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Functional Vanilloid Receptors in Cultured Normal Human Epidermal Keratinocytes

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Vanilloid receptor subtype 1, VR1, is an ion channel that serves as a polymodal detector of pain-producing chemicals such as capsaicin and protons in primary afferent neurons. Here we showed that both capsaicin and acidification produced elevations in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in cultured human epidermal keratinocytes. The capsaicin- and acidification-evoked increases in $[Ca^{2+}]_i$ were inhibited by capsazepine, an antagonist to VR1. VR1-like immunoreactivity was observed in the cells. These findings suggest that functional VR1-like protein is present and functions as a sensor against noxious chemical stimuli, such as capsaicin or acidification, in epidermal keratinocytes.

Key Words: **capsaicin; proton; VR1; keratinocytes; intracellular Ca^{2+} concentration; capsazepine.**

Capsaicin, an active ingredient in hot chili peppers, selectively activates nociceptive afferents (1). Vanilloid receptor subtype 1 (VR1), a receptor responsible for capsaicin, protons (acidification) and noxious heat (>43°C), has been cloned from rat dorsal root ganglia (2, 3). VR1 is a nonselective cation channel with high permeability for divalent cations, especially for Ca^{2+}, and is localized in a subpopulation of primary afferent neurons (2, 4). Mice lacking VR1 showed no vanilloid-evoked pain behavior and little thermal hyperalgesia induced by tissue-injury (5). These findings suggest a critical role for VR1 in the detection of pain in the primary afferent neurons. Recently, VR1 has been reported to be present in other tissues such as brain (6, 7), kidney (7), bronchial epithelial cells (8) and even in keratinocytes in the epidermis (9). It is, however, not known whether the VR1 in these tissues is functional, and nothing is known about its physiological significance there.

The skin is the largest organ of the body and is the first site of exposure various external stimuli. In other words, the skin is an interface between the body and the environment. It protects water-rich internal organs from harmful environmental factors such as dryness, chemicals and UV irradiation. Recently, we demonstrated that calcium dynamics may play an important role in the homeostasis of skin epidermis which is the outermost part of skin tissue (10). Furthermore, various environmental and neuroendocrinological factors influence on the ion flux through epidermal keratinocytes (11). Thus, the skin should have sensors for such external signals. Functional receptors for various neurotransmitters such as acetylcholine (12, 13) and glutamate (14) are also present in keratinocytes. Various environmental stimuli or neurotransmitters can influence the influx of ions, including Ca^{2+}, in the skin (12, 14, 16). Ca^{2+} dynamics play an important role in the homeostasis of the skin epidermis, the outermost part of skin tissues, an increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in response to external stimuli results in epidermal cell differentiation (11, 17). These findings raise the possibility that epidermal keratinocytes have functional Ca^{2+}-permeable receptors, similar to neuronal VR1, that are activated by environmental noxious stimuli such as capsaicin or acidification. In the present study, we show the existence of functional Ca^{2+}-permeable VR1-like protein in cultured normal human epidermal keratinocytes (NHEK), and demonstrate that both capsaicin and acidification produce increases in $[Ca^{2+}]_i$ via capsazepine-sensitive VR1.

METHODS

Cells and cell culture. Normal human epidermal keratinocytes (NHEK) were obtained as cryopreserved first-passage cells from neonatal foreskins (Kurabo, Osaka, Japan). The cells were plated on collagen-coated coverslips, then cultured in serum-free keratinocyte

Abbreviations used: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; NHEK, normal human epidermal keratinocytes; VR1, vanilloid receptor subtype 1.

