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Calcium and Vitamin D Increase mRNA Levels for the Growth Control hIK1 Channel in Human Epidermal Keratinocytes but Functional Channels are Not Observed.

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Calcium and Vitamin D increase mRNA levels for the growth control hIK1 channel in human epidermal keratinocytes but functional channels are not observed

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Abstract

Background: Intermediate-conductance, calcium-activated potassium channels (IKs) modulate proliferation and differentiation in mesodermal cells by enhancing calcium influx, and they contribute to the physiology of fluid movement in certain epithelia. Previous reports suggest that IK channels stimulate proliferative growth in a keratinocyte cell line; however, because these channels indirectly promote calcium influx, a critically unique component of the keratinocyte differentiation program, an alternative hypothesis is that they would be anti-proliferative and pro-differentiating. This study addresses these hypotheses.

Methods: Real-time PCR, patch clamp electrophysiology, and proliferation assays were used to determine if human IK1 (hIK1) expression and function are correlated with either proliferation or differentiation in cultured human skin epidermal keratinocytes, and skin biopsies grown in explant culture.

Results: hIK1 mRNA expression in human keratinocytes and skin was increased in response to anti-proliferative/pro-differentiating stimuli (elevated calcium and Vitamin D). Correspondingly, the hIK1 agonist 1-EBIO inhibited keratinocyte proliferation suggesting that the channel could be anti-proliferative and pro-differentiating. However, this proliferative inhibition by 1-EBIO was not reversed by a panel of hIK1 blockers, calling into question the mechanism of 1-EBIO action. Subsequent patch clamp electrophysiological analysis failed to detect hIK1 channel currents in keratinocytes, even those expressing substantial hIK1 mRNA in response to calcium and Vitamin D induced differentiation. Identical electrophysiological recording conditions were then used to observe robust IK1 currents in fibroblasts which express IK1 mRNA levels comparable to those of keratinocytes. Thus, the absence of observable hIK1 currents in keratinocytes was not a function of the electrophysiological techniques.

Conclusion: Human keratinocyte differentiation is stimulated by calcium mobilization and influx, and differentiation stimuli coordinately upregulate mRNA levels of the calcium-activated hIK1 channel. This upregulation is paradoxical in that functional hIK1 channels are not observed in cultured keratinocytes. It appears, therefore, that hIK1 does not contribute to the functional electrophysiology of primary human keratinocytes, nor intact human skin. Further, the results indicate caution is required when interpreting experiments utilizing pharmacological hIK1 modulators in human keratinocytes.

Background

Intermediate conductance, calcium-activated potassium channels (IKs) stimulate proliferative growth in T-cells and fibroblasts [1,2], via their ability to maintain a negative membrane potential, thus enhancing the electromotive driving force for calcium entry [3], a mitogenic requirement in these cells. IKs have also been suggested to modulate secretion and cell volume in epithelial cells from the gut and airways [4-7], but it is not known if these channels also exert a growth control function in either isolated epithelial cells or intact epithelia. Unlike in T-cells and fibroblasts, stimulated calcium entry in human epidermal keratinocytes is associated with cessation (rather than enhancement) of proliferation, as well as promotion of the differentiation program [8]. Therefore, although across different cell types IKs may be expected to produce equivalent electrophysiological effects including the enhancement of calcium entry, in epithelial cells typified by keratinocytes this action may serve to limit proliferative activity and promote differentiation, the converse of IK function in T-cells and fibroblasts.

In support of a growth regulatory role for IK channels in human epidermal keratinocytes, the immortalized, non-tumorigenic human keratinocyte cell line HaCaT has been shown to express human IK1 (hIK1) [9]. Interestingly, prolonged exposure of HaCaT to the IK activator, 1-EBIO, was reported to downregulate IK expression, an effect correlated with proliferative inhibition [10]. These results, although complicated by agonist induced channel expression changes, suggest a pro-proliferative role for IK in keratinocytes. Further, although potassium conductances have been reported in studies of primary, non-immortalized keratinocytes, it is unclear if these conductances are due to IK [11,12]. Thus, the exact physiological role of IK channels in primary human keratinocytes and in intact skin remains undetermined.

Methods

Cell Culture

Adult human epidermal keratinocytes (Cascade Biologics, Inc., Portland, OR or Cambrex Bio Science, Walkersville, MD) were cultured per the supplier's instructions in EpiLife Medium with Human Keratinocyte Growth Supplement (bovine pituitary extract, 0.2% v/v; bovine insulin, 5 μg/ml; hydrocortisone, 0.18 μg/ml; bovine transferrin, 5 μg/ml and human epidermal growth factor, 0.2 ng/ml)(Cascade Biologics) in a 5% CO_2, 37°C incubator. Basal calcium concentration was 0.06 mM. Stock cell cultures were passaged so as to maintain density at less than 70%, and cultures were not used for more than six weeks after direct receipt from the supplier (typically within 5–10 passages). For gene expression and physiological studies keratinocytes were plated at densities of approximately 30–40% confluence at the start of the experiment. Medium was then refreshed with or without various agents for chronic exposure experiments. Medium and additions were replenished every other day thereafter for the duration of the experiment.

Cell proliferation and toxicity assays

Keratinocytes were seeded in a 96-well plate at 10×10^3/well in standard growth medium. After 2–4 hours (to allow attachment to substrate) test agents and 1 μCi ^{3}H-thymidine (Perkin Elmer Life Sciences/NEN Life Sciences, Boston, MA) were added. Approximately 24 hours later cells were harvested for scintillation counting of incorporated thymidine, expressed as CPM (each experimental group represents mean ± SEM of triplicate samples). The CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit (Promega, Madison, WI) was used to measure potential cytotoxic actions of test compounds.

