Unexpected Down-regulation of the hIK1 Ca\(^{2+}\)-activated K\(^{+}\) Channel by Its Opener 1-Ethyl-2-benzimidazolinone in HaCaT Keratinocytes

Inverse Effects on Cell Growth and Proliferation*

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We used a combination of electrophysiological and cell and molecular biological techniques to study the regulation and functional role of the intermediate conductance Ca\(^{2+}\)-activated K\(^{+}\) channel, hIK1, in HaCaT keratinocytes. When we incubated cells with the hIK1 opener, 1-ethyl-2-benzimidazolinone (1-EBIO), to investigate the cellular consequences of prolonged channel activity, an unexpected down-regulation of channels occurred within a few hours. The same effect was produced by the hIK1 openers chlorzoxazone and zoazolamine and was also observed in a different cell line (C6 glioma cells). After 3 days of treatment with 1-EBIO, mRNA levels of hIK1 were substantially diminished and no channel activity was detected. Down-regulation of hIK1 was accompanied by a loss of mitogenic activity and a strong increase in cell size. After withdrawal of 1-EBIO, hIK1 mRNA and channel activity fully recovered, and the cells resumed mitogenic activity. Our data present evidence for a novel feedback mechanism of hIK1 expression that appears to result from the paradoxical action of its pharmacological activator during prolonged application. Because the down-regulation of hIK1 bears immediate significance on the biological fate of keratinocytes, 1-EBIO and related compounds might emerge as potent tools to influence the proliferation of various non-excitable cells endowed with IK channels.

Unlike small conductance and big conductance Ca\(^{2+}\)–activated K\(^{+}\) channels (SK and BK, respectively), \(\tau\) intermediate conductance Ca\(^{2+}\)–activated K\(^{+}\) channels (IK) are exclusively expressed in non-excitable cells, including fibroblasts, endothelial cells, secretory epithelial cells, immature smooth muscle cells, T-lymphocytes, and erythrocytes. The activity of IK channels has been implicated in the regulation of secretion, cellular migration, and in the proliferation of mitogenically active cells (for review see Ref. 1 and 2).

We recently demonstrated the expression of hIK1 (hSK4) mRNA in the human keratinocyte cell line, HaCaT (3). In perforated-patch whole-cell recordings, the extracellular mediator ATP produced a prominent and long-lasting hyperpolarization of HaCaT cells through a signaling pathway that involves IP\(_3\)-mediated Ca\(^{2+}\) release and subsequent activation of IK channels. Because ATP has been shown to promote proliferation of keratinocytes (4), it is likely that the mitogenic effect of ATP is at least partially mediated by the activation of hIK1. In support of this notion, we found that the levels of hIK1 mRNA declined as HaCaT cells began to differentiate (3).

This finding is consistent with observations from other types of non-excitable cells, which corroborate a link between IK channel activity and cellular proliferation. For example, unstimulated T cells express a low number of IK channels, whereas stimulation of T cells with mitogens or specific antigens results in increased IK channel density (5–8). In a myogenic fibroblast cell line, the mitogenic action of basic fibroblast growth factor (bFGF) was linked to the up-regulation of IK channels. Vice versa, the execution of the myogenic program was associated with a decline in IK channel transcripts (10, 11). Finally, IK conductance was much higher in proliferating smooth muscle cells compared with differentiated cells (12).

Given their apparent involvement in the regulation of cellular proliferation, IK channels might emerge as a prime drug target to manipulate the mitogenic behavior of non-excitable cells under various conditions. Indeed, pharmacological suppression of IK channels by charybdotoxin (ChbTx) or clotrimazole inhibited the mitogen-induced conversion of resting T cells to activated, proliferating T cells (9, 13). In a myogenic fibroblast cell line, suppression by ChbTx of IK channels abrogated the mitogenic effect of bFGF (10). Likewise, ChbTx and clotrimazole inhibited the proliferative response of human umbilical vein endothelial cells to the angiogenic factors bFGF and vascular endothelial growth factor, both of which up-regulate IK1 transcripts in the same cells (14).

