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Calcium - a central regulator of keratinocyte differentiation in health and disease

Regular keratinocyte differentiation is crucial for the formation of an intact epidermal barrier and is triggered by extracellular calcium. Disturbances of epidermal barrier formation and aberrant keratinocyte differentiation are involved in the pathophysiology of several skin diseases, such as psoriasis, atopic dermatitis, basal and squamous skin cancer, and genetic skin diseases such as Darier's disease and Olmstedt syndrome. In this review, we summarize current knowledge about the underlying molecular mechanisms of calcium-induced differentiation in keratinocytes. We provide an overview of calcium's genomic and non-genomic mechanisms to induce differentiation and discuss the calcium gradient in the epidermis, giving rise to cornified skin and lipid envelope formation. We focus on the calcium-sensing receptor, transient receptor potential channels, and STIM/Orai as the major constituents of calcium sensing and calcium entry in the keratinocytes. Finally, skin diseases linked to impaired differentiation will be discussed, paying special attention to disturbed TRP channel expression and TRP channel mutations.

Key words: keratinocyte, differentiation, calcium, TRP channels

Keratinocyte differentiation in health

Skin consists of three different layers, called the subcutis, the dermis and the epidermis. The epidermis is the outermost layer and the place of keratinocyte differentiation [1]. In the epidermis, keratinocyte proliferation is restricted to the basal cell layers [2], whereas more superficial layers differentiate [3] and finally die. After mitosis in the basal layer, keratinocytes differentiate across the epidermis toward the stratum corneum. During differentiation, several keratinocyte layers develop in the epidermis, starting with the stratum basale and then the stratum spinosum, the stratum granulosum and the stratum corneum, expressing distinctive marker genes at each differentiation stage *(figure 1)* [4, 5]. Keratins are mainly expressed by basal (keratin 5 and keratin 14) and spinous keratinocytes (keratin 1 and keratin 10; see also *figure 1*). Transglutaminase and involucrin are generated in spinous keratinocytes, whereas granular keratinocytes produce loricrin and filaggrin [6]. Besides these proteins, keratinocytes in the stratum granulosum synthesize sphingolipid precursors, which are stored in lamellar bodies. These lamellar bodies fuse with the plasma membrane during the differentiation process and release lipid precursors into the extracellular space in the stratum granulosum and corneum. After enzymatic processing, they are incorporated in lipid lamellar membranes, which embed together with the cornified envelope keratin-filled corneocytes, forming the permeability barrier [7].

The main trigger for keratinocyte differentiation is calcium. In 1980, Hennings *et al.* demonstrated that keratinocyte differentiation and proliferation depend on extracellular calcium concentration: After elevating extracellular calcium from 0.09 mM to 1.2 mM, the differentiation of murine keratinocytes began immediately, accompanied by a decline in DNA, RNA and protein synthesis. In contrast, more than 90% proliferate in the presence of 0.09 mM calcium [8]. These findings were later confirmed for human keratinocytes [9]. The mechanisms by which calcium induces differentiation are multifarious and include genomic and non-genomic pathways [4]. An example of a non-genomic mechanism is desmosome formation: In the presence of 1.2 mM extracellular calcium, murine keratinocytes form desmosomes as early as five minutes later. After two to five hours, desmosomes are symmetrical and functional [8, 10]. Desmosomes give mechanical strength to the epidermis but might also provide a signaling complex for differentiating keratinocytes [11].

Genomic mechanisms involve the activation of calcium-responsive promoters such as activator protein 1 (AP1) sites. AP1 sites are found in the involucrin [12] and in the keratin 1 gene [13, 14]. Both genes code for differentiation markers expressed in response to high extracellular calcium. It remains to be elucidated whether other differentiation-related genes contain calcium-sensitive promoters. Using a subtraction hybridization technique, Seo *et al.* found that 290 genes up-regulated in response to calcium. Most were differentiation related, whereas genes involved in metabolism, DNA repair, transcription, and translation decreased [15].

In the epidermis, extracellular calcium is provided by a calcium gradient with peaking calcium concentrations in the granular layer and a steep drop-off in the stratum corneum,

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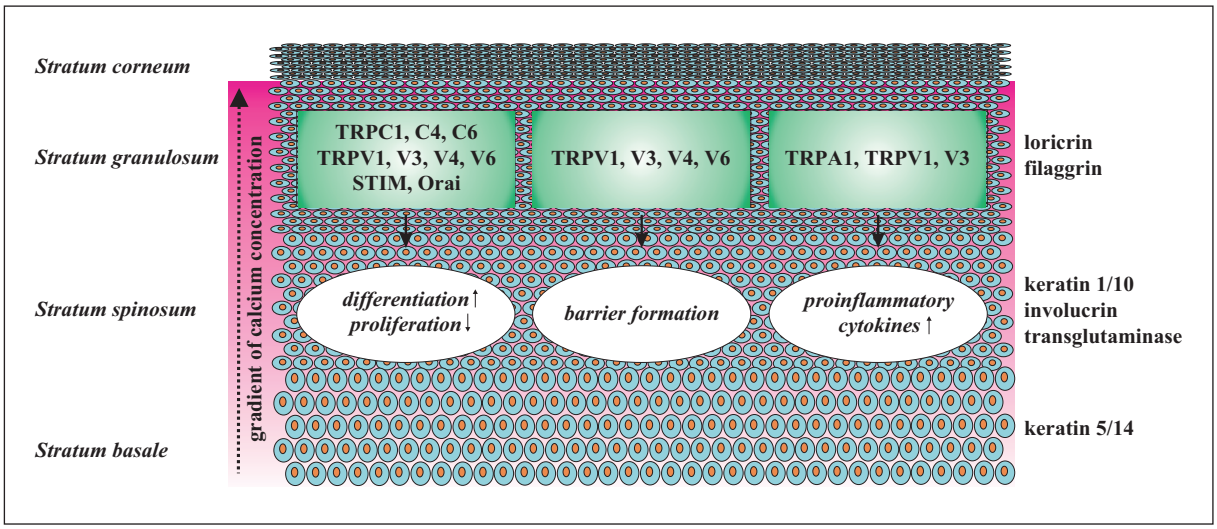

