment and the body, serving as a hydrophobic barrier essential for protection against infection from the outside and dehydration from the inside. The skin barrier function is achieved by keratinocytes in the epidermis, which consists of a basal layer and several differentiated layers covered with a cornified layer at the surface. The main barrier function is achieved by a cornified layer-dependent hydrophobic barrier, which is composed of a continuous sheet of protein-enriched cells embedded in an extracellular non-polar lipid layer. In addition to this, a barrier mediated by cell-cell junctions (19) beneath the cornified layer, including tight junctions (TJs) and adherens junctions (AJs), has recently been highlighted as an essential structure of the skin barrier function (20). An extensive epidermal water loss was observed in mice lacking claudin-1, the main component of the TJ complex, despite an apparently normal cornified layer function (21). Moreover, mice lacking E-cadherin specifically in the developing epidermis, the main component of the AJ complex, phenocopied the claudin-1-deficient mice (22). Thus, proper formation of AJs and TJs is indispensable for the skin barrier function preventing dehydration. In support of this, some skin diseases with barrier disruption are related to AJs and TJs abnormality; lack of extracellular domain of E-cadherin or absence of claudin-1 protein has been reported in

The TRPV4 Channel Contributes to Intercellular Junction Formation in Keratinocytes

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Hailey-Hailey disease (23) or in neonatal ichthyosis and sclerosing cholangitis syndrome (24), respectively. The formation of cell-cell junctions in keratinocytes critically depends on rearrangement of actin fibers during the differentiation process (25–27), which can be initiated by an increase in extracellular Ca(2+)

(28). In fact, the formation of a Ca(2+) gradient in the epidermis has been reported in vivo with higher extracellular and intracellular Ca(2+) levels in the differentiated cell layers (28, 29). It has been suggested that some Ca(2+)-permeable TRP channels are involved in keratinocyte differentiation (30, 31). However, the contribution of the ion channels to the cell-cell junction formation has not been elucidated. In seeking a potential role of TRPV4 in keratinocytes, we found that the channel interacts with β-catenin and E-cadherin, both of which are basic components of AJs. We investigated the significance of TRPV4 in cell-cell junction development and the integrity of intercellular permeability by comparing skin and primary keratinocytes obtained from wild-type and TRPV4-deficient mice. The involvement of TRPV4 in the increase of intracellular Ca(2+at physiological skin temperatures and in Rho activation was also examined, both of which are important for regulation of the actin cytoskeleton in skin. Finally, TRPV4 localization and junction structure among keratinocytes were investigated.

EXPERIMENTAL PROCEDURES

Antibodies and Plasmids—The following antibodies were used: mAb anti-β-catenin (BD Transduction Laboratories), mAb anti-GFP (MBL, Nagoya, Japan), mAb anti-FLAG (Sigma), mAb anti-HA (Roche Applied Science), mAb anti-E-cadherin (ECCD2) (a generous gift from Dr. M. Takeichi, RIKEN Center for Developmental Biology (CDB), Kobe, Japan), pAb anti-claudin-1 (IBL, Takasaki, Japan), pAb anti-occludin (Zymed Laboratories Inc.), pAb anti-TRPV4 (prepared against an N-terminal peptide of TRPV4 (32)), pAb anti-rat TRPV4 (33), and mAb anti-RhoA (Cytoskeleton, Inc.). The specificity of two anti-TRPV4 antibodies is described elsewhere (32, 33). β-Catenin and mouse TRPV4 cDNA were generously provided by Dr. M. Takeichi and Dr. M. Caterina, respectively. Mouse TRPV4 cDNA for a rescue experiment was introduced into a DsRed-Express vector (Clontech).

