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Calcium regulation of keratinocyte differentiation

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Abstract

Calcium is the major regulator of keratinocyte differentiation *in vivo* and *in vitro*. A calcium gradient within the epidermis promotes the sequential differentiation of keratinocytes as they traverse the different layers of the epidermis to form the permeability barrier of the stratum corneum. Calcium promotes differentiation by both outside–in and inside–out signaling. A number of signaling pathways involved with differentiation are regulated by calcium, including the formation of desmosomes, adherens junctions and tight junctions, which maintain cell–cell adhesion and play an important intracellular signaling role through their activation of various kinases and phospholipases that produce second messengers that regulate intracellular free calcium and PKC activity, critical for the differentiation process. The calcium receptor plays a central role by initiating the intracellular signaling events that drive differentiation in response to extracellular calcium. This review will discuss these mechanisms.

Keywords

cadherin; calcium; calcium receptor; catenin; keratinocyte; phospholipase; protein kinase C; Src kinase

Microanatomy of the epidermis

The epidermis is composed of four functionally different layers of keratinocytes at different stages of differentiation **(Figure 1)**. The thickness of these layers varies in different sites, different species and under different conditions. The basal layer (stratum basale) rests on the basal lamina separating the dermis and epidermis. This layer contains the epidermal stem cells. These cells proliferate, providing the cells for the upper differentiating layers. They are large, columnar cells forming intercellular attachments with adjacent cells through desmosomes and adherens junctions. Desmogleins (Dsg) 2 and 3 and desmocollins (Dsc) 2 and 3 are the dominant cadherins in the desmosomes of the basal layers [1], whereas both P- and E-cadherins participate in the adherens junctions of these cells. However, as the keratinocytes move out of the basal layer and begin to differentiate, Dsg 1 and Dsc 1 become the dominant desmosomal cadherins, whereas P-cadherin is no longer produced as E-cadherin becomes the dominant cadherin in adherens junctions [1]. Furthermore, the expression of proteins (claudins and occludins) that form the tight junctions is initiated [2]. An asymmetric distribution of integrins on their lateral and basal surface enables their attachment to the basal lamina and adjacent cells [3–5] and helps to regulate their proliferation and subsequent differentiation [6]. Keratinocytes in the stratum basale express

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the keratins K5 and K14 [7]. As the cells leave the stratum basale, they switch from producing K5/K14 to producing keratins K1 and K10 in the stratum spinosum [8]. Cells of this layer also begin to produce involucrin [9], a component of the cornified envelope, and the enzyme, transglutaminase-K, which is responsible for the ε-(γ-glutamyl)lysine crosslinking of involucrin and other substrates into the insoluble cornified envelope [10]. The stratum granulosum, which is above the stratum spinosum, is so named because of the presence of electron-dense keratohyalin granules [11]. It is the uppermost nucleated layer. The larger of the two granule types contains profilaggrin, precursor of filaggrin, first thought to function as a bundling protein for the keratin filaments, and more recently thought to play a role in maintaining the water content of the epidermis, as it is degraded into smaller peptides with osmotic properties [12]. The smaller granules contain loricrin, a major component of the cornified envelope [13]. The stratum granulosum also contains lamellar bodies and lipid-filled vesicles responsible for secreting their lipid content (and lipid-processing enzymes) into the junction of the stratum granulosum and the stratum corneum [14]. The stratum corneum is the outermost layer of the epidermis. In this layer, the cornified envelope fully matures, surrounding the bundled keratin filaments and attached to the lipid envelope contributed by the processing of the lipids secreted by the lamellar bodies. The net result is a resilient impermeable structure protecting the viable layers underneath [15].

Calcium forms a steep gradient within the epidermis, with the highest concentration in the stratum granulosum [16]. Although initial studies with ion-capture cytochemistry and proton-induced x-ray emission indicated that the calcium levels were lowest in the stratum basale, more recent studies with phasor representation of fluorescent lifetime imaging microscopy demonstrated that the calcium content of cells in the stratum basale was variable, with a number of the keratinocytes having substantial amounts of calcium relative to the keratinocytes of the stratum spinosum. Furthermore, these recent studies demonstrated that the calcium was essentially all intracellular [17]. Therefore, in the subsequent discussion of the calcium switch *in vitro*, it is important to bear in mind that the authors do not have precise measurements of the extracellular calcium concentration in the intact epidermis. It would not, for example, be appropriate to assume that the extracellular calcium concentration in the epidermis is the same as that in the bloodstream. The higher levels of calcium in some of the cells of the stratum basale versus the stratum spinosum may contribute to initiating the differentiation process, as keratinocytes from patients with Hailey–Hailey disease (mutations in the Golgi calcium pump, ATP2C1) or from patients with Darier's disease (mutations in the endoplasmic reticulum (ER) calcium pump, ATP2A2) have lower calcium content in their stratum basale with decreased cell adhesion and disruption of the transition from the basal expression of K14 to that of K10 [18,19]. When the barrier is disrupted, the calcium gradient is lost [20], with an increase in lamellar body secretion and decreased expression of the differentiation markers loricrin, profilaggrin and involucrin [21]. Exposure of the skin to a high extracellular calcium concentration (Cao) following barrier disruption restores the expression of these marker genes, whereas exposure to a low Cao maintains the lowered expression of these differentiation markers [21]. As will be clear in the section that follows, this *in vivo* study is consistent with the more extensive studies of the response of keratinocytes to Cao *in vitro*.