Although these studies examined the cellular consequences of blocking IK channels, the recent advent of IK channel openers (reviewed in Ref. 1) now raises the question of how these compounds affect the pattern of cellular proliferation and differentiation. Do positive modulators of IK channels just act opposite to IK channel blockers, promoting proliferation and delaying differentiation? We report here the unexpected finding that prolonged exposure (3 days) of HaCaT keratinocytes to the prototype IK channel opener 1-ethyl-2-benzimidazolinone (1-EBIO) (15) caused a dramatic down-regulation of the channels at the protein and mRNA level. This novel feedback con-
Down-regulation of IK Channels by IK Channel Openers

Cell Culture—HaCaT keratinocytes (16) and C6 glioma cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (FCS) and antibiotics (penicillin/streptomycin 100 units/ml). 1–2 × 10^5 cells were seeded into 3.5-cm culture dishes and incubated for 24 h in the plating medium to allow for cell attachment and spreading on the dish. The plating medium was subsequently replaced by DMEM/5% FCS. K⁺ channel openers dissolved in Me₂SO or MeSO alone were added at this time. Cells were further cultured with a daily medium change and analyzed at different time points. 1-EBIO was purchased from Tocris (Cologne, Germany), medium, serum, and antibiotics were from Invitrogen (Karlsruhe, Germany).

Proliferation Assay—To determine the rate of HaCaT cell proliferation, cells were seeded in 3.5-cm dishes as described above. Growth medium was changed every day. At different time points after seeding, cells were detached with trypsin (0.25%/EDTA (0.5 mM)) and counted in a daily medium change and analyzed at different time points. 1-EBIO was purchased from Tocris (Cologne, Germany), chlorzoxazone (CZ), and zoxazolamine (ZOX) from Sigma (Munich, Germany). Medium, serum, and antibiotics were from Invitrogen (Karlsruhe, Germany).

EXPERIMENTAL PROCEDURES

Cell Lysis and Immunoblotting—Preparation of total cell lysates was performed as described previously (19), and protein concentrations were determined using the BCA kit (Pierce, Rockford, IL). Proteins were separated by SDS-polyacrylamide gel electrophoresis (10%) and transferred to nitrocellulose membranes. Membranes were incubated with the primary antibodies followed by alkaline phosphatase-conjugated secondary antibodies. Antibody-binding proteins were detected with the nitro blue tetrazolium/5-bromochloro-3-indolyl phosphate detection system (Promega, Madison, WI). The following antibodies were used: mouse monoclonal antibodies directed against keratin 10 (Dako, Glostrup, Denmark), keratin 14 and involucrin (NeoMarkers, Fremont, CA), and β-actin (Sigma, Munich, Germany). Secondary antibodies were from Promega.

Immunocytochemistry—Cells were washed twice with ice-cold PBS and fixed in acetone/methanol 1:1 for 20 min at −20 °C. Endogenous peroxidase activity was blocked with 3% H₂O₂ at room temperature. After blocking unspecific binding sites with 3% bovine serum albumin (BSA) in PBS, cells were incubated overnight at 4 °C with antibodies against keratin 10 or keratin 14 (see above), with a mouse monoclonal antibody against KG6 (NeoMarkers) or with a rabbit polyclonal antibody directed against E-cadherin (kindly provided by Rolf Kemler, Freiburg, Germany). Culture dishes were incubated at 4 °C overnight with the primary antibodies and rinsed three times with PBS and once with PBS/3% BSA. After a 2-h incubation with a peroxidase-coupled anti-mouse IgG (Promega, Mannheim, Germany) at room temperature, cells were washed three times with PBS, washed once with ddH₂O, and stained using the 3-amin-9-ethylcarbazole staining kit (Vector Laboratories, Burlingame, CA).

