TRPV6 Is a Ca$^{2+}$ Entry Channel Essential for Ca$^{2+}$-induced Differentiation of Human Keratinocytes

Received for publication, December 12, 2006, and in revised form, June 4, 2007. Published, JBC Papers in Press, June 5, 2007, DOI 10.1074/jbc.M611398200

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Ca$^{2+}$ is an essential factor inducing keratinocyte differentiation due to the natural Ca$^{2+}$ gradient in the skin. However, the membrane mechanisms that mediate calcium entry and trigger keratinocyte differentiation had not previously been elucidated. In this study we demonstrate that Ca$^{2+}$-induced differentiation up-regulates both mRNA and protein expression of a transient receptor potential highly Ca$^{2+}$-selective channel, TRPV6. The latter mediates Ca$^{2+}$ uptake and accounts for the basal [Ca$^{2+}$], in human keratinocytes. Our results show that TRPV6 is a prerequisite for keratinocyte entry into differentiation, because the silencing of TRPV6 in human primary keratinocytes led to the development of impaired differentiated phenotype triggered by Ca$^{2+}$. The expression of such differentiation markers as involucrin, transglutaminase-1, and cytokeratin-10 was significantly inhibited by small interfering RNA-TRPV6 as compared with differentiated control cells. TRPV6 silencing affected cell morphology and the development of intercellular contacts, as well as the ability of cells to stratify. 1,25-Dihydroxyvitamin D3, a cofactor of differentiation, dose-dependently increased TRPV6 mRNA and protein expression in human keratinocytes. This TRPV6 up-regulation led to a significant increase in Ca$^{2+}$ uptake in both undifferentiated and differentiated keratinocytes. We conclude that TRPV6 mediates, at least in part, the pro-differentiating effects of 1,25-dihydroxyvitamin D3 by increasing Ca$^{2+}$ entry, thereby promoting differentiation. Taken together, these data suggest that the TRPV6 channel is a key element in Ca$^{2+}$/1,25-dihydroxyvitamin D3-induced differentiation of human keratinocytes.

Human epidermis is the largest tissue of the body responsible for barrier function and is primarily composed of keratinocytes. Keratinocytes are arranged in highly organized, specialized layers according to their functions and the programmed life cycle. Proliferating keratinocytes comprise the stratum basale. As a result of proliferation, keratinocytes leave the stratum basale, moving toward the exterior with the onset of differentiation in the stratum spinosum. Differentiation is completed in the stratum granulosum, thereby constituting the enucleated stratum corneum, which plays the major role of permeability barrier.

A number of auto- and paracrine factors drive keratinocyte differentiation. Calcium and 1,25-dihydroxyvitamin D3, an active metabolite of vitamin D, play important roles in this process (1). Cytosolic Ca$^{2+}$ signaling is well known to control a wide array of cell functions, ranging from short-term responses such as contraction and secretion to long-term regulation of cell growth and proliferation (2). The epidermis has a calcium gradient, with the lowest concentrations in the stratum basale and the highest in the stratum granulosum, where the proteins critical for barrier function are produced (3, 4). The keratinocyte response to extracellular calcium, i.e. “calcium switch” that induces differentiation, is not fully understood. This response was considered to be, at least partly, mediated by a calcium receptor identified in keratinocytes (5). Indeed, the epidermis of the currently available mouse model with a knocked-out full-length calcium receptor has lower levels of terminal differentiation markers and decreased ability of extracellular calcium to raise intracellular Ca$^{2+}$ concentration (6). Nevertheless, this knock-out did not prevent the cells from differentiation to the full extent, suggesting that other mechanisms may be involved.

Calcium-permeable channels are potential candidates participating in the increased influx of calcium into keratinocytes (7, 8). Of particular interest is a transient receptor potential superfamily of cation channels that includes a remarkable spectrum of channels mediating a variety of sensory and receptor-induced signals (9, 10). Within this superfamily, a member of transient receptor potential subfamily V member 6, TRPV6, is a highly selective Ca$^{2+}$ channel that has already been shown to be expressed in several tissues, including the intestine, kidney, pancreas, prostate, and testis (11–13). TRPV6 is permeable for Ca$^{2+}$, an essential factor inducing keratinocyte differentiation due to the natural Ca$^{2+}$ gradient in the skin.