Cell Preparation and Biochemistry—COS-7 cell culture and cDNA transfection of the cells were carried out as described previously (34). Primary keratinocytes were obtained from newborn wild-type mice, TRPV4-deficient mice (35), and TRPV3-deficient mice (13) and cultured in MCDB 153 medium containing 5 μg/ml insulin, 0.4 μg/ml hydrocortisone, 14.1 μg/ml phosphorlyethanolamine, 10 ng/ml epidermal growth factor (all from Sigma), 10 μg/ml transferrin (Funakoshi, Tokyo, Japan), 40 μg/ml bovine pituitary gland extract (Kyokuto, Tokyo, Japan), 25 μg/ml gentamicin, 50 units/ml penicillin, 50 μg/ml streptomycin, and 1% fetal bovine serum. After incubating the cells in this medium at 37 °C for 24 h, the medium was changed to one without fetal bovine serum. We termed the fetal bovine serum-free medium “0 mM Ca2+ medium,” although it contained 0.04 mM CaCl2. The cells were further incubated for 36–60 h, and a Ca(2+) switch was performed by adding 2 mM CaCl2 at 33 °C. We paid attention to mouse age (P2) and the cell density of suspension (1.0 × 10⁶/ml) for keeping experimental conditions constant because both factors correlate with cell viability and proliferation, which influence cell density on the cover glass. Immunoprecipitation and immunoblotting using membrane fraction were performed as described previously (34). For preparing the membrane fraction, the cells were washed with ice-cold phosphate-buffered saline and resuspended in TNE buffer (10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, complete EDTA-free protease inhibitor mixture (Roche Applied Science), 1 mM Na3VO4). Samples were centrifuged for 5 min at 10,000 × g. The pellets were resuspended in TNE buffer with 1% Nonidet P-40 and sonicated for 30 s. Following centrifugation at 100,000 × g for 60 min, the protein concentrations of the supernatants were adjusted and used as a membrane fraction. In rescue experiments, TRPV4 cDNA linking DsRed cDNA at its C terminus was transfected with Lipofectamine (Invitrogen) before a Ca(2+) switch.

Immunofluorescence Study—Keratinocytes obtained from P2 mice were plated on cover glasses (Warner Instruments, 12 mm) or a Transwell system (Costar, pore size 0.4 μm). The cells were fixed with paraformaldehyde (4%) and permeabilized with 0.5% Triton X-100. Samples were incubated with primary antibodies for 90 min at room temperature and then incubated with Alexa Fluor-conjugated secondary antibodies (Molecular Probes) for 40 min at room temperature. Actin fiber was stained with Alexa Fluor-phalloidin. Back skin from newborn mice was fixed with paraformaldehyde (4%), dehydrated with 20% followed by 30% sucrose, and embedded in OCT compound. The skin sections (16 μm) were stained in a similar manner as described above. Nuclei were stained with propidium iodide. The images were obtained using a fluorescent microscope (Olympus, IX71) or a confocal fluorescent microscope (Zeiss, Axiovert 200M). For measurement of the thickness of the cornified layer and epidermal layers, three positions (approximately left, middle, and right) were randomly picked up from skin section images, and the thickness was measured by the ImageJ software (National Institutes of Health). The averaged value from each image was taken as a thickness of each sample regardless of variable thickness.

Quantification of Actin Fiber Formation and Cell Stratification—Immunofluorescent images obtained 4 and 24 h after Ca(2+) switch were processed by the ImageJ software. Actin fiber formation was evaluated as (phalloidin-positive area) (mean pixel intensity) (arbitrary unit). The cell stratification level was evaluated as (E-cadherin-positive area)/(total image area) × 100 (%). Data were represented as box-and-whisker plots, where sample minimum, lower quartile, median, mean, upper quartile, and sample maximum are indicated (see Fig. 3).

Rho Activation Assay—Rho activity was evaluated with a Rho activation assay Biochem Kit (Cytoskeleton, Inc.) following the manufacturer’s instructions. Briefly, total cell lysates (300 μg of protein) were collected from keratinocyte cultures on 10-cm dishes, and active Rho (GTP binding form) was pulled down by Rhotekin-Rho binding domain (RBD)-conjugated beads at 4 °C for 1 h. The precipitates were separated in 12% SDS-polyacrylamide gel and transferred to Immobilon P polyvinylidene difluoride membranes (Millipore). Rho was detected with mouse anti-RhoA first antibody and anti-mouse IgG-horse radish per-
oxidase. The chemiluminescent signal was visualized with x-ray film (Kodak). The band intensity was measured and normalized to each input using ImageJ software.