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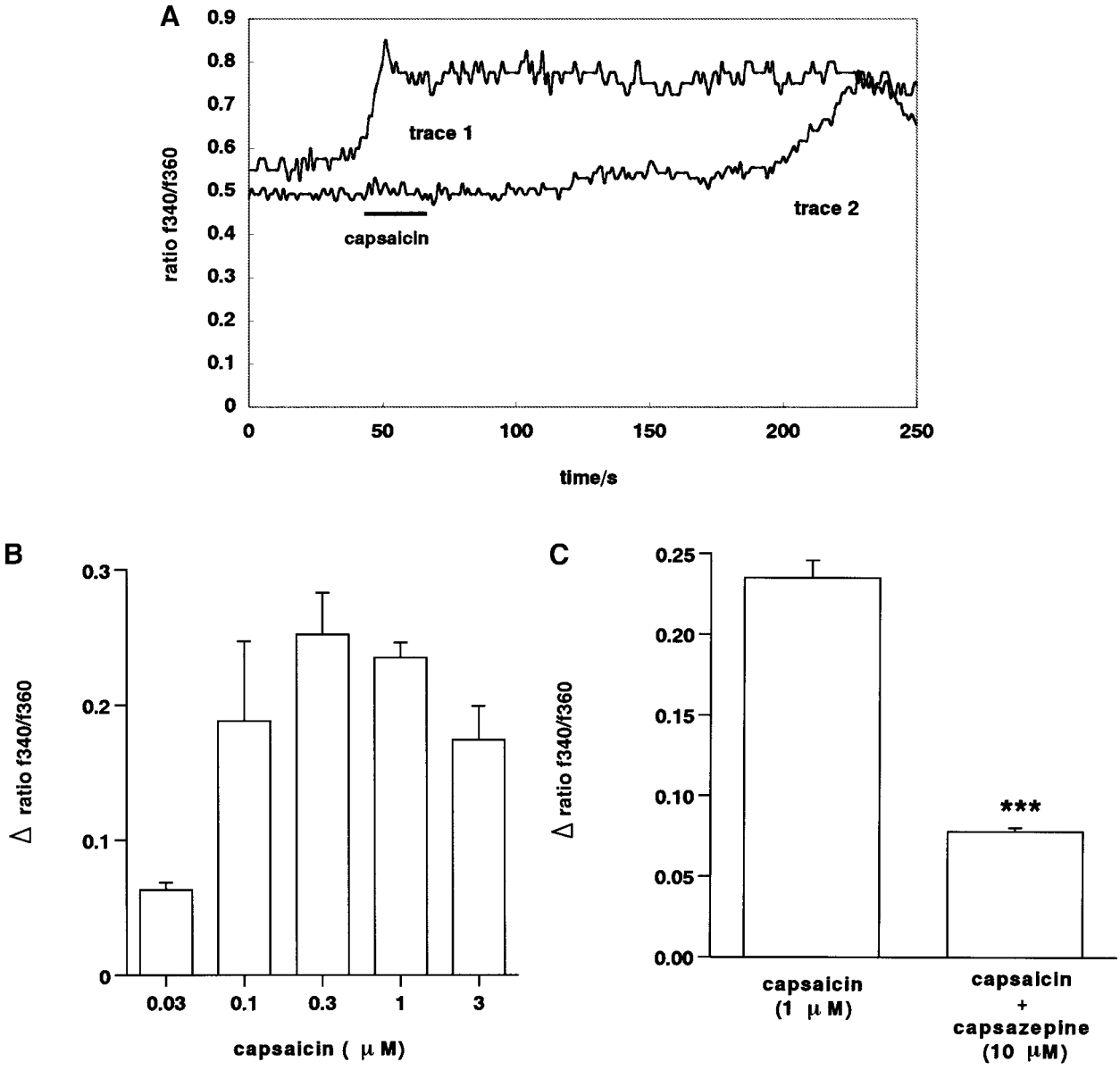

FIG. 1. Effects of capsaicin on intracellular calcium concentration in NHEK. (A) Two types of response to capsaicin (1 μM) applied for 30 s were shown by NHEK. Trace 1 shows the fast response (<30 s) and trace 2 shows the slow response (30–180 s). (B) Concentration dependence of the capsaicin-evoked peak rise in $[Ca^{2+}]_i$ in NHEK. Capsaicin was added to the BSS after recording the basal level of $[Ca^{2+}]_i$. (C) Effect of capsazepine (10 μM) on the response to capsaicin (1 μM) in NHEK (***$P < 0.001$ by Student's *t*-test).

growth medium, consisting of Humedia-KB2 (Kurabo, Osaka, Japan) supplemented with bovine pituitary extract (0.4% v/v), human recombinant epidermal growth factor (0.1 ng/ml), insulin (10 μg/ml), hydrocortisone (0.5 μg/ml), gentamicin (50 μg/ml), and amphotericin-B (50 ng/ml). The medium was replaced every 2–3 days.

