INTERCELLULAR CALCIUM-MEDIATED CELL SIGNALING IN KERATINOCYTES CULTURED FROM PATIENTS WITH NF1 OR PSORIASIS

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OULU 2002
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Academic Dissertation to be presented with the assent of the Faculty of Medicine, University of Oulu, for public discussion in the Auditorium of the Department of Anatomy and Cell Biology, on September 27th, 2002, at 12 noon.

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Oulu, Finland
2002

Abstract

Neurofibromatosis type 1 syndrome (NF1) is caused by mutations of the NF1 gene. The NF1 protein (neurofibromin) contains a domain which is related to the GTPase-activating protein (GAP) and accelerates the switch of active Ras-GTP to inactive Ras-GDP. The NF1 protein has been referred to as a tumor suppressor, since the cells of malignant schwannomas of NF1 patients may display a loss of heterozygosity of the NF1 gene. Psoriasis is characterized by hyperproliferation of the epidermis and by down-regulated levels of NF1 mRNA and protein. Ca^{2+} is an universal signal transduction element modulating cell growth and differentiation. Many cell types coordinate their activities by transmitting waves of elevated intracellular calcium levels from cell to cell. The propagation of calcium waves had not been studied previously in human keratinocytes. Thus, the aim of the present study was to find out which pathways may play a role in Ca^{2+} signaling at different extracellular calcium concentrations in NF1 and psoriatic keratinocytes versus normal control keratinocytes. The results demonstrated that NF1 and psoriatic keratinocytes have a tendency to form cultures characterized by altered Ca^{2+}-mediated cell signaling compared to normal keratinocytes. Specifically, the main route of calcium-mediated signaling was gap-junctional in normal keratinocytes. In contrast, ATP-mediated calcium signaling predominated and capacitative calcium influx was defective in NF1 and psoriatic keratinocytes. The results of the present study suggest that mutations of the NF1 tumor suppressor gene or lowered levels of NF1 protein/mRNA may eventually lead to altered intercellular communication.

Keywords: calcium, connexins, cytoskeleton, intercellular junctions, keratinocytes, neurofibromatosis, NF1 tumor suppressor, psoriasis, signal transduction
To my family
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Oulu, September 2002

Timo Korkiamäki
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cADPr</td>
<td>cyclic adenosine diphosphate ribose</td>
</tr>
<tr>
<td>CLSM</td>
<td>confocal laser scanning microscopy</td>
</tr>
<tr>
<td>Cx26</td>
<td>connexin 26</td>
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<tr>
<td>Cx32</td>
<td>connexin 32</td>
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<td>Cx40</td>
<td>connexin 40</td>
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<td>Cx43</td>
<td>connexin 43</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetra-acetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethyleneglycol-bis(β-aminoethyl ether)-N,N,N’,N’.-tetraacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyantate</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase-activating protein</td>
</tr>
<tr>
<td>GJJC</td>
<td>gap-junctional intercellular communication</td>
</tr>
<tr>
<td>CRAC</td>
<td>Ca²⁺ release-activated Ca²⁺ channel</td>
</tr>
<tr>
<td>Hepes</td>
<td>N-(2-hydroxyethyl)piperazine-N´-(2-ethanesulphonic acid)</td>
</tr>
<tr>
<td>µM</td>
<td>micromolar, 10⁻⁶ µmol/l</td>
</tr>
<tr>
<td>NF1</td>
<td>type 1 neurofibromatosis</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PMCA</td>
<td>plasma membrane calcium ATPase</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>PPADS</td>
<td>pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid tetrasodium</td>
</tr>
</tbody>
</table>
PDGF  platelet-derived growth factor
SERCA  sarco(endo)plasmic calcium ATPase
SOC    store-operated calcium channel
TBS    tris-buffered saline
TGF β1 transforming growth factor β1
TRITC  tetramethyl-rhodamine isothiocyanate
List of original publications

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:


II  Korkiamäki Timo, Ylä-Outinen Heli, Koivunen Jussi, and Peltonen Juha. An Intact Actin-Containing Cytoskeleton is Required for Capacitative Calcium Entry, but not for ATP-Induced [Ca²⁺]i, Signaling in Cultured Human Keratinocytes. Submitted.

III  Korkiamäki Timo, Ylä-Outinen Heli, Koivunen Jussi, and Peltonen Juha. The Effect of Different Extracellular Calcium Concentrations ([Ca²⁺]e) on Calcium Mediated Cell Signaling in Keratinocytes Cultured from Normal Control Persons and from Patients with Type 1 Neurofibromatosis (NF1). Submitted.


*These authors contributed equally to this study.
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References
1 Introduction

Calcium is an universal signal transduction element modulating cell growth and differentiation (Lodish et al. 2000). The calcium levels outside cells are 10,000 times higher than free intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)). However, free [Ca\(^{2+}\)]\(_i\) is the physiologically active form of calcium (Rasmussen 1988). The level of free intracellular calcium is regulated and maintained as low as (~100 nM) in resting cells through the action of a number of binding proteins and ion exchange mechanisms (Clapham 1995 a and b, Putney 1999). On stimulation this level can rise globally to in excess of 1 µM. This increase can be generated from sources both within and outside the cell. Endoplasmic reticulum (ER) is a major site for sequestered Ca\(^{2+}\) ions. The entry of external Ca\(^{2+}\) into cells is controlled by several mechanisms, including voltage-operated channels, receptor-operated channels that open in response to binding of an extracellular ligand, and store-operated channels that open after depletion of intracellular Ca\(^{2+}\) stores. A coupling has been demonstrated between the filling state of the intracellular calcium stores and the plasma membrane calcium channel activity (Putney 1986 and 1990). Thus, a subset of calcium channels have been termed store-operated calcium channels (SOCs) (Berridge & Irvine 1984, Pietrobon et al. 1990, Parekh & Penner, 1997). The trigger for SOC activation is decreased Ca\(^{2+}\) in the ER lumen (Putney & Bird 1993, Parekh & Penner 1997). Together, the coordinated regulation of internal release and external influx results in an extremely complex increase in [Ca\(^{2+}\)]\(_i\) in terms amplitude, duration, frequency and spatial patterning.

Many cell types coordinate their activities by transmitting waves of elevated intracellular calcium levels from cell to cell. This phenomenon is called as intercellular calcium wave. Intercellular calcium waves are stimulus evoked cellular responses in which propagated waves of cytoplasmic Ca\(^{2+}\) increases spread from one cell to another. Intercellular calcium waves are considered to serve as a pathway to long-range signaling. Intercellular calcium waves have been studied in many different cell types, such as neurons, smooth muscle cells, and osteoblastic cells (Xia & Ferrier 1992, Charles et al. 1996, Young et al. 1996). Two mechanisms of intercellular calcium waves have been identified. On the one hand, the generation of intercellular calcium waves relies on the paracrine activity of ATP. Mechanical stimulation or physiological damage of cells results in a release of ATP, which activates purinergic receptors on neighboring cells (Osipchuk & Cahalan 1992). This, in turn, leads to generation of IP\(_3\), and release of Ca\(^{2+}\) from
intracellular stores (Dubyak & el-Moatassim 1993). Secondly, calcium waves have been
demonstrated to spread from cell to cell via gap junctions. Diffusion of IP₃ through gap-
junctional structures has been shown to mediate the release of Ca²⁺ of its IP₃-sensitive
stores in neighboring cells (Sanderson et al. 1990, Boitano et al. 1992, Sneyd et al.
1995). Cell-cell interactions, and especially gap-junctional intercellular communication
(GJIC), seem to play crucial roles in cell regulation, differentiation, development, and

NF1 refers to type 1 neurofibromatosis syndrome, which has been linked with
mutations in the NF1 gene (Gutmann et al. 1991, Marchuk et al. 1991, Gutmann et al.
1997). The hallmarks of NF1 include pigmented café au lait spots of the epidermis,
cutaneous neurofibromas with a potential to be transformed to malignant sarcomas, and
hamartomas of the iris (Riccardi 1981, Gutmann et al. 1997). The NF1 protein
(neurofibromin) has been referred to as a tumor suppressor since the cells of the
malignant tumors of neurofibromatosis patients may display a loss of heterozygosity of
the NF1 gene (Legius et al. 1993, Metheny et al. 1995). However, the molecular
functions of NF1 protein are not fully known. NF1 protein acts as a GTPase activating
protein and thus accelerates the switch of active the GTP-bound “active” Ras to inactive
GDP bound form in various cell types (Xu et al. 1990, Basu et al. 1992, Bollag &
McCormick 1992, DeClue et al. 1992). Furthermore, the cytoskeleton has been
demonstrated to be abnormal in cells cultured from patients with NF1 (Peltonen et al.

NF1 protein is associated with endoplasmic reticulum (ER), which is also the major
site of Ca²⁺ storage. Due to this, the present studies were performed in order to investigate
the role of NF1 in calcium mediated cell signaling. For comparison, Ca²⁺-signaling was
studied in keratinocytes cultured from patients with psoriasis. Psoriasis is a skin disease,
in which a disturbance in Ca²⁺-dependent cellular differentiation has been postulated. 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 into vascular endothelial cells (Holda & Blatter
1997). Thus, the aim of the present study was also to investigate which pathways may
play a role in Ca²⁺ signaling in NF1 and control keratinocytes. Keratinocytes were
sublimated in the presence of inhibitors of gap-junctional or ATP-mediated
communication in order to determine which pathways are operative in Ca²⁺ signaling
between these cells. Given the importance of Ca²⁺ influx for a variety of cellular
processes, such as Ca²⁺ oscillations (Tsien & Tsien 1990, Berridge 1993), secretion
(Parekh & Penner 1995), and enzymatic regulation (Chiono et al. 1995), the results are
likely to be of widespread importance to a plethora of physiological processes.
2 Review of the literature

2.1 Neurofibromatosis type 1

Neurofibromatosis type 1 syndrome (NF1) is caused by mutations of the \textit{NF1} gene. It is a common cancer-predisposing syndrome with an estimated incidence of about 1 in 3500 individuals worldwide (Gutmann \textit{et al.} 1997, Friedman 1999a, Friedman 1999b, Gutmann 2001). The disease is also known as von Recklinghausen’s syndrome, and it is autosomal in nature. NF1 has been called a neurocutaneous disorder, but symptoms of the disease do occur in many other organ systems as well. The clinical picture varies from mild to severe, even in patients carrying the same mutation of the NF1 gene (Friedman 1999a, Friedman 1999b, Gutmann 2001). Thus, other genes may modify the natural history of the disease, and \textit{p53} gene mutations, for example, have been shown to increase its severity (Easton \textit{et al.} 1993, Cichowski \textit{et al.} 1999, Vogel \textit{et al.} 1999, Vogel & Parada 1998).

There are additional features that occur more frequently in NF1 patients than in the general population. For example, 30 to 60% of patients suffer from learning disabilities, which most commonly affect the visuospatial integration skills (Riccardi 1981, Legius \textit{et al.} 1994). The common complications of NF1 patients also include short stature (25-35% of patients), scoliosis (12-20%), and headache (20%). Rare complications (in less than 5% of patients) include epilepsy, hydrocephalus, macroencephaly, cardiovascular problems, and an increased risk for malignancies (e.g. malignant peripheral nerve sheath tumors, neurilemmomas, juvenile chronic myelogenous leukemias, or pheochromocytomas) (Riccardi 1981, Stumpf \textit{et al.} 1988, Xu \textit{et al.} 1992a, Legius \textit{et al.} 1993, Shannon \textit{et al.} 1994, Korf 2000, Lin \textit{et al.} 2000, North 2000, Hamilton \textit{et al.} 2001).

