J. Membrane Biol. 132, 201–209 (1993)

The Journal of
Membrane Biology
© Springer-Verlag New York Inc. 1993

Ion Channels are Linked to Differentiation in Keratinocytes

Theodora M. Mauro†*, R. Rivkah Isseroff†, Richard Lasarow†, and Pamela A. Pappone‡
†Department of Dermatology, School of Medicine, and ‡Department of Animal Physiology, University of California, Davis, Davis, California 95616

Summary. In vivo and in vitro, keratinocyte differentiation is linked with increased extracellular Ca^{2+}. In order to correlate ion channels with cell differentiation and investigate keratinocyte membrane responses to Ca^{2+}, keratinocyte single channel currents were studied using the patch-clamp technique. The most frequently observed channel was a 14 pS nonspecific cation channel. This channel was permeable to Ca^{2+} and activated by physiological concentrations of Ca^{2+}. We also found a 35 pS Cl^- channel whose open probability increased with depolarization. Finally, a 70 pS K^+ channel was seen only in cell-attached or nystatin-permeabilized patches. We correlated channel types with staining for involucrin, an early marker of keratinocyte differentiation. While the nonspecific cation channel and Cl^- channel were seen in both involucrin positive and involucrin negative cells, all channels in which the K^+ channel activity was present were involucrin positive. Membrane currents through these channels may be one pathway by which signals for keratinocyte proliferation or differentiation are sent.

Key Words keratinocyte · patch clamp · involucrin · ion channels · calcium

Introduction

Human keratinocytes in culture respond to altered concentrations of extracellular cations with predictable changes in rates of proliferation and expression of proteins characteristic of mature keratinocytes. Increased extracellular Ca^{2+}, the most extensively studied of these ions, inhibits proliferation and induces protein markers of differentiation (Hennings, Holbrook & Yuspa, 1983; Watt, Mattey & Garrod, 1984; Rubin & Rice, 1986). In vivo, the epidermis establishes a gradient from low to high extracellular Ca^{2+} as cells move from the basal layer to the stratum granulosum (Menon, Grayson & Elias, 1985). This gradient is disturbed in the abnormal epidermal differentiation of psoriasis (Menon & Elias, 1990), implying that keratinocyte response to extracellular Ca^{2+} has physiologic significance.

In cultured mouse and human keratinocytes, raised extracellular Ca^{2+} induces an increase in Ca^{2+} influx and intracellular Ca^{2+} (Hennings et al., 1989; Pillai & Bikle, 1991; Reiss, Lipsey & Zhou, 1991), stimulates a voltage-sensitive Cl^- current (Mauro, Pappone & Isseroff, 1990), and inhibits voltage-insensitive currents carried through a nonspecific cation channel (Galietta et al., 1991). The rise in intracellular Ca^{2+} is probably mediated both through IP_3-dependent intracellular release and influx of extracellular Ca^{2+} (Jaken & Juspa, 1988; Tang et al., 1988; Kruszewski et al., 1991; Reiss et al., 1991). In this study we characterize single channel currents in keratinocytes that may underlie the membrane responses to Ca^{2+}. We also correlate the presence of specific ion channels with the presence of involucrin, a marker of differentiation (Watt, 1983). Membrane currents through these channels may be one pathway by which signals for keratinocyte proliferation or differentiation are sent.

Materials and Methods

Cell Culture

Epidermis was isolated from newborn human foreskin (Rheinwald & Green, 1975), dissociated with trypsin, and plated in modified MCDB 153 medium containing 0.07 mM Ca^{2+} and bovine pituitary extract (Boyce & Ham, 1985), termed keratinocyte growth medium (KGM, Clonetics, Boulder, CO). At confluence, keratinocytes were dissociated with trypsin and replated on 35 mm plastic plates in which a labeled grid had been imprinted (Mecanex, Switzerland). Initial plating density was 1×10^3-1×10^5 cells/ml. Passage 1–3 cells were studied 1–5 day after plating.

* *Present address of corresponding author:* Dermatology Service (190), San Francisco Veterans Administration Medical Center, 4150 Clement Street, San Francisco, California 94121.

Table 1. Bath solution (mM)

Solutions	NaCl	Na-Glutamate	KCl	CaC12	EGTA[¥]	NMDG-Cl
Control	136	0	5	0.07	0	0
Gluta-mate	0	136	5	0.07	0	0
High K^+	41	0	100	0.07	0	0
NMDG[a]	0	0	0	0.07	0	141
50 nM Ca^{2+b}	136	0	5	0.92	2	0
250 nM Ca^{2+b}	136	0	5	1.62	2	0
500 nM Ca^{2+b}	136	0	5	1.78	2	0

[a] N-methyl D-glucamine
[b] calculated
[¥] Ethylene glycol-bis-(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid.
All bath solutions contained (in mM): 14 $NaHCO_3$, 28 N-tris[hydroxymethyl] methyl-2-aminoethane sulfonic acid (TES), 10 glucose and NaOH to adjust to pH 7.40 (approximately 10).

Electrophysiology

Solutions

Concentrations (in mM) of the bath solutions are listed in Table 1. The control solution is modeled after the KGM culture medium. As directed by Boyce and Ham (1985), the pH of bathing solutions was adjusted to 7.4 with NaOH before $NaHCO_3$ was added. N-tris [hydroxymethyl] methyl-2-amino ethane sulfonic acid (TES) buffer was used to buffer pH as other buffers have been reported to block Cl^- flux (Hanrahan & Tabarchani, 1990). Solutions were bubbled with a 5% CO_2/95% O_2 mixture. All solutions were pH 7.4, 330 mOsm.

For cell-attached or inside-out patches, the standard pipette solution was the control bath solution. For nystatin permeabilized patches (Horn & Marty 1988), the pipette contained (in mM): 150 K-aspartate, 20 KCl, 10 tetramethyl ammonium hydroxide (TMA)-TES and 50 μg/ml nystatin, pH 7.25, 330 mOsm. For studies of Ca^{2+} permeation of the nonspecific cation channel, the pipette contained (in mM): 100 $CaCl_2$, 28 TMA-TES, TMA-OH to adjust the pH to 7.4.