Ca^{2+} imaging in single keratinocytes. Changes in $[Ca^{2+}]_i$ in single cells were measured by the fura-2 method (18) with minor modifications (19). In brief, the culture medium of cells grown on a coverslip was replaced with balanced salt solution (BSS) of the following composition (mM): NaCl 150, KCl 5, $CaCl_2$ 1.8, $MgCl_2$ 1.2, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes) 25 and D-glucose 10 (pH 7.4). The cells were loaded with fura-2 by incubation with 5 μM fura-2 acetoxymethyl ester (fura-2AM) (Molecular Probes Inc., Eugene) at room temperature (20–22°C) in BSS for 45 min, then washed with BSS and further incubated for 15 min to allow deesterification of the loaded dye. The cover slip was mounted on an inverted epifluorescence microscope (TMD-300, Nikon, Tokyo, Japan) equipped with a 75 W xenon-lamp and band-pass filters of 340 and 360 nm wavelengths. Measurements were carried out at room temperature. Image data, recorded using a high-sensitivity silicon intensifier target camera (C-2741-08, Hamamatsu Photonics, Hamamatsu, Japan) were analyzed using a Ca^{2+}-analyzing system (Furusawa Laboratory Appliance, Kawagoe, Japan).

Generation of antipeptide antisera. Rabbit polyclonal antiserum against the VR1 N-terminal sequence RASLDSEESESPPQENSC, which is also found in the rat VR1 protein (20), and blocking peptide corresponding to this sequence were purchased from Neuromics (Minneapolis, MN). The antiserum was previously reported to cross