2.1.1 \textit{NF1} gene

The NF1 gene is in chromosome 17q11.2 spanning over 350 kb of genomic DNA (Marchuk \textit{et al.} 1991, Li \textit{et al.} 1995, Viskochil 1999a) with 60 exons organized into four clusters separated by four large introns (Cawthon \textit{et al.} 1990, Viskochil \textit{et al.} 1990, Wallace \textit{et al.} 1990). The NF1 gene is ubiquitously expressed in humans, resulting in 11-
18 kb NF1 mRNA and many alternatively spliced variants (Skuse & Cappione 1997). The NF1 protein contains a domain which is related to the GTPase-activating protein (GAP) and accelerates the switch of GTP-bound form of Ras to inactive GDP-bound form of Ras (Viskochill 1999b). The NF1 coding region contains an even longer homology to the yeast IRA1 and IRA2 proteins, which are regulators of the Ras-cAMP pathway (Ballester et al. 1990, Buchberg et al. 1990, Martin et al. 1990, Xu et al. 1990a, Xu et al. 1990b). Another region, comprising exons 19, 11-17, contains homology to the ATP-binding and cAMP-dependent protein kinase (PKA) recognition sequences (Fahsold et al. 2000). Sequences coding for tubulin-binding sites locate within and in the vicinity of the GAP-related domain (GRD) (Bollag et al. 1993, Scheffzek et al. 1998). The mutation rate of the NF1 gene is one of the highest known to occur in the human genome, 3.1-6.5 x 10⁻⁶ (Vogel & Motulsky 1997), and about 50% of all NF1 patients lack a family history of the disease (Huson & Hughes 1994). In general, mutational analysis of the NF1 gene is complex due to the large size of the gene, the existence of pseudogenes and the great diversity of the lesions (Legius et al. 1992, Kehrer-Sawatzki et al. 1997, Regnier et al. 1997, Luijten et al. 2000). Mutations of the NF1 gene are distributed along the whole genomic region, and only a few possible mutational hot spots have been suggested so far (Shen et al. 1996, Boulandet et al. 2000, Fahsold et al. 2000, Messiaen et al. 2000). There are germ line mutations in NF1. Somatic mutations or LOH have also been shown in many NF1-associated malignancies as well as in cancers of non-NF1 patients (Xu et al. 1992a, Andersen et al. 1993, Johnson et al. 1993, Legius et al. 1993, Shannon et al. 1994). LOH has also been described in some of the benign neurofibromas (Colman et al. 1996, Sawada et al. 1996, Däschner et al. 1997, Eisenberth et al. 2000). Furthermore, a few cases of somatic mosaicism have been described at the molecular level (Lazaro et al. 1995, Colman et al. 1996, Wu et al. 1997).

### 2.1.2 NF1 protein

The NF1 protein has been referred as a tumor suppressor, as the cells of malignant schwannomas of neurofibromatosis patients may display a loss of heterozygosity of the NF1 gene (Legius et al. 1993, Metheny et al. 1995). In fact, NF1, which affects 1 in 3500 individuals, is a common heritable disease that is associated with an increased risk for different cancers (Riccardi 1981, Blatt et al. 1986). Interestingly, somatic mutations of the NF1 gene have also been found in malignant tissues of otherwise healthy persons. Specifically, mutations of the NF1 gene have been found, for example, in colon adenocarcinoma, myelodysplastic syndrome, and anaplastic astrocytoma (Li et al. 1992, Johnson et al. 1993). Furthermore, the levels of NF1 protein and/or mRNA have been reported to be altered in certain proliferative diseases, such as transitional cell carcinoma, basal cell carcinoma, astrocytoma, pheochromocytoma, and psoriasis (Gutmann et al. 1995a & 1996, Hermonen et al. 1995, Peltonen et al. 1995, Aaltonen et al. 1999).

Molecular cloning of the entire coding sequence of the NF1 gene and subsequent analysis of the corresponding peptide sequence have led to the recognition of the NF1 protein as a regulator of Ras (Xu et al. 1990b, Gutmann et al. 1991, Marchuk et al. 1991, Bollag & McCormick 1992). The NF1 protein contains a domain that is related to the
GTPase-activating protein (GAP) and accelerates the switch of active Ras-GTP to inactive Ras-GDP in various cell types (Xu et al. 1990, Basu et al. 1992, Bollag & McCormick 1992, DeClue et al. 1992). Alternative splicing results in the formation of different isoforms of NF1 mRNAs and the translation of different NF1 protein isoforms (types I-IV) (Gutmann et al. 1995b). The biological significance of the different isoforms is poorly understood. Both type I and type II isoforms of NF1 protein have been shown to affect Ras inhibition, although type II is less potent (Gutmann et al. 1995b).

The NF1 protein is ubiquitously expressed, but the protein levels vary in different tissues and in developmental or functional states (Gutmann et al. 1991, Daston et al. 1992, Daston & Ratner 1992). The NF1 protein contains 2818 amino acids with a calculated molecular mass of 327 kDa (Wallace et al. 1990, Marchuk et al. 1991). The NF1 protein has been shown to contain six potential phosphorylation sites for serine/threonine kinases and one for tyrosine kinases (Marchuk et al. 1991). The protein kinases phosphorylating the NF1 protein have not been characterized in detail (Boyer et al. 1994, Izawa et al. 1996, Tokuo et al. 2001). The NF1 protein is thought to play crucial roles in Ras- and cAMP-dependent protein kinase A (PKA)-associated signaling pathways. In Ras-signaling it acts as a negative regulator of the p21ras-signaling pathway, similarly to mammalian GAP and yeast IRA1 and IRA2, due to its intrinsic GAP-activity (Ballester et al. 1990, Buchberg et al. 1990, Xu et al. 1990b, Marchuk et al. 1991, Scheffzek et al. 1998). NF1 GRD spans 250-400 amino acids, the residues 1198-1530 (25-40 kDa), representing only 10% of the protein sequence (Martin et al. 1990, Xu et al. 1990a, Andersen et al. 1993, Scheffzek et al. 1998). NF1 GRD accelerates the intrinsic activity of Ras-GTPase, resulting in active Ras-GTP to be converted into an inactive GDP form (Bollag & McCormick 1992). Many NF1-deficient tumors have been shown to contain elevated levels of Ras-GTP and/or Ras-dependent signaling pathways (Basu et al. 1992, DeClue et al. 1992, Bollag et al. 1996, Guha et al. 1996, Klesse & Parada 1998, Feldkamp et al. 1999, Ingram et al. 2001). Recently, several studies have demonstrated that, in some cell types, the cAMP/PKA pathway may be regulated by the NF1 protein rather than Ras (Guo et al. 1997, The et al. 1997, Fieber 1998, Guo et al. 2000, Kim et al. 2001, Tokuo et al. 2001).
The functions of NF1 tumor suppressor. NF1 protein acts as a negative regulator of the Ras. The GRD of the NF1 protein accelerates the switch of active Ras-GTP to an inactive Ras-GDP form. The NF1 protein has also been shown to interact with cytoskeleton and to be involved in adenylyl cyclase/PKA pathway. Growth signals (ligand coupled to receptor) activate guanine nucleotide exchange factors (GEFs), which enables GTP binding to Ras-proteins. Signaling pathways downstream of Ras-GTP include phosphatidylinositol-3-kinase (PI3-kinase), Ral-Rac-Rho-, and raf-MEK-ERK kinase cascades.

It is of interest to note that GAP activity is not enough to explain the entire function of the NF1 protein. For example, a melanocyte cell line cultured from neurofibromatosis patients or melanoma and neuroblastoma cell lines cultured from patients without NF1 display lowered NF1 protein levels but normal Ras-GTP levels (Johnson et al. 1993, Griesser et al. 1995). On the other hand, overexpression of NF1 protein causes inhibition of growth without a change in the Ras-GTP levels in cultured NIH 3T3, melanoma, and colon carcinoma cells (Johnson et al. 1994). Furthermore, in drosophila, NF1 protein does not seem to be a regulator of the Ras pathway but to interact with the PKA-pathway (Guo et al. 1997, The et al. 1997).

Some studies have shown that NF1 protein can complement the function of IRA proteins, which regulate the Ras/cAMP pathway in yeast cells (Ballester et al. 1990, Xu et al. 1990a). The GTPase activities of NF1 protein and GAP are differentially regulated: dodecyl maltoside and some lipids (e.g. arachidonic acid, phosphatidic acid, stearic acid, oleic acid, phosphatidylinositol-4,5-bisphosphate, some n-6 and n-3 polyunsaturated fatty acids) inhibit the activity of NF1 protein more than GAP (Bollag & McCormick 1991, Golubic et al. 1991, Golubic et al. 1992, Han et al. 1991, Golubic et al. 1998). The
association of NF1 protein with tubulin has also been shown to inhibit the GTPase-stimulating activity of NF1 protein (Bollag et al. 1993, Gregory et al. 1993, Xu & Gutmann 1997).

Subcellular localization of the NF1 protein and its co-localization with other molecules have given clues of its putative functions. NF1 protein has been shown to associate with the microtubular and microfilamentous cytoskeleton, and even sequentially with both within the same cell (Bollag et al. 1993, Gregory et al. 1993, Xu & Gutmann 1997, Gutmann et al. 2001, Li et al. 2001). Furthermore, interaction between NF1 protein and syndecan and paxillin (the scaffold for molecules associating with focal adhesions) has been suggested (Hsueh et al. 2001, Li et al. 2001). In addition, NF1 protein has been found to occur in the mitochondria, endoplasmic reticulum and nucleus in association with both the membrane and cytosolic fractions (DeClue et al. 1991, Hattori et al. 1991, Daston et al. 1992, Daston & Ratner 1992, Golubic et al. 1992, Hattori et al. 1992, Gregory et al. 1993, Nordlund et al. 1993, Roudebush et al. 1997, Hsueh et al. 2001, Li et al. 2001). Taken together, the NF1 protein seems to be involved in the p21ras and cAMP/PKA pathways. An additional functional spectrum for the NF1 protein might be achieved with alternative splicing, phosphorylation, or regulatory molecules. Thus, it is not surprising that the NF1 protein seems to have pleiotropic effects on cell behavior (Vogel et al. 1995, Largaespada et al. 1996, Gutman et al. 2001).

2.1.3 Ras proteins


The functions of activated Ras proteins are pleiotropic, depending on the cell type, the duration of pathway activation, and the developmental status (Campbell et al. 1998, reviewed by Olson & Marais 2000, Schlessinger 2000). For example, the activation of Ras-dependent signaling pathways has been shown to regulate cell cycle and apoptosis and to affect the organization of the cytoskeleton, the formation of cell junctions, and cell movements (Nobes & Hall 1999, Holzer et al. 2001, Vasioukhin et al. 2001,
Walsh & Bar-Sagi 2001). Oncogenic mutations of the ras gene are present in approximately 30% of all human cancers (Adjei 2001). Furthermore, tumor invasion, metastasis, and angiogenesis require controlled degradation of extracellular matrix (ECM), and increased expression of matrix metalloproteinases (MMPs) is associated with tumor invasion and the metastasis of malignant tumors of different histogenetic origins (Ala-aho et al. 2002, Vihinen & Kahari 2002). Protein kinase C (PKC) has been shown to activate the ERK1,2-signaling pathway, and the mitogen-activated ERK1,2 pathway has been shown to be the major activator of collagenase-1 (MMP-1) gene expression (Westermarck & Kahari 1999).

2.1.4 NF1 tumor suppressor and cytoskeleton

Some evidence suggested a link between neurofibromatosis, or NF1 protein, and the organization of the cellular cytoskeleton. Specifically, a cell culture study before the cloning of the NF1 gene demonstrated that the cytoskeleton of cells cultured from neurofibromas is characterized by haphazard organization of intermediate filaments and actin-containing stress fibers (Peltonen et al. 1984). Interaction of NF1 protein with microtubules has been demonstrated (Bollag et al. 1993, Gregory et al. 1993).

Cell cultures provide an abundant source of human keratinocytes and a well-documented cell differentiation model that can be modified by raising the calcium concentration of the culture medium. A previous study has demonstrated a phenomenon in which an increased Ca^{2+} concentration of the cell culture medium induces a rapid organization of NF1 protein into finger-like projections extending from the perinuclear region to the subplasmalemmal region in differentiating keratinocytes (Koivunen et al. 2000). A recent study has also shown an association between NF1 protein and the intermediate filament cytoskeleton. Double immunolabelings with antibodies to NF1 protein and a panel of cytoskeletal components and cell adhesion molecules demonstrated a transient association of NF1 protein with cytokeratin 14 (CK14) (Koivunen et al. 2000). These findings suggest that the NF1 tumor suppressor exerts part of its effects through organization of intermediate filaments, which is a prerequisite for the formation of selected cell-cell and cell-matrix junctions characterized in cultured keratinocytes.

