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Zinc Released from Injured Cells Is Acting via the Zn^{2+}-sensing Receptor, ZnR, to Trigger Signaling Leading to Epithelial Repair*

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A role for Zn^{2+} in accelerating wound healing is established, yet, the signaling pathways linking Zn^{2+} to tissue repair are not well known. We show that in the human HaCaT keratinocytes extracellular Zn^{2+} induces a metabotropic Ca^{2+} response that is abolished by silencing the expression of the G-protein-coupled receptor GPR39, suggesting that this Zn^{2+}-sensing receptor, ZnR, is mediating the response. Keratinocytic-ZnR signaling is highly selective for Zn^{2+} and can be triggered by nanomolar concentrations of this ion. Interestingly, Zn^{2+} was also released following cellular injury, as monitored by a specific non-permeable fluorescent Zn^{2+} probe, ZnAF-2. Chelation of Zn^{2+} and scavenging of ATP from conditioned medium, collected from injured epithelial cultures, was sufficient to eliminate the metabotropic Ca^{2+} signaling. The signaling triggered by Zn^{2+}, via ZnR, or by ATP further activated MAP kinase and induced up-regulation of the sodium/proton exchanger NHE1 activity. Finally, activation of ZnR/GPR39 signaling or application of ATP enhanced keratinocytes scratch closure in an *in vitro* model. Thus our results indicate that extracellular Zn^{2+}, which is either applied or released following injury, activates ZnR/GPR39 to promote signaling leading to epithelial repair.

Following injury, keratinocytes are exposed to diverse extracellular stimuli such as growth factors, cytokines, and matrix components, resulting in stimulation of cellular proliferation and migration. The release of these factors at the wound site leads to renewal of the epithelial layer in the damaged area (1). Zinc is found in the intracellular and extracellular matrix, in its free or protein-bound form and was shown to accumulate in skin tissues following injury (2). Furthermore, topical addition of zinc, in ointments or bandages, was known for many years and is also used in modern medicine to accelerate wound healing and the re-epithelialization process (2–5). Consistent with a key role for Zn^{2+} in promoting wound healing, the manifestation of dietary or genetic Zn^{2+} deficiency are severe skin lesions and impaired wound healing that can be reversed by Zn^{2+} supplementation (6, 7). Whereas the role of Zn^{2+} transporters in diseases linked to dyshomeostasis of this ion has been described (8–13) cellular mechanisms linking Zn^{2+} to keratinocytes proliferation and migration are not well understood.

Several studies have highlighted a role for Zn^{2+} in cellular signaling (12, 14). Of particular importance is the activation of mitogen-activated protein kinase (MAPK)[2] and PI3 kinase pathways, leading to enhanced cell proliferation and survival (15–20). In epidermal tissues, higher levels of zinc have been correlated with higher mitotic activity (21). It was further shown that application of Zn^{2+} accelerates the migration of keratinocytes via modulation of integrin receptors expression or interaction with metalloproteases (22–25). The effect of Zn^{2+} in enhancing cell proliferation is synergistic with intracellular Ca^{2+} rise, indicating that their effects are mediated through a common pathway (26, 27). Importantly, Zn^{2+} affects cell proliferation rate prior to changes in its intracellular levels, suggesting that the extracellular Zn^{2+} pool mediates signaling leading to epithelial repair (28).

We have previously identified a Zn^{2+}-sensing receptor (ZnR) active in keratinocytes and other epithelial cells (16, 29). ZnR activity was manifested by robust increase in intracellular Ca^{2+} concentrations triggered by extracellular Zn^{2+} (29). Subsequent studies have found that in neurons ZnR activity is mediated by the G-protein-coupled receptor, GPR39 (30). We demonstrate here that a ZnR/GPR39 is activated in keratinocytes, by exogenous Zn^{2+} or by conditioned medium collected from injured cells, and up-regulates signaling and ion transport pathways necessary for wound healing.

EXPERIMENTAL PROCEDURES

Cell Culture—The HaCaT cell line, a spontaneously transformed, non-tumorigenic human keratinocyte cell line (31) was cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 4 mM L-glutamine, and 0.5 μg/ml antibiotic penicillin streptomycin solution (Biological Industries, Israel).

Fluorescent Imaging of Ca^{2+}, Zn^{2+}, or pH—Fluorescent imaging measurements were acquired and analyzed using AIW 4 (INDEC BioSystems) and analyzed in Microsoft Excel. Graphs presented are representative graphs of at least three independent experiments. The fluorescent signal is shown as

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[2] The abbreviations used are: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; NHE, Na^+/H^+ exchanger; ZnR, Zn^{2+}-sensing receptor; Ca^{2+}_i, intracellular Ca^{2+}; pH_i, intracellular pH; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; BSA, bovine serum albumin.

the percentage of the averaged baseline signal (R_0) acquired during the first 20 s of each measurement ($R/R_0 \times 100$), as previously described (29, 30). The rate of fluorescent change was determined using Kaleidagraph and averaged over at least three independent experiments.

For $Ca^{2+}{}_i$ imaging, cells were incubated for 30 min, with 5 μM Fura-2 acetomethyl ester (TEF-Lab) in 0.1% BSA in Ringer's solution containing: 120 mM NaCl, 2.7 mM KCl, 0.8 mM $MgCl_2$, 2 mM Hepes, 0.75 mM glucose, 1.8 mM $CaCl_2$. For pH_i measurements, cells were loaded with the pH-sensitive dye 2′, 7′-bis-(2-carboxyethyl)-5,6 carboxyfluorescein acetoxy methyl-ester (1.25 μM BCECF-AM, TEF-Lab) for 12 min. Following dye loading, the cells were washed in Ringer's solution, and the cover slides were mounted into an imaging chamber. To buffer the low Zn^{2+} concentrations, Ringer's solutions contained 100 μM of CaEGTA, which has affinity to Zn^{2+} that is six orders of magnitude higher than to Ca^{2+} or Mg^{2+} (32). Concentration of free-Zn^{2+} were determined using Webmaxcstandard software. Intracellular pH was calibrated by perfusing the cells with high potassium Ringer's solution (120 mM KCl replacing 120 mM NaCl) containing 5 μM nigericin, at extracellular pH levels: 6.5, 7.0, 7.5, and 8.0 (20).

To monitor extracellular Zn^{2+} release, the cell impermeant fluorescent dye ZnAF-2 (2 μM, K_d = 2.7 nM for Zn^{2+}, Sigma-Aldrich) (33), was employed. ZnAF-2 was excited at 480 nm and imaged with 535-nm long pass filter.

Statistical analysis was performed using Student's *t* test, applied following Levene's test for homogeneity of variances, or ANOVA analysis as relevant. Multiple comparisons were performed by student-Newman-Keuls or Dunnet method as appropriate. Non-parametric test included Mann Whitney and Kruskal Wallis test. Multiple comparisons followed by Fisher's protected level of significance method, were adjusted for the non-parametric setting. The data are presented as mean ± S.E. *, $p < 0.05$; **, $p < 0.01$.

GPR39 Silencing using siRNA—For gene-silencing experiments, cells were cotransfected with either of the silencing plasmids, 3 μg of siGPR39 or siT1R3 or a scrambled (siControl) siRNA construct (Sigma-Aldrich) in 35-mm plates, using the transfection reagent DreamfectGold (OZBiosciences) as described by the manufacturer. Cells were imaged 48 h after transfection. Analysis of cells co-transfection with YFP indicated that more than 90% of the cells were transfected using this reagent. The target sequence of the GPR39 for siRNA was CCATGGAGTTCTACAGC ATtt and that of T1R3 was CUUAGGA UGAAGGGGGACUtt.

Desensitization Protocol—Cells seeded on glass coverslips were incubated for 30 min with 50 μM Zn^{2+}, concentrations that induced the Ca^{2+} response, concomitant with the Fura-2-loading procedure, at room temperature. Because the BSA may chelate the Zn^{2+} and change the effective amount of this ion in the solution, the Ringer's solution during loading did not contain BSA. In some of the experiments the cells were then allowed to recover in BSA containing Ringer's solution. For the prolonged recovery periods, cells were loaded immediately prior to imaging. Fluorescence imaging was performed as above to study the Zn^{2+}-dependent Ca^{2+} response.

NH_4Cl Prepulse Paradigm for NHE1 Activity—BCECF-loaded cells were exposed to 30 mM NH_4Cl in Ringer's solution, resulting in alkalinization of the cytoplasm, then Na^+-free (NMDG replacing Na^+) and NH_4Cl- free Ringer's solution was applied, resulting in cellular acidification. Recovery of pH_i, representing NHE activity, was apparent when cells were perfused with nominally Ca^{2+}-free Ringer's solution, containing 120 mM Na^+ (20).

