Keratinocyte K^+ Channels Mediate Ca^{2+}-Induced Differentiation

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K^+ channel activation has been associated with growth or differentiation in many cells. We have previously identified a 70-pS K^+ channel that was found only in differentiated involucrin-positive cells. In this study we examined the role of K^+ channels in Ca^{2+}-induced keratinocyte differentiation. Consistent with our previous report, we found that a K^+ conductance developed only in cells cultured in high extracellular Ca^{2+}. Addition of charybdotoxin or verapamil blocked these K^+ channels and inhibited Ca^{2+}-induced differentiation, as assessed by cornified envelope formation or transglutaminase activity. These results suggest that K^+ channel activation is necessary for Ca^{2+}-induced differentiation. Finally, we used ^{125}I-labeled charybdotoxin to demonstrate the presence of K^+ channels in intact human and mouse epidermis, hair follicles, and eccrine glands, indicating that these channels are found in keratinocytes both *in vitro* and *in vivo*. Thus K^+ channels may moderate Ca^{2+} influx in more differentiated keratinocytes and may play a central role in keratinocyte differentiation. ***Key words: ion channels/hair follicle/charybdotoxin/verapamil. J Invest Dermatol 108:864–870, 1997***

Although intracellular Ca^{2+} has been the focus of most studies that examine the effects of ion fluxes on keratinocyte differentiation, K^+ fluxes also have been shown to modulate Ca^{2+}-induced keratinocyte differentiation. For example, the Ca^{2+}-induced trigger to differentiate occurs only when intracellular K^+ concentrations are high (Hennings *et al*, 1983a, 1983b). Furthermore, application of K^+ channel openers such as diazoxide increases intracellular Ca^{2+}, suggesting that keratinocytes contain both K^+ channels and a non-voltage–activated Ca^{2+} influx pathway (Xiong and Harmon, 1995). Finally, K^+ ions are also important for epidermal permeability barrier repair, a late event in epidermal differentiation (Lee *et al*, 1994).

K^+ channel activity was not measured in the original (Mauro *et al*, 1990; Galietta *et al*, 1991a) studies of keratinocytes, probably because these studies (i) used methods that removed intracellular components and (ii) focused on undifferentiated cells. A K^+ conductance was noted in short-circuit current studies of differentiated keratinocytes (Kansen *et al*, 1992) and a later study that correlated the presence of ion channels with keratinocyte differentiation localized K^+ channels only in involucrin-positive cells, affording the first clue that K^+ channels may develop as keratinocytes commit to differentiation (Mauro *et al*, 1993). In the current study, we found that K^+ channels develop as keratinocytes differentiate and are present in epidermis. In addition, we found that these K^+ currents were necessary for continued keratinocyte differentiation, as K^+ channel blockade inhibited Ca^{2+}-induced keratinocyte differentiation.

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Abbreviations: KGM, keratinocyte growth medium; NSCC, nonspecific cation channel.

MATERIALS AND METHODS

Cell Culture Epidermis was isolated from newborn human foreskin and dissociated with trypsin, and keratinocytes were plated in keratinocyte growth medium (KGM, Clonetics, San Diego, CA; Boyce and Ham, 1985). Second-passage keratinocytes were plated on 60-mm plates for morphologic studies and on 35-mm plastic plates in which a labeled grid had been imprinted (Mecanex, Nyon, Switzerland) for patch-clamp studies. All cells were studied before cultures had reached confluence.

Electrophysiology

Solutions The standard external solution (KGM Ringer) was 136 mM NaCl, 5 mM KCl, 0.03 mM $CaCl_2$, 28 mM *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (Sigma, St. Louis, MO), 14 mM $NaHCO_3$, NaOH to adjust the pH to 7.4, and 10 mM glucose (pH 7.40, 330 milliosmolal). The solution was gassed with a 95% O_2/5% CO_2 mixture during the experiments. The bath was exchanged with solutions containing charybdotoxin, apamin, tetraethylammonium, and verapamil through a port in the perfusion chamber. Currents were recorded in the perforated-patch or nystatin-permeabilized whole-cell and outside-out patch configurations (Horn and Marty, 1988; Levitan and Kramer, 1990). The pipette solution contained 130 mM potassium aspartate, 20 mM KCl, 10 mM tetramethyl ammonium hydroxide/*N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, and 50 mg nystatin per ml (pH 7.25, 330 milliosmolal).

Patch-Clamp Recording Patch pipettes were pulled in two steps from borosilicate glass (Sutter Instruments, Novato, CA) on a Brown-Flaming puller (Sutter Instruments) to a resistance of 1–2 Mohm. Single-channel and whole-cell currents were measured with an Axopatch 200 patch amplifier (Axon Instruments, Foster City, CA). Currents were filtered at 2–10 kHz with an 8-pole low-pass Bessel filter, digitized by TL-1 DMA interface (Axon Instruments), sampled at 20 kHz, and stored on a computer using the Axotape program for single-channel data (version 2.0, Axon Instruments) and the pClamp program (version 6.0, Axon Instruments) for whole-cell data. Capacitance compensation was performed with the circuitry built into

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the Axopatch 200. Currents were analyzed with the pClamp program (version 6.0, Axon Instruments).