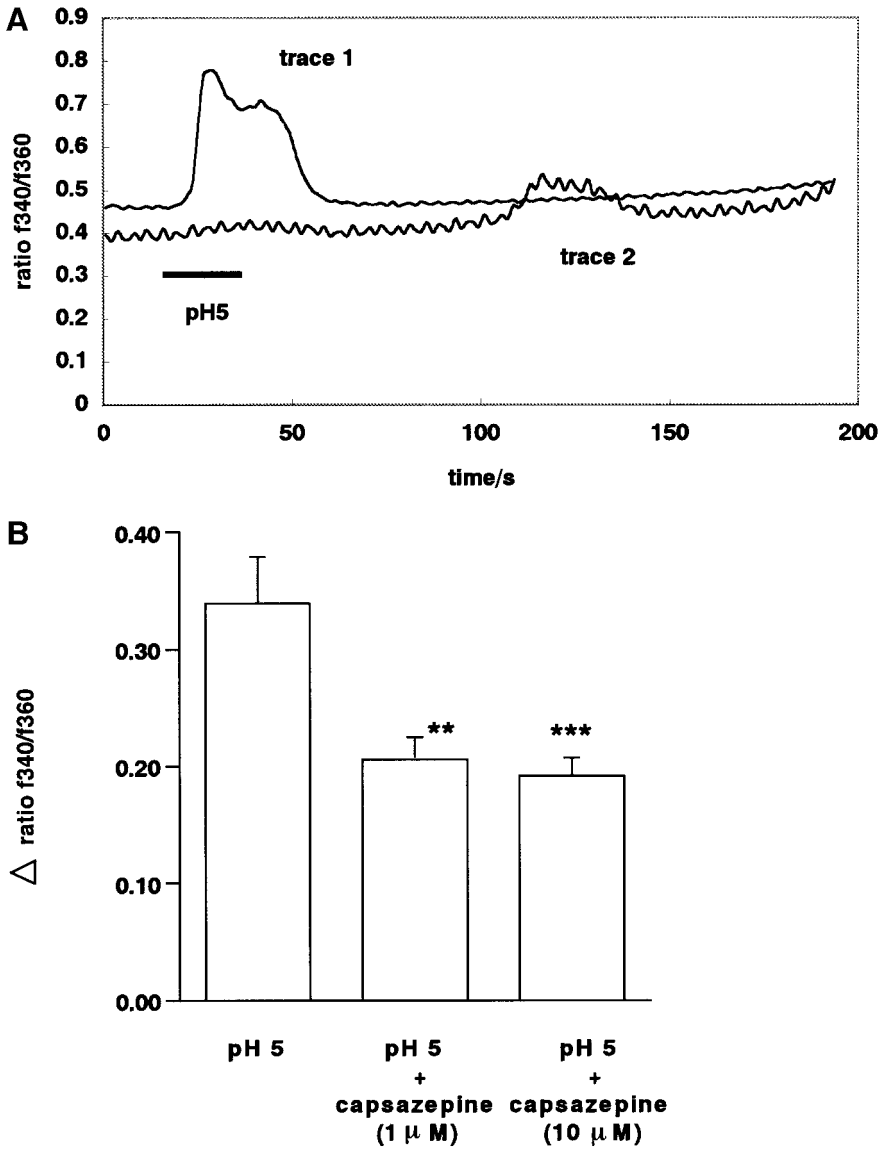

FIG. 2. Effects of acidification (pH 5.0) on intracellular calcium concentration in NHEK. (A) Two types of response to pH 5.0 applied for 30 s were shown by NHEK. Trace 1 shows the fast response (<30 s) and trace 2 shows the slow response (30–180 s). (B) The response of NHEK to pH 5.0 showed partial inhibition by capsazepine (1, 10 μM) (**$P < 0.01$ and ***$P < 0.001$ by Scheffe's test).

react with the human dorsal root ganglion (7). Each antiserum was diluted 1:500 with blocking solution (3% bovine albumin PBS solution supplemented with 10% heat-inactivated goat serum and 0.4% Triton X-100). Fluorescent secondary antibodies were purchased from Molecular Probes, Inc. (Alexa Fluor 488 and 594, anti-guinea pig and anti-rabbit IgG conjugate, Molecular Probes, Inc., Eugene, OR). The secondary antibodies were also diluted 1:500 with the blocking solution. The blocking peptides were reconstituted with 200 μl of PBS solution. For blocking the antibody/antigen binding, the peptide was used at a final concentration of 10 μM, as suggested by the manufacturer.

Immunocytochemistry. Cultures were fixed with methanol for 20 min and soaked in PBS solution. Next the cells were blocked with blocking solution for 1 h at room temperature, and then they were covered with diluted antiserum solution and kept at 4°C overnight. The cells were washed with PBS solution containing 0.05% Tween 20 for 15 min 3 times and covered with the secondary fluorescent antibody solution for 1 h at room temperature. Then the cells were washed with PBS solution containing 0.05% Tween 20 for 15 min 3 times, and mounted with Vectashield (Vectashield with Dapi, Vector Laboratories, Inc., Burlingame, CA). The cells were observed and photographs were taken within 6 h.

Statistics. Experimental results are expressed as means ± SE, and the statistical significance of differences were determined by using Student's *t* test, or by using Scheffe's test.

RESULTS

Changes in $[Ca^{2+}]i$ in NHEK

Figure 1A shows typical changes in $[Ca^{2+}]_i$ in a single NHEK in response to 1 μM capsaicin. About 12% of cells (83 out of 690 cells tested) responded to capsaicin.