Permeation Assay—For the Lucifer yellow assay, newborn mice (P0) were immersed in 1 mM Lucifer yellow/phosphate-buffered saline for 1 h followed by quick freezing. For the toluidine blue assay, P0 mice were dehydrated in 25, 50, 75, and 100% MeOH for 1 min followed by immersion in 0.1% toluidine blue O/phosphate-buffered saline for 20 h. The cornified layer was disrupted by soaking mice in 100% acetone for 15 min prior to dehydration. For the biotin diffusion assay, 10 mg/ml EZ-LinkSM Sulfo-NHS-LC Biotin (Promega), phosphate-buffered saline, 1 mM CaCl2 was injected into the dermis on the back of P0 mice. The skin was quickly frozen after 30 min. Transepidermal water loss (TEWL) was measured on the shaved back skin of anesthetized male mice (7–10 weeks) for four times using a TEWL meter (AS-V100RS, AsahiBioMed, Tokyo, Japan) at 25 °C and in 50–55% humidity. After applying acetone-soaked cotton on the shaved back skin for 1 min, the skin was warmed until the surface temperature was increased more than 33 °C. Then, TEWL was measured again on the same area four times. Each averaged value was considered as a datum from an individual mouse. For the dextran permeation assay, P2 primary keratinocytes (6 × 105) were cultured on a Transwell insert in 0 mM Ca2+ medium for 36 h. Twenty-four h after a Ca2+ switch, medium in the insert was replaced with 1 mg/ml fluorescein isothiocyanate-dextran (Sigma, molecular weight 40,000). Fluorescein isothiocyanate fluorescence in the bottom well was measured using a microplate reader. The permeability of dextran was calculated as fluorescent intensity/collected volume × total volume in the bottom well (arbitrary unit).

Transmission Electron Microscopy—After a Ca2+ switch was performed (for 24 h at 37 °C), keratinocytes cultured on coverslips were fixed with 2.5% glutaraldehyde, 2% formaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) followed by postfixation with 1% OsO4 in the same buffer. The subsequent procedure was the same as the one above, including washing and dehydration before embedding the specimen. The blocks were sliced into ultrathin sections, stained with uranyl acetate and lead citrate, and visualized using a transmission electron microscope (H-11001; Hitachi, Tokyo, Japan) at 80 kV.

RESULTS

TRPV4 Binds to β-Catenin at Cell-Cell Junctions—To clarify the physiological function of TRPV4 in the skin, we used a yeast two-hybrid system and a human keratinocyte cDNA library to search for TRPV4-binding proteins. The N-terminal cytoplasmic domain of TRPV4 was found to bind to the armadillo repeat domain of β-catenin (Fig. 1A and B). These data are consistent with the notion that β-catenin is predominantly located beneath the plasma membrane together with E-cadherin (39, 40). We found the binding of TRPV4 and β-catenin in both a heterologous expression system (Fig. 1C) and primary keratinocytes obtained from neonatal mice (Fig. 1D). β-Catenin co-precipitated with TRPV4 was hardly observed in keratinocytes, which might be due to weak competency of the TRPV4 antibody for immunoprecipitation, relatively low expression level of TRPV4, or both. We also observed that the two proteins interacted with E-cadherin in the membrane fraction of keratinocytes (Fig. 1D, far right), suggesting that TRPV4 is located in the AJs. By contrast, TRPV3, another heat-sensitive TRP channel expressed in keratinocytes (41), did not bind to β-catenin in the membrane fractions (supplemental Fig. S1A) despite its abundant expression in skin. The complex formation was also examined in Madin-Darby canine kidney cells, which are known to express TRPV4. Interaction among TRPV4, β-catenin, and E-cadherin was recognized in the membrane fraction (supplemental Fig. S1B). These results indicate that TRPV4 is specifically localized with AJ components at the plasma membrane.

Loss of TRPV4 Impaired Intercellular Junction-dependent Barrier Function in the Skin—Skin from wild-type and TRPV4-deficient mice was used to determine whether the loss of TRPV4 in keratinocytes altered epidermal structure. The latter had a significantly thicker cornified layer (14.0 ± 0.5 μm in the wild-type mice, n = 7, and 19.7 ± 0.7 μm in the TRPV4-deficient mice, n = 7; p < 0.0001) (Fig. 2A and supplemental Fig. S2A). Except for the cornified layers, the thickness of the epidermal layers in the two genotypes was indistinguishable (30.8 ± 0.9 μm in the wild-type mice, n = 16, and 29.05 ± 0.8 μm in the TRPV4-deficient mice, n = 16; p = 0.12). There were no obvious differences between wild-type and TRPV4-deficient mice in their expression of several proteins related to cytoskeleton and intercellular adhesion, either at the mRNA or at the protein level (Supplemental Fig. S2, A and B).