2.2 Psoriasis

The clinical features of psoriatic epidermis and the related histopathologic changes are considered to be primarily dependent on the biologic potential of the keratinocyte to express the psoriatic phenotype (Pittelkow 1998). Psoriasis is also recognized at the functional level, however, where the interaction of keratinocytes with lymphocytes generates the psoriatic lesion. Thus, psoriasis is characterized both by an independent keratinocyte disorder indicating basal hyperproliferation and by a stratum corneum barrier function disorder as well as interaction with, for instance, lymphocytes, which provide inflammatory factors (cytokines) attenuating the development and maintenance
of psoriatic lesion (Strange et al. 1993, Bata-Csorgo et al. 1995).

A certain \( \text{Ca}^{2+} \) gradient is necessary for normal terminal differentiation of keratinocytes and provides an explanation for the existence of the \( \text{Ca}^{2+} \) gradient \textit{in vivo}. Loss of this gradient has been observed in psoriatic epidermis \textit{in vivo}, favoring enhanced proliferation (Menon & Elias 1991). The rise in \( [\text{Ca}^{2+}] \), after an increase in the extracellular calcium (\([\text{Ca}^{2+}]_e\)) concentration appears to be a prerequisite for keratinocyte differentiation (Hennings et al. 1980, Sharpe et al. 1989, Bikle et al. 1996). Elevation of \([\text{Ca}^{2+}]_i\), alone does not apparently determine keratinocyte differentiation, but it acts with other intracellular signals to promote differentiation (Jones & Sharpe 1994, Poumay & Pittelkow 1995). There is a basic defect in the proliferation of psoriatic skin, as the phenotypically normal skin of psoriatic persons is also diseased (Harper et al. 1978, Krueger et al. 1981).

### 2.2.1 Psoriasis and NF1

Psoriasis is characterized by hyperproliferation of the epidermis. It is also characterized by a down-regulated levels of NF1 mRNA and protein (Peltonen et al. 1995). In normal skin, neurofibromin is particularly abundant in the basal keratinocytes of epidermis. In marked contrast to normal epidermis, active psoriatic lesions are characterized by a weak immunosignal for types I and II neurofibromin in the basal cell layer of the epidermis. Previously lesional, clinically healed areas displayed variable, yet clearly detectable, expression of neurofibromin. Previous results demonstrate that the epidermis of psoriatic lesions displays reduced immunostaining for type I and II neurofibromins compared to normal epidermis, and that neurofibromin immunoreactivity is partially restored upon clinical healing of the lesions (Peltonen et al. 1995).

### 2.2.2 Psoriasis and calcium

Previous studies have suggested that a \( \text{Ca}^{2+} \) gradient exists in the epidermis, so that basal and spinous cells are in a \( \text{Ca}^{2+} \) environment substantially below the serum \( \text{Ca}^{2+} \) levels, while the granular layer \( \text{Ca}^{2+} \) content is very high (Menon et al. 1985). The factors that maintain this gradient \textit{in vivo} are not known, but their existence supports the validity of the \( \text{Ca}^{2+} \) effect \textit{in vitro}. The expression of specific differentiation markers is temporally and spatially restricted to very precisely determined \( \text{Ca}^{2+} \) concentrations (Yuspa et al. 1989). Thus, a certain \( \text{Ca}^{2+} \) gradient is necessary for normal terminal differentiation and provides an explanation for the existence of the \( \text{Ca}^{2+} \) gradient \textit{in vivo}. Loss of this gradient has been observed in psoriatic epidermis \textit{in vivo}, which favors enhanced proliferation (Menon & Elias 1991). The rise in \([\text{Ca}^{2+}]_i\), after an increase in \([\text{Ca}^{2+}]_e\), appears to be a prerequisite for keratinocyte differentiation (Hennings et al. 1980, Sharpe et al. 1989, Bikle et al. 1996). Elevation of \([\text{Ca}^{2+}]_i\), alone does not apparently determine keratinocyte differentiation, but it acts with other intracellular signals to promote differentiation (Jones & Sharpe 1994, Poumay & Pittelkow 1995).
Changes in intracellular calcium levels can be analyzed with the use of ion-sensitive indicators, whose light emission reflects the local concentration of the ion. Some of these indicators are luminescent (emitting light spontaneously), while others are fluorescent (emitting light on exposure to light). Fluorescent Ca$^{2+}$ indicators have been synthesized that bind Ca$^{2+}$ tightly and are excited at slightly longer wavelengths when they are free of Ca$^{2+}$ than when in their Ca$^{2+}$-bound form. By measuring the ratio of fluorescence intensity at two excitation wavelengths, the concentration ratio of the Ca$^{2+}$-bound indicator to the Ca$^{2+}$-free indicator can be determined. This method provides an accurate measurement of the free Ca$^{2+}$ concentration. Two indicators of this type, called quin-2 and fura-2, are widely used for second-by-second monitoring of changes in intracellular Ca$^{2+}$ concentrations in the different parts of a cell viewed in a fluorescence microscope. Because the ratio between the pair on wavelengths is used the fluorescence variations due to uneven cell thickness and dye content are normalized. This is essential when quantitating [Ca$^{2+}$] in single cells under the microscope. Ratios are converted to [Ca$^{2+}$], values using a calibration curve obtained from dye in buffers of known [Ca$^{2+}$], with viscosity comparable to cytoplasm. Fura-2 can be esterified with acetoxymethyl groups to form a membrane-permanent derivate. This ester, fura-2/AM can be hydrolysed by cytoplasmic esterases to regenerate and trap the dye in cytosol. This trick for loading large numbers of cells without damaging their plasma membranes is a major advantage of fura-2.

Ca$^{2+}$ is an universal signal transduction element in cells modulating cell growth and differentiation (Lodish et al. 2000). The calcium levels outside cells are 10 000 times higher than free intracellular Ca$^{2+}$. However, free [Ca$^{2+}$] is the physiologically active form of calcium (Rasmussen 1988). The level of free intracellular calcium ([Ca$^{2+}$]) is regulated and maintained as low (~100 nM) through the action of a number of binding proteins and ion exchange mechanisms. Each cell has a unique set of Ca$^{2+}$ signals to control its function. Ca$^{2+}$ signal transduction is based on rises in free cytosolic Ca$^{2+}$ concentration. Ca$^{2+}$ can flow from the extracellular space or be released from intracellular stores. The endoplasmic reticulum (ER) is a major site for sequestered Ca$^{2+}$ ions. Recent studies indicate that the Golgi apparatus may also be a Ca$^{2+}$ store in keratinocytes (Hu et al. 2000, Sudbrak et al. 2000). Ca$^{2+}$ is accumulated into intracellular stores by means of Ca$^{2+}$ pumps and released by inositol 1,4,5-trisphosphate (IP$_3$) via IP$_3$ receptors and by cyclic adenosine diphosphate ribose (cADPr) via ryanodine receptors (Clapham 1995a and b, Putney 1999). A connection has been demonstrated between the filling status of the intracellular calcium stores and the plasma membrane calcium channel activity (Putney 1986 and 1990). Extracellular Ca$^{2+}$ enters the cell through various types of plasma-
membrane Ca\(^{2+}\) channels. Soluble proteins, such as calmodulin, contribute to the buffering of cell Ca\(^{2+}\), but membrane-intrinsic transporting proteins are more important. Ca\(^{2+}\) is transported across the plasma membrane (channel, pump, Na\(^+\)/Ca\(^{2+}\) exchanger) and across the membranes of organelles.

External signals arriving at the cell engage plasma membrane receptors to initiate cell signaling pathways. One of the end results is increased intracellular calcium concentration. On stimulation this level can rise globally to in excess of 1 \(\mu\)M. This increase can be generated from sources both within and outside the cell. The formation of IP\(_3\) is the focal point for two major pathways, one initiated by a family of G protein-linked receptors and the other by receptors linked by tyrosine kinases either directly or indirectly. These separate receptor mechanisms are coupled to energy-requiring transducing mechanisms which activate phospholipase C (PLC) to hydrolyse the lipid precursor phosphatidylinositol 4,5-biphosphate to generate both DAG and IP\(_3\). The latter then binds to an IP\(_3\) receptor (IP\(_3\)R) to mobilize stored calcium and to promote an influx of external calcium.

**Calcium homeostasis**

![Diagram of calcium homeostasis](image)

Fig. 2. Representation of calcium homeostasis in a single cell. Extracellular Ca\(^{2+}\) enters the cell through plasma membrane Ca\(^{2+}\) channels and leaves the cell using Ca\(^{2+}\) pumps and Na\(^+\)/Ca\(^{2+}\) exchangers. Endoplasmic reticulum (ER) is a major site for sequestered Ca\(^{2+}\) ions. Ca\(^{2+}\) is accumulated in intracellular stores by means of Ca\(^{2+}\) pumps and released by inositol 1,4,5-trisphosphate (IP\(_3\)) via IP\(_3\) receptors (IP\(_3\)R) and by cyclic adenosine diphosphate ribose (cADPr) via ryanodine receptors (RyR). Store-operated calcium channels (SOCs) open in response to depletion of the (ER) Ca\(^{2+}\) stores. Calcium influx factor (CIF) has postulated to mediate the signal from IP\(_3\)R to the plasma membrane store-operated calcium channels (SOCs).
Many important aspects of cellular physiology are regulated by the free cytosolic Ca\(^{2+}\) concentration. The intracellular Ca\(^{2+}\) signal is regulated in space, amplitude, and frequency. In both excitatory and nonexcitable cells, Ca\(^{2+}\) increases have a complex temporal and spatial arrangement (e.g. oscillations and waves) (Lechleiter et al. 1991, Thomas et al. 1991). Such global Ca\(^{2+}\) signals have been suggested to result from spatially and temporally coordinated recruitment of subcellular Ca\(^{2+}\) release units (Bootman & Berridge 1995, Lipp & Niggli 1996). These units represent the elementary building blocks of Ca\(^{2+}\) signaling, but the principles underlying their recruitment are largely unknown (Berridge 1997). Examples of elementary Ca\(^{2+}\) release events are the “Ca\(^{2+}\) sparks” in cardiac muscle cells (Cheng et al. 1993, Lipp & Niggli 1994, López- López et al. 1995) and the Ca\(^{2+}\) puffs in Xenopus oocytes (Yao et al. 1995), PC12 cells (Reber & Schindelholz 1996), and HeLa cells (Bootman et al. 1997). Such elementary Ca\(^{2+}\) release events are highly localized signals of short duration (Bootman & Berridge 1995, Lipp & Niggli 1996), which dissipate rapidly owing to diffusion in the cytoplasm and sequestration into the intracellular stores.

Elementary Ca\(^{2+}\) signals seem to have two functions. They can either activate processes in the Ca\(^{2+}\) microdomain in the immediate vicinity of the Ca\(^{2+}\) channel or, by recruiting other channels throughout the cell, they can activate processes through a global increase in [Ca\(^{2+}\)]. To avoid the toxic nature of Ca\(^{2+}\) cells use either low-amplitude Ca\(^{2+}\) signals or, more usually, transient Ca\(^{2+}\) signals, known as Ca\(^{2+}\) oscillations. Cells use either amplitude modulation or frequency modulation to decode information from Ca\(^{2+}\) signals. For example, the frequency of Ca\(^{2+}\) oscillations is used to optimize gene expression. To use frequency modulation based signaling, cells have developed decoders that respond to the frequency and duration of the Ca\(^{2+}\) signal, the best known example being calmodulin-dependent protein kinase II (De Koninck & Schulman 1998). One molecule that is sensitive to changes in intracellular calcium levels is Ras. The small GTPase operates as a binary molecular switch, and regulates cell proliferation and differentiation. The release of internally stored Ca\(^{2+}\) can lead to Ras activation (Rosen et al. 1994, Lockyer et al. 2001). In most cases this has been achieved through the addition of reagents such as thapsigargin and ionomycin, which do not release Ca\(^{2+}\) with the same spatio-temporal patterning as physiological stimuli. The plasma membrane has been considered to be the crucial location for Ras regulation, but new data is emerging to indicate that endomembrane Ras might also be important for compartmentalized Ras-signaling (Chiu et al. 2002). Complex Ca\(^{2+}\) signals might influence the spatio-temporal control of Ras activation-deactivation (Cullen & Lockyer 2002).

### 2.3.3 Regulation of intracellular calcium

[Ca\(^{2+}\)] increases can be generated from sources both within and outside the cell: Ca\(^{2+}\) release from intracellular calcium stores and secondly Ca\(^{2+}\) influx across the plasma membrane. The regulated entry of Ca\(^{2+}\) across the plasma membrane is an essential and ubiquitous signaling mechanism in both electrically excitable and nonexcitable cells.

