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Acidic pH-activated Cl^- Current and Intracellular Ca^{2+} Response in Human Keratinocytes

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The layers of keratinocytes form an acid mantle on the surface of the skin. Herein, we investigated the effects of acidic pH on the membrane current and $[Ca^{2+}]_c$ of human primary keratinocytes from foreskins and human keratinocyte cell line (HaCaT). Acidic extracellular pH ($pH_e \leq 5.5$) activated outwardly rectifying Cl^- current ($I_{Cl,pH}$) with slow kinetics of voltage-dependent activation. $I_{Cl,pH}$ was potently inhibited by an anion channel blocker 4,4`-diisothiocyanostilbene-2,2`-disulphonic acid (DIDS, 73.5% inhibition at 1 μM). $I_{Cl,pH}$ became more sensitive to pH_e by raising temperature from 24°C to 37°C. HaCaT cells also expressed Ca^{2+}-activated Cl^- current ($I_{Cl,Ca}$), and the amplitude of $I_{Cl,Ca}$ was increased by relatively weak acidic pH_e (7.0 and 6.8). Interestingly, the acidic pH_e (5.0) also induced a sharp increase in the intracellular $[Ca^{2+}]$ ($\triangle[Ca^{2+}]_{acid}$) of HaCaT cells. The $\triangle[Ca^{2+}]_{acid}$ was independent of extracellular Ca^{2+}, and was abolished by the pretreatment with PLC inhibitor, U73122. In primary human keratinocytes, 5 out of 28 tested cells showed $\triangle[Ca^{2+}]_{acid}$. In summary, we found $I_{Cl,pH}$ and $\triangle[Ca^{2+}]_{acid}$ in human keratinocytes, and these ionic signals might have implication in pathophysiological responses and differentiation of epidermal keratinocytes.

Key Words: Keratinocyte, Extracellular pH, Anion channel, Intracellular calcium, pH-activated Cl- current

INTRODUCTION

The human skin has an acidic surface; the acidity of stratum corneum reaches to pH 4.5 and becomes normal pH at deeper layer of epidermis. The major function of the 'acid mantle' has been suggested to be an antimicrobial one. However, recent studies suggested that the acid mantle is also critical for the permeability barrier homeostasis and normal integrity (Hachem et al, 2003; Fluhr et al, 2004). Multiple mechanisms have been suggested as the origin of the acid mantle; 1) fatty acids, urocanic acids, and lactic acids generated from various enzymes (Fluhr et al, 2001), and 2) active generation of proton by Na^+/H^+ exchangers in keratinocytes (Behne et al, 2002).

Although the acidity such as pH 5.0 is unlikely to be present in the normal internal environment of our body, the keratinocytes might be occasionally exposed to such an acidic pH. For example, 'Sting test' is applied for screening patients with 'sensitive skin'. A fraction of the population is believed to have sensitive skin showing stinging, itching, desquamation, papules, and whealing to various skin agents (Willis et al, 2001). To identify a 'sensitive' person, a variety of test methods have been used, and the Sting test among them is widely used (Frosch et al, 1977). For the Sting test, skin reactions to 10% (w/v) lactic acid are evaluated. Since the pKa of lactic acid is 3.85, it is supposed that the Sting test might elicit further and deeper acidification of epidermis in addition to the acidic mantle in stratum corneum.

In this background, the cellular responses of keratinocytes to the acidic pH are worthwhile to be investigated, and here we focused on the membrane ion channels and intracellular Ca^{2+} signals. Among various ion channels, the anion channels are becoming appreciated as important players controlling cell volume, cell cycle and apoptosis (Okada et al, 2004; Lang et al, 2005; Wang et al, 2007). In the present study, therefore, we investigated the effects of acidic extracellular pH (pH_e) on the anionic currents and cytoplasmic concentration of Ca^{2+} ($[Ca^{2+}]_c$) in the primary human keratinocytes and immortalized human keratinocyte (HaCaT).

METHODS

Cell culture

HaCaT cells were cultured in Dulbecco's modified Eagle's media (DMEM) supplemented with penicillin (400 U/ml),

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ABBREVIATIONS: $I_{Cl,pH}$, acidic pH-activated Cl^- current; $\triangle[Ca^{2+}]_{acid}$, acidic pH-induced intracellular Ca^{2+} increase; $I_{Cl,Ca}$, Ca^{2+}-activated Cl^- current, pH_e, extracellular pH.

streptomycin (50 μg/ml), and 10% FBS in a humidified 5% CO_2 atmosphere at 37°C. Human primary keratinocytes obtained from foreskins were cultured in keratinocyte growth media (Clonetics, San Diego, CA) primarily as described previously (Boyce et al, 1983), which was composed of MCDB 153 medium supplemented with epidermal growth factor (10 ng/ml), bovine pituitary extract (70 μg/ml), hydrocortisone (0.5 μg/ml), penicillin (100 μg/ml), streptomycin (100 μg/ml), and fungizone (0.25 μg/ml). Third-passage keratinocytes were used.