Ca²⁺—Photometry—HaCaT cells were loaded with the fluorescent dyes via their membrane-permeant AM esters. Stock solutions of 0.9 mM Calcium Green-1 AM and 1 mM Fura Red AM (Molecular Probes, Eugene, OR) were prepared by dissolving 50 µg of each dye in 20 µl of Me₂SO plus 20 µl of Phorun F-127 20% (Molecular Probes). Prior to loading, HaCaT cells were rinsed three times with HEPES-buffered saline (HBS, see below). The cell cultures were then incubated for 30 min at 37 °C in 3 ml of HBS to which 20 µl of stock solution had been added (final concentrations: Calcium Green-1, 6 µM; Fura Red, 7 µM) and were washed thoroughly after preincubation. All optical recordings were performed in HBS containing (in mM) 118 NaCl, 3 KCl, 1.5 CaCl₂, 1 MgCl₂, 20 sodium glutamate, 6 Hepes, and 10 d-glucose (pH 7.4) at room temperature.

Culture dishes were mounted on an upright microscope (Olympus BX50WI, objective: Olympus UPLPlanFI 40xW) to which a confocal laser-scanning (Bio-Rad MRC 1024) and an independent custom-made photometric system (20) were attached. The specimen was illuminated at 485 ± 30 nm in intervals of 5 s. Two photodiodes collected fluorescent light emitted from the cell layer at wavelengths 530 ± 30 nm (emission Calcium Green) and 660 ± 50 nm (emission Fura Red) simultaneously and produced voltage signals that were proportional to fluorescence emission. Electrophysiological signals were linearly amplified prior to PC-based data digitization (DT2812, Data Translation, Marlboro, MA).

Data were monitored with a modified version of the program QTRAC (21). Further analysis and ratio (r = F₅₀⁵/F₆₆₀) calculations were performed using the scientific spreadsheet program Origin (Microcal, Northampton, MA). Ratio values are given as normalized r = R/R₀, i.e. divided by the mean ratio value before drug administration. The time course of Ca²⁺ transients was fitted with Origin 4.1.

RESULTS

Short-term and Long-term Effects of K⁺ Channel Openers on Hyperpolarizing Response to ATP—Whole-cell recordings were performed on HaCaT cells, an immortalized, non-tumorigenic human keratinocyte cell line that maintains partial differentiation capacity in vitro and full differentiation capacity in vivo (16). Fig. 1 compares the voltage response of pre-confluent HaCaT cells to the physiological stimulus ATP, to its non-hydrolysable analogue, ATP[S], and to the three IK channel openers 1-EBIO, chlorzoxazone (CZ), and zoxazolamine (ZOX). As we have shown before (3), ATP (10 µM, n = 28) produced a biphasic voltage change, consisting of an initial, transient depolarization due to the opening of Ca²⁺-activated Cl⁻ channels and non-selective cation channels, followed by a strong hyperpolarization due to the opening of ChTx-sensitive hIK1 channels (Fig. 1A). ATP[S] (10 µM, n = 3) gave rise to virtually identical voltage trajectories (Fig. 1B). 1-EBIO (1 mM, n = 14), CZ (1 mM, n = 17) and ZOX (1 mM, n = 8) all led to a marked and ChTx-sensitive hyperpolarization of the membrane potential (Fig. 1, C–E), demonstrating their pharmacological efficacy and their functional coupling to IK channels in HaCaT cells.