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The abbreviations used are: TRPV6, transient receptor potential subfamily V member 6 channel; hPK, human primary keratinocyte; IVL, involucrin; KRT10, cytokeratin 10; TGM1, transglutaminase 1; VDR, vitamin D3 receptor; PBS, phosphate-buffered saline; DMEM, Dulbecco’s modified Eagle’s medium; siRNA, small interfering RNA.

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$^{*}$This work was supported in part by grants from Institut National de la Santé et de la Recherche Médicale, Ministère de l’Education Nationale, and the “Pierre Fabre” pharmaceutical company. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

$^{1}$Supported by the Ministère de l’Education Nationale and Region Nord Pas-de-Calais.

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monovalent and divalent cations (14), with high selectivity for Ca$^{2+}$ (15), and has been shown to be involved in Ca$^{2+}$ (re)absorption in Ca$^{2+}$-transporting tissues, including the small intestine and kidney (11). Furthermore, in these tissues TRPV6 expression is directly up-regulated by 1,25-dihydroxyvitamin D3 (16).

Interestingly, 1,25-dihydroxyvitamin D3 promotes keratinocyte differentiation via similar pathways as calcium (17). The 1,25-dihydroxyvitamin D3 receptor (VDR) is critical for the genomic actions of 1,25-dihydroxyvitamin D3 (18). VDR-null mice have also been shown experimentally to exhibit a defect in epidermal differentiation, demonstrated by reduced levels of involucrin (IVL), profilaggrin, and loricrin, as well as the loss of keratin granules (19). Thus, 1,25-dihydroxyvitamin D3 also appears to be one of the key regulators of keratinocyte differentiation in vivo.

Together, calcium and 1,25-dihydroxyvitamin D3 regulate the programmed differentiation process by sequentially turning on and off the genes that produce the elements required for differentiation, as well as activating the enzymes directly involved. However, it is not known yet which of these targets play the role of extracellular Ca$^{2+}$ sensors or triggers during Ca$^{2+}$-induced differentiation. We therefore investigated the role of the TRPV6 channel in Ca$^{2+}$-induced differentiation of human keratinocytes.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The HaCaT human keratinocyte cell line, kindly provided by Prof. B. Dufy, CNRS UNR 5543, Bordeaux, France, was cultured in Dulbecco’s medium (DMEM) (Invitrogen) supplemented with 10% fetal calf serum, containing 0.07 mM Ca$^{2+}$, 100 μg/ml kanamycin, and 2 mM L-glutamine. Human primary keratinocytes

![Table 1: Primers and siRNA](image)

| No. | Name, NCBI accession no. | Forward (5’...-3’) | Backward (5’...-3’) | Expected size bp |
|-----|-------------------------|-------------------|--------------------|-----------------|
| 1.  | TRPV6, NM_018646         | ATGGTGATGCGGCTCATCAGTG | GTAGAAAGCTGCCTGCTTCCG | 255             |
| 2.  | KRT10, NM_000421         | TACACACTGTAATATGCTCAAT | ATTGGCAGAATTTCAGGT | 260             |
| 3.  | IVL, NM_005547           | TCTTAAAAGTGCCCCAGGAAGCA | TCTGATCCTGGTCCCCAGGGTTT | 292             |
| 4.  | TGM1, NM_000359          | GATCCGATTCACCTGGATTTCC | TCCCTCTGTCCCCTCCATACCATCAAT | 304             |
| 5.  | VDR, NM_000376           | CCAAGTTCTCGAAGTATGTT | GTTCCTTCTGGTGAAGAAGAA | 383             |
| 6.  | β-actin, NM_001101       | 5’-CAGCAGGAGCGAGCAATCTTT-3’ (dTdT) | 5’-TTGCAAGGTCTCAAACGATGTC-3’ (dTdT) | 209             |
| 7.  | TRPV6 siRNA              | 5’-CCUCUCUUCAGCGAAGAGG (dTdT) - 3’ (position 111–129 from ATG) | 8.  | TRPV6 siRNA-1 | 5’-GACUCUCUUCAGCGAAGAGG (dTdT) - 3’ (position 825–843 from ATG) |
(hPKs) were obtained from adult skin after breast reduction surgery as previously described (20), according to the rules of the Medical Center Ethics Committee, Centre Hospitalier Régional Universitaire Lille.