We pursued the possibility that TRPV4 might affect skin barrier function because the thick cornified layer observed in the TRPV4-deficient skin was similar to the phenotype previously reported in mice lacking either claudin-1 (21) or E-cadherin (22), both of which showed severe damage in the barrier functions. To assess the cornified layer-dependent barrier functions, dye permeation into the skin was examined at the whole animal level. Lucifer yellow did not permeate through the cornified layer in either wild-type or TRPV4-deficient mice (Fig. 2B). Toluidine blue staining of the skin after immersion was indistinguishable between wild-type and TRPV4-deficient mice.
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![Diagram showing binding of TRPV4 to β-catenin and E-cadherin.](image)

**FIGURE 1. Binding of TRPV4 to β-catenin and E-cadherin.** A, schematic representation of the structure of TRPV4 and β-catenin. The N-terminal domain of TRPV4 (N1) interacts with the armadillo repeat domain of β-catenin (β3, β3). A, ankyrin repeat domain; TM, transmembrane domain; C, fragment of TRPV4. B, left, fragments of TRPV4 (N1, N2, and C) and β-catenin (β1c) expressed in COS-7 cells. Nonidet P-40-soluble fractions were immunoprecipitated (IP) with an anti-FLAG antibody and immunoblotted (IB) with an anti-β-catenin antibody (anti-β-catenin) (top). N1 binds to β1c (asterisk). A nonspecific band (25 kDa) was in the input (5% of total lysates). Right, the fragment of TRPV4 (N1) and β-catenin fragments (β1c, β2c, β3c, and β4c) expressed in COS-7 cells. Nonidet P-40-soluble fractions were immunoprecipitated with anti-FLAG and immunoblotted with anti-β-catenin or anti-GFP antibody (anti-GFP). Lack of interaction between N1 and β4c indicates N1 binding to β3c. C, TRPV4 interacts with β-catenin in a heterologous expression system. COS-7 cells were transfected with pEGFP (GFP) + β-catenin (β-catenin/HA) (lane 1), rat TRPV4/EGFP (V4/GFP) + β-catenin/HA (lane 2), or V4/GFP + pCAN-HA (HA) (lane 3). The membrane fractions were immunoprecipitated with an anti-GFP antibody (left panels) and immunoblotted with an anti-β-catenin antibody or an anti-HA antibody. Black and red arrowheads denote precipitated TRPV4 and β-catenin co-precipitated with TRPV4, respectively. Right panels show input (total fraction). D, TRPV4 interacts with β-catenin in mouse keratinocytes. Left, each membrane fraction was immunoprecipitated with an anti-IgG antibody (anti-IgG) (lane 1), an anti-N terminus TRPV4 antibody (anti-TRPV4) (lane 2), or an anti-β-catenin antibody (lane 3); the immunoprecipitated samples were then immunoblotted with an anti-β-catenin or anti-TRPV4 antibody. The arrowhead indicates TRPV4 co-precipitated with β-catenin. C, TRPV4 co-precipitated with TRPV4 is hardly observed (see “Results”). Right, the far right lane on the left panel was reblotted with an anti-E-cadherin antibody (anti-E-cad). The arrowhead indicates E-cadherin.

mice (Fig. 2C, acetone (−)). These data suggest that the cornified layer-dependent hydrocellar barrier was intact in the TRPV4-deficient skin. We next tested the cell-cell junction-dependent permeability by disrupting the cornified layer with acetone. Dye permeation increased more in TRPV4-deficient than in wild-type mice following acetone treatment (Fig. 2C, acetone (+)), suggesting that the cell-cell junction-dependent barrier is impaired by TRPV4 deficiency. To confirm this, the diffusion of small molecules in the intercellular space was compared. After biotin injection into the dermis on the back, the intercellular diffusion was evident regardless of genotypes, but it was prevented at TJs among the differentiated cells located beneath the cornified layer of wild-type skin (Fig. 2D). By contrast, biotin permeated through the TJs in TRPV4-deficient skin, reaching the apical side of denucleated cells. This TJ dysfunction was not observed in wild-type keratinocytes (Fig. 2D). These results indicate that the tightness of the cell-cell junction depends on TRPV4. Because this barrier is important to restrict dehydration (21, 22), TEWL values were examined on the whole animal level. With intact skin, TEWL values were similar among wild-type, TRPV4-deficient, and TRPV3-deficient mice (Fig. 2e, acetone (−)). However, after the cornified layer was damaged (acetone (+)), TEWL values increased significantly more in TRPV4-deficient mice than in wild-type or TRPV3-deficient mice. TEWL values in TRPV3-deficient mice were indistinguishable from those in wild-type mice, indicating that the deterioration in TJ-dependent permeability is specifically attributed to TRPV4 deficiency and that the channel is important for the integrity of intercellular junctions.