Figure 1. Calcium entry mechanisms in keratinocyte physiology. The cartoon visualizes the different layers of the epidermis (stratum basale, stratum spinosum, stratum granulosum, and stratum corneum), the appearance of differentiation markers (keratin 1, 5, 10, and 14, involucrin, transglutaminase, filaggrin, and loricrin) and the calcium gradient along the different epidermal layers. TRP channels as well as STIM/Orai-involved differentiation in the epidermis, barrier formation and release of proinflammatory cytokines are summarized.

as demonstrated using a variety of techniques such as ion capture cytochemistry, microbeam proton induced x-ray emission or calcium-sensitive fluorescent dyes in murine and human skin [16-19]. In contrast, very low extracellular calcium levels are demonstrated in the basal layer, where low calcium keeps keratinocytes proliferating. Recent studies by Behne *et al*. [20] and Celli *et al.* [21] used a combination of two-photon microscopy, fluorescence lifetime imaging and phasor analysis in *ex vivo* unfixated skin biopsies of the epidermis to determine the intracellular calcium concentration. They confirmed previous findings showing that calcium concentrations in the upper layers of the viable epidermis are higher than in the stratum corneum. However, the calcium gradient was not as steep as previously described, and most epidermal calcium was found in intracellular organelles such as the endoplasmic reticulum or the Golgi apparatus. The authors suggested that keratinocytes might not only differentiate simply by responding to extracellular calcium but also by changing the composition of plasma membrane ion channels or by changing the intracellular or plasma membrane calcium sensing capacity.

The mechanisms of the formation and maintenance of the calcium gradient are still under investigation. In rodents, the calcium gradient develops before birth [22]. Gradient maintenance is achieved by tight junctions, preventing calcium loss [21]. The calcium gradient is vulnerable to skin damage, as injuring murine skin by tape-stripping markedly decreased the calcium gradient [23, 24]. A decreased calcium gradient results in reduced levels of the differentiation markers profilaggrin, loricrin and involucrin [25]. In addition, barrier insult by tape-stripping also resulted in a mobilization of calcium from intracellular calcium stores [20]. These findings are not surprising, as graded levels of extracellular calcium elicit a graded differentiation response in keratinocytes, the buffering of intracellular calcium prevents the terminal differentiation of keratinocytes, and the expression of early and late differentiation markers is controlled by intracellular calcium compartments [19]. However, a more comprehensive understanding of barrier development and maintenance is still lacking.

Which receptors and ion channels take part in differentiation?

The calcium-sensing receptor (CaSR)

Elevated extracellular calcium concentrations and keratinocyte differentiation are closely linked. Keratinocytes sense changes in the extracellular calcium concentration via the G-protein coupled calcium-sensing receptor (CaSR) [26]. The CaSR is predominantly expressed in the suprabasal keratinocyte layers. Several groups have provided evidence for the expression of the CaSR in primary human keratinocytes isolated from foreskin as well as in gingival keratinocytes from adult patients or primary neonatal keratinocytes [27-29]. The CaSR is involved in the mobilization of intracellular calcium as well as E-cadherin-mediated cell adhesion. Both pathways are important for calcium-mediated keratinocyte differentiation [5, 30]. Full-length CaSR was found to be essential for calcium-induced keratinocyte differentiation, as its ablation by the cDNA technique reduced the response to extracellular calcium and decreased the differentiation markers involucrin and transglutaminase [31]. These findings were confirmed by transgenic $CaSR^{-/-}$ mice displaying ultrastructural changes in the epidermis and reduced levels of loricrin and filaggrin [32]. However, these animals died early (mostly 5-7 days after birth), making the characterization of the skin phenotype in adulthood impossible. Therefore, Tu *et al*. [5] generated a transgenic mouse model with a keratinocyte-specific knockout of CaSR in the stratum basale. These animals were characterized by reduced epidermal differentiation, reflected in a reduced expression of the late

differentiation markers involucrin, loricrin, and filaggrin. In contrast, keratin 1 and keratin 5 mRNA expression was not altered in the epidermis of these animals. Furthermore, the epidermis of these animals was marked by a delay in the formation of the lipid envelope, as demonstrated by a reduced number of lamellar bodies in the stratum granulosum as well as in the stratum corneum and disturbed formation of the permeability barrier in prenatal development. In adulthood, the skin permeability barrier is only significantly impaired after stress induced by dietary Ca^{2+} restriction. Recently, Popp *et al.* [29] investigated the role of the CaSR on the Wnt/ß-catenin signaling pathway, which plays an important role in proliferation, survival and cell-fate specification. They demonstrated that high extracellular calcium leads to the differentiation of keratinocytes via the activation of the CaSR, which depends on Wnt5a biosynthesis and secretion. However, the calcium channel involved in this process remains unclear.

The signal transduction of CaSR via Gαq involves the generation of inositol phosphates: Co-localization and immunoprecipitation studies have revealed that the calcium-sensing receptor is linked to phospholipase Cβ1 (*figure 2*), the IP_3 receptor, and the Golgi apparatus [33], suggesting the generation of inositol phosphates and diacylglycerol (DAG). Inositol-3,4,5-trisphosphate releases calcium from intracellular stores in a ligand-dependent manner and results in the depletion of the internal calcium stores, such as through the endoplasmatic reticulum activating stromal interaction molecules (STIM) and Orai. Evidence for an interaction between the CaSR and STIM/Orai was provided by Fatherazi *et al.* [27, 34] (*figure 2*). DAG directly stimulates the transient receptor potential (TRP) channels of the canonical subfamily (TRPC) [35, 36]. Direct interaction between the CaSR and TRPC channels was demonstrated by Fatherazi *et al.* [34] for TRPC4 channels and by Müller *et al.* [37]. The CaSR is also stimulated via the interaction of $G\alpha_{12/13}$, filamin A, and RhoGEF Fyn/Src kinases. Fyn/Src kinases phosphorylate catenins, promoting the formation of E-cadherin/catenin complex at the cell membrane and the activation of PI3K. PI3K, in turn, activates PLCγ1 to further increase intracellular calcium-triggering differentiation [38].