ERK1/2 Phosphorylation by Western Blotting—HaCaT cells were grown to confluency in 60-mm dishes and then starved overnight for serum. Following treatment, with Zn^{2+} and inhibitors, cells were disrupted in lysis buffer and protein concentrations in the supernatant were determined using the Bio-Rad assay. Cytosolic fractions (20 μg) were separated on 10% SDS-PAGE followed by immunoblotting. Antibodies against doubly phosphorylated ERK1/2 (1:5000, Sigma), β-actin (1:40,000) or total ERK (1:5000) were detected and quantified digitally using Chemimager 5 (Alpha-Innotech, Labtrade) (20). Densitometry analysis was performed using ImageJ software. The values presented are normalized to β-actin or total ERK. Each graph represents an average of at least three independent experiments.

In Vitro Scratch Assay—Cells were seeded at 2×10^4 cells/well in 96-well plates and grown to confluency and deprived of serum for 24 h. A scratch was performed using 1 ml plastic pipette tip (~2 mm in width), as previously described (34), in 37 °C Ringer's solution and subsequently washed extensively with DMEM (supplemented with 1% FBS and 100 μM CaEGTA). Agonists (Zn^{2+} or ATP) were applied following scratching for 15 min in Ringer's solution, the PLC inhibitor U73122 (1 μM, 30 min) or NHE1 inhibitor cariporide (0.5 μM, 60 min) were applied prior to scratching. The Ringer's solution was then replaced back to DMEM (1% FBS, 100 μM CaEGTA). The rate of scratch closure was determined by acquiring bright field images immediately after performing the scratch, and after 24 h. Area measurements of the cell-free region were performed using ImageJ software, and the ratio of denuded areas at these times are presented.

Conditioned Medium—Conditioned medium was produced from confluent monolayers of HaCaT cells seeded on 60-mm dishes. HaCaT cells were grown to confluency and were then starved for serum for 24 h, to remove exogenous growth factors. The monolayers were washed with Ringer's solution, scraped with a rubber policeman into 100 μl of Ringer's solution, and the supernatant was collected. Cellular debris was removed by centrifugation (10 min, 1500 rpm). The supernatant was frozen (−80 °C) until used.

RESULTS

ZnR Signaling in HaCaT Keratinocytes—We first sought to determine if Zn^{2+} activates the ZnR pathway in HaCaT cells. The intracellular Ca^{2+} ($Ca^{2+}{}_i$) response in HaCaT cells loaded with the Ca^{2+}-sensitive dye Fura-2 was determined following application of 50 μM free-Zn^{2+} (150 μM Zn^{2+} in the presence of 100 μM CaEGTA) in Ringer's solution. A rapid Zn^{2+}-dependent increase of Fura-2 fluorescence was observed in the presence or absence of extracellular Ca^{2+} (Fig. 1*A*), consistent with our previous findings in primary cultures of normal human

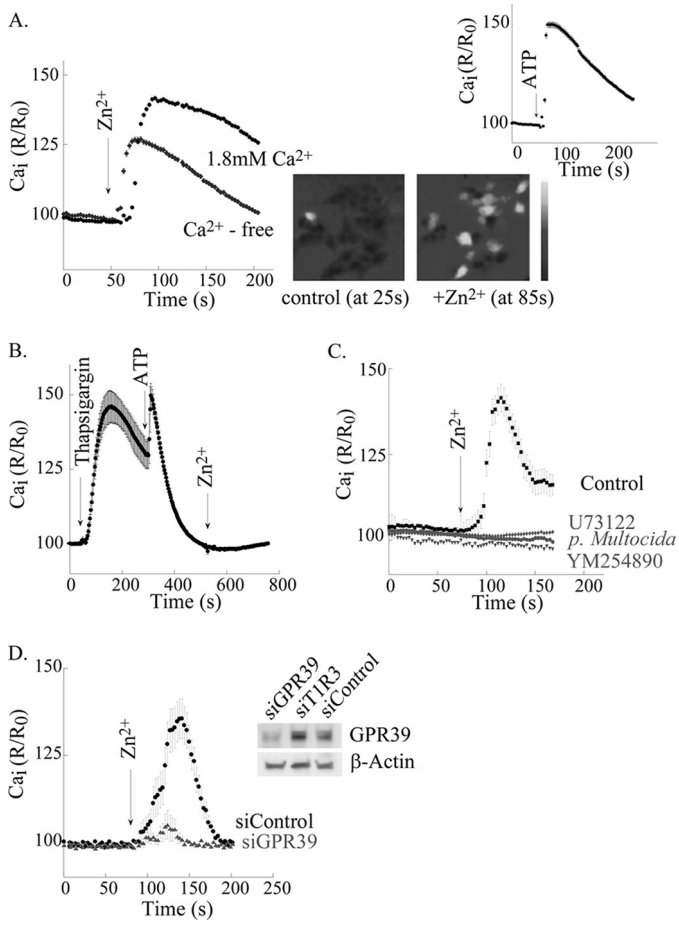

FIGURE 1. **Extracellular Zn^{2+} triggers a ZnR-dependent $Ca^{2+}{}_i$ response mediated by the G_q protein-coupled receptor GPR39 in HaCaT keratinocytes.** *A*, application of extracellular Zn^{2+} (50 μM) to Fura-2 loaded HaCaT cells. A robust rise in Fura-2 fluorescence following application of Zn^{2+} is shown both in the absence or presence of extracellular Ca^{2+}. To the *right*, fluorescent images of the culture before and after application of Zn^{2+}. The $Ca^{2+}{}_i$ rise triggered by ATP (100 μM) is shown (*top right*). *B*, application of Zn^{2+} following depletion of $Ca^{2+}{}_i$ stores using thapsigargin (200 nM) and ATP (100 μM) in nominally Ca^{2+}-free Ringer's solution, notice that following application of Zn^{2+} no rise in fluorescence is seen for the same time period shown in *A*. *C*, Zn^{2+}-dependent $Ca^{2+}{}_i$ response was monitored in cells pretreated with the PLC inhibitor, U73122 (3 μM), the $G\alpha_q$ inhibitor, YM-254890 (1 μM), and following treatment with *Pasteurella multocida* toxin (12 h). Inhibitors of the IP_3 pathway or depletion of Ca^{2+} stores blocked the Zn^{2+}-dependent Ca^{2+} rise. *D*, Zn^{2+} (100 μM)-dependent Ca^{2+} response in HaCaT cells transfected with siGPR39, or a scrambled control siRNA (*siControl*). *Inset* shows immunoblot of the expression level of GPR39 in HEK293 cells transfected with GPR39 plasmid (0.33 μg/60 mm plate) together with the indicated siRNA constructs: either siGPR39, siControl, or siT1R3 aimed to silence a non-related GPCR. Silencing of GPR39 eliminated the Zn^{2+}-dependent response. ($n = 5$ for each treatment).

keratinocytes (29). The Zn^{2+}-dependent $[Ca^{2+}{}_i]$ rise was similar to the response triggered by ATP (100 μM), a purinergic agonist (see inset). Because Fura-2 is sensitive to Zn^{2+} ($K_d = 2$ nM), permeation of this ion can also trigger a fluorescent signal rise. To test this hypothesis Ca^{2+} stores were first depleted using the Ca^{2+} pump inhibitor thapsigargin (200 nM) and ATP (100 μM) in nominally Ca^{2+}- free Ringer's solution. Application of Zn^{2+} (100 μM) following Ca^{2+} stores depletion, failed to elicit an increase in Fura-2 fluorescence (Fig. 1*B*), indicating that the response is mediated by Zn^{2+}-dependent release of $Ca^{2+}{}_i$. The PLC inhibitor U73122 (1 μM) blocked the Zn^{2+}-dependent $[Ca^{2+}{}_i]$ rise (Fig. 1*C*), whereas its inactive analogue, U73343, did not attenuate this response (not shown). Similarly, pretreatment of HaCaT cells with *Pasteurella multocida* toxin, which induces uncoupling of PLC from $G\alpha_q$ or pretreatment with the $G\alpha_q$ inhibitor YM-254890 (1 μM) (35, 36) also abolished the Zn^{2+}-dependent $[Ca^{2+}{}_i]$ rise (Fig. 1*C*).

The orphan GPCR, GPR39, was recently linked to ZnR signaling in neurons (30), we therefore asked if GPR39 also mediates keratinocytic-ZnR activity. Transfection of HaCaT cells with an siRNA (siGPR39) construct targeted to silence GPR39 expression (see *inset* in Fig. 1*D*) was followed by elimination of the Zn^{2+}-dependent $[Ca^{2+}{}_i]$ response, yet a scrambled siRNA construct (siControl) did not attenuate this response (Fig. 1*D*). These results suggest that extracellular Zn^{2+} activates a $G\alpha_q$-protein coupled zinc-sensing receptor in HaCaT cells that is mediated by GPR39 (16, 29, 36).