Keratinocytes are a special case concerning the relationship between extracellular calcium concentration and cellular differentiation. Previous studies have shown that
changes in the concentration of extracellular calcium affect the balance between proliferation and differentiation in epidermal keratinocytes (Yuspa et al. 1989, Menon et al. 1992). Specifically, extracellular Ca\textsuperscript{2+} leads to a rapid increase in [Ca\textsuperscript{2+}], and IP\textsubscript{3} production as a result of activation of the Ca\textsuperscript{2+}-sensing receptor. Extracellular calcium can thus influence intracellular calcium levels. Elevation of [Ca\textsuperscript{2+}] above 0.03 mM (calcium switch) inhibits proliferation and induces the onset of terminal differentiation. One early response to the elevation of extracellular calcium is an increase in [Ca\textsuperscript{2+}], (Sharpe et al. 1989). Blocking of the rise in [Ca\textsuperscript{2+}], with an intracellular calcium chelator blocks the ability of extracellular calcium to induce differentiation (Li et al. 1995). After the calcium switch, the levels of IP\textsubscript{3} and DAG also increase rapidly (Jaken & Yuspa 1988, Bikle & Pillai 1993). This is subsequently followed by elevated expression of differentiation-related genes, such as involucrin (Rice & Green 1979) and transglutaminase (Thacher & Rice 1985, Gibson et al. 1996), a substrate and an enzyme, respectively, required for cornified envelope formation. Previous studies (Filvaroff et al. 1994, Bikle et al. 1996, Tu et al. 1999) suggest the involvement of the extracellular calcium-sensing receptor (CaR) in mediating calcium signaling during keratinocyte differentiation. Activation of CaR with calcium or other polyvalent cations activates the phospholipase C-signaling pathway, resulting in the generation of inositol 1,4,5-trisphosphate and the release of calcium from intracellular stores (Garrett et al. 1995, Chattopadhyay et al. 1996). Human keratinocytes express full-length CaR and an alternatively spliced forms of CaR lacking exon 5 (AltCaR) (Oda et al. 1998). Unlike the full-length CaR, AltCaR fails to mediate the acute IP\textsubscript{3} response to [Ca\textsuperscript{2+}]. The full-length CaR message is maximally expressed in undifferentiated keratinocytes, but its level decreases as the cells differentiate. On the other hand, the message levels of AltCaR remain relatively unchanged throughout differentiation (Oda et al. 1998). These changes in CaR expression are consistent with the reduction in the [Ca\textsuperscript{2+}] and IP\textsubscript{3} responses to [Ca\textsuperscript{2+}] during differentiation and further support the role for CaR in keratinocyte differentiation (Oda et al. 1998).

Ca\textsuperscript{2+} signaling in electrically excitable and nonexcitable cells differs in a fundamental way; whereas excitable cells in many cases use voltage-gated Ca\textsuperscript{2+} channels to regulate Ca\textsuperscript{2+} entry, nonexcitable cells rely on voltage-independent Ca\textsuperscript{2+} channels. Several classes of voltage-independent Ca\textsuperscript{2+} channels have been discovered, including receptor-operated, ligand-operated, G protein-coupled, and store-operated channels (reviewed by Penner et al. 1993, Fasolato et al. 1994, Felder et al. 1994). Store-operated channels (SOCs), defined as channels that open in response to depletion of the endoplasmic reticulum (ER) Ca\textsuperscript{2+} stores, represent one of the most ubiquitous mechanisms for triggering Ca\textsuperscript{2+} influx in nonexcitable cells. Hypothalamic peptides called orexins (Sakurai et al. 1998) or hypocretins (de Lecea et al. 1998) mediate their effects through G protein-coupled receptors called OX\textsubscript{1} and OX\textsubscript{2} receptors (Sakurai et al. 1998). A previous study has shown that the orexin OX\textsubscript{1} receptor activates the Ca\textsuperscript{2+} influx pathway necessary for coupling to phospholipase C in Chinese hamster ovary cells (Lund et al. 2000). On the other hand, PLC-\gamma is also activated by PI3 kinase-induced formation of Ptd Ins (3,4,5) \textsubscript{P}, which serves as a binding target for the PH-domain of PLC\gamma (Falasca et al. 1998). One of the end results is the activation of two receptor-mediated pathways for stimulating the formation of IP\textsubscript{3} and DAG and the subsequent elevation of [Ca\textsuperscript{2+}]. The regulation of Ca\textsuperscript{2+} in the nucleus, where some important Ca\textsuperscript{2+}-sensitive processes reside, is debated issue (Bootman et al. 2000). Finally, if the control of cellular Ca\textsuperscript{2+} homeostasis somehow fails
(excess penetration), mitochondria “buy time” by precipitating inside Ca$^{2+}$ and phosphate. If the injury persists, Ca$^{2+}$-induced cell death eventually ensues (Bootman et al. 2000).

Over the past several years, electrophysiological and molecular biological studies have demonstrated the existence of a diverse family of store-operated channels. The best-characterized SOCs have many salient features. The essential defining feature of these channels is activation by a variety of stimuli that deplete stores, independently of the Ca$^{2+}$ released during the depletion process. These stimuli include agonists to phosphoinositide-linked receptors, intracellular IP$_3$, Ca$^{2+}$ ionophores, inhibitors of SERCA-type Ca$^{2+}$ ATPases, and intracellular dialysis with buffered solutions containing low concentrations of Ca$^{2+}$ (<50 nM). Because the content of intracellular Ca$^{2+}$ stores is still difficult to measure and correlate with inflow activation, the identification of a current as being store-operated relies entirely on indirect inference. For this reason, it is essential to test as many methods of store depletion as possible. Activation by IP$_3$ suggests that the agonist
receptor is not directly coupled to channel activation. Activation by ionophores and SERCA inhibitors, which deplete stores without generating IP3, implies that the channels respond to depletion rather than directly to IP3. However, because most of these agents increase the net Ca\(^{2+}\) flux from stores, it is also important to demonstrate current activation under conditions of constant [Ca\(^{2+}\)]. For example, powerful evidence for a store-dependent mechanism is obtained from current activation by intracellular Ca\(^{2+}\) chelators, which are thought to deplete stores by reducing [Ca\(^{2+}\)] and interrupting a futile cycle of ongoing Ca\(^{2+}\) leak and reuptake by stores. These activation criteria have been met for a number of store-operated channels. A second feature common to all SOCs described to date is a lack of voltage-dependent gating, although this need not be a distinctive feature. Despite the lack of voltage-dependent gating, the membrane potential, nevertheless, plays several important roles in regulating the Ca\(^{2+}\) influx through SOCs. First, depolarization inhibits Ca\(^{2+}\) entry by reducing the driving force for Ca\(^{2+}\) entry. Second, hyperpolarization promotes rapid inactivation of Ca\(^{2+}\) release-activated Ca\(^{2+}\) (CRAC) channels by Ca\(^{2+}\), presumably by raising the local [Ca\(^{2+}\)] at the inactivation sites (Hoth & Penner 1992 and 1993, Zweifach & Lewis 1996). Hyperpolarization may enhance CRAC channel activity through effects on Ca\(^{2+}\)-dependent potentiation (Zweifach & Lewis 1996).

### 2.3.4 Capacitative calcium influx

The original idea that calcium might enter cells through a capacitative mechanism was first introduced by Jim Putney (Putney 1986). The somewhat surprising hypothesis was that calcium entry was regulated by the filling status of the calcium stores. Analogously to a capacitor in an electrical circuit, the calcium stores prevent entry when they are charged up but begin to promote entry as soon as the stored calcium has been discharged. This capacitative entry mechanism is present in many cells and has properties that are very similar in different cells (Putney 1990, Meldolesi et al. 1991, Penner et al. 1993, Fasolato et al. 1994, Putney & Bird 1994). Capacitative calcium entry can be switched on by a variety of stimuli, such as normal agonists or pharmacological agents, all of which share the property of releasing stored calcium. Examples include calcium-mobilizing agonists (Parker & Miledi 1987, Lupu-Meiri et al. 1993, Parekh et al. 1993, Zweifach & Lewis 1993, McDonald et al. 1993, Petersen & Berridge 1994, Byron & Taylor 1995), the calcium-mobilizing second messenger inositol 1,4,5- triphosphate (IP3) (McDonald et al. 1993, Petersen & Berridge 1994, Hoth & Penner 1992 and 1993, Yao & Parker 1993, DeLisle et al. 1995), the calcium ionophore ionomycin (Hoth & Penner 1992, Morgan & Jacob 1994), inhibitors of the endoplasmic reticulum (ER) pumps, such as thapsigargin (Lupu-Meiri et al. 1993, Zweifach & Lewis 1993, Petersen & Berridge 1994, Mason et al. 1991) and cyclopiazonic acid (Mason et al. 1991, Demaurex et al. 1992), or simply incubation of cells in Ca\(^{2+}\)-free conditions (Petersen & Berridge 1994, Hoth & Penner 1992 and 1993). All this evidence indicates that the entry of external calcium is somehow controlled by the calcium content of the ER. Previous studies have investigated thapsigargin-induced calcium mobilization in keratinocytes (Jones & Sharpe 1994, Harmon et al. 1996).
Numerous studies utilizing agents that inhibit the Ca\(^{2+}\)-ATPase responsible for Ca\(^{2+}\) storage within the endoplasmic reticulum (e.g. thapsigargin) have demonstrated that Ca\(^{2+}\) store depletion provides a full and sufficient signal for the activation of a capacitative calcium influx (Putney, 1993 and 1997). However, the nature of the signal which links the depletion of ER calcium stores to the opening of the capacitative Ca\(^{2+}\) channels remains unknown. Several potential mechanisms have been suggested for the coupling of the calcium content of ER and the permeability of the plasma membrane to calcium: Initially, physiological contact between the ER membrane and the plasma membrane calcium channels was proposed (Putney 1986, Irvine 1990). Furthermore, intermediates of IP\(_3\) metabolism, small G proteins, a factor synthesized in calcium-depleted ER, also known as calcium influx factor-hypothesis (CIF-hypothesis), cytochromes P450, tyrosine kinases, protein kinases C and/or protein kinase A, and the cytoskeleton have been suggested to play a role in the coupling between the calcium content of ER and the permeability of the plasma membrane to calcium (Luckhoff & Clapham 1992, Alvarez et al. 1992, Parekh et al. 1993, Randriamapita & Tsien 1993, Lee et al. 1993, Striggow & Bohnensack 1994, Petersen & Berridge 1996, Gregory & Barritt 1996, Holda & Blatter 1997, Ribeiro et al. 1997). More specifically, the mechanisms proposed to explain how information is transferred from the endoplasmic reticulum to the plasma membrane fall into two main lines of deduction (for reviews, see Putney & Bird 1993, Berridge 1995b). One model describes a direct protein-protein association between the IP\(_3\) receptor in the endoplasmic reticulum and the capacitative entry channel. In this model, the IP\(_3\) receptor would undergo a conformational change, which would allow its interaction with the capacitative Ca\(^{2+}\) channel and its opening (Ribeiro et al. 1997). Another possible explanation is that a diffusible messenger is released as a consequence of Ca\(^{2+}\) store depletion, which would subsequently stimulate the Ca\(^{2+}\) influx channel to open (Holda & Blatter 1997). The first model implies close physical interaction between the endoplasmic reticulum and the plasma membrane mediated by, for example, the cytoskeleton. No direct interaction between the ER and the plasma membrane would be required for a diffusible factor to act. Indirect evidence has suggested that the actin cytoskeleton may mediate physical interaction between IP\(_3\) receptors and the plasma membrane (Rossier et al. 1991, Ribeiro et al. 1997). This interaction might be important for calcium signaling and for capacitative calcium entry.

Experimental approaches to analyze the signal involved in store-operated calcium entry have been considerably advanced by the discovery of mammalian homologs of the Drosophila cation channel transient receptor potential (TRP). Some members of the TRP family can be expressed to Ca\(^{2+}\)-permeable channels that enable SOCs; other members form channels activated independently of stores. TRP proteins may be an essential part of endogenous Ca\(^{2+}\) entry channels but so far expression of most TRP cDNAs has not resulted in restitution of channels found in any mammalian cells, suggesting the requirement for further unknown subunits (Zitt et al. 2002).