Electrophysiology

HaCaT cells were transferred into a bath mounted on the stage of an inverted microscope (IX-70, Olympus, Osaka, Japan). The bath (0.15 ml) was superfused with K^+-free Tyrode's solution at 5 ml/min, and voltage clamp experiments were performed at room temperature (22~25°C). Patch pipettes with a free-tip resistance of about 2.5 MΩ were connected to the head stage of a patch-clamp amplifier (EPC-9, HEKA elektronik, Germany). Liquid junction potentials were corrected with an offset circuit before each experiment. A conventional whole-cell clamp was achieved by rupturing the patch membrane after making a giga-seal. Voltage data were low pass filtered (5 kHz), stored in a Pentium-grade computer and analyzed using Origin version 7.0 (Microcal Software Inc., Northampton, MA, USA).

Measurement of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$)

Keratinocytes were loaded with the Ca^{2+}-sensitive fluorescent indicator fura-2-AM (2 μM, 15 min) in K^+-free Tyrode's solution at room temperature, and then the unloaded indicators were washed out with fresh solution. The recordings of $[Ca^{2+}]_c$, was performed with a microfluorometric system consisting of an inverted fluorescence microscope (Eclipse-2000, Nikon, Tokyo, Japan) interfaced with DeltaRAM ratiometry system (Photon Technology International, Lawrenceville, NJ, USA) (IX-70, Olympus). Light was provided by a 75-W xenon lamp (Ushino, Tokyo, Japan). A chopper wheel alternated the light path to monochromators filtering specific wavelengths of light (340 and 380 nm). As a measure of $[Ca^{2+}]_c$, the ratio of the fluorescence emissions at 340 nm and 380 nm excitation ($R_{340/380}$) is presented.

Measurement of intracellular pH (pH_i)

For the measurement of pH_i, keratinocytes were exposed to acetoxymethyl ester form of 2,7-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF-AM, 2 μM, K^+-free Tyrode's solution) for 15 min at room temperature to load the cells with the fluorescent dye. Unloaded dye was washed twice with fresh K^+-free Tyrode's solution. BCECF-loaded cells were allowed to adhere to the base of a superfusion chamber mounted on the Eclipse-2000 inverted microscope interfaced with DeltaRAM ratiometry system. Cells were excited at 488 nm and 440 nm, and emitted fluorescence was measured at 530 nm. Intracellular pH was estimated by *in situ* calibration of the ratios of fluorescence at 488 nm to that at 440 nm (F_{488}/F_{440}) according to the nigericin-high K^+ method (Silver, 1998).

Solutions and chemicals

The K^+-free Tyrode's solution with the following composition [(in mM) 140 NaCl, 4 CsCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, 5 MES, 10 glucose and 10 sucrose at pH 7.4 (titrated with NaOH)] was superfused during all whole-cell patch clamp recordings. The CsCl pipette solution contained (in mM) 140 CsCl, 5 EGTA, 10 HEPES, 1 $MgCl_2$ and 5 MgATP at pH 7.2 (titrated with CsOH). In preliminary studies and also in the experiments shown in Fig. 1A, the CsCl pipette solution contained 130 CsCl, 20 BAPTA, 10 HEPES, 1 $MgCl_2$ and 5 MgATP at pH 7.2 (titrated with CsOH). The activation of ICl,pH was not different between these two conditions. For NMDG-Cl pipette solution, CsCl was totally replaced with equimolar NMDG-Cl. DIDS and Niflumic acid were purchased from Sigma (St. Louis, MO). Cell culture media, antibiotics and fetal bovine serum (FBS) were purchased from Gibco. The calcium-sensitive indicator Fura-2AM was obtained from Molecular Probes (Carlsbad, CA, USA).

Statistics

Statistical significance was determined using the Student's *t*-test. Results are presented as means±SEM. All p-values quoted are two-tailed and significance was accepted when $p < 0.05$.

RESULTS

Acidic pH_e-activated anionic current in human primary keratinocytes

We first examined the effect of pH_e on the membrane current of human primary keratinocyte using CsCl pipette solution in the whole-cell configuration. Currents were elicited by stepping from a holding potential of −40 mV to a series of test potentials from −100 mV to +100 mV in 20 mV increments. In control condition (pH 7.4), the membrane conductance was very small (7±0.97 pA/pF at 100 mV, n=6). By changing pH_e from 7.4 to 4.2, an immediate increase of membrane conductance was observed. The acidic pH_e-induced-current showed outwardly rectifying current-voltage relation (I-V curve) and time-dependent activation at depolarizing voltages (Fig. 1A). The same type of outwardly rectifying current was induced by acidic pH_e in HaCaT cells (Fig. 1B). To elucidate the acid-sensitivity of $I_{Cl,pH}$ in HaCaT cells, pH_e was changed to 6.5, 6.0, 5.5, 5.0 and 4.2 (Fig. 1B (b)). A discernible activation of $I_{Cl,pH}$ was observed from pH_e 5.5 and below.

