Altered Calcium-Mediated Cell Signaling in Keratinocytes Cultured from Patients with Neurofibromatosis Type 1

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Capacitative calcium entry and calcium wave propagation were studied in keratinocytes cultured from control persons and patients with type 1 neurofibromatosis. The cells were stimulated mechanically in the presence of inhibitors of gap-junctional or ATP-mediated communication to determine which pathways are operative in Ca^{2+} signaling between these cells. Keratinocytes cultured from patients with type 1 neurofibromatosis (NF1) had a tendency to form cultures with markedly altered calcium-related signaling characteristics. Specifically, the resting Ca^{2+} levels, intracellular Ca^{2+} stores, capacitative calcium influx, and gap-junctional signal transduction were defective in NF1 keratinocytes. Western transfer analysis revealed apparently equal connexin 43 protein levels in normal control and in NF1 keratinocytes. Indirect immunofluorescence, however, demonstrated that connexin 43 was relatively evenly distributed in NF1 cells and did not form typical gap-junctional plaques between keratinocytes. Furthermore, the speed of the calcium wave was reduced in NF1 cells compared to normal keratinocytes. The results demonstrate that keratinocytes cultured from patients with NF1 display altered calcium-mediated signaling between cells. (*Am J Pathol* 2002, 160:1981–1990)

NF1 refers to type 1 neurofibromatosis, which has been linked with mutations of the *NF1* gene.^{1–3} Mutations of the *NF1* gene can cause haploinsufficiency.⁴ The hallmarks of NF1 include pigmented café au lait spots of the epidermis, cutaneous neurofibromas, and hamartomas of the iris.^{3,5} Furthermore NF1 is associated with osseous malformations, learning defects, intellectual handicap, and predisposition to selected malignant transformations. The *NF1* gene has been referred to as a tumor-suppressor gene because cells of malignant tumors of

neurofibromatosis patients may display loss of heterozygosity of the *NF1* gene.⁶ *NF1* has also been referenced as a histogenesis control gene because NF1 is often associated with dysplasia, such as osseous malformations, and because normal human and mouse tissue repair has been shown to involve activation of the *NF1* gene.^{7–9} However, the molecular functions of the NF1 protein are not entirely known. NF1 protein has been shown to accelerate the switch of active RasGTP to inactive RasGDP in various cell types.^{10–13} NF1 protein has been shown to interact with tubulin,¹⁴ and we have recently shown that the NF1 protein forms a short-lasting, high-affinity link with bundles of intermediate filaments of the cytoskeleton during the formation of cellular contacts in differentiating keratinocytes.¹⁵ Furthermore, the cytoskeleton has been demonstrated to be abnormal in different types of cells cultured from patients with NF1.^{15,16}

An intact cytoskeleton is an integral part of the mechanism for calcium-mediated cell signaling. Disruption of cytoskeletal microfilaments with cytochalasin D has been shown to inhibit capacitative calcium entry in vascular endothelial cells.¹⁷ The present study used cultures of human keratinocytes that can be used as a well-documented cell differentiation model, and that have been successfully used for studies on expression and functions of the NF1 protein.¹⁵ Furthermore, keratinocytes have been demonstrated to propagate intercellular calcium waves.¹⁸

Ca^{2+} is the most common signal transduction element in modulating cells, eg, cell growth and differentiation.¹⁹ The level of free intracellular calcium ($[\text{Ca}^{2+}]_i$) is regulated and maintained low (~ 100 nmol/L) through the

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action of a number of binding proteins and ion exchange mechanisms.^{20–22} The endoplasmic reticulum (ER) is a major site for sequestered Ca^{2+} ions. It is of interest to note that a previous study has demonstrated neurofibromin localization to smooth ER in neurons.²³ A coupling has been demonstrated between the filling state of the intracellular calcium stores and the plasma membrane calcium-channel activity.^{24,25} Thus, a subset of calcium channels has been termed store-operated calcium channels (SOCs).^{26–28}

In electrically nonexcitable cells, activation of cell-surface receptors that stimulate IP_3 production evokes a biphasic increase in cytosolic-free Ca^{2+} . Receptor-induced Ca^{2+} signals comprise two interdependent components—rapid Ca^{2+} release from Ca^{2+} stores in the ER and Ca^{2+} entry through slowly activating plasma membrane SOCs.²⁹ The trigger for SOC activation is decreased Ca^{2+} concentration in ER lumen.^{28,30}

Many cell types coordinate their activities by transmitting waves of elevated intracellular calcium levels from cell to cell. Intercellular calcium waves have been studied in many different cell types such as neurons, smooth muscle cells, and osteoblastic cells.^{31–33} Two mechanisms for calcium waves have been identified. On the one hand, generation of calcium waves relies on autocrine and paracrine activity of ATP. Mechanical stimulation of cells results in the release of ATP that activates purinergic receptors on neighboring cells.³⁴ This in turn triggers the release of IP_3 and intracellular calcium.³⁵ Secondly, calcium waves have been shown to spread from cell to cell via gap junctions. Diffusion of IP_3 through gap-junctional pores has been shown to mediate release of IP_3 -sensitive intracellular calcium stores in neighboring cells.^{36–38} Cell-cell interactions, and especially gap-junctional intercellular communication, seem to play crucial roles in cell regulation, differentiation, development, and cancer formation.^{39–41} The absence or reduced number of gap junctions and gap-junctional intercellular communication have been observed in a large number of human and animal tumor cell lines.^{39,42}