Cells were cultured at 37°C in a humidified atmosphere with 5% CO2 in air. The medium was changed three times a week, and cultures were split by treating the cells with 0.25% trypsin (in PBS) for 5 min at 37°C prior to confluency. For the experiments, cells were seeded in 6-well plates for PCR and Western blotting and on glass coverslips for immunocytochemistry and calcium imaging.

For the 1,25-dihydroxyvitamin D3 studies, cells were treated with EtOH as a solubilizer for 1,25-dihydroxyvitamin D3.

Throughout this report, the terms “undifferentiated” and “differentiated” keratinocytes are used as follows. Cells were cultured in a complete DMEM (HaCaT) or in a Defined-Keratinocyte serum-free medium (SFM) (hPK) containing 0.07 mM Ca2+/H11001 (undifferentiated) or 1.8 mM Ca2+/H11001 (differentiated), corresponding to the differentiation state of these cells. Generally, for the differentiated phenotype, cells were allowed to attach for 24 h after trypsinization, and then a 0.07 mM Ca2+/H11001-containing DMEM (or a Defined-Keratinocyte SFM) was replaced by DMEM with 1.8 mM Ca2+/H11001 (or a Defined-Keratinocyte SFM with 1.8 mM Ca2+/H11001). After 24–48 h of incubation in the latter medium, cells were considered differentiated and their state was confirmed by PCR and Western blotting of corresponding markers.

Human embryonic kidney cells were cultured in complete DMEM supplemented with 10% fetal bovine serum.

Reverse Transcription PCR—Total RNA was isolated using the guanidinium thiocyanate-phenol-chloroform extraction procedure (21).

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Reverse Transcription PCR—Total RNA was isolated using the guanidinium thiocyanate-phenol-chloroform extraction procedure (21). After DNase I (Invitrogen) treatment to eliminate genomic DNA, 2 μg of total RNA was reverse-transcribed into cDNA at 42°C,
using random hexamer primers (PerkinElmer) and MuLV reverse transcriptase (PerkinElmer) in a 20-μl final volume followed by PCR as described below. The PCR primers used to amplify TRPV6, INV, TGM1, KRT10, VDR, and β-actin cDNAs are specified in Table 1. PCR was performed on the reverse transcription-generated cDNA using a GeneAmp PCR System 2400 thermal cycler (PerkinElmer). To detect different cDNAs, PCR was performed by adding 1 μl of reverse transcription template to a mixture of (final concentrations) 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 2.5 mM MgCl₂, 200 μM each dNTP, 600 nM sense and antisense primers, and 1 unit of AmpliTaq Gold (PerkinElmer) in a final volume of 25 μl. DNA amplification conditions included an initial 5-min denaturation step at 95 °C (which also activated the Gold variant of Taq polymerase) and 33 cycles as follows: 30 s at 95 °C, 30 s at 59 °C, 30 s at 72 °C, and finally 7 min at 72 °C. Density was measured using “Quantity one” software (Bio-Rad), and the data were analyzed using Origin 7.0 (Microcal Software Inc., Northampton, MA).

siRNA Cell Transfection—Human keratinocytes were transfected overnight with 200 nM siRNA-TRPV6/well in a six-well plate using “Gene porter 2” (Gene Therapy Systems, Inc.) in a final volume of 1 ml. Ready-to-use siRNA-TRPV6 (processing option A4) was synthesized by Dharmacon Research Inc. (see Table 1). Functional non-coding siRNA 1 (Dharmacon Research Inc.) was used as a control.

Immunocytochemistry—Cells grown on glass coverslips were washed once with PBS and incubated with cholera toxin subunit B Alexa Fluor® 488 conjugate (1/2000; Molecular Probes) for 15 min and then washed once with PBS and fixed in 3.5% paraformaldehyde in PBS, PBS-glycine (30 mM) was used to quench the reaction, and the subsequent permeabilization was obtained using 0.1% Triton X-100. The cells were washed again in PBS and subjected to a conventional immunostaining procedure. Alexa Fluor® 546 or 488 goat anti-rabbit IgGs (1/4000; Molecular Probes) were used as secondary antibodies for TRPV6, or KRT10 and IVL staining, respectively. Fluorescence was analyzed on a Carl Zeiss Laser Scanning Systems LSM 510 connected to a Zeiss Axiovert 200M with a 1.4 numerical aperture oil immersion lens at room temperature. Both channels were excited, collected separately, and then merged to examine colocalization using Carl Zeiss LSM Image Examiner software.