Electrophysiology

Standard patch clamp techniques were used to attempt to record hIK1 currents in keratinocytes, and these procedures as applied to IK1 recordings from fibroblast cells have been extensively described ([13-15], and references therein). To facilitate patch seal formation and recording cells were rounded by brief (1–2 min) exposure to trypsin/EDTA, after which they were switched to the bath solution (in mM): 138 NaCl, 9 KCl, 1 $CaCl_2$, 1 $MgCl_2$, and 10 HEPES. The patch pipette solution was 150 KCl, 1 $MgCl_2$, 10 HEPES, with free calcium adjusted via addition of EGTA/calcium as described below. All solutions were at pH 7.4 and between 320–325 mOsm. Pipette resistances were between 2–4 MΩ. Cells were held at -70 mV and stepped to 0 mV (for 100 msec) in the presence of elevated intracellular calcium in order to activate hIK1 or mIK1 channels. Two approaches were used to elevate intracellular calcium to activate calcium-dependent currents. Either the free calcium concentration of the patch pipette solution was set to 10 μM (1 mM EGTA with 10 mM calcium), or the pipette solution contained only 0.1 mM EGTA (see above) and intracellular calcium was increased by extracellularly applying 10 μM calcium ionophore (A23187) for 1–2 minutes. Compounds including A23187 were acutely applied to patch clamped cells by dissolving them in the bath solution, and then pressure ejecting the solution from blunt-tipped micropipettes positioned about 5–10 μM from the cell. All recordings were performed at ambient temperatures (21–23°C).

Skin biopsies

Skin biopsies (5 mm punches) were obtained from healthy donors via the Cooperative Human Tissue Network, Midwestern Division (Columbus, OH). For immediate RNA isolation biopsies were placed into RNALater (Ambion, Austin, TX) at the time of donation and shipped overnight in this solution. For experiments on living tis-

sue biopsies were placed into EpiLife alone (with 1.4 mM calcium) and shipped overnight on ice. Upon arrival in the lab living biopsies were trimmed of extraneous subdermal and dermal tissue, and were cultured in 6-well culture plates in EpiLife (with 1.4 mM calcium) in a 5% CO_2, 37°C incubator. Medium with or without test agents was refreshed daily. All skin biopsies were obtained under procedures approved by the Institutional Review Board of the Evanston Northwestern Healthcare Research Institute.

RNA Isolation

Total RNA was extracted from cultured human keratinocytes and skin biopsies with the RNAqueous phenol-free RNA isolation kit (Ambion, Austin, TX). RNAlater (Ambion) preserved skin samples were homogenized in RNAqueous lysis buffer with an IKA Works Ultra-Turrax T8 dispersing instrument (IKA Works, Wilmington, NC). Cell debris were removed from the homogenates by centrifuging for two to three minutes at 12,000 g. RNA was subsequently purified according to manufacturer's instructions. Keratinocytes were washed twice with Dulbecco's Phosphate Buffered Saline then homogenized with lysis buffer directly in culture dishes using a cell scraper. Keratinocyte RNA was purified as per instructions of manufacturer. RNA concentrations were determined by measuring absorbance at 260 nm. Ratios of absorbance at 260/280 nm yielded values between 1.8 and 2.1. All samples were DNase treated for at least 1 hr prior to reverse transcription.

RT-PCR

cDNA was synthesized by reverse transcribing 0.5–2 μg of total RNA using Power Script reverse transcriptase (BD Biosciences Clontech, Palo Alto, CA) in a 40 μL reaction. Briefly, samples were pre-incubated at 70°C with 20 ng of random decamers (Ambion) for two minutes, cooled on ice then incubated at 42°C for 90 minutes. Reactions were terminated with a final 15°C incubation for 15 minutes.

Expression levels of the targeted mRNA were quantified by fluorescent real-time polymerase chain reaction using an ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA). Real-time PCR reactions were performed in triplicate in a total volume of 30 μL containing 1 μL of cDNA, 15 μL of Universal PCR Master Mix (Applied Biosystems), 300–900 nM of each forward and reverse primer and 200 nM of each probe. Gene specific Taq-Man primers and probes were designed and synthesized using the Primer Express software package (Applied Biosystems). Primer and probe sequences for hIK1 were (5'-3'): GTGAACTCCATGGTGGACATCTC (forward), CGGTGTGAGCTGCTCAGATTC (reverse), ATGCACATGATCCTGTATGACCTGCAGC (probe); and, for mIK1: TGCCTTCCTCCTTTGTCTTATTGT (forward), AGCCCGTTGTCAGTCATGAAC (reverse), TCTTCCATGCCAAGGAGGTCCAGC (probe). Probes were labeled with the fluorescence reporter FAM at the 5' end, and the fluorescence quencher TAMRA at the 3' end. GAPDH was detected using primers and a VIC labeled probe purchased from Applied Biosystems as a Pre-developed Taq-Man Assay Reagent. PCR reaction conditions were as follows: two minutes at 50°C to activate uracil-N-glycosylase (Amp-Erase UNG) 95°C for 5 minutes to deactivate UNG, 40 cycles at 95°C for 15 seconds and 60°C for one minute. The relative quantification of mRNA, described as either fold change from control or % of GAPDH, was determined by the comparative Ct method where the target is normalized to the endogenous reference GAPDH. The threshold Ct value is the cycle number arbitrarily selected from the logarithmic phase of the PCR curve where an increase in fluorescence can be detected above background. The ΔCt is determined by subtracting the Ct of GAPDH from the Ct of the target (ΔCt = Ct-target – Ct-GAPDH). The relative value of target to endogenous reference is described as % of GAPDH = $2^{-\Delta Ct}$. Fold change from control = $2^{-\Delta\Delta Ct}$ where ΔΔCt = ΔCt-control – ΔCt-treatment.

To perform end-point PCR for 1281 bp of the 1284 bp coding sequence of hIK1 PCR reactions were run in a reaction volume of 50 μL using Advantage-GC 2 Polymerase with 15 μL of GC melt (BD Biosciences Clontech, Palo Alto, CA), 1X GC 2 PCR Buffer, 200 μM dNTP, 0.4 μM of each primer and 5 μL of cDNA. PCR reaction parameters were as follows: 94°C for 3 minutes, 35 cycles of 94°C for 15 seconds and 68°C 3 minutes, and a final extension at 68°C for 3 minutes. PCR for the amplification of GAPDH mRNA was performed in the same manner except 30 cycles were used and the GC melt was omitted. Primer sequences for hIK1 were (5'-3'): ATGGGCGGGGATCTGGTGCTT (forward), CTTGGACTGCTGGCTGGGTTC (reverse). GAPDH primers (Maxim Biotech, Inc., San Francisco, CA) were (5'-3'): TGCTGGCGCTGAGTACGTCGT (forward), GTGGAGGAGTGGGTGTCGCTG (reverse) yielding a PCR product of 615 bp. PCR products were analyzed on a 1.5% agarose gel stained with ethidium bromide.