We then studied if ZnR-dependent metabotropic Ca^{2+} rise will activate the MAP kinase pathway that is critical in promoting wound healing (37). Phosphorylation of extracellular-regulated kinase (ERK) 1/2 was determined in HaCaT cells treated with 50 μM Zn^{2+} (applied for 0 to 120 min, as indicated). Zn^{2+}-dependent ERK1/2 phosphorylation was already evident after 10 min and peaked at 60 min (Fig. 2*A*). Application of ATP resulted in a similar temporal pattern of ERK1/2 phosphorylation (Fig. 2*B*). Zn^{2+}-induced ERK1/2 phosphorylation was blocked by the MEK inhibitor, U0126 (1 μM) (Fig. 2*C*). The $G\alpha_q$ inhibitor YM-254890 (1 μM, 30 min) attenuated Zn^{2+}-induced ERK1/2 phosphorylation by about 40% (Fig. 2*C*), suggesting that activation of the IP_3 pathway by ZnR is partially mediating the Zn^{2+}-dependent ERK1/2 activation. Intracellular Ca^{2+} rise activates PKC and PI3K pathways and may thereby enhance cellular proliferation (38–40). Application of the PKC inhibitor, bisindolylmaleimide I (BI, 10 nM), the PI3K inhibitor, wortmannin (100 nM), or both inhibitors, attenuated the Zn^{2+}-dependent ERK1/2 phosphorylation by about 50% (Fig. 2*D*), suggesting that the two pathways converge to mediate the Zn^{2+}-dependent activation of MAPK.

High Affinity and Selectivity of the Keratinocytic-ZnR—Because the concentration of free Zn^{2+} in the epidermis is estimated to be in the nanomolar range (2) we performed a dose-response analysis to determine if the affinity of the keratinocytic-ZnR is physiologically relevant. Zinc, at concentrations between 0.5 nM and 100 μM (free-Zn^{2+} buffered with EGTA, see "Experimental Procedures") was applied to HaCaT cells loaded with Fura-2. Amplitude of the $[Ca^{2+}{}_i]$ responses

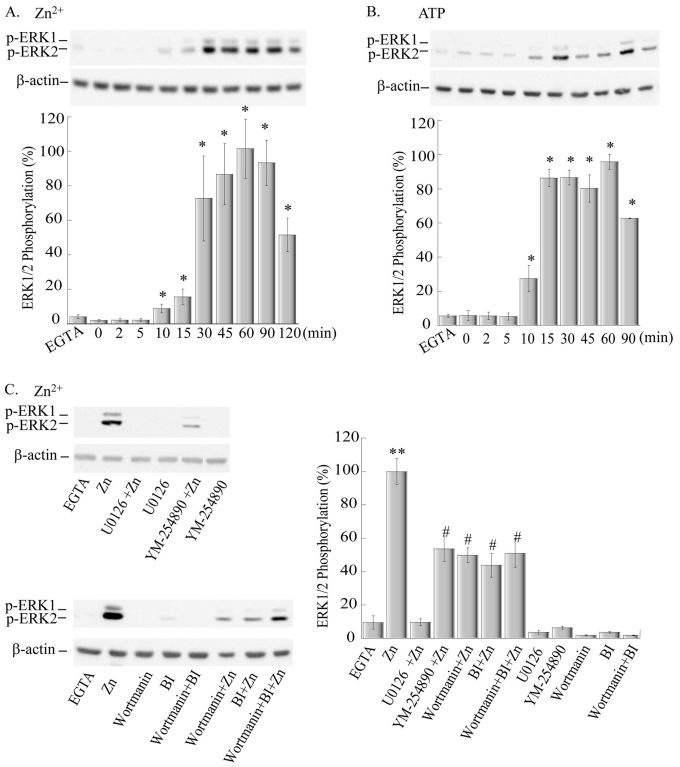

FIGURE 2. **ZnR activates the MAPK pathway in HaCaT keratinocytes.** Immunoblot of ERK1/2 phosphorylation following application of either 50 μM Zn^{2+} or the extracellular Zn^{2+} chelator, CaEGTA to chelate residual Zn^{2+} (*A*), or 100 μM ATP (*B*) for the indicated times. Quantification of the phosphorylation level normalized to β-actin expression is shown in *lower panels*. *C*, Zn^{2+}-dependent ERK1/2 phosphorylation was determined in the presence of the MEK inhibitor U0126 (1 μM); the $G\alpha_q$ inhibitor, YM-254890 (1 μM); the PI3K inhibitor, wortmannin (100 nM), or the PKC inhibitor, BI (10 nM) as indicated. Quantification of ERK1/2 phosphorylation normalized to β-actin expression, shown to the *right*, indicating that inhibition of either the IP_3, MAPK, or PI3K pathways reduced the Zn^{2+}-dependent ERK1/2 phosphorylation. ($n = 5$ for each treatment; *, $p < 0.05$; **, $p < 0.001$ compared with control; #, $p < 0.05$ compared with Zn^{2+}).

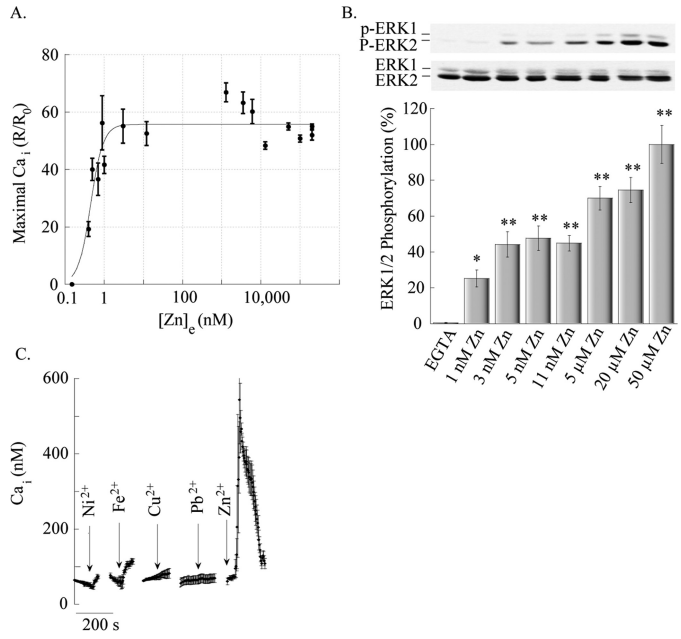

FIGURE 3. **Affinity and selectivity of the ZnR to Zn^{2+} in HaCaT keratinocytes.** *A*, dose-response of the intracellular Ca^{2+} rise triggered by extracellular Zn^{2+} in HaCaT cells. The maximal Ca_i^{2+} response (monitored as in 1) following application of the indicated Zn^{2+} concentration was averaged and plotted *versus* the applied free-Zn^{2+} concentration (see "Experimental Procedures"). *B*, cells were treated with varying concentrations of Zn^{2+} and then ERK1/2 phosphorylation was monitored. Quantification of ERK1/2 phosphorylation normalized to total ERK1/2 levels is shown in *bottom panel* ($n = 4$ for each concentration; *, $p < 0.05$; **, $p < 0.001$ compared with EGTA). Zn^{2+}-dependent ERK1/2 phosphorylation was already evident in the presence of 1 nM Zn^{2+} indicating that the keratinocytic ZnR has a high affinity to Zn^{2+}. *C*, cells were exposed to the indicated divalent ions, while monitoring the $Ca^{2+}{}_i$ response. None of the other heavy metals applied triggered a significant rise in $Ca^{2+}{}_i$, indicating that the ZnR response is Zn^{2+} selective.

triggered by Zn^{2+} were fitted using the Michaelis-Menten equation and exhibited a $K_{0.5}$ of 450 ± 50 pM (Fig. 3*A*). We then asked whether MAPK signaling could also be triggered by similar low Zn^{2+} concentrations. Cells were treated with the same Zn^{2+} concentrations for 30 min. The stimulatory effect of Zn^{2+} on ERK1/2 phosphorylation was already apparent upon addition of 1 nM and was 3-fold higher at 50 μM Zn^{2+} (Fig. 3*B*). Thus, the affinity of the keratinocytic-ZnR for Zn^{2+} is well within the physiological range of Zn^{2+} levels in the epidermis.

Other trace elements, such as copper, are also used *in vivo* to enhance wound healing (24). To determine the metal selectivity of the ZnR, $Ca^{2+}{}_i$ responses, following the administration of Ni^{2+}, Fe^{2+}, Cu^{2+}, and Pb^{2+} in the presence of CaEGTA allowing free-ion concentration of 5, 30, 10, and 6 μM, respectively, were compared with the response triggered by 10 μM Zn^{2+} (Fig. 3*C*). All the cations tested, but Zn^{2+}, failed to evoke a significant change in $[Ca^{2+}{}_i]$, indicating that the keratinocytic ZnR is selectively activated by this ion, in agreement with ZnR/GPR39 selectivity in other cell types (29, 41).

Zn^{2+} Released from Injured Cells Is Sufficient to Activate ZnR Signaling—The high affinity and the specificity of the keratinocytic-ZnR suggest that endogenous Zn^{2+} which may be released upon cellular injury can act as a paracrine agonist via the ZnR. To determine if Zn^{2+} is released upon injury in the keratinocyte model, a scratch was made across a monolayer of HaCaT cells while monitoring changes in the fluorescence of the non-permeant Zn^{2+}-selective fluorescent dye ZnAF-2 (2 μM, (42)). A sharp rise in the extracellular fluorescence was observed immediately following formation of the scratch (Fig. 4*A*). This signal was largely reduced in the presence of the extracellular Zn^{2+} chelator, CaEDTA (100 μM, Fig. 4*B*). Whereas CaEDTA can also chelate other ions the selectivity of Zn-AF-2 to Zn^{2+} (43) indicates that this ion is indeed released following injury. Thus, our findings suggest that Zn^{2+} is released or secreted from cells upon injury.