The acidic pH_e-induced-current was largely abolished by replacing Cl^- with 145 mM gluconate in the bath solution (Fig. 1B(a)) whereas unaffected by total replacement of intracellular and extracellular monovalent cations with $NMDG^+$ (data not shown). Such results indicated that the acidic pH_e activated Cl^- current ($I_{Cl,pH}$). Hereafter, further characterization of $I_{Cl,pH}$ was performed in HaCaT cells with K^+-free Tyrode's bath solution and CsCl pipette solution. The $I_{Cl,pH}$ channels were also permeable to other halide anions (Br^- and I^-) as well as to Cl^-. The outwardly rectify-

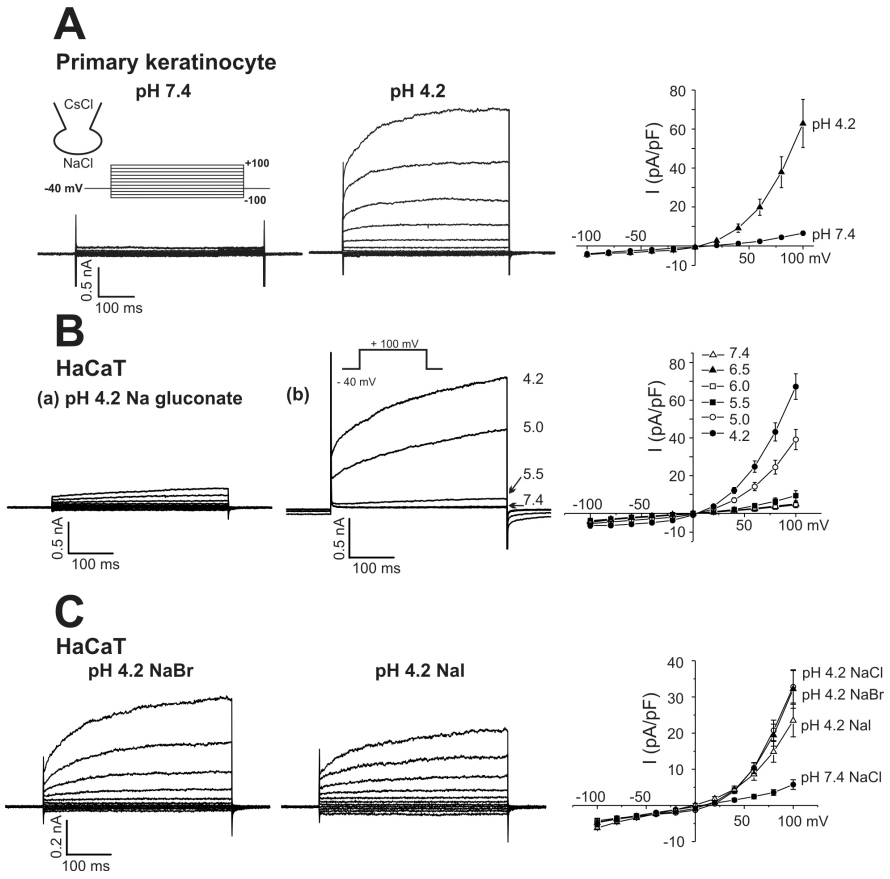

Fig. 1. Activation of outwardly rectifying Cl^- current by acidic pH_e. Representative current traces obtained from primary keratinocytes (A) and HaCaT cells (B) by step-like pulses. The membrane voltage was held at −40 mV, and incremental step-like pulses from −100 to 100 mV (20 mV intervals, 400 ms duration, see *inset*). The currents (B-a) were obtained with Na-gluconate solution in the bath (pH 4.2). (B-b) Representative current traces obtained by step-like pulses under different pH_e. The current to voltage relations (I-V curves), measured at the end of each pulse, are shown in right panels. Step like pulses were applied in different conditions of extracellular anions (pH 4.2 Br^-, and I^-). Current traces presented in (C) were obtained from the same cell, respectively. I-V curves of $I_{Cl,pH}$ obtained in NaCl, NaBr, and NaI solutions are shown in the right panel of (C) Each symbol represents mean±SEM of current amplitudes normalized to cell capacitance (pA/pF).