Based on their strong effect on the resting membrane potential, all three channel openers appeared as suitable tools to study how the prolonged pharmacological activation of IK channels influences the pattern of proliferation and differentiation in HaCaT cells. Before doing so, however, we performed a series of control experiments to prove the continued activation of IK channels during prolonged application of the channel openers. HaCaT cells were cultured in the presence of 1 mM 1-EBIO, and the resting membrane potential and the electrophysiological response to ATP (10 µM) were measured after drug incubation periods of variable duration (3 h to 3 days). To our surprise, incubation with 1-EBIO for just 3 h was already sufficient to produce a dramatic desensitization of IK channels, so that application of ATP (10 µM) failed to give rise to the typical hyperpolarization (Fig. 2, A and B). As a consequence,
the depolarizing action of ATP, which is quickly reversed by IK channel opening under control conditions, only gradually declined. After 7-h incubation with 1-EBIO, activation of IK channels by ATP was almost completely lost, as indicated by the plateau-like depolarization evoked by ATP (Fig. 2, A and B).

When keratinocytes were grown for 3 days in the presence of 1 mM 1-EBIO, they showed a markedly depolarized resting membrane potential (−19 ± 2 mV, n = 9, Fig. 2C) compared with control cells (−53 ± 4 mV, n = 9). These data are consistent with the depolarizing effect of ChbTx in normal HaCaT keratinocytes (3), suggesting that tonic activity of ChbTx-sensitive IK channels contributes to their resting membrane potential. Cells grown in the presence of 0.1 mM 1-EBIO showed only a slightly depolarized membrane potential (−41 ± 5 mV, n = 8). The presence of the solvent alone (Me2SO, 0.1%) did not influence resting membrane potential (−52 ± 2 mV, n = 8).

**FIG. 1.** ATP and IK channel openers induce ChbTx-sensitive hyperpolarization of the membrane potential. Voltage responses were measured using the perforated-patch variation of the whole-cell recording technique. A and B, the extracellular signaling molecule ATP (10 μM) and its non-hydrolyzable analogue ATPγS (10 μM) produced virtually identical, biphasic voltage changes, in which a brief depolarization was followed by a pronounced hyperpolarization. The latter was completely reversed by the IK channel inhibitor ChbTx (100 nM). C–E, the IK channel openers 1-EBIO (1 mM), CZ (1 mM), and ZOX (1 mM) produced a rapid shift of the membrane potential in the hyperpolarizing direction, which was sensitive to ChbTx (100 nM).

**FIG. 2.** Loss of hyperpolarizing response to ATP during prolonged application of IK channel openers. A, representative voltage traces demonstrating the gradual decline of the hyperpolarizing action of ATP (10 μM) at 3 h and at 7 h of 1-EBIO (1 mM) pretreatment. Note that, as the ATP-activated IK conductance disappears, the depolarizing effect of ATP prevails, giving rise to a plateau-like shift of the membrane potential in the depolarizing direction. B, summary of the experiments on the time dependence of decline of the ATP response. Voltage axis indicates maximal voltage deviation from resting membrane potential (broken line) that was induced by ATP (10 μM) at the various time points of 1-EBIO (1 mM) treatment (control cells, n = 12; 1-EBIO-treated cells, n = 13). C, application of 10 μM ATP produced no hyperpolarization in cells cultured for 3 days in the presence of 1-EBIO (1 mM, red trace), CZ (1 mM, green trace) or ZOX (1 mM, blue trace), whereas control cells showed the typical biphasic change of membrane potential (black trace). Note that 3 days of treatment with IK channel openers also caused a shift of resting membrane potential in the depolarizing direction suggesting that the activity of IK channels contributes to the resting membrane potential under normal conditions. D, histogram summarizing the experiments illustrated in C. All data were obtained after 3-day incubation with the different compounds or the solvent (Me2SO) alone. Although the ATP-induced voltage shift in the hyperpolarizing direction (given as ΔVm) was not affected by Me2SO or a low concentration of 1-EBIO (0.1 mM), 1 mM 1-EBIO, 1 mM CZ, and 1 mM ZOX almost completely abrogated this effect. *, p < 0.05; **, p < 0.001.
FIG. 3. Cytosolic Ca\(^{2+}\) response to ATP in 1-EBIO-treated HaCaT keratinocytes. Changes in cytosolic Ca\(^{2+}\) were measured using ratiometric photometry. A, representative traces showing the ATP-induced Ca\(^{2+}\) rise in control cells (black trace) and in cells treated with 1-EBIO (1 mM) for 3 days (gray trace). B, histogram summarizes decay time constant of Ca\(^{2+}\) signals after ATP (10 \(\mu\)M) application in control cells, in cells treated with Me\(_2\)SO alone, and in cells treated with 1-EBIO (0.1 mM or 1 mM). Duration of treatment was 3 days. **, \(p < 0.001\).