The aim of the present study was to investigate which pathways may play a role in Ca^{2+} signaling in normal and NF1 keratinocytes. The cells were stimulated mechanically in the presence of inhibitors of gap-junctional or ATP-mediated communication to determine which pathways are operative in Ca^{2+} signaling between these cells. Given the importance of Ca^{2+} influx for a variety of cellular processes, such as Ca^{2+} oscillations,^{43,44} secretion,⁴⁵ and enzymatic regulation,⁴⁶ our results are likely to be of widespread importance to a plethora of physiological processes. The present results suggest that the pathogenesis of NF1 may involve impaired calcium-mediated cell signaling.

Materials and Methods

Tissue Samples and Cell Culture

Cell cultures were initiated from skin samples from a total of eight healthy volunteers (age, 29 to 61 years) and from

a total of eight volunteering patients with NF1 (age, 23 to 40 years) obtained from operations performed for cosmetic and therapeutic reasons at the Department of Dermatology, University of Oulu, or the Department of Surgery, University of Turku, Finland, with the appropriate approval of the Joint Ethical Committee of the Oulu University Hospital and the Joint Ethical Committee of the Turku University Hospital and the University of Turku. Primary cultures of keratinocytes were established from skin samples by a modification of a method previously described.⁴⁷ Keratinocytes (second to eighth passage) were maintained in serum-free, low-calcium keratinocyte growth medium (KGM; Gibco BRL/Life Technologies, Gaithersburg, MD). Before experimentation, two identical groups of cultures were seeded and grown in KGM, which contains a low (<0.1 mmol/L) calcium concentration until $\sim 40\%$ confluency for thapsigargin experiments, and to high confluency for cell signaling analysis.

Intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) Measurements by Fluorescence Ratio Imaging

For intracellular Ca^{2+} measurements by fluorescence ratio imaging analysis the cells were incubated in the loading medium containing 1 $\mu\text{mol/L}$ fura-2/AM and 0.1% pluronic, F-127 (Molecular Probes, Eugene, OR) in balanced salt solution, (10 mmol/L HEPES, pH 7.4, 120 mmol/L NaCl, 4 mmol/L KCl, 1 mmol/L KH_2PO_4 , 1 mmol/L MgCl_2 , 5 mmol/L glucose, 0.05 mmol/L CaCl_2) for 30 minutes. A dispersing agent, pluronic F-127, was included in the loading medium to facilitate the efficient loading of keratinocytes with fura-2/AM. The cells were washed and incubated in defined keratinocyte growth medium (Gibco BRL/Life Technologies) containing 1.8 mmol/L of Ca^{2+} for 30 minutes to eliminate any unhydrolyzed fura-2/AM. Alternatively the cells were incubated in keratinocyte growth medium containing 3.5 mmol/L of heptanol, a gap junction blocker (Sigma, St. Louis, MO), or 100 $\mu\text{mol/L}$ of suramin, an ATP-receptor antagonist (Calbiochem, La Jolla, CA) for intracellular calcium-mediated cell-signaling experiments. Loaded keratinocytes on the Belco coverslips were placed on the stage of a Nikon TMD Diaphot inverted microscope surrounded by a plastic incubation hood (Nikon, Tokyo, Japan) and the cells were covered with 1 ml of prewarmed ($+37^\circ\text{C}$) defined keratinocyte growth medium containing 5 mmol/L of EGTA⁴⁸ or 1.8 mmol/L of Ca^{2+} , and in cell-signaling experiments medium containing 3.5 mmol/L of heptanol (Sigma) or 100 $\mu\text{mol/L}$ of suramin, the inhibitor of P_2 -purinergic receptors (Calbiochem, La Jolla, CA),^{49–51} alternatively. The chamber was kept at $+37^\circ\text{C}$. To study intracellular calcium stores and capacitative calcium influx, a stock solution of 100 $\mu\text{mol/L}$ of thapsigargin (Molecular Probes) was added to the medium to result in a final concentration of 1 $\mu\text{mol/L}$. The peak $[\text{Ca}^{2+}]_i$ elevation was measured in 20 to 25 cells from each selected field. For cell-signaling experiments, wounds were made in the confluent keratinocyte monolayer with a single scratch in a speed of ~ 2 mm/second with a fresh pipette tip, which removed a four to eight cell-wide path (~ 100 to