Capacitative calcium entry plays a central role in many aspects of cell signaling. For example, the Ca\(^{2+}\) oscillations that have been recorded in many cells are maintained by the small but constant influx of external calcium (Berridge 1990 and 1993). Capacitative calcium influx has also been implicated in the function of osteoclasts (Zaidi et al. 1993), the regulation of adenylate cyclase (Cooper et al. 1994), regulatory volume decrease (Tinel 1994), phototransduction in Drosophila photoreceptors (Hardie 1991, Ranganathan...
et al. 1991, Minke & Selinger 1991, Hardie & Minke 1992, Selinger et al. 1993), and the activation of mitogenesis in lymphocytes (McDonald et al. 1993) and in fibroblasts (Lee et al. 1993). The control of proliferation is a particularly interesting example because certain forms of immunodeficiency may result from a defect in calcium entry (Partiseti et al. 1994). Also, there was a marked increase in capacitative calcium entry in simian virus 40 (SV-40)-transformed Swiss 3T3 cells (Newcomb et al. 1993). Furthermore, previous studies have shown that an intact actin-containing cytoskeleton is necessary for the activation of store-operated Ca\(^{2+}\) channels (Wang et al. 2002, Bishara et al. 2002, Rafferty et al. 1994).

2.3.5 Calcium signaling and disease

Mutations or abnormalities in one of the above mentioned Ca\(^{2+}\)-transporting proteins may lead to disease. Two skin diseases are caused by Ca\(^{2+}\) pump mutations (Hailey-Hailey disease and Darier disease). Hailey-Hailey disease (HHD, MIM 16960) is inherited in an autosomal dominant manner and characterized by persistent blisters and erosions of the skin (Burge 1992). Hailey-Hailey disease is caused by mutations in the ATP2C1 gene encoding a novel Ca\(^{2+}\) pump (Sudbrak et al. 2000). The ATP2C1 gene encodes a new class of P-type Ca\(^{2+}\) transport ATPase, which is related to the sarco(endo)plasmic calcium ATPase (SERCA) and the plasma membrane calcium ATPase (PCMA) families of Ca\(^{2+}\) pumps. Impaired intercellular adhesion and epidermal blistering also occur in patients with Darier disease (DD, MIM 124200), which is caused by mutations in the ATP2A2 gene, a gene encoding a sarco/endoplasmic reticulum (ER)-Golgi calcium pump (Richard et al. 1990). Recent evidence from Hailey-Hailey disease patients indicates that the Golgi apparatus may also be an important Ca\(^{2+}\) store in keratinocytes (Hu et al. 2000, Sudbrak et al. 2000).

Skeletal-muscle pathology may be caused by abnormal ryanodine receptors (malignant hyperthermia, porcine stress syndrome, central core disease), plasma membrane Ca\(^{2+}\) channels (hypokalemic periodic paralysis, muscular dysgenesis in mice, paraneoplastic Lambert-Eaton myasthenia syndrome), or Ca\(^{2+}\) pumps (Brody disease). Neurologic disorders may be related to altered function of plasma-membrane Ca\(^{2+}\) channels (episodic ataxia type 2, spinocerebellar ataxia type 6, familial hemiplegic migraine, glutamate excitotoxicity, tottering, leaner, lethargic and stargazer mice), IP3 receptors (Lowe's oculocerebrorenal syndrome, manic depression, Alzheimer's disease, opisthotonos mice) and Ca\(^{2+}\) pumps (deafwaddler mouse and wriggle mouse sagami). Incomplete X-linked congenital stationary night blindness is caused by a mutation in the plasma membrane Ca\(^{2+}\) channels in rods and cones (for a review see Missiaen et al. 2000).
2.4 Intercellular calcium waves

2.4.1 Gap-junctional calcium wave

2.4.1.1 Gap junctions

Gap junctions are intercellular channels, which are involved in cell-cell communication (Burge 1994, Richard 2000). Gap junctions are pervasive intercellular membrane channels that directly connect cells on tissues and organs, allowing inorganic ions and other small water-soluble molecules (<1 kDa) to pass from one cell to another and thereby coupling cells electrically and metabolically (Goodenough et al. 1996, Kumar & Gilula 1996). Gap junction biogenesis requires assembly of six subunit proteins, connexin, into a hexameric connexon and their trafficking to the plasma membrane. 14 distinct connexins have been described in rodents, and all appear to have similar arrangement in the membrane (Goodenough et al. 1996).

Fig. 4. Connexin structure. The cylinders represent transmembrane domains (M1-M4). The loops between the first and second, as well as the third and fourth, transmembrane domains are predicted to be extracellular (E1 and E2, respectively).
Interactions between connexons from juxtaposed cells result in functional gap junction channels. Each gap junction pore is formed by a juxtaposition of two intercellular hemichannels in neighboring cells, which interact to span the plasma membranes of two adjacent cells and directly join the cytoplasm of one cell to that of another. Each hemichannel is composed of a hexameric array of connexins (Goodenough et al. 1996, Saez et al. 1993). Many different types of connexins can join to form a wide diversity of gap-junctional channels, depending on the tissue and the functional status of the cell (Goliger & Paul 1994, Wiszniewski et al. 2000).

Fig. 5. Possible arrangements of connexons to form gap junction channels. Connexons consist of six subunits, connexins. A connexon may be homomeric, i.e. composed of six identical connexin subunits, or heteromeric, i.e. including more than one species of connexins. Connexons associate end to end to form a double-membrane gap junction channel. The channel may be homotypic with identical connexons or heterotypic with two different connexons. (The figure has been modified from Kumar & Gilula 1996).

Gap junctions have diverse integrative functions, including roles in development, synchronous contraction of the heart, regulation of exocytosis, and control of cell growth (Goodenough et al. 1996). Gap junctions are numerous in all layers of the epidermis and have an important role in the coordination of keratinocyte growth and differentiation (Salomon et al. 1994, Tada & Hashimoto 1997). Mutations in connexins or defective production of gap junctions are associated with deafness (Kelsell et al. 1997), Charcot-Marie-Tooth X-linked neuropathy (Bergoffen et al. 1993), malignancy (Budunova 1994), and cataractogenesis (Gong et al. 1997).

A spectrum of drugs have been shown to inhibit gap-junctional communication with variable degrees of efficacy and specificity. These include volatile anesthetics, such as halothane (Nedegaard 1994), the straight-chain alcohols heptanol and octanol (Donahue et al. 1995, Nedegaard 1994), anandamide (Venance et al. 1995), and glycerrhetinic acid...
(Davidson & Baumgarten 1995). Although a variety of drugs have been used to block gap-junctional communication, the specificity of the blockers remains to be solved.

### 2.4.1.2 Connexins as tumor suppressors

The possible role of GIJC in suppressing the growth of malignant cells was first suggested by Stoker (Stoker 1967). More recent evidence of a direct role of GIJC in tumor suppression has come from a series of experiments in which connexin genes were transfected into GIJC-deficient malignant cell lines, to study the effect of connexins on cell growth in vitro and in vivo. Thus, human liver tumor cells transfected with the Cx32 gene showed reduced tumour growth (Eghbali et al. 1991). Similarly, rat glioma cells (Naus et al. 1992) and chemically transformed mouse fibroblasts (Rose et al. 1993) transfected with the Cx43 gene did not produce tumour growth. The ability to suppress tumors is related to specific connexin types. HeLa cells transfected with the Cx26 gene were completely non-tumorigenic and showed a reduced growth rate in vitro, while the Cx40 and Cx43 genes did not have a significant tumor-suppressive effect on the same cells, despite an increase in GIJC (Mesnil et al. 1994). The Cx26 gene has previously been proposed to be a tumor-suppressor gene of human mammary cancers (Lee et al. 1991). The Cx26 gene appears to be the major connexin gene expressed in the cervix. These results suggest that connexin genes exert a differential cell growth control effect, depending on the cell type in which they are expressed.

In certain systems, there is a good correlation between the expression of specific connexin genes and susceptibility to carcinogenesis. For example, the putative tumor suppressor gene, Cx26, is expressed in the liver to different degrees in different species, increasing in the order rat < mouse < guinea pig (Kuraoka & Shibata 1993). The resistance of these rodent species to liver carcinogenesis increases in the same order (Bannasch 1983), suggesting that the extent of Cx26 gene expression is associated with resistance to the induction of liver carcinogenesis.

Previous studies have shown that many different connexins are expressed in human skin. For example, connexin 26 (Cx 26) and connexin 43 (Cx43) are the main connexins in skin. Connexin 26 has been shown to be significantly and connexin 43 slightly upregulated in psoriatic skin in vivo (Labarthe et al. 1998). It has been suggested that psoriatic keratinocytes compensate for their insufficient need of intercellular communication by increasing connexin 26 channels. Connexin 26 upregulation has also been shown to be a feature of keratinocyte differentiation not only in hyperproliferative skin but also in buccal and vaginal epithelium (Lucke et al. 1999). Connexin 43 is widely expressed in different tissues, including brain, heart, kidney, smooth muscle, ovary, and some epithelia, and it is actually the most abundant connexin (Goodenough et al. 1996, Musil et al. 1990). On cell contact, connexons from neighboring cells may form gap junctions in a matter of minutes or even seconds, suggesting that connexons pre-exist in the plasma membrane (Rook et al. 1990).
2.4.1.3 Regulation of gap-junction activity by calcium

Like conventional ion channels, individual gap-junction channels do not remain continuously open; instead, they flip between open and closed states. The permeability of gap junctions is rapidly (within seconds) and reversibly decreased by experimental manipulations that decrease cytosolic pH or increase the cytosolic concentration of free Ca\(^{2+}\). These observations indicate that gap-junction channels are dynamic structures that are gated. They can undergo a reversible conformational change that closes the channel in response to changes in the cell. When a cell is damaged, its plasma membrane can become leaky. Ions present at high concentration in the extracellular space, such as Ca\(^{2+}\), then move into the cell. The influx of Ca\(^{2+}\) into the sick cell causes its gap-junction channels to close immediately, effectively isolating the cell and preventing damage from spreading in this way. Elevated levels of cytoplasmic Ca\(^{2+}\) in the vicinity of gap junctions thus result in closure of the channel (Rose & Loewenstein 1975, Crow et al. 1994, Lazrak & Peracchia 1993, Unwin & Ennis 1983), but the mechanisms are poorly understood. Such effects may involve soluble factors (Nicholson et al. 1998), intermediates, such as calmodulin (Torok et al. 1997, Peracchia et al. 1996), or possibly accessory adhesion proteins (Jongen et al. 1991). The gating of gap junction channels is also regulated by H\(^{+}\) ions, cAMP, voltage, and phosphorylation by protein kinase C (Goodenough et al. 1996).

Calcium waves are propagated intercellularly via gap junctions (Boitano et al. 1992). Two hypotheses concerning the role of calcium in the operating mechanism of gap junction intercellular channels have been discussed. According to the first, the connexon hemichannels in the plasma membrane, prior to assembly into gap junctions, are in a closed configuration since they exist in a high (~5 \(\mu\)M) sub-plasma-membrane Ca\(^{2+}\) environment. Other studies have shown that the “free” connexon hemichannels in the plasma membrane are closed (Li et al. 1996). As these hemichannels accrete and dock with partner channels in juxtaposed cells, they enter into or generate a lower intracellular Ca\(^{2+}\) environment at this confined gap junction microdomain, which is more favorable for the opening of the coupled hemichannels. According to the second hypothesis, a uniformly high sub-plasma-membrane Ca\(^{2+}\) level exists in cells, including the areas directly beneath gap junctions. Such a relatively high \([\text{Ca}^{2+}]_{\text{pm}}\) would favor a closed configuration of gap junctions (Rose & Loewenstein 1975). In this scheme, an auxiliary proteins associated with the gap junction is thought to mediate channel opening. The present studies do not distinguish between these two hypothesis, but any mechanism proposed must also take into account other central questions concerning intercellular signaling across gap junctions, especially the way in which Ca\(^{2+}\) waves propagate between cells (Boitano et al. 1992). With regard to the question of whether connexons are in a closed configuration during their intracellular trafficking, it seems unlikely that the hemichannels are shut by the low ambient cytoplasmic Ca\(^{2+}\) levels.