Calcium switch

Calcium is the best-studied prodifferentiating agent for keratinocytes. As noted above, the calcium gradient in the epidermis plays a role in epidermal differentiation. However, much of the understanding of the role of calcium in keratinocyte differentiation comes from *in vitro* studies. Some care must be taken in extrapolating the *in vitro* results to the *in vivo* situation, since the dermis clearly affects keratinocyte differentiation as evidenced by the

fact that hair follicles will not develop in the absence of certain dermal structures (dermal papilla). Nevertheless, much has been learned about calcium-regulated differentiation from these *in vitro* studies, that is applicable to the *in vivo* situation. Keratinocytes in low calcium concentrations (e.g., 0.03 mM) proliferate but fail to differentiate into a stratified layer. When switched to calcium concentrations above 0.1 mM (the calcium switch), the differentiation process is initiated, involving both genomic and nongenomic pathways. The cells rapidly undergo morphologic changes with the development of cell–cell contacts that are critical for the differentiation process [22]. This is mediated by the redistribution to the membrane of desmoplakin to form desmosomes, occludins and claudins to form tight junctions, and E-cadherin with its associated catenins and kinases to form adherens junctions. As will be discussed subsequently, these membrane complexes provide not only adhesion between cells but also a signaling complex that participates in changes in actin distribution and sustained increases in intracellular calcium (Cai) [23–25]. These translocations to the membrane are dependent on the actin network in that cytochalasin blocks these events [24–27], but are rapid and not dependent on new protein synthesis. However, with the sustained increase in Cai, the cells begin to express in sequential fashion K1 and K10, involucrin and transglutaminase-I and loricrin and filaggrin, in that order [28–32]. A number of these genes (e.g., involucrin and *K1*) have known response elements, such as activator protein 1 (AP-1) sites for calcium and phorbol esters, acting at least in part by PKC activation. This regulation requires hours and is genomic [30–36]. Members of the Fos/Jun families bind to these sites following the calcium switch, but details of the actual mechanisms by which calcium controls the transcription of these genes remain elusive. A change in integrin expression accompanies the differentiation process and may be essential for keratinocytes to leave the stratum basale for the upper layers [37]. The response of keratinocytes to the calcium switch is multiphasic. The initial spike in Cai after an increase in Cao represents release from intracellular stores and is mediated by the calcium receptor (CaR), which will be discussed at length subsequently. However, it is the sustained increase in calcium that is critical for differentiation [27,30,32–34,38–41]. As the keratinocytes differentiate and the Cai increases, they become less sensitive to changes in Cao. The increase in Cai following the calcium switch is blocked by lanthanum, suggesting a requirement for calcium entry to sustain the Cai [42,43]. A number of different channels have been found in keratinocytes that may function as calcium channels (L-type calcium channels do not appear to be present) [44–48], with recent attention to being paid transient receptor potential vanilloid channel V6 (TRPV6) and the transient receptor potential cation (TRPC)1, 3 and 4 channels that may function as store-operated channels [49–51]. As will be discussed subsequently, several of these channels are regulated by phospholipase C (PLC)-γ1 and the CaR. Agents such as ATP that stimulate only the initial increase of Cai from intracellular stores do not promote differentiation [52–55].

A number of pathways are critical for the calcium response, and these will be reviewed prior to discussing the role of the CaR.

Phospholipase C

The calcium switch stimulates phosphoinositide metabolism, providing critical second messengers for the differentiation process [56–59]. The main enzymes involved are PLC β and γ, which hydrolyze phosphatidylinositol bisphosphate (PIP_2) to inositol trisphosphate (IP_3) and diacylglycerol (DAG). Both calcium and the active metabolite of vitamin D, $1,25(OH)_2D$, induce these enzymes [60,61]. Following the calcium switch, the rise in Cai and in IP_3 and DAG are both immediate and prolonged. As noted above, other agents such as ATP raise Cai and IP_3 levels as effectively as calcium, at least acutely, and yet do not stimulate differentiation [55], presumably owing to the transient nature of the increase in Cai. The prolonged increase in IP_3 and Cai is due to calcium activation of PLC-γ1, as it is

blocked by an antisense PLC-γ1 construct [61], whereas the initial increase in IP_3 and Cai after the calcium switch appears to be mediated by PLC-β, as it is not blocked by the antisense PLC-γ1 construct but is blocked by the general PLC inhibitor U73122 and knockdown (by siRNA) of PLC-β1 [XIE Z, BIKLE DD, UNPUBLISHED DATA]. The critical role for PLC-γ1 in mediating calcium-induced differentiation is demonstrated by the ability of the PLC-γ1 antisense construct to prevent the calcium induction of differentiation markers such as involucrin and transglutaminase [61]. Furthermore, the PLC-γ1 antisense construct, as well as U73122, block the influx of calcium into the cells through putative store-operated channels TRPC1 and 4 following depletion of the intracellular stores with thapsigargin [50]. The mechanism leading to PLC-γ1 activation by calcium will be covered in the section describing the E-cadherin/catenin complex.

