Intracellular Proton-mediated Activation of TRPV3 Channels Accounts for the Exfoliation Effect of α-Hydroxyl Acids on Keratinocytes

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Background: Little is known about how α-hydroxy acids (AHAs) widely cause exfoliation to expose fresh skin cells.

Results: Transient receptor potential vanilloid 3 (TRPV3) channel in keratinocytes is potently activated by intracellular acidification induced by glycolic acid.

Conclusion: TRPV3-mediated proton-sensing and cell death in keratinocytes may serve as a molecular basis for the cosmetic use of AHAs.

Significance: We describe a novel mechanism by which TRPV3 is activated by intracellular protons.

α-Hydroxyl acids (AHAs) from natural sources act as proton donors and topical compounds that penetrate skin and are well known in the cosmetic industry for their use in chemical peels and improvement of the skin. However, little is known about how AHAs cause exfoliation to expose fresh skin cells. Here we report that the transient receptor potential vanilloid 3 (TRPV3) channel in keratinocytes is potently activated by intracellular acidification induced by glycolic acid. Patch clamp recordings and cell death assay of both human keratinocyte HaCaT cells and TRPV3-expressing HEK-293 cells confirmed that intracellular acidification led to direct activation of TRPV3 and promoted cell death. Site-directed mutagenesis revealed that an N-terminal histidine residue, His-426, known to be involved in 2-aminoethyl diphenylborinate-mediated TRPV3 activation, is critical for sensing intracellular proton levels. Taken together, our findings suggest that intracellular protons can strongly activate TRPV3, and TRPV3-mediated proton sensing and cell death in keratinocytes may serve as a molecular basis for the cosmetic use of AHAs and their therapeutic potential in acidic pH-related skin disorders.

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terminus of TRPV3 that cause Olmsted syndrome, characterized by bilateral mutilating palmoplantar keratoderma and periarticular keratotic plaques with severe itching at lesions (35). All these investigations indicate that overactive TRPV3 mediates an important role in skin physiology and pathophysiology. Furthermore, the TRPV3-activator carvacrol has been shown to boost collagen expression in the skin (36). Interestingly, glycolic acid also promotes collagen synthesis in skin (7, 9, 37), indicating a role for TRPV3 in glycolic acid-mediated processes (7, 9, 37). Taken together, these observations raise the question of whether AHAs can affect the skin physiology by directly modulating the function of TRPV3 channel. To understand the molecular mechanism by which topical AHAs affect the epidermis and keratinization of the skin, we investigated the proton effect on TRPV3 function.

Here we report that glycolic acid can strongly activate the TRPV3 channel. This activation is partially mediated by intracellular protons that act on His-426, located at the distal N terminus. Our findings demonstrate a novel gating mechanism by which TRPV3 is directly activated by intracellular acidification, likely accounting for the cosmetic effect of AHAs on keratinization of the skin.

**MATERIALS AND METHODS**

cDNA Constructs and Cell Culture—Human keratinocyte HaCaT cells and HEK-293 cells were cultured in a DMEM supplemented with 10% FBS at 37 °C with 5% CO2. They were passaged every 6–24 h, and plated onto glass coverslips coated with 0.1 mg/ml of poly-1-lysine for improving cell adhesion and subsequent patch recordings. Transient transfections were made using Lipofectamine 2000 (Invitrogen). TRP channel cDNAs, including mouse TRPV1, mouse TRPV2, mouse TRPV3, TRPV3-E631K/D641K, and TRPV3-H426N mutants, human TRPV3, mouse TRPV4, and the TRPV4-N456H/W737R mutant were fused at the C terminus with the cDNA encoding an eYFP, as previously described (38). Attachment of eYFP, used as a marker for identification of transfected cells did not affect channel function. For each transfection, 1 μg of individual cDNA were used and for the coexpression of TRPV3 and TRPV3-E631K/D641K, equal amounts of each cDNA (1 μg) were used. All mutants were generated by overlap PCR and confirmed by DNA sequencing. Electrophysiological experiments were performed between 24 and 48 h after transfection.