We next assessed if the release of endogenous Zn^{2+} following injury of epithelial cultures triggers ZnR activation. We therefore monitored the Ca^{2+} response following application of conditioned medium obtained from mechanically injured cells (see "Experimental Procedures"). Application of conditioned medium was followed by a massive $[Ca^{2+}{}_i]$ rise (Fig. 4, *C* and *D*) and ERK1/2 phosphorylation (Fig. 4*E*) in HaCaT cultures. Because conditioned medium may also contain ATP that can trigger a Ca^{2+} rise (44), the same experiment was conducted in the presence of the ATP scavenger apyrase (0.66 units/ml). In the presence of apyrase, application of conditioned medium triggered an attenuated $Ca^{2+}{}_i$ response (about 50 ± 3% of the control, Fig. 4, *C* and *D*), suggesting that while ATP is activating metabotropic $Ca^{2+}{}_i$ signaling, a significant

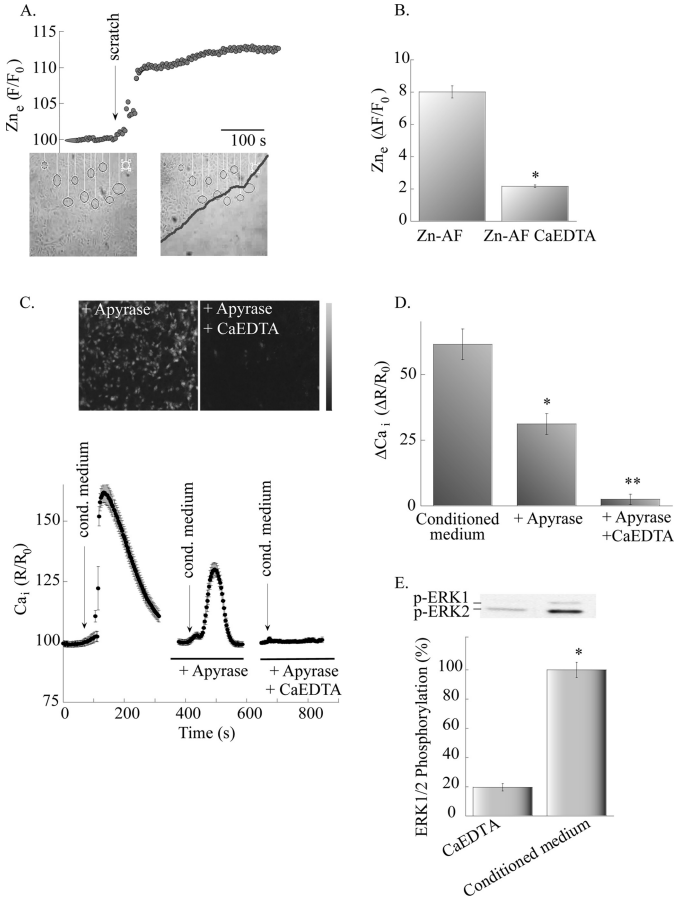

FIGURE 4. **Activation of ZnR signaling by endogenous Zn^{2+}, released during cellular injury.** *A*, changes in fluorescence of the cell impermeable Zn^{2+}-sensitive dye ZnAF-2 (2 μM), at the indicated time a scratch was performed with a pipette tip (see "Experimental Procedures"). The scratching was followed by robust rise in ZnAF-2 fluorescence. Images of cell culture before and after the scratch are shown below, and the circles show regions of interest used for averaging ZnAF-2 fluorescence. *B*, averaged ZnAF-2 response following injury in the absence or presence of the non-permeable Zn^{2+} chelator CaEDTA ($n = 4$, *, $p < 0.05$). *C*, supernatant obtained from injured cultures (conditioned medium, see "Experimental Procedures") was applied alone or in the presence of the ATP scavenger, apyrase (0.66 units/ml), or apyrase with the extracellular Zn^{2+} chelator, CaEDTA (100 μM) to intact HaCaT cells loaded with Fura-2-AM. Fluorescent images of the Ca^{2+} response in these cultures following application of the conditioned medium as indicated are shown above the graph. *D*, averaged intracellular Ca^{2+} signals from *C* are shown ($n = 5$; *, $p < 0.05$; **, $p < 0.01$). Application of both CaEDTA and apyrase eliminated the Ca^{2+} rise triggered by application of conditioned medium. *E*, conditioned medium was applied (10 min) to HaCaT cell cultures and ERK1/2 phosphorylation was monitored. ERK1/2 phosphorylation is significantly increased upon application of the conditioned medium ($n = 3$; *, $p < 0.05$).

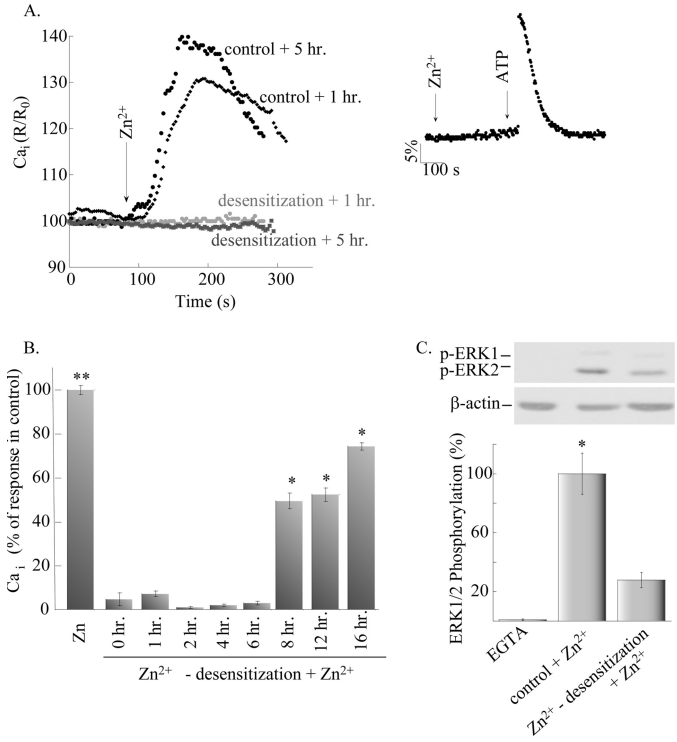

FIGURE 5. **ZnR desensitization by Zn^{2+}.** *A*, HaCaT cells were pretreated with 50 μM Zn^{2+} for 30 min and allowed to recover (see "Experimental Procedures"). At 1 or 5 h later Zn^{2+} (50 μM) or ATP (100 μM, see *inset*) was re-applied while monitoring the $Ca^{2+}{}_i$ response. Cells that were pretreated with Zn^{2+} did not respond to re-application of Zn^{2+}, while a robust Ca^{2+} signal was monitored following application of ATP. *B*, averaged Ca^{2+} response to Zn^{2+} re-applied at the indicated times following desensitization ($n = 3$, *, $p < 0.05$). *C*, phosphorylation of ERK1/2 in response to re-application of Zn^{2+} at 5 h following pre-application of Zn^{2+}, quantification normalized to β-actin expression is shown below ($n = 5$, *, $p < 0.05$). Similar to the effect on Ca^{2+} signaling, desensitization by Zn^{2+} attenuated the subsequent Zn^{2+}-dependent phosphorylation of ERK1/2.

residual response is maintained in its absence. To determine if Zn^{2+} mediates this residual response, we applied conditioned medium containing both the extracellular Zn^{2+} chelator CaEDTA (100 μM) and apyrase. This resulted in complete elimination of the Ca^{2+} response (about 10 ± 2% compared with control, Fig. 4*D*), suggesting that the residual activity was triggered by Zn^{2+}. Indeed, the residual response to application apyrase-treated conditioned medium ($\Delta R/R_0$ of 31 ± 4%) is similar to the response triggered by application of 50 μM Zn^{2+} ($\Delta R/R_0$ of 33 ± 2%). Although CaEDTA can also chelate other heavy metals, it is unlikely that these ions triggered the $Ca^{2+}{}_i$ rise because of the metal selectivity of ZnR response (Fig. 3*C*).