Adenosine dependent calcium pumps residing in the plasma membrane and intracellular membranes, such as the endoplasmic reticulum, Golgi or mitochondria, also regulate intracellular calcium. They buffer excess cytosolic calcium by pumping out calcium through the plasma membrane (the plasma membrane Ca^{2+} ATPase and the Na^+/Ca^{2+} exchanger) or into intracellular storage sites, such as the endoplasmic reticulum via Ca^{2+} ATPase (SERCA) (*figure 2*).

TRP channels

Keratinocytes express a plethora of different calcium channels, among them members of the superfamily of TRP channels. Most of them are non-selective ion channels. Mammalian TRP channels fall into six subfamilies on the basis of amino acid sequence homology: the TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPA (ankyrin), TRPP (polycystin), and TRPML (mucolipin) groups. So far, members of the TRPC, TRPV, TRPM,

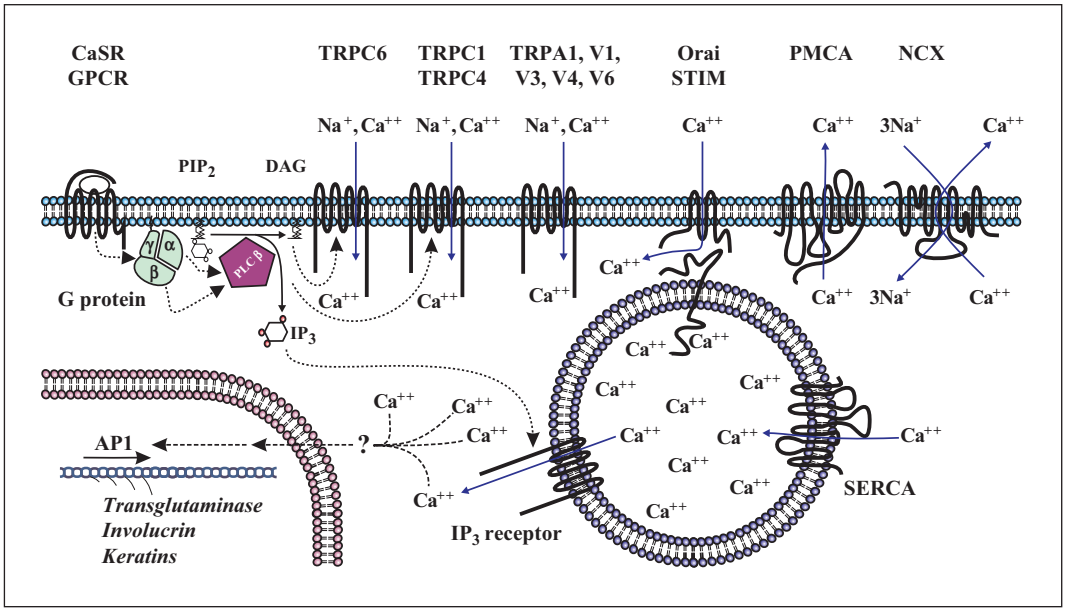

Figure 2. Calcium homeostasis in keratinocytes. The calcium-sensing receptor (CaSR) as GPCR senses extracellular calcium changes. Phospholipase C β isoforms activated subsequently to CaSR-dependent G-Protein stimulation results in the breakdown of phosphatidyl inositol-4,5-bisphosphate (PIP_2) leading to the formation of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 mediates calcium release from endoplasmic calcium stores by activation IP_3 receptors in a ligand-dependent manner. DAG directly activates TRPC6, whereas TRPC1 and TRPC4 are activated by signals downstream of PLC activation. TRPV1, V3, V4 mediate calcium entry in keratinocytes in temperature- or ligand-dependent manner. The STIM/Orai complex controls the calcium filling of the intracellular storage compartment, which is filled by the activity of the sarcoplasmic/endoplasmic calcium ATPases (SERCA). Intracellular calcium concentrations are additionally controlled by the plasmemembrane calcium ATPase (PMCA) and the sodium-calcium exchanger (NCX).

and TRPA subfamilies have been identified in human keratinocytes [39-44]. Their physiological functions in the epidermis are summarized in *figure 1*.