Western Blotting—Semiconfluent HaCat or hPK cells were treated with an ice-cold lysis buffer containing 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, 1% Nonidet P-40, and protease inhibitor mixture from Sigma. The lysates were centrifuged at 15,000 g and 4 °C for 20 min, with a sample buffer containing 125 mM Tris-HCl, pH 6.8, 4% SDS, 5% β-mercaptoethanol, 20% glycerol, 0.01% Bromphenol blue, and boiled for 5 min at 95 °C. Total protein samples were subjected to 8–10% SDS-PAGE and transferred to a nitrocellulose membrane by semi-dry Western blotting (Bio-Rad Laboratories). The membrane was blocked in a 5% milk TNT buffer (Tris-HCl, pH 7.5, 140 mM NaCl, and 0.05% Tween 20) overnight and then probed using specific rabbit polyclonal anti-TRPV6 antibody (1/200; Alomone Labs).
Calcium Imaging—Cells were plated onto glass coverslips and loaded with 4 μM Fura-2 AM in the growth medium at room temperature for 45 min. Recordings were performed in Hanks’ buffered salt solution containing (in mM) 140 NaCl, 5 KCl, 2 MgCl₂, 0.3 Na₂HPO₄, 0.4 KH₂PO₄, 4 NaHCO₃, 5 glucose, and 10 HEPES, adjusted to pH 7.4 with NaOH. CaCl₂ was adjusted to 0.07 or 1.8 mM, depending on the experiment. The coverslips were then placed in a perfusion chamber on the microscope stage. Fluorescence images of the cells were recorded with a video image analysis system (Quanticell). Fura-2 fluorescence at an emission wavelength of 510 nm was recorded by exciting the probe alternately at 340 and 380 nm. The signal ratio at 340/380 nm was converted into [Ca²⁺]ᵢ levels using an in vitro calibration.

Statistics—Data were expressed as mean ± S.D. Statistical analyses were carried out using Student’s unpaired t-tests (two-tailed). p < 0.05 indicates statistical significance.

RESULTS

Ca²⁺-induced Differentiation and TRPV6 Expression in Human Keratinocytes—Two types of human keratinocytes were selected for our study: HaCaT, a cell line similar in functional competence to normal keratinocytes (22), and human primary keratinocytes (hPK). We initially studied the mRNA expression of keratinocyte differentiation-specific markers such as IVL, transglutaminase 1 (TGM1), and cytokeratin 10 (KRT10) under semiconfluent conditions in the presence of 0.07 or 1.8 mM Ca²⁺ in DMEM (Fig. 1A). Little, if any, IVL transcript was detected in 0.07 mM Ca²⁺, whereas IVL mRNA levels increased in the differentiated state. Similarly, TGM1 levels, encoding a rate-limiting enzyme for cornified envelope formation, increased with Ca²⁺-induced differentiation of HaCaT keratinocytes. KRT10 was especially interesting as a marker of HaCaT differentiation because it was undetectable under 0.07 mM Ca²⁺ conditions and appeared following Ca²⁺-induced differentiation. Thus, cells kept in the medium containing 0.07 mM Ca²⁺ were considered undifferentiated (U), whereas those kept in the medium containing 1.8 mM Ca²⁺ were considered differentiated (D). This model was justified by using hPK stained for KRT10 (green) and nuclei (blue) to show the ability of hPK to differentiate by expressing KRT10 and forming a supralayer under the above conditions (Fig. 1B).

The expression of TRPV6 mRNA in HaCaT cells is apparently differentiation-dependent (Fig. 1). We observed an almost 5-times increase in TRPV6 mRNA in HaCaT cells kept in 1.8

TRPV6 Drives Differentiation of Human Keratinocytes

Reagents—All reagents were purchased from Sigma unless otherwise specified.