Differentiation Markers

Cornified Envelopes After exposure to K^+ channel blockers, normal human keratinocytes were incubated in [^{35}S]methionine/cysteine for 48 h (King *et al*, 1986). Ionomycin at 5 μM was added at 46 h, 2 h before assaying for cornified envelopes. Cells were solubilized in 2% SDS and placed in 4% SDS/40 mM dithiothreitol in boiling water for 30 min. The SDS-insoluble pellet was washed with 0.1% SDS/0.1% dithiothreitol, and the radioactivity incorporated into the detergent-insoluble cornified envelopes was determined by scintillation counting. To determine total protein synthesized, an aliquot of cell lysate was precipitated with 10% trichloroacetic acid on ice for 30 min, washed with 5% trichloroacetic acid, and quantitated by scintillation counting. The percent cornified envelopes was calculated as percent cpm/total protein cpm $\times$ 100. Data are presented as the mean $\pm$ SEM. Statistical analysis was performed with the analysis of variance test.

Transglutaminase Activity of membrane-bound transglutaminase was determined as described by Pillai *et al* (1990). Briefly, after harvesting the keratinocytes into 50 mM Tris(hydroxymethyl)aminomethane hydrochloride with 5 mM ethylenediamine tetraacetic acid (pH 8.0), homogenates were sonicated and centrifuged at 11,000 rpm for 1 h. The resulting pellets were resuspended by brief sonication in 50 mM Tris(hydroxymethyl)aminomethane hydrochloride/5 mM ethylenediamine tetraacetic acid/1% Triton X100, and a protein assay was performed (Bio-Rad, Hercules, CA). A 100-μl portion of this suspension was then incubated for 60 min in 50 mM Tris(hydroxymethyl)aminomethane hydrochloride, 1 mg dimethylcasein per ml, 5 mM dithiothreitol, 10 mM $CaCl_2$, and 1 μCi of ^{3}H-labeled putrescine at 37°C (final volume, 1 ml). After precipitation with 10% trichloroacetic acid, the amount of ^{3}H-labeled putrescine incorporated into casein was determined by scintillation spectroscopy. Transglutaminase activity is expressed as picomoles of ^{3}H-labeled putrescine incorporated into casein per mg of protein per min. Statistical analysis was performed with the analysis of variance test.

Intracellular Ca^{2+} Measurements

Attached Cells Keratinocytes grown on glass coverslips were incubated in 6.25 μM cell-permeant Fura-2 acetomethoxy ester plus 0.1% Pluronic (Molecular Probes, Eugene, OR) at room temperature for 15–30 min, rinsed for 30 min in KGM Ringer, and placed in a superfusion chamber mounted on an inverted microscope (Nikon, Garden City, NY). Intracellular Ca^{2+} was monitored constantly by using a ratiometric method (Paradiso *et al*, 1987). Briefly, cells were illuminated each 10 s with 200-ms flashes of 340- and 390-nm light. The resultant fluorescence at >510 nm was measured with a charge-coupled device camera, then ratioed, and stored by using the program, Fluor (Universal Imaging, West Chester, PA). Calibration was performed at the end of each experiment according to the formula for Ca^{2+} (nanomolar): $[(F_{exp} - F_{min})/(F_{max} - F_{exp})] \times K_d$, where F_{max} was obtained by adding 5 μM ionomycin; F_{min} was obtained by adding a solution containing 0 mM Ca^{2+}, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid, and 0.01% Triton; and the K_d for Fura-2 is 220 nM (Li *et al*, 1993). Alternatively, calibration was performed by first applying ionomycin to a final concentration of 5 μM and then superfusing the cells with KGM Ringer containing 50 nM and 500 nM Ca^{2+}. Both methods yielded similar results. Data are presented as the mean $\pm$ SEM. Statistical analysis was performed with the two-tailed Student's t test.

Suspended Cells Keratinocytes were trypsinized and resuspended at a concentration of 1.0–1.5 $\times$ 10^6 cells per ml in KGM Ringer. Calcium measurements were made on a Perkin-Elmer 650–40 fluorimeter, using an emission maximum of 526 and an excitation maximum of 506. Because this fluorimeter cannot measure ratioable dyes such as Fura-2, cells were incubated at 37°C with 1 μM Fluo-3 for 30 min, washed three times, and suspended in phosphate-buffered saline. Relative intracellular calcium concentrations were calculated according to the formula: $(F_{exp} - F_{min})/(F_{max} - F_{exp})$. Autofluorescence of keratinocytes was <5% at these wavelengths.

^{125}I-Labeled Charybdotoxin Labeling of Skin Sections Cryostat sections (5 μm) of unfixed human and mouse skin frozen in liquid nitrogen were thaw-mounted onto glass slides. Sections were labeled with ^{125}I-labeled charybdotoxin, as described by Gehlert and Gackenheimer (1993). The sections were washed for 10 min with 50 mM Trizma buffer, pH 7.40, containing 100 mM NaCl and 0.1% bovine serum albumin and then incubated with ^{125}I-labeled charybdotoxin (DuPont-NEN, Boston, MA) for 1 h. The sections were fixed with 5% glutaraldehyde in phosphate-buffered saline, washed in phosphate-buffered saline and distilled water several times, and dried in a desiccator. To assure the specificity of charybdotoxin binding, control sections were incubated in 100-fold excess of unlabeled charybdotoxin. Autoradiography using Ilford K5 emulsion was performed according to Wisden and Morris (1994).

