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Endothelin-2/vasoactive Intestinal Contractor via ROCK regulates transglutaminase 1 on differentiation of mouse keratinocytes

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Abstract

We previously found that endothelin-2/vasoactive intestinal contractor (ET-2/VIC) greatly increased in mouse epidermis after birth. In the present study, we evaluated whether ET-2/VIC expression was associated with the calcium-induced differentiation of cultured mouse keratinocytes. The differentiation induction was revealed by morphological change, cornified envelope (CE) formation, and involucrin and transglutaminase 1 (TG 1) expressions. ET-2/VIC gene expression and peptide production subsequently increased in the induction of the differentiation. We also found that Y-27632, a Rho-associated coiled–coil forming protein serine/threonine kinase (ROCK) inhibitor, suppressed up-regulation of ET-2/VIC gene expression, the induction of morphological change, the CE formation, and TG 1 expression, but not involucrin expression. These results indicate new three findings, (1) ET-2/VIC expression increases and has potential as a differentiation marker, (2) ET-2/VIC expression is mediated by ROCK, and (3) the ROCK regulated TG 1 expression, on the calcium-induced differentiation of mouse keratinocytes.

Keywords: Differentiation; Endothelin-2 (ET-2)/vasoactive intestinal contractor (VIC); Keratinocyte; Transglutaminase 1 (TG 1); Rho-associated coiled–coil forming protein serine/threonine kinase (ROCK)

Endothelin-2 (ET-2) and vasoactive intestinal contractor (VIC) are members of the endothelin (ET) vasoconstrictor peptide family, where VIC is thought to be the mouse and rat orthologous peptide of the human ET-2. The structures of VIC and ET-2 differ from that of ET-1 by three and two amino acid residues, respectively [1]. ET-2/VIC gene expression has been observed at high levels in ovary and uterus as well as in stomach and intestine [1]. We previously reported that ET-2/VIC gene expression increased significantly during embryonic development [1]. We also found a greater increase in ET-2/VIC, ET receptor type A (ET-A), and ET receptor type B (ET-B) production in mouse epidermis and dermis after birth than before birth [2]. These observations suggest that ET-2/VIC also plays an important role in skin development after birth.

The actions and the expression patterns of ET family peptides and their receptors in skin have been studied. ET-1 increased by ultraviolet B (UVB) in keratinocytes was reported to stimulate melanocyte proliferation [3]. UVB also was reported to up-regulate ET-1, ET-A, and ET-B in cultured human keratinocytes [4]. ET-2 gene expression, on the other hand, was reported to be down-regulated by UVB-induced apoptosis in human keratinocytes [5]. Furthermore, ET-A binding by ET-1 was reported to promote the proliferation of keratinocytes from human foreskin [6]. ET-B was reported to be the main receptor in melanocyte development during embryogenesis

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[7]. These reports indicate that the ET system is closely associated with skin functions. However, little is known about the regulation of the ET system in keratinocyte differentiation.

In the present study, we evaluated whether the ET system, specially, ET-2/VIC, is associated with differentiation in cultured mouse neonatal epidermal keratinocytes. We found that ET/VIC has a novel function in mouse skin.

Materials and methods

Reduction of calcium from FBS with Chelex-100 resin. To reduce the calcium concentration in FBS, we used Chelex-100 resin (200–400 mesh, sodium form, Bio-Rad Laboratories Ltd., Hercules, CA). Chelex 100 resin (2 g) was mixed and stirred with 50 mL distilled water at room temperature, and the pH was adjusted to 7.5 by addition of HCl. The slurry was filtered, and 50 mL FBS was added to the washed resin. After stirring for 1 h, the FBS was separated from the resin by centrifuging. The supernatant was sterilized by passage through a filter membrane. The calcium concentration in FBS treated with Chelex 100 resin was measured with Calcium *C*-test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The calcium concentrations of untreated FBS and treated FBS with Chelex 100 resin were 4.035 ± 0.005 mM ($n = 2$) and 0.087 ± 0.061 mM ($n = 4$), respectively.