Reagents and chemicals

The following compounds were prepared as stocks for application to cell cultures or single cells after appropriate dilution in either culture medium or physiological saline, respectively. VitD (calcitriol; 1,25-$(OH)_2$-D_3, Sigma Corp., St. Louis, MO) in DMSO; A23187 in DMSO (Sigma); 50 mM 1-EBIO (1-ethyl-2-benzimidazolinone, Tocris Cookson Inc., Ellisville, MO) in ethanol; 25 mM clotrimazole (Sigma) in ethanol; 10 μM charybdotoxin (Tocris) in 10 mM acetic acid with 0.1% BSA; 1 mM TRAM-34 in DMSO. TRAM-34 was a generous gift of Dr. Heike Wulff (UC Davis School of Medicine). Correspond-

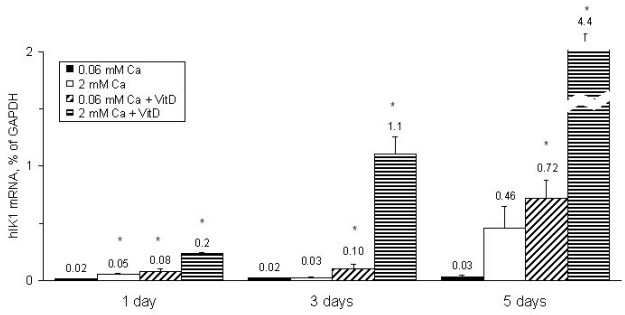

Figure 1

hIK1 mRNA expression is upregulated in human epidermal keratinocytes under culture conditions which are anti-proliferative/pro-differentiating. Keratinocytes were grown in either proliferative (0.06 mM calcium), or anti-proliferative, pro-differentiating culture conditions (2 mM calcium, 1 μM VitD, or both) for 1, 3, and 5 days. Total mRNA was harvested and processed for real-time PCR analysis. hIK1 mRNA levels are expressed as % of GAPDH mRNA, with data values at the top of each respective column. Each column represents the mean ± SEM for 2 to 6 cultures treated as indicated. The far right data column is broken to allow scaling of the *y*-axis appropriate to the lower expression values. * indicates significant difference from 0.06 mM calcium treatment at the indicated number of days.

ing vehicle controls showed no effect on cell proliferation, cell toxicity, electrophysiology, or gene expression.

Data Analysis

Numerical results are given as mean ± SEM unless otherwise noted. One-way ANOVA was used for comparisons of treated groups to a control no-treatment group, as well as for pairwise comparisons among treatment groups. The 95% confidence interval was used to assess significance for these analyses.

Results and Discussion

hIK1 mRNA expression in proliferating and differentiating human keratinocytes

We examined whether or not hIK1 mRNA is expressed in primary human epidermal keratinocytes, and if this expression is altered by addition of VitD at 1 μM, a concentration which is maximal for anti-proliferative/pro-differentiating effects [16], or elevation of extracellular calcium from 0.06 to 2.0 mM. Both of these perturbations induce expression of differentiation genes, with elevated calcium also producing profound morphological changes consistent with the onset of the differentiation program [17,18]. Both elevated calcium and VitD significantly increased hIK1 mRNA levels within 24 hours and these increases were maintained over the course of five days exposure (figure 1). The combination of VitD and elevated calcium produced synergistic increases in hIK1 expression at days 3 and 5. These results are consistent with the hypothesis that in human keratinocytes hIK1 may be both anti-proliferative and a positive regulator of the differentiation process.

The effect of VitD on hIK1 levels was also assessed in cells that had been previously exposed to elevated extracellular calcium, simulating what might occur in intact skin when VitD is therapeutically applied in part because of its pro-differentiating properties. In this experiment hIK1 expression in basal calcium (0.06 mM) was 0.029% of GAPDH, and after 48 hours in 2 mM calcium the value was 0.068%, similar to the result shown in figure 1. In cells that were treated with 2 mM calcium for 24 hours followed by addition of 1 μM VitD for another 24 hours hIK1 expression was 0.165%, again comparable to results shown in figure 1 for 24 hour calcium and VitD exposure. GAPDH levels, which were used to normalize gene expression, varied little in response to calcium and/or VitD (GAPDH Ct difference between untreated and treated cells of 0.2 ± 0.2, N = 9 determinations), further substantiating the robustness of the hIK1 upregulation results.

hIK1 mRNA expression in human skin biopsies

To directly assess the significance of the cell culture results to intact skin, hIK1 mRNA was assayed in human skin biopsies. To assess the variability of baseline hIK1 expression among skin samples, sets of three skin punch biopsies from each of three donors were placed in RNALater at the donation site. hIK1 mRNA expression in these biopsies ranged from 0.10 to 0.21% of GAPDH expression, with the values being quite consistent among biopsies from a given donor as well as between donors (figure 2A). Skin hIK1 expression values were somewhat higher than those observed in cultured keratinocytes growing under basal conditions (0.02% of GAPDH, 0.06 mM calcium no added VitD). However, keratinocytes treated with VitD alone or calcium and VitD expressed hIK1 at levels comparable to those in skin. mRNA levels for involucrin (INV) and transglutaminase-1 (TG-1), two contributors to and markers of keratinocyte differentiation, also showed little inter or intradonor variability. Expression levels for INV (9.4 ± 1.6% of GAPDH, N = 9) and TG-1 (2.7 ± 0.4%, N = 9) in the biopsies were higher than levels observed in untreated cultured keratinocytes (INV 4.9 ± 0.7%, TG-1 0.74 ± 0.19%). However, treatment of cultured keratinocytes with calcium, VitD or both agents elevated levels of INV (3.1-fold) and TG-1 (2.5-fold) into the ranges observed for skin biopsies.