Patch-Clamp Recording

Patch pipettes were pulled in two steps from Borosilicate glass (Sutter Instruments, Novato, CA) on a Brown-Flaming puller (Sutter Instruments, Novato, CA). The pipette tips were fire-polished to resistances of 3–4 MΩ. Following seal formations of 5–20 GΩ, single channel currents were recorded in cell-attached, inside-out or nystatin-permeabilized outside-out configurations (Hamill et al., 1981; Horn & Marty 1988). Single channel currents were measured with a List L/M EPC 7 patch amplifier (List-Medical, Germany), Cheshire Data Interface (Indec, Sunnyvale, CA), and LSI 11/73 computer (Digital Equipment, Marlboro, MA), using programs from R. Lewis, Stanford University. Currents were filtered at 1 KHz with an eight-pole low-pass Bessel filter (Frequency Devices, Haverhill, MA) and stored on videotape.

Single channel currents were analyzed and open times calculated using the pCLAMP programs (version 5.5.1, Axon Instruments). The reversal potential (E_R) and slope conductances were obtained by linear regression fitting of amplitudes from current amplitude histograms. Relative ion permeabilities at different membrane potentials were determined from the Goldman-Hodgkin-Katz potential equation. Channel open times were calculated by the pCLAMP program using a nonlinear least-squares curve-fitting routine to fit a single exponential function to the open times distribution. Open probabilities were determined from records in which only one channel was apparent as the amount of time the channel was open/total amount of recorded time as measured by the pStat program. Statistical significance was determined using One Way Analysis of Variance (ANOVA) tests. All results are presented as the mean ± SEM.

Involucrin Staining

After patch-clamp experiments, the location of the previously studied keratinocyte on the labeled grid was noted. Cells were washed with the control bath solution and stored at 5°C until staining in Michel's solution, which contained (in mM): 100 K-citrate, 100 $MgSO_4$, 100 N-ethyl maleimide, dissolved in distilled water.

Cells were stained for involucrin using an immunogold technique (Parenteau, Ekert & Rice, 1987). Briefly, the plates were washed with phosphate-buffered saline (PBS), fixed with 0.1% formalin, rinsed again in PBS, and permeabilized with 0.1% saponin. They were incubated with 5% normal autologous goat serum and 0.1% bovine serum albumin (BSA), rinsed, and incubated for 1 hr with antibody to involucrin (gift of R. Rice, UC Davis), diluted 1 : 500 in PBS. Excess antibody was washed away, and cells were then incubated with 1 : 40 Auro probe Goat anti-rabbit IgG coupled to colloidal gold (Amersham). The keratinocytes were rinsed again in PBS and fixed in 2% gluteraldehyde solution.

The cells were washed with distilled water and incubated with silver (Amersham) to enhance the colloidal gold staining. The keratinocyte whose currents had been studied was identified by its grid location and was examined for involucrin staining. The keratinocyte was scored as involucrin positive if there was uniform staining throughout the entire cell. Plates were stained in parallel, using the same procedure without the primary antibody as a negative control. Some cells detached from the plate after the experiments. Therefore, the number of cells stained is less than the number of cells studied with the patch clamp.

Results

A total of 55 cells was studied. A 14 pS nonspecific cation channel was the only channel found in 23 patches. This channel passed Na^+ and K^+ equally well, and displayed a significant permeability to Ca^{2+}. This channel was sensitive to physiological concentrations of Ca^{2+}, opening more frequently after increasing Ca^{2+} in the solution bathing the cytoplasmic side of the membrane. A 35 pS Cl^- channel was noted in 20 patches. This channel was voltage sensitive, opening more frequently with depolarization. Finally, a 70 pS K^+ channel was seen in 12 patches. This channel was voltage insensitive. It was studied with nystatin-permeabilized patches as it disappeared rapidly when studied in excised inside-out patches. Perhaps because of the larger surface area of the nystatin-permeabilized patch, the nonspecific cation channel was also seen in several of the patches with K^+ channels. While the nonspecific cation channel and Cl^- channel were seen in both involucrin positive and negative cells, all cells in which K^+ channel activity was present were involucrin positive.

Nonspecific Cation Channel

The most frequently observed channel in excised inside-out patches was a cation-selective, 14 pS (±0.6, pS, $n = 10$) channel (Fig. 1*A*). When K^+ was substituted for Na^+ in the bath solution (Fig. 1*B*), the slope conductance measured 15 pS (±0.8 pS, $n = 5$), and the reversal potential shifted from an average of 2.6 mV (±1.4 mV) to −5.6 mV (±3.1 mV), indicating that this channel did not distinguish between Na^+ and K^+ ($P_{Na}/P_K = 0.96$). E_R shifted 35 mV (±2.40 mV) positive when Na^+ was replaced by the large cation N-methyl D-glucamine (NMDG) (Fig. 1*C*), identifying it as a cation-selective channel ($P_{NMDG}/P_{Na} = 0.25$, $n = 4$). Substitution of glutamate for Cl^- had no effect on single channel current amplitude or E_R (*data not shown*), also suggesting that this channel is not permeable to anions.