Preparation of keratinocytes from newborn mouse skin. ICR neonates (1 day old) were purchased from Japan Clea (Tokyo, Japan). The present study was approved by the Animal Care Committee at the National Institute of Advanced Industrial Science and Technology (AIST).

The skin, removed from the trunk between the limbs, was incubated by floating onto 0.25% trypsin-1 mM ethylenediamine tetraacetic acid (EDTA) solution for 16 h at 4 °C. Epidermal sheets were mechanically separated from the dermis with forceps and then were gently shaken and repeatedly pipetted with Pasteur pipette to generate a single cell suspension in phosphate-buffered saline (PBS) containing 0.02% EDTA. Undissociated cell clusters were removed by filtering through steel mesh (Ikemoto Scientific Technology Co., Ltd., Tokyo, Japan). The keratinocytes were obtained by centrifuging the epidermal cell suspension.

Cell culture. The cells were cultured in a normal 10-cm dish (Becton-Dickinson, San Jose, CA) containing the culture medium (low calcium, at a final concentration of 0.06 mM), which consisted of S-MEM (calcium free, Invitrogen Corp., Carlsbad, CA) supplemented with 9% Chelex 100 resin-treated FBS as described above, 0.1 mM non-essential amino acids, 2 mM-glutamine, 0.05 mM $CaCl_2$, 4 ng/mL epidermal growth factor, and antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B), at 37 °C in a humidified atmosphere of 5% CO_2 in air. The cells were passed two to three times a week with dispase solution (2 U/mL) sterilized by passage through filter membrane.

To induce differentiation, the cells were incubated in the differentiation medium (high calcium, at a final concentration of 2.0 mM), which consisted of minimum essential medium (MEM) supplemented with 10% FBS and the antibiotics listed above.

Observation of morphological change. The cells were seeded in 3- or 6-cm dishes containing 2 or 3 mL culture medium, respectively. After the cell cultures reached a semiconfluent stage, the culture medium was replaced with fresh differentiation medium. After a period of incubation, observation of morphological change was carried out with a phase-contrast microscope.

RT-PCR. Cells were seeded and cultured, and cell differentiation was induced as described above. After a period of incubation, the cells were washed. Preparation of lysate and total RNA, quantification and conversion of total RNA, and PCR were carried as described previously [8]. Table 1 shows the PCR conditions used for the amplification procedure and the oligonucleotide primer sets. The intensities of ET-2/VIC, ET-1, ETA, and ETB were normalized to the endogenous reference 18S rRNA. These four primer sets for ET-2/VIC, ET-1, ETA, and ETB used in the present study span each introns [1]. No band from genomic DNA contamination was detected in all RT-PCR experiments.

Western blot (WB) analysis. Cells were seeded, cultured, and induced to differentiation as described above. After a period of incubation, the cells were recovered by lysing with 0.5 mL of a sample buffer (50 mM Tris–HCl, pH 8.0, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, and 1 mM phenylmethanesulfonyl fluoride) and by cell scraper. The recovered cells were homogenized with the Physcotron homogenizer (Microtec Co., Ltd., Chiba, Japan) for 1 min on ice and then centrifuged at 10,000*g* for 15 min at 4 °C. An aliquot of the supernatant was taken to determine protein content using the BCA Protein Assay kit (Pierce Biotechnology, Inc., Rockford, IL). An equal amount of protein (20–50 μg) was recovered by precipitation using 1 mM HCl–acetone from a portion of the supernatant. The protein was lysed in a sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) buffer (0.5 M Tris–HCl, pH 6.8, 10% SDS, 5% 2-mercaptoethanol (2-ME), and 10% glycerol) and boiled at 100 °C for 1 min. The protein was subjected to 10% SDS–PAGE and electroblotted on a polyvinylidene fluoride (PVDF) membrane. The PVDF membrane was probed with one of the following primary antibodies: rabbit anti-involucrin polyclonal antibody (1:1000; Covance Research Products Inc., Berkeley, CA) or rabbit anti-transglutaminase 1 (TG 1) polyclonal antibody (1:200; H-87, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The membranes were incubated with AP-conjugated goat anti-mouse (Bio-Rad Laboratories Ltd.) or goat anti-rabbit (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) secondary antibodies (1:2500 dilution). Protein on the membrane was visualized by incubating with the colorimetric substrates nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Immunostaining. Cells were seeded in 3.5-cm dishes with glass on the bottom containing 2 mL culture medium. The cells were cultured, and cell differentiation was induced as described above. After a period of incubation, the cells were fixed with 4% paraformaldehyde at room temperature for 30 min. The cells were treated with 0.3% H_2O_2 at 4 °C for 5 min to inactivate internal HRP. The cells were incubated with a rabbit polyclonal antibody against ET-2/VIC (1:64 dilution, Yanaihara Institute Inc., Shizuoka, Japan) at 4 °C for 24 h. Thereafter, the cells were incubated with a