ZnR Undergoes Profound Desensitization by Zn^{2+}—An important hallmark of GPCRs is functional desensitization by their ligands, a process designed to regulate intracellular signaling and to prevent excessive stimulation (19, 45). Because unlike many other ligands Zn^{2+} is not degraded nor quickly removed from the extracellular region (46), effective desensitization of ZnR is of particular relevance. Pre-exposure of HaCaT cells to Zn^{2+} (50 μM, 30 min) resulted in complete inhibition of the Zn^{2+}-dependent $[Ca^{2+}{}_i]$ response, monitored immediately after or up to 6 h following the desensitization process (Fig. 5, *A* and *B*). The pretreatment with Zn^{2+} did not reduce the ATP-dependent response indicating that the IP_3 pathway remained intact (see *inset* in Fig. 5*A*). The Zn^{2+}-dependent Ca^{2+} response recovered to 75 ± 2% of the control only 16 h following pre-exposure to Zn^{2+} (Fig. 5*B*). Thus, following the pre-application of Zn^{2+} the ZnR undergoes complete and prolonged functional desensitization. We then used the desensitization paradigm (50 μM Zn^{2+} for 30 min) and monitored Zn^{2+}-dependent ERK1/2 phosphorylation at 5 h following desensitization. The pre-exposure to Zn^{2+} largely inhibited the Zn^{2+}-induced ERK1/2 phosphorylation (Fig. 5*C*). This suggests that a functional ZnR is required for mediating Zn^{2+}-dependent signaling in HaCaT keratinocytes.

The Keratinocytic-ZnR Regulates the Sodium-Proton Exchanger, NHE1—As the NHE exchangers are regulated by MAPK activation (47) and enhance cell proliferation and

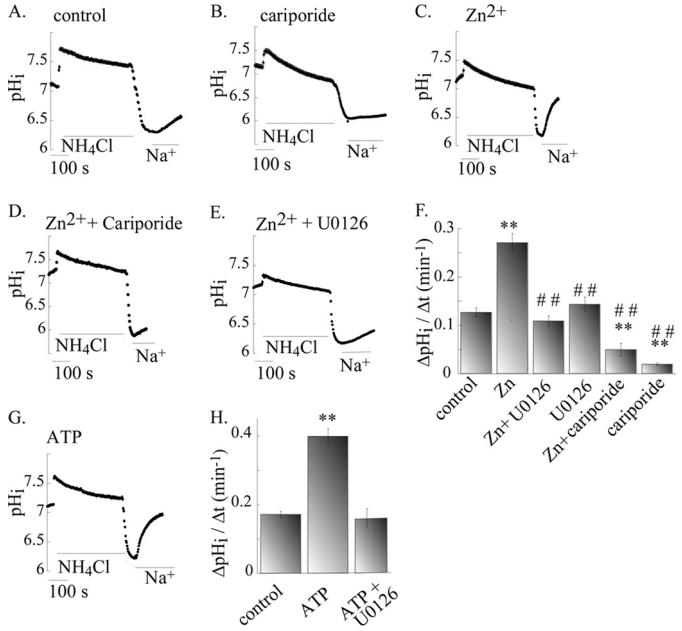

FIGURE 6. **Stimulation of Na^+/H^+ exchange by Zn^{2+} via the ZnR, in HaCaT keratinocytes.** HaCaT cells loaded with the pH-sensitive dye, BCECF-AM (1 μM), were acidified by NH_4Cl pre-pulse protocol (see "Experimental Procedures") upon addition of extracellular Na^+ (at time indicated by the *bar*) alkalinization is observed and the rate of pH_i recovery during this phase represents NHE activity. The pH_i recovery rate was measured in either: (*A*) control cells (*B*) in the presence of the NHE1 inhibitor, cariporide (1 μM), (*C*) following application of Zn^{2+} (50 μM, 15 min), (*D*) application of Zn^{2+} in the presence of cariporide (0.5 μM), or (*E*) application of Zn^{2+} in the presence of the ERK1/2 inhibitor, U0126 (1 μM). *F*, averaged rates of pH_i recovery, associated with NHE1 activity, of *A–E*. *G*, NH_4Cl prepulse protocol was performed following application of 100 μM ATP (10 min). *H*, averaged rate of pH_i recovery following application of ATP in the presence or absence of U0126. ($n = 7$ for each treatment, **, $p < 0.01$ compared with control, ##, $p < 0.01$ compared with Zn^{2+}).

migration (48) we asked if ZnR stimulates NHE activity. Basal activity of the Na^+/H^+ exchanger in HaCaT cells, loaded with the pH-sensitive dye BCECF, was determined by monitoring the rate of pH_i recovery following acid load induced by NH_4Cl prepulse. Addition of Na^+ following the acidification, to control cells was followed by rapid pH_i recovery to resting level (Fig. 6*A*, 0.17 ± 0.01 pH units/min). This recovery phase was completely inhibited following application of the NHE1 blocker, cariporide (0.5 μM, Fig. 6*B*, 0.02 ± 0.01 pH units/min). The pH_i recovery rate was then determined following pre-application of Zn^{2+} (50 μM, 15 min), a paradigm which does not lead to Zn^{2+} permeation but activates the ZnR (see Fig. 1*B*). ZnR activation was followed by enhanced pH_i recovery rate (Fig. 6*C*, 0.27 ± 0.02 pH units/min), that was also inhibited by cariporide (Fig. 6*D*, 0.05 ± 0.02 pH units/min) indicating that ZnR is up-regulating the activity of NHE1. To determine if NHE1 activation is mediated by the MAPK pathway (47, 49) the ERK1/2 inhibitor U0126 (1 μM) was added 10 min before application of Zn^{2+}. The addition of this ERK1/2 inhibitor resulted in inhibition of the stimulatory effect of Zn^{2+} on NHE1 activity (Fig. 6*E*).

We then studied the role of the purinergic agonist ATP in regulating NHE1 activity. Application of ATP (100 μM, 10 min) was also followed by enhanced NHE1 activity (Fig. 6*G*, 0.4 ± 0.02 pH units/min) that was reversed by the inhibition of the MAPK pathway using U0126 (1 μM, Fig. 6*H*, 0.16 ± 0.03 pH units/min). Thus, both the ZnR- and ATP-mediated signaling upregulate the rate of NHE1-dependent recovery from acid load via the MAPK pathway.

ZnR Activity Promotes in Vitro Scratch Closure—Our results (Fig. 4) indicate that Zn^{2+} is released from injured cells, such transient release of extracellular Zn^{2+} may activate the ZnR. To study whether ZnR activity underlies Zn^{2+}-dependent enhanced wound healing, an *in vitro* scratch assay was performed on confluent HaCaT cultures and the rate of closure was determined by comparing the cell denuded areas immediately and 24 h following formation of the scratch. To fully activate the ZnR response, as determined by the dose response analyses (Fig. 4), cultures were treated with 50 μM Zn^{2+} for 15 min. Application of Zn^{2+}, using the ZnR activation paradigm, was followed by an increase in the closure rate, which was about 20% higher than that of CaEGTA-treated cells (Fig. 7*A*), that was used to chelate residual Zn^{2+} in the solutions. Application of ATP, also enhanced scratch closure (by 30% compared with control). Because extracellular Ca^{2+} may alter the balance between differentiation and proliferation of keratinocytes (31, 50) we assessed the scratch closure rate in the presence of extracellular Ca^{2+} (1.8 mM). Similar Zn^{2+}-dependent closure rates were measured in the presence or absence of $Ca^{2+}{}_e$, (Fig. 7*A*) indicating that at least during the first 24 h, extracellular Ca^{2+} does not affect scratch closure rates. To determine if keratinocytic-ZnR signaling is triggering the enhanced scratch closure, the PLC and NHE1 inhibitors were applied prior to addition of Zn^{2+}. The PLC inhibitor (U73122, 1 μM), completely blocked the Zn^{2+}-dependent enhancement of scratch closure (Fig. 7*A*). Application of the NHE1 inhibitor, cariporide (0.5 μM) also reduced Zn^{2+}-mediated enhancement of the scratch closure rate, to a level similar to the control (Fig. 7*A*). Thus, our results (Fig. 7*B*) indicate that Zn^{2+}, via ZnR signaling, activates the IP_3 pathway and up-regulates NHE1 and thereby enhances scratch closure.

GPR39 Is Essential for Mediating the Effects of Zn^{2+} in HaCaT Keratinocytes—GPR39 silencing resulted in elimination of the Zn^{2+}-dependent Ca^{2+} response (Fig. 1) suggesting that this receptor mediates ZnR signaling in keratinocytes. We therefore asked if ZnR/GPR39 is mediating the effects of Zn^{2+} on NHE1 activity and scratch closure. HaCaT cells were transfected with the siGPR39 or siControl constructs and the NH_4Cl prepulse paradigm was applied in BCECF-loaded cells. Following the transfection basal activity of NHE1 in the HaCaT cells was completely attenuated to a similar level with both siRNA constructs (Fig. 8*A*). Cells were then treated with Zn^{2+} (as in Fig. 6) and pH_i recovery from acid load was monitored. While in the control cells Zn^{2+} strongly enhanced pH_i recovery (0.04 ±