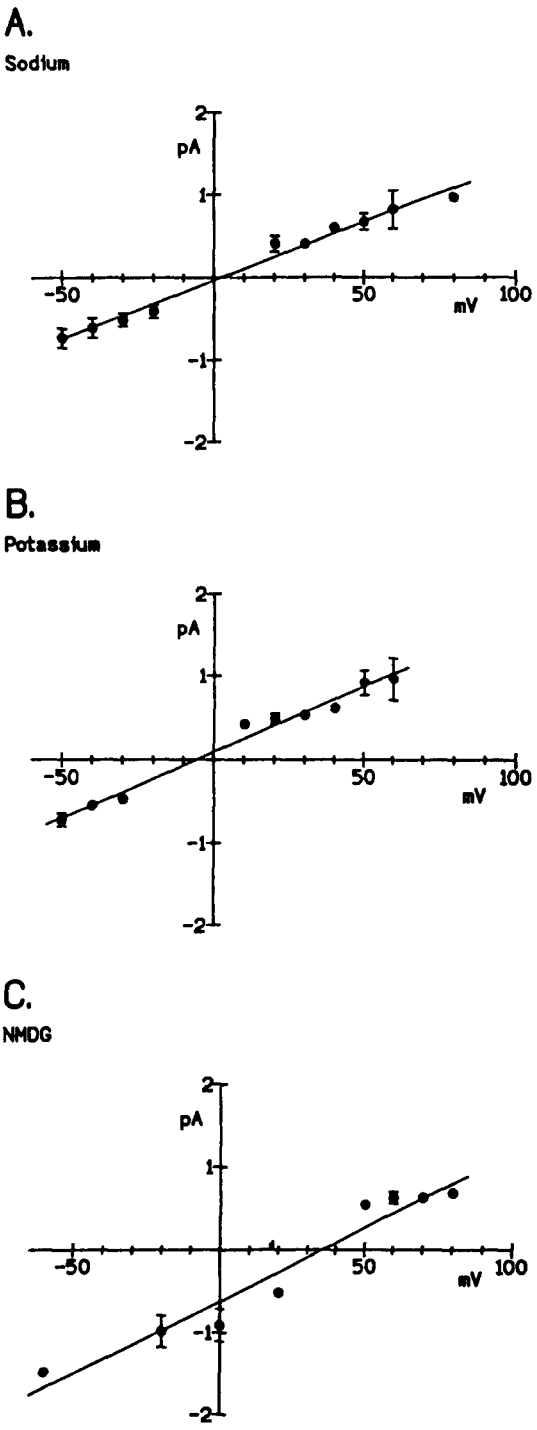

Fig. 1. Characteristics of the 14 pS nonspecific cation channel. Data from excised inside-out patches. (*A*) Current-voltage curve with symmetric NaCl solutions in bath and pipette (14 pS, E_R = 2.6 mV, $n = 10$) (*see* Table 1 for composition). (*B*) Current-voltage curve when 100 mM KCl was substituted for NaCl in the bath (internal) solution (15 pS, $E_R = -5.6$ mV, $n = 5$). The pipette (external) solution remained the control bath solution. (*C*) Current-voltage curve when internal NaCl was replaced with NMDG-Cl (16 pS, $E_R = 35$ mV, $n = 4$). Pipette solution remains the control bath solution. In this and following figures, standard error bars are smaller than the characters for some data points.

Calcium Activates the Nonspecific Cation Channel

Human keratinocytes cultured in low (0.03–0.07 mM Ca^{2+}) calcium media have intracellular Ca^{2+} concentrations in the range of 100–150 nM (Sharpe, Gillespie & Greenwell, 1989; Pillai & Bikle, 1991). Lower values of intracellular Ca^{2+}, approximately 30 nM, have been reported for mouse keratinocytes grown in low Ca^{2+} medium (Hennings et al., 1989). Intracellular Ca^{2+} levels rise after raising extracellular Ca^{2+} and, in human keratinocytes, continue to rise in the range of 500 nM after the cells reach confluence (Pillai & Bikle, 1991). The nonspecific cation channel could be affected by changes in intracellular Ca^{2+}, or could act to raise intracellular Ca^{2+} by allowing Ca^{2+} entry. Our experiments were therefore directed to exploring the effects of intracellular Ca^{2+} on the nonspecific cation channel. To investigate whether the nonspecific cation channel is sensitive to physiological intracellular Ca^{2+} concentrations, channel open probabilities were calculated from recordings made with different Ca^{2+} concentrations bathing the cytoplasmic side of the membrane. The channel opened more frequently with cytoplasmic Ca^{2+} concentrations of 500 nM or 70 μM than at 50 nM or 250 nM (Fig. 2*A*). These results suggest that the channel opens at the low cytoplasmic Ca^{2+} concentrations seen in keratinocytes grown in low Ca^{2+} medium and imply that the channel openings are influenced by the cytoplasmic Ca^{2+} concentration. Open probability of this channel did not appear to depend on voltage (*data not shown*).

Calcium Permeates the Nonspecific Cation Channel

Since the nonspecific cation channel was active at physiological Ca^{2+} concentrations, we tested whether Ca^{2+} ions could pass through it. Permeability to Ca^{2+} was tested using excised inside-out patches (Fig. 2*B*). The pipette (extracellular) solution contained 100 mM $CaCl_2$, while the bath (cytoplasmic) solution (control solution, *see* Table 1) contained Na^+ as the major cation. Under these conditions, Ca^{2+} is the major carrier of inward current through this channel. Fitting the inward current amplitudes with linear regression produced a calculated reversal potential of 0 mV and a slope conductance of 10 pS (±0.9 pS, $n = 4$) demonstrating that Ca^{2+} ions can pass through this channel. Similar results were obtained with 100 mM $CaCl_2$ in the pipette and 160 mM Na-aspartate as the cytoplasmic solution, further indicating that these are cation currents carried by Ca^{2+} (*data not shown*).