Application of 10 \(\mu\)M ATP to control cells hyperpolarized the membrane potential by \(-22 \pm 4\) mV \((n = 9)\), which was not different from the effect in Me\(_2\)SO (0.1%)-treated cells \((\Delta V_m = -24 \pm 2\) mV, \(n = 8\) Fig. 2, C and D). Incubation with 1 mM 1-EBIO virtually abrogated any effect of ATP on IK channel activity \((\Delta V_m = -3 \pm 1\) mV, \(n = 8\) Fig. 2, C and D). By contrast, incubation with 1-EBIO at the lowest concentration (0.1 mM) did not alter the hyperpolarizing response to ATP \((\Delta V_m = -25 \pm 3\) mV, \(n = 8\) Fig. 2D). This agrees with the observation that, during acute application in control cells, 0.1 mM 1-EBIO displayed only a weak electrophysiological effect (data not shown).

To determine whether the strong and unexpected down-regulation of the channels is a common pharmacological feature of IK channel openers or represents a peculiarity of 1-EBIO, we repeated the above experiments using CZ (1 mM) and ZOX (1 mM), which were only recently identified as IK channel openers (22, 23). A 3-day incubation with CZ or ZOX led to an almost complete disappearance of the hyperpolarizing action of ATP. \(\Delta V_m\) was \(-5 \pm 1\) mV \((n = 4)\) in CZ-treated cells and \(-4 \pm 2\) mV \((n = 3)\) in ZOX-treated cells (Fig. 2, C and D). In addition, both substances produced a depolarization of the resting membrane potential (ZOX: \(-35 \pm 2\) mV, \(n = 3\); CZ: \(-42 \pm 5\) mV, \(n = 4\)). This effect was weaker than that of 1 mM 1-EBIO \((-19 \pm 2\) mV, \(n = 9\)), suggesting that 1-EBIO caused the most complete down-regulation of IK channels.

Is the availability of functional IK channels in the membrane affected in a similar way, when HaCaT cells are exposed for a longer time to a natural stimulant of IK channel activity, such as ATP? This scenario might occur, for example, after skin wounding when large amounts of ATP are released into the extracellular space. To test this possibility, HaCaT cells were incubated for 3 days with the stable ATP analogue, ATP\(_s\) (10 \(\mu\)M), which, as shown above (Fig. 1B), fully reproduces the electrophysiological action of ATP. In striking contrast to the down-regulation produced by the three IK channel openers, preincubation with ATP\(_s\) did not diminish the responsiveness of HaCaT cells to a single application of ATP, which under this condition hyperpolarized the membrane potential by \(-33 \pm 4\) mV \((n = 4,\) data not shown). This suggests that prolonged channel activity will not per se initiate subsequent down-regulation, unless induced by an IK channel opener that binds to the channel or a closely associated protein of the channel complex.