Statistics—Data were expressed as mean ± S.D. Statistical analyses were carried out using Student’s unpaired t-tests (two-tailed). p < 0.05 indicates statistical significance.
TRPV6 Drives Differentiation of Human Keratinocytes

TRPV6 Is a Ca\(^{2+}\) Uptake Channel Contributing to Increase in [Ca\(^{2+}\)], following Keratinocyte Differentiation—To investigate the contribution of TRPV6, as a Ca\(^{2+}\)-selective channel, to a calcium influx into keratinocytes during Ca\(^{2+}\)-induced differentiation, we recorded the response of the keratinocytes to acute changes in [Ca\(^{2+}\)], from 0 to 1.8 mm by measuring intracellular calcium levels ([Ca\(^{2+}\)]) in cells pretreated with the control and anti-TRPV6 siRNAs (previously validated on the protein level, Fig. 2A). Both undifferentiated and differentiated HaCaT (uHaCaT and dHaCaT), as well as hPK (uhPK and dhPK), cells responded to the acute changes in [Ca\(^{2+}\)], by increases in [Ca\(^{2+}\)]. This effect was significantly suppressed in the cells treated with siRNA-TRPV6 (Fig. 2, B–F). We also checked whether this acute calcium uptake was specific to TRPV6 by using anti-TRPC7 oligo-RNA (Fig. 2G). This anti-TRPC7 oligo-RNA induced the extinction of the corresponding protein but the acute response to the [Ca\(^{2+}\)] increase was not affected, suggesting that TRPV6 plays a specific role in this process. TRPV5, a Ca\(^{2+}\)-selective TRP channel very similar to TRPV6, was not expressed in either keratinocyte species (data not shown), indicating that the above effects were explicitly confined to TRPV6.

These data suggest that TRPV6 is a constitutively open channel responsible for mediating the cytoplasmic Ca\(^{2+}\) increase in response to an increase in extracellular Ca\(^{2+}\) concentrations, i.e. a “Ca\(^{2+}\) switch”.

TRPV6 Is Essential for Keratinocyte Differentiation—The effects of TRPV6 silencing on Ca\(^{2+}\)-induced differentiation of human keratinocytes were studied using the same set of differentiation markers. Both HaCaT and hPK cells grown at 0.07 mm Ca\(^{2+}\) were transfected with siRNA-TRPV6, incubated for 3 days, and then subjected to a Ca\(^{2+}\) switch for 24 h. The effects of TRPV6 silencing on Ca\(^{2+}\)-induced differentiation of human keratinocytes, indicated by the expression of differentiation-specific markers, are shown in Fig. 3. TRPV6 silencing significantly decreased mRNA levels of IVL, TGM1, and KRT10 as compared with non-transfected differentiated hPK and HaCaT cells treated with Ca\(^{2+}\) under the same conditions (Fig. 3, A and B). Quantitative histograms depicting the effects of TRPV6 silencing on the expression of differentiation markers normalized to their levels in undifferentiated keratinocytes are shown in Fig. 3, C and D. Western blotting of the key differentiation

mm Ca\(^{2+}\) for 3 days (the numbers under TRPV6 panel indicate the relative intensities of the bands) as compared with the 0.07 mm Ca\(^{2+}\) condition. Immunoblotting with anti-hTRPV6 antibody (verified using human embryonic kidney cells stably overexpressing TRPV6, Fig. 1C) detected significant increases in TRPV6 protein in both HaCaT and hPK cells following differentiation (Fig. 1, D and E). However, it should be noted that when loaded with more than 20 μg of total protein/well the enhanced chemiluminescence method lost its linearity, compromising quantifications. To confirm our data HaCaT and hPK cells were immunostained with cholera toxin (green), used as a plasma membrane marker, and TRPV6 (red) (Fig. 1F). This experiment (together with Western blot and PCR techniques) confirmed the significant increase in TRPV6 protein levels after Ca\(^{2+}\)-induced differentiation.

Thus, the expression pattern of differentiation markers such as IVL, TGM1, and KRT10 correlated with the differentiation state of the keratinocytes. TRPV6 is important for keratinocyte differentiation and, to a certain extent, may also represent a differentiation marker.

FIGURE 5. The effects of 1,25-dihydroxyvitamin D3 on expression of TRPV6 mRNA in undifferentiated (A) and differentiated (B) HaCaT cells. Semiconfluent cells were incubated with 0.1–500 nM 1,25-dihydroxyvitamin D3 for 3 days. Total mRNA was isolated, reverse-transcribed, and subjected to PCR. C, graph representing quantitative up-regulation of TRPV6 mRNA in response to 1,25-dihydroxyvitamin D3. D, Western blot of TRPV6 protein levels in control undifferentiated HaCaT and cells incubated with 100 nM 1,25-dihydroxyvitamin D3 for 3 days. E, a separate series of experiments showing TRPV6 mRNA expression for subconfluent HaCaT cells and the corresponding histogram (F). All data were normalized to control, taken as 1. Asterisks denote statistical significance; p < 0.05, for at least three experiments.
markers such as IVL and KRT10 was also used to confirm the effects of TRPV6 silencing on protein levels (Fig. 3E).