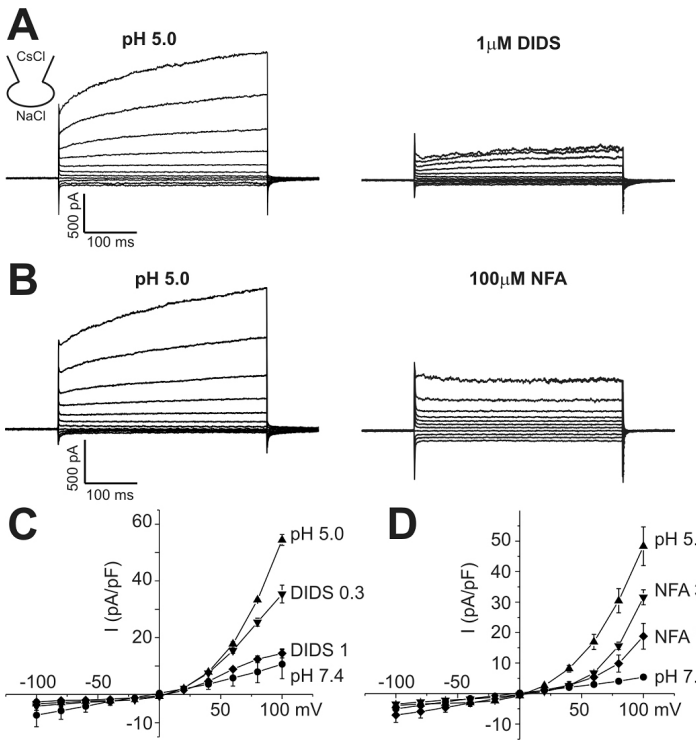

Fig. 2. Effects of anion channel blockers on $I_{Cl,pH}$. (A, B) Representative current traces obtained by step-like pulses, same as in Fig. 1. $I_{Cl,pH}$ was activated by pH_e 5.0, and DIDS (1 μM) or niflumic acid (NFA, 100 μM) was added (right panels). (C, D) I-V curves of $I_{Cl,pH}$ activated by pH_e 5.0 and effects of DIDS (0.3 μM and 1 μM) or niflumic acid (30 μM and 100 μM). Each symbol represents mean±SEM (n=5, respectively) of current amplitudes normalized to the cell capacitance (pA/pF).

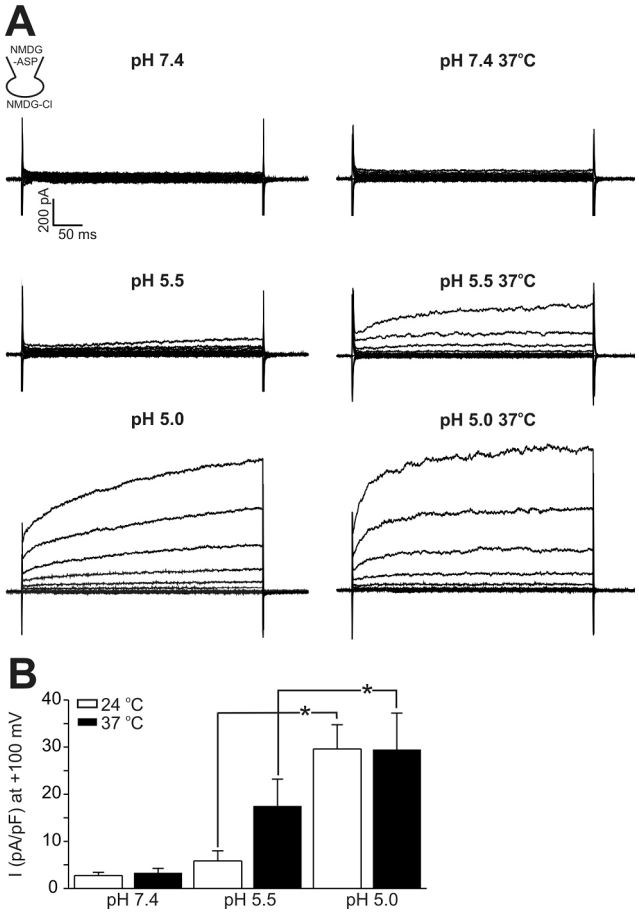

Fig. 3. Effects of bath temperature on $I_{Cl,pH}$ activation in HaCaT cells. (A) Representative current traces obtained by step like pulses, same as in Fig. 1. Current traces at pH 7.4, 5.5, and 5.0 were recorded under room temperature (24°C, left panels) and 37°C (right panels). The raise of temperature alone had insignificant effect on membrane conductance, whereas substantial increase was shown at pH 5.5. At pH 5.0, high temperature increased the activation kinetics with similar peak amplitude. All current trances shown were obtained from the same cell. (B) Mean amplitudes of normalized $I_{Cl,pH}$ (pA/pF) measured at 100 mV under different pH_e and temperature were compared (n=8) *indicate p-value $<$ 0.05.