To determine the dynamics of hIK1 expression, hIK1 expression levels were measured as a function of time after biopsy collection and subsequent growth in explant cul-

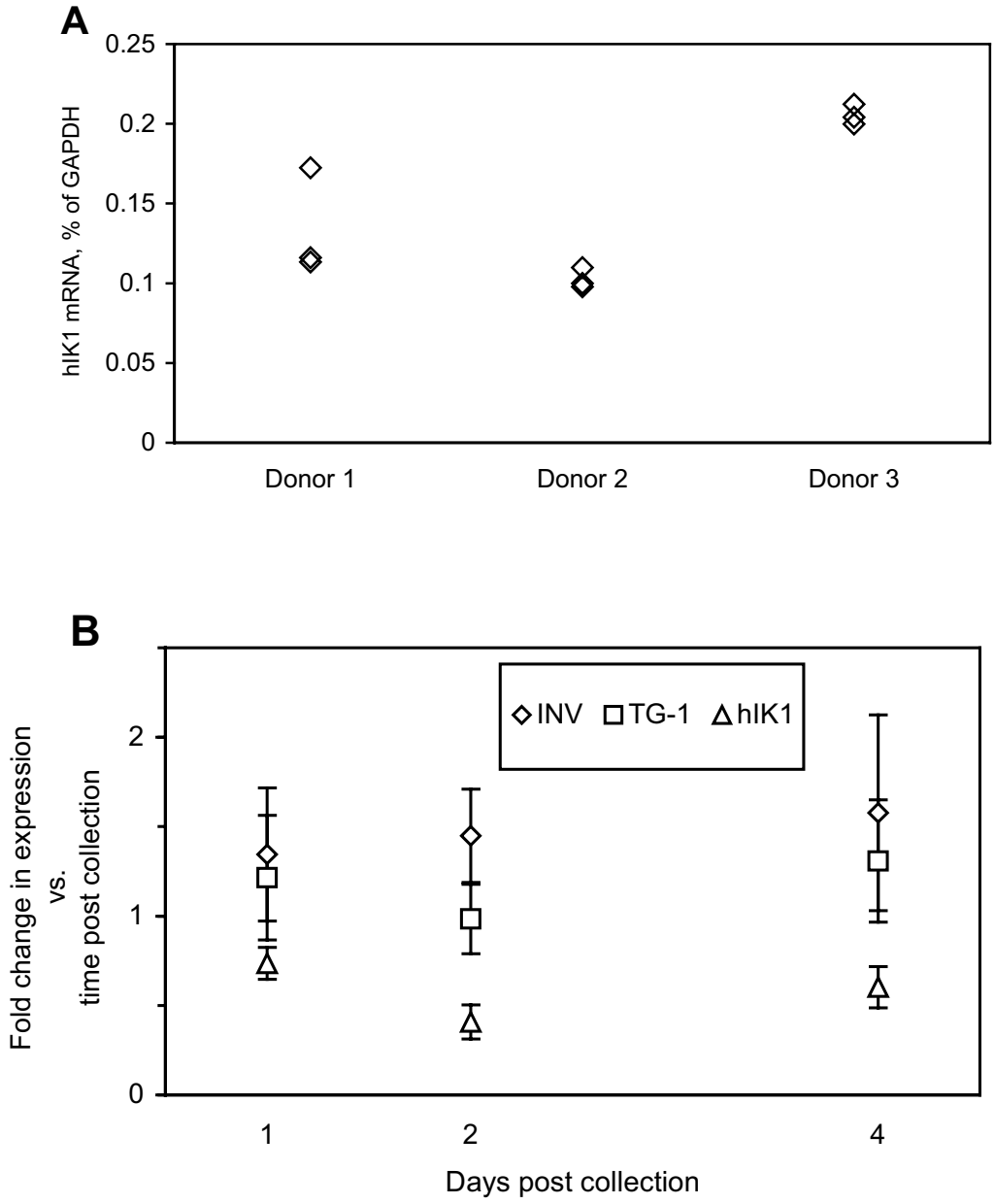

Figure 2

Consistent hIK1 mRNA levels among biopsies from individuals and donor groups are maintained in explant culture. Real-time PCR analysis of mRNA expression levels (normalized to GAPDH) in human skin biopsies from healthy donors. (a) data points are hIK1 mRNA levels from three biopsies from each of three donors with mRNA harvesting performed at time of biopsy collection. There were no statistically significance differences in hIK1 expression among the three donors. (b) effects of culture time on gene expression in explant biopsies assessed by measuring mRNA levels from biopsies preserved in RNALater at time of collection, and assessing fold changes from these levels in sibling biopsies shipped overnight in culture medium (1 day post collect), or shipped and kept in culture for 1 and 2 additional days (2 and 4 days post collect). Expression of involucrin (INV) and transglutaminase (TG-1) are shown for comparison with hIK1. Each data point is mean ± SEM for 3 biopsies. For any given gene there were no statistically significant differences in expression when comparing data from days one, two, and four.