We also investigated whether TRPV6 silencing had an influence on keratinocyte morphology. Light microscopy examination showed that TRPV6 silencing significantly affected the ability of the cells to change their shape and develop cell-to-cell interactions (Fig. 4Aa). Black arrows indicate the closing of cell-to-cell contacts among three adjacent cells. As can be seen from Fig. 4Aa, in low Ca\(^{2+}\) conditions the membranes of the cells are not closed, leaving enough spare space among them. In high Ca\(^{2+}\) conditions the membranes are in complete contact with one another. TRPV6 knock down also impairs the ability of the cells to form complete closed contacts among three adjacent cells. The cell shape is also likely to be affected by TRPV6 knock down, which prevents the cells switching from well rounded to flattened form allowing assembly of monolayer epidermal sheets. The other important criterion in assessing keratinocyte differentiation is the ability of keratinocyte monolayers to stratify. Immunocytochemistry of the confluent HaCaT cells co-stained with anti-TRPV6 (red) and anti-KRT10 or anti-IVL antibody (green) revealed that the ability of keratinocytes to form a supralayer of differentiated cells was impaired (Fig. 4, Ab and Ac). The quantitative effects of TRPV6 silencing are demonstrated in the corresponding histogram (Fig. 4B). These results suggest that TRPV6 is an essential component in inducing human keratinocyte differentiation.

**1,25-Dihydroxyvitamin D3 Increases TRPV6 Expression in HaCaT Cells**

Having demonstrated the importance of extracellular calcium levels for the expression of differentiation markers and TRPV6, we then studied the effects of 1,25-dihydroxyvitamin D3, one of the key autocrine and paracrine keratinocyte differentiation regulators. The effects of 1,25-dihydroxyvitamin D3 (0.1–500 nM) on TRPV6 mRNA expression levels in undifferentiated and differentiated semiconfluent human keratinocytes are shown in Fig. 5. HaCaT cells were kept in either 0.07 or 1.8 mM [Ca\(^{2+}\)]\(_o\) for at least 3 days and then incubated with the corresponding concentration of 1,25-dihydroxyvitamin D3 for 3 days. 1,25-Dihydroxyvitamin D3 increased the expression of TRPV6 mRNA transcripts in human keratinocytes in a concentration-dependent manner (Fig. 5, A and B). It is noteworthy that in their undifferentiated
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FIGURE 7. TRPV6 contributes to basal [Ca\(^{2+}\)], in human keratinocytes. Basal [Ca\(^{2+}\)], was measured in undifferentiated (A, C) and differentiated (B, D) hPK (A, B) and HaCaT (C, D) cells. Cells were transfected either with siRNA 1 or siRNA-TRPV6, incubated for 3 days, and then treated with 100 nM 1,25-dihydroxyvitamin D3 for 48 h. Cells were loaded with Fura-2AM in the same incubation medium and subjected to calcium imaging. Asterisks denote statistical significance; *, p < 0.01; **, p < 0.05; n = 300 cells.

A.

state (Fig. 5A) HaCaT cells were more sensitive to 1,25-dihydroxyvitamin D3 than when they were differentiated (Fig. 5B). The corresponding quantitative plot (Fig. 5C) shows the effect of 1,25-dihydroxyvitamin D3 on TRPV6 mRNA expression. The protein level was also increased, as confirmed by Western blotting (Fig. 5D).

A separate series of experiments showing TRPV6 mRNA expression in subconfluent HaCaT cells was performed. Fig. 5E shows the effects of Ca\(^{2+}\) 0.1 and 100 nm 1,25-dihydroxyvitamin D3 on TRPV6 mRNA levels in subconfluent HaCaT cells. Under undifferentiated low Ca\(^{2+}\) (0.07 mM) conditions, 1,25-dihydroxyvitamin D3 was capable of increasing TRPV6 expression to an extent very similar to that of differentiated high Ca\(^{2+}\) (1.8 mM) HaCaT cells (Fig. 5F). However, 1,25-dihydroxyvitamin D3 was unable to initiate the expression of the keratinocyte differentiation marker KRT10, which was still exclusively dependent on Ca\(^{2+}\)-induced keratinocyte differentiation.