RESULTS

K^+ Channel Characteristics

Whole-Cell Characteristics The keratinocyte K^+ current was linear with voltage **(Fig 1)**, indicating that it was not voltage-gated. Since 70-pS K^+ channels are present only in differentiating keratinocytes, (Mauro *et al*, 1993), we hypothesized that differentiating keratinocytes would respond to changes in extracellular K^+, whereas undifferentiated keratinocytes would not. We found that the reversal potential of whole-cell currents from keratinocytes grown in 0.07 mM Ca^{2+} did not change when extracellular K^+ was raised to 100 mM (n = 10), but in cells grown in 1.2 mM Ca^{2+} for 4 d, culture conditions that maximize the expression of K^+ channels **(Table I)**, the reversal potential shifted +11.18 $\pm$ 3.11 mV (n = 11) **(Fig 1*A*)**. When the extracellular K^+ was changed from 5 mM to 0 K^+, the reversal potential shifted in the opposite direction, −9.60 $\pm$ 0.95 mV (n = 5) **(Fig 1*B*)**.

To test the pharmacology of the K^+-sensitive currents, we applied the following K^+ channel antagonists: tetraethylammonium, an agent that blocks a wide variety of K^+ channels; charybdotoxin, a blocker specific for K channels, including large and intermediate conductance Ca^{2+}-activated K^+ channels and some voltage-gated K^+ channels; apamin, an intermediate and small conductance Ca^{2+}-activated K^+ channel blocker; verapamil, well-known as a Ca^{2+} channel blocker but also effective in blocking epithelial K^+ channels in trachea (Galietta *et al*, 1991b) and alveolae (DeCoursey, 1995). Voltage-sensitive Ca^{2+} channels are not present in keratinocytes at this stage of differentiation (Mauro *et al*, 1995). Treatment with 10 nM charybdotoxin **(Fig 1*C*)** or 10 μM verapamil **(Fig 1*D*)** decreased whole-cell currents. The observed reversal potential for the blocked conductances measured −30 $\pm$ 12 mV for cells exposed to charybdotoxin (n = 6) and −32 $\pm$ 16 mV for cells exposed to verapamil (n = 7), more positive than the calculated K^+ equilibrium potential of −84 mV. The difference in calculated reversal potential and observed reversal potentials is probably due to the imperfect selectivity of this channel for K^+ (Mauro *et al*, 1993). Neither 10 mM tetraethylammonium nor 1 μM apamin changed whole-cell currents (n = 5).

Keratinocytes possess voltage and Ca^{2+}-sensitive Cl^- currents (Mauro *et al*, 1990, 1993; Kansen *et al*, 1992). In voltage-clamped keratinocytes, Cl^- currents measured at +60 mV decreased 33 $\pm$ 9% (n = 5) when extracellular K^+ was switched from 5 to 100 mM. Since the membrane potential is held constant during these experiments, these data suggest that raised extracellular K^+ decreased Cl^- current by decreasing intracellular Ca^{2+}.

Single-Channel Studies In single-channel studies, K^+ channels were identified by their characteristic 70-pS conductance. These channels were studied by using the nystatin-permeabilized outside-out patch clamp technique (Levitan and Kramer, 1990). Application of charybdotoxin or verapamil decreased the open probability of the K^+ channel **(Fig 2)**. These experiments and the whole-cell experiments detailed above demonstrated that the K^+ channels could be blocked with charybdotoxin and verapamil. We then used these agents to determine the role of K^+ channels in controlling intracellular Ca^{2+} and modulating keratinocyte differentiation

Intracellular Ca^{2+} Is Controlled by Extracellular K^+ in Differentiating but Not in Proliferating Keratinocytes Because Ca^{2+} enters cultured keratinocytes through a voltage-insensitive channel (NSCC) (Mauro *et al*, 1995), we were able to test for the presence of K^+ channels by measuring intracellular Ca^{2+} in response to changes in extracellular K^+. If K^+ channels are present in sufficient quantities to modulate membrane potential, raising the extracellular K^+ to 100 mM will depolarize the cell, decreasing the driving force for Ca^{2+} entry and lowering intracellular Ca^{2+}.