PLC-γ1 contains several important domains that are critical for its regulation [62–65]. The Src homology domain 2 (SH2) domains are responsible for binding PLC-γ1 to phosphotyrosines such as those found in a number of growth factor receptor kinases, for example the EGF receptor. The EGF receptor phosphorylates PLC-γ1 at three tyrosines (771, 783 and 1254 [66]), at least two of which (Y783 and Y1254) are required for maximal activation. A pleckstrin homology (PH) domain is found in the N-terminal portion of the molecule, with two half PH domains on either side of the SH2 and SH3 domains. These PH domains and the SH2 domains enable PLC-γ1 binding to phosphatidylinositols in the membrane [63–65], in particular phosphatidylinositol trisphosphate (PIP_3). Calcium activates PLC-γ1 via PIP_3 in the absence of tyrosine phosphorylation of PLC-γ1 [67], an important function of the E-cadherin/catenin complex, as will be discussed in a subsequent section. The means by which PLC-γ1 is activated dictates whether PLC-γ1 mediates growth factor-stimulated proliferation [68] or calcium-induced differentiation [67].

Protein kinase C

The rise in DAG and Cai following the calcium switch results in PKC activation. Studies of the role of PKC in keratinocyte differentiation utilize phorbol esters [54,56,69–73]. These compounds are capable of stimulating differentiation of keratinocytes at least *in vitro* even in low Cao conditions, although their effects can be potentiated by Cao [69–73]. Furthermore, PKC inhibitors block a number of effects of phorbol esters and Cao on keratinocyte differentiation [74,75]. However, phorbol esters and calcium differ in at least some aspects of their impact on differentiation. Phorbol esters, for example, do not stimulate K1 and K10 expression [76,77], unlike their effects on later differentiation markers, such as involucrin, loricrin and filaggrin. Cao and phorbol esters also differ in their patterns of protein phosphorylation [58,78,79], and, importantly, phorbol esters do not activate the PLC pathway [56,80]; rather, the PLC pathway activated by Cao results in PKC activation via generation of DAG [56,79,80]. Moreover, phorbol esters, at least in other cells, can interfere with PLC activation [81,82]. Nevertheless, PKC activation plays an important role in the mechanism by which calcium promotes keratinocyte differentiation.

There are a large number of PKC isozymes in the epidermis, generally products of different genes under different modes of regulation and distribution within the epidermal layers [83–87]. Of the classic PKC enzymes, only PKC-α is found in the keratinocyte. Classic PKC enzymes are characterized by their activation by calcium, phorbol esters and DAG. Three novel PKC enzymes, PKC-δ, ε and η, characterized by their responsiveness to phorbol esters and DAG but not calcium, are present in keratinocytes. The keratinocyte also expresses PKC-ζ, an atypical PKC that does not respond to calcium or phorbol esters. Different agents promoting differentiation may utilize different PKC isozymes. Several studies, including this one, showed that blocking the expression of PKC-α with antisense oligonucleotides prevented Cao induction of a number of differentiation markers [87,88].

However, not all studies have reached this conclusion. In particular, PKC-δ has been shown in some studies to be the most critical PKC for keratinocyte differentiation, whereas PKC-α overexpression was found to block calcium-induced differentiation [36]. These disparities remain unresolved, but may result from differences in species or between experimental approaches using overexpression versus reduction of the protein of interest.

As alluded to previously, activation of PKC leads to activation of transcription factors in the Fos/Jun families that probably mediate the effects of calcium, phorbol esters and DAG on keratinocyte differentiation [53,54,89–93]. These transcription factors bind to AP-1 sites in the regulatory regions of the genes that they regulate [94]. In addition to c-Fos and c-Jun, Fra-1, Fra-2, Jun B and Jun D are found in keratinocytes, and their distribution in the epidermis is both cell- and species-specific [95]. The best-studied gene in this regard is involucrin, in which the distal AP-1 site (critical for both phorbol ester and calcium regulation) binds Fra-1, Jun B and Jun D following PKC activation [96]. Surprisingly, a dominant negative mutant of c-Jun that blocks c-Jun/Fos-regulated prolactin expression [97] actually promotes transcription of involucrin [77], indicating that these Fos/Jun factors may have both stimulatory and inhibitory actions on the genes that they regulate.

E-cadherin–catenin complex

As noted in the discussion of the response of the keratinocyte to the calcium switch, cell–cell contacts are established. These consist of adherens junctions, tight junctions and desmosomes [6,24]. These contacts serve not only as mechanisms for cellular adhesion but also as important signaling complexes. Cao plays a critical role in enabling the intercellular contacts, although the precise role played by Cao in the binding of the extra-cellular domains of the proteins remains unclear [1,98]. What does seem clear is that the strength of these intercellular bonds is regulated by the intracellular portions of these complexes. The E-cadherin–catenin complex, the major component of adherens junctions, is the best studied with respect to its signaling function, and plays a key role in the mechanism by which Cao stimulates differentiation [99]. Within minutes of the calcium switch, E-cadherin is translocated to the plasma membrane in a complex with α, β, γ and p120 catenins, RhoA, Src family kinases, PI3K and phosphatidylinositol 4 phosphate 5 kinase 1α (PIP5K1α) [100–108]. E-cadherin is expressed throughout the epidermis, unlike P-cadherin, which is expressed only in the basal layer [109]. Deletion of E-cadherin prevents the formation of adherens junctions and impairs differentiation of the keratinocytes, although cell–cell adhesion persists, presumably owing to the continued expression of P-cadherin and proteins involved with desmosome and tight junction formation [102,105,110]. The results *in vivo* with E-cadherin deletion are somewhat discrepant, with one report demonstrating the maintenance of adherens junctions, although the epidermis was hyperplastic and lacked full expression of terminal differentiation markers [111], whereas a second report demonstrated a high rate of perinatal mortality not found in the earlier report, with breakdown of the permeability barrier and loss of the catenins [112].