**Ca\(^{2+}\) Signaling and Down-regulation of IK Channels—**In HaCaT cells, extracellular ATP binds to P2Y2 receptors, leading to inositol 1,4,5-trisphosphate formation and subsequent release of intracellular Ca\(^{2+}\), which then activates IK channels (3, 24). Is it conceivable that sustained application of 1-EBIO somehow interferes with this pathway, so that the fading voltage response to ATP would be secondary to changes in receptor signaling, rather than reflect a down-regulation of the channel itself? To examine whether P2Y2 receptor activation in 1-EBIO-treated keratinocytes still produces Ca\(^{2+}\) mobilization, we measured intracellular Ca\(^{2+}\) responses to ATP (10 \(\mu\)M) in control cells, and in cells cultured for 3–4 days in 0.1 or 1 mM 1-EBIO, or in Me\(_2\)SO alone. Because we used ratiometric photometry with two fluorescent Ca\(^{2+}\) indicators (Calcium Green/ Fura Red), we cannot calculate absolute intracellular Ca\(^{2+}\) concentrations from changes in the fluorescence signal. Previous studies using the indicators Fura-2 or Indo-1 to obtain quantitative measurements of intracellular Ca\(^{2+}\) reported average resting levels of 78–145 nM in HaCaT cells, with ATP producing an average increase of intracellular Ca\(^{2+}\) by 580 nM (25, 26). In our hands, ATP induced qualitatively very similar cytosolic Ca\(^{2+}\) increases with rapid onset kinetics in all four cell groups (Fig. 3A), indicating that P2Y2 signaling was not impaired by sustained 1-EBIO treatment. It is worth noting, however, that the decline of the intracellular Ca\(^{2+}\) signal was dramatically accelerated after hIK1 down-regulation (Fig. 3A).

In control cells, Me\(_2\)SO-treated cells, and low 1-EBIO-treated cells, the Ca\(^{2+}\) signal decreased slowly with almost identical...
Fig. 5. Expression of hIK1 mRNA is strongly reduced in 1-EBIO-treated HaCaT keratinocytes. A, total RNA was prepared from HaCaT keratinocytes cultured for 3 days in control medium or in medium containing 0.1% MeSO, 0.1 mM 1-EBIO or 1 mM 1-EBIO. Samples of 20 µg were subjected to RNase protection analysis using an antisense probe to hIK1. Hybridization of the same RNAs with a GAPDH antisense probe served as a loading control. RNA (20 µg) was used as a negative control. 1000 cpm of the hybridization probes was loaded in the lanes labeled “probe” and used as a size marker. B, the signal intensities were determined by phosphorimaging and normalized to the GAPDH signal intensity. The signal intensity of non-treated cells was arbitrarily set at 100%. *, p < 0.05; **, p < 0.001.

Fig. 6. 1-EBIO inhibits keratinocyte proliferation. A, HaCaT cells were seeded in 3.5-cm dishes and cultured for 3 days in DMEM/5% FCS with or without MeSO, 1-EBIO, or ATP-γS as indicated (see “Experimental Procedures”). They were subsequently trypsinized and counted in duplicate dishes using 0.04% trypan blue to monitor cell viability. The number of cells at day 3 after plating in relation to the number of seeded cells is shown. B, HaCaT cells grown for 3 days in medium with and without 1-EBIO (1 mM) were stained with an antibody to Ki67. Note the reduced number of stained cells after treatment with 1-EBIO. The scale bar in B indicates 100 µm. **, p < 0.001.

suggests that prolonged application of 1-EBIO leads to a virtually complete down-regulation of channel expression, irrespective of the cell type under study.

Loss of hIK1 Channel Activity Is Associated with Down-regulation of hIK1 mRNA Levels—The fading response to ATP and 1-EBIO in cells incubated with IK channel openers might possibly involve alterations of channel phosphorylation, channel internalization, and degradation, and/or reduction of the mRNA levels. Because the latter is arguably the most incisive mechanism of negative feedback control, we wondered whether the electrophysiologically determined loss of functional IK channels is a result of reduced hIK1 mRNA levels. We therefore used RNase protection assays to analyze hIK1 mRNA levels under the various experimental conditions. Total RNA was isolated after culturing HaCaT cells for 3 days under control conditions, in MeSO (0.1%) alone, or in 1-EBIO (0.1 or 1 mM), hIK1 mRNA levels were markedly reduced in cells treated with 1 mM 1-EBIO for 3 days and slightly reduced in cells grown in 0.1 mM 1-EBIO (Fig. 5A). In cells treated with MeSO alone, mRNA levels were not different from control. The signal intensities of the bands corresponding to transcripts of hIK1 and to transcripts of the housekeeping gene GAPDH were determined by phosphorimaging. The signal intensity of hIK1 was then normalized to that of GAPDH. The histogram of Fig. 5B summarizes the relative change of normalized hIK1 mRNA under the different experimental conditions, based on the results from eight independent RNase protection assays. These
data indicate that prolonged application of IK channel openers leads to a pronounced reduction of hIK1 mRNA levels.