Table 1
Specific primers, PCR conditions, and the amplification products

Genes	Primers (sense) (antisense)	PCR conditions	Amplification Products (bp)
ET-2/VIC	5′-CTGCGTTTTCGTCGTTGCT-3′ 5′-TGCAGCTCATGGTGTTATCTCTTC-3′	30 cycles (denaturation, 94 °C for 30 s; annealing, 60 °C for 30 s; extension, 72 °C for 30 s)	422
ET-1	5′-TTCCCGTGATCTTCTCTCTGCT-3′ 5′-TCTGCTTGGGAGAAATTCCA-3′	30 cycles (denaturation, 94 °C for 30 s; annealing, 60 °C for 30 s; extension, 72 °Cfor 30 s)	370
ET-A	5′-GCTGGTTCCCTCTTCACTTAAGC-3′ 5′-TCATGGTTGCCAGGTTAATGC-3′	35 cycles (denaturation, 94 °C for 30 s; annealing, 62 °C for 30 s; extension, 72 °C for 30 s)	129
ET-B	5′-TGTGCTCTAAGTATTGACAGATATCGAG-3′ 5′-GGCTGTCTTGTAAAACTGCATGA-3′	35 cycles (denaturation, 94 °C for 30 s; annealing, 62 °C for 30 s; extension, 72 °C for 30 s)	240
18S rRNA	5′-CGGCTACCACATCCAAGGAA-3′ 5′-GCTGGAATTACCGCGGCT-3′	20 cycles (denaturation, 94 °C for 30 s; annealing, 58 °C for 30 s; extension, 72 °C for 30 s)	187

goat anti-rabbit IgG HRP-conjugated secondary antibody (1:500 dilution, Jackson ImmunoResearch Laboratories Inc.) at 37 °C for 30 min. The cells were visualized by 0.2 mg/mL 3,3′-diaminobenzidine dissolved in 0.1 M phosphate buffer (pH 6.4) containing 0.06% H_2O_2.

Cornified envelope (CE) assay. The cornified envelope is well known as one of markers of terminal differentiation. Cells were recovered and the total protein (TP) content was measured as described in WB analysis. The cells were pelleted by centrifugation at 10,000*g* for 15 min. The cell pellets were resuspended with 0.5 mL CE buffer (0.1 M Tris–HCl, pH 9.0, containing 2% SDS and 1% 2-ME). Soluble protein was removed twice by boiling for 5 min with 0.5 mL of the CE buffer and centrifuged at 10,000*g* for 5 min. Insoluble protein was rinsed three times with the sample buffer for WB. The insoluble protein as CE resuspended with 0.5 mL of the sample buffer for WB was homogenized as described above. An aliquot of the CE suspention was taken to determine protein content as described above. CE protein content was calculated as a percentage of TP content.