The chain of events leading to the formation of the E-cadherin–catenin complex following the calcium switch is discussed later. When activated by calcium, the CaR, in turn activates RhoA [113]. This leads to activation of Src family kinases, Fyn and Src, in particular, responsible for the tyrosine phosphorylation of the catenins and their binding to E-cadherin [67,100,101,105,108,114]. Surprisingly, this does not require an increase in Cai, in that the Cai chelator BAPTA fails to block calcium-induced E-cadherin–catenin complex formation, although, as noted earlier, the increase in Cai is required for subsequent differentiation [115]. p120 catenin binds to the juxtamembrane region of E-cadherin and stabilizes the complex [67,116]. Knockdown of p120 catenin leads to loss of E-cadherin and β- and α-catenin, with epidermal hyperplasia and chronic inflammation [117] similar to that seen in the E-cadherin null mouse reported by Tinkle *et al.* [111]. β- and γ- (phakoglobin) catenins

bind separately to the catenin-binding domain of the E-cadherin tail. Through their N-termini, they bind α-catenin, which in turn links the complex to the underlying actin cytoskeleton [118]. In mouse keratinocytes, PI3K binds to p120 and γ-catenin [102], but in human keratinocytes and most other cells, p120 and β-catenin serve as the anchors for PI3K binding [105]. In human keratinocytes, γ-catenin plays little role in the formation or maintenance of the E-cadherin–complex, and its deletion has little impact on Cao-induced differentiation, unlike deletion of the other catenins and E-cadherin itself [105].

There are a number of PI3Ks with different regulatory and catalytic subunits and distinct modes of regulation [119]. Class 1A enzymes are composed of one of four regulatory subunits (p85-α and -β, p55 and p50), and one of three catalytic subunits (p110-α, -β and -δ). The regulatory subunits bind to activated receptor tyrosine kinases or other phosphotyrosine-containing proteins via their SH2/SH3 domains. Within the E-cadherin complex, this binding is to the catenins that enable phosphorylation of the regulatory subunit, releasing and thus activating the catalytic subunit. Class 1B PI3K has one known catalytic subunit, p110γ, and one known regulatory subunit, p101. This isoform is activated by the β/γ subunits of G proteins, such that agonists of G protein-coupled receptors, including CaR, may also activate PI3K by this mechanism. Inhibition of PI3K activity blocks the ability of calcium to activate PLC-γ1 and induce differentiation [67,105].

PIP5K1α is also bound to the E-cadherin–catenin complex following the calcium switch, and this binding is also dependent on β-catenin [106]. Deletion of PIP5K1α *in vitro* blocks both PI3K and PLC-γ1 activity following the calcium switch. Furthermore, deletion of PIP5K1α blocks the acute rise in Cai following Cao, ATP or ionomycin, indicating a reduction in Cai stores. As with the inhibition of PI3K, this results in an inhibition of Cao-induced differentiation.

The model the authors propose that links the E-cadherin–catenin complex to calcium-induced differentiation is shown in **Figure 2**. p120 and β catenin when bound to E-cadherin recruit PI3K and PIP5K1α to the membrane. PIP5K1α phosphorylates phosphatidylinositol phosphate to PIP_2, and PI3K phosphorylates PIP_2 to PIP_3. PIP_3 activates PLC-γ1, which in turn hydrolyzes PIP_2 to IP_3 and DAG. These second messengers release calcium from intracellular stores and activate PKC. The rise in Cai and activation of PKC contribute to the induction of genes that are responsible for the differentiation process. Deletion of PIP5K1α results in a reduction of PIP_2 production, thus limiting the ability of any of the PLCs to produce IP_3 and DAG, and limiting the amount of PIP_3 that can be produced by PI3K. Deletion or inactivation of PI3K prevents PIP_3 formation, thus blocking calcium-induced activation of either PLC-γ1 or Akt (also known as protein kinase B, which is dependent on PIP_3 for its recruitment to the membrane and subsequent phosphorylation by PI3K). The net result is that formation of the E-cadherin complex following the calcium switch is critical for the pathways, in particular the PKC- and PLC-mediated pathways, required for differentiation. CaR plays a key role in initiating these events in response to Cao.