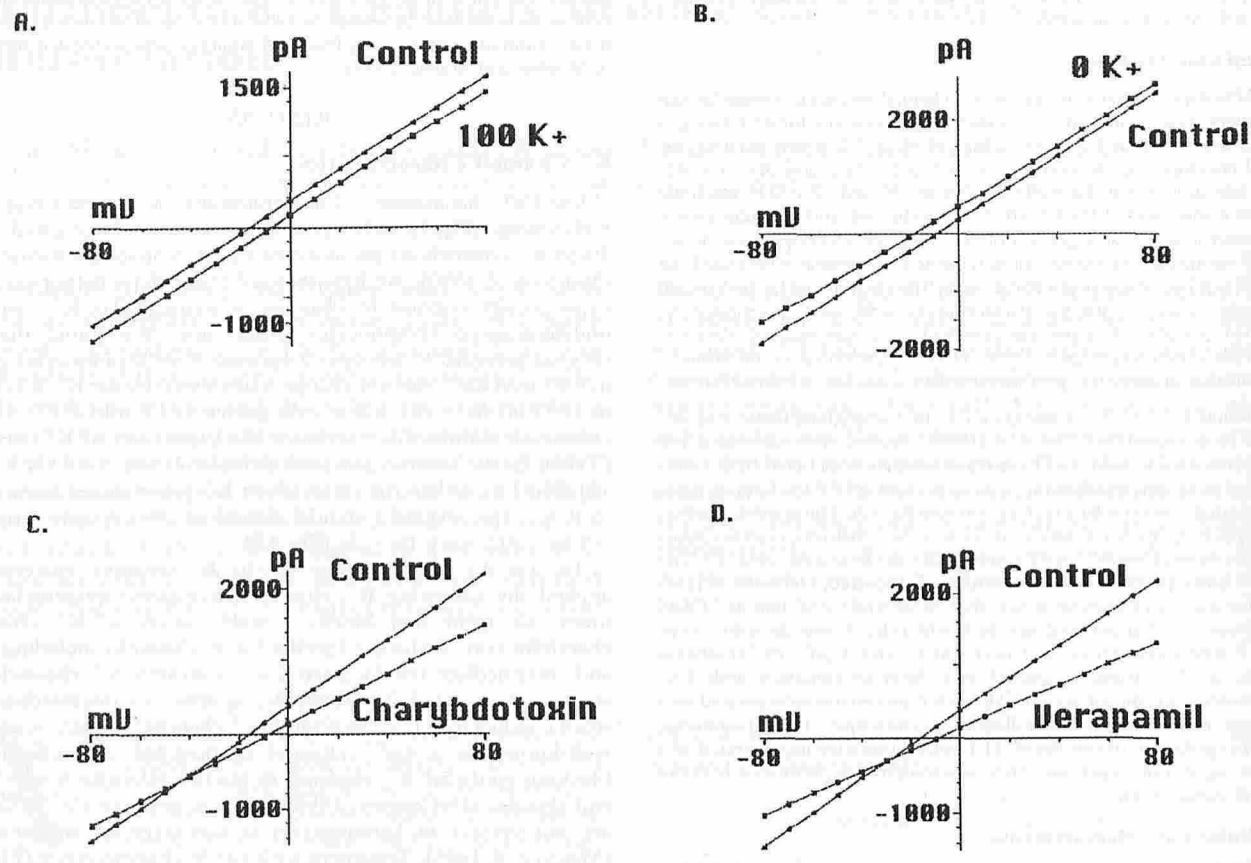

Figure 1. Whole-cell currents in differentiated keratinocytes. Current-voltage relationship. Peak total current was measured at each voltage. Normal human keratinocytes were plated in 35-mm plates into which a grid had been imbedded and cultured in KGM medium containing 1.2 mM Ca^{2+} for 4–5 d. The cell membrane potential was stepped from the holding potential of −40 mV to potentials between −80 and +80 mV in increments of 10 mV. Duration of the voltage steps was 60 ms. Currents contain both voltage-sensitive and voltage-insensitive components. *(A)* The control bath solution (KGM Ringer, see *Materials and Methods*) containing 5 mM KCl and 136 mM NaCl was replaced with 100 mM KCl and 41 mM NaCl. Average value for 11 experiments is given in the text. *(B)* The control bath solution (KGM Ringer) containing 5 mM KCl and 136 NaCl was replaced with 0 mM KCl and 141 mM NaCl. Average value for 5 experiments is given in the text. *(C)* Charybdotoxin at 10 nM was added to the control bath solution (KGM Ringer). Average value for 6 experiments is given in the text. *(D)* Verapamil at 10 μM was added to the control bath solution (KGM Ringer). Average value for 7 experiments is given in the text.

A K^+ conductance sufficient to control intracellular Ca^{2+} was present in half of the cells exposed to 1.2 mM Ca^{2+} for 2 d and in most keratinocytes exposed to 1.2 mM Ca^{2+} for 4 d **(Table I)**. When the superfusing solution was switched from KGM Ringer (containing 136 mM Na^+ and 5 mM K^+) to a buffer containing 41 mM Na^+ and 100 mM K^+, differentiating cells (i.e., keratinocytes grown in 1.2 mM Ca^{2+} for 4–5 d) responded to raised extracellular K^+ with a reversible decrease in intracellular Ca^{2+} **(Fig 3)**. Average intracellular Ca^{2+} in these cells fell from 249 ± 21 nM to 163 ± 21 nM ($p < 0.01$, $n = 16$). Conversely, when the superfusing solution was switched from KGM Ringer to a buffer containing 141 mM Na^+ and O K^+, intracellular Ca^{2+} increased from 237 ± 22 to 337 ± 33 nM ($p < 0.01$, $n = 8$).

Table I. Keratinocyte Response to Extracellular K^+ Increases as Cells Differentiate

	Cells in Which Ca_i Decreased by >25% (No. responding/total no.)[b]	
Days in Culture[a]	0.07 nM Ca^{2+}	1.2 mM Ca^{2+}
1	0/9	4/15
2	0/10	10/20
3	1/13	20/28
4	1/19	13/16

[a] Normal human keratinocytes were cultured in KGM containing 0.07 or 1.2 mM Ca^{2+} for 1–4 d.

[b] After loading of the cells with Fura-2 (see *Materials and Methods*), intracellular Ca^{2+} (Ca_i) was measured in KGM Ringer containing 5 mM K^+ and after the substitution of a superfusing solution containing 100 mM K^+. Cells were judged to respond to extracellular K^+ if their intracellular Ca^{2+} decreased by at least 25% after exposure to 100 mM K^+.