2.4.2 Extracellular ATP-mediated calcium wave

Intercellular calcium waves are stimulus evoked cellular responses in which propagated waves of cytoplasmic Ca\(^{2+}\) increases spread from one cell to another. Intercellular calcium
waves are considered to serve as a pathway to long-range signaling. The generation of intercellular calcium waves relies partly on the autocrine activity of ATP. Mechanical stimulation of cells results in release of ATP, which activates purinergic receptors on neighboring cells (Osipchuk & Cahalan 1992). Extracellular ATP binds to P2 purinergic receptors, which constitute a large family of receptors that are ion channels (P2X purinoceptors) or couple to G proteins (P2Y purinoceptors). The activation of purinergic receptors, in turn, triggers the release of IP$_3$ and intracellular calcium stores (Dubyak & el-Moatassim 1993). Suramin and reactive blue are P$_{2Y}$-purinoceptor antagonists. One P$_{2X}$ antagonist is pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid tetrasodium (PPADS). An anti-trypanosomal and anti-filarial drug suramin was used to block P$_{2Y}$-purinergic receptors in the present study (Dunn & Blakeley 1988, Jørgensen et al. 1997, Cotrina et al. 1998). Suramin also has other effects. It alters phosphoinositide synthesis, inhibits growth factor receptor binding in HT-29 cells (Kopp & Pfeiffer 1990), and inhibits melanoma heparanase and invasion (Nakajima et al. 1991).

2.4.3 Cytoskeleton and calcium signaling

Depletion of intracellular Ca$^{2+}$ stores activates a pathway for Ca$^{2+}$ influx across the plasma membrane, which has been termed capacitative Ca$^{2+}$ entry (Putney 1986). Numerous studies utilizing reagents that inhibit the Ca$^{2+}$-ATPase responsible for Ca$^{2+}$ storage within the endoplasmic reticulum (e.g. thapsigargin) have demonstrated that Ca$^{2+}$ store depletion provides a full and sufficient signal for the activation of capacitative Ca$^{2+}$ entry (Putney 1993 and 1997). However, the nature of the signal linking pool depletion to the opening of the capacitative Ca$^{2+}$ influx pathway remains unknown. Two general mechanisms have been proposed to explain how information is transferred from the endoplasmic reticulum to the plasma membrane (reviewed Putney & Bird 1993, Berridge 1995). One model describes a direct protein-protein association between the IP$_3$ receptor in the endoplasmic reticulum and the capacitative entry channel; after IP$_3$ binding and release of stored Ca$^{2+}$, the IP$_3$ receptor would undergo a conformational change, which allows for its interaction with the capacitative Ca$^{2+}$ channel, resulting in its opening. According to the other proposal, a diffusible messenger, released and/or formed as a consequence of Ca$^{2+}$ pool depletion, would stimulate the Ca$^{2+}$ influx pathway to open. The first model implies a close physical interaction between the endoplasmic reticulum and the plasma membrane; on the other hand, such a direct interaction would not be required for a diffusible factor to act. Indirect evidence has earlier been presented by Ribeiro et al. 1997 to show that there is physical interaction between the IP$_3$ receptors and the plasma membrane that involves the actin cytoskeleton, and the authors speculated that this interaction might be important in signaling capacitative calcium entry (Rossier et al. 1991).
3 Aims of the present study

The purpose of the present study was to elucidate calcium-mediated cell signaling in NF1 and psoriatic keratinocytes. The specific aims were as follows:

1. To study intracellular calcium stores, capacitative calcium influx, and the activation of store-operated calcium channels in normal, NF1 and psoriatic keratinocytes.

2. To study mechanical stimulation-induced intercellular calcium waves in normal, NF1 and psoriatic keratinocytes in the presence of inhibitors of gap-junctional or ATP-mediated communication.

3. To elucidate the effect of an intact cell cytoskeleton on the capacitative calcium influx in normal and NF1 keratinocytes.

4. To study the effect of different extracellular calcium concentrations on calcium-mediated cell signaling in normal, NF1 and psoriatic keratinocytes.

5. To assess the potential role of altered calcium-mediated cell signaling in keratinocytes cultured from patients with psoriasis.
4 Materials and Methods

4.1 Materials

The materials used are shown summarized below in the Tables 1 and 2, and they have been described in detail in the original publications (I-IV).

Table 1. The tissues and cell lines used are listed below and have been described in detail in the original publications (I-IV). All tissue samples were collected with appropriate approvals from the ethical committees of the respective universities and university hospitals.

<table>
<thead>
<tr>
<th>Tissue samples</th>
<th>Used in</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human adult skin (from 22 individuals, aged 29-61 yrs)</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td>Skin of NF1 patients (from 22 individuals, aged 23-40 yrs)</td>
<td>I, II, III</td>
</tr>
<tr>
<td>Lesional and nonlesional skin of psoriasis patients, (from 6 individuals, aged 34-63 yrs)</td>
<td>IV</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cell cultures</th>
<th>Used in</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human adult keratinocytes</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td>Keratinocytes cultured from NF1 patients</td>
<td>I, II, III</td>
</tr>
<tr>
<td>Keratinocytes cultured from psoriasis patients, lesional and nonlesional</td>
<td>IV</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Substances used in vitro</th>
<th>Source</th>
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</thead>
<tbody>
<tr>
<td>Fura-2/AM (Molecular Probes)</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td>Pluronic F-127 (Molecular Probes)</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td>Thapsigargin (Molecular Probes)</td>
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</tr>
<tr>
<td>Heptanol (Sigma)</td>
<td>I, III, IV</td>
</tr>
<tr>
<td>Suramin (Calbiochem)</td>
<td>I, III, IV</td>
</tr>
<tr>
<td>ATP (Calbiochem)</td>
<td>I, II, IV</td>
</tr>
<tr>
<td>Nocodazole (Calbiochem)</td>
<td>II</td>
</tr>
<tr>
<td>Cytochalasin D (Calbiochem)</td>
<td>II</td>
</tr>
</tbody>
</table>

*Department of Surgery, University of Turku; †Department of Dermatology, University of Oulu, and Department of Surgery, University of Turku; ‡Department of Dermatology, University of Oulu
Table 2. The following antibodies were used in immunolabeling or Western blotting analyses. Phalloidin was used to visualize actin.

<table>
<thead>
<tr>
<th>Primary antibodies</th>
<th>Description</th>
<th>Source / Reference</th>
<th>Used in</th>
</tr>
</thead>
<tbody>
<tr>
<td>Connexin 43 (Im, W)</td>
<td>Mouse Mab</td>
<td>Zymed</td>
<td>I</td>
</tr>
<tr>
<td>Connexin 43 (W)</td>
<td>Rabbit Pab</td>
<td>Zymed</td>
<td>I</td>
</tr>
<tr>
<td>α-tubulin (Im)</td>
<td>Mouse Mab</td>
<td>Sigma</td>
<td>II</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>Phalloidin Alexa Fluor 488 conjugated</td>
<td>Molecular Probes</td>
<td>II</td>
</tr>
</tbody>
</table>

Abbreviations: Immunolabeling (Im); Western blotting analyses (W), polyclonal antibody (Pab); monoclonal antibody (Mab).

4.2 Methods (I-IV)

The methods used in the thesis are shown summarized below (Table 3), and they have been described in detail in the original publications (I-IV).

Table 3. The methods used in the original communications (I-IV).

<table>
<thead>
<tr>
<th>Methods</th>
<th>Used in</th>
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<tbody>
<tr>
<td>Cell cultures</td>
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<tr>
<td>Establishing and culturing of keratinocytes</td>
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</tr>
<tr>
<td>Indirect immunofluorescence labeling</td>
<td>I, II</td>
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<tr>
<td>Labeling of actin filaments</td>
<td>II</td>
</tr>
<tr>
<td>Western blot</td>
<td>I</td>
</tr>
<tr>
<td>Fluorescence and microscopic techniques</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td>Light microscopy</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td>Conventional fluorescence microscopy</td>
<td>I</td>
</tr>
<tr>
<td>Differential interference contrast microscopy</td>
<td>II</td>
</tr>
<tr>
<td>Confocal laser scanning microscopy</td>
<td>II</td>
</tr>
<tr>
<td>Digital image analysis</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td>Disruption of the cell cytoskeleton</td>
<td>II</td>
</tr>
<tr>
<td>Statistical analysis</td>
<td>I, II, III, IV</td>
</tr>
</tbody>
</table>
5 Results

5.1 Intracellular calcium stores (I, III, IV)

In order to elucidate intracellular calcium stores in keratinocytes, the extracellular Ca$^{2+}$ was chelated with EGTA and the cells were treated with thapsigargin. The exposure of cells to thapsigargin results in elevation of intracellular free Ca$^{2+}$, which reflects the release of calcium stores from the ER, but not an influx of extracellular Ca$^{2+}$. The results demonstrated that NF1 and psoriatic keratinocytes displayed reduced calcium stores of the ER compared to normal cells after exposure to thapsigargin, when the influx of extracellular calcium was inhibited.

5.2 Capacitative calcium influx at different extracellular calcium concentrations (I, III, IV)

Capacitative calcium influx was investigated after exposure of the cells to thapsigargin. Fluorescence ratio imaging was performed using fura-2 as a calcium-sensitive probe. When the cells are maintained in a medium containing different extracellular concentrations, 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. The results demonstrated that the effect of thapsigargin on the intracellular Ca$^{2+}$ levels of NF1 and psoriatic keratinocytes was significantly weaker compared to normal control cells. This difference was even more pronounced at high extracellular calcium concentrations. The resting Ca$^{2+}$ levels were also lower in NF1 and psoriatic keratinocytes compared to normal cells. The results suggest that capacitative calcium influx is altered in NF1 and psoriatic keratinocytes compared to normal control cells.
5.3 Effect of extracellular ATP on normal, NF1, and psoriatic keratinocytes (I, II, IV)

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

5.4 Activation of store-operated calcium channels (I)

In order to elucidate the activation of store-opetared calcium channels \(\text{Mn}^{2+}\) quench analysis were performed. \(\text{Mn}^{2+}\) can enter cells via SOCs that open after release of intracellular \(\text{Ca}^{2+}\) stores. The end result of \(\text{Mn}^{2+}\) influx is reduction of fura-2 fluorescence, which is because of replacement of \(\text{Ca}^{2+}\) from the calcium sensitive probe. When \(\text{Mn}^{2+}\) alone was applied to the cells and fura-2 fluorescence quenching was monitored, a slow decline of fluorescence was detected in normal control cells. The quenching under these resting conditions may reflect slow leakage of \(\text{Mn}^{2+}\) into the cells. Quenching of fura-2 fluorescence was more moderate in NF1 keratinocytes under the same experimental conditions.
conditions, suggesting slow entry of Mn\textsuperscript{2+} into these cells. Application of thapsigargin in the presence of extracellular Mn\textsuperscript{2+} resulted in different effects on normal keratinocytes compared to cells cultured from patients with type 1 neurofibromatosis. Specifically, the application of thapsigargin resulted in fast and marked quenching of cytosolic fura-2 fluorescence in normal cells due to influx of extracellular Mn\textsuperscript{2+} into the cells. In contrast, there was first an increase of cytosolic fura-2 fluorescence followed by fluorescence quenching in NF1 keratinocytes under the same experimental conditions.

The exposure of keratinocytes to ATP in the presence of extracellular Mn\textsuperscript{2+} resulted in fast and marked quenching of cytosolic fura-2 fluorescence in normal cells, but not in NF1 keratinocytes. In the same experimental conditions, NF1 keratinocytes responded to extracellular ATP by transiently elevating cytosolic fura-2 fluorescence before enhanced fluorescence quenching due to increased Mn\textsuperscript{2+} entry.

The results suggest reduced influx of extracellular Mn\textsuperscript{2+} into NF1 keratinocytes. The results also 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\textsuperscript{2+} stores and down-regulated signal from ER to SOCs, this might explain defective capacitative calcium influx in these cells.