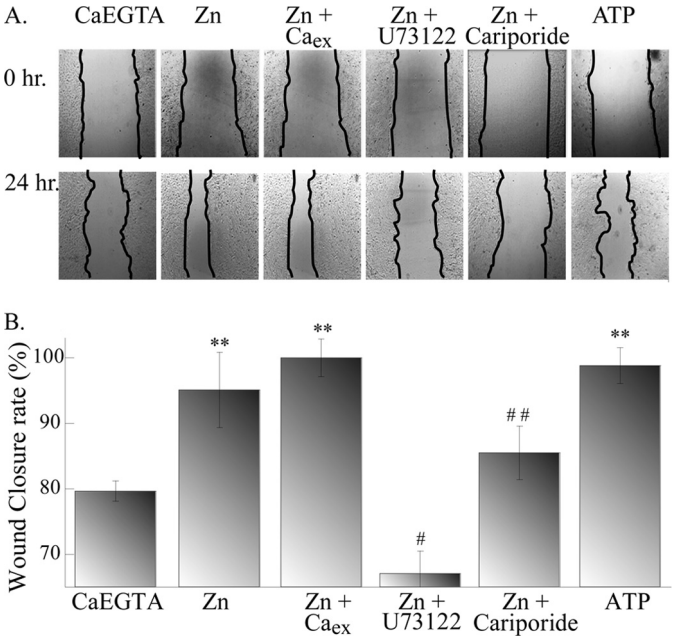

FIGURE 7. **Extracellular Zn^{2+} via the ZnR promotes *in vitro* scratch closure.** *A*, scratch closure assay was performed on confluent monolayers of HaCaT cells and the scratch closure rate was determined by comparing the denuded area immediately and 24 h later (see "Experimental Procedures"). This assay was repeated in cells treated with CaEGTA or Zn^{2+} (50 μM) in the presence of: low, 0.05 mM, or high, 1.8 mM, Ca^{2+} concentration in the extracellular Ringer's solution; the PLC inhibitor, U73122 (1 μM); the NHE1 inhibitor, cariporide (0.5 μM), or ATP (100 μM). *B*, averaged rates of scratch closure during 24 h following injury, normalized to the rate monitored in Zn^{2+}-treated cultures, is shown. ($n = 8$ for Zn^{2+} and 4 for all other treatments, **, $p < 0.01$ compared with CaEGTA; #, $p < 0.01$ compared with Zn^{2+}).

0.01 pH units/min *versus* 0.32 ± 0.04 pH units/min following Zn^{2+} treatment), in siGRP39-treated cells application of Zn^{2+} failed to upregulate the activity of NHE1 (0.04 ± 0.02 pH units/min *versus* 0.06 ± 0.02 pH units/min following Zn^{2+} treatment). These results are in agreement with the pharmacological demonstration of the major role of ZnR in Zn^{2+}-dependent up-regulation of NHE1 (Fig. 6) and further indicate that Zn^{2+}, via ZnR/GPR39 up-regulates NHE1 activity.

Finally, we determined the effect of Zn^{2+} on scratch closure rate following GPR39 silencing. HaCaT cells were transfected with the siGPR39 or siControl constructs and the *in vitro* scratch assay was performed on confluent cultures as described in Fig. 7. Scratch closure rates were significantly enhanced following application of Zn^{2+} treatment to cultures transfected with siControl (by about 70% compared with control cells treated with CaEGTA, Fig. 8*B*). In contrast, Zn^{2+} application did not significantly enhance scratch closure rate in the siGPR39 transfected cultures. Following transfection with either the siControl or siGPR39 constructs, the rate of recovery in control cells, not treated with Zn^{2+}, was largely attenuated compared with the naïve cultures, consistent with the low basal activity of NHE1 (Fig. 8*A*). Our results therefore suggest that ZnR/GPR39 is essential for mediating the effects of Zn^{2+} on scratch closure.

DISCUSSION

We demonstrate here that extracellular Zn^{2+}, at the concentrations found in the epidermis, acts as a signaling molecule, triggering Ca^{2+} release from thapsigargin-sensitive stores. The response to Zn^{2+} is mediated through a $G\alpha_q$-coupled receptor, GPR39, which activates PLC and the IP_3 pathway, similar to the ZnR-response in colonocytes and neurons (20, 30, 41). Analysis of the Zn^{2+}-dependent Ca^{2+} response, indicated that the keratinocytic-ZnR has high affinity and selectivity for Zn^{2+}. Importantly, we show that following epithelial injury endogenous Zn^{2+} is released and may act as a paracrine agonist triggering ZnR signaling leading to activation of intracellular signaling pathways associated with wound healing.

Our results demonstrate ZnR-dependent activation of the MAPK pathway in keratinocytic cells. Activation of this pathway triggers the majority of the early genes induced in a scratch model and is essential for wound healing (51–53). Interestingly, a long-lasting, marked increase in ERK1/2 phosphorylation was apparent following a brief exposure to Zn^{2+}. Sustained ERK1/2 phosphorylation may be, upon certain conditions, referred to as a precursor of cell death (54, 55). However, in HaCaT cells upon exposure to UVA, sustained ERK1/2 phosphorylation via activation of metalloproteinases may serve as a survival signal suppressing apoptosis (56). The enhanced migration of HaCaT cells, monitored using the *in vitro* scratch assay, following keratinocytic-ZnR activation suggests that ZnR-dependent ERK1/2 phosphorylation leads to cell growth. ZnR-dependent activation of ERK1/2 was partially mediated by PKC and the PI3K pathway, suggesting that cross-talk between these pathways may mediate the effects of Zn^{2+} on epithelial repair. These signaling pathways are also involved in enhanced epithelial breast cancer cell proliferation following activation of the bradykinin receptor (57, 58) and in melanoma cell growth (59, 60).

The NHE1, once thought to merely regulate pH_i, was also shown to enhance cell proliferation and migration (48, 61–64), for example, by promoting the G2/M transition (65) and cell volume (66) or via its functional interaction with cytoskeletal elements (67). Our results show that ZnR- or ATP-dependent metabotropic Ca^{2+} signaling induce up-regulation of NHE1 activity and thereby enhance the recovery of intracellular pH from acid load. Moreover, inhibition of the Zn^{2+}-induced NHE1 up-regulation by cariporide resulted in attenuated migration and scratch closure. Phosphorylation of NHE1 by ERK1/2 plays a pivotal role in the activation of the exchanger by growth factors, hormone stimulation (47, 68) and also by Zn^{2+} (20). Inhibition of the MAPK pathway resulted in attenuation of the stimulatory effect of Zn^{2+} via the ZnR on NHE1 also in keratinocytes.

In agreement with other works (69, 70), we show that the Na^+/H^+ exchanger NHE1 is activated in skin cells following