In contrast, few keratinocytes grown in 0.07 mM Ca^{2+} developed a K^+ conductance, regardless of the culture duration **(Table I)**. In these undifferentiated keratinocytes, intracellular Ca^{2+} rose from 159 ± 11 to 170 ± 14 nM (not significant, $n = 16$) after substitution of 100 mM K^+ in the bath solution. Our findings that the intracellular Ca^{2+} response develops as keratinocytes are cultured in 1.2 mM Ca^{2+} suggests that a K^+ conductance develops as these cells differentiate.

K^+ Channel Block Decreases Intracellular Ca^{2+} When 10 μM verapamil was applied to attached keratinocytes cultured 4 d in 1.2 mM Ca^{2+}, intracellular Ca^{2+} fell from 237 ± 19 to 177 ± 13 nM (mean ± SEM, $n = 10$, $p < 0.05$). Since we used a perfused system to measure rapid changes in intracellular Ca^{2+}, addition of charybdotoxin in this set of experiments was not feasible.

To test whether the block of K^+ channels causes a persistent decrease in intracellular Ca^{2+} in less differentiated keratinocytes,

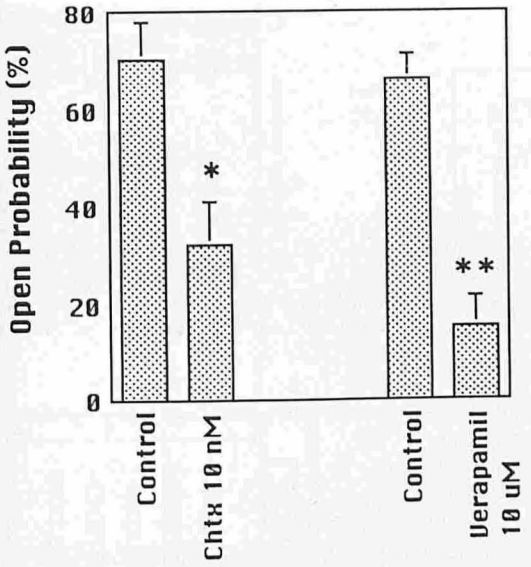

Figure 2. Charybdotoxin and verapamil decrease the K^+ channel opening probability. K^+ single-channel currents were studied by using the nystatin-permeabilized outside-out configuration. After baseline currents were recorded (control, n = 15), 10 nM charybdotoxin (n = 16) or 10 μM verapamil (n = 16) was added to the bath. Open probability was calculated as described in *Materials and Methods*. *Error bars*, SEM. *$p < 0.005$; **$p < 0.001$. Statistical significance was calculated with the two-tailed Student's t test.

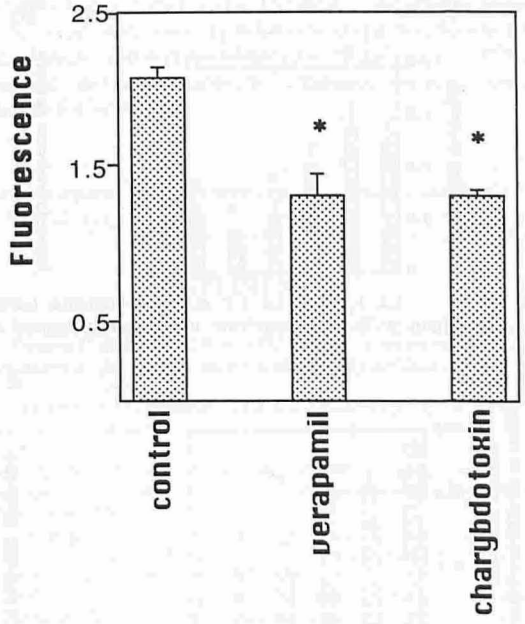

Figure 4. Charybdotoxin and verapamil decrease intracellular calcium. Cultured keratinocytes were grown to 50% confluence in 0.07 mM Ca^{2+} KGM medium and then switched to KGM medium containing 1.2 mM Ca^{2+} with a single application of 10 nM charybdotoxin or 10 μM verapamil. Cells switched to 1.2 mM Ca^{2+} without drugs were used as controls. After 24 h, cells were washed, harvested, and loaded with Fluo-3, and relative intracellular Ca^{2+} was measured (see *Materials and Methods*). *Error bars*, SEM (n = 3). *$p < 0.05$. Statistical significance was calculated with the analysis of variance test.

we measured intracellular Ca^{2+} in keratinocytes grown in 0.07 mM Ca^{2+} and then switched to 1.2 mM Ca^{2+} for 24 h with or without the addition of 10 μM verapamil or 10 nM charybdotoxin. A single addition of either agent to the culture medium caused a significant decrease in intracellular Ca^{2+} 24 h after application **(Fig 4)**. Since keratinocytes differentiate in response to raised intracellular Ca^{2+}, the ability of K^+ channels to control intracellular Ca^{2+} may be one mechanism by which K^+ channels modulate keratinocyte differentiation.