Inhibitors. We evaluated whether regulation of ET-2/VIC gene expression was affected by inhibition of the differentiation induction of keratinocytes. Signal pathway such as mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK) and Rho-associated coiled–coil forming protein serine/threonine kinase (ROCK) was indicated to associate with differentiation of keratinocytes [9,10]. Therefore, we evaluated the impact of PD98059 (Sigma–Aldrich Co., St. Louis, MO), a MEK inhibitor, or Y-27632 (Wako Pure Chemical Industries, Ltd.), a ROCK-specific inhibitor, respectively, on the up-regulation of ET-2/VIC gene expression in the differentiation of mouse keratinocytes. PD98059 or Y-27632 dissolved in dimethyl sulfoxide (DMSO) was added at a final concentration of 20 μM to the culture medium. A final concentration of DMSO was 0.2% (v/v). The cells were preincubated at 37 °C for 30 min with the culture medium in the presence of DMSO alone or the inhibitor and then the medium was replaced with the culture medium or the differentiation medium in the presence of DMSO alone or the inhibitor.

Statistical analysis. Data represent means ± SD values. Results were analyzed by one-way ANOVA and Scheffé's *F*-test or Student's *t*-test to identify significant differences between treatments. *P* values lower than 0.05 were considered significant.

Results

Calcium-induced differentiation of mouse keratinocytes

Exposure of mouse keratinocytes to calcium at a high concentration induced clear morphological changes and decreased distinct spaces between cells within 24 h (data not shown). To reveal the differentiation of keratinocytes, we analyzed the expression of differentiation markers by Western blot (WB) analysis and CE formation. After the culture medium was replaced with the differentiation medium for 72 h, expression of involucrin and TG 1 was evaluated. TG 1, participating CE formation through mediation of a calcium dependant enzyme such as calpain, is catalyzing an intermolecular bond formation between proteins such as involucrin. Involucrin and TG 1 were up-regulated and CE content increased, and treatment with the differentiation medium for 72 h caused more than 8-fold increases in these levels (Table 2).

Early-phase up-regulation of ET-2/VIC gene expression in the ET system

To define an involvement of the ET system in the calcium-induced differentiation, we evaluated the gene expression of ET families, ET-2/VIC and ET-1, and ET receptors, ET-A and ET-B, in the mouse keratinocytes. Differentiation for more than 24 h and more than 3 h caused a significant decrease in ET-1 and ET-A gene expression, respectively (data not shown). No ET-B band was detected for the mouse keratinocytes (data not shown). On the contrary, ET-2/VIC gene expression significantly increased after incubation for 1, 3, 6, and 12 h (data not shown). Greatly increases in ET-2/VIC gene expression were observed after 20 min and after 40 min in the differentiation medium (Fig. 1A).

Table 2
Induction of involucrin, transglutaminase 1 (TG 1), and cornified envelope (CE) formation[a]

	Involucrin	TG 1	CE
Low Ca	1.00 ± 0.44	1.00 ± 0.08	1.00 ± 1.96
High Ca	9.44* ± 1.40	14.69* ± 3.36	8.39* ± 2.89

Values represent means ± SD for three dishes. The asterisk indicates significantly different values (*, $P < 0.05$) according to Scheffé's *F*-test.
[a] The value is expressed relative to that of the low Ca.

Immunodetection of up-regulation of intracellular ET-2/VIC peptide

To confirm the expression of ET-2/VIC peptide in keratinocytes, we tried to detect specific immunoreactive ET-2/VIC in keratinocytes in mouse epidermis using the specific antibody raised against ET-2/VIC [2]. Neither the presence