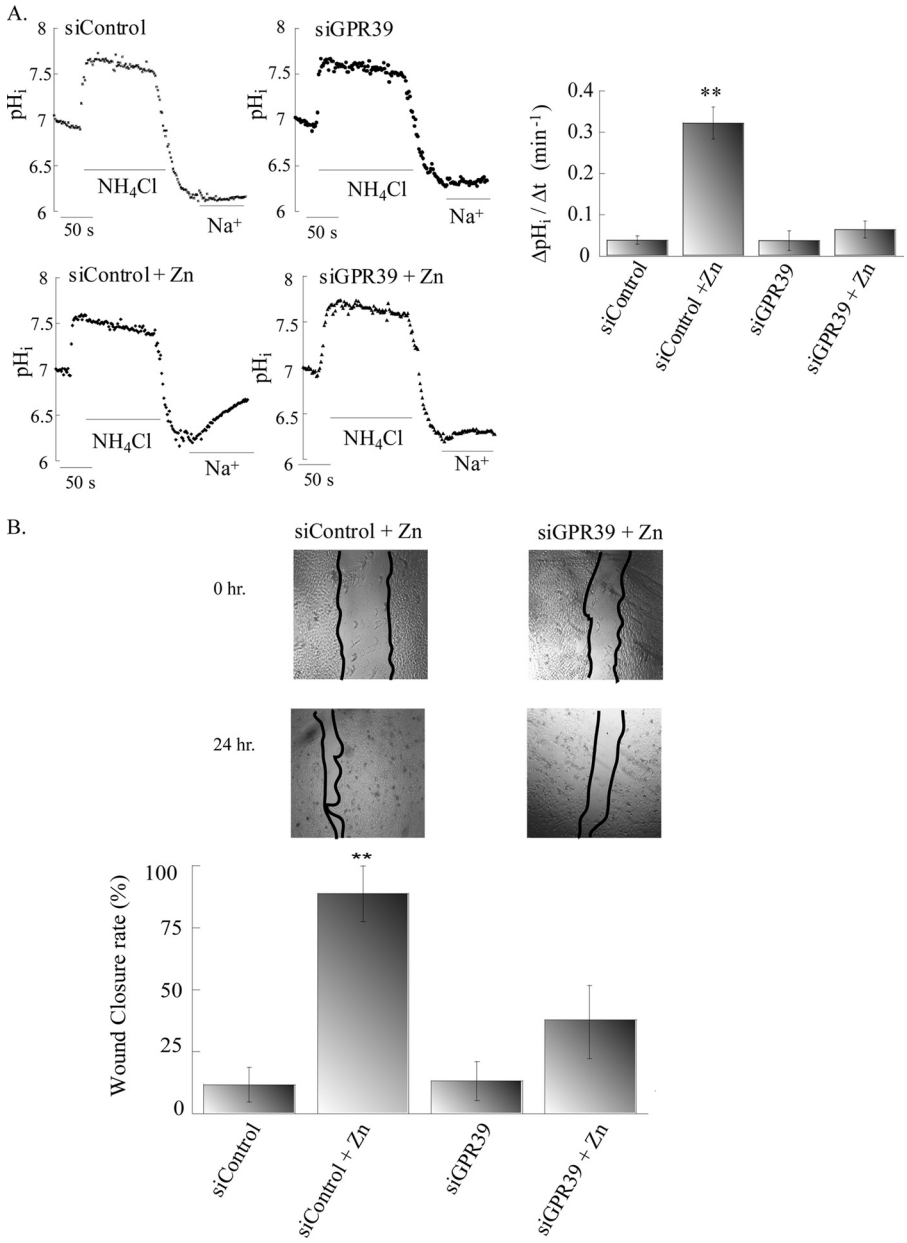

FIGURE 8. **ZnR/GPR39 mediates the Zn^{2+}-dependent up-regulation of NHE1 activity and scratch closure.** *A*, HaCaT cells were transfected with siGPR39 or siControl constructs and the NH_4Cl prepulse paradigm was performed as in Fig. 6, recovery rate was measured in cells treated with Zn^{2+} or in control cells. Averaged rates of pH_i recovery, representing NHE1 activity are shown to the *right* ($n = 5$ for each treatment, **, $p < 0.01$ compared with siControl cells without Zn^{2+}). *B*, *in vitro* scratch assay was performed as in Fig. 7 in cultures transfected with siControl or siGPR39. Cultures were treated with Zn^{2+} or CaEGTA, and injured areas were monitored immediately and 24 h following scratching. Averaged rates of scratch closure recovery normalized to the recovery rate of control cells treated with Zn^{2+} are shown below ($n = 7$ for each treatment, **, $p < 0.01$ compared with siControl cells without Zn^{2+}).

cellular acidification. The robust up-regulation of NHE1 activity by Zn^{2+} in keratinocytes suggests an important physiological role for this ion in pH regulation. The acidic surface, pH of 6.0, found in the upper layer of the skin is essential for formation of the permeability barrier (70, 71). Zn^{2+} deficiency enhances the inflammatory response (72) and a prominent role for this ion in wound healing was suggested to be an antimicrobial effect. A beneficial effect of Zn^{2+} may therefore require strengthening of the proton gradient and the epithelial permeability barrier that is regulated by NHE1 activity or expression (70, 73, 74). Our results suggest that following skin injury, the release of Zn^{2+} or its topical application may enhance formation of the barrier, and thereby, exert an anti-inflammatory effect.

Another molecule, apart from Zn^{2+}, that is released from epithelial cultures is ATP (44), which can trigger a metabotropic Ca^{2+} response mediated via the purinergic receptors (52, 75, 76). Our results show that both Zn^{2+} and ATP are released following injury and can independently induce Ca^{2+} signaling and activation of the MAPK pathway leading to up-regulation of NHE1 activity. Interestingly, activation of the ZnR pathway in salivary gland cells induces the release of ATP (36), this may provide a mechanism for the release of this molecule that will further enhance the metabotropic Ca^{2+} signaling. The role of ATP in wound healing however is still controversial. Activation of the purinergic receptor and subsequent Ca^{2+} release were shown to induce EGFR transactivation and lead to enhanced wound closure (77, 78). Another study however, claimed that ATP inhibited serum-induced ERK1/2 and PI3K activation and attenuated cell migration (79). Our results, as well as others (52), show that ATP activates the MAPK pathway leading for example to enhance airway epithelial cell migration (75). Importantly, our results indicate that the downstream signaling triggered by ATP induced up-regulation of NHE1 activity and enhanced *in vitro* scratch closure. Our results further support a role for ATP in promoting wound healing, as chelation of Zn^{2+} and scavenging of ATP eliminated the Ca^{2+} response triggered by conditioned medium. This indicates that following injury, Zn^{2+} and ATP are two major mediators of the Ca^{2+} signaling that is essential for wound healing.

The physiological significance of the keratinocytic-ZnR activity mediated by GPR39 is demonstrated by the involvement of its downstream signaling pathway in epithelial proliferation and migration. Using the highly selective probe ZnAF-2, we further show release of Zn^{2+} to the extracellular medium following keratinocyte injury, indicating that transient changes in the concentration of extracellular Zn^{2+} occur dur-

ing this process. The concentration of Zn^{2+} released following a scratch or in the conditioned medium was sufficient to activate the high affinity ZnR-dependent Ca^{2+} signaling in keratinocytic cultures. Although the effects of Zn^{2+} on wound healing were previously described, our results provide the first mechanistic link between Zn^{2+} and this process via the specific ZnR/GPR39. Generation of agonists that will activate, but will not desensitize, the ZnR may in the future provide an effective approach to accelerate wound healing.