Electrophysiology—Whole cell and single-channel currents were recorded using a HEKA EPC10 amplifier with PatchMaster software (HEKA). Patch pipettes were pulled from borosilicate glass and fire polished to a resistance of ~2 MΩ. Membrane potential was held at 0 mV unless stated otherwise. Currents were elicited by a protocol consisting of a 300-ms step to +80 mV, followed by a 300-ms step to −80 mV at 1-s intervals. Current amplitude was analyzed at +80 mV. During whole cell recording the capacity current was minimized by the amplifier circuitry, and the series resistance was compensated by 80%. All experiments were conducted at ~22 °C. The dose-response relationship and the closing rate were determined using a solution exchanger RSC-200 with seven separate tubes to deliver different concentrations of protons. The tube number sent by the solution exchanger was fed into an analog input port of the EPC10 patch-clamp amplifier and recorded simultaneously with current. The stable current amplitude at different concentrations was recorded.

**Solutions and Chemicals**—Both pipette solution and bath solution contained 130 mM NaCl and 0.2 mM EDTA. Glycolic acid solution contained 100 mM glycolic acid, 30 mM NaCl, and 0.2 mM EDTA. For solutions with pH > 6.0, 3 mM HEPES was added; for solutions with pH ≤ 6.0, HEPES was replaced with 3 mM MES. All the chemicals, including 2-APB for electrophysiological experiments were from Sigma.

Cell Death Assay—HEK-293 cells and HaCaT cells grown in 6-well culture plates were randomly divided into different groups and then treated with 20 mM glycolic acid with or without modifying agents at pH 7.4 or 5.5. 12 h later, cells were washed three times with PBS and incubated in PBS containing Hoechst or propidium iodide (PI) for 10 min. Cell images of four random fields of view demonstrating total cells (Hoechst-stained nuclei) and dead cells (PI-stained nuclei) were taken and counted on a microscope (Olympus IX71) using a digital camera. Cell death was determined by averaging the percentage of dead cells (PI/Hoechst staining) in each plate.

Data Analysis—All data are shown as mean ± S.E. G-V curves were fitted to a single Boltzmann function.

\[
\frac{G}{G_{\text{max}}} = \frac{1}{1 + e^{\frac{V - V_{\text{half}}}{zF/RT}}} \tag{1}
\]

Where \(G/G_{\text{max}}\) is the normalized conductance, \(z\) is the equivalent gating charge, \(V_{\text{half}}\) is the half-activation voltage, \(F\) is the Faraday’s constant, \(R\) is the gas constant, and \(T\) is the temperature in Kelvin. The relative conductance \((G/G_{\text{max}})\) was obtained by normalizing the tail currents (after 2-ms hyperpolarization to −100 mV from a given voltage) to the maximum tail current. The dose-response relationship was fitted to the Hill equation.

\[
\frac{I_x}{I_{\text{max}}} = \frac{[X]^n}{EC_{50} + [X]^n} \tag{2}
\]

Where \(I_x\) is the steady-state TRPV current in the presence of concentration \([X]\), \(I_{\text{max}}\) is the maximal current amplitude. \(EC_{50}\) is the concentration for the half-maximal effect and is replaced by \(IC_{50}\) if the effect is inhibitory. Statistical significance, determined by Student’s *t* test, is indicated as: *, \(p < 0.05\); **, \(p < 0.01\); ***, \(p < 0.001\); and n.s., no significant difference.

**RESULTS**

Glycolic Acid Activates TRPV3 Expressed in HEK-293 Cells—To examine whether TRPV3 can be directly activated by proton released from glycolic acid, we conducted patch clamp recordings of HEK-293 cells transfected with TRPV3 cDNA. Whole cell recordings of channel-expressing cells gave rise to a robust response to extracellular glycolic acid (100 mM, pH 5.5) (Fig. 1A, left panel). The glycolic acid effect developed slowly, and could be washed out slowly after switching to a normal bath solution. Subsequent application of TRPV3 channel opener 2-APB (300 μM) elicited an instantaneous TRPV3 current from the same cells, confirming the presence of TRPV3 channels. The glycolic acid-induced current was only present at acidic pH levels.
Application of the same concentration of glycolic acid buffered at pH 7.4 failed to activate TRPV3, whereas a robust TRPV3 current was seen upon subsequent application of 2-APB (Fig. 1A, right panel). These observations indicate that extracellular glycolic acid at low pH (5.5 but not 7.4) caused the activation of TRPV3.