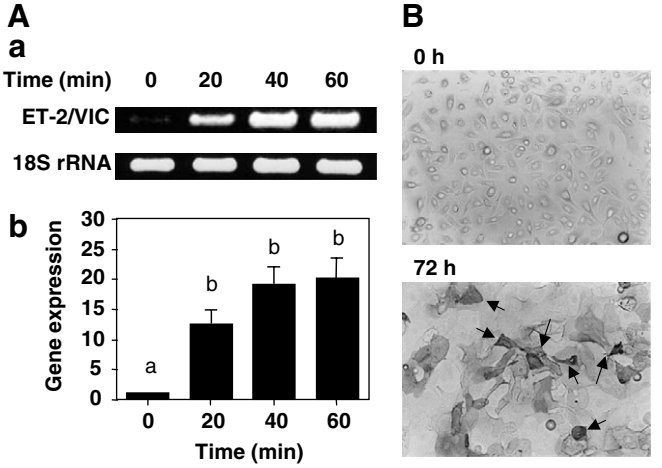

Fig. 1. ET-2/VIC gene expression and immunodetection of intracellular ET-2/VIC peptide in the calcium-induced differentiation of mouse keratinocytes. (A) The cells were treated with the differentiation medium including 2.0 mM calcium for the indicated times. (a) PCR was carried out under the conditions shown in Table 1. (b) The intensities of ET-2/VIC were normalized to that of 18S rRNA. Values represent means ± SD for three dishes. Different letters (ab) indicate significantly different values ($P < 0.05$) according to Scheffé's *F*-test. (B) Immunostaining of ET-2/VIC peptide production in the calcium-induced differentiation of mouse keratinocytes. Some of typical ET-2/VIC antibody-positive cells were indicated by arrows. The cells were photographed with a microscope (200×).

nor the production of ET-2/VIC peptide were detected by WB with the antibody employed in the present study (data not shown). However, ET-2/VIC peptide expression evaluated by immunostaining with the same antibody was detected in mouse keratinocytes treated with the differentiation medium for 72 h but not 0 h (Fig. 1B).

Regulation of ET-2/VIC on the inhibition of the calcium-induced differentiation of mouse keratinocytes

To reveal participation of regulation of ET-2/VIC gene expression to the differentiation induction of keratinocytes, we evaluated the gene expression and differentiation treated with PD98059 and Y-27632 as known inhibitors for MEK and ROCK signaling pathways, respectively, which are indicated to associate with differentiation of keratinocytes. PD98059 did not at all affect morphological changes (data not shown). On the other hand, Y-27632 clearly inhibited the morphological changes by induced by calcium for 24 h in mouse keratinocytes (Fig. 2A) and significantly inhibited up-regulation of ET-2/VIC gene expression for 1 h after replacement with the differentiation medium (Fig. 2B).

Inhibitory effect of Y-27632 on CE formation, involucrin and TG 1 expression in the calcium-induced differentiation of mouse keratinocytes

To reveal the inhibitory effect of Y-27632 on the differentiation, we evaluated expressions of marker proteins such as involucrrin, TG 1, and CE formation in the Y-27632 treated-keratinocytes. Y-27632 did not inhibit up-regulation of involucrin expression in the mouse keratinocytes treated with the high calcium medium for 72 h (Fig. 3A). However, Y-27632 significantly decreased the expression of TG 1 and formation of CE in the calcium-induced differentiation for 72 and 120 h, respectively (Fig. 3A and B).

Discussion

Homeostasis of epidermis is regulated and protected by many humoral factors. ET-1 production in keratinocytes is known to be increased by UVB, and it is considered an important factor in melanocyte proliferation and increase of melanin production [3]. However, little is known of the physiological and biological actions of ET-2/VIC in skin, even though ET-2 has been reported to be down-regulated by UVB-induced apoptosis in human keratinocytes [5]. We previously reported a greater increase in ET-2/VIC production in mouse epidermis and dermis after birth than before birth [2], suggesting that ET-2/VIC plays an important role in skin development. Recently, we found that ET-2/VIC gene expression and peptide production were up-regulated in the differentiation induced by cobalt chloride in PC12 cells [8]. Up-regulation of ET-2/VIC expression may be associated with cell differentiation in skin. To reveal the hypothesis, we evaluated whether ET-2/VIC is associated with differentiation using culture of epidermal keratinocytes from newborn mouse skin.