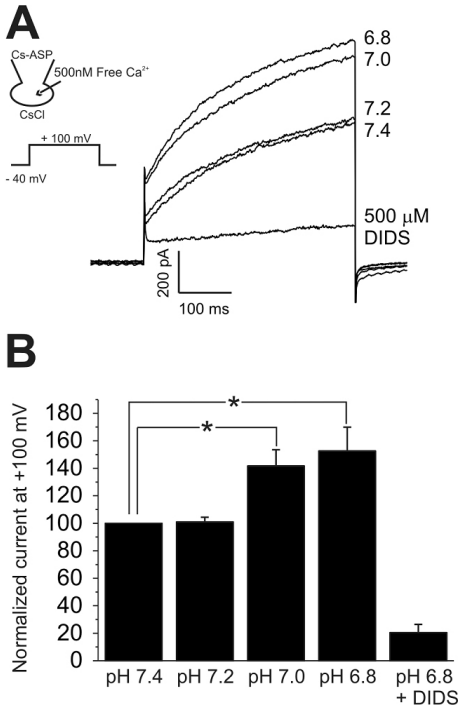

Fig. 4. Ca^{2+}-activated Cl^- current ($I_{Cl,Ca}$) of HaCaT cells and effects of acidic pH_e. (A) Outward currents with slowly activating kinetics were induced by dialyzing cells against Ca^{2+}-clamped CsCl solution (see *inset*). Lowering pH_e to 7.0 and 6.8 increased the amplitude of outward current. After testing pH_e effects, the inhibition of outward current by 500 μM DIDS was confirmed at pH_e 6.8. (B) Summary of the effects of pH_e on $I_{Cl,Ca}$ of HaCaT cells. In each cell, the current amplitudes measured at the end of step-pulse were normalized to the control amplitude (pH_e 7.4), and mean±SEM values were plotted (n=8). *indicate p-value $<$ 0.05.

ing currents were similarly acivated when extracellular Cl^- was replaced with Br^- or I^- (Fig. 1C). From the observed reversal potentials of I-V curves (−8.0±1.98 mV, 7.3±1.90 mV and 13.4±2.42 mV, for Cl^- (n=8), Br^- (n=8), I^- (n=5), respectively), it could be concluded that the permeability for the $I_{Cl,pH}$ channel is $I^- \geq Br^- \geq Cl^-$.

The effects of pharmacological blockers on $I_{Cl,pH}$ was tested at pH_e 5.0. $I_{Cl,pH}$ was sensitively inhibited by 4,4`- diisothiocyanostilbene-2,2`-disulphonic acid (DIDS, 73.5% inhibition at 1 μM, n=5, Fig. 2). Niflumic acid, another class of anion channel blocker, showed only partial inhibition of $I_{Cl,pH}$ (62.2% of inhibition at 100 μM, n=5, Fig. 2). The other type of anion channel blocker, NPPB, was not tested because NPPB became precipitated at pH 5.0.

Although keratinocytes are lying at the outermost surface of human body, the epidermal temperature might increase close to the core temperature or above, depending on the environmental situation. Therefore, we tested whether the pH threshold of $I_{Cl,pH}$ activation could be altered by raising the bath temperature (Fig. 3). To exclude the activation of other ionic conductance (e.g. thermosensitive nonselective cationic current), cations in the bath and pipette solutions were replaced with $NMDG^+$ (symmetrical NMDG-Cl solution). Also, the pH of warmed NMDG-Cl solution was separately titrated to 5.5 at 37°C, because the pH could be changed by raising the bath temperature. Again, only small amplitudes of $I_{Cl,pH}$ were induced by pH_e 5.5 at room temperature (24°C), which was 17.7% of $I_{Cl,pH}$ at pH_e 5.0. In contrast, a substantial amplitude of $I_{Cl,pH}$ was induced by pH_e 5.5 at 37°C, which was 52.7% of the $I_{Cl,pH}$ induced by pH_e 5.0. The amplitude of $I_{Cl,pH}$ induced by pH_e 5.0 was not different between recordings under 24 and 37°C, although the activation kinetics on step depolarization became faster (Fig. 3).