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REFERENCES

1. Jacinto, A., Martinez-Arias, A., and Martin, P. (2001) *Nat. Cell Biol.* **3,** E117–123
2. Lansdown, A. B., Mirastschijski, U., Stubbs, N., Scanlon, E., and Agren, M. S. (2007) *Wound Repair Regen.* **15,** 2–16
3. Barceloux, D. G. (1999) *J. Toxicol. Clin. Toxicol.* **37,** 279–292
4. Lansdown, A. B. (1996) *Lancet* **347,** 706–707
5. Schwartz, J. R., Marsh, R. G., and Draelos, Z. D. (2005) *Dermatol. Surg.* **31,** 837–847; discussion 847
6. Jensen, S. L., McCuaig, C., Zembowicz, A., and Hurt, M. A. (2008) *J. Cutan. Pathol.* **35,** Suppl. 1, 1–13
7. Takahashi, H., Nakazawa, M., Takahashi, K., Aihara, M., Minami, M., Hirasawa, T., and Ikezawa, Z. (2008) *J. Dermatol. Sci.* **50,** 31–39
8. Andrews, G. K. (2008) *Biochem. Soc. Trans.* **36,** 1242–1246
9. Kambe, T., and Andrews, G. K. (2009) *Mol. Cell Biol.* **29,** 129–139
10. Kambe, T., Weaver, B. P., and Andrews, G. K. (2008) *Genesis* **46,** 214–228
11. Besecker, B., Bao, S., Bohacova, B., Papp, A., Sadee, W., and Knoell, D. L. (2008) *Am. J. Physiol. Lung Cell Mol. Physiol.* **294,** L1127–L1136
12. Hogstrand, C., Kille, P., Nicholson, R. I., and Taylor, K. M. (2009) *Trends Mol. Med.* **15,** 101–111
13. Taylor, K. M. (2008) *Biochem. Soc. Trans.* **36,** 1247–1251
14. Zhao, L., Chen, W., Taylor, K. M., Cai, B., and Li, X. (2007) *Biochem. Biophys. Res. Commun.* **363,** 82–88
15. Hershfinkel, M. (2006) in *Molecular Biology of Metal Homeostasis and Detoxification* (Tamas, M., and Martinoia, E., eds) springer-verlag Berlin
16. Hershfinkel, M., Silverman, W. F., and Sekler, I. (2007) *Mol. Med.* **13,** 331–336
17. Murakami, M., and Hirano, T. (2008) *Cancer Sci.* **99,** 1515–1522
18. Kaisman-Elbaz, T., Sekler, I., Fishman, D., Karol, N., Forberg, M., Kahn, N., Hershfinkel, M., and Silverman, W. F. (2009) *J. Cell Physiol.* **220,** 222–229
19. Dubi, N., Gheber, L., Fishman, D., Sekler, I., and Hershfinkel, M. (2008) *Carcinogenesis* **29,** 1692–1700
20. Azriel-Tamir, H., Sharir, H., Schwartz, B., and Hershfinkel, M. (2004) *J. Biol. Chem.* **279,** 51804–51816
21. Rothstein, J. D., Martin, L., Levey, A. I., Dykes-Hoberg, M., Jin, L., Wu, D., Nash, N., and Kuncl, R. W. (1994) *Neuron* **13,** 713–725
22. Ravanti, L., and Kahari, V. M. (2000) *Int. J. Mol. Med.* **6,** 391–407
23. Pirila, E., Korpi, J. T., Korkiamaki, T., Jahkola, T., Gutierrez-Fernandez, A., Lopez-Otin, C., Saarialho-Kere, U., Salo, T., and Sorsa, T. (2007) *Wound Repair Regen.* **15,** 47–57
24. Tenaud, I., Leroy, S., Chebassier, N., and Dreno, B. (2000) *Exp. Dermatol.* **9,** 407–416
25. Tenaud, I., Saiagh, I., and Dreno, B. (2009) *J. Dermatolog. Treat.* **20,** 90–93
26. Huang, J. S., Mukherjee, J. J., Chung, T., Crilly, K. S., and Kiss, Z. (1999) *Eur. J. Biochem.* **266,** 943–951
27. Kiss, Z., Crilly, K. S., and Tomono, M. (1997) *FEBS Lett.* **415,** 71–74
28. MacDonald, R. S., Wollard-Biddle, L. C., Browning, J. D., Thornton, W. H., Jr., and O'Dell, B. L. (1998) *J. Nutr.* **128,** 1600–1605
29. Hershfinkel, M., Moran, A., Grossman, N., and Sekler, I. (2001) *Proc. Natl. Acad. Sci. U.S.A.* **98,** 11749–11754
30. Besser, L., Chorin, E., Sekler, I., Silverman, W. F., Atkin, S., Russell, J. T., and Hershfinkel, M. (2009) *J. Neurosci.* **29,** 2890–2901
31. Boukamp, P., Petrussevska, R. T., Breitkreutz, D., Hornung, J., Markham, A., and Fusenig, N. E. (1988) *J. Cell Biol.* **106,** 761–771
32. Kay, A. R. (2004) *BMC Physiol.* **4,** 4, http://www.biomedcentral.com/1472-6793/4/4
33. Hirano, T., Kikuchi, K., Urano, Y., Higuchi, T., and Nagano, T. (2000) *Angew. Chem. Int. Ed. Engl.* **39,** 1052–1054
34. O'Toole, E. A., Marinkovich, M. P., Peavey, C. L., Amieva, M. R., Furthmayr, H., Mustoe, T. A., and Woodley, D. T. (1997) *J. Clin. Invest.* **100,** 2881–2891
35. Taniguchi, M., Suzumura, K., Nagai, K., Kawasaki, T., Takasaki, J., Sekiguchi, M., Moritani, Y., Saito, T., Hayashi, K., Fujita, S., Tsukamoto, S., and Suzuki, K. (2004) *Bioorg. Med. Chem.* **12,** 3125–3133
36. Sharir, H., and Hershfinkel, M. (2005) *Biochem. Biophys. Res. Commun.* **332,** 845–852
37. Thuraisingam, T., Xu, Y. Z., Eadie, K., Heravi, M., Guiot, M. C., Greemberg, R., Gaestel, M., and Radzioch, D. (2010) *J. Invest. Dermatol.* **130,** 278–286
38. Chiu, T., Santiskulvong, C., and Rozengurt, E. (2005) *Am. J. Physiol. Gastrointest. Liver Physiol.* **288,** 182–194
39. Rozengurt, E. (2007) *J. Cell Physiol.* **213,** 589–602
40. Breitkreutz, D., Braiman-Wiksman, L., Daum, N., Denning, M. F., and Tennenbaum, T. (2007) *J. Cancer Res. Clin. Oncol.* **133,** 793–808
41. Yasuda, S., Miyazaki, T., Munechika, K., Yamashita, M., Ikeda, Y., and Kamizono, A. (2007) *J. Recept. Signal Transduct. Res.* **27,** 235–246
42. Crivat, G., Kikuchi, K., Nagano, T., Priel, T., Hershfinkel, M., Sekler, I., Rosenzweig, N., and Rosenzweig, Z. (2006) *Anal. Chem.* **78,** 5799–5804
43. Hirano, T., Kikuchi, K., Urano, Y., and Nagano, T. (2002) *J. Am. Chem. Soc.* **124,** 6555–6562
44. Sammak, P. J., Hinman, L. E., Tran, P. O., Sjaastad, M. D., and Machen, T. E. (1997) *J. Cell Sci.* **110,** 465–475
45. Kohout, T. A., and Lefkowitz, R. J. (2003) *Mol. Pharmacol.* **63,** 9–18
46. Taylor, K. M., Morgan, H. E., Johnson, A., and Nicholson, R. I. (2004) *Biochem. J.* **377,** 131–139
47. Snabaitis, A. K., Yokoyama, H., and Avkiran, M. (2000) *Circ. Res.* **86,** 214–220
48. Fliegel, L. (2005) *Int. J. Biochem. Cell Biol.* **37,** 33–37
49. Haworth, R. S., McCann, C., Snabaitis, A. K., Roberts, N. A., and Avkiran, M. (2003) *J. Biol. Chem.* **278,** 31676–31684
50. Micallef, L., Belaubre, F., Pinon, A., Jayat-Vignoles, C., Delage, C., Charveron, M., and Simon, A. (2009) *Exp. Dermatol.* **18,** 143–151
51. Fitsialos, G., Chassot, A. A., Turchi, L., Dayem, M. A., LeBrigand, K., Moreilhon, C., Meneguzzi, G., Busca, R., Mari, B., Barbry, P., and Ponzio, G. (2007) *J. Biol. Chem.* **282,** 15090–15102
52. Yang, L., Cranson, D., and Trinkaus-Randall, V. (2004) *J. Cell Biochem.* **91,** 938–950
53. Gazel, A., Nijhawan, R. I., Walsh, R., and Blumenberg, M. (2008) *J. Cell Physiol.* **215,** 292–308
54. Ho, Y., Samarasinghe, R., Knoch, M. E., Lewis, M., Aizenman, E., and DeFranco, D. B. (2008) *Mol. Pharmacol.* **74,** 1141–1151
55. Sarfaraz, S., Afaq, F., Adhami, V. M., Malik, A., and Mukhtar, H. (2006) *J. Biol. Chem.* **281,** 39480–39491
56. Han, W., and He, Y. Y. (2009) *Photochem. Photobiol.* **85,** 997–1003
57. Campbell, M., and Trimble, E. R. (2005) *Circ. Res.* **96,** 197–206
58. Greco, S., Storelli, C., and Marsigliante, S. (2006) *J. Endocrinol.* **188,** 79–89
59. Sinnberg, T., Lasithiotakis, K., Niessner, H., Schittek, B., Flaherty, K. T., Kulms, D., Maczey, E., Campos, M., Gogel, J., Garbe, C., and Meier, F. (2009) *J. Invest. Dermatol.* **129,** 1500–1515
60. Lasithiotakis, K. G., Sinnberg, T. W., Schittek, B., Flaherty, K. T., Kulms, D., Maczey, E., Garbe, C., and Meier, F. E. (2008) *J. Invest. Dermatol.* **128,** 2013–2023
61. Orlowski, J., and Grinstein, S. (2004) *Pflugers Arch.* **447,** 549–565
62. Putney, L. K., Denker, S. P., and Barber, D. L. (2002) *Annu. Rev. Pharmacol. Toxicol.* **42,** 527–552
63. Baumgartner, M., Patel, H., and Barber, D. L. (2004) *Am. J. Physiol. Cell Physiol.* **287,** C844–C850

64. Schneider, L., Stock, C. M., Dieterich, P., Jensen, B. H., Pedersen, L. B., Satir, P., Schwab, A., Christensen, S. T., and Pedersen, S. F. (2009) *J. Cell Biol.* **185,** 163–176
65. Putney, L. K., and Barber, D. L. (2003) *J. Biol. Chem.* **278,** 44645–44649
66. Lang, F., Foller, M., Lang, K., Lang, P., Ritter, M., Vereninov, A., Szabo, I., S, M. H., and Gulbins, E. (2007) *Methods Enzymol.* **428,** 209–225
67. Denker, S. P., and Barber, D. L. (2002) *J. Cell Biol.* **159,** 1087–1096
68. Malo, M. E., Li, L., and Fliegel, L. (2007) *J. Biol. Chem.* **282,** 6292–6299
69. Sarangarajan, R., Shumaker, H., Soleimani, M., Le Poole, C., and Boissy, R. E. (2001) *Biochim. Biophys. Acta* **1511,** 181–192
70. Hachem, J. P., Behne, M., Aronchik, I., Demerjian, M., Feingold, K. R., Elias, P. M., and Mauro, T. M. (2005) *J. Invest. Dermatol.* **125,** 790–797
71. Darlenski, R., Sassning, S., Tsankov, N., and Fluhr, J. W. (2009) *Eur. J. Pharm. Biopharm.* **72,** 295–303
72. Knoell, D. L., Julian, M. W., Bao, S., Besecker, B., Macre, J. E., Leikauf, G. D., DiSilvestro, R. A., and Crouser, E. D. (2009) *Crit. Care Med.* **37,** 1380–1388
73. Stock, C., Cardone, R. A., Busco, G., Krahling, H., Schwab, A., and Reshkin, S. J. (2008) *Eur. J. Cell Biol.* **87,** 591–599
74. Stuwe, L., Muller, M., Fabian, A., Waning, J., Mally, S., Noel, J., Schwab, A., and Stock, C. (2007) *J. Physiol.* **585,** 351–360
75. Wesley, U. V., Bove, P. F., Hristova, M., McCarthy, S., and van der Vliet, A. (2007) *J. Biol. Chem.* **282,** 3213–3220
76. Gomes, P., Srinivas, S. P., Vereecke, J., and Himpens, B. (2005) *Invest. Ophthalmol. Vis. Sci.* **46,** 104–113
77. Yin, J., Xu, K., Zhang, J., Kumar, A., and Yu, F. S. (2007) *J. Cell Sci.* **120,** 815–825
78. Boucher, I., Yang, L., Mayo, C., Klepeis, V., and Trinkaus-Randall, V. (2007) *Exp. Eye. Res.* **85,** 130–141
79. Taboubi, S., Milanini, J., Delamarre, E., Parat, F., Garrouste, F., Pommier, G., Takasaki, J., Hubaud, J. C., Kovacic, H., and Lehmann, M. (2007) *Faseb J.* **21,** 4047–4058