150 μm). The pipette tip removed most cells from the wound. The peak $[\text{Ca}^{2+}]_i$ elevation was measured in 10 to 25 cells in the second cell row from the wound edge. Dynamic video imaging was performed using the MCID/M2 system (Imaging Research Inc., Brock University, Ontario, Canada) installed in an Intel 403E microcomputer linked to an Image 1280 image processor (Matrox, Dorval, Quebec, Canada). Fura-2/AM-loaded keratinocytes were excited with a 100 W xenon lamp using both 340 DF 10-nm and 380 DF 13-nm interference filters (Omega Optical Inc., Brattleboro, VT) mounted in a computer-driven filter wheel (MAC 2000; Ludl Electronic Products Ltd., Hawthorne, NY). A Nikon ND 8 filter was used to reduce the excitation light to a level that does not damage the cells during UV irradiation. The emitted light was then allowed to pass through the 400-nm dichroic mirror and the 510 BW 40-nm interference filter (Nikon). The objective used in the experiments (Nikon CF Fluor DL air) was $\times 20$, and the resulting images were guided to a Dage 72E CCD camera (Dage-MTI Inc., Michigan City, MI) coupled to a videoscope charge-coupled device image intensifier (model KS-1381, Videoscope International, Ltd., Washington, DC). Calibration of Ca^{2+} -dependent fluorescence was performed by sequential saturation of the dye with 15 to 40 $\mu\text{mol/L}$ of ionomycin ($\pm 10 \text{ mmol/L}$ CaCl_2) to maximum fluorescence (F_{max}), followed by chelation of Ca^{2+} to minimum fluorescence (F_{min}) with 7.5 mmol/L of EGTA plus 60 mmol/L of Tris, pH 10.5. Ratio fluorescence with alternate 340/380 excitation at 5-second intervals was used for validation of each set of experiments. Standard formulae were used for the calculation of $[\text{Ca}^{2+}]_i$ using a K_d of fura-2 for Ca^{2+} of 224 nmol/L .⁵² All of the images (640×512 pixels) were acquired by real-times at 340-nm and 380-nm wavelengths with an interval of 5 seconds. The total number of ratio images was 80 in capacitative calcium experiments and 20 or 40 in intracellular calcium-mediated cell-signaling experiments. All of the frames were displayed in pseudocolors characterizing $[\text{Ca}^{2+}]_i$.

Assessment of Ca^{2+} Influx by the Mn^{2+} Quench of Cytosolic Fura-2 Fluorescence

Keratinocytes were loaded with the AM ester of fura-2 as above. Loaded keratinocytes on the Belco coverslips were placed on the stage of a Nikon TMD Diaphot inverted microscope surrounded by a plastic incubation hood and the cells were covered with 1 ml of prewarmed ($+37^\circ\text{C}$) defined keratinocyte growth medium containing 1.8 mmol/L of MnCl_2 . The chamber was kept at $+37^\circ\text{C}$. To study capacitative calcium influx, a stock solution of 100 $\mu\text{mol/L}$ of thapsigargin (Molecular Probes) was added to the medium to result in a final concentration of 1 $\mu\text{mol/L}$. In the extracellular ATP experiments, ATP (100 $\mu\text{mol/L}$ final concentration) (Calbiochem) was added to the culture medium during the measurements. Mn^{2+} quench of human keratinocyte cytosolic fura-2 fluorescence was monitored using the MCID/M2 system (Imaging Research Inc., Brock University, Ontario, Canada) installed in an Intel 403E microcomputer linked to an Image 1280 image processor (Matrox; Dorval, Quebec,

Canada). Fura-2/AM-loaded keratinocytes were excited with a 100 W xenon lamp using both 340 DF 10-nm and 380 DF 13-nm interference filters (Omega Optical Inc.) mounted in a computer-driven filter wheel (MAC 2000; Ludl Electronic Products Ltd.). A Nikon ND 8 filter was used to reduce the excitation light to a level that does not damage the cells during UV irradiation. The emitted light was then allowed to pass through the 400-nm dichroic mirror and the 510 BW 40-nm interference filter (Nikon). The objective used in the experiments (Nikon CF Fluor DL air) was $\times 20$, and the resulting images were guided to a Dage 72E CCD camera (Dage-MTI Inc.) coupled to a videoscope CCD image intensifier (model KS-1381). All of the images (640×512 pixels) were acquired by real time at 340-nm and 380-nm wavelengths with an interval of 10 seconds. The total number of ratio images was 80 in Mn^{2+} quench experiments. The fura-2 fluorescence was measured in 20 to 25 cells from each selected field. From the channels of 340-nm and 380-nm wavelengths we plotted and evaluated the total fura-2 fluorescence intensity. By this way we will observe a slow or rapid decline of fura-2 fluorescence depending on the magnitude of the Mn^{2+} influx.

Antibodies

Primary antibody used in immunolabeling was monoclonal mouse anti-connexin-43 (Cx43) (Zymed Laboratories, Inc., San Francisco, CA). Monoclonal and polyclonal anti-Cx43 was used for Western blotting (Zymed Laboratories). Secondary antibody used in indirect immunohistochemistry was tetramethyl-rhodamine isothiocyanate-conjugated swine anti-mouse (DAKO A/S, Glostrup, Denmark). Peroxidase-linked sheep anti-mouse IgG and donkey anti-rabbit IgG antibodies (Amersham Life Sciences, Little Chalfont, England) were used as secondary antibodies in Western analysis.