Augmentation of Ca^{2+}-activated Cl^- current by acidic pH

In keratinocytes, the expression of Ca^{2+}-activated Cl^- current ($I_{Cl,Ca}$) has previously been reported (Koegel and Alzheimer, 2001). Therefore, we also tested the effects of acidic pH_e on $I_{Cl,Ca}$ by using CsCl pipette solution with 500 nM free Ca^{2+}. Under this condition, the step depolarizing pulses revealed outwardly rectifying currents with time-de-

pendent activation kinetics, which was consistent with the known properties of $I_{Cl,Ca}$. The amplitudes of $I_{Cl,Ca}$ was also increased by acidic pH_e. The augmentation of $I_{Cl,Ca}$ was observed from pH 7.0, which was significantly more sensitive than the activation of $I_{Cl,pH}$ (Fig. 4). pH below 5.5 was not tested for $I_{Cl,Ca}$ due to the additional activation of $I_{Cl,pH}$. Although the overall shapes of currents were similar between $I_{Cl,Ca}$ and $I_{Cl,pH}$, the comparison of I-V curves showed that $I_{Cl,Ca}$ has relatively larger inward current at negative voltages. As for the pharmacological sensitivity, $I_{Cl,Ca}$ was much less sensitive to DIDS than $I_{Cl,pH}$; no inhibition by 5 μM DIDS, and 500 μM of DIDS was required to inhibit 83% of $I_{Cl,Ca}$ (Fig. 4B, n=8). We also tested and found that niflumic acid had only a weak inhibitory effect on $I_{Cl,Ca}$ (inhibition by 43 % at 100 μM, n=3, data not shown).

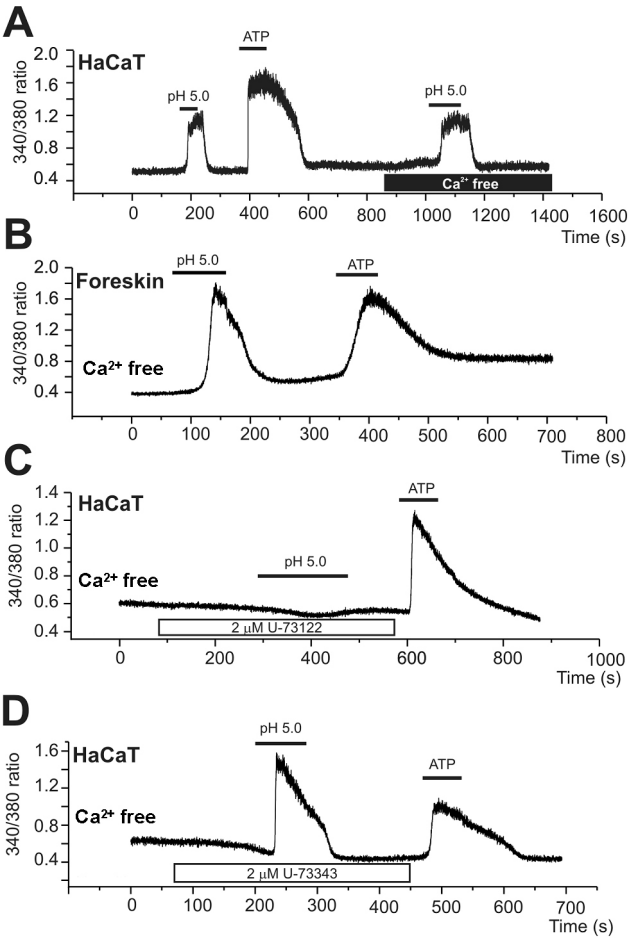

Fig. 5. Acidic pH_e-induced increase of $[Ca^{2+}]_c$ ($\triangle[Ca^{2+}]_{acid}$). (A, B) Representative traces of the fluorescence ratio ($F_{340/380}$) and the responses of HaCaT (A) and primary keratinyocyte (B) to pH_e 5.0 and ATP (100 μM). The $\triangle[Ca^{2+}]_{acid}$ was persistent in the absence of extracellular Ca^{2+} (A). (C, D) Inhibition of $\triangle[Ca^{2+}]_{acid}$ by PLC inhibitor (U73122, 2 μM)), whereas no effect of the negative analogue (U73343, 2 μM).

Activation of PLC-mediated store Ca^{2+} release by acidic pH_e

Finally, we spectrofluorimetrically examined the effects of acidic pH_e on $[Ca^{2+}]_c$ by using fura-2. The change of bath pH from 7.4 to 5.0 elevated $[Ca^{2+}]_c$ in a reversible manner (Fig. 5). After returning to the control pH, the application of 100 μM ATP induced similar increase in $[Ca^{2+}]_c$ (Fig. 5A). The acid-induced increase of $[Ca^{2+}]_c$ ($\triangle[Ca^{2+}]_{acid}$) was still observed in the absence of extracellular Ca^{2+}, indicating the release from intracellular Ca^{2+} stores (Fig. 5A). The most common pathway of stored Ca^{2+} release is phospholipase C (PLC)-dependent generation of IP_3 and subsequent activation of IP_3 receptors in endoplasmic reticulum. In the present study, the $\triangle[Ca^{2+}]_{acid}$ was completely and reversibly suppressed by the pretreatment with 2 μM U73122, an inhibitor of PLC, whereas not by a negative analogue U73343 (Fig. 5B and C). The $\triangle[Ca^{2+}]_{acid}$ was consistently observed in HaCaT cells (19 out of total 20 tested HaCaT cells), whereas the proportion of cells showing $[Ca^{2+}]_{acid}$ was only 18 % in the primary human keratinocytes (5 out of 28 tested cells, Fig. 5B).