Calcium receptor

The acute response of the keratinocyte to calcium resembles that of the parathyroid cell [120], which senses Cao via a seven-transmembrane domain, the GTP-binding protein-coupled CaR [121,122]. This receptor was originally discovered in the parathyroid gland, but is found in a number of other tissues, including the keratinocyte [41,123–126]. The human CaR is composed of 1078 amino acids with a number of glycosylation sites that appear to be important for function. Moreover, the keratinocyte and a number of other tissues (bone, cartilage and kidney) produce an alternatively spliced variant of the CaR (CaRalt) as they differentiate; this variant lacks exon 5 and so would be missing residues

461–537 in the extracellular domain [123,124]. At least in the skin, CaRalt appears to have little function and may even act as a dominant negative. The original global knockout of CaR was produced using a neomycin cassette inserted in exon 5, so that this mouse continued to express (and actually increased the expression of) CaRalt. Nevertheless, these mice died within a few weeks of severe hyperparathyroidism and hypercalcemia. The authors have recently developed a mouse model in which exon 7, which codes the transmembrane domain and cytoplasmic tail of CaR, is floxed, allowing it to be deleted in the tissue of our choice [127]. By deleting CaR only in keratinocytes, the authors have avoided the systemic problems of hyperparathyroidism and hypercalcemia in the global CaR null model. These mice demonstrated a reduction in differentiation markers and permeability barrier function in the epidermis, defects more pronounced in mice ingesting a low calcium diet [128]. Turksen and Troy, however, overexpressed the CaR in keratinocytes and demonstrated accelerated development of the barrier during embryologic development [129].

Blocking the expression of CaR with an antisense construct in keratinocytes decreases the ability of calcium to raise Cai and induce the involucrin and transglutaminase-I genes [115,125]. There are several mechanisms by which CaR mediates calcium-induced increases in Cai, thereby promoting differentiation. CaR in the plasma membrane responds to Cao with activation of PLC, which in turn hydrolyzes PIP_2 to IP_3 and DAG as discussed earlier. IP_3 then releases calcium from intracellular stores, but probably also stimulates calcium influx. The activation of PLC occurs by at least two mechanisms **(Figure 3)**. CaR is a G-protein-coupled receptor that directly activates PLCs, most likely through Gq. This mechanism would activate both PLC-β and -γ. As noted earlier, this is the mechanism favored for PLC-β activation and is not blocked by inhibition of PLC-γ1 activity or expression. This mechanism has received little study in keratinocytes, and its role in Cao-induced differentiation is unknown. However, the failure of ATP, which increases Cai transiently presumably by this mechanism, does not stimulate differentiation. PLC-γ1 activation following the calcium switch requires the E-cadherin–catenin complex as described previously. Blocking CaR expression prevents the calcium-induced formation and stabilization of the E-cadherin–catenin complex and the subsequent activation of PLC-γ1 [115]. The mechanism is as follows: Cao induces the formation of a complex containing CaR, filamin A and RhoA with the E-cadherin complex [113]. RhoA is activated in the process, in turn activating the Src family kinases Fyn and Src, leading to tyrosine phosphorylation of the catenins and their binding to E-cadherin. This enables recruitment of PIP5K1α and PI3K, and activation of PLC-γ1 via PIP_3 as described earlier. Filamin A is a scaffolding protein that links CaR with RhoA. Interference with the binding of CaR to filamin A blocks calcium-stimulated RhoA activation and formation of the E-cadherin–catenin complex, with subsequent inhibition of keratinocyte differentiation [113]. Blocking RhoA activity did not prevent the initial rise in Cai following the calcium switch, unlike deletion of CaR, although it did prevent the prolonged increase in Cai. These results are consistent with the hypothesis that the mechanism for the sustained increase in Cai necessary for differentiation requires PLC-γ1 activation via the E-cadherin–catenin complex.

However, most of the CaR is located intracellularly in the ER and trans-Golgi, the major sources of Cai [130]. In the ER, the CaR is primarily unglycosylated, unlike the forms in the trans-Golgi and plasma membrane [130]. In the trans-Golgi, the CaR forms a complex with PLC-γ1, the IP_3 receptor and the Golgi calcium pump (SPCA1, the gene product of *ATP2C1*) [130]. Both the ER and Golgi fractions are capable of ATP-stimulated calcium uptake and IP_3-stimulated calcium release [130]. Blocking CaR expression, through stimulating the production of both the endoplasmic reticulum (ER) and Golgi calcium pumps (SERCA2 and SPCA1, respectively), reduces the intracellular stores and limits the

amount of releasable calcium following the addition of Cao, ionomycin or thapsigargin [130]. The importance of Cai stores in regulating the differentiation process is illustrated by diseases such as Dariers disease and Hailey–Hailey disease that likewise have reduced levels of calcium in their ER and Golgi, respectively, and impaired calcium-stimulated differentiation due to mutations in their respective calcium pumps SERCA2 and SPCA1 [131,132]. One explanation for the reduction in Cai stores is that PLC-γ1 is required for the activation of store-operated channels (in particular TRPC-1 and -4) in keratinocytes as previously noted [50]. TRPC1 forms a complex with PLC-γ1 and the IP_3 receptor [50], and its knockdown impairs Cao-induced increases in Cai and the expression of differentiation markers [50,51]. The CaR is required for calcium activation of PLC-γ1 and, as such, would be expected to be required for calcium influx through the TRPC channels, in addition to its presumed role in causing IP_3-mediated release of calcium from intracellular stores.