**TRPV4 Deficiency Causes Abnormal Actin Organization and Stratification—**Cell-cell junctions among keratinocytes develop with increased extracellular Ca²⁺ (28), during which the clustering of E-cadherin/β-catenin/α-catenin is accompanied by actin organization via direct binding. This leads to dynamic changes in cell shape and stratification with maturation of cell-cell junctions (25, 26, 42). Therefore, we examined those processes in primary keratinocytes upon the addition of high levels of extracellular Ca²⁺ (from 0 to 2 mM, a Ca²⁺ switch; note that the 0 mM Ca²⁺ medium substantially includes 0.04 mM Ca²⁺). There were no obvious differences in actin fibers or β-catenin/E-cadherin localization between wild-type and TRPV4-deficient keratinocytes without a Ca²⁺ switch (0 h) (Fig. 3A, upper). It has been reported that a Ca²⁺ switch elicits E-cadherin accumulation at the periphery and actin fiber-rich filopodia projections among adjacent cells, leading to formation of early cell-cell contacts (27). Such actin remodeling and intercellular contact formation were clearly observed 4 h after the Ca²⁺ switch in wild-type cells, which was recognized by a single row of E-cadherin immunoreactivity at sites of cell-cell contact (Fig. 3, A and B, upper and supplemental Fig. S3). Actin fiber organization further progressed, cell stratification became prominent between 8 and 24 h (Fig. 3A, upper), and sites of cell-cell contacts colocalized with cortical actin (the surrounding belt of actin) 12 h after Ca²⁺ switch in wild-type keratinocytes (Fig. 3B, upper). In contrast, actin organization and cell stratification were poorly developed in TRPV4-deficient cells when compared with those in wild-type cells (Fig. 3A, lower, and supplemental Fig. S4). The cell stratification at 24 h was obviously delayed in TRPV4-deficient keratinocytes when compared with wild-type cells, as recognized by fewer overlapping areas among cells. TRPV4-deficient cells showed two rows of E-cadherin at cell-cell borders at 4 h, and a single row appeared after 12 h (Fig. 3B, lower), which seems similar to that observed in wild-type cells after 4 h.
At 48 h, wild-type keratinocytes displayed strong stratification with well-developed cortical actin visible as a honeycomb network. Both of these features were poorly developed in TRPV4-deficient cells (Fig. 3E and supplemental Fig. S5). TRPV3-deficient keratinocytes showed phenotypes similar to those in wild-type cells 48 h after the Ca\textsuperscript{2+} switch (Fig. 3E). These observations clearly demonstrate that TRPV4-mediated actin organization and cell-cell contact development are important for mature stratification. The abnormalities in TRPV4-deficient keratinocytes were rescued by transfection of TRPV4 cDNA (Fig. 3F and supplemental Fig. S5B). Twelve h after the Ca\textsuperscript{2+} switch, adjacent cells transfected with TRPV4 cDNA exhibited stratification and cortical actin formation, whereas cells lacking TRPV4 did not advance beyond the actin fiber formation step and showed comparatively less stratification (see also Fig. 3A, 12 h). These results support the idea that TRPV4 is involved in the actin remodeling, which is important for development and maturation of cell-cell contacts induced by the Ca\textsuperscript{2+} switch.

We tested whether pharmacological inhibition of TRPV4 could lead to a similar phenotype like TRPV4-deficient keratinocytes. In the presence of ruthenium red (RR, a broad TRP channel blocker), Ca\textsuperscript{2+} switch-induced actin fiber formation was less than that in the absence of RR in wild-type cells (Fig. 3G). In contrast, the actin fiber formation was not influenced by RR treatment in TRPV4-deficient cells, suggesting that the process of cell-cell junction formation involves TRPV4 activation during a Ca\textsuperscript{2+} switch. Next, we attempted to address whether activation of TRPV4 could accelerate Ca\textsuperscript{2+} switch-induced development of cell-cell junctions. 4\alpha-PDD (a TRPV4 activator) induced robust Ca\textsuperscript{2+} increase in wild-type keratinocytes, but not in TRPV4-deficient cells, as reported previously (3) (supplemental Fig. S5A). Unexpectedly, however, treatment of keratinocytes with Ca\textsuperscript{2+} and 4\alpha-PDD dramatically perturbed actin organization and cell stratification (supplemental Fig. S6B). It seems that the influence of 4\alpha-PDD is TRPV4-independent but extracellular Ca\textsuperscript{2+}-dependent because the same phenomena were observed in both TRPV4-deficient and TRPV3-deficient keratinocytes and because the perturbation did not occur in the absence of the Ca\textsuperscript{2+} switch (supplemental Fig. S6C). Accordingly, we did not pursue the effects of pharmacological activation of TRPV4 on the actin cytoskeleton and cell-cell junction.