Indirect Immunofluorescence Labeling

Keratinocyte cultures were maintained in KGM containing a high-calcium concentration (1.8 mmol/L) for 2 hours and subsequently fixed in 100% methanol at -20°C for 10 minutes. To prevent nonspecific binding, the samples were preincubated in phosphate-buffered saline (PBS) supplemented with 1% bovine serum albumin for 15 minutes. Primary antibody was diluted in 1% bovine serum albumin-PBS, and incubated at 4°C for 20 hours. After five 10-minute washes in PBS, the cells were incubated with the secondary antibody at 20°C for 1 hour and washed five times in PBS. The samples were observed and photographed with a Leitz Aristoplan epifluorescent microscope filter for tetramethyl-rhodamine isothiocyanate fluorescence.

Western Transfer Analysis

Approximately 80% confluent keratinocyte cultures were grown on 21.5-cm² Petri dishes for Western analysis. Before the extraction cells were rinsed once with PBS

supplemented with protease inhibitors (Complete, Mini, EDTA-free Protease Inhibitor Cocktail Tablets, 1 tablet/10 ml; Boehringer Mannheim GmbH, Mannheim, Germany) and then extracted with RIPA buffer (1× PBS, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate) supplemented with protease inhibitors (see above). Cells were scraped from the dishes with a rubber policeman and incubated on ice for 30 minutes, then cell lysate was centrifuged at $15,000 \times g$ at 4°C for 10 minutes to separate insoluble fraction. Protein concentrations of soluble fraction were detected with DC Protein Assay (Bio-Rad Laboratories, Hercules, CA). Loading buffer (3×)⁵³ was added to each sample to a final concentration of 1×. Ten μg of each preparation was loaded on 7.5% sodium dodecyl sulfate-polyacrylamide gel. After electrophoresis, proteins were electrophoretically transferred to Immobilon-P filter (Millipore Corporation, Bedford, MA). The filter was blocked with swine serum containing 0.5% Tween-20, and then immunolabeled with Cx43 monoclonal and Cx43 polyclonal antibodies in 3% bovine serum albumin/PBS and 0.5% Tween-20 at 4°C overnight. The filter was washed with PBS and 0.5% Tween-20 for 25 minutes with three changes of the washing buffer. Peroxidase-linked sheep anti-mouse IgG and donkey anti-rabbit IgG antibodies (Amersham Life Sciences) were used as secondary antibodies in 3% bovine serum albumin/PBS and 0.5% Tween-20 at room temperature for 1 hour and then the filter was washed for 25 minutes as above. Proteins were detected with ECL (Amersham Life Sciences) and the filter was exposed to autoradiographic film (Eastman Kodak, Rochester, NY).

Statistical Analysis

Statistical analyses were performed using the PC version of the SPSS Inc. Professional Statistics, Release 7.5 (Chicago, IL). Differences between control and NF1 keratinocytes were compared using independent sample's *t*-test, Mann-Whitney test, or paired samples *t*-test. The chosen level of significance was $P < 0.05$.

Results

Capacitave Calcium Influx of Normal and NF1 Keratinocytes

Capacitave calcium influx was first investigated after exposure of the cells to thapsigargin, an inhibitor of ER Ca^{2+} -ATPase. Fluorescence ratio imaging was performed using fura-2 as a calcium-sensitive probe. When the cells are maintained in a medium containing high (1.8 mmol/L) calcium concentration, treatment of the cells with thapsigargin results in both the release of calcium from endoplasmic calcium stores and subsequent influx of extracellular calcium through SOCs.^{20,54} This cascade is called capacitave calcium influx, and the final outcome is an elevated cytoplasmic Ca^{2+} concentration. The present study demonstrated that the effect of thapsigargin on intracellular Ca^{2+} levels of NF1 keratinocytes was significantly weaker compared to normal cells (Figure 1,

A, G, and H; and Table 1). The resting Ca^{2+} levels were also lower in NF1 cells compared to normal cells.

When the extracellular Ca^{2+} is chelated with EGTA, the exposure of cells to thapsigargin results in elevation of intracellular free Ca^{2+} that reflects the release of calcium stores from the ER, but not an influx of extracellular Ca^{2+} .⁵⁵ The results of the present study demonstrated that NF1 keratinocytes displayed reduced calcium stores of the ER compared to normal cells after exposure the cells to thapsigargin when the influx of extracellular calcium was inhibited (Table 1).

Effect of Extracellular ATP on Normal and NF1 Keratinocytes

When ATP was added to the culture medium, both normal control and NF1 keratinocytes responded with a marked increase in $[\text{Ca}^{2+}]_i$. The rise in $[\text{Ca}^{2+}]_i$ was significantly more pronounced in NF1 keratinocytes compared to normal control cells (Figure 1C and Table 1). This finding suggests that the P_2 -purinergic signaling is functioning both in normal control and NF1 keratinocytes.