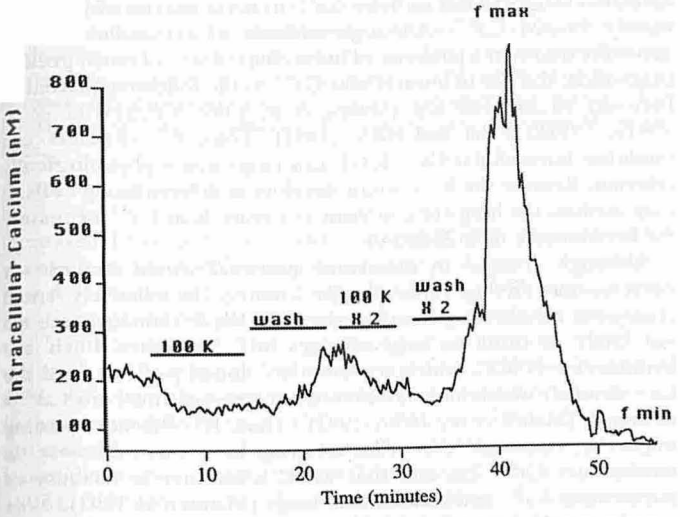

Figure 3. Extracellular K^+ controls intracellular Ca^{2+} in differentiating keratinocytes. Intracellular Ca^{2+} was measured in single Fura-2-loaded keratinocytes grown in 1.2 mM Ca^{2+} for 4 d. At the times indicated, the K^+ concentration of the superfusing solution was changed from 5 mM K^+ (KGM Ringer) to 100 mM K^+. Intracellular Ca^{2+} returned to baseline values when KGM Ringer was reapplied (wash), indicating that the effect of extracellular K^+ on intracellular Ca^{2+} was reversible. Calibration was performed at the end of the experiments by applying 5 μM ionomycin to obtain an F_{max} and then applying an ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid-containing solution to obtain the F_{min}. Average values for 16 cells are given in the text.

K^+ Currents Are Necessary for Ca^{2+}-Induced Differentiation To test whether K^+ channels were important in modulating Ca^{2+}-induced differentiation, we applied charybdotoxin, apamin, or verapamil to keratinocytes cultured in 1.2 mM Ca^{2+}. Charybdotoxin blocked the increase in transglutaminase activity and synthesis of cornified envelopes normally induced by 1–2 mM Ca^{2+} at the same concentration (10 nM) that blocked the K^+ channel **(Fig 5*A,B*)**. Verapamil at 1–10 μM also inhibited Ca^{2+}-induced transglutaminase and cornified envelope synthesis **(Fig 5 *C,D*)**, but 1 μM apamin, which did not block the K^+ channel, had no effect on Ca^{2+}-induced synthesis of transglutaminase or cornified envelopes **(Fig 5*E,F*)**. These studies show that blockade of K^+ channels inhibits Ca^{2+}-induced differentiation.

K^+ Channels Are Present in the Epidermis ^{125}I-labeled charybdotoxin labeled normal epidermis in both human and mouse skin **(Fig 6*A,B*)**. Hair follicle keratinocytes and all viable layers of the epidermis, from the basal layer to the stratum granulosum, were labeled. Charybdotoxin-sensitive cells, such as eccrine gland (Samman *et al*, 1993), and muscle **(Fig 6*D*)** (Wade and Sims, 1993), also stained with the ^{125}I-labeled charybdotoxin. Fibroblasts in the dermis, which do not bind charybdotoxin, (Rane, 1991) were not labeled **(Fig 6*A,B*)**. This binding was specific, because unlabeled charybdotoxin inhibited labeling by the radioactive compound **(Fig 6*C*)**.

Since charybdotoxin is a specific agent for K^+ channels, these experiments suggest that the K^+ channels found in *in vitro* experiments have their counterparts in the epidermis *in vivo*.