Summary

Cao regulation of keratinocyte differentiation is well established *in vivo* and *in vitro*. Calcium signals by both outside–in and inside–out mechanisms. Central to both mechanisms is the CaR. Although Cao is necessary to facilitate the intercellular adhesion mediated by the extracellular domains of the proteins forming the desmosomes, adherens junctions and tight junctions, it is the intracellular signaling that results in stability of these complexes and promotes the differentiation process. CaR, through RhoA and Src family kinases, enables the formation of a stable E-cadherin–catenin complex in the membrane that recruits two important enzymes, PI3K and PIP5K1α, to the membrane where they maintain levels of PIP_3 and PIP_2 that respectively activate and serve as substrates for PLC-γ1. When cleaved by PLC-γ1, PIP_2 produces two important signaling molecules, IP_3 and DAG. These in turn stimulate the release of calcium from intracellular stores and activate PKC. PLC-γ1 is also required for the activation of store-operated channels by which Cai stores are replenished. The prolonged rise in Cai coupled with PKC-regulated transcription factors in the Fos/Jun families regulate transcription of the genes required for keratinocyte differentiation. In addition, the CaR forms a complex with the IP_3 receptor and the Golgi calcium pump, possibly to regulate the uptake and release of calcium from these intracellular stores. With the development of the keratinocyte-specific CaR-null mouse, one can expect the rapid progress in understanding calcium-regulated keratinocyte differentiation to continue.

Expert commentary

Calcium is a major regulator of keratinocyte differentiation. The CaR is central to the mechanism by which calcium affects this role. However, most of the information comes from *in vitro* studies. Although a calcium gradient exists in the epidermis, its role *in vivo* in the differentiation process is less clear. The development of the conditional CaR-null mouse that enables specific deletion of the CaR in keratinocytes will help answer the degree to which the effects of calcium *in vitro* can be translated to the *in vivo* situation. In addition, very few studies are available on the role of calcium in hair follicle cycling. Whether calcium affects this process to the degree and by the same mechanisms that it regulates epidermal differentiation remains to be determined.

Five-year view

Separating the inside–out and outside–in mechanisms by which calcium regulates keratinocyte differentiation should play an increasingly important role in future research in this area. The role of the desmosomes, adherens junctions and tight junctions not only as the means of attaching cells and/or regulating paracellular transport of fluids and ions but also as intracellular signaling complexes will become better appreciated and studied. Currently, the E-cadherin–catenin complex has received the most study in this regard, but it is likely

that the desmosomes and tight junctions play similar roles. In addition, the mechanisms by which the CaR regulates not only events at the plasma membrane but also the handling of calcium by the ER and Golgi have received little attention and remain wide open for future investigation.

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Key issues

- Keratinocytes cultured in low calcium concentrations remain proliferative; when switched to high calcium concentrations (the calcium switch), the cells begin to differentiate and form intercellular contacts important for the differentiation process.
- Calcium initiates the differentiation by outside–in and inside–out mechanisms. Specifically, calcium enables the formation of desmosomes, adherens junctions and tight junctions that bind cells together in the presence of calcium, and stimulates the calcium receptor (CaR) to initiate intracellular mechanisms required for differentiation.
- The E-cadherin–catenin complex, the major component of adherens junctions, exemplifies the role that the intercellular contacts play, as it helps bind cells together while also serving as a scaffold for intracellular processes required for differentiation.
- The sustained increase in intracellular calcium (Cai) following the calcium switch is required for differentiation; transient increases in Cai do not suffice.
- When activated by calcium, the CaR increases Cai by stimulating phospholipase C (PLC) activity leading to hydrolysis of phosphatidylinositol bisphosphate to form two second messengers, inositol trisphosphate (IP_3) and diacylglycerol, important for keratinocyte differentiation. IP_3 stimulates the release of calcium from intracellular stores, and diacylglycerol stimulates PKC.
- Two PLCs play different roles in the response of the keratinocyte to calcium. PLC-β is primarily responsible for the initial spike in Cai, whereas PLC-γ1 is primarily responsible for the sustained increase in Cai both by increasing IP_3 levels to release Cai and by increasing calcium influx through store-operated channels.
- PLC-β is activated by Gq as a direct result of CaR binding to calcium, whereas PLC-γ1 is activated via the CaR-mediated formation of the E-cadherin–catenin complex that brings together the enzymes required for phosphatidylinositol triphosphate production, the direct activator of PLC-γ1 following the calcium switch.