Mn^{2+} Quench Analysis of Normal and NF1 Keratinocytes

Further evidence for down-regulated capacitave calcium influx in NF1 keratinocytes was derived from Mn^{2+} quench experiments. Mn^{2+} can enter cells via SOCs that open after release of intracellular Ca^{2+} stores.²⁵ The end result of Mn^{2+} influx is reduction of fura-2 fluorescence, which is because of replacement of Ca^{2+} from the calcium-sensitive probe. When Mn^{2+} alone was applied to the cells and fura-2 fluorescence quenching was monitored, a slow decline of fura-2 fluorescence was detected in normal control cells. Quenching of fura-2 fluorescence was more moderate in NF1 keratinocytes under the same experimental conditions demonstrating slow entry of Mn^{2+} into these cells. Application of thapsigargin in the presence of extracellular Mn^{2+} resulted in different effects in normal keratinocytes compared to cells cultured from patients with type 1 neurofibromatosis. Specifically, application of thapsigargin resulted in a fast and marked quench of cytosolic fura-2 fluorescence in normal cells because of influx of extracellular Mn^{2+} into the cells (Figure 2A). In contrast, there was first an increase of cytosolic fura-2 fluorescence followed by fluorescence quenching in NF1 keratinocytes under the same experimental conditions (Figure 2B).

In analogy, the exposure of the keratinocytes to ATP in the presence of extracellular Mn^{2+} resulted in a fast and marked quench of cytosolic fura-2 fluorescence in normal cells (Figure 2A), but not in NF1 keratinocytes (Figure 2B). Instead, NF1 keratinocytes responded to extracellular ATP by transiently elevating cytosolic fura-2 fluorescence before an enhanced fluorescence quenching because of increased Mn^{2+} entry (Figure 2B).

Figure 1. Analysis of intracellular calcium-mediated cell signaling in normal and NF1 keratinocytes. Experiments were performed in high (1.8 mmol/L) $[Ca^{2+}]_o$. The mean increase in $[Ca^{2+}]_i$ was analyzed in 20 to 25 cells in each experiment. The mean peaks of $[Ca^{2+}]_i$ are presented as one solid line in **A–F**. **A:** Capacitative calcium influx in normal and NF1 keratinocytes. Ca^{2+} stores of the ER were mobilized with thapsigargin, which subsequently causes an influx of extracellular Ca^{2+} through SOCs. This cascade is called capacitative calcium influx, and the final outcome is an elevated cytoplasmic Ca^{2+} concentration. The results demonstrate that the effect of thapsigargin on intracellular Ca^{2+} levels of NF1 keratinocytes is significantly weaker (**H**) compared to normal cells. Note also lower resting Ca^{2+} levels in NF1 keratinocytes. **B:** Release of calcium stores from ER with thapsigargin before wounding inhibited the propagation of calcium waves in normal and NF1 keratinocytes. This finding indicates that both types of keratinocytes required release of intracellular calcium stores to propagate intercellular calcium waves. **C:** $[Ca^{2+}]_i$ transient after application of ATP to normal and NF1 keratinocytes. The rise in $[Ca^{2+}]_i$ was more pronounced in NF1 keratinocytes compared to normal cells. **D:** $[Ca^{2+}]_i$ transient after mechanical wounding in normal and NF1 keratinocytes. $[Ca^{2+}]_i$ transiently increases in 20 to 30 rows of cells at the edge of the wound after mechanical scratching. The rise in $[Ca^{2+}]_i$ was significantly lower (**H**) in NF1 keratinocytes compared to normal cells. **E:** $[Ca^{2+}]_i$ transient in cells treated with heptanol, an inhibitor of gap-junctional signaling. When gap-junctional signaling was blocked with 3.5 mmol/L of heptanol and keratinocyte monolayers were mechanically wounded, the rise of $[Ca^{2+}]_i$ in normal keratinocytes was slightly reduced (**H**). In contrast, the inhibitory effect of heptanol on the propagation of calcium wave in NF1 keratinocyte cultures was less pronounced. **F:** $[Ca^{2+}]_i$ transient in keratinocytes treated with suramin, an inhibitor of P_2 -purinergic receptors. When P_2 -purinergic signaling was blocked with 100 μ mol/L of suramin and keratinocyte monolayers were mechanically wounded, the $[Ca^{2+}]_i$ rise in normal keratinocytes was significantly higher compared to NF1 keratinocytes (**H**). Combined information from **E** and **F** indicates that gap-junctional signaling is the main component of intercellular calcium waves in normal keratinocytes, whereas NF1 keratinocytes propagate mainly ATP-dependent calcium waves. **G:** Image analysis data of normal and NF1 keratinocytes treated with thapsigargin as in **A**. Panels consist of frames taken at 5-second intervals visualizing cytosolic fura-2 fluorescence. **Yellow box** indicates start of thapsigargin treatment. Frame width, $\sim 100 \mu$ m. **H:** Statistical analysis of mean elevation of $[Ca^{2+}]_i$ in normal and NF1 keratinocytes under different experimental conditions presented in **A, D–F**. The number of cells is 20 to 25 per experiment, in each of *n* experiments. Statistical differences: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