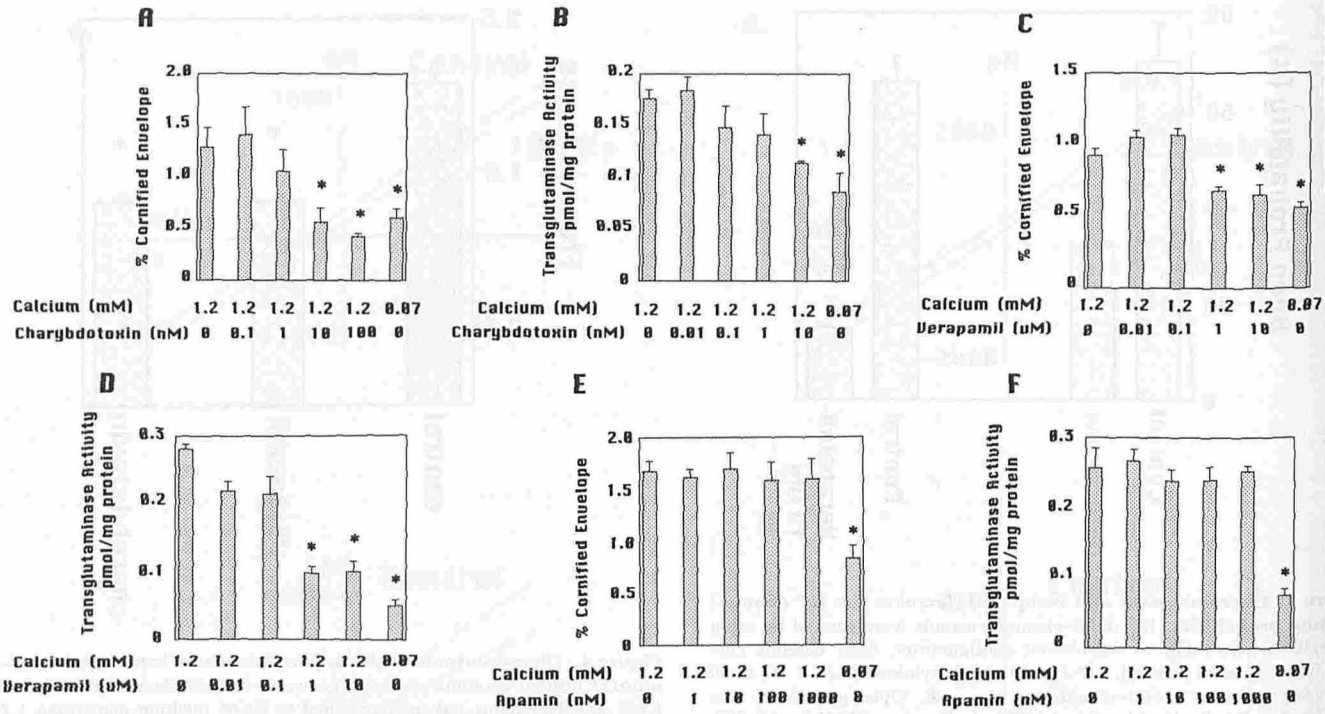

Figure 5. K^+ Channel blockers inhibit Ca^{2+}-induced differentiation. Keratinocytes were grown in 0.07 mM Ca^{2+} KGM to 50% confluence and then switched to 1.2 mM Ca^{2+} with or without various concentrations of charybdotoxin *(A,B)*, verapamil *(C,D)*, or apamin *(E,F)*. After 48 h, cornified envelope formation and transglutaminase synthesis were measured (see *Materials and Methods*). Control and experimental groups were measured in triplicate. Cells grown in 0.07 mM Ca^{2+} were used as controls. *$p < 0.05$. *Error bars*, SEM ($n = 3$ for each data point).

DISCUSSION

Experiments studying keratinocyte intracellular Ca^{2+} regulation (Xiong and Harmon, 1995) and epidermal barrier repair (Lee *et al*, 1994) suggest that a K^+ conductance develops as keratinocytes differentiate. Therefore, we tested whether the K^+ channel's ability to modulate intracellular Ca^{2+} increases as keratinocytes differentiate. Intracellular Ca^{2+} measurements using Ca^{2+}-sensitive dyes showed that intracellular Ca^{2+} is controlled by extracellular K^+ concentrations in cells cultured in 1.2 mM Ca^{2+}. A similar response to K^+ was reported in rabbit corneal epithelial cells, in which raised extracellular K^+ caused a decrease in intracellular Ca^{2+} (Rich and Rae, 1995).

Intracellular Ca^{2+} decreased when K^+ channels were blocked with verapamil or charybdotoxin, but apamin had no effect. When these agents were applied to keratinocytes grown in 1.2 mM Ca^{2+}, charybdotoxin and verapamil blocked the synthesis of cornified envelopes and transglutaminase. Thus, agents that block this channel inhibited Ca^{2+}-induced differentiation, but apamin did not inhibit differentiation. These data suggest that K^+ channels are likely to be physiologically important in the control of keratinocyte differentiation.

Unlike many K^+ channels, the keratinocyte K^+ channel is highly but not perfectly selective for K^+. This property was measured directly in previous single-channel studies (Mauro *et al*, 1993), in which the permeability ratio of Na^+ to K^+ was 0.08. K^+ channels with similar selectivity occur in the epithelial cell line T_{84} (Tabcharani *et al*, 1994) or after mutations in the external mouth of the pore region in voltage-gated K^+ channels (Kirsch *et al*, 1995). Mutations within the pore region seem to cause a more drastic loss of K^+ selectivity (Navarro *et al*, 1996). Finally, the eag mutant of *Drosophila* is permeable to both K^+ and Ca^{2+} (Bruggemann *et al*, 1993), suggesting an additional mechanism whereby K^+ channel opening might allow Ca^{2+} influx.

K^+ channels are linked to differentiation and secretion in many cells, including T lymphocytes (DeCoursey *et al*, 1987; Price *et al*, 1989), renal epithelial cells (Teulon *et al*, 1992), and brown fat, glial, and melanoma cells (Puro *et al*, 1989; Nilius and Wohlrab, 1992; Pappone and Ortiz-Miranda, 1993). The experiments presented above demonstrate that extracellular K^+ concentrations can mediate changes in intracellular Ca^{2+} of keratinocytes of approximately 90 nM Ca^{2+}. Although addition of extracellular Ca^{2+} generally results in a peak rise of intracellular Ca^{2+} of much greater magnitude, the rise in intracellular Ca^{2+} in the following plateau is between 30 and 100 nM (Sharpe *et al*, 1989; Kruszewski *et al*, 1991a, 1991b; Pillai and Bikle, 1991). Thus, K^+ channels can modulate intracellular Ca^{2+} levels in a range that is physiologically relevant. Because the K^+ current develops in differentiating cells, it may mediate the long-term increase in intracellular Ca^{2+} necessary for keratinocyte differentiation.