TABLE 1

Response to Capsaicin and pH5 in NHEK

	$[Ca^{2+}]_i$ response Δ ratio (f340/f360)	Type of $[Ca^{2+}]_i$ increase responding (responding/tested)	
		Type 1	Type 2
cap 30 nM	0.063 ± 0.005	1/30 (3.3%)	1/30 (3.3%)
cap 100 nM	0.118 ± 0.059	2/67 (3.0%)	14/67 (20.9%)
cap 300 nM	0.252 ± 0.031	11/146 (7.5%)	5/146 (3.4%)
cap 1 μM	0.235 ± 0.011	4/323 (1.2%)	33/323 (10.2%)
cap 3 μM	0.174 ± 0.025	6/124 (4.8%)	6/124 (4.8%)
cap 1 μM + caz 10 μM	0.078 ± 0.011	0/64 (0%)	0/64 (0%)
pH 5	0.337 ± 0.038	25/35 (71.4%)	7/35 (28.6%)
pH 5 + caz 1 μM	0.206 ± 0.019	23/61 (37.7%)	2/61 (3.3%)
pH 5 + caz 10 μM	0.192 ± 0.015	40/65 (61.5%)	6/65 (9.2%)

The capsaicin-evoked elevation in $[Ca^{2+}]_i$ in the cells consisted of two types of responses, i.e., a fast (<30 s, trace 1) and a slow (30–180 s, trace 2) onset of Ca^{2+} response. Over 8% of cells showed the slow onset response (Table 1). Capsaicin evoked rises in $[Ca^{2+}]_i$ in a concentration-dependent manner over a concentration range from 30 nM to 3 μM, and caused a maximal response at 300 nM. Both the fast- and slow-onset Ca^{2+} responses were abolished by 1 μM capsazepine, a competitive antagonist to vanilloid receptor VR1 (Fig. 1C). In addition, the capsaicin-evoked elevation in $[Ca^{2+}]_i$ was abolished by the removal of extracellular $[Ca^{2+}]_i$.

Next we investigated the effect of acidification on the changes in $[Ca^{2+}]_i$ in NHEK. Acidification (pH 5) also evoked an increase in $[Ca^{2+}]_i$ in about 80% of cells (28/35 cells tested). Like the slow-onset capsaicin-evoked Ca^{2+} response (Fig. 1A, trace 2), the acidification-evoked elevation in $[Ca^{2+}]_i$ was very slow and it started 30–180 s after stimulation. The acidification-evoked $[Ca^{2+}]_i$ increase was abolished by 10 μM capsazepine (Fig. 2B). The proportion of acidification-responding cells was higher than that of the capsaicin-responding cells (Table 1).

The very-slow-onset Ca^{2+} response to acidification might be a secondary response mediated by other extracellular molecules released in response to acidification. Since ATP and glutamate are known to be released and cause rises in $[Ca^{2+}]_i$ in keratinocytes (21, 22), we examined the effects of antagonists to glutamate- or P2-receptors on the acidification-evoked Ca^{2+} response. Neither suramin (100 μM) nor glutamate antagonists (a mixture of AP5 (100 μM), CNQX (30 μM) and MCPG (100 μM) affected the acidification-evoked response (control; 0.337 ± 0.038, $n = 30$, suramin; 0.284 ± 0.023, $n = 35$, glutamate antagonists, 0.263 ± 0.021, $n = 20$).

Immunoreactivity of VR1

To examine the expression of VR1, we performed an immunocytochemical study in NHEK. Figure 3 shows staining with anti-VR1 antiserum (red) and its merged image with that of DAPI-staining (blue). This image revealed that the anti-VR1 antiserum stained the NHEK membrane. The staining with anti-VR1 antiserum was reduced by the blocking peptide (data not shown). VR1-like protein was shown to be expressed constitutively in NHEK.

DISCUSSION

We demonstrated here that both capsaicin and acidification produced increases in $[Ca^{2+}]_i$ in cultured NHEK. These elevations in $[Ca^{2+}]_i$ were inhibited by incubation of the cells with Ca^{2+}-free solution or by capsazepine, an antagonist to VR1. The concentration-response curve for the capsaicin-evoked Ca^{2+} response in NHEK was similar to that for capsaicin-activated currents in DRG neurons (23). We also showed the localization of VR1-like immunoreactivity in the cell membrane (Fig. 3) and the expression of VR1 mRNA by RT-PCR (9) in NHEK. All these findings strongly suggest that NHEK possess functional Ca^{2+}-permeable VR1 or a VR1-like protein that can be activated by either capsaicin or acidification.