Table 1. Intracellular Calcium Stores, Extracellular Calcium Influx, and Cell-Signaling Pathways in Normal and NF1 Keratinocytes

	Increase in $[Ca^{2+}]_i$ (nM), mean $\pm$ S.D. (<i>n</i> experiments)			
	Normal cells		NF1 cells	
	EGTA	1.8 mM $[Ca^{2+}]_e$	EGTA	1.8 mM $[Ca^{2+}]_e$
Thapsigargin	40 $\pm$ 8 (16)	107 $\pm$ 42 (16)	19 $\pm$ 13* (20)	56 $\pm$ 18* (20)
ATP		243 $\pm$ 14 (16)		278 $\pm$ 16** (16)
Wounding		164 $\pm$ 56 (32)		110 $\pm$ 36* (50)
Wounding + heptanol		68 $\pm$ 8 (24)		78 $\pm$ 23 (30)
Wounding + suramin		121 $\pm$ 17 (16)		56 $\pm$ 17*** (20)

NF1 keratinocytes have lower intracellular calcium stores, reduced extracellular calcium influx, and lower wound-induced Ca^{2+} peaks compared to normal keratinocytes. These data indicate that ATP-dependent communication is enhanced and gap-junctional communication is reduced in NF1 keratinocytes compared to normal keratinocytes.

The number of cells is 20 to 25 per experiment, in each of *n* experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

$[Ca^{2+}]_e$, extracellular calcium concentration.

Wound-Induced Calcium Wave in Normal and NF1 Keratinocytes

In further studies, we analyzed wound-induced calcium waves of normal and NF1 keratinocytes. The results revealed that the rise of $[Ca^{2+}]_i$ after mechanical stimulation was significantly ($P < 0.05$) lower in NF1 keratinocytes compared to normal cells (Figure 1, B and D; Figure 3; and Table 1). When endoplasmic calcium stores were released with thapsigargin before wounding, neither normal nor NF1 keratinocytes displayed an in-

crease in $[Ca^{2+}]_i$, and calcium waves could not be induced (Figure 1B).

Gap-Junctional and P_2 -Purinergic Receptor-Mediated Calcium-Signaling Routes in Normal and NF1 Keratinocytes

Intercellular calcium waves were further analyzed in the presence of heptanol, a gap-junctional signaling inhibitor,⁵⁰ or suramin, an inhibitor of P_2 -purinergic receptors.⁵¹ Even though heptanol is not an entirely specific agent as a gap-junctional blocker, we chose to use heptanol as a gap-junctional inhibitor since it has been previously used successfully in various cell lines, including keratinocytes.^{18,49,50} Involvement of ATP as the sole source of Ca^{2+} wave propagation has been demonstrated in gap-junctional signaling-deficient rat basophilic leukemia cells,³⁶ with complete inhibition of the intercellular Ca^{2+} waves by suramin. Similar studies in mammary tumor cells, which exhibit gap-junctional signaling, have also indicated that release of ATP is involved in the propagation of mechanically simulated Ca^{2+} waves.⁵⁶ Interestingly, the results of the present study demonstrated that the main routes of calcium-mediated signaling were different in normal and NF1 keratinocytes. Specifically, the main route of calcium-mediated signaling in normal keratinocytes was gap junctional. In contrast, ATP-mediated calcium signaling predominated in NF1 keratinocytes (Figure 1, E, F, and H; and Table 1).

Figure 2. Mn^{2+} quench in normal and NF1 keratinocytes. **A:** Application of Mn^{2+} to defined keratinocyte growth medium resulted in Mn^{2+} quench of cytosolic fura-2 fluorescence in normal human keratinocytes apparently reflecting influx of Mn^{2+} into the cells. **B:** In NF1 keratinocytes, Mn^{2+} quench of cytosolic fura-2 fluorescence was less pronounced when Mn^{2+} alone was added to the growth medium. Application of Mn^{2+} and thapsigargin to cells resulted in a fast and marked quench of cytosolic fura-2 fluorescence in normal keratinocytes. In contrast, there was a slight and a transient increase of cytosolic fura-2 fluorescence in NF1 keratinocytes under the same experimental conditions. The results suggest that the capacitative calcium influx in NF1 keratinocytes is at least in part altered because of lowered activation of SOCs. Application of Mn^{2+} and extracellular ATP resulted in a fast and marked quench of cytosolic fura-2 fluorescence in normal cells. There was a transient increase of cytosolic fura-2 fluorescence in NF1 keratinocytes under the same experimental conditions. The y axis represents fluorescence in arbitrary units. Fluorescence tracings represent eight experiments.