From the above results, it was asked whether the acute responses, such as $I_{Cl,pH}$ and $\triangle[Ca^{2+}]_{acid}$, might have been due to changes in intracellular pH. Therefore, we tested how fast the acidic pH_e changes the intracellular pH (pH_i), by using BCECF fluorescence ratio ($R_{490/440}$). In contrast with the almost immediate activation of $I_{Cl,pH}$ and $\triangle[Ca^{2+}]_{acid}$, the change of pH_i in response to acidic pH_e was slow and did not reach a steady-state until 10 min after changing pH_e (Fig. 6).

DISCUSSION

In this study, we examined the effects of acidic pH_e on the anion channels and $[Ca^{2+}]_c$ in human keratinocytes. Relatively strong acidic pH_e (<5.5) directly activated $I_{Cl,pH}$, and the pH threshold was lowered by raising bath temperature. Although the less acidic conditions (pH_e 7.0 and 6.8) did not directly activate $I_{Cl,pH}$, they nevertheless increased the amplitude of $I_{Cl,Ca}$. Interestingly, the strong

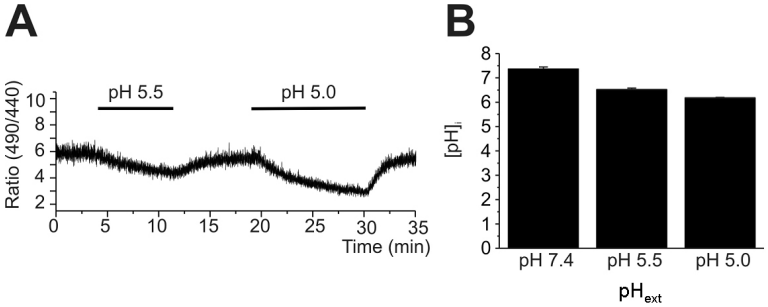

Fig. 6. Slow decrease of intracellular pH (pH_i) in response to extracellular acidification. (A) A representative trace of BCECF emission ratio ($F_{490/440}$) measured in HaCaT cell. pH_e was changed from 7.4 to 5.5. or to 5.0 as indicated. (B) Summary of calibrated pH_i (mean± SEM, n=8) of control (pH_e 7.4) and 10 min after pH_e changes (5.5 and 5.0).

acidic pH (5.0) also activated PLC-dependent store Ca^{2+} release in HaCaT cells, although the $\triangle[Ca^{2+}]_{acid}$ was not consistently found in the human primary keratinocytes.

Acidic pH-activated Cl^- currents

The acidic pH-activated outward rectifying anion current similar to $I_{Cl,pH}$ was firstly reported in rat Sertoli cells (Auzanneau et al, 2003) and subsequently in various types of cell lines (HEK-293, CHO, HeLa, PC-12, Caco-2 and HBE), hippocampal astrocytes and cardiac myocytes (Nobles et al, 2004; Lambert et al, 2005; Yamamoto et al, 2006; Wang et al, 2007). The properties of $I_{Cl,pH}$ in human keratinocytes are very similar to those reported in the above literatures; activation at pH_e below 5.5 (room temperature), time-dependent kinetics, outward rectification, and high sensitivity to DIDS blocking (Nobles et al, 2004; Lambert et al, 2005; Yamamoto et al, 2006; Wang et al, 2007). In this respect, $I_{Cl,pH}$ appears to be broadly expressed in mammalian cells, like volume-activated Cl^- current ($I_{Cl,vol}$). Actually, $I_{Cl,pH}$ and $I_{Cl,vol}$ seem to share some properties and to interconvert at combined hypotonicity and acidic pH; i.e. different manifestations of the same channel (Nobles et al, 2004). However, more recent study by Lambert & Oberwinkler (2005) showed that $I_{Cl,pH}$ and $I_{Cl,Vol}$ are distinct populations of anion channels with different properties such as Mg^{2+} sensitivity, steepness of I-V relation, voltage-dependent inactivation and some pharmacological sensitivity. Unfortunately, the properties of $I_{Cl,pH}$ do not match with any of the cloned Cl^- channels, and the molecular identity of this widely expressed channel remains to be elucidated.