TRPC channels

In 2005, Cai *et al.* investigated the mRNA and protein expression of TRPC channels in human gingival keratinocytes [45]. Undifferentiated human gingival keratinocytes express TRPC1, TRPC4, TRPC5, TRPC6, and TRPC7 mRNA and proteins. Calcium-induced differentiation induced a biphasic response and increased TRPC channel expression during the first two days, which declined afterwards [34, 39]. In contrast to Cai *et al*. [39], Pani *et al*. and our group also detected TRPC3 channels in epidermal skin sections, using immunofluorescence as well as in HaCaT keratinocytes detected by Western blotting and PCR [46, 47]. TRPC1 as well as TRPC6 are expressed *in vivo* in the human gingival epithelia [39]. Therefore, all human TRPC channels seem to be expressed in keratinocytes. However, the physiological role of every TRPC channel in keratinocytes is not fully clear yet. Fatherazi provided evidence for the role of TRPC4 channels in human gingival keratinocytes [34]. Calcium influx was markedly delayed and reduced after the siRNA mediated knockdown of TRPC4. The preservation of the transient response and the absence of the physiological sustained elevation of intracellular calcium concentration argues for the contribution of TRPC4 to the latter [34]. Similar results were obtained for TRPC1 knockdown in gingival keratinocytes, supporting previous data from Cai *et al.* [39, 48]. The essential role of TRPC1 and TRPC4 in keratinocyte differentiation was also confirmed in experiments using HaCaT keratinocytes: the siRNA-mediated down-regulation of TRPC1 and TRPC4 reduced the expression of the differentiation markers transglutaminase 1 and involucrin at the mRNA level [49]. From our point of view, besides data for TRPC1 and TRPC4 channels, only for TRPC6 channels are there convincing data regarding their physiological function in keratinocytes. Our group demonstrated that they play an essential role in calcium influx and keratinocyte differentiation mediated by high extracellular calcium [37]. We detected TRPC6 channel expression in HaCaT keratinocytes, in primary keratinocytes derived from adult skin and in human skin biopsies using various experimental approaches, such as PCR, Western blot or immunohistochemistry. Our group showed that the activation of TRPC6 channels by the specific activator hyperforin is sufficient to induce keratinocyte differentiation, monitored by changes in the expression of K1, K10, transglutaminase 1 and involucrin. SiRNA-mediated TRPC6 knockdown strongly attenuates keratinocyte differentiation mediated by high extracellular calcium [37]. Interestingly, TRPC6 expression is up-regulated by the application of secondary plant compounds, such as hyperforin or betulin in HaCaT keratinocytes, as well as skin biopsies by the constituents of St. John's wort or birch extract, which are traditionally used to treat skin diseases [37, 50].

TRPV

Whereas several TRPC channels detected in keratinocytes are clearly involved in differentiation [34, 39, 48], the role of the TRPV1, TRPV2, TRPV3, TRPV4, and TRPV6 channels are only partly understood and somewhat contradictory, due to the variety of activation mechanisms, such as heat, cold, pressure and a variety of chemical compounds [51]. In general, TRPV1, TRPV2, TRPV3, and TRPV4 are believed to take part in thermo sensation in mice and humans [52, 53]. At first glance, it is astonishing that TRPV channels, which are known for their relevance in thermal nociception in the neuronal system, are also expressed in non-neuronal cells. At least for TRPV3, co-culture experiments demonstrate crosstalk between keratinocytes and dorsal root ganglion neurons, communicating temperature information via ATP and purinoreceptors [54].

In 2001, TRPV1 expression on human keratinocytes was first discovered by the immunofluorescense of punch biopsies from healthy volunteers as well as primary keratinocytes, and TRPV1 mRNA was isolated from primary keratinocytes [55]. Although TRPV1 function was not explored in this work, the authors speculated that TRPV1 expression in the skin might contribute either to the detection of environmental factors or to keratinocyte differentiation [55]. TRPV1-mediated calcium influx in cultured human keratinocytes and HaCaT keratinocytes inhibited proliferation and induced apoptosis [52, 56-58] TRPV1 activation was also associated with the calcium-dependent expression and secretion of COX2, prostaglandin E2 and interleukin 8, indicating that keratinocytes take an active part in skin inflammation [59, 60]. TRPV1 channels also seem to be involved in photo-mediated skin aging mediated by collagen destruction via the induction of membrane metalloprotease 1 (MMP1) [58]. Lee *et al*. [58] applied UV irradiation in HaCat keratinocytes and showed that UV irradiation-mediated calcium influx and the induction of MMP1 was blocked by the TRPV1 blocker capsazepine. These findings were confirmed by a study using hairless mice. The TRPV1-specific blocker 5'-iodoresiniferatoxin inhibited the UV irradiation-induced increase in MMPs as well as in proinflammatory cytokines such interleukin 1β, 2, 4 and tumor necrosis factor α. It also reduced UV-induced skin thickening [61].

There is little evidence for TRPV2 in human keratinocytes except in the study of Radtke *et al*., who demonstrated TRPV2 in human skin by immunostaining, and no data exist regarding the physiological role of TRPV2 in keratinocytes [52].

TRPV3 channels play a pivotal role in heat nociception [40] and participate in skin inflammation and itching [62]. The intraplantar injection of intermediates of the mevalonate pathway causes nociceptive behavior in rodents to be absent after TRPV3 knockdown [63]. However, TRPV3 channels are also involved in the formation and regulation of the physical-chemical skin barrier [64, 65]. Cheng *et al*. [66] reported altered keratinocyte differentiation in neonates, represented by an increase in the thickness of the keratin 1 and 10 positive layer. Keratin 1 and 10 are expressed in differentiating keratinocytes. As keratinocytes undergo cornification while moving towards the skin surface, keratin 1 and 10 expressions decline and loricrin expression increases. In $TRPV3^{-/-}$ mice, loricrin expression was enhanced. Importantly, TRPV3 channels are co-expressed in a functional signaloplex with the endothelial growth factor (EGF) receptor as well as with TGFα, both key players in the regulation of the epidermal barrier [66]. In addition, TRPV3 activation using the TRPV3

activators camphor and 2-aminoethoxydiphenyl borate (2-APB) controls keratinocyte migration and wound healing, probably via the release of nitiric oxide, which depends on intracellular acidification [67]. These findings and the identification of mutations in the TRPV3 gene leading to a severe skin disease, Olmstedt syndrome (see below), clearly show the important role of the TRPV3 channel function in skin physiology [65].

Besides TRPV3 channels, TRPV4 channels are also connected to skin barrier maintenance. The activation of TRPV4 using 4α-phorbol 12,13-didecanoate or a temperature above 33 °C, the physiological skin temperature, enhances tight junction formation in differentiated keratinocytes and thereby an augmented skin barrier [41]. TRPV4 co-localizes with β-catenin and E-cadherin, indicating that TRPV4 is situated in keratinocyte adherence junctions. In line with these results, TRPV4-specific knockdown decreased epidermal barrier functions by reducing junction formation [41]. In accordance with these findings, the calcium-induced reorganization of the cytoskeleton and stratification are impaired in TRPV4-deficient keratinocytes, leading to impaired stratification. Usually, the TRPV4 protein is located at cell-cell junctions, these are defective in TRPV4-deficient keratinocytes [68].