The Velocity of the Calcium Wave in Normal and NF1 Keratinocytes

Intercellular Ca^{2+} waves induced by mechanical stimulation have been studied in many cell types. For example, mechanical stimulation of glial cells evoked an intercellular Ca^{2+} wave, with a mean propagation rate of 13.9 $\mu\text{m}/\text{second}$.⁵⁷ Mechanical strain applied to confluent prostate cancer cells induced an intercellular Ca^{2+} wave spreading with a velocity of 15 $\mu\text{m}/\text{second}$.⁵⁸ Previous studies have not addressed the velocity of calcium waves in human keratinocyte cultures.

Figure 3. The velocity of calcium wave in normal and NF1 keratinocytes. The wound site is at the **left** of each panel. Intracellular calcium levels were observed with 10-second intervals after mechanical wounding of keratinocyte cultures. Subsequently, cine-loop videos were generated based on the primary data. Calcium waves in NF1 keratinocytes generally proceeded at a rate of $\sim 11.8 \pm 2.5 \mu\text{m}/\text{second}$ ($n = 30$). The velocity of the calcium wave was $\sim 16.8 \pm 2.5 \mu\text{m}/\text{second}$ ($n = 32$) in normal keratinocytes. The speed of the calcium wave was significantly lower in NF1 keratinocytes than in normal cells ($P < 0.001$). Distinct gap-junctional signaling routes were noted in the normal cell monolayers where the intracellular calcium signal proceeded at a higher velocity. Distinct gap-junctional signaling routes were not detected as clearly in NF1 keratinocytes compared to normal cells. **Green pentagons** at the **bottom** represent the keratinocytes that were elevating their intracellular calcium levels faster compared to other cells in the monolayer.

In the present study, further analysis demonstrated that the intercellular Ca^{2+} wave velocity was $\sim 16.8 \mu\text{m}/\text{second}$ in normal keratinocyte cultures. The speed of the calcium wave was higher in cultures of normal keratinocytes than in NF1 cell cultures ($P < 0.001$) (Figure 3). Intercellular calcium waves in cultures of NF1 keratinocytes progressed on average at the rate of $\sim 11.8 \mu\text{m}/\text{second}$. Distinct gap-junctional signaling routes were de-

Figure 4. Cx43 in normal and NF1 keratinocyte cultures. Keratinocytes maintained in a high-calcium concentration for 2 hours were fixed in methanol and immunolabeled with antibody to Cx43. Normal cells displayed a distinct organization of Cx43 to gap-junctional plaques (**white arrows**). NF1 keratinocytes lacked formation of gap-junctional plaques and Cx43 is mostly associated with cytoskeletal filaments. Higher magnification **insets** represent the cellular distribution of Cx43 in normal and NF1 keratinocyte cultures. Western blotting demonstrated apparently equal levels of Cx43 in normal and NF1 keratinocytes as evaluated by two different Cx43-specific antibodies. Scale bar, $10 \mu\text{m}$.

tected in the normal cell monolayers but not as clearly in NF1 keratinocytes (Figure 3).

Detection of Cx43 in Normal and NF1 Keratinocytes

Indirect immunofluorescence labeling of normal keratinocytes revealed a distinct organization of Cx43 to gap-junctional plaques (Figure 4). In contrast, NF1 keratinocytes were characterized with mostly cytoplasmic immunoreaction for Cx43, often in association with cytoskeletal filaments. Western blotting demonstrated apparently equal levels of Cx43 in normal and NF1 keratinocytes (Figure 4).

Discussion

The results demonstrated that coordinated Ca^{2+} responses in human keratinocyte cultures can take place through the activation of receptors by ATP-mediated sig-

naling, or through gap junctions between contacting cells. Furthermore, the results of the present study indicate that different components of intercellular Ca^{2+} signaling pathways predominated in normal *versus* NF1 keratinocytes. NF1 keratinocytes had low intracellular resting Ca^{2+} levels and reduced capacitative calcium influx. This may cause slowing down of the speed of the calcium wave, and a relatively moderate peak of $[\text{Ca}^{2+}]_i$ in NF1 keratinocytes compared to normal cells. In addition, Ca^{2+} stores were lower in NF1 keratinocytes compared to normal control cells. In further experiments, ATP was applied to the culture medium. The rise in $[\text{Ca}^{2+}]_i$ was more pronounced in NF1 keratinocytes compared to normal control cells. This finding suggests that NF1 keratinocytes are more sensitive to P_2 -purinergic stimulation than normal control cells. Furthermore, NF1 keratinocytes responded to wounding with a higher transient in $[\text{Ca}^{2+}]_i$ in heptanol experiments compared to normal cells. These results strengthen the view that P_2 -purinergic signaling was enhanced in NF1 keratinocytes. In addition, ATP and heptanol experiments collectively suggest that the changes observed in $[\text{Ca}^{2+}]_i$ were real and the moderate peaks of $[\text{Ca}^{2+}]_i$, usually seen in NF1 keratinocytes were not a result of altered uptake or distribution of the indicator, fura-2.