Glycolic Acid Induces TRPV3-like Currents in Human Keratinocytes—To confirm that endogenous TRPV3 channels could be activated by glycolic acid, we performed whole cell patch clamp recordings of HaCaT cells, a human keratinocyte cell line. Consistent with reports that TRPV3 is highly expressed and functional in a small subset of keratinocytes (14, 25, 39), application of 300 μM 2-APB evoked a TRPV3-like current in 8 of 53 HaCaT cells (Fig. 1B, left panel), whereas most of the recorded cells did not respond to 2-APB (or glycolic acid at pH 5.5) (Fig. 1B, right panel). To further confirm the identity of this current, we used a nonspecific voltage-dependent antagonist of TRP channels, ruthenium red. 10 μM Ruthenium red blocked the 2-APB-induced inward current, but potentiated the outward current, consistent with characteristic ruthenium

FIGURE 1. Glycolic acid-induced activation of TRPV3 in HaCaT and HEK-293 cells. A, representative whole cell recordings of HEK-293 cells expressing TRPV3 in the presence of 100 mM glycolic acid at pH 5.5 (left panel) or pH 7.4 and 300 μM 2-APB (right panel). B, left panel, representative whole cell TRPV3-like currents were evoked by 300 μM 2-APB and 100 mM glycolic acid at pH 5.5 in HaCaT cells; as a control, cells that had no response to 2-APB also had no response to glycolic acid (right panel). C, sequence alignment between the pore regions of TRPV1 and TRPV3. The Glu-601 pH site in TRPV1 and its corresponding Glu-610 in TRPV3 are highlighted in red. D, time course of whole cell TRPV3 currents evoked by a MES-buffered solution at pH 5.5 and subsequent application of 300 μM 2-APB. After washout of 2-APB, a slow developing current was evoked by the pH 5.5 solution (left panel). Comparison of current traces labeled a and b obtained at the time indicated showed the remaining channel activity of TRPV3 by 2-APB (right panel).
Proton Activation of TRPV3

A representative current trace was recorded from an inside-out patch facing solutions of various pH levels (left panel). The current traces labeled as a to e were obtained at the time indicated (right panel). B, dose-response curve for proton activation of TRPV3. Baseline-subtracted currents were normalized to steady-state currents at pH 5.5. The smooth curve represents a fit of the Hill equation with pH 1/2 = 6.1 ± 0.01, and Hill slope at 1.7 ± 0.5 (n = 5). C, inside-out patch recordings of single TRPV3 channel expressed in HEK293 cells in response to different pH values. D, a representative current trace recorded from an outside-out patch in response to a bath solution of pH 5.5 (left). The current traces labeled as a and b were obtained at the time indicated (right panel).

Because a glutamate residue (at the extracellular end of S5) involved in extracellular proton activation of TRPV1 is also conserved in TRPV3, we wondered whether extracellular application of glycolic acid could activate TRPV3 through a similar mechanism (41) (Fig. 1C). To test the effect of extracellular protons on TRPV3, we utilized an acidic bath solution (pH 5.5) buffered by MES, a membrane impermeable acid. Application of this solution for at least 5 min failed to induce a current response in cells expressing TRPV3 (Fig. 1D). Subsequent application of 2-APB (300 μM) induced a robust, rapid TRPV3 current activation from the same cells, confirming the presence of the channel (Fig. 1D). This observation indicates that extracellular proton cannot activate the TRPV3 channel, consistent with a previous report (16). Surprisingly, when a MES-buffered acidic bath solution (pH 5.5) was used after 2-APB application, a slowly developing current very similar to that elicited by glycolic acid was observed (Fig. 1D). We noticed that after 2-APB treatment, the TRPV3 channels maintained a low activity level (Fig. 1D, right). As TRPV3 is a nonselective and calcium-permeable cation channel with substantial proton permeability, protons can pass through the open channel to induce cytosolic acidosis (42). The substantial permeation effect raised the possibility that protons could be activating TRPV3 from the intracellular side. Intracellular activation of TRPV3 by proton would be consistent with results from the glycolic acid experiments, because that glycolic acid is a weak AHA acid, and in solution it exists as an equilibrium of the charged and neutral forms: HG ↔ H⁺ + G⁻. At low pH levels (high [H⁺]), the equilibrium shifts toward the left and glycolic acid exists in the neutral, membrane permeable form; whereas at high pH levels (low [H⁺]), the equilibrium shifts toward the right and glycolic acid exists in the charged, membrane impermeable form. Once the protonated neutral glycolic acid diffuses across the cell membrane into the cell, it re-equilibrates to release a free proton, causing intracellular acidification (12, 43). Can intracellular acidification activate TRPV3? To address this question, we performed inside-out patch clamp recordings of HEK-293 cells expressing TRPV3.