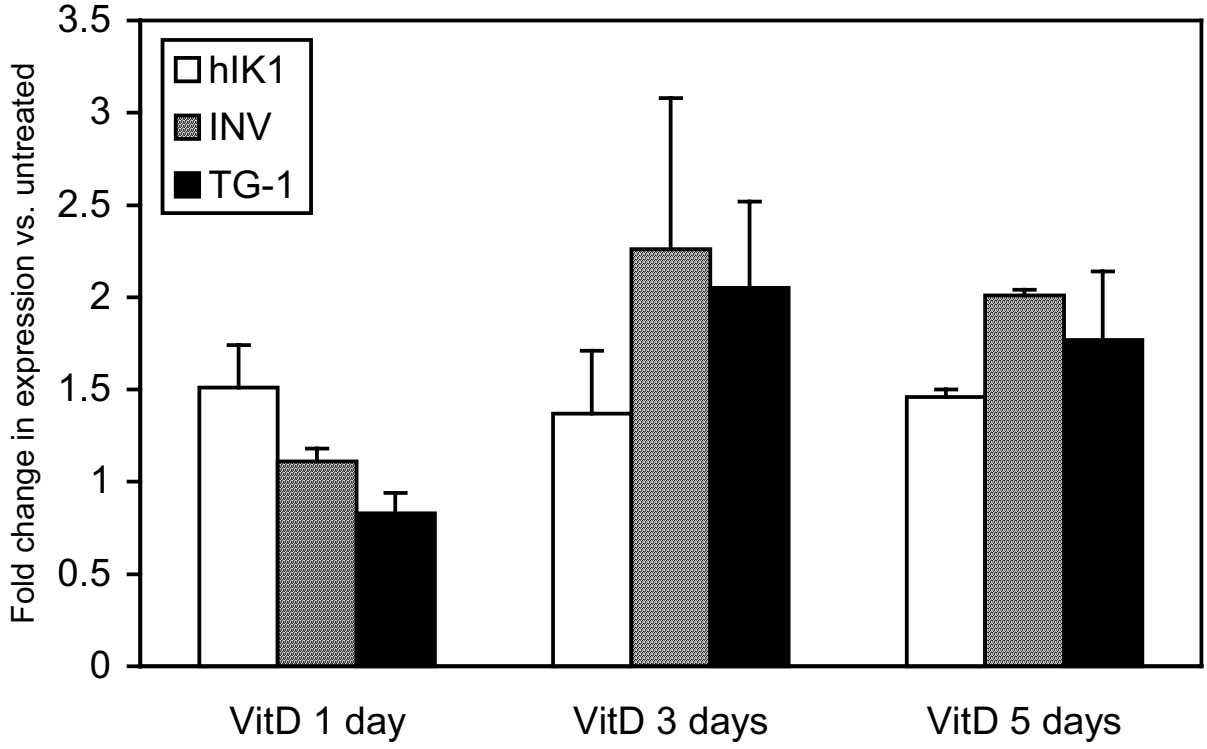

Figure 3
VitD modestly upregulates mRNA for hIK1 (and INV, TG-1) in explant cultured skin biopsies. Real-time PCR analysis of mRNA expression levels (normalized to GAPDH) in human skin biopsies from healthy donors. Each column represents the mean ± SEM fold change in mRNA levels for three treated biopsies relative to three untreated, time-matched controls. Changes in gene expression did not reach statistical significance.

ture (figure 2B). Medium always contained 1.4 mM calcium, a requirement for survival of explant skin cultures [19]. At 2 days post collection (1 day shipping, 1 day in culture) hIK1 expression had decreased to 40% of its initial value, but with an additional 2 days in culture expression had recovered to 60% of its initial value. TG-1 expression showed a similar decrease followed by an increase with culture time, while INV increased insignificantly. Skin biopsies contain dermal fibroblasts that may express hIK1. Although we trimmed much of the dermal tissue from the biopsies used for this study, we cannot completely discount some contribution of dermal fibroblasts to the hIK1 expression results.

We examined the effect of VitD exposure over time on skin explant hIK1 expression. In response to 1 μM VitD in the culture medium hIK1 expression increased slightly to approximately 1.5-fold of the untreated condition, and remained elevated for the duration of the experiment (figure 3). INV and TG-1 expression remained relatively unchanged at day one, followed by approximately 2-fold increases at days three and five. Thus the ability of VitD to enhance hIK1 mRNA expression in explant biopsies was less than for cultured keratinocytes.

Effect of the hIK1 activator 1-EBIO on keratinocyte proliferation

The increase in hIK1 expression under anti-proliferative/pro-differentiating growth conditions (calcium, VitD) was surprising in that increased IK channel expression has typically been correlated with proliferative stimuli and growth [1,2]. In addition, contact induced proliferative inhibition in the HaCaT keratinocyte line correlated with decreased hIK1 expression [9], and pharmacological downregulation of hIK1 correlated with mitogenic inhibition [10]. However, differentiation marker expression did

not increase, and it must be noted that proliferative inhibition is not necessarily coupled to enhanced cell differentiation. Our results correlating increased hIK1 expression with keratinocyte differentiation are, however, consistent with the general physiological model for this channel's function, namely to enhance calcium influx. Thus, in keratinocytes where calcium influx is a differentiation signal, hIK1 would be predicted to have anti-proliferative and/or pro-differentiating functions.

To more directly test this hypothesis that hIK1 activation would be inhibitory towards keratinocyte proliferation, we looked at the effect of the hIK1 activator 1-EBIO on tritiated thymidine incorporation, a measure of mitogenic activity. Indeed, 1-EBIO inhibited mitogenesis with an apparent IC_{50} slightly greater than 100 μM (figure 4), close to the EC_{50} for 1-EBIO activation of the cloned hIK1 channel (74–84 μM, [20,21]). This effect was not due to 1-EBIO induced cytotoxicity, as an LDH release based assay showed no cytotoxicity up to 300 μM 1-EBIO (data not shown). Surprisingly, however, mitogenic inhibition due to 1-EBIO could not be reversed by any of a panel of hIK1 blockers, including ChTX (0.2 μM), clotrimazole (0.3 μM), and TRAM-34 (0.1 and 1 μM) [1,2,22]. The blockers themselves did not inhibit mitogenesis except for a modest effect of TRAM-34 (<30% inhibition) at the relatively high concentration of 1 μM (400–500 times the IC_{50} for IK block). The inability of these blockers to reverse mitogenic inhibition by 1-EBIO called into question whether hIK1 was indeed the target of 1-EBIO's anti-proliferative action. Thus, these results required a functional examination of hIK1 activity in primary keratinocytes.

Electrophysiological evaluation of hIK1 functional expression

To directly correlate expression and pharmacological data with hIK1 channel function, we attempted to record hIK1 currents in keratinocytes. Whole cell patch clamp recordings were performed on a total of 41 keratinocytes. Only recordings with seal resistances greater than 5 GΩ were deemed acceptable for data acquisition. The number of cells recorded for each treatment condition, and the ranges of cell capacitances were as follows: 4 untreated, 12–19 pF; 4 at 2 mM calcium for 3 days, 18–35 pF; 14 at 1 μM VitD for 3 days, 14–40 pF; 11 at 1 μM VitD for 5 days, 27–58 pF; 5 at 1 μM VitD and 2 mM calcium for 3 days, 34–62 pF; 3 at 1 μM VitD and 2 mM calcium for 5 days 45–61 pF. The increase in cell capacitances (i.e., cell area) in response to the differentiating agents calcium and VitD is consistent with the increase in cell size observed when cells withdraw from the cell cycle and differentiate. Intracellular calcium in cells was increased via either the patch pipette solution (10 μM free calcium) or by external application of 10 μM A23187 for 1–2 minutes, but hIK1 currents, as evidenced by increased outward currents at 0 mV, were not observed under either condition (figure 5). In 17 cells challenged with A23187, current amplitudes at -70 and 0 mV were -42.3 ± 32.2 and -3.2 ± 5.6 pA respectively before challenge, and -48.3 ± 29.2 and -4.4 ± 5.7 pA after. An additional 21 cells exposed to 10 μM free calcium via the patch pipette had current amplitudes of -54.1 ± 45.2 pA and -1.5 ± 6.6 pA at -70 and 0 mV respectively, again showing no significant calcium induced current increase at 0 mV as would be expected for IK activation. Three of the cells in the 5 day VitD group were also simultaneously exposed to the IK agonist 1-EBIO (100 μM) and 10 μM internal free calcium, but again no hIK1 currents were evoked (current amplitude at 0 mV of -2.0 ± 4.2 pA). Inside-out patches also were made from 3 additional cells treated with VitD and calcium for 5 days, but no hIK1 channel activity was observed in these patches. We found no evidence of voltage-gated currents in keratinocytes at any test potentials investigated from -40 to 80 mV (data not shown).