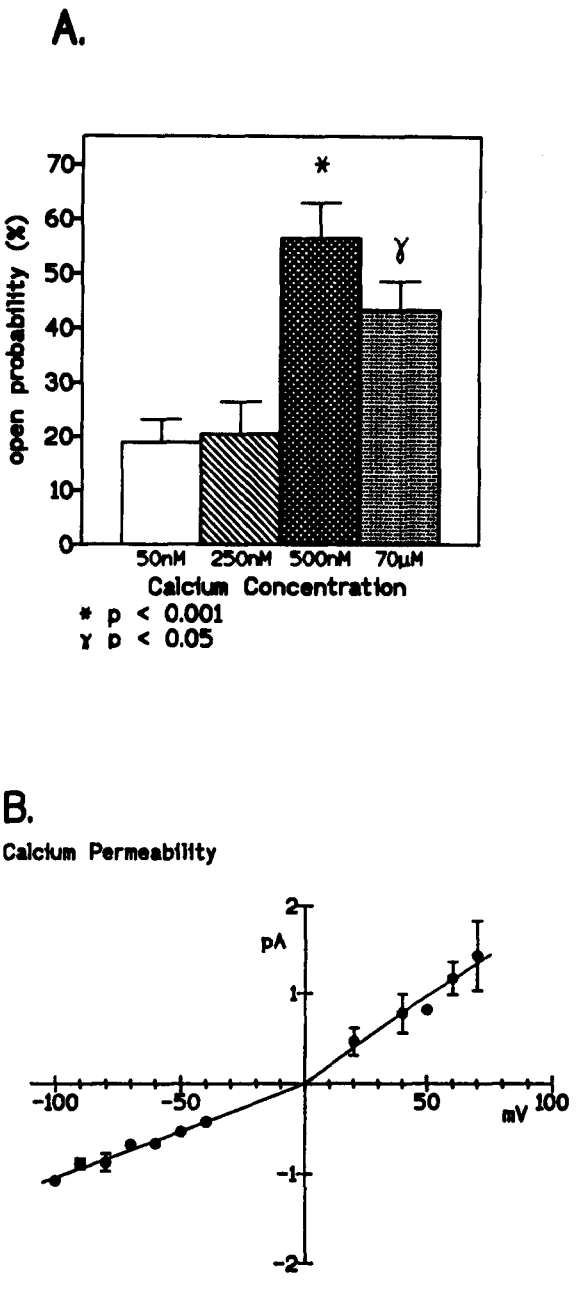

Fig. 2. Ca^{2+} sensitivity and permeability of the nonspecific cation channel. (*A*) Open probability of the nonspecific cation channel with varying Ca^{2+} concentrations in the solutions bathing the cytoplasmic side of the membrane. Experiments were done with excised inside-out patches with cytoplasmic Ca^{2+} concentrations of 50 nM ($n = 4$), 250 nM ($n = 1$), 500 nM ($n = 4$), or 70 μM ($n = 4$) with currents measured over a voltage range of −60 to +60 mV. The pipette contained the control solution with 70 μM Ca^{2+}. Data were analyzed using the ANOVA test. Groups are significantly different from 50 nM as noted. (*B*) Current-voltage curves with 70 μM Ca^{2+} bathing the cytoplasmic face. The pipette contained (in mM): 100 $CaCl_2$, 28 TMA-TES, TMA-OH to adjust the pH to 7.4. Slope conductances for inward currents (10 ± 0.8 pS) and outward currents (19 ± 2.9 pS) were determined separately, by linear regression fitting of the amplitudes from current amplitude histograms.

Chloride Channel

In whole cell studies, the major voltage-sensitive current activated by depolarization is carried by Cl^- (Mauro et al., 1990). In the present experiments we identified a 35 pS (±1.3 pS, n = 11) Cl^--selective channel in inside-out and cell-attached patches (Fig. 3*A*, *C*). When glutamate was substituted for Cl^- in the cytoplasmic solution (Fig. 3*A*), the reversal potential shifted from −2 mV (±1.3 mV) to −23 mV (±4.0 mV)(P_{glut}/P_{Cl} = 0.4), demonstrating that outward current through this channel normally is carried largely by Cl^- ions. The single channel conductance did not change with glutamate substitution (33 ± 2.1 pS, n = 4). Unlike the keratinocyte whole cell current and the single channel Cl^- current reported by Galietta et al. (1991), this channel did not rectify. However, the channel open probability increased with depolarization (Fig. 3*B*), implying that the rectification seen in whole cell studies may be due to increased channel opening at depolarized potentials. Since this was the only channel seen that carried Cl^- in both the cell-attached and inside-out configurations, and since these cells were studied under identical conditions to our previous whole cell studies, we believe that this channel carries the Cl^- current observed in whole cell studies of keratinocytes.

When studied in the cell-attached configuration (Fig. 3*C*), the channel conductance was 38 pS (±2.6 pS, n = 3), not significantly different from the conductance of the channel in the excised inside-out patches (p = 0.3). The reversal potential in the cell-attached mode measured −15 mV, relative to the cell's resting potential.

Potassium Channel

A 70 pS (±6.1 pS, n = 9) K^+-selective channel was seen in cell-attached and nystatin-permeabilized patches (Fig. 4*A*, *B*). This channel disappeared within several minutes when the cell-attached patch was excised from the cell (n = 3). Since the K^+ channel was seen only in cell-attached or nystatin-permeabilized patches, it may require intracellular organelles or cytoplasmic components which are lost when the patch is excised. With the standard solution for nystatin-permeabilized patches (150 mM KCl and 20 mM K-aspartate) in the pipette and 5 mM KCl in the bath, the reversal potential obtained by fitting the outward currents was −65 mV (±6.4 mV) (calculated reversal potential for K^+ = −87 mV). When the bath solution was changed to 100 mM K^+, the conductance measured 84 pS (±9.7 pS, n = 4) and the reversal potential shifted to −2 mV