Intracellular Acidification Activates TRPV3 Expressed in HEK-293 Cells—A low, basal TRPV3 activity was observed when inside-out patches were excised and exposed to a pH 7.4 bath solution (Fig. 2A). Decreasing the bath pH from 7.4 to 5.5 resulted in a gradual increase of TRPV3 current in a pH-dependent manner (Fig. 2, A and B), indicating that intracellular acidification could indeed directly activate the channel. Activation of TRPV3 current could be seen at pH 6.5 and reached a maximum at approximately pH 5.5 (Fig. 2, A and B). Single channel recordings of TRPV3 current further confirmed that intracellular protons increased the open channel probability (Fig. 2C).
Further acidification (such as pH 4.5) led to current inhibition, which was partially due to reduction of the single-channel conductance (Fig. 2C and see below). In contrast, when we performed outside-out patch clamp recordings, we observed that extracellular proton did not activate any TRPV3 current. Instead, there was a slight inhibition of the basal TRPV3 activity, which was apparently due to a reduction of the single-channel current amplitude (Fig. 2C). The observation further confirms that TRPV3 activation by proton originates from the intracellular side. As additional controls, we tested other temperature-sensitive TRPV channels (TRPV1, TRPV2, and TRPV4) and confirmed that they were not activated by intracellular protons.

Intracellular Protons Are a Potent Activator for TRPV3

To test the efficiency of proton in activating TRPV3, we conducted single-channel recordings in the inside-out configuration at a wide pH range to evaluate the permeation effect of proton that partially masked its gating effect. These recordings showed that protons dose dependently inhibited the single-channel current amplitude (Fig. 3A). At physiological pH, the single-channel conductance was estimated to be 157.7 ± 4.0 pS (n = 5), similar to that previously reported (19, 38). At pH 5.5, intracellular protons reduced the single-channel conductance by almost 50%, to 84.1 ± 6.9 pS (Fig. 3B), indicating that the gating effect was indeed partially masked. For this reason, we used the pH-dependent single-channel conductance to adjust the macroscopic currents as shown in Fig. 3C. The corrected current amplitudes were much higher after removing the permeation effect, reflecting a significant gating effect of intracellular protons. For comparison, at a saturating concentration (300 μM) of 2-APB, TRPV3 activation reached an open probability of about 73%, as estimated by noise analysis (44). Based on the relative current amplitudes of the proton-elicited current and the 2-APB-elicited current, at pH 5.5 proton potentiated TRPV3 to an open probability of about 90% (Fig. 3D). We also observed that intracellular protons activated large currents in HEK-293 cells transfected with the human TRPV3 channel, indicating that activation by protons is a conserved property of TRPV3 channels (Fig. 3E). Thus, our data demonstrate that intracellular protons are a strong activator for TRPV3.

Off-response Property of Proton-induced Activation of TRPV3—Our results showed that pH-induced TRPV3 activation appeared to saturate at pH 5.5 (Fig. 2B). Further decreasing bath pH to 5 or 4.5 resulted in suppression of the steady-state TRPV3 current in a dose-dependent manner (Fig. 4, A and B), indicating a dominant inhibitory effect on conductance. More interestingly, at these low pH levels (high [H⁺]) robust acidic-induced transient off-responses were clearly observed upon removal of the acid, and the amplitude of the off-response almost reached the same level as the current at pH 5.5 (Fig. 4A). This implies that the open probability of TRPV3 at pH 4.5 could reach the maximum level, but inhibition in single-channel conductance and other potential proton-sensitive site(s) contributed to the decline of overall current amplitude. Removal of conductance inhibition transiently revealed the full scale of the acti-
vation effect of protons. The off-response thus reflected the dual effects of intracellular protons on TRPV3, indicating the off gating transition is much slower than the speed of the acid-base neutralization reaction.