**Down-regulation of hIK1 Channels Leads to Increased Cell Volume and Inhibits Proliferation**—Because the expression of IK channels is closely associated with, or even causally linked to cellular proliferation (see the introduction), we wondered how the strong down-regulation of the channel would alter the normal pattern of growth and differentiation in HaCaT cells. To analyze proliferation of 1 mM 1-EBIO-treated HaCaT keratinocytes, cells were seeded in culture dishes, and the number of cells was determined after 3 days in the presence of 1-EBIO (0.1 and 1 mM), ATP of cells was determined after 3 days in the presence of 1-EBIO treatment on HaCaT keratinocytes (control cells

**Down-regulation of hIK1 and Associated Cellular Effects Are Reversible**—To determine the reversibility of the cellular effects of hIK1 down-regulation, 1-EBIO was withdrawn from the medium and the cells were cultured for 3 more days under standard conditions. In electrophysiological measurements (Fig. 8, A and B), we did not observe a significant difference between the cell groups with respect to the ATP-induced maximal hyperpolarization (control: \(-73 \pm 2 \text{ mV}, n = 5\); Me\(_2\)SO: \(-73 \pm 2 \text{ mV}, n = 4\); 0.1 mM 1-EBIO: \(-71 \pm 2 \text{ mV}, n = 6\); 1 mM 1-EBIO: \(-74 \pm 2 \text{ mV}, n = 6\)). This finding agrees well with the results of RNase protection assays (n = 4), which demonstrated complete recovery of hIK1 mRNA levels in 1-EBIO-treated cells at 3 days post treatment (97 \pm 16\% of level in control cells). Finally, cell counts (n = 8) showed that, upon withdrawal of the K\(^+\) channel opener from the medium and subsequent re-expression of hIK1, mitogenic activity was re-ignited, leading to a massive increase in the proliferation rate (Fig. 8C). Because the other cell groups (n = 8 per column) had already grown to confluence at this time point, they showed only little further increase in cell number.

**DISCUSSION**

We report here the novel and unexpected finding that, depending on the duration of their application, the IK channel openers 1-EBIO, CZ, and ZOX all exerted exactly opposite effects on hIK1 channels of HaCaT keratinocytes: Although application of the drugs for minutes produced a prominent and rapidly reversible hyperpolarization, as expected for a K\(^+\) channel opener, exposure for hours and days led to a complete disappearance of functional hIK1 channels, as indicated by the loss of the hyperpolarizing response to extracellular ATP.
Down-regulation of IK Channels by IK Channel Openers

The various mechanisms of short-term and long-term desensitization, including down-regulation at the transcriptional level, have been studied in detail for G protein-coupled receptors (32) and ligand-activated ion channels (33–35). In addition, Levitan et al. (36) showed that membrane depolarization by 50 mM extracellular K\(^+\) causes rapid inhibition of the transcription of the Kv1.5 channel gene, which was maximal at 3 h. However, the complete disappearance of channel activity and the concomitant decline of channel transcripts during prolonged application of a direct channel opener is, to our knowledge, a novel observation.