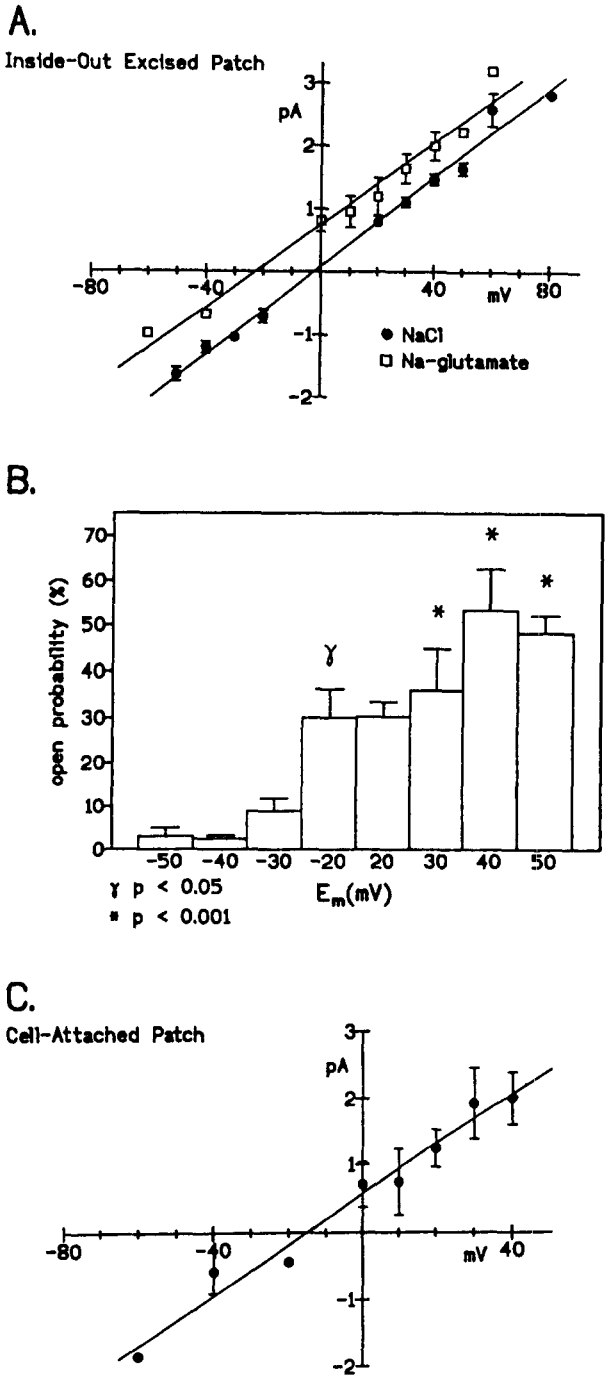

Fig. 3. Characteristics of the chloride channel. (*A* and *B*) Data from excised inside-out patches. (*A*) Current-voltage curves when the cytoplasmic NaCl solution (slope = 35 pS, E_R = −2 mV, n = 11) was replaced with Na-glutamate (slope = 33 pS, E_R = −23 mV, n = 4) (*see* Table 1 for composition of solutions). The pipette contained the control bath solution. (*B*) Channel open probability of increases with depolarization. Bath and pipette solutions were the control solution. Open probabilities were calculated, as described in Materials and Methods. Groups are significantly different from −40 mV as noted. (*C*) Current-voltage relationship from cell-attached single channel recordings. Membrane potential is measured relative to cell resting potential. The pipette contained the control bath solution.

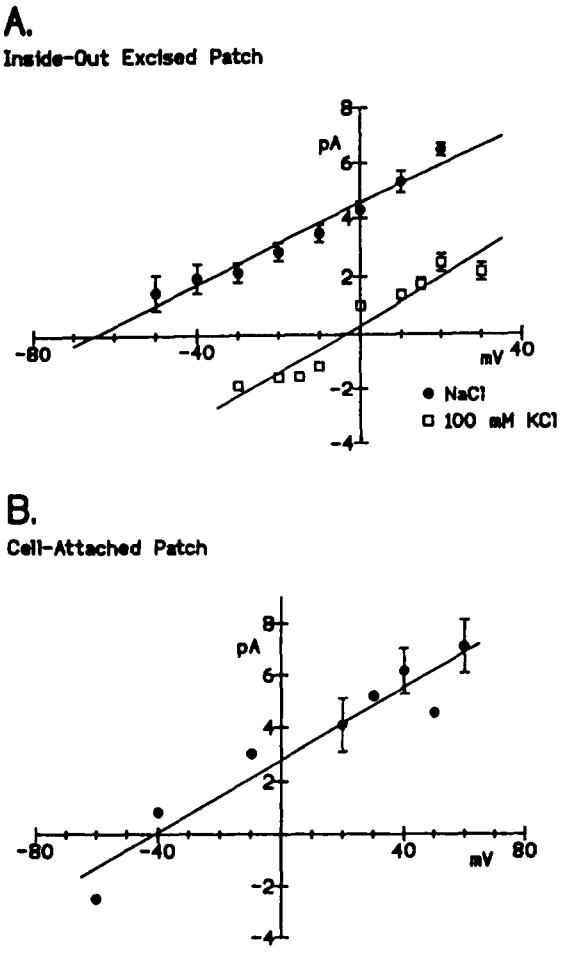

Fig. 4. Characteristics of the 70 pS potassium channel. (*A*) Current-voltage curves from single channel currents recorded from nystatin-permeabilized outside-out patches. The bath solution was the control bath solution (*see* Table 1). The pipette contained (in mM): 150 K-aspartate, 20 KCl, 10 TMA-TES and 50 μg/ml nystatin, pH 7.25, 330 mOsm. The NaCl (●) bath solution (slope = 70 pS, E_R = −65 mV, n = 9) is replaced by one (□) containing 100 mM K^+ (slope = 84 pS, E_R = −2 mV, n = 4). (*B*) Current-voltage relationship of cell-attached single channel recordings (slope = 68 pS, E_R = −41 mV, n = 3). Membrane potential is measured relative to cell resting potential. The pipette contained the control bath solution (*see* Table 1).

(±0.6 mV), indicating that this channel is highly but not perfectly selective for K^+ (P_{Na}/P_K = 0.08). This channel was also studied in the cell-attached mode (Fig. 4*B*). In this configuration, the conductance was 68 pS (±8.8 pS, n = 2), similar to that seen in the nystatin-permeabilized outside-out configuration. The channel's reversal potential was −41 mV, relative to the cell. Thus the K^+ channel's reversal potential is significantly more negative than the Cl^- channel's reversal potential or normal resting potential. Open probability of the K^+ channel did not vary with voltage in 0.07 mM extracellular Ca^{2+} (*data not shown*).