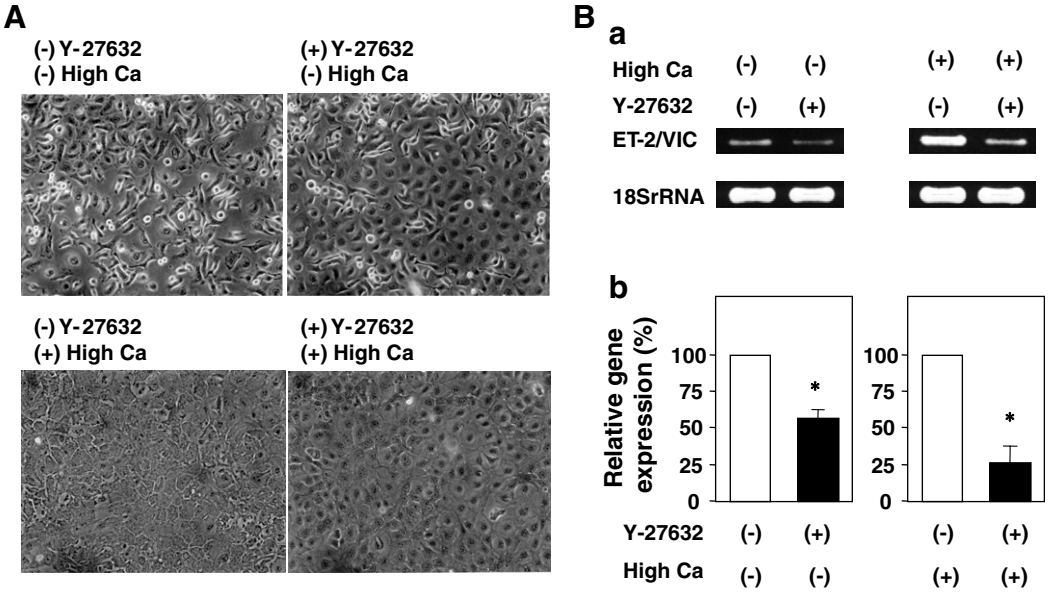

Fig. 2. Involvement of ROCK signaling pathway on induction of morphological changes and ET-2/VIC gene expression in the calcium-induced differentiation of mouse keratinocytes. Cells were incubated with the culture medium (0.06 mM calcium: (−) High Ca) or the differentiation medium (2.0 mM calcium: (+) High Ca) in the presence of DMSO alone (Y-27632 (−)) or Y-27632 (Y-27632 (+)). (A) After the incubation for 24 h, morphological changes were observed by phase-contrast microscopy (200×). (B) After incubation for 1 h, ET-2/VIC gene expression was evaluated by RT-PCR. (a) PCR products were subjected to agarose gel electrophoresis. (b) The intensities of ET-2/VIC were normalized to that of 18S rRNA and were expressed relative to that of no addition of Y-27632. Values represent means ± SD for three dishes. The asterisks indicate significantly different values (*, $P < 0.05$) according to Student's *t*-test.