To better clarify the off-response of TRPV3, we conducted comparative measurements for the off-response of TRPV1 induced by removal of the extracellular acidic solution from outside-out patches (45, 46). A small and rapid off-response was observed in TRPV1 after acid exposure (pH 4.5). The time constant of the closing process was less than 310 ms (Fig. 4, C and D), consistent with the observation of the transient gating process of TRPV1 (47). We then measured the closing rate of the proton-induced gating of TRPV3 in the presence of a pH 4.5 bath solution. The closing rate was significantly slower, with a time constant of 7.2 ± 2.2 s (n = 6) (Fig. 4C). Activation of TRPV3 displays a unique property known as sensitization due to hysteresis of opening the gate (14–16, 48, 49). It is likely that the strong hysteresis of TRPV3 gating leads to the noticeably slower off-response.

**Leftward Shift of the Voltage-dependent Activation Curve of TRPV3 by Intracellular Protons**—TRPV3 activation exhibits weak voltage dependence in a nonphysiological, highly depolarized voltage range. However, other stimuli such as temperature change or binding of ligands can shift the voltage-dependent activation range toward physiologically relevant potentials to gate the channel (50–52). We therefore tested the effect of intracellular proton on voltage-dependent activation of TRPV3. In inside-out patches, TRPV3 currents were elicited using a voltage step protocol ranging from −100 to +300 mV at different levels of intracellular pH (Fig. 5A). At pH 7.4, there was very little channel activity even at a membrane potential of +300 mV, and the
midpoint of voltage activation \((V_{1/2})\) was estimated as 182.7/11006 2.0 mV (Fig. 5B). Increasing the proton concentration to pH 5.5 induced a large increase of TRPV3 current (Fig. 5A, right panel) and a significant leftward shift of the activation curve, with the \(V_{1/2}\) value shifted to 94.4/11006 5.2 mV (Fig. 5B). The shift resulted in enhanced channel activity at physiological voltages. These data suggest that, similar to heat and 2-APB, intracellular proton can increase TRPV3 activity by both direct activation and a shift of the voltage dependence of the channel.

**Reduction of Intracellular Proton-activated Currents in a TRPV3-H426N Mutant**—To identify residues responsible for intracellular proton activation of TRPV3, we focused on amino acids having side chain \(pK_a\) values in a physiologically relevant range (i.e. His, Glu, and Asp residues that have a \(pK_a\) value of 6.0, 4.1, and 4.0, respectively). The His residues are good candidates because His has a \(pK_a\) value closest to the half-maximal pH (pH\(_{1/2}\)) for TRPV3 activation. Based on the sequence alignment (Fig. 6A), His-426 in TRPV3 is unique, as the corresponding residue in the intracellular insensitive TRPV1, TRPV2, or TRPV4 is Asn. We noticed that His-426 in TRPV3 is important for 2-APB activation (53), and decided to determine whether His-426 is also critical for the response of the channel to intracellular protons. For the TRPV3-H426N mutant, the 2-APB response was deficient, but the mutant retained normal camphor sensitivity (53). To evaluate the response of mutant TRPV3 to intracellular acidification, we compared the current induced by proton at pH 5.5 to that by 8 mM camphor (Fig. 6B). The current amplitude ratio between them was calculated to be 1.51 for wild-type TRPV3, versus 0.46 for the H426N mutant, indicating a partial loss of proton activation for the H426N mutant and the role of His-426 in pH sensing (Fig. 6C).

To further investigate whether protons and 2-APB used the same pathway to influence TRPV activation, we generated a 2-APB-sensitive TRPV4 mutant by introducing a histidine mutation at the equivalent position of His-426 and an addition of Trp to Arg mutation at position 737 (TRPV4-N456H/ W737R) (53), and tested the proton sensitivity of this TRPV4 mutant. The TRPV4-N456H/W737R mutant and wild-type TRPV3 showed a similar dose-response relationship to 2-APB (EC\(_{50}\) of 42.7/11006 0.3 and 58.7/11006 0.8 M, respectively) (Fig. 6D). However, the TRPV4-N456H/W737R mutant channel showed no activation response to intracellular acidification (Fig. 6E).