5.5 Propagation of intercellular calcium waves at different extracellular calcium concentrations (I, III, IV)

In further studies, we analyzed wound-induced calcium waves of normal and NF1 keratinocytes at different extracellular calcium concentrations using fluorescence ratio imaging. The results revealed that the rise of [Ca\textsuperscript{2+}] following mechanical stimulation was lower in NF1 and psoriatic keratinocytes compared to normal control cells. The difference was even more pronounced at high (1.8 mM) extracellular calcium concentration. It was concluded that calcium wave progression was altered in NF1 and psoriatic keratinocytes compared to normal control cells.
5.5.1 Role of gap-junctional signaling in calcium wave propagation (I, III, IV)

In order to elucidate gap-junctional signaling, intercellular calcium waves were further analyzed in the presence of heptanol, a gap-junctional signaling inhibitor, or suramin, an inhibitor of P₂-purinergic receptors. The results demonstrated that the main routes of calcium-mediated signaling were different in normal versus NF1 and psoriatic keratinocytes. Specifically, the main route of calcium-mediated signaling was gap junctional in normal keratinocytes. In contrast, ATP-mediated calcium signaling predominated in NF1 and psoriatic keratinocytes. Indirect immunofluorescence labeling of normal keratinocytes revealed a distinct organization of Cx43 to gap-junctional plaques. In contrast, NF1 keratinocytes were characterized by a mostly cytoplasmic immunoreaction for Cx43, often in association with cytoskeletal filaments. Western blotting demonstrated apparently equal levels of Cx43 in normal and NF1 keratinocytes. The results indicate that gap-junctional signaling was defective in NF1 and psoriatic keratinocytes.

Fig. 7. Intercellular calcium-mediated cell signaling at various extracellular concentrations. Keratinocytes were stimulated mechanically in the presence of inhibitors of gap-junctional or ATP-mediated communication in order to determine which pathways are operative in Ca²⁺ signaling in normal control versus NF1 keratinocytes.
5.5.2 Role of extracellular ATP in calcium wave propagation (I, III, IV)

Intercellular calcium waves were further analyzed in the presence of suramin, an inhibitor of P_2−purinergic receptors. 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 and psoriatic keratinocytes. This finding suggests that P_2 purinergic signaling is functioning in NF1 and psoriatic keratinocytes, and these cells may thereby partly compensate for their defective gap-junctional signaling.

5.5.3 Velocity of intercellular calcium wave (I, III)

In order to elucidate intercellular calcium wave velocity, wound-induced calcium waves of normal and NF1 keratinocytes were analyzed using fluorescence ratio imaging. The results demonstrated that the intercellular Ca^{2+} wave velocity was ~16.8 µm/s at high [Ca^{2+}], and ~13.2 µm/s at low [Ca^{2+}], in normal keratinocyte cultures. Previous studies have not addressed the velocity of calcium waves in human keratinocyte cultures. The speed of the calcium wave was higher in cultures of normal keratinocytes than in NF1 cell cultures (p<0.001 at high [Ca^{2+}], and p<0.05 at low [Ca^{2+}]). Average intercellular calcium waves in cultures of NF1 keratinocytes progressed at a rate of ~11.8 µm/s at high [Ca^{2+}], and ~10.9 µm/s at low [Ca^{2+}]. Distinct gap-junctional signaling routes were detected as evaluated by cine-loop videos in the normal cell monolayers but less clearly in NF1 keratinocytes. The results indicate that gap-junctional signaling was defective in NF1 keratinocytes.

5.5.4 Intracellular calcium stores and intercellular calcium wave propagation (I, III, IV)

Calcium wave propagation was investigated after exposure of the cells to thapsigargin. When endoplasmic calcium stores were released with thapsigargin prior to wounding, neither normal nor NF1 keratinocytes displayed an increase in [Ca^{2+}], and calcium waves could not be induced. The results indicate that intracellular calcium stores are required in calcium wave propagation.
5.6 Disruption of cytoskeletal filaments and calcium signaling (III)

5.6.1 Disruption of actin microfilaments with cytochalasin D (III)

Normal and NF1 keratinocytes were treated with cytochalasin D or nocodazole to depolymerize the actin microfilaments or microtubules, respectively. Cytochalasin D and nocodazole induced significant alterations in cellular morphology. To verify the specificity of cytochalasin D and nocodazole, a double-labeling fluorescence protocol was used to visualize the actin and tubulin cytoskeletons. The filamentous actin and the microtubules were intact and distributed throughout the cytoplasm in control cells. Pretreatment of cells with cytochalasin D or nocodazole specifically depolymerized either the actin stress fibers or the microtubules, respectively. In cytochalasin D-treated cells, the actin bundles were replaced by a punctate accumulation of actin in the cytoplasm. In nocodazole-treated cells, the depolymerized tubulin redistributed throughout the cytoplasmic compartment.

5.6.2 Treatment of cells with thapsigargin and calcium imaging (III)

In further studies, the effect of cytoskeleton-disrupting agents on capacitative calcium influx was investigated. Thapsigargin was used to release the intracellular calcium stores and, subsequently, to activate the influx of extracellular \( \text{Ca}^{2+} \) through SOCs. In normal control keratinocytes, thapsigargin induced a rapid and transient increase in \([\text{Ca}^{2+}]_i\). In control experiments, NF1 keratinocytes were treated with vehicle alone and the \( \text{Ca}^{2+} \) stores of the ER were also mobilized with thapsigargin. In control NF1 keratinocytes, thapsigargin induced a slow or moderate increase in \([\text{Ca}^{2+}]_i\). The \( \text{Ca}^{2+} \) stores of the ER were mobilized with thapsigargin after treatment of the cells with cytochalasin D. The results demonstrated that the capacitative calcium influx in cytochalasin D-treated normal keratinocytes is significantly weaker compared to normal control cells as evaluated by rise in \([\text{Ca}^{2+}]_i\). In cytochalasin D-treated NF1 keratinocytes, the kinetics and activation and the magnitude of thapsigargin-induced \( \text{Ca}^{2+} \) mobilization were indistinguishable from NF1 keratinocytes treated with DMSO alone.

5.6.3 Treatment of cells with extracellular ATP and calcium imaging (III)

In order to elucidate the effect of disruption of actin microfilaments on intracellular calcium signaling, the cells were treated with ATP. Extracellular ATP acts through purinergic receptors to couple to phospholipase \( \text{C}_\beta \) activation. In control normal cells and
in NF1 keratinocytes, ATP induced a rapid and transient increase in \([\text{Ca}^{2+}]\). In cytochalasin D-treated cells, the kinetics, activation, and magnitude of ATP-induced \(\text{Ca}^{2+}\) mobilization were apparently indistinguishable from those in control cells. The responses observed in calcium dynamics were apparently the same in nocodazole-treated cells compared to keratinocytes treated with cytochalasin D. The results clearly demonstrate that cytochalasin D blocked capacitative \(\text{Ca}^{2+}\) entry when ER \(\text{Ca}^{2+}\) stores were depleted with thapsigargin. In contrast to the effects on thapsigargin-induced \([\text{Ca}^{2+}]\), signaling, neither the release nor the entry of \(\text{Ca}^{2+}\) in response to extracellular ATP was significantly affected by cytoskeletal disruption in normal or NF1 keratinocytes. The results suggest that microfilaments play a crucial role for functional capacitative \(\text{Ca}^{2+}\) entry in cultured keratinocytes.
6 Discussion

6.1 Intracellular calcium stores

The endoplasmic reticulum-associated Ca\(^{2+}\) stores were investigated by fluorescence ratio imaging following exposure of the cells to thapsigargin. When extracellular Ca\(^{2+}\) was chelated with EGTA, exposure of the cells to thapsigargin resulted in an elevation of intracellular free Ca\(^{2+}\). This reflects the depletion of the intracellular calcium stores released from the endoplasmic reticulum. Thapsigargin is also a weak skin tumor suppressor (Lowry et al. 1996, Serpi et al. 1999). In the present study, the short-term effects of thapsigargin were studied on the calcium transient in keratinocytes. The incubation times in some previous studies, however, were hours to weeks. Concentrations exceeding 30 nM are toxic to keratinocytes in the long run (Li et al. 1995, Lowry et al. 1996). The results demonstrated that NF1-deficient keratinocytes displayed reduced intracellular calcium stores compared to control cells. NF1 and psoriatic keratinocytes had low intracellular resting Ca\(^{2+}\) levels and lower intracellular Ca\(^{2+}\) stores compared to normal control cells. This may cause some slowing down of the calcium wave and a relatively moderate peak of [Ca\(^{2+}\)] in NF1 and psoriatic keratinocytes compared to normal cells. In addition, the reduced release of intracellular calcium may partly explain the defective activation of store-operated calcium channels in the plasma membrane.

6.2 Defective capacitative calcium influx

The results showed that NF1-deficient keratinocytes were characterized by a reduced elevation of [Ca\(^{2+}\)], compared to normal cells when treated with thapsigargin. This finding may be partly due to the reduced endoplasmic calcium stores of NF1 cells and/or the inadequate signal from the endoplasmic reticulum to the store-operated calcium channels in NF1 keratinocytes.

Further evidence of defective capacitative calcium influx was derived from Mn\(^{2+}\) quenching experiments. Fura-2 fluorescence was investigated in 20 to 25 cells from each selected field. From the channels of 340 nm and 380 nm wavelengths, the total fura-2...
fluorescence intensity was plotted and evaluated. When Mn\(^{2+}\) alone was applied to the cells and fura-2 fluorescence quenching was monitored, a decline of fura-2 fluorescence was observed in normal control cells. Somewhat more moderate quenching of fura-2 fluorescence was detected in NF1 keratinocytes under similar experimental conditions. This suggests a reduced influx of extracellular Mn\(^{2+}\) into NF1 keratinocytes.

Thapsigargin induced fast and marked quenching 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 similar conditions. In analogy, the exposure of normal keratinocytes to ATP in the presence of extracellular Mn\(^{2+}\) resulted in fast and marked quenching of cytosolic fura-2 fluorescence, but not in NF1 keratinocytes. Instead, NF1 keratinocytes responded to extracellular ATP by transiently enhanced cytosolic fura-2 fluorescence, which was followed by fluorescence quenching due to increased Mn\(^{2+}\) entry. In response to thapsigargin, the intracellular calcium stores are released in NF1 cells, which causes increased fura-2 fluorescence. The capacitative calcium influx in NF1 cells seems to be defective, and the influx of Mn\(^{2+}\) is delayed. This would explain the transient increase in the trace recording Mn quenching. The same rationale applies to the findings showing a transient increase of fura-2 fluorescence after the application of ATP to NF1 keratinocytes. The results suggest that NF1 keratinocytes may be particularly sensitive to ATP since fura-2 fluorescence quenching was more pronounced with ATP compared to thapsigargin experiments. If NF1 keratinocytes exhibit lower intracellular Ca\(^{2+}\) stores and a downregulated signal from ER to SOCs, this might explain the defective capacitative calcium influx in these cells. However, the results demonstrate that the downregulated capacitative calcium influx in NF1 keratinocytes is, at least in part, due to a defective signal for the activation of SOCs.

### 6.3 Intercellular calcium wave propagation

Mechanical wounding was used to induce intercellular calcium waves. The results revealed that the rise of [Ca\(^{2+}\)] following mechanical stimulation at different extracellular calcium concentrations was lower in NF1 and psoriatic keratinocytes compared to normal control cells. When the intracellular calcium stores were depleted with thapsigargin prior to wounding, neither control nor NF1-deficient keratinocytes displayed an increase in [Ca\(^{2+}\)], and no calcium waves could be induced. This finding indicates that both types of keratinocytes required release of intercellular calcium stores to propagate intercellular calcium waves.

Based on previous studies, the intracellular Ca\(^{2+}\) response induced by mechanical stimulation has been divided into two phases. Upon stimulation, [Ca\(^{2+}\)], is first increased within the stimulated cell. This is called the “primary” response, and it is followed by radial propagation of the calcium response away from the stimulated cell, called the “secondary” response. Several studies have shown that an influx of extracellular Ca\(^{2+}\) into the stimulated cell contributes to the primary response, while the secondary response, which involves the propagation of the Ca\(^{2+}\) wave across cell boundaries, appears to be mediated by the IP\(_3\)-dependent release of Ca\(^{2+}\) from intracellular pools, without requiring extracellular Ca\(^{2+}\). In the present study, the propagation of mechanically induced Ca\(^{2+}\)
waves was not inhibited by incubation in Ca\(^{2+}\)-free media, supporting the theory that the secondary response in the surrounding cells relies mainly on release of intracellular Ca\(^{2+}\).