**Physiological Skin Temperature Induces TRPV4-mediated Ca\textsuperscript{2+} Influx and Rho Activation**—In keratinocyte differentiation, signals mediated by the Rho family of small GTPases are involved in actin organization and cell-cell contact formation and in cortical actin formation for the maintenance of E-cadherin-mediated adhesion (43–47). Following the Ca\textsuperscript{2+} switch, sustained Rho activation was observed in wild-type keratinocytes between 4 and 12 h, similar to a previous report (46), whereas Rho activation was significantly lower in TRPV4-deficient cells throughout the experiments (Fig. 4A). Rho activity in TRPV3-deficient keratinocytes was comparable with that in wild-type cells. These data indicate that Rho activity is regulated downstream of TRPV4 after the Ca\textsuperscript{2+} switch, leading to promotion of actin remodeling.
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FIGURE 3. Actin remodeling and stratification in primary keratinocytes following a Ca\(^{2+}\) switch. A, time course (0–24 h) of the cell-cell junction formation following a Ca\(^{2+}\) switch in WT (upper) and TRPV4-deficient (TRPV4-KO, lower) cells. Actin (green), \(\beta\)-catenin (blue), and E-cadherin (red) are shown as merged images (see supplemental Figs. S3 and S4 for separated images). Similar results were obtained from seven independent experiments. B, formation of intercellular contacts was delayed in TRPV4-deficient keratinocytes. Accelerated formation of intercellular contacts was manifest in WT at 4, 8, and 12 h. Each panel is magnified from a portion of a panel shown in A. Bar = 50 \(\mu\)m. V4KO, TRPV4-deficient. C, actin fiber formation was significantly weaker in TRPV4-deficient keratinocytes 4 (left) and 24 (right) h after the Ca\(^{2+}\) switch. The amount of F-actin was evaluated as (phalloidin-positive area) \(\times\) (the mean pixel intensity) (arbitrary unit), \(n = 6\). D, stratification level was significantly weaker in TRPV4-deficient keratinocytes 24 h after the Ca\(^{2+}\) switch. The level was evaluated with E-cadherin-positive area (maximum image size = 100\%), \(n = 5\)–6. *, \(p < 0.05\) versus WT. **, \(p < 0.01\) versus WT for C and D. E, keratinocytes 48 h after the Ca\(^{2+}\) switch. Strong cell stratification and cortical actin formation were apparent in WT and TRPV3-deficient (TRPV3-KO) keratinocytes, but not in TRPV4-deficient cells (see supplemental Fig. S5 for separated images). Similar results were obtained from 3–6 independent experiments. Bar = 50 \(\mu\)m. F, cell stratification and cortical actin formation were accelerated in TRPV4-deficient cells transfected with TRPV4 cDNA (12 h after the Ca\(^{2+}\) switch) (see supplemental Fig. S5 for separated images). TRPV4 (green), actin (red), and \(\beta\)-catenin (white) are shown. Note that two TRPV4-transfected cells show stratification recognized by \(\beta\)-catenin staining. Early cell-cell contacts and progressive formation of actin fibers are observed in TRPV4-deficient cells. Bar = 50 \(\mu\)m. G, actin fiber formation was weaker in the presence of RR in WT, but not in TRPV4-deficient cells. F-actin 24 h after the Ca\(^{2+}\) switch with (+) or without (−) 5 \(\mu\)M RR is shown. Similar results were obtained from three independent experiments. Bar = 50 \(\mu\)m.

A previous report claims that Rho activity is induced by an increase in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)) (48), which could mediate Ca\(^{2+}\)-induced morphological changes and cell-cell contact formation in keratinocytes (27, 30, 31). Therefore, we measured [Ca\(^{2+}\)]\(_{i}\), after a Ca\(^{2+}\) switch at physiological skin temperature (−33 °C). A significantly higher [Ca\(^{2+}\)]\(_{i}\), was observed in wild-type keratinocytes than in TRPV4-deficient cells between 8 and 24 h after the Ca\(^{2+}\) switch at 33 °C, whereas no such difference was observed at room temperature in either genotype (Fig. 4B). These results, together with RR effects on actin cytoskeleton (Fig. 3G) in wild-type cells, suggest that the greater increase in [Ca\(^{2+}\)]\(_{i}\), in wild-type keratinocytes is caused by warm temperature-activated TRPV4, which may regulate intracellular signaling including Rho activity. The time course consistently matched with that of Rho activation, changes in actin cytoskeleton, and formation of cell-cell junctions upon a Ca\(^{2+}\) switch (Figs. 3 and 4A).