The fact that we introduced 2-APB sensitivity but not proton sensitivity into TRPV4 implies that the mechanisms for pH sensing and 2-APB sensing are distinct. Hence our data suggest that His-426 in TRPV3 is involved in sensing both 2-APB and proton, whereas additional residues may participate in either proton binding or subsequent conformational changes.

**Glycolic Acid Induces Cytotoxicity in Both HEK-293 Cells Expressing TRPV3 and HaCaT Cells**—Based on the observation that glycolic acid potentially activates TRPV3 channel via intracellular acidosis, we asked whether TRPV3 could mediate glycolic acid-induced cell toxicity. We applied glycolic acid to HEK-293 cells expressing TRPV3 and measured cell viabilities.

**FIGURE 6. TRPV3-H426N mutation affects proton activation.** A, sequence alignment of the N-terminal MPR of mTRPV1, mTRPV2, mTRPV3, and mTRPV4. The amino acid histidine involved in 2-APB sensing is indicated in red. B, representative currents obtained from inside-out patches with HEK-293 cells expressing wild-type (WT) TRPV3 (left panel) or mutant TRPV3 H426N (right panel) evoked by pH 5.5 solution, 8 mM camphor, or 300 \(\mu\)M 2-APB. C, a comparison of the ratio between the current evoked by pH 5.5 solution and current evoked by 8 mM camphor from WT TRPV3 and the TRPV3-H426N mutant, \(p < 0.001, n = 3–4\). D, concentration-response curve of the 2-APB activated response in WT TRPV3 and TRPV4-N456H/W737R mutant (left panel), \(n = 5\). Representative whole cell currents obtained from HEK-293 cells expressing WT TRPV3 (middle panel) and the TRPV4-N456H/W737R mutant (right panel) evoked by various concentrations of 2-APB. E, a representative current trace recorded from an inside-out patch evoked by pH 5.5 solution and currents evoked by 300 \(\mu\)M 2-APB in HEK-293 cells expressing the TRPV4-N456H/W737R mutant.
using a PI staining assay. Cell death was determined by quantification of Hoechst-stained cells (total) versus PI-labeled cells (dead) after treatment with 20 mM glycolic acid for 12 h. Exposure to glycolic acid at pH 5.5 showed a significant increase in cell death with HEK-293 cells expressing TRPV3, as compared with cells expressing eYFP (negative control) (Fig. 7B). As expected, cells expressing TRPV3 and treated with glycolic acid at pH 7.4 did not show a significant increase in cell death. Although there is a lack of highly selective TRPV3 inhibitor, the toxicity was greatly attenuated by application of 10 μM ruthenium red, a nonspecific TRP channel blocker, or by co-transfection of the TRPV3-E631K/D641K double mutant (54, 55) that exerted a strong dominant-negative effect on rescuing the overactive TRPV3 channel (Fig. 7, A and B). These results are consistent with the idea that glycolic acid induces cell damage by activating TRPV3. In addition, we tested the effect of TRPV3

**FIGURE 7. Contribution of TRPV3 activation to glycolic acid-mediated cell death in HEK293 and HaCaT cells.** A, sequence alignment of pore helix and selectivity filter for mTRPV1, mTRPV2, mTRPV3, and mTRPV4. The conserved negatively charged residues causing dominant-negative effects when substituted with lysine are indicated in red or blue (left panel). Typical normalized currents to cell membrane capacitance evoked by 300 μM 2-APB and measured by a voltage ramp from −100 to +100 mV in 100 ms from cells expressing wild-type TRPV3 and TRPV3-E631K/D641K (TRPV3-DN) mutant alone or co-expressing wild-type TRPV3 and TRPV3-DN mutant at 1:1 ratio (right panel). B, top panels show representative images of Hoechst staining (upper images) and PI labeling (lower images) of HEK-293 cells expressing eYFP, TRPV3 alone, or co-expressing TRPV3 and TRPV3-DN mutant at 1:1 ratio following he indicated interventions. The bottom panel shows a bar graph of statistical analysis, n = 3. C, representative images of Hoechst staining and PI labeling of HaCaT cells following the indicated interventions (left panels), and statistical analysis of cell death quantified by PI labeling over Hoechst staining cells in each corresponding group (left images), n = 4. Statistical significance is indicated as: *, p < 0.05; **, p < 0.01; n.s., no significant difference.
activation on HaCaT cells. As shown in Fig. 7C, HaCaT cells treated with glycolic acid at pH 5.5 showed increased cell death, as compared with HaCaT cells at pH 7.4, consistent with the data obtained with HEK-293 cells expressing TRPV3. Thus, our results confirmed the involvement of TRPV3 in mediating the cellular actions of glycolic acid. Our findings are also consistent with the anti-proliferative effects of glycolic acid in HaCaT cells (56) as well as the report that TRPV3 activation induces cell death in human outer root sheath keratinocytes (27).