TRPV6 is associated with keratinocyte differentiation. Recent studies have indicated that TRPV6 is pivotal for calcium-induced differentiation, as the siRNA knockdown of TRPV6 reduced the expression of differentiation markers triggered by high extracellular calcium. TRPV6 channel mRNA and protein expression was also increased in human primary keratinocytes as well as in HaCaT keratinocytes after the triggering of keratinocyte differentiation with high extracellular calcium. Interestingly, TRPV6-deficient keratinocytes are characterized by the loss of close contacts among adjacent cells, and they lose the ability to flatten. 1,25-dihydroxyvitamin D3 is one of the key autocrine and paracrine keratinocyte differentiation regulators [70]; it upregulates TRPV6 expression dose-dependently, suggesting a link between 1,25-dihydroxyvitamin D3-induced differentiation and TRPV6 [69].

TRPA and TRPM

So far, the mammalian TRPA family includes one member, TRPA1, which has been detected on keratinocytes. TRPA1 is activated by a multitude of natural compounds [71] and low temperatures. In the epidermis, TRPA1 is mainly localized in the basal layers. The activation of TRPA1 using icilin induces the expression of genes involved in differentiation, proliferation and transcription, indicating that TRPA1 regulates keratinocyte differentiation. Furthermore, the activation of TRPA1 causes the expression of two inflammatory cytokines, interleukin 1-α and 1-β, reflected by mRNA expression [44] and the secretion of the proinflammatory eicosanoid prostaglandin E2 [72]. The relevance of TRPA1 for skin inflammation was also demonstrated in a mouse model where the topical application of the TRPA1 agonist cinnamaldehyde caused ear edema and promoted leukocyte infiltration. Both effects were blocked by ruthenium red, a rather unselective TRPA1 blocker [73]. In a study using dorsal root ganglions, it was shown that dibutyl phthalate, which is used as a plasticizer for plastic, activates TRPA1 [74]. The authors speculated that dibutyl phthalate, which is also present in house dust, might contribute to skin hypersensitivity reactions.

Undifferentiated keratinocytes respond to temperatures below 20 °C by activating TRPA1, resulting in a marked calcium influx. Low temperature-evoked calcium influx is almost absent in differentiated keratinocytes. There was no difference in TRPA1 expression as assessed by mRNA and protein expression [75]. However, the meaning of this result for keratinocyte biology is not understood yet.

To date, eight TRPM channels have been described [76]. In keratinocytes, there is only evidence for TRPM8, which has been detected by immune fluorescence throughout the living part of murine epidermis. TRPM8 mRNA is also apparent in human keratinocytes. In the same study, the activation of TRPM8 using menthol or WS12 was demonstrated to accelerate barrier recovery after barrier disruption by tape-stripping using a mouse model. This effect was prevented by the TRPM8 blocker ruthenium red [43]. In contrast to TRPA1, TRPM8 does not seem to contribute to phthalate-induced skin hypersensitivity, as it is not activated by dibutyl phthalate [77].

STIM and Orai

In the past, an increasing interest in store-operated calcium entry mechanisms emerged that was further pushed with the discovery of STIM and Orai molecules. Since the 1980s, it has been understood that inositol triphosphate mediates calcium release from the intracellular stores via IP_3 receptor activation [78]. However, the molecular mechanism for refilling intracellular stores remained elusive. In 2005, siRNA techniques revealed that STIM1 and STIM2 act as calcium sensors in the ER membrane [79]. Their knockdown in various cell systems markedly reduces store-operated calcium (SOCE) entry [79, 80]. So far, three different STIM molecules have been discovered [81]. As STIM molecules contain only one transmembrane domain [81], it is clear that there must be another protein forming the calcium-permeable pore. Vig *et al.* identified Orai1 and Orai2 as the missing calcium channel in the plasma membrane [82]. The current idea of store-operated calcium entry is that STIM1 and STIM2 sense decreasing calcium concentrations in the ER via their luminally located calcium-binding EF hand. With low ER calcium concentrations, STIM1 translocates to the plasma membrane, where it forms "punctae" together with Orai molecules, which activates the calcium current via Orai [81].

In keratinocytes, calcium-induced differentiation evokes inositol triphosphate generation, which empties intracellular calcium stores and activates calcium entry via the plasma membrane. Early work from Ross *et al.* [83] showed that ectopically expressed STIM1 translocates and forms punctae in HaCaT keratinocytes and might be involved in agonist-induced calcium entry using lysophosphatidic acid, adenosine triphosphate (ATP) or uridine triphosphate (UTP). Recent work from Numaga-Tomita and Putney demonstrated that HaCaT keratinocytes express STIM1 and Orai1 [84]. Thapsigargin-induced SOCE as well as stimulation of the Ca^{2+}-selective Ca^{2+} release-activated Ca^{2+}-current I_{CRAC} by a divalent cation-free extracellular solution was significantly reduced upon the siRNA-mediated knockdown of STIM1 and Orai1. Importantly, calcium influx induced by high extracellular Ca^{2+}