To assure that the recording conditions were satisfactory for detecting hIK1 activity, we used the same solutions and reagents to evoke mouse IK1 (mIK1) currents in *ras*-transformed NIH3T3 cells (figure 5). Currents due to mIK1, a homolog of hIK1 [23], were detected in all four cells tested, with current amplitudes ranging from 0.78 nA to 1.68 nA (figure 5). These experiments were performed on the same day that identical recordings failed to detect hIK1 in keratinocytes treated for 3 days with calcium and VitD. The fact that all *ras*-transformed NIH3T3 cells tested responded with large magnitude mIK1 currents is consistent with previous reports from several groups, including our own, concerning these cells [24,25].

mRNA levels for mIK1 and hIK1 were measured from cell cultures made in parallel to those used for electrophysiology. hIK1 levels in keratinocytes treated for 5 days with calcium and VitD were approximately half of those for mIK1 in *ras*-transformed NIH3T3 cells. This level of hIK1 expression was entirely consistent with the initial expression studies shown in figure 1. By comparison, channel mRNA levels in wild type NIH3T3 and untreated keratinocytes were considerably lower. It is important to note, however, that despite the 19-fold difference in mIK1 mRNA levels between wild type (0.41%) and *ras*-transformed (7.97%) NIH3T3 cells, wild type cells still display readily observable whole cell mIK1 currents at approximately 10% of the amplitude of *ras*-transformed cells [24,25]. Further, the mIK1 mRNA level in wild type 3T3 cells is exceeded by hIK1 mRNA levels in keratinocytes from the following treatment groups: VitD for 5 days; VitD and calcium for 3 days; VitD and calcium for 5 days. Therefore, although IK currents can be observed in 3T3 cells expressing mIK1 mRNA at approximately 0.4% of GAPDH, no IK currents were observed in any of the 19

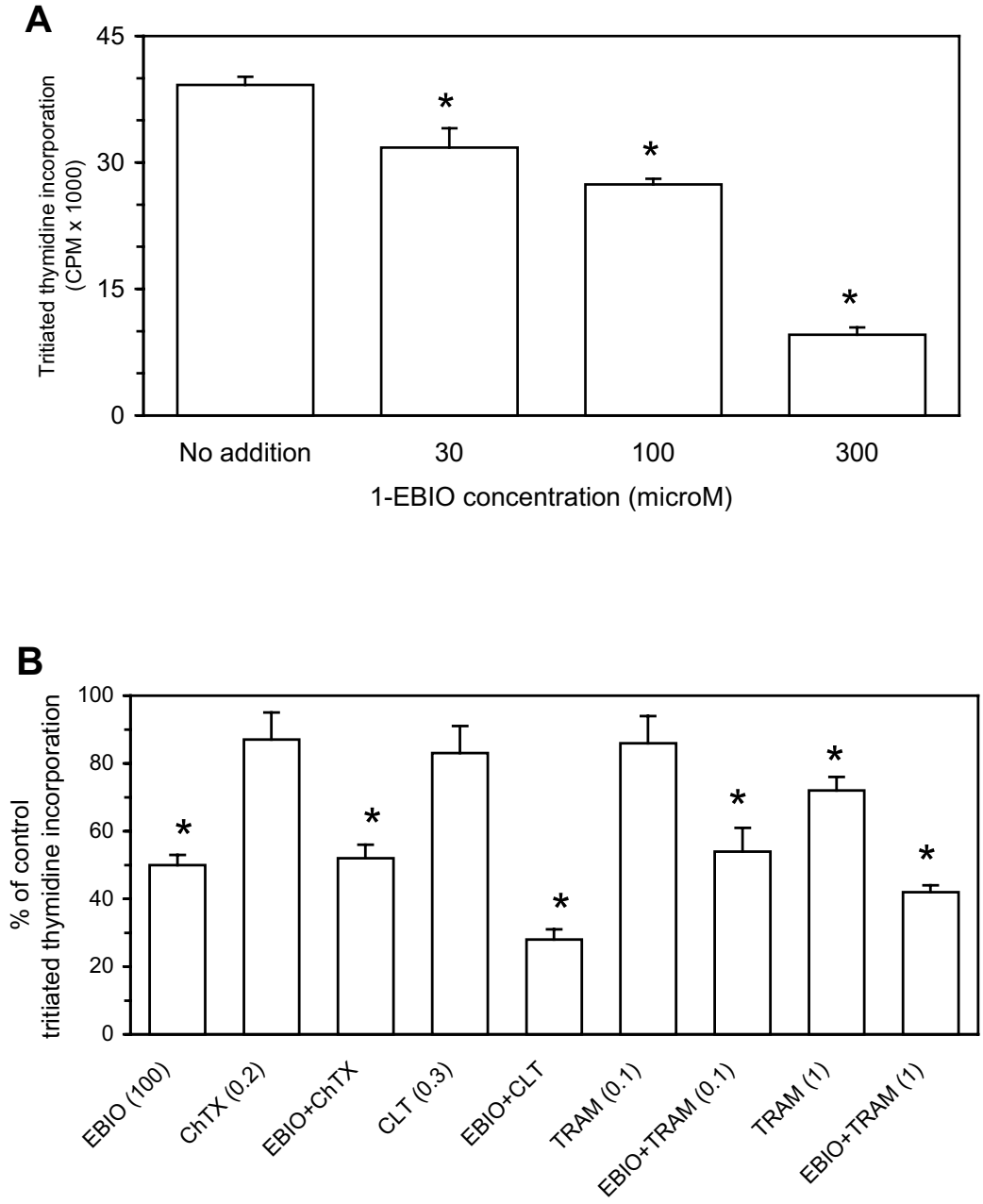

Figure 4

Inhibition of keratinocyte mitogenesis by the hIK1 agonist 1-EBIO is not reversed by hIK1 blockers charybdotoxin (ChTX), clotrimazole (CLT) and TRAM-34 (TRAM). Keratinocyte mitogenesis was assessed by measuring tritiated-thymidine incorporation over a 24 hour period. (A) single experiment, representative of four total, showing dose-dependence of 1-EBIO inhibition of mitogenic activity. Mitogenic inhibition was significant (*) at the concentrations of 1-EBIO tested (ANOVA, 95% confidence interval). (B) Cumulative data showing failure of hIK1 blockers ChTX, clotrimazole, and TRAM-34 to reverse the inhibitory effect of 100 μM 1-EBIO on keratinocyte mitogenesis. Percent difference in thymidine incorporation versus untreated control cells plotted with each column representing mean ± SEM of 3–4 experiments. Numbers next to drugs indicate μM concentrations. 100 μM 1-EBIO, with or without hIK1 blockers present, caused significant reductions in thymidine incorporation relative to untreated control cells (indicated by asterisks). That is, blockers did not reverse inhibition due to 1-EBIO which would be expected if 1-EBIO was acting via agonism of hIK1. TRAM-34 itself caused significant proliferative inhibition but only at 1 μM.