DISCUSSION

Weak acids from normal cellular metabolites, whereas not toxic under physiological conditions, can lead to intracellular acidification in a concentration and extracellular pH-dependent fashion (12, 43, 57–59). In this study, we demonstrated that glycolic acid-induced intracellular acidification could lead to TRPV3 activation. We suggest that glycolic acid may cause intracellular acidification in two ways (Fig. 8). In its neutral form, glycolic acid may cross the cell membrane to reach the cytosol where it re-equilibrates and releases the proton. Alternatively, protons can pass through the open TRPV3 channel or other proton-permeant channels. These two processes may work cooperatively in TRPV3-expressing cells, as an increase in intracellular proton concentration through the first pathway would activate TRPV3 to promote the second pathway. As TRPV3 has a high Ca\(^{2+}\) permeability, elevated TRPV3 activity may cause intracellular Ca\(^{2+}\) overload that affects many aspects of cellular physiology and eventually causes cell death. Our findings reveal a novel mechanism by which TRPV3 is regulated by intracellular acidification through a coupling between cytosolic proton and Ca\(^{2+}\). This mechanism may help explain the cosmetic effect of weak acids on proliferation and an early phase of apoptosis in keratinocytes for skin renewal (Fig. 8). Therefore, pH sensing by TRPV3 may serve as an important mechanism in medical applications of topical AHAs such as glycolic acid on the skin.

The detailed molecular mechanism underlying intracellular proton activation of TRPV3 remains to be elucidated. Our results suggest that His-426 plays an important role in proton-mediated activation of TRPV3. His-426 resides in the N-terminal membrane-proximal region (MPR), located between the last ankyrin repeat domain and the first transmembrane domain. MPR is an important region involved in different modes of TRPV1–4 activation. Especially, His-426 in the MPR of TRPV3 plays a critical role in 2-APB-induced channel activation (53). The corresponding amino acid, His-378, in the MPR of TRPV1 has been shown to affect the intracellular alkalization-induced activation (60). Yao et al. (61) recently reported that MPR might be the heat sensor of TRPVs, as swapping MPRs between TRPV channels led to a switch of the temperature sensitivity. These results suggest MPR is a shared functional element important for the activation of TRPV channels. Protonation of His-426 in the MPR is thus expected to affect TRPV3 activity by directly interacting with the activation process. However, we cannot rule out other potential site(s) besides His-426 that may be involved in the proton sensing.

Besides providing a viable explanation of the cosmetic effect of topical AHAs, what is the physiological and pathophysiological significance of TRPV3 proton sensing? Variations in the pH level occurs in normal physiological processes such as respiratory and metabolic acidosis and alkalosis; it can be much more severe under pathological conditions such as tissue damage, inflammation, and ischemia (62). Cell acidification can serve as a concomitant of cell death (63) and a means to detect painful conditions, especially in deep tissues where the temperature is expected to remain constant (64). A number of TRP channels have been shown to be quite sensitive to changes in extracellular pH (65, 66). Although intracellular acidification-mediated inhibition has been observed in TRPV5 and TRPM2 channels (67, 68), our findings represent the first demonstration of intracellular acidification-mediated activation by a member of the TRPV channel family.