Table 2. Involucrin staining of keratinocytes

Channel Type	Positive	Significance	Negative
NSCC	4	NS	9
Cl^- Channel	5	NS	7
K^+ Channel	6	P = 0.02	0

Individual keratinocytes were examined for involucrin staining after electrophysiological study. Keratinocytes were stained and scored, as shown in Materials and Methods. Significance was calculated by Fisher's Exact Test.

Involucrin Staining

In whole cell studies of keratinocyte currents (Mauro et al., 1990), marked heterogeneity was noted not only in resting currents of keratinocytes but also in whole cell current response to extracellular Ca^{2+}. Since the presence of ion channels, particularly K^+ channels, is associated with proliferation and differentiation in other cell types (Lee et al., 1986; DeCoursey et al., 1987; Teulon et al., 1992), we attempted to correlate the presence of ion channels with involucrin, a differentiation marker in keratinocytes. Involucrin staining of identified, patch-clamped keratinocytes was performed after patch-clamp study and the results are summarized in Table 2. The nonspecific cation channel and Cl^- channel were seen in both involucrin-positive and involucrin-negative cells. In contrast, all cells in which the K^+ channels were found were involucrin positive. These results suggest that the presence of K^+ channels may be associated with the capacity to differentiate.

Discussion

This report describes three channels commonly seen in keratinocytes grown in low calcium medium. The nonspecific cation channel was permeable to Ca^{2+}, and opened more frequently with increasing Ca^{2+} concentration of the solution bathing the cytoplasmic side of the membrane. It may thus function as an alternative mechanism for increasing intracellular Ca^{2+}. The Cl^- channel was voltage sensitive, opening more frequently with depolarization. Since the whole-cell Cl^- current is the major voltage-sensitive current, this channel may control the membrane potential of the cell, thereby increasing or decreasing the driving force for Ca^{2+} entry. A voltage-insensitive K^+ channel was seen less frequently. These channels may be important in cell differentiation, as they were found only in involucrin-positive keratinocytes which have the potential to differentiate.

The most common channel seen in our studies was a small nonspecific cation channel. This channel was present in both involucrin-positive and involucrin-negative cells, did not select between K^+ and Na^+, and displayed a significant Ca^{2+} permeability. Such channels may function to allow entry of extracellular Ca^{2+} (Matthews, Neher & Penner, 1989; Nilius & Riemann, 1990; Puro, 1991), either in response to growth factors (Puro, 1991), or in concert with IP_3 mediated Ca^{2+} current (Matthews et al., 1989, Hoth & Penner, 1992). Nonspecific cation channels can be activated by changes in intracellular Ca^{2+}, with widely varying sensitivities reported (Maruyama & Petersen, 1984; Galietta et al., 1991). In keratinocytes, a similar nonspecific cation channel was reported to be sensitive to cytoplasmic Ca^{2+}. Channel activity was seen at 200 μM–2 mM cytoplasmic Ca^{2+}, but not at 1 μM (Galietta et al., 1991). Channel open probabilities at these Ca^{2+} concentrations are not reported in that paper, so it is difficult to compare directly the Ca^{2+} sensitivity of the NSCC's described in that paper and this report. Keratinocytes used in that study were cultured and patch-clamped under different experimental conditions than those in our study, which may explain the differing Ca^{2+} sensitivity. We have not fully studied the concentration necessary for activation, but have seen channel activity at cytoplasmic Ca^{2+} concentrations as low as 50 nM Ca^{2+}. This activity increases as the cytoplasmic Ca^{2+} is raised to 500 nM or 70 μM Ca^{2+}. Thus, we believe this channel is active with Ca^{2+} concentrations in the physiological range.

The current-voltage relationship of the chloride channel in this report is linear, in contrast to the outwardly rectifying whole cell current (Mauro et al., 1990). In this study, we have demonstrated that the open probability of the chloride channel increases with depolarization. Thus, we believe that the rectification seen in whole cell studies is the result of the voltage-dependent increase in channel opening, rather than intrinsic rectification of the channel itself. We were concerned that the HEPES buffer used in our earlier study had induced an artifactual current rectification, as this buffer has subsequently been shown to block epithelial cell Cl^- currents in a voltage-dependent manner which is relieved by depolarization (Hanrahan & Tabarchini, 1990). Thus, inward currents would be blocked more effectively than outward currents, leading to apparent rectification. However, we have repeated our whole cell studies using the buffer used for our single channel measurements, TES, and have found the same outward rectification of the whole cell Cl^- currents seen in earlier experiments (*data not shown*). The rectification seen in the whole cell currents is, therefore, not due to currents blocked by the HEPES buffer, but is more likely due to the voltage sensitivity of the channel itself.

It is unclear whether the single channel data reported in this study and by Galietta et al. (1991) describe the same Cl^- channel. If it is the same channel described, the difference in current rectification is unexplained. A similar discrepancy, as yet unresolved, exists in studies of the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel, where early studies reported outwardly rectifying current-voltage curves, but later studies demonstrated a linear current-voltage relationship (Anderson et al., 1991).

When cells were studied using the permeabilized patch technique, a 70 pS voltage-insensitive K^+-selective channel was seen. This channel disappeared when the cell-attached patch was removed from the membrane, and was not seen in excised inside-out patches, implying that it requires some intracellular component, such as a second messenger, for its activity. In contrast to the nonspecific cation and chloride channel discussed above, the K^+ channel was seen only in involucrin-positive cells. Involucrin staining is seen in more differentiated keratinocytes in vivo, and cells with the potential to differentiate in vitro (Watt, 1982). Since K^+ channels are associated with involucrin-positive cells, they may take part in signaling the cell to differentiate. The area of membrane sampled by a patch pipette is small, and it is possible that cells staining negative for involucrin also contained K^+ channels in their membranes. However, the fact that all the cells in which K^+ channels were found were involucrin positive argues that K^+ channels may be important in, or a marker for, cells with the potential to differentiate.