TRPV4 Contributes to Formation of the Normal Intercellular Junction-dependent Barrier—At the final step of keratinocyte differentiation, mature cell-cell junctions, including TJs and AJs, are formed at the uppermost region (apical side) between adjacent stratified cells (42, 47), which overlaps with the cortical actin (Fig. 3 and supplemental Fig. S5). To determine whether TRPV4 is also located within the same region, TRPV4 immunoreactivity was examined after a Ca\(^{2+}\) switch, although the expression level is modest (Fig. 1). TRPV4 colocalized with \(\beta\)-catenin and E-cadherin (Fig. 5, A and B) 24 h after the Ca\(^{2+}\) switch, consistent with the results confirmed by biochemical analysis (Fig. 1 and supplemental Fig. S1). TRPV4 was located at cell-cell borders, on the apical side, areas that overlapped with TJ localization (Fig. 2D) and the honeycomb network of cortical actin (Fig. 3A).

Interestingly, the majority of TRPV4 localized on the apical side of the keratinocytes, whereas faint signal was observed within the stratified areas beneath the cortical actin network. These results strongly indicate that TRPV4 colocalizes with the AJ complex, especially at the apical side of the cells where the intercellular junction barrier is formed (Fig. 2).

Clear localization of TRPV4 at the cell-cell junction barrier prompted us to test whether the intercellular junction structure is affected by TRPV4 because TRPV4 deficiency caused imma-
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In the present study, we have provided evidence that the temperature-sensitive TRPV4 channel promotes cell-cell junction formation in skin keratinocytes, thereby regulating intercellular integrity. Many temperature-sensitive TRP channels are now regarded as polymodal receptors in various types of cells, where those channels evoke multiple signals in response to the changes in physiological contexts. TRPV3 and TRPV4 (12, 13), both of which are expressed in skin keratinocytes, could have distinct roles because they are activated by a similar range of temperatures in the same type of cells. Indeed, TRPV3 is primarily involved in warm temperature-evoked ATP release from keratinocytes (14), and its mutation causes abnormality in hair growth and allergic dermatitis in the skin (15, 16). On the other hand, we found that TRPV4, but not TRPV3, associates with the AJ complex (Fig. 1). Recent reports that TRPV4 is located in close proximity to the actin cytoskeleton in a human keratinocyte cell line (49, 50) strongly support our findings. TRPV4-mediated maturation of intercellular junctions (Figs. 2, 3, and 5) is physiologically significant because the skin barrier prevents dehydration and its dysfunc-
tion can lead to death. However, unlike the mice lacking clau-
din-1 or E-cadherin (21, 22), TRPV4-deficient mice survive,
probably because of the normal expression levels of AJ- and
TJ-related proteins (supplemental Fig. S2). The leaky barrier
might be masked by compensation, such as the thickened cor-
nified layer observed in TRPV4-deficient cells (Fig. 2). Never-
thless, TRPV4-AJ complex formation may be universally
important. TRPV4-mediated functions such as intercellular
permeability (6, 7), the hypotonic response (49), and the
mechanical stretch response (9) are directly relevant to
cytoskeletal/morphological changes. Thus, our findings would
provide a new aspect to clarify the mechanisms of the TRPV4-
mediated physiological responses.

There are at least two ways in which TRPV4 might partici-
pate in AJ formation. In the first, TRPV4 might simply serve as a scaffold component to stabilize the AJ complex. Because TRP channels are thought to consist of tetramer, TRPV4 can promote clustering of E-cadherin/β-catenin complexes surrounding the channel, which might contribute to augmenting tight-
ness of the cell-cell junction in the skin. In the second, TRPV4
may provide Ca\textsuperscript{2+} from the extracellular space, thereby facili-
tating signaling pathways to enhance junction formation. It has
been reported that Rho GTPase, a main regulator of the actin
cytoskeleton in a human keratinocyte cell line (49, 50)
would provide a new aspect to clarify the mechanisms of the TRPV4-
mediated physiological responses.