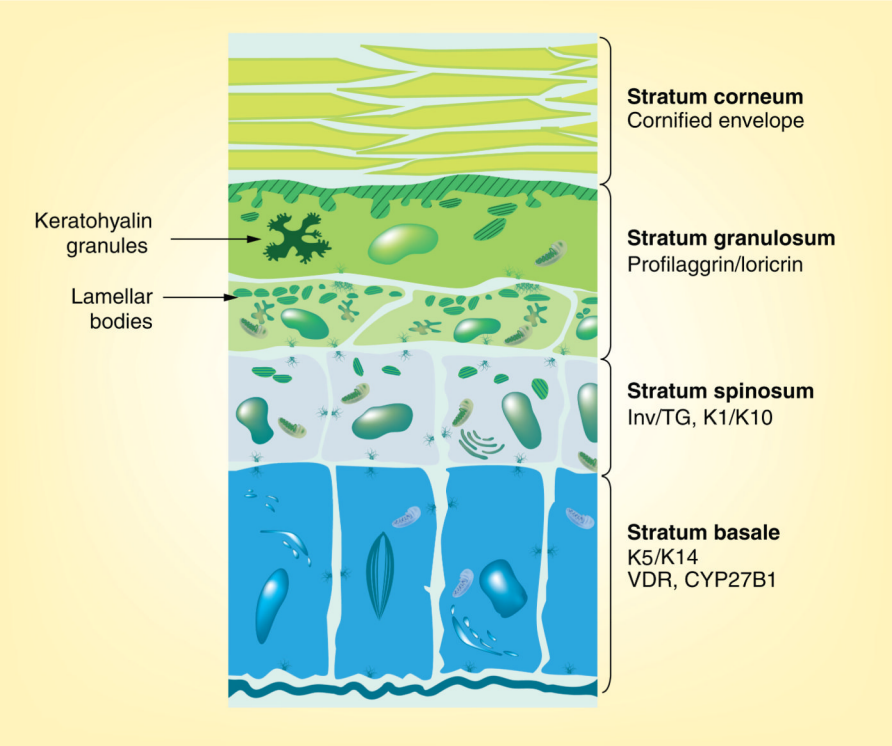

Figure 1. Microanatomy of the epidermis

The epidermis is composed of four functionally different layers that may vary in thickness depending on location and species. The stratum basale rests on the basal lamina and contains the stem cells. This layer is distinguished by its production of keratins 5 and 14. The VDR and the 25OHD-1α CYP27B1 responsible for producing the active metabolite of vitamin D ($1,25[OH]_2D$) are found in the highest concentration in this layer. Differentiation is initiated as the cells move from the stratum basale to the stratum spinosum, where involucrin, transglutaminase-I and the keratins K1 and K10 are expressed. In the next layer, the stratum granulosum, profilaggrin and loricrin are produced and packaged in keratohyalin granules. This layer is also where lipids for the waterproofing of the permeability barrier are produced and packaged into lamellar bodies. The stratum corneum is the enucleated layer critical for barrier function containing the cornified envelope within and the lipid matrix without the cells.

VDR: Vitamin D receptor.

Adapted with permission from [133].

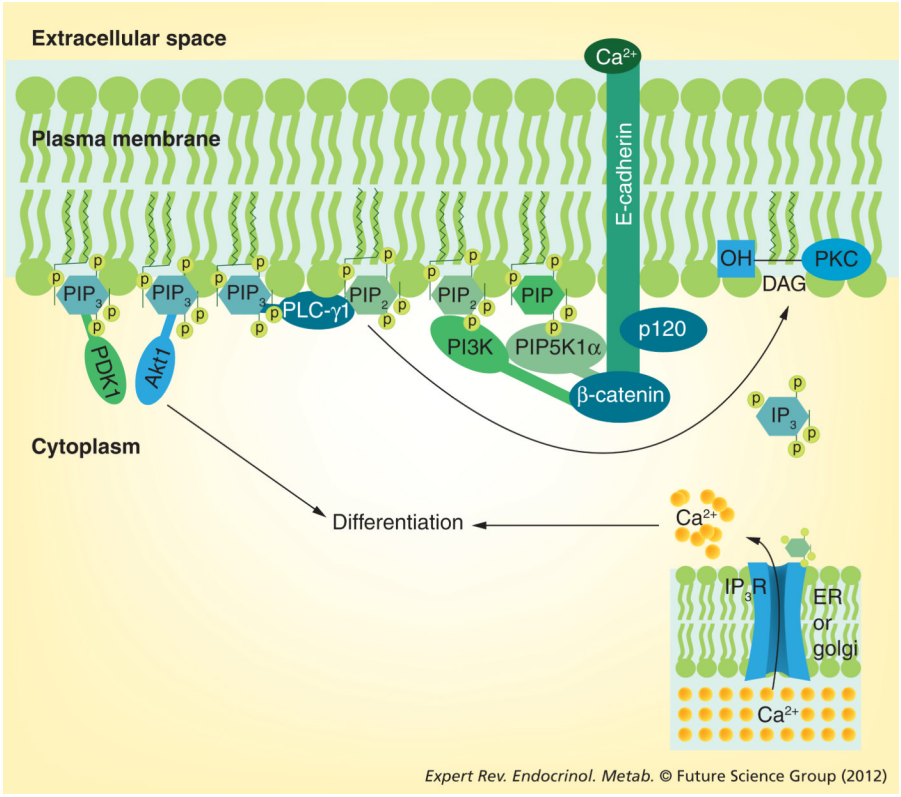

Figure 2. Signaling by the E-cadherin–catenin complex

Following the calcium switch, the E-cadherin–catenin complex forms in the plasma membrane. Extracellular calcium serves to link the extracellular domains of E-cadherin together, forming the adherens junctions (outside–in signaling). Within the cell, p120- and β-catenins bind to the juxtamembrane and cytoplasmic tail, respectively, of E-cadherin. γ-catenin (not shown) competes with β-catenin for this site, but in human keratinocytes it does not appear to have a major role in calcium-induced keratinocyte differentiation. α-catenin (not shown) attaches to β-catenin and links the complex to the cytoskeleton. β-catenin also serves as the binding site for PIP5K1α and PI3K, enzymes that phosphorylate PIP to PIP_2 and PIP_2 to PIP_3, respectively. PIP_3 activates PLC-γ1, which hydrolyzes PIP_2 to IP_3 and DAG, leading to the release of calcium from intracellular stores and activation of PKC, respectively. PIP_3 also serves as a binding site for Akt and PDK1, which also contribute to the differentiation process.