(1.8 mM) via the CaSR was substantially reduced by the siRNA-mediated knockdown of STIM1 and Orai1. STIM1 and Orai1 are required for the suppression of keratinocyte proliferation as well as calcium-induced differentiation mediated high extracellular Ca^{2+}, assessed by the diminished formation of the early differentiation marker keratin 1 [84]. *In vivo* experiments using a polyclonal antibody against the intracellular C-terminus of Orai1 showed that Orai1 is highly expressed in keratinocytes of the stratum basale and the stratum spinosum of mouse epidermis. Interestingly, Gwack *et al*. [86] generated Orai1$^{-/-}$ mice, which were characterized by thinner skin and fewer elongated keratinocytes (skin isolated from the calvaria and snout of the animals). The animals also showed sporadic hair loss resembling the cyclical alopecia described in mice with a keratinocyte-specific deletion of the Cnb1 gene (encoding the regulatory subunit of calcineurin, calcineurin B1). Jans *et al.* [85] demonstrated that STIM1 and Orai1 are involved in lysophophatic acid-induced intracellular calcium mobilization in primary keratinocyte cultures. Lysophosphatidic acid is a potent bioactive phospholipid that improves wound closure following topical application to experimental wounds in rats and mice. Moreover, using siRNA-mediated STIM1 knockdown and overexpression of the dominant negative Orai1 mutant R91W, which substantially attenuates Orai1-mediated Ca^{2+} entry, the authors showed that STIM1 and Orai1 are involved in lysophosphatidic acid-induced keratinocyte migration [85]. These results suggest that Orai1 and STIM1 play an important role in skin physiology.

Notably, there is evidence for interactions between Orai1 and TRPC channels in keratinocytes, as suggested by the comprehensive review of Saul *et al*. [87]. They suggested that TRPV3 and TRPV4 are likely to work together with Orai1 in a keratinocyte-mediated skin barrier and might provide the necessary Ca^{2+} influx. Fatherazi *et al*. [34] provided evidence for the involvement of TRPC4 channels in high extracellular Ca^{2+}-induced I_{CRAC}-like channels followed by a larger, non-specific component. Knocking down TRPC4 using siRNA resulted in a decrease of the I_{CRAC}-like channel. However, the non-specific component was not identified. Saul *et al*. [87] pointed out the possibility that I_{CRAC} derives from STIM/Orai and that TRPC4 might regulate I_{CRAC} activity.

Adenosine dependent calcium pumps residing in the plasma membrane and intracellular membranes, such as the endoplasmic reticulum, Golgi or mitochondria, also regulate intracellular calcium. They buffer excess cytosolic calcium by pumping out calcium through the plasma membrane (the plasma membrane Ca^{2+} ATPase and the Na^{+}/Ca^{2+} exchanger) or into intracellular storage sites such as the endoplasmic reticulum via Ca^{2+} ATPase (SERCA) (*figure 2*). These mechanisms might also be altered in skin diseases such as Darier's disease.

Disturbed calcium regulation in disease

Given the crucial role of TRP channels in keratinocyte differentiation, it is highly likely that they are involved in certain skin disorders. Indeed, there is mounting evidence that aberrant TRP channel expression and function might contribute to several skin diseases associated with altered keratinocyte differentiation and proliferation. In this review, we focus on psoriasis, atopic dermatitis and basal cell carcinoma, as well as on the genetic skin diseases Darier's disease and Olmstedt syndrome *(table 1).* Itching and pain are not discussed here, as excellent overviews on the involvement of TRP channels in the pathogenesis of itching and pain exist [88, 89].

Psoriasis

Psoriasis is a chronic inflammatory disease affecting the skin, scalp and joints. The most common form, plaque psoriasis, is characterized by erythematous plaques covered with silvery scales [90]. Generally, epidermal thickening and excessive keratinocyte proliferation occur [91]. The prevalence of psoriasis is approximately 2% in Central Europe and a genetic predisposition is evident [90]. Psoriasis is considered an organ-specific, T cell-driven inflammatory disease and T cells play a dominant pathogenic role in the initiation and maintenance of this disease (see also the excellent and comprehensive reviews of Cai *et al*. [92] and Chong *et al*. [93]). In recent years, psoriasis research has mainly focused on immune function and inflammation. However, in the 1980s, ion capture cytochemistry studies provided the first evidence for a defective calcium gradient in psoriatic skin [17]. These findings pointed to the involvement of additional mechanisms, which might be related to a defective calcium response during keratinocyte differentiation and proliferation. Indeed, psoriatic keratinocytes show a diminished response after calcium store depletion with thapsigargin, compared to healthy keratinocytes, suggesting a flawed store-operated calcium entry [94]. In line with these findings, Leuner *et al*. found reduced mRNA and protein expression of all TRPC channels in keratinocytes and skin biopsies isolated from psoriasis patients. This is also reflected in impaired differentiation, as shown by decreased differentiation markers and enhanced proliferation [47]. Importantly, these defects were detected in non-lesional and lesional skin from punch biopsies of psoriasis patients. Furthermore, the incubation of psoriasis keratinocytes with the TRPC6 activator hyperforin partly restored differentiation and proliferation defects. Further studies are needed to characterize the role of TRP channels in psoriasis pathogenesis and analyze whether reduced TRPC expression is a cause or a consequence of psoriasis. Given the crucial contribution of cytokines in the pathogenesis of psoriasis, reduced TRPC expression might be a result of the interaction between immunological cells and keratinocytes via cytokines. Unpublished data from our laboratory supports this hypothesis. Of note, the intake of calcium channel inhibitors in hypertension treatment is associated with psoriasiform skin lesions [95, 96]. On the other hand, TRPV1 activation in outer root sheath keratinocytes leads to the increased expression of cytokines such as interleukin 1ß [56], suggesting bidirectional interference between TRP channels and inflammation. In addition, several TRP channels, such as TRPC5, TRPC5, TRPM4 and TRPM7 channels, regulate the production and release of cytokines from T cells [97] and might thereby be involved in the inflammation process.

Table 1. Skin diseases with involvement of the TRP channels.