Of the 10 keratinocytes with K^+ channels we attempted to stain for involucrin, 6 remained on the plates to be studied. It is impossible to know whether the detached cells were involucrin positive or negative. However, keratinocytes which stain positively for involucrin have been shown to have decreased substrate adhesiveness (Watt & Green, 1982), implying that the cells may have detached because they were involucrin positive. Indeed, the proportion of detached cells was higher for keratinocytes with K^+ channels (4/10 cells) than those with Cl^- channels (3/15 cells) or nonspecific cation channels exposed to the usual 0.07 mM Ca^{2+} concentration (4/17 cells).

The low Ca^{2+} KGM media (70 μM) produces a heterogeneous population of cells; although reported to be predominantly basaloid, it does allow for the generation of involucrin-positive cells (Pillai et al., 1990). Half of the keratinocytes studied stained positively for involucrin, which is a greater proportion than expected for keratinocytes grown in low Ca^{2+} media. The most likely explanation for

this finding was our plating technique which produced isolated single cells. Single keratinocytes are preferable for electrophysiology, but they tend to be larger and large cells are more likely to be involucrin positive (Watt & Green, 1981, Watt, Jordan & O'Neill, 1988).

There are several mechanisms by which these membrane channels may be involved in keratinocyte differentiation. Intracellular Ca^{2+} concentration and Ca^{2+} flux seem to be key in keratinocyte differentiation, and there are roles that each of the channels described above might play in influencing Ca^{2+} entry. Activation of the Cl^- or K^+ conductances might stimulate a sustained calcium influx by hyperpolarizing the membrane and increasing the driving force for Ca^{2+} entry. The nonspecific cation channel may afford a parallel pathway for Ca^{2+} entry, especially later in differentiation as the intracellular Ca^{2+} levels rise above 250 nM.

Other functions not related to Ca^{2+} entry may also be significant. K^+ channels are important in the proliferation of several cell types (Decoursey et al., 1987; Nilius & Wohlrab, 1992; Pappone & Ortiz-Miranda, 1992; Teulon et al., 1992), and, given the dependence of the "Ca^{2+}-switch" in keratinocytes on extracellular K^+ (Hennings et al., 1983), these channels may play a central role in keratinocyte differentiation. K^+ channels regulate cell volume in kidney epithelial cells (Ubl et al., 1988) and in lens cells, where cell elongation and differentiation is dependent on an increase in cell volume (Parmalee & Beebe, 1988). Such changes in cell shape or size have been shown to alter differentiation in keratinocytes (Watt & Green, 1981; Watt et al., 1988). Functional K^+ channels are necessary for proliferation and protein synthesis in T lymphocytes (Sabath et al., 1986), and progressive increases in intracellular K^+ concentration stimulate, then inhibit protein synthesis in oocytes (Horowitz & Lau, 1988). Since intracellular K^+ increases as keratinocytes differentiate (Hennings et al., 1983), it may influence protein synthesis in a similar fashion in keratinocytes.

In this report we have described three types of ion channels in cultured keratinocytes grown under conditions which generate proliferative cells. The small nonspecific cation channel is activated by Ca^{2+}, the Cl^- channel is activated by depolarization, and the K^+ channel requires intracellular constituents for activation. The K^+ channel is seen only in involucrin-positive keratinocytes, i.e., cells which have committed to differentiate. Each of these channels may play a role in keratinocyte growth or differentiation.

This work was supported in part by a National Institutes of Health grant K08 AR01853-03 and a National Science Foundation grant DCB-9009915 (to T.M.M.); National Institutes of Health Research Career Development Award K04 ARO 1803 and AR 39031 (to R.R.I.) and a National Institutes of Health grant GM-44840 (to P.A.P.).

References

Anderson, M.P., Rich, D.P., Gregory, R.J., Smith, A.E., Welsh, M.J., 1991. Generation of cAMP-activated chloride currents by expression of CFTR. *Science* **251**:679–682

Boyce, S.T., R.G. Ham. 1985. Normal human epidermal keratinocytes. *In:* In Vitro Models for Cancer Research. M. Webber and L. Sekely, editors. pp. 245–274. CRC, Boca Raton, FL

DeCoursey, T.E., Chandy, K.G., Gupta, S., Cahalan, M.D. 1987. Mitogen induction of ion channels in murine T lymphocytes. *J. Gen. Physiol.* **89**:405–420

Galietta, L.J., Barone, V., DeLuca, M., Romeo, G. 1991. Characterization of chloride and cation channels in cultured human keratinocytes. *Pfluegers Arch.* **418**:18–25

Hamill, O.P., Marty, A., Neher, E., Sakmann, B., Sigworth, F. 1981. Improved patch-clamp techniques for high resolution current recorded from cells and cell-free membranes. *Pfluegers Arch.* **391**:85–100

Hanrahan, J.W., Tabarchani, J.A. 1990. Inhibition of an outwardly rectifying anion channel by HEPES and related buffers. *J. Membrane Biol.* **116**:65–77

Hennings, H., Holbrook, K.A., Yuspa, S.H. 1983. Factors influencing calcium-induced terminal differentiation in cultured mouse epidermal cells. *J. Cellular Physiol.* **116**:265–281

Hennings, H., Kruszewski, Yuspa, S.H., Tucker, R.W. 1989. Intracellular calcium alterations in response to increased external calcium in normal and neoplastic keratinocytes. *Carcinogenesis* **10**:777–780

Horn, R., Marty, A. 1988. Muscarinic activation of ionic currents measured by a new whole cell recording method. *J. Gen. Physiol.* **92**:145–159

Horowitz, S.B., Lau, Y.-T. 1988. A function that relates protein synthetic rates to potassium activity in vivo. *J. Cellular Physiol.* **135**:425–434

Hoth, M., Penner, R. 1992. Depletion of intracellular calcium stores activates a calcium current in mast cells. *Nature* **355**:353–355

Jaken, S., Yuspa, S.H. 1988. Early signal for keratinocyte differentiation: role of Ca^{2+} -mediated inositol lipid metabolism in normal and neoplastic cells. *Carcinogenesis* **9**:1033–1038