TRPV6 Channel Is Responsible for the Enhanced Ca\(^{2+}\) Uptake Induced by 1,25-Dihydroxyvitamin D3—Having demonstrated that 1,25-dihydroxyvitamin D3 up-regulated the TRPV6 expression responsible for Ca\(^{2+}\) uptake in keratinocytes, we investigated whether TRPV6 mediated the pro-differentiating effects of 1,25-dihydroxyvitamin D3. Cells were pretransfected with siRNA-TRPV6 72 h before the experiment and treated with 1,25-dihydroxyvitamin D3 for 48 h (Fig. 6). siRNA-TRPV6 significantly inhibited the keratinocyte response to acute changes in [Ca\(^{2+}\)], from 0 to 1.8 mM. All the cells, irrespective of their differentiation state, responded to the acute changes in [Ca\(^{2+}\)], by increasing [Ca\(^{2+}\)]. This effect was significantly suppressed by pretreatment with siRNA-TRPV6 (Fig. 6, B–E), confirming that 1,25-dihydroxyvitamin D3-induced Ca\(^{2+}\) uptake was mediated by TRPV6. In another series of experiments, we recorded the basal [Ca\(^{2+}\)] level in human keratinocytes. The increase in [Ca\(^{2+}\)], evoked in both undifferentiated and differentiated HaCaT and hPK cells by 100 nM 1,25-dihydroxyvitamin D3 was suppressed by pretreating the cells with siRNA-TRPV6 (Fig. 7). Contrary to hPK, where the basal [Ca\(^{2+}\)], in the low calcium medium was much higher (Fig. 7A), siRNA-TRPV6 decreased basal [Ca\(^{2+}\)], in differentiated (Fig. 7D), but not undifferentiated (Fig. 7C), HaCaT keratinocytes. It is evident that TRPV6 contributes to steady state [Ca\(^{2+}\)], in human keratinocytes. Thus, TRPV6 mediates 1,25-dihydroxyvitamin D3-regulated Ca\(^{2+}\)-influx into human keratinocytes.

DISCUSSION

In this study, we report three major findings: 1) Ca\(^{2+}\)-induced differentiation of human keratinocytes involves up-regulation of the TRPV6 Ca\(^{2+}\)-selective cationic channel as a prerequisite for keratinocyte differentiation; 2) TRPV6 mediates the acute response of keratinocytes to the “calcium switch”; 3) 1,25-dihydroxyvitamin D3, a keratinocyte differentiation cofactor, increases TRPV6 expression and potentiates Ca\(^{2+}\) uptake.

TRPV6 Channel Is a Likely Candidate for Mediating Ca\(^{2+}\)-induced Differentiation—In the present work for the first time we identified TRPV6 in human keratinocytes as a functional Ca\(^{2+}\)-mediated channel with an expression pattern dependent on differentiation state. Indeed, although TRPV6 expression, regulation, and functions in intestine (13), kidney (23, 24), and cancerous prostate (25, 26) had been previously studied, the role of TRPV6 in the epidermis, where calcium plays a major role in cell physiopathology, was previously unknown.

Our results showed that Ca\(^{2+}\)-triggered differentiation involved TRPV6, a Ca\(^{2+}\)-selective cationic channel. TRPV6 is present in undifferentiated keratinocytes at comparatively low levels, and [Ca\(^{2+}\)], i.e. Ca\(^{2+}\) switch, induces its up-regulation in differentiated keratinocytes. The data on TRPV6 silencing indicated that human keratinocytes pretransfected with siRNA-TRPV6 developed a significantly different phenotype from that of control differentiated cells. We also report evidence that TRPV6 affects the differentiation of human keratinocytes by inhibiting the expression of IVL, TGM1, and KRT10, crucial components required for differentiation. It is noteworthy that initiation of gene transcription for these components is Ca\(^{2+}\)-dependent (1, 3, 17). This supports the hypothesis that TRPV6 is a prerequisite for keratinocyte entry into differentiation. We showed that TRPV6 was actively involved in Ca\(^{2+}\) uptake, acting as a Ca\(^{2+}\) entry channel in both hPK and HaCaT cell models. The impact of TRPV6 silencing on cell morphology and the ability of keratinocytes to form supralayers of differentiated cells is intriguing. Indeed, TRPV6 silencing clearly has a signif-
Ca\(^{2+}\)-induced and 1,25-Dihydroxyvitamin D3-regulated Ca\(^{2+}\) Influx into Keratinocytes Is Mediated by TRPV6—The response of keratinocytes to calcium is complex. Ca\(^{2+}\) switch results in a sustained increase in basal [Ca\(^{2+}\)]\(_{i}\), which activates a number of calcium-dependent activating protein-1 (AP-1) transcription factors such as JunB, JunD, Fra1, and Fra2 required to drive differentiation-specific genes, e.g. IVL (29) and TGM1 (1). On the contrary, agents like ATP and epidermal growth factor that only stimulate a transient increase in [Ca\(^{2+}\)]\(_{i}\), are unable to stimulate keratinocyte differentiation (30).