Although widely expressed, $I_{Cl,pH}$ seems to have physiological implication only under limited situations since the *in vitro* activation of $I_{Cl,pH}$ occurs at very acidic pH such as 5.0 or below. Even in the inflammatory sites where local accumulation of lactic acid and short chain fatty acids produce acidic environment, the pH of exudates is above 6.0 (Menkin, 1958). Therefore, the *in vivo* activation of $I_{Cl,pH}$ would be possible only at extreme inflammation and ischemia that lead to cell death. In this respect, the presence of $I_{Cl,pH}$ in keratinocytes could have intriguing physiological implication. As mentioned in *Introduction,* human skin forms 'acid mantle' near the surface of epidermis, and skin is frequently exposed to chemical substances with acidic pH like cosmetics with preservatives and lactic acid solution (pH, ~4.0) used for 'Sting test' (Frosch et al, 1977). Although it is not certain whether the pH of deeper epidermis can be actually lowered to below pH 5.0, it is likely that keratinocytes *in vivo* might be exposed to the threshold pH to activate $I_{Cl,pH}$, especially when combined with raised temperature.

Facilitation of $I_{Cl,Ca}$ by acidic pH

In contrast to the pH threshold for $I_{Cl,pH}$, the augmentation of $I_{Cl,Ca}$ was observed at less acidic pH_e. Previous studies in other cells also showed that alkaline pH_e decreases $I_{Cl,Ca}$ [18] and acidic pH_e enhances $I_{Cl,Ca}$ (Hirayama et al, 2002). Therefore, when combined with the $[Ca^{2+}]_c$ activating stimuli (e.g. ATP), the acidic pHe would facilitate the anionic conductance to increase, subsequently evoking various cellular responses such as volume changes. The enhancement of $I_{Cl,Ca}$ by less acidic pH_e would contribute to the increase of anionic conductance of keratinocytes in broad ranges of pH_e.

Acidic pH induces release of stored Ca^{2+}

The recruitment of stored Ca^{2+} by acidic pH_e (Fig 5) might have physiological implication with regard to the interplay between epidermal pH_e gradient and the Ca^{2+}-mediated differentiation of keratinocytes. It is well known that the proliferation and differentiation of keratinocytes in epidermis are regulated by Ca^{2+} signals. For examples, changes in the concentration of extracellular calcium affect the balance between proliferation and differentiation in epidermal keratinocytes; elevation of the extracellular calcium concentration (calcium switch) inhibits proliferation and induces the onset of terminal differentiation (Yuspa et al, 1989; Menon et al, 1992). Ca^{2+} sensing receptors (CaSR) coupled with PLC/$InsP_3$ pathway and Ca^{2+}-selective TRPV6 channels in keratinocytes have been suggested as candidate mechanisms mediating the 'calcium switch' into the increase of $[Ca^{2+}]_c$ (Tu, 2001; Lehen'kyi et al, 2007). Although still remains controversial, it is suggested that the epidermis has a calcium gradient, with the lowest concentrations in the stratum basal and the highest in the stratum granulosum (Elias et al, 2002).

The inhibition of $\triangle[Ca^{2+}]_{acid}$ by U73122 suggests that PLC/IP_3 pathway is activated during acidic stimulus. However, it is still unclear whether a specific type of G protein-coupled receptor is activated or the pH_e change somehow directly activates PLC in keratinocytes. The decrease of pH_i by external acidification occurs very slowly and incompletely (Fig. 6). Therefore, it is not likely than acidic pH_e activates the PLC/$InsP_3$ by intracellular acidification. The phenomenon of $InsP_3$-mediated Ca^{2+} release evoked by acidic pH_e has previous been reported in human fibroblasts, coronary endothelium, neuroblastoma and umbilical smooth muscle cells (Smith et al, 1989). Recently, a group of proton-activated GPCRs, called OGR1, G2A and TDAG8, has been reported (Ludwig et al, 2003; Murakami et al, 2004; Ishii et al, 2005). Among them, OGR1 and G2A are coupled with PLC/$InsP_3$-mediated Ca^{2+} release pathway (Ludwig et al, 2003; Murakami et al, 2004). However, compared with the pH-dependence of $\triangle[Ca^{2+}]_{acid}$ in keratinocytes, the activation of OGR1 and G2A occurs at much higher pH_e ranges (7.2~6.8), where the $[Ca^{2+}]_c$ of keratinocytes does not change. Therefore, different type of pH_e-sensing mechanism(s) or GPCR seems to be involved in $\triangle[Ca^{2+}]_{acid}$ of keratinocytes.

In summary, we found functional expression of $I_{Cl,pH}$ in human keratinocytes and confirmed the enhancement of $I_{Cl,Ca}$ by acidic pH_e. Also, the strong acidic pH evokes PLC-dependent store Ca^{2+} release in human keratinocytes. Differing from other tissues in the body, epidermal keratinocytes are likely to be exposed to strong acidic environment. The specific roles of $I_{Cl,pH}$ and $\triangle[Ca^{2+}]_{acid}$ in keratinocytes physiology would be interesting topics of future study.

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