Based on our RNase protection assay data, it seems likely that the reduction in hIK1 mRNA levels as a result of reduced transcription and/or mRNA stability is responsible for the long-term down-regulation of the channel. Turnover rates of voltage-dependent Na\(^+\) and Ca\(^{2+}\) channels are relatively slow (\(t_{1/2} \approx 1\) day) (37, 38), but the half-life of some K\(^-\) channels might be substantially shorter, as demonstrated for Kv1.5 channels (\(t_{1/2} \approx 4\) h) (39). Although the turnover of hIK1 in HaCaT cells remains to be determined, it hence appears conceivable that down-regulation of the mRNA upon continued drug application might also be responsible for the lack of channel activity at earlier time points. However, the fact that we already observed a reduced response to ATP within 3 h suggests that 1-EBIO also induces a conformational change in the channel protein that leads to more rapid internalization and/or degradation.

Down-regulation of hIK1 channels had three distinct cellular consequences in HaCaT keratinocytes when compared with cells grown under standard culture conditions for the same period of time, namely (i) inhibition of proliferation, (ii) increase in cell size, and (iii) impaired expression of the differentiation marker K10. The association of hIK1 down-regulation with inhibited cellular proliferation and the parallel recovery of both parameters is consistent with a growing body of evidence proposing a causal link between IK channel activity and cellular proliferation (see the introduction). Because expression of the early differentiation marker K10 is also diminished in 1-EBIO-treated cells, the reduced proliferation is obviously not accompanied by a premature onset of differentiation in these cells. Previous studies have implicated K\(^+\) channels and Cl\(^-\) channels of unknown molecular identity in the proliferation and differentiation of keratinocytes (40–42). Our findings suggest that hIK1 is a key player in these processes.

What is the relationship between the down-regulation of hIK1 channels and the pronounced increase in cellular volume, and how could this possibly be linked to the cessation of proliferation? A role of IK channel in regulatory volume decrease in response to hypotonic cell swelling is still disputed. Although IK channels are currently believed not to contribute to regulatory volume decrease of the widely studied Ehrlich cells (43, 44), this does not necessarily hold for other cell types (reviewed in Ref. 1). In sickle cell anemia, for example, the increased IK conductance of erythrocytes has been established as a major cause of their salt loss and dehydration. This is in agreement with studies from secretory epithelial cells, in which IK channels have been implicated in the generation and maintenance of the ion gradients required for secretion (see above). Although HaCaT cells do not have an appreciable secretory function, the down-regulation of functional IK channels might impair extrusion of osmotically active substances and thus lead to a substantial gain in cellular volume. We do not think, however, that the large increase in cell size arises from uncontrolled, pathological swelling. Such a process should inflict lasting damage on the cell, but we found that 1-EBIO-treated cells resumed their normal proliferation rate once the drug was withdrawn from the culture medium. Is the gain in cell volume causally linked to the concomitant decline in mitogenic activity? Rouzaire-Dubois et al. (45) have recently proposed two mechanisms that might account for this highly inverse relationship. First, cell volume changes may alter the concentration of cellular components involved in the expression or activity of cell cycle regulating proteins. Second, cytoskeleton rearrangements due to cell volume changes may affect the protein kinases or phos-
Down-regulation of IK Channels by IK Channel Openers

Because IK channels are not only essential for cell proliferation but play also a pivotal role in epithelial secretion (see the introduction), IK channel openers are currently under consideration as potentially beneficial agents in diseases such as cystic fibrosis and chronic obstructive pulmonary disease (1). Given the virtually identical down-regulation of IK channels in two independent cell lines (HaCaT and C6 glioma), our data send a strong note of caution regarding the clinical usefulness of IK channel openers as secretion-stimulation compounds. Based on their inverse effect during prolonged application, the compounds might actually worsen the symptoms of cystic fibrosis and chronic obstructive pulmonary disease. In striking contrast to their original purpose, K channel openers might counteract central pathophysiological processes. Examples include epithelial and endothelial hyperproliferative disorders, autoimmune diseases, and graft-versus-host reactions.

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