DISCUSSION

In the present study, we have provided evidence that the
temperature-sensitive TRPV4 channel promotes cell-cell junc-
tion formation in skin keratinocytes, thereby regulating inter-
cellular integrity. Many temperature-sensitive TRP channels
are now regarded as polymodal receptors in various types of
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[Ca^{2+}]_i at physiological skin temperature and changing Rho activity and the progress of cell-cell junction formation (Fig. 3) in the two genotypes. Inhibition of Rho A or its effector Rho-activated kinase is sufficient to perturb development of cell-cell contacts in keratinocytes (47), and Rho-activated kinase reportedly up-regulates Rho E, which is important for cell stratification and whose depletion delays initiation of keratinocyte differentiation (52). These reports support that TRPV4-induced Rho activation is one of the key elements in the development of cell-cell junctions via actin organization. Further analysis is needed to determine upstream signaling for Ca^{2+}-dependent Rho activation. In keratinocytes, [Ca^{2+}]_i regulation involving a cytosolic Ca^{2+} store is known to be mediated through membrane proteins such as Ca^{2+}-sensing receptors (53, 54), TRPC1 (30) and TRPV6 (31), which may explain a considerable increase in [Ca^{2+}]_i in the absence of TRPV4 after a Ca^{2+} switch (Fig. 4). However, our results demonstrate that such a [Ca^{2+}]_i increase was insufficient to overcome TRPV4 deficiency. Additionally, TRPV3, another possible molecule causing Ca^{2+} influx under physiological skin temperature, did not seem to participate in the formation and function of the cell-cell junctions (Figs. 2–5). It is tempting to speculate that Ca^{2+} influx via TRPV4 plays a specific role in regulating the cell-cell junction formation in the AJ complex, whereas increases in [Ca^{2+}]_i, via Ca^{2+}-sensing receptors, TRPC1 and TRPV6, contributes to expression of differentiation-related genes (30, 31, 55).

One might ask how TRPV4-AJ complex controls the integrity of intercellular junctions. Predominant localization of TRPV4 at the apical side of keratinocytes suggests that TRPV4 must be located in close proximity to the TJ barrier to exert its regulatory roles (Fig. 5). There are evidences that abnormal AJs drastically impair the TJ-dependent barrier (22, 40), suggesting that TRPV4 deficiency-induced AJ abnormalities could lead to impaired functionality of the TJ-dependent intercellular barrier. TJs are also regulated by Rho through interaction between the actin cytoskeleton and TJ components (56). Thus, TRPV4-induced Rho activation possibly strengthens the cell-cell junction barrier as well as actin remodeling. Additional studies are necessary to clarify the mechanisms regulating the spatial distribution of TRPV4. Such information might reveal the general dynamics of the TRPV4-AJ complex.

TRPV4 is reportedly involved in the permeability of epithelial monolayers such that TRPV4 activation increases both transcellular and paracellular conductance in a mouse mammary cell line (57). In the latter pathway, TRPV4 was proposed to be involved in down-regulation of TJ protein expression. Another report (58) demonstrated that 4α-PDD increased lung endothelial permeability, which was blocked by RR. The effect of TRPV4 on barrier functions observed in those reports contradicts that obtained in our own study. For instance, expression of cell adhesion-related genes was not significantly altered by TRPV4 deficiency (supplemental Fig. S2), and TRPV4 activation instead increased the integrity of intercellular perme-
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ability (Figs. 2 and 5). Both reports, however, utilized 4α-PDD to activate TRPV4, by which we observed the strong side effects on cytoskeletal reorganization and the morphology during the Ca²⁺ switch (supplemental Fig. S6), making it difficult to discuss the differences. TRPV4/Ca²⁺-dependent signaling and TJ/AJ barrier structure should be different between monolayered cells used in the previous reports and multilayered keratinocytes in our study. For instance, the [Ca²⁺]i increase in endothelial cells disassemble VE-cadherin junctions, thereby increasing intercellular permeability (59). Thus, the molecular mechanisms of TRPV4-regulated barrier integrity may vary depending on the cell, which requires further clarification.

The cell-cell junction-dependent barrier is indispensable for restricting dehydration (20–22). Given that TRPV4 activity can be modulated by skin temperature, we speculate that enhanced TRPV4 activation at high temperature would protect against excess water loss by the barrier enforcement. In contrast, dried skin at cold temperatures might be partially explained by low TRPV4 activity, although there is no direct evidence. Currently, no skin diseases with TRPV4 malfunction have been reported; our results thus raise the possibility that manipulating TRPV4 activity, although there is no direct evidence. Currently, skin at cold temperatures might be partially explained by low excess water loss by the barrier enforcement. In contrast, dried skin at cold temperatures might be partially explained by low TRPV4 activity, although there is no direct evidence. Currently, no skin diseases with TRPV4 malfunction have been reported; our results thus raise the possibility that manipulating TRPV4 activity, although there is no direct evidence. Currently, skin at cold temperatures might be partially explained by low excess water loss by the barrier enforcement. In contrast, dried skin at cold temperatures might be partially explained by low excess water loss by the barrier enforcement. In contrast, dried

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