Mn^{2+} quench analyses were performed to analyze the influx of divalent cations into keratinocytes through SOCs. This technique is based on the effect that extracellularly applied Mn^{2+} ions quench fura-2 fluorescence when Mn^{2+} ions flow across the plasma membrane into the cytoplasm.⁵⁹ When Mn^{2+} alone was applied to the cells and fura-2 fluorescence quenching was monitored, we observed a decline of fura-2 fluorescence in normal control cells. A somewhat more moderate quenching of fura-2 fluorescence was detected in NF1 keratinocytes under the same experimental conditions. This data suggests reduced influx of extracellular Mn^{2+} into NF1 keratinocytes.

Thapsigargin induced a fast and marked quench of cytosolic fura-2 fluorescence in normal cells. In contrast, NF1 keratinocytes responded to thapsigargin treatment with an increase of cytosolic fura-2 fluorescence and subsequent quenching under the same conditions. In analogy, the exposure of the keratinocytes to ATP in the presence of extracellular Mn^{2+} resulted in a fast and marked quench of cytosolic fura-2 fluorescence in normal cells, but not in NF1 keratinocytes. Instead, NF1 keratinocytes responded to extracellular ATP by transiently elevating cytosolic fura-2 fluorescence that was followed by fluorescence quenching because of increased Mn^{2+} entry. In a previous study, enhanced fluorescence quenching after a transient $[\text{Ca}^{2+}]_i$ elevation was noted when mesangial cells were exposed to arginine vasopressin.⁶⁰ In our study, we explain increased fura-2 fluorescence in Figure 2B in the following manner: in response to thapsigargin the intracellular calcium stores are released in NF1 cells that causes increased fura-2 fluorescence (Figure 2B). Because the capacitative calcium influx in the NF1 cells seems to be defective and the influx of Mn^{2+} is delayed, this would explain transient increase in the trace recording Mn quench. The same

rationale applies to Figure 2B, in which transient increase of fura-2 fluorescence was observed after application of ATP to NF1 keratinocytes. The results suggest that NF1 keratinocytes may be particularly sensitive to ATP because fura-2 fluorescence quenching was more pronounced with ATP compared to thapsigargin experiments. If NF1 keratinocytes exhibit lower intracellular Ca^{2+} stores and down-regulated signal from ER to SOCs, this might explain defective capacitative calcium influx in these cells. However, the results demonstrate that down-regulated capacitative calcium influx in NF1 keratinocytes is at least in part because of defective signal for activation of SOCs. Additional studies are needed to investigate the potential role for the coupling of calcium content of ER and the permeability of the plasma membrane to calcium.

The results of the present study demonstrated that the main routes of calcium-mediated signaling were different in normal and NF1 keratinocytes. Specifically, the main route of calcium-mediated signaling in normal keratinocytes was gap junctional. In contrast, ATP-mediated calcium signaling predominated in NF1 keratinocytes. The speed of the calcium wave was higher in cultures of normal keratinocytes than in NF1 cell cultures ($P < 0.001$). Distinct gap-junctional signaling routes were detected in the normal cell monolayers but not as clearly in NF1 keratinocytes. We suggest that lack of organization of Cx43 into gap-junctional plaques in NF1 tumor suppressor-deficient cells, at least in part, leads to altered calcium-mediated cell signaling.

Taken together, the results of the present study demonstrate that NF1 keratinocytes have a tendency to form cultures characterized with altered Ca^{2+} -mediated cell signaling compared to normal keratinocytes. Mutations of the *NF1* gene and the subsequent haploinsufficiency can thus eventually lead to altered intercellular communication. Specifically, the intercellular calcium wave progression and Ca^{2+} -mediated cell signaling was defective in NF1 keratinocytes compared to normal cells. Keratinocytes acquired intracellular calcium stores to propagate intercellular calcium waves. Intracellular calcium stores and extracellular calcium influx were down-regulated in NF1 keratinocytes. Resting Ca^{2+} levels were lower in NF1 keratinocytes compared to normal control cells. Gap-junctional signaling was defective and NF1 keratinocytes propagated ATP-dependent calcium waves that required activation of P_2 -purinergic receptors. Western transfer analysis revealed apparently equal Cx43 protein levels in normal control and in NF1 keratinocytes. Indirect immunofluorescence, however, demonstrated that Cx43 was relatively evenly distributed in NF1 cells and did not form typical gap-junctional plaques between keratinocytes.

A conclusion that mutations of the *NF1* gene can finally lead to altered calcium-mediated cell signaling would be warranted by further studies using cells from, for example, transgenic animals carrying conditional knockout mutations of the *NF1* gene. Ca^{2+} waves are thought to be important in regulating a wide range of cellular processes, such as brain function.⁶¹ We speculate that if altered calcium-mediated cell signaling is characteristic to other cell types in addition to keratinocytes, this might

in part explain selected features of the NF1 syndrome, such as learning defects. Our observations on altered calcium-mediated cell signaling in keratinocytes focus further interest toward other cell types including neurons to approach the causes of findings associated with NF1.

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