Intercellular calcium waves were further analyzed in the presence of suramin, an inhibitor of P\(_2\)-purinergic receptors, and heptanol, a gap-junctional signaling inhibitor. An anti-trypanosomal and anti-filarial drug suramin was used to block P\(_2\)-purinergic receptors (Dunn & Blakeley 1988, Jørgensen et al. 1997, Cotrina et al. 1998). Suramin also has other effects on the mechanism that affects cell differentiation apart from the inhibition of P\(_2\)-purinergic receptors. It alters phosphoinositide synthesis and inhibits growth factor receptor binding in HT-29 cells (Kopp & Pfeiffer 1990, Jørgensen et al. 1997, Cotrina et al. 1998). Suramin also has other effects on the mechanism that affects cell differentiation apart from the inhibition of P\(_2\)-purinergic receptors. It alters phosphoinositide synthesis and inhibits growth factor receptor binding in HT-29 cells (Kopp & Pfeiffer 1990, Jørgensen et al. 1997, Cotrina et al. 1998). Suramin also has other effects on the mechanism that affects cell differentiation apart from the inhibition of P\(_2\)-purinergic receptors. It alters phosphoinositide synthesis and inhibits growth factor receptor binding in HT-29 cells (Kopp & Pfeiffer 1990, Jørgensen et al. 1997, Cotrina et al. 1998). Suramin also has other effects on the mechanism that affects cell differentiation apart from the inhibition of P\(_2\)-purinergic receptors. It alters phosphoinositide synthesis and inhibits growth factor receptor binding in HT-29 cells (Kopp & Pfeiffer 1990, Jørgensen et al. 1997, Cotrina et al. 1998).

Long-chain alcohols octanol and heptanol have been used as gap-junctional signaling blockers in various cell lines in several studies (Johnston et al. 1980, Kimura et al. 1995, Jørgensen et al. 1997, Cotrina et al. 1998). These agents can affect the junctional membrane, and their action is rapid and reversible (Kimura et al. 1995). Heptanol has been reported also to have an effect on the cardiac potassium current (Niggli 1989), the calcium inward current (Niggli 1989) and the sodium current (Nelson & Makielski 1991). Even though heptanol is not an entirely specific gap-junctional blocker, we chose to use heptanol as a gap-junctional inhibitor since it has been earlier used successfully in various cell lines. Interestingly, the present results 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-deficient keratinocytes. This finding suggests that there is P\(_2\)-purinergic signaling in NF1 and psoriatic keratinocytes, and the cells may thereby partly compensate for their defective gap-junctional signaling.

The results collectively demonstrate that NF1 and psoriatic keratinocytes have a tendency to form cultures characterized by altered Ca\(^{2+}\)-mediated cell signaling compared to normal keratinocytes at different extracellular calcium concentrations. The differences were most pronounced at high [Ca\(^{2+}\)].

### 6.4 Role of intact cytoskeleton in calcium signaling

The aim of the further studies was to investigate the contribution of the cytoskeleton to capacitative Ca\(^{2+}\) entry in human keratinocytes. Disruption of actin filaments with cytochalasin D resulted in profound alterations of both cell morphology and physiology. Previous studies have shown that microfilaments play a crucial role in functional capacitative Ca\(^{2+}\) entry in cultured human endothelial cells (Holda & Blatter 1997). Thus, the communication between the endoplasmic reticulum and the plasma membrane involved in signaling capacitative Ca\(^{2+}\) entry may depend on a structural connection between the plasma membrane calcium channels and an intact actin-containing cytoskeleton. The present results clearly demonstrate that cytochalasin D, a drug that disrupts the microfilaments of the cytoskeleton, blocked capacitative Ca\(^{2+}\) entry when ER Ca\(^{2+}\) stores were depleted with thapsigargin. In contrast to the effects on thapsigargin-
induced [Ca\(^{2+}\)]\(_i\) signaling, neither the release nor the entry of Ca\(^{2+}\) in response to extracellular ATP was significantly affected by cytoskeletal disruption. These findings rule out a number of causes for the block of the thapsigargin response in cells treated with cytochalasin D. Specifically, reduced Ca\(^{2+}\) stores, a defective signal for the activation of store-operated calcium channels, or augmented Ca\(^{2+}\) buffering by Ca\(^{2+}\) pumps are not likely explanations for the block of the thapsigargin response after disruption of the actin cytoskeleton. Additionally, the failure of cytoskeletal disruption to affect ATP-induced Ca\(^{2+}\) entry indicates that neither an intact cytoskeleton nor cytoskeletal-dependent processes are required for the activation of ATP-dependent calcium mobilization in cultured human keratinocytes.

In an earlier study on cultured human umbilical venous endothelial cells (HUVEC), cytochalasin D was shown to block thapsigargin-induced Ca\(^{2+}\) entry, but not ATP-induced Ca\(^{2+}\) release (Holda & Blatter 1997). The authors interpreted their findings as supportive of the conformational coupling model in endothelial cells (Berridge 1995b). Thus, the results on keratinocytes in the present study are essentially the same as described for endothelial cells. However, the use of NIH3T3 fibroblasts results in markedly different findings compared to those on keratinocytes or endothelial cells (Ribeiro et al. 1997). Specifically, the authors suggest that an intact cytoskeleton is required for agonist-induced [Ca\(^{2+}\)]\(_i\) signaling, but not for capacitative calcium entry in NIH3T3 cells. The results on NIH3T3 cells suggested that cytoskeletal disruption alters the spatial relationship between phospholipase C and IP\(_3\) receptors, impairing phospholipase C-dependent calcium signaling. Capacitative calcium entry was not altered under experimental conditions, indicating that the coupling between the depletion of intracellular calcium stores and calcium entry does not depend on a precise structural relationship between the intracellular stores and plasma membrane calcium channels in NIH3T3 cells.

The reason for these markedly different results is not clear. However, alteration of the association between the plasma membrane and the endoplasmic reticulum may disrupt the spatial relationship required for signaling in the same way as IP\(_3\)-mediated signaling was disrupted in a study where NIH3T3 fibroblasts were used, but not in HUVECs. Previous studies have shown that the signal for capacitative calcium entry is rapidly inactivated (Hughes et al. 1988) and, at least in the case of the Xenopus oocyte, has a limited range of action (Petersen & Berridge 1996). Diffusible messengers are required both for the release of Ca\(^{2+}\) and for the activation of calcium entry, and the functional domains of these messengers may be differentially dependent on cell structure in different cell types.

Some evidence suggested a link between neurofibromatosis, or NF1 protein, and the organization of the cellular cytoskeleton. Specifically, a cell culture study before the cloning of the NF1 gene demonstrated that the cytoskeleton of cells cultured from neurofibromas is characterized by haphazard organization of intermediate filaments and actin-containing stress fibers (Peltonen et al. 1984, Koivunen et al. 2000). Interaction of NF1 protein with microtubules has been demonstrated in previous studies (Bollag et al. 1993, Gregory et al. 1993).

The results demonstrate that, in cultured keratinocytes, disruption of the cytoskeleton blocks thapsigargin-elicited [Ca\(^{2+}\)]\(_i\) mobilization. Capacitative calcium entry was altered by cytoskeletal dismantling, suggesting that the coupling between calcium pool depletion
and capacitative calcium entry depends on a precise structural relationship between the ER and plasma membrane calcium channels. Our results demonstrate that the signaling mechanism for capacitative Ca\(^{2+}\) entry is dependent on cell morphology and on the integrity of the actin cytoskeleton. Our observation that IP\(_3\)-dependent release of Ca\(^{2+}\) remained intact in the presence of cytochalasin D provides further evidence to show that a signaling pathway that depends on a diffusible messenger (IP\(_3\)) was functional when the cytoskeleton became disrupted. If the diffusible factor model for capacitative calcium influx was correct, one might assume that capacitative Ca\(^{2+}\) entry would not have been inhibited in the presence of cytochalasin D, since IP\(_3\) diffusion did not appear to be altered. The present results, however, do support the conformational-coupling model for capacitative Ca\(^{2+}\) entry following store depletion. Our data suggest that signaling cannot occur without maintenance of close proximity between the intracellular stores and the plasma membrane Ca\(^{2+}\) channels and that microfilaments play a crucial role in functional capacitative Ca\(^{2+}\) entry in cultured keratinocytes.

6.5 Altered calcium-mediated cell signaling in psoriasis

Intercellular communication through gap junctions is important in coordinating cell signaling. Connexins, which are gap junction proteins, may facilitate selective assembly or permeability of gap junctions, influencing the distribution of metabolites between cells. Moreover, a decrease of communication between cells may lead to uncontrolled growth, and connexin genes may function as tumor suppressor genes (Lee et al. 1991, Huang et al. 1998). Focally evoked calcium waves have been thought to propagate by gap-junction-mediated intercellular passage of chemical signals (Sanderson et al. 1994). Calcium waves can also proceed across cell-free areas in astrocytes (Hassinger et al. 1996, Cotrina et al. 1998), indicating that there is another extracellular autocrine mode of Ca\(^{2+}\) signaling. Connexin 26 has been shown to be significantly and connexin 43 slightly upregulated in psoriatic skin in vivo (Labarthe et al. 1998). It has been suggested that psoriatic keratinocytes compensate for their match of communication requirements by increasing connexin 26 channels. Connexin 26 upregulation has also been shown to be a feature of keratinocyte differentiation not only in hyperproliferative skin but also in buccal and vaginal epithelium (Lucke et al. 1999). The results of the present study provide additional proof of the altered gap junctional signaling in psoriatic keratinocytes.
The results showed the rise of [Ca\(^{2+}\)] following mechanical stimulation at different extracellular calcium concentrations to be lower in psoriatic keratinocytes compared to normal control cells. They also showed that both lesional and nonlesional keratinocytes of psoriatic patients have an intrinsic error of calcium metabolism. The results hence suggest that psoriatic keratinocytes have lower intracellular calcium stores compared to normal keratinocytes, and the activation of store-operated calcium channels and capacitative calcium entry is downregulated. Wounding of the monolayer produced a significant rise in [Ca\(^{2+}\)] in control keratinocytes in contrast to psoriatic keratinocytes, indicating defective intercellular coupling of these cells. The blocking of gap-junctional intercellular signaling with heptanol and P\(_2\)-purinergic receptors with suramin showed that gap-junctional communication is significantly defective in psoriatic keratinocytes, and the cells compensate for the altered gap-junctional intercellular signaling with ATP-mediated signaling.
7 Conclusions

The present results demonstrate that NF1 and psoriatic keratinocytes have a tendency to form cultures characterized by altered Ca\textsuperscript{2+}-mediated cell signaling compared to normal keratinocytes. The results of the present study suggest that mutations of the NF1 tumor suppressor gene or lowered levels of NF1 protein/mRNA may eventually lead to altered intercellular communication.

1. Intracellular calcium stores and capacitative calcium influx were lower in NF1 and psoriatic keratinocytes compared to normal control cells. Resting Ca\textsuperscript{2+} levels were lower in NF1 keratinocytes compared to normal control cells. Different components of intercellular Ca\textsuperscript{2+} signaling pathways predominated in normal versus NF1 keratinocytes. Specifically, gap-junctional signaling was defective and NF1-deficient keratinocytes propagated ATP-dependent calcium waves that required activation of P\textsubscript{2y} purinergic receptors. Gap-junctional signaling routes were distinctly detectable in the normal cell monolayers, but less distinct in NF1 keratinocytes.

2. Disruption of the cytoskeleton blocked thapsigargin-elicited [Ca\textsuperscript{2+}], mobilization in cultured keratinocytes but did not alter the ATP-induced Ca\textsuperscript{2+} transient. Capacitative calcium entry was altered by cytoskeletal dismantling, suggesting that the coupling between calcium pool depletion and capacitative calcium entry depends on a precise structural relationship between the ER and plasma membrane calcium channels.

3. Calcium-mediated signaling was altered in NF1 keratinocytes compared to normal cells. The differences were even more pronounced at high extracellular calcium concentrations.

4. Altered calcium dynamics was seen both in lesional and nonlesional keratinocytes. Psoriatic keratinocytes had lower intracellular calcium stores compared to normal keratinocytes. Activation of store-operated calcium channels and capacitative calcium influx was downregulated in psoriatic keratinocytes compared to normal control cells. The rise of [Ca\textsuperscript{2+}], following mechanical stimulation was lower in psoriatic keratinocytes compared to normal control cells at different extracellular calcium concentrations. The blocking of gap-junctional intercellular signaling and P\textsubscript{2y} purinergic receptors showed that the functioning of gap junctions is significantly altered in psoriatic keratinocytes, and the cells compensate for this with purinergic signaling.
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