Although capsaicin at low concentrations (30–100 nM) produced an elevation of $[Ca^{2+}]_i$ in NHEK as well as in DRG neurons (24), the fraction of NHEK that were capsaicin-responders was much smaller than that of responders among DRG neurons (Table 1). The VR1-like protein in NHEK might be present in only a subset of the cells. The heterogeneous expression of VR1-like immunoreactivity in NHEK may support this idea (Fig. 3). It is also possible that VR1-like protein in NHEK also might be regulated by intracellular factors such as protein kinase C (25) and protein kinase A (26) may become more functional when activated by such kinases, as suggested for VR1 in DRG neurons.

The time-course of the capsaicin- and acidification-evoked Ca^{2+} responses was also different from that seen in DRG neurons (24). The onset of these responses was much slower than that seen in DRG neurons (Figs. 1A and 2A). Such slow Ca^{2+} responses to capsaicin or acidification raise the possibility that capsaicin or acidification evokes a rise in $[Ca^{2+}]_i$ in NHEK secondarily to the release of some chemical mediators such as ATP and glutamate. In fact, functional glutamate (14) and ATP (21) receptors are present in keratinocytes. However, neither suramin, an antagonist to P2 receptors, nor antagonists to glutamate receptors (a mixture of CNQX, AP5 and MCPG) affected the capsaicin-evoked Ca^{2+} responses. Thus, the secondary effects of capsaicin or acidification on the $[Ca^{2+}]_i$ elevation seem to be negligible. Rather, capsaicin or protons appear to pro-

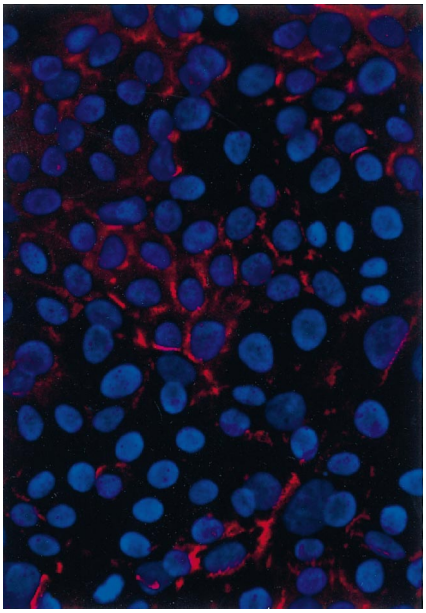

FIG. 3. Immunocytochemical detection of VR1 peptides in cultured NHEK. Merged image of staining with VR1 antiserum (red) and DAPI staining (blue).

duce an increase in $[Ca^{2+}]_i$ by acting directly on VR1-like receptors in NHEK. The reason for such a slow-onset of the Ca^{2+} response in the cells remains unknown. Recently, a novel human vanilloid receptor-like protein, VRL-2, has been identified. This receptor is localized in non-neuronal tissues such as kidney and airway epithelia (27). The Ca^{2+} responses seen in NHEK might be mediated by homologues of VR1, such as VRL-2 with different activation profiles. The existence of splice variants of VR1 may also account for the slow Ca^{2+} response (7, 28).

The physiological significance of VR1-like protein in NHEK remains to be clarified. Because the epidermis can be an interface between the body and the environment, VR1-like proteins might play a role as a sensor against environment factors such as protons, harmful heat, dryness and UV radiation. The existence of several receptors which were natively found neuronal cells is also localized in skin (13, 14, 16, 29). The relationships between differentiation and calcium influx mediating these receptors were reported in keratinocytes. The higher increase of $[Ca^{2+}]_i$ in the cells results in the differentiation and reduction of proliferation of the cells. VR1-like protein in keratinocytes may function as a primary sensor against harmful noxious stimuli, and may protect internal organs by stimulating reepithelialization.

In summary, we demonstrated the presence of functional Ca^{2+}-permeable VR1-like proteins in NHEK and suggested the important physiological role of these proteins as an initial sensor for noxious chemical stimuli. VR1-like protein in the cells could provide a basis for studying the regulatory mechanisms underlying the differentiation or proliferation of keratinocytes.

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