Disease	Impaired Channels/ Receptors	Therapeutic Effects by Agonist/antagonist
Psoriasis Chronic inflammatory skin disorder, characterized by hyperproliferative keratinocytes and reduced differentiation [91] Disturbed calcium gradient [17] Reduced calcium response in psoriatic keratinocytes [94]	TRPC1, TRPC3-7 Reduced expression of all TRPC channels in psoriasis [47]	Hyperforin Agonist at TRPC6 [106] Sufficient to induce keratinocyte differentiation [37]
Atopic dermatitis Chronic inflammatory skin disease with dry skin in characteristic location [100]	TRPC6 [104]	Hyperforin Reduces T cell and peripheral blood mononuclear cell activation [107] Topical treatment alleviates atopic dermatitis [108]
	TRPV3 Increased activity causes atopic dermatitis in mice [112]	-
Basal cell and squamous cell carcinoma Aberrant keratinocyte differentiation	CaSR Uncoupling from CaSR to differentiation [125]	NPS-467 [134] Effect on skin diseases not yet established
	TRPC1 Reduced expression [49]	-
	TRPC4 Reduced expression [49]	-
Darier's disease Acantholysis and disturbed keratinization [117] Defect type 2 sarco(endo)plasmatic reticulum Ca^{2+}-ATPase [118]	TRPC1 Increased expression, compensatory mechanisms [46, 120]	-
Olmstedt syndrome Hyperkeratotic plaques [129]	TRPV3 Constitutively active, high intracellular calcium concentrations cause apoptosis [127,	-

Atopic dermatitis

Atopic dermatitis appears most often in children, with a prevalence ranging from 10-20% for children and 1-3% in adults in the United States [98]. Patients with this chronic inflammatory disease present with dry skin in characteristic locations and pruritus [99, 100]. Earlier, atopic dermatitis was understood as a pure keratinocyte disorder but in the last two decades, emerging evidence has put forward a more comprehensive view of the disease as resulting from a complex interplay between immunological cells and keratinocytes. In particular, the contribution of Th2 cytokines such as interleukin 4 or 13 has been intensively investigated, highlighting their relevance for barrier integrity [101, 102]. Furthermore, environmental influence and genetic disposition play a large role [98, 103]. In atopic dermatitis, the role of three TRP channels, TRPC6, TRPV1, and TRPV3, has been discussed.

It has been speculated that TRPC6, whose activation is sufficient to induce keratinocyte differentiation [37], might be reduced in atopic dermatitis, giving rise to impaired keratinocyte differentiation, which is a key feature of atopic dermatitis [104]. It is likely that other TRPC channels might contribute to atopic dermatitis pathogenesis as well, given their importance in keratinocyte differentiation [34, 48, 49, 105]. TRPC channel expression is diminished in psoriasis [47], hinting at a similar role in atopic dermatitis. Indeed, some smaller clinical investigations of atopic dermatitis patients have demonstrated the positive effects of an ointment containing hyperforin, a selective TRPC6 activator [106]. Keratinocytes from subjects treated with the hyperforin ointment showed a reduced ability to stimulate the proliferation of T cells and peripheral blood mononuclear cells [107]. In a randomized, placebo-controlled clinical investigation, a hyperforin-containing ointment alleviated atopic dermatitis lesions significantly better than the vehicle alone [108]. This study supports the hypothesis that other natural compounds modifying the TRP channel will prove efficient in the treatment of skin disorders, but further preclinical and clinical work needs to be done.

TRPV1 is involved in histamine-induced itching [109], suggesting a contribution to highly pruritogenic skin diseases. Investigating TRPV1 expression in a mouse model of atopic dermatitis, Yun *et al*. found that TRPV1 expression was up-regulated [110]. Subsequently, the application of a TRPV1 antagonist alleviated scratching behavior in this mouse model and decreased calcium influx via TRPV1. Importantly, the TRPV1 antagonist also ameliorated bar-

rier integrity, as measured by transepidermal water loss and the level of the differentiation markers filaggrin and loricrin [110, 111]. In contrast, the activation of TRPV1 in human keratinocytes by anandamide, a prototypic endocannabinoid, via the CB_1 receptor, diminished proliferation and triggered apoptosis dose-dependently, for which reason it was suggested for the treatment of hyperproliferative skin disorders [57]. Furthermore, increased TRPV1 expression is associated with skin disorders linked to aged skin [42].

Spontaneously hairless mice develop an atopic dermatitis-like skin disease with pruritus. Further investigations revealed mutations in the TRPV3 gene linking TRPV3 to pruritus and atopic dermatitis [112, 113]. These results were partly confirmed in a $TRPV3^{-/-}$ mouse model. In this model, dry skin was induced by an aceton diethylether mixture and water. However, there was no difference in the development of dry skin between the knockout mice and their littermates, whereas the $TRPV3^{-/-}$ mice showed reduced scratching behavior [114]. These mice were characterized by an intracutaneous and systematic increase in pro-inflammatory cytokines and nerve growth factor (NGF), which are also involved in the pathogenesis of atopic dermatitis in humans. These findings were also replicated in cultured mouse keratinocytes using TRPV3 activators such as eugenol, leading to IL-1α release [115]. Importantly, TRPV3 channels are sensitized by pro-inflammatory mediators, which might further trigger skin inflammation. Recently, the endogenous omega-3 lipid metabolism product 17(R)-resolvin D1 was shown to specifically inhibit TRPV3 channels, thereby mediating anti-inflammatory effects [116]. These findings imply a role for TRPV3 in pruritus and atopic dermatitis; however, its role in humans needs to be defined.

Darier's disease

Darier's disease is a severe genetic skin disease characterized by acantholysis, abnormal keratinization and the presence of rounded keratinoctyes [117]. The causal mutation is located in the *ATP2A2* gene and is autosomal-dominantly passed on [117]. The *ATP2A2* gene encodes for the type 2 sarco(endo)plasmatic reticulum Ca^{2+}-ATPase (SERCA), which is responsible for intracellular calcium homeostasis by pumping cytosolic calcium back in the endoplasmatic reticulum [118, 119]. Most mutations disrupt functional domains, thereby depleting intracellular calcium stores and provoking disturbed calcium homeostasis [117]. Interestingly, TRPC1 expression is up-regulated in epidermal keratinocytes from Darier's patients. Moreover, HaCaT keratinocytes in which SERCA2 expression was silenced with the siRNA technique displayed increased TRPC1 expression. This, in turn, was associated with reduced apoptosis [46]. The same group speculated that the up-regulation of TRPC1 might be a compensating mechanism [120]. A similar effect has been discussed for Hailey-Hailey disease, which is caused by mutations in the *ATP2C1* gene encoding for the Golgi or secretory pathway Ca^{2+}-ATPases (SPCA1). Again, the result is disturbed calcium sequestration [121], which might cause the elevated expression of TRP channels.