Although changes in membrane potential would increase or decrease the driving force for Ca^{2+} entry, the relatively small changes in membrane potential induced by the K^+ conductance are not likely to result in large changes in Ca^{2+} influx. Both the keratinocyte NSCC, which acts as a Ca^{2+} influx pathway, and the Cl^- channel, which also modifies membrane potential, are Ca^{2+}-activated (Mauro *et al*, 1990, 1993). Thus, K^+ channel opening might hyperpolarize the cells, resulting in a small increase in intracellular Ca^{2+}. Because the NSCC is sensitive to increase of intracellular Ca^{2+} in the nanomolar range (Mauro *et al*, 1993), even a small rise in intracellular Ca^{2+} would activate these channels, amplifying Ca^{2+} influx. Any membrane depolarization induced by NSCC opening could be offset by the activation of the Ca^{2+}-sensitive Cl^- channel that contributes to the resting potential of these cells (Mauro *et al*, 1990, 1993). Ca^{2+} influx may induce differentiation through activation of enzymes such as mitogen-activated protein kinase (Rosen *et al*, 1994), activation of the guanine nucleotide binding protein ras (Huang and Rane, 1994; Rosen *et al*, 1994), or up-regulation of the immediate early genes c-fos or c-jun (Cavalie *et al*, 1994).

Although the intracellular calcium response to extracellular K^+

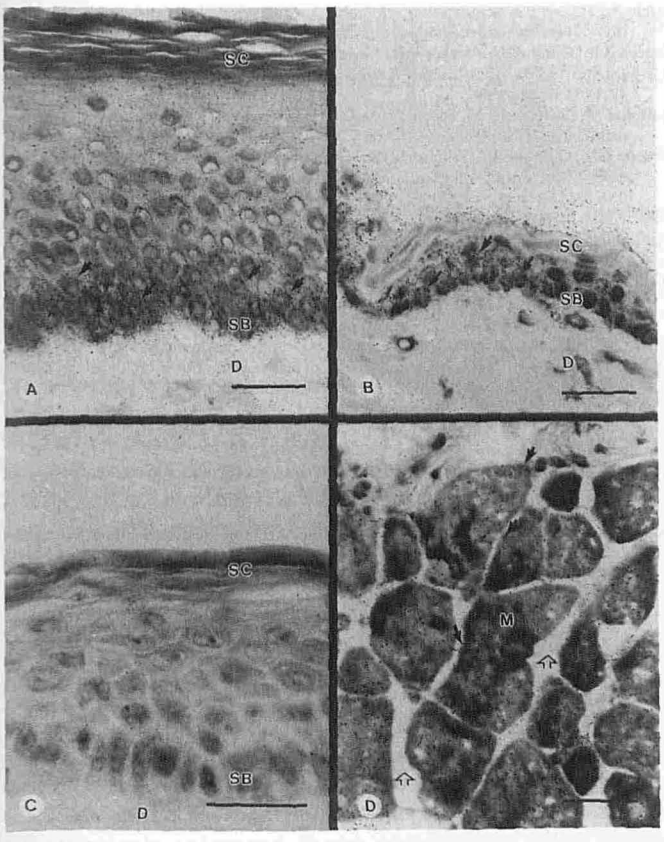

Figure 6. K^+ Channels are found in human and mouse skin. *(A)* Human skin; *(B)* hairless mouse skin. K^+ channels are localized as ^{125}I-labeled charybdotoxin binding sites (see *Materials and Methods*). ^{125}I-labeled charybdotoxin-binding sites are present throughout the epidermis. Stratum corneum and dermis show a low level of nonspecific (background) staining. *Scale bar*, 50 μm. *(C)* Specificity of localization of K^+-channels in human skin using ^{125}I-labeled charybdotoxin. Sections were incubated with ^{125}I-labeled charybdotoxin in a 100-fold excess of unlabeled charybdotoxin. *Scale bar*, 50 μm. *(D)* Localization of K^+ channels in the muscle fibers of hairless mice. ^{125}I-labeled charybdotoxin-binding sites are present every muscle fiber, whereas the connective tissue between the muscle fibers (arrowheads) shows a low level of nonspecific (background) staining. *Scale bar*, 50 μm. SC, stratum corneum; SB, stratum basale; D, dermis; M, muscle fiber.

increased throughout the period measured (4–5 d), blockade of K^+ channels inhibited differentiation as early as 2 d after exposure to raised extracellular Ca^{2+}, implying that K^+ channels could control keratinocyte differentiation even before their maximal effects on Ca^{2+} influx occurred. Thus, other mechanisms through which K^+ channels might influence cell differentiation should also be considered. K^+ channels might regulate cell volume, known to control keratinocyte differentiation (Watt and Green, 1981), influencing growth either by its actions on the cell cycle (Deutsch, 1990) or on intracellular Na^+ concentration (DuBois and Rouzaire-DuBois, 1993). Alternatively, K^+ channels may mediate cell differentiation by controlling membrane potential (Grimley and Aszalos, 1987; Chiu and Wilson, 1989), possibly acting through modification of integrin-mediated cell outgrowth (Arcangeli *et al*, 1993). Finally, K^+ channels may mediate cell growth and differentiation indirectly, by affecting the rates of protein synthesis (Lau *et al*, 1988) or production of a growth factor such as interleukin-2 (Price *et al*, 1989).

Based on the effects on intracellular Ca^{2+}, we postulated that K^+ channel density would increase as the cells differentiated from the basal layer to the stratum granulosum. In contrast, we found that labeling was uniform through the viable layer of the epidermis. These results suggest that some additional factor(s) is required to activate K^+ channels. This hypothesis is supported by electrophysiologic studies in cultured keratinocytes (Mauro *et al*, 1993), which demonstrated that keratinocyte K^+ channels required intracellular components for activation.

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