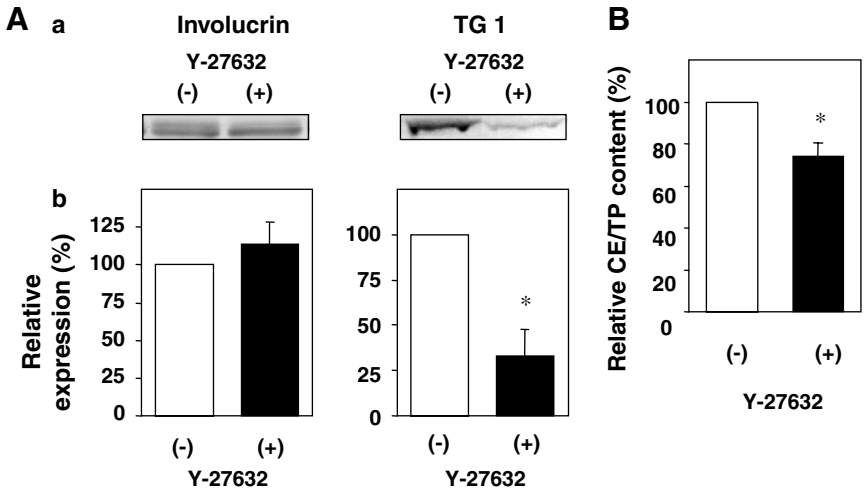

Fig. 3. Involucrin and TG 1 expressions, and CE formation were dependent on ROCK signaling in the calcium-induced differentiation of mouse keratinocytes. Cells were incubated in the presence of DMSO alone (Y-27632 (−)) or Y-27632 (Y-27632 (+)) for 72 h (for WB) and 120 h (for CE). (A) WB analysis of involucrin and TG 1. (a) The membranes were further incubated with an AP-conjugated secondary antibody. (b) The intensity is expressed relative to that of no addition of Y-27632. Values represent means ± SD for three dishes. The asterisks indicate significantly different values (*, $P < 0.05$) according to Student's *t*-test. (B) CE/total protein (TP) content was expressed relative to that of no addition of Y-27632. Values represent means ± SD for three dishes. The asterisks indicate significantly different values (*, $P < 0.05$) according to Student's *t*-test.

In the present culture system, the biochemical differentiation of keratinocytes was confirmed by formation of CE, expression of involucrin and TG 1 (Table 2), widely used differentiation markers. CE formation, expressions of involucrin and TG 1 were significantly up-regulated after 72 h of incubation in the differentiation medium (Table 2). Therefore, we could confirm that the keraticocytes had the ability to both proliferate and induce differentiation.

This is the first report to identify up-regulation of ET-2/VIC expression in the differentiation of keratinocytes induced by calcium (Fig. 1A). Our previous studies showed, through immunostaining, that the use of a highly specific ET-2/VIC antibody eliminates ET-1 interference [8,11] and that ET-2/VIC peptide is produced in mouse skin [2] and intestine [11]. The present immunocytochemical study shows that ET-2/VIC peptide production is also up-regulated in the calcium-induced differentiation of cultured mouse keratinocytes (Fig. 1B). ET-2/VIC may be not only a marker but also an important factor in the differentiation of keratinocytes. The present study and our previous study of cobalt-induced differentiation in PC12 cells associated with ET-2/VIC expression [8] strongly suggest that ET-2/VIC is associated with the differentiation in some types of cells.

To reveal participation of regulation of ET-2/VIC expression to the differentiation induction of keratinocytes, we searched reagents to inhibit the differentiation induction of the mouse keratinocytes. Signal pathway such as MEK has been reported to associate with differentiation of keratinocytes [9]. Y-27632, a specific inhibitor of Rho-associated coiled–coil forming protein serine/threonine kinase (ROCK), is reported to inhibit Ca^{2+} sensitization through ROCK [12]. Rho activity has been reported to increase and promote cell–cell adhesion in calcium-induced differentiation of keratinocytes [13]. Block of ROCK by Y-27632 inhibits differentiation of keratinocytes induced by single-cell suspension, indicating that ROCK participates in the differentiation of keratinocytes [10]. Therefore, we evaluated whether PD98059, an inhibitor for MEK, and Y-27632 affected calcium-induced differentiation of mouse keratinocytes. In the present study, we found that the morphological changes induced by calcium were inhibited by Y-27632 (Fig. 2A), and that Y-27632 significantly inhibited up-regulation of the ET-2/VIC gene in calcium-induced differentiation of mouse keratinocytes (Fig. 2B), but PD98059 did not affect the effect (data not shown). Furthermore, Y-27632 significantly decreased the expression of TG 1 and formation of CE, but not involucrin (Fig. 3). These results suggest that the ROCK regulated TG 1 expression and participation of ET-2/VIC to calcium-induced differentiation of keratinocytes; up-regulation of the ET-2/VIC gene expression mediated by ROCK regulates TG 1 on the formation of CE in calcium-induced differentiation of mouse keratinocytes.

ET-1 and ET-A gene expression were down-regulated and ET-B gene was not detected in the present experimental conditions (data not shown), whereas ET-B expression was confirmed to be detected in isolated mouse skin prior to culture (data not shown). The detailed mechanisms of extracellular high calcium-induced and ET-2/VIC-mediated signaling through ET receptors in the differentiation of keratinocytes deserve further study.

Acknowledgments

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