We showed that 1,25-dihydroxyvitamin D3 dose-dependently increased TRPV6 expression, especially in undifferentiated keratinocytes. These results suggest that transcription of the trpv6 gene may be directly activated by VDR. Despite the fact that the VDR-response element in the human trpv6 gene failed to match that in mice (31), recent work by Wang et al. (32) revealed VDR-response elements in the human trpv6 gene promoter. The requirement for other transcriptional factors, such as the retinoic acid receptor, to be recruited by VDR (33) seems plausible and will require further investigation.

It should be noted that 1,25-dihydroxyvitamin D3-induced TRPV6 expression was comparable with that of differentiated keratinocytes. These results suggest that 1,25-dihydroxyvitamin D3 is an important regulator or cofactor, rather than a trigger of keratinocyte differentiation.

We showed that the 1,25-dihydroxyvitamin D3-induced increase in both Ca\(^{2+}\) uptake and basal [Ca\(^{2+}\)]\(_{i}\), was substantially attenuated by siRNA-TRPV6. This suggests that TRPV6 plays a key role in 1,25-dihydroxyvitamin D3-induced Ca\(^{2+}\) influx in human keratinocytes irrespective of their differentiation state. Consequently, 1,25-dihydroxyvitamin D3 enhances Ca\(^{2+}\)-induced differentiation by increasing the ability of keratinocytes to uptake Ca\(^{2+}\) via TRPV6.

Thus, the high Ca\(^{2+}\) selectivity of the TRPV6 channel represents an essential component in Ca\(^{2+}\) influx, especially in 1,25-dihydroxyvitamin D3-induced Ca\(^{2+}\) influx into keratinocytes. Up-regulation of the TRPV6 channel in differentiated cells is an important contributory component in Ca\(^{2+}\)-entry, thereby promoting differentiation. Visualization of the TRPV6 channel by immunocytochemistry not only confirmed the levels obtained by PCR and Western blotting but also indicated an increased TRPV6 expression on the plasma membrane in differentiated keratinocytes, suggesting the mechanism for TRPV6 translocation to the membrane in response to the Ca\(^{2+}\) switch. Indeed, a number of TRP channels have been shown to be regulated by the "translocation" mechanism in response to various stimuli. Bezzerides et al. (34) demonstrated the rapid translocation of TRPC5 from vesicles held in reserve just under the plasma membrane, which dramatically increased membrane-associated TRPC5 channels and functional TRPC5 current. Bindels and co-workers (35) showed that TRPV6-mediated current in human embryonic kidney 293 cells was dependent on S100A10-annexin complex routing TRPV6 to the plasma membrane and highlighted the important role of Rab11a in trafficking TRPV6 to the plasma membrane (36). However, other protein partners are also involved in regulating TRPV6 activity (for review see Ref. 37). The TRPV6 regulation mechanism, like extracellular signal-triggered translocation to the human keratinocyte plasma membrane, is of potential interest and will be studied in the near future. Furthermore, in undifferentiated hPK, we observed a preferential localization of TRPV6 at the site of pseudopodes, suggesting that TRPV6 plays a role in cell movement that is known to be Ca\(^{2+}\)-dependent (38).

Finally, a very recent work by Hediger and co-workers (39) showed that the skin of trpv6 KO mice had fewer, thinner layers of stratum corneum, decreased total Ca\(^{2+}\) content, and loss of the normal Ca\(^{2+}\) gradient. In addition, 20% of all trpv6 KO animals developed alopecia and dermatitis. In conclusion, our study strongly suggests that TRPV6 channel is a major player in the control of human keratinocyte differentiation and may therefore be responsible for skin pathologies.
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