Besides being expressed in skin keratinocytes, TRPV3 is also expressed in various areas of the nervous system such as dorsal root ganglion, trigeminal ganglion, spinal cord, and brain (14, 16). Acidosis is a noxious condition associated with inflammation, ischemia, or defective acid containment. Among several acid-sensitive ion channels, acid-sensing ion channels and TRPV1 have been proposed to sense acid-mediated nociception (69–71). Acid-sensing ion channels detect moderate decreases in extracellular pH (72), whereas TRPV1 is activated only by severe acidosis resulting in pH values below 6 (41). Both acid-sensing ion channels and TRPV1 exhibit very rapid desensitization and only produce a transient current response (73–75). How neurons can detect long lasting acid environments remains largely unknown. Although TRPV3 cannot sense the extracellular protons directly, we show in the present study that it can sensitively report intracellular acidification. Because TRPV3 mediates responses to warm temperatures above 33 °C, TRPV3 confers a basal activity at body temperatures that might allow protons to enter the cell and further activate the channel at elevated extracellular proton levels, thus acting as a primary

**Proton Activation of TRPV3**

![Diagram of Proton Activation of TRPV3](attachment:image.png)
Proton Activation of TRPV3

pH sensor. Interestingly, Miyamoto et al. (42) recently reported that TRPV3 regulates nitric-oxide synthesies in the skin through a pH-dependent fashion. The activation of TRPV1 or other proton-permeable channels that are coexpressed with TRPV3 in many cell types may further facilitate cytosolic acidification (76), supporting a role for TRPV3 in acid sensing, acid-induced pain, and acid-evoked feedback regulation of homeostatic reactions. For these reasons, TRPV3 may be an attractive target for the development of analgesic drugs to relieve acidic pain (77).

An interesting observation of the present study was that increasing the concentration of intracellular protons beyond pH 5.5 evoked little TRPV3 current, but a robust transient response was observed when the acidic solution was replaced. This off-response has been suggested to have a potential association with sour taste sensation in mammals (21, 78–81). We showed here that this off-response is predominantly due to the removal of inhibition effects of intracellular protons on single-channel conductance. It reveals that the gating process underlying recovery from low pH-induced activation is quite slow. Our data also suggest that the strong inhibition of the TRPV3 current at highly acidic pH levels may not be accounted for by simple conductance inhibition, indicating that an inhibitory effect of proton on channel gating may also exist. A second pH sensing domain or general charge effects on the TRP domain may contribute to this inhibition (82).

In summary, we have demonstrated that intracellular protons can strongly activate TRPV3, revealing a coupling between cytosolic protons and calcium. When the channel is sensitized, extracellular proton can efficiently pass through the channel pore to further open the channel, so that TRPV3 can detect both intracellular and extracellular acidosis under physiological or pathophysiological conditions. The sensitivity of TRPV3 to acidosis may explain the cosmetic effect of topical AHAs and provide a new target for pain medication and human skin diseases caused by TRPV3 gain-of-function mutations (35).

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Proton Activation of TRPV3

I. Introduction

The transient receptor potential vanilloid 3 (TRPV3) ion channel is a voltage-dependent, permeation-gated channel that is activated by a variety of stimuli, including acidic pH, pyrophosphate, and mechanical stimuli. TRPV3 was first identified as a gene mutated in a syndrome characterized by hyperalgesia and hyperthermia, Olmsted syndrome. Despite its name, TRPV3 is not as sensitive to capsaicin as its closely related homologue, TRPV1.

II. Acidic Activation

Acidic pH is one of the most potent activators of TRPV3. The channel is activated in the pH range of 5.0 to 6.5, with an apparent pH 

III. Pyrophosphate Activation

Pyrophosphate is a novel pain-producing molecule that activates TRPV3. It is a pyrophosphate anion and is a potent activator of TRPV3 in a pH range of 5.0 to 6.5.

IV. Mechanical Activation

Mechanical stimulation, such as repetitive stimulations, can activate TRPV3. The channel responds to mechanical stimuli in a similar manner to TRPV1.

V. Role in Pain Sensation

TRPV3 plays a role in the sensation of pain. It is involved in the transmission of nociceptive signals and is expressed in sensory neurons that mediate pain.

VI. Conclusion

TRPV3 is a versatile channel that is activated by a variety of stimuli, including acidic pH, pyrophosphate, and mechanical stimuli. Its activation by these stimuli highlights its potential role in pain sensation and other physiological processes.
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