Basal cell and squamous cell carcinoma

Skin cancers are divided into two groups: the melanoma type and the non-melanoma type. Basal cell cancers account for 65-70% of non-melanoma skin cancers [122]. Another non-melanoma skin cancer is squamous cell carcinoma, with its premalignant form, actinic keratosis, accounting for more than 250,000 new cases in the United States annually [123]. Both cancer subtypes originate in the basal layer of the epidermis [124], occurring primarily in sun-exposed areas. Enhanced oxidative stress and the release of inflammatory mediators contribute to tumor development and both cancer types are characterized by aberrant keratinocyte differentiation, which could be associated with altered TRP channel function and expression. In basal cell carcinoma, reduced differentiation is associated with the lack of TRPC1 and TRPC4 protein *in vitro*, leading to diminished calcium entry after calcium-induced differentiation and subsequently to failed differentiation [49]. Pillai *et al*. investigated the response of several squamous cell carcinoma cell lines to 1.2 mM calcium. They found that these cell lines still responded to high extracellular calcium concentrations but failed to differentiate, as assessed by involucrin synthesis and cornified envelope formation, suggesting an uncoupling of differentiation and calcium-induced differentiation [125]. Recently, Fusi *et al.* [124] showed that TRPA1 protein and mRNA expression were significantly increased in skin biopsies from patients with solar keratosis, a premalignant form of non-melanoma skin cancer. In contrast, the TRPV4 protein and mRNA levels were down-regulated in lesional skin biopsies obtained from patients suffering from basal cell carcinoma, solar keratosis or squamous cell carcinoma. The authors also found a mechanism mediating this alteration in TRPV4 expression. They demonstrated that TRPV4 stimulation using the TRPV4 agonist 4α-phorbol-12,13-didecanoate evoked a dose-dependent release of interleukin 8, which in turn down-regulated TRPV4 mRNA in HaCaT keratinocytes [124]. Importantly, the loss of TRPV4 expression seems to be associated with the transition from healthy skin to a cancer phenotype. These findings are supported by recent experiments indicating that UVB exposure leads directly to TRPV4 activation in primary mouse keratinocytes and demonstrating enhanced TRPV4 expression in human skin overexposed to UV [126]. External topical application of the TRPV4 inhibitor GSK205 significantly attenuated UVB-induced skin tissue injury. These findings provide a pathogenic link between TRP channels and inflammation in skin cancers probably induced by overexposure to UV light.

Olmstedt syndrome

Olmstedt syndrome is a genetic disease [127, 128] that presents with sharply confined hyperkeratotic plaques on the palms and soles. The plaques are also found around the mouth and on the eyelids [129]. Other clinical findings include flexion deformities of the fingers, localized alopecia and leukokeratosis of the tongue [129]. The clinical symptoms are variable but typically severe and disabling [130]. Extracutaneous manifestations are uncommon and include mental retardation, deafness, joint

laxity, osteopenia, osteolysis, secondary infections and squamous cell carcinoma [131]. Most cases are sporadic but both autosomal-dominant and X-linked recessive inheritance have been reported and associated with mutations in the *TRPV3* gene in the 573 and 692 positions, leading to a gain-of-function phenotype mediated by a constitutively active ion channel [132]. Consequently, larger inward currents are detected compared to the wild type. The resulting increased intracellular calcium concentrations cause apoptosis and thereby the characteristic hyperkeratosis seen in patients with Olmstedt syndrome [127]. In 2014, several new *TRPV3* mutations were characterized, such as a homozygous recessive p.Trp521Ser mutation in a two-year-old girl [131], a heterozygous p.Trp692Cys missense mutation in a 10-year-old boy [130], and a heterozygous, recessive p.Gly568Cys missense mutation [133].

Conclusion

We discussed calcium-induced keratinocyte differentiation with the focus on TRP and Orai/STIM channels. TRPC1, TRPC4, TRPC5, TRPV 1, TRPV3, TRPV4 and TRPV6, as well as STIM/Orai, are essential for the induction of keratinocyte differentiation and the inhibition of keratinocyte proliferation. Furthermore, TRPV1, TRPV3, TRPV4, and TRPV6 are involved in skin barrier formation. These functions point to an essential role of these ion channels for the formation and maintenance of the epidermis and suggest that the dysfunction of these ion channels might contribute to skin diseases. Preliminary preclinical and clinical data underline the role of TRPC channels, especially TRPC6 channels, in the pathophysiology of psoriasis and atopic dermatitis. TRPA1, TRPV1, and TRPV3 are involved in the release of proinflammatory cytokines from keratinocytes, which might play a role in the pathophysiology of psoriasis and atopic dermatitis. TRP channels, such as TRPC1 or TRPV3, have been linked to rare genetic diseases such as Darier's disease and Olmstedt syndrome. These findings might help to define new targets for the treatment of various skin diseases and might accelerate the development of new treatment strategies. Therefore, TRP channels might emerge as a hitherto underestimated and rather new drug target for the treatment of skin diseases.
However, a deeper understanding of skin diseases such as psoriasis or atopic dermatitis with TRP channel contribution is mandatory, as it remains unclear whether altered TRP channel expression or function is a cause or a consequence. In particular, the interplay of cytokines and differentiation-related ion channels in inflammatory skin diseases deserves further elucidation. ■

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