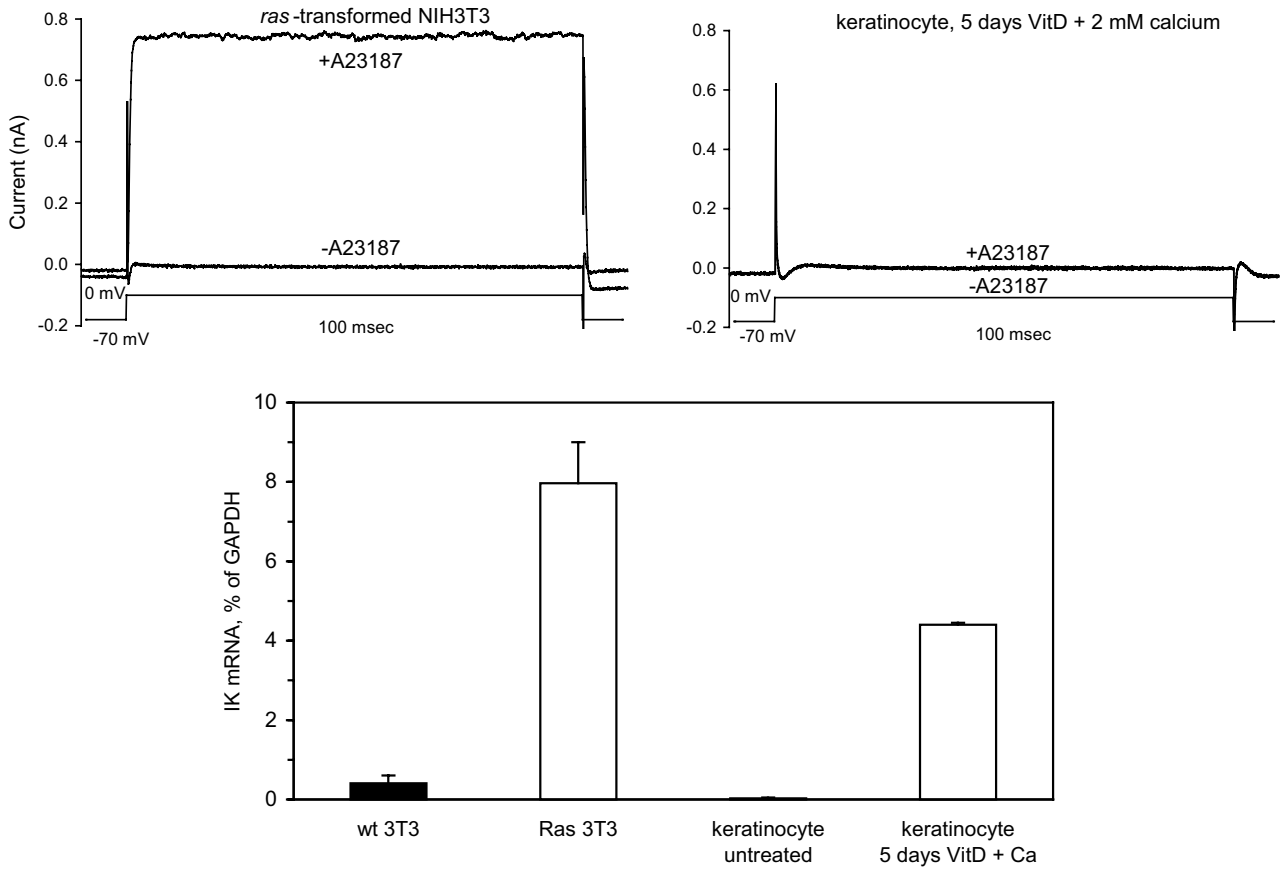

Figure 5

Both NIH3T3 cells and calcium/VitD-treated keratinocytes have abundant IK1 mRNA but only NIH3T3 display IK1 currents. Top panels show whole cell patch clamp recordings from a keratinocyte (treated 5 days with 2 mM calcium and 1 μM VitD) and *ras*-transformed NIH3T3 fibroblast. Each panel comprises records taken prior to and then during 1–2 minute application of 10 μM A23187, a calcium ionophore which allows IK channel activity to be observed during voltage steps to 0 mV (voltage protocol shown beneath current records). mIK1 current is readily observed in *ras*-transformed NIH3T3 cells in response to A23187, while hIK1 activity is absent from keratinocytes. Bottom panel shows real-time PCR analysis of IK mRNA levels from sibling cultures to those used for the patch clamp recordings (mIK1 for NIH3T3, hIK1 for keratinocytes). Each column represents mean ± SEM of two cultures. For comparison filled columns show mRNA levels for wild type (wt, non-transformed) NIH3T3 cells and untreated keratinocytes (0.06 mM calcium, no VitD).

keratinocytes from cultures which express hIK1 mRNA at 0.7 to 4.4% of GAPDH.

Why are no functional hIK1 channels observed despite the fact that hIK1 mRNA is abundant and appears to be physiologically regulated?