Kruszewski, F.H., Hennings, H., Yuspa, S.H., Tucker, R.W. 1991. Regulation of intracellular free calcium in normal murine keratinocytes. *Am. J. Physiol.* **261**:C767–C773

Lee, S.C., Sabath, D.E., Deutsch, C., Prystowsky, M.B. 1986. Increased voltage-gated potassium conductance during interleukin-2 stimulated proliferation of a mouse helper T lymphocyte clone. *J. Cell Biol.* **102**:1200–1208

Maruyama, Y., Petersen, O.H. 1984. Single calcium-dependent cation channels in mouse pancreatic acinar cells. *J. Membrane Biol.* **81**:83–87

Matthews, G., Neher, E., Penner, R. 1989. Second messenger-activated calcium influx in rat peritoneal mast cells. *J. Physiol.* **418**:105–130

Mauro, T., Pappone, P.A., Isseroff, R.R. 1990. Extracellular calcium affects the membrane currents of cultured human keratinocytes. *J. Cellular Physiol.* **143**:13–20

Menon, G., Grayson, S., Elias, P. 1985. Ionic calcium reservoirs in mammalian epidermis: ultrastructural localization by ion-capture cytochemistry. *J. Invest. Dermatol.* **54:**508–512

Menon, G.K., Elias, P.M. 1990. Ultrastructural localization of calcium in psoriatic and normal human epidermis. *Arch. Dermatol.* **127:**57–63

Nilius, B., Riemann, D. 1990. Ion channels in human endothelial cells. *Gen. Physiol. Biophys.* **9:**89–112

Nilius, B., Wohlrab, W. 1992. Potassium channels and regulation of proliferation of human melanoma cells. *J. Physiol.* **445:**537–548

Pappone, P.A., Ortiz-Miranda, S. 1992. Blockers of voltage-gated K^+ channels inhibit proliferation of cultured brown fat cells. *Am. J. Physiol. (in press)*

Parenteau, N.L., Ekert, R.L., Rice, R.H. 1987. Primate involucrins: antigenic relatedness and detection of multiple forms. *Proc. Natl. Acad. Sci. USA* **84:**7571–7575

Parmalee, J.T., Beebe, D.C. 1988. Decreased membrane permeability to potassium is responsible for the cell volume increase that drives lens fiber cell elongation. *J. Cellular Physiol.* **134:**491–496

Pillai, S., Bikle, D.D., Mancianti, M.-L., Cline, P., Hincenbergs, M. 1990. Calcium regulation of growth and differentiation of normal human keratinocytes: modulation by differentiation competence by stages of growth and extracellular Ca^{2+}. *J. Cellular Physiol.* **143:**294–302

Pillai, S., Bikle, C.D. 1991. Role of intracellular-free calcium in the dornified envelope formation of keratinocytes: differences in the mode of action of extracellular calcium and 1,25 dihydroxyvitamin D_3. *J. Cellular Physiol.* **146:**94–100

Puro, D.G. 1991. A calcium-activted, calcium-permeable ion channel in human glial cells: modulation by basic fibroblast growth factor. *Brain Res.* **548:**329–333

Reiss, M., Lipsey, L.R., Zhou, Z.L. 1991. Extracellular calcium-dependent regulation of transmembrane calcium fluxes in murine keratinocytes. *J. Cellular Physiol.* **147:**281–291

Rheinwald, J.G., Green, H. 1975. Serial cultivation of strains of human keratinocytes: The formation of keratinizing colonies from single cells. *Cell* **6:**331–344

Rubin, A., Rice, R. 1986. Differential regulation by retinoic acid and transglutaminase in cultured neoplastic and normal human keratinocytes. *Cancer Res.* **46:**2356–2361

Sabath, D.E., Monos, D.S., Lee, S.C., Deutsch, C., Prystowsky, M.B. 1986. Cloned T-cell proliferation and synthesis of specific proteins are inhibited by quinine. *Proc. Natl. Acad. Sci. USA* **83:**4739–4743

Sharpe, G.R., Gillespie, J.I., Greenwell, J.R. 1989. An increase in intracellular free calcium is an early event during differentiation of cultured keratinocytes. *FEBS Lett.* **254:**25–28

Tang, W., Ziboh, V., Isseroff, R.R., Martinez, D. 1988. Turnover of inositol phopholipids in cultured murine keratinocytes: possible involvement of inositol triphosphate in cellular differentiation. *J. Invest. Dermatol.* **90:**37–43

Teulon, J., Ronco, P.M., Geniteau-Legendre, M., Baudouin, B., Estrade, S., Cassingena, R., Vandewalle, A. 1992. Transformation of renal tubule epithelial cells is associated with emergence of Ca^{2+}- insensitive K^+ channels and altered mitogenic sensitivity to K^+ channel blockers. *J. Cellular Physiol.* **151:**113–125

Ubl, J., Murer, H., Kolb, H. 1988. Ion channels activated by osmotic and mechanical stress in membranes of opossum kidney cells. *J. Membrane Biol.* **104:**223–232

Watt, F. 1983. Involucrin and other markers of keratinocyte terminal differentiation. *J. Invest. Dermatol.* **81:**1005–1035

Watt, F., Green, H. 1981. Involucrin staining is correlated with cell size in human epidermal cultures. *J. Cell. Biol.* **90:**738–742

Watt, F., H. Green. 1982. Stratification and terminal differentiation of cultured epidermal cells. *Nature* **295:**434–436

Watt, F., Mattey, D., Garrod, D. 1984. Calcium-induced reorganization of desmosomal components in cultured human keratinocytes. *J. Cell. Biol.* **99:**2211–2215

Watt, F., Jordan, P.W., O'Neill, C.H. 1988. Cell shape controls terminal differentiation of human epidermal keratinocytes. *Proc. Natl. Acad. Sci. USA* **85:**5576–5580

Received 25 September 1992