DAG: Diacylglycerol; ER: Endoplasmic reticulum; IP_3: Inositol trisphosphate; IP_3R: Inositol trisphosphate receptor; P: Phosphorylation; PIP: Phosphatidylinositol phosphate; PIP_2: Phosphatidylinositol bisphosphate; PIP_3: Phosphatidylinositol trisphosphate; PIP5K1α: Phosphatidylinositol 4 phosphate 5 kinase 1α.

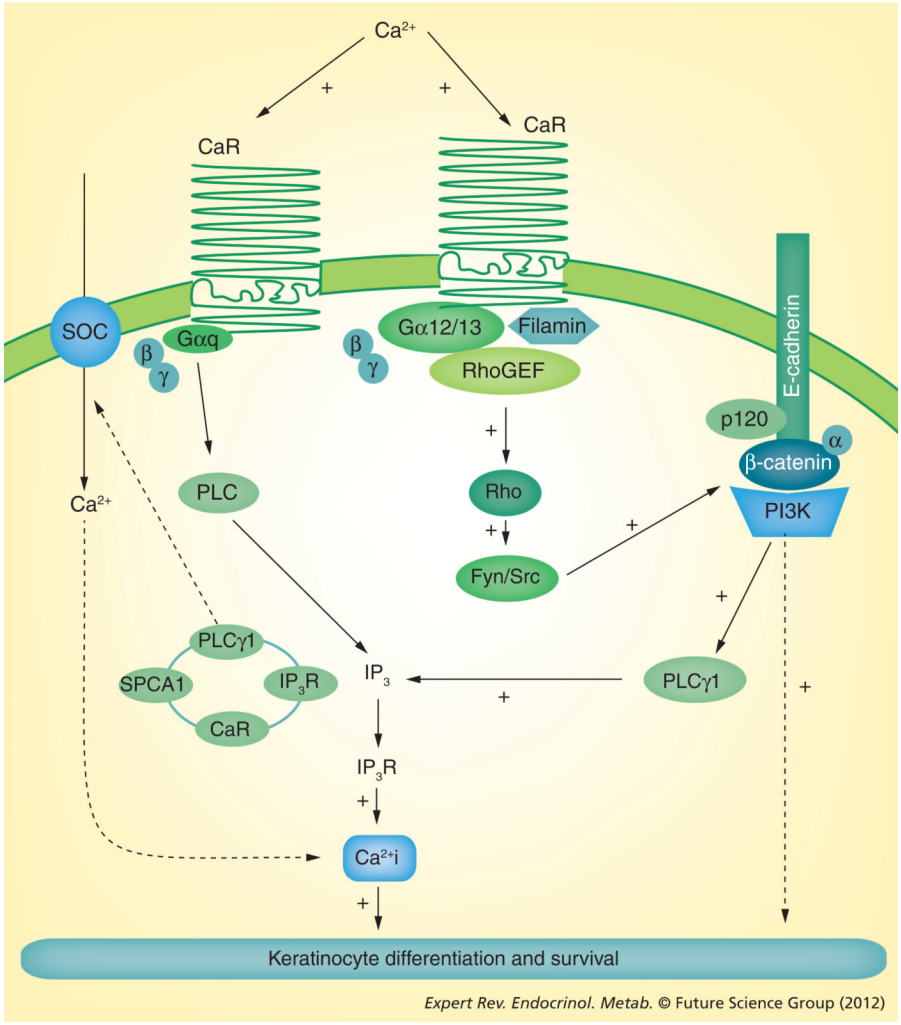

Figure 3. The role of the calcium receptor in calcium-induced differentiation

Calcium stimulates intracellular signaling (inside–out signaling) through its binding to CaR. In one pathway, CaR activates the G protein Gαq, leading to PLC activation and generation of IP_3 with the release of calcium from intracellular stores. This is the predominant mechanism for the initial spike in Ca^{2+}i concentration following the calcium switch. CaR also activates Rho that, in turn, activates the src kinases Src and Fyn to tyrosine phosphorylate the catenins, enabling their binding to the E-cadherin–catenin complex. As noted in the legend of **Figure 2**, this results in PLC-γ1 activation, leading to a sustained increase in Ca^{2+} i concentration both by release of calcium from intracellular stores and by stimulating the influx of calcium through store-operated channels. The latter role may be played by PLC-γ1 as part of the complex in Golgi with the Golgi calcium pump SPCA1, CaR and the IP_3 receptor.

Ca^{2+}i: Intracellular calcium; CaR: Calcium receptor; IP_3: Inositol trisphosphate; IP_3R: Inositol trisphosphate receptor; PLC: Phospholipase C; SOC: Store-operated channel.