Our results showing 1-EBIO inhibits keratinocyte proliferation suggest an involvement of hIK1 in this process, but the hIK1 blocker ChTX failed to reverse this inhibition. 1-EBIO has also been shown to modulate cell growth in the HaCaT keratinocyte cell line, but 1-EBIO and other benzimidazolones also affect chloride (and other) conductances [26,27]. which are known to be present in HaCaT [9,28]. Although the presence of chloride channels in primary keratinocytes has not been addressed, it is possible that 1-EBIO affects proliferation in these cells and HaCaT through channels other than hIK1, or through some target yet to be determined. In addition, the electrophysiological effects of 1-EBIO and indeed the presence of hIK1 currents in HaCaT cells have not been subjected to direct voltage clamp analysis [9,10]., therefore, a critical electrophysiological comparison of these cells with pri-

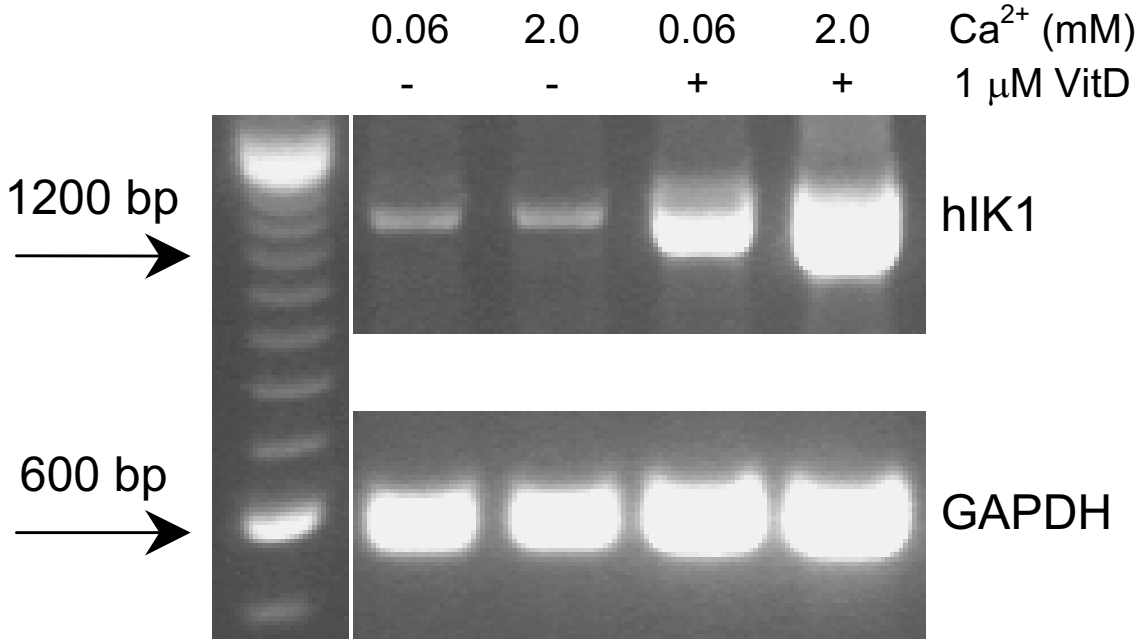

Figure 6
End-point PCR for the full-length protein coding sequence of hIK1 confirms real-time PCR results. Conventional end-point RT-PCR amplifying the coding sequence (1281 bp of 1284) of hIK1 was performed on keratinocytes grown for five days in either proliferative (0.06 mM calcium) or anti-proliferative, pro-differentiating (2 mM calcium, 1 μM VitD) culture conditions. GAPDH, 615 bp of the complete coding sequence, is shown as a loading control. Full-length hIK1 message is upregulated by VitD and more so by combining VitD with calcium, exactly as for the real-time PCR results (see figure 1).

mary keratinocytes is not yet possible. Thus, it appears that although hIK1 is expressed at the mRNA level in primary human keratinocytes as well as skin, functional channels are not formed, and thus cell growth studies utilizing hIK1 pharmacophores must be interpreted with caution.

At present we have no mechanistic explanation for why primary keratinocytes express hIK1 mRNA but lack any apparent functional channels. One purely technical possibility is that channels are produced but require cellular conditions for activity that we could not reproduce during the patch clamp experiments. However, IK channels are quite functionally robust and all reported recording conditions are straightforward and virtually identical, even in cells as diverse as fibroblasts, T-cells, microglia, and smooth muscle. A more interesting possibility is that hIK1 channels are produced in keratinocytes but they localize to organellar membranes and not the plasma membrane. This hypothetical intracellular hIK1 would not be detected with conventional patch clamp techniques, and it could be both sensitive to lipophilic compounds like 1-EBIO while being protected from peptide toxins (e.g., ChTX). Despite this intriguing possibility a physiological function for intracellularly localized IK channels is also uncertain. Another possibility is that our TaqMan based PCR was detecting a truncated [29] or splice variant hIK1 [30] which is electrophysiologically non-functional. To address this possibility, the hIK1 mRNA upregulation experiment shown in figure 1 was repeated, using end-point PCR for the full-length hIK1 sequence instead of the real-time method (which relies on detection of less than 100 bp out of the coding sequence). Figure 6 shows that exposure to calcium or VitD for 5 days significantly increases levels of full-length hIK1 mRNA. Further, commercial sequencing (SeqWright Inc., Houston, TX) of the PCR products showed the base content to be >90% identical to the reported hIK1 sequence, the non-matching

10% being due to sequencing ambiguities and incomplete sequencing of the 3' and 5' ends. Most importantly, sequencing showed no evidence for alternative first exons as has been reported for the closely related SK3 channel to encode non-functional, dominant negative variants [30,31]. Finally, similar to our results with hIK1, calcium-regulated expression of mRNA for certain P2Y purinergic receptor subtypes in human keratinocytes has been reported coincident with a lack of functional receptors [32].

Conclusions

The critical role for calcium influx in keratinocyte proliferation and differentiation strongly indicates an electrophysiological component to these processes, and hIK1 has been modeled to serve an essential role in controlling calcium mobilization in other non-neuronal, non-muscle cell types. However, our results suggest that the functional electrophysiology of primary human keratinocytes, and possibly intact human skin, does not include hIK1. Further, the lack of functional hIK1 channels suggests caution must be used when interpreting results with IK1 pharmacophores in human epidermal keratinocyte cells and skin.

Abbreviations

ChTX, charybdotoxin; 1-EBIO, 1-ethyl-2-benzimidazolinone; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IK, intermediate-conductance calcium-activated potassium channel; INV, involucrin; TRAM-34, 1- [(2-chlorophenyl)diphenylmethyl]-1H-pyrazole; VitD, 1,25-$(OH)_2$-D_3; TG-1, transglutaminase-1.

Competing Interests

None declared.

Authors' Contributions

SGR conceived of the study and with SR and MK directed its design and coordination. VM did the PCR analyses, and AB, WQ and SGR performed the cell proliferation and patch clamp experiments. SGR wrote the manuscript with significant input from AB, SR and VM. All authors read and approved the final manuscript.

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