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Disruption of the Vertical Calcium Gradient in Murine Epidermis by a Potent Vitamin D_3 Analogue, KH 1060

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The hormonal forms of vitamin D_3 (1,25-dihydroxyvitamin D_3 and synthetic vitamin D_3 analogues) are potent regulators of keratinocyte growth and stimulators of keratinocyte differentiation. Recent experiments *in vitro* on cultured keratinocytes indicate that Ca^{2+} may be a second messenger mediating the effects related to the induction of keratinocyte differentiation by the hormonal forms of vitamin D_3. In this study we employed the technique of ion capture cytochemistry to investigate the effects of a potent vitamin D_3 analogue, KH 1060 (20-epi-22-oxa-24*a*-homo-26,27-dimethyl-1,25-dihydroxyvitamin D_3), on the distribution of bound intracellular and extracellular calcium in murine epidermis *in vitro*. Topical treatment of the skin with KH 1060 (0.4 mmol/l) resulted in the development of epidermal hyperplasia and hyperkeratosis. We observed that epidermis treated with KH 1060 contained fewer calcium deposits in the upper epidermal strata (both intra- and extracellularly) than the control skin. This phenomenon was rapid and occurred after only a single application of KH 1060. Calcium distribution in the basal cell layer was not affected. We propose that observed reduction in the quantity of calcium deposits was caused by the release of sequestered calcium from the intracellular stores and cellular Ca^{2+} uptake, leading eventually to the increase in the intra-cellular concentration of ionized calcium. The ability of the active vitamin D_3 compounds to release Ca^{2+} may be important for their activity in psoriasis.

Key words: analogues; calcium gradient; cholecalciferols; differentiation; epidermis; proliferation.

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1,25-dihydroxyvitamin D_3 and other vitamin D_3 compounds demonstrating hormonal activity (collectively referred to as the vitamin D compounds, VDs) are potent regulators of keratinocyte growth and stimulators of epidermal cell differentiation. VDs suppress keratinocyte growth *in vitro* (1, 2) and have been used clinically in the treatment of psoriasis, where they inhibit excessive epidermal proliferation (3–6). However, in certain situations VDs paradoxically stimulate keratinocyte growth (7–9). In our laboratory we have developed a simple model of epidermal hyperplasia in hairless mice induced by a very potent vitamin D_3 analogue, KH 1060 (10–12). Single or repeated topical applications of this compound in nano- or micromolar concentrations invariably stimulate keratinocyte proliferation and differentiation, leading to epidermal hyperplasia and hyperkeratosis. Using this model we were able to show the involvement of the protein kinase C and the oncogene product c-fos as mediators of epidermal hyperplasia (13). Other researchers have reported activation of similar signalling pathways by VDs in cultured keratinocytes and in other cell types (14–20). These observations led to the development of the hypothesis that some of the biological effects of VDs are mediated by various second messengers (such as protein kinase C) rather than, as traditionally viewed, by the vitamin D receptor operating as a transcription factor (21).

Ionised calcium (Ca^{2+}) is the most important second messenger in eukaryotic cells and an increase in the intracellular Ca^{2+} concentration is an endpoint of activation of the majority of different membrane receptors. In non-excitable cells, such as keratinocytes, the Ca^{2+} mobilization is achieved *via* activation of the receptor-coupled phospholipases (phospholipase Cβ for G-protein receptors and the phospholipase Cγ for receptor tyrosine kinases), generation of inositole triphosphate and calcium release from the intracellular stores (22). Additionally, keratinocytes seem to possess a membrane receptor for calcium which is activated in the presence of high extracellular calcium concentration leading to calcium release from intracellular stores followed by the capacitative entry of Ca^{2+} from the outside (23). The net result is the sustained elevation of cytoplasmic Ca^{2+} concentration, an effect which causes keratinocyte differentiation (24).

There is evidence that VD stimulates Ca^{2+} response in many cell types. For instance, 1,25-dihydroxyvitamin D_3 has been shown to cause a release of inositol triphosphate or generate calcium fluxes in myoblasts (25), skeletal muscle cells (20), pancreatic β cells (26), osteoblasts (27), or intestinal cells (28). However, the existence and role of VD-dependent calcium signalling in keratinocytes is still a matter of debate. Some authors have succeeded in demonstrating that in cultured keratinocytes VD increases the cytoplasmic Ca^{2+} concentration and argue that calcium is responsible for the VD-induced keratinocyte differentiation (29). However, other research groups have been unsuccessful in demonstrating the Ca^{2+}-mobilizing activity of VD (30). To further investigate this problem we used ion-capture biochemistry to determine the effects of KH 1060 on calcium distribution in keratinocytes in murine epidermis.

MATERIAL AND METHODS

KH 1060 (20-epi-22-oxa-24*a*-homo-26,27-dimethyl-1,25-dihydroxyvitamin D_3) was obtained from the Chemical Research Department of Leo Pharmaceutical Products. The 0.4 mmol/l solution was prepared in the buffered isopropyl alcohol, as described (10). Female hairless C3H mice (strain hr/hr C3H/Tif Bom, Bommice, Denmark, weight approximately 20 g) were divided into two groups of 10 animals in each, and treated topically with 50 ml of the KH 1060 solution or the vehicle, once daily. After 1, 2, 3, 5, and 14 days of treatment, two mice from the experimental and control groups were sacrificed, and two

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Table I. *Effects of topical KH 1060 on the intensity of cytochemical calcium labelling in murine epidermis*

Structure		Days of treatment (labelling intensity)[1]					
		0[2]	1	2	3	5	14
Basal cell layer	intracellular	+	+	+	+	+	+
	extracellular	+	+	+	+	+	+
Upper spinous and granular cell layers	intracellular	3+	2+	+	+	+	+
	extracellular	2+	2+	2+	+	+	+
Cornified layers		0	0	0	0	0	0

[1]0: no labelling; +: 1–20 precipitates/100 μm^2; 2+: 21–100 precipitates/100 μm^2; 3+: multiple precipitates and conglomerates. [2]Control labelling in the vehicle-treated animals. The calcium staining did not differ throughout the treatment period in this group.

2 mm punch biopsies were sampled from the dorsal skin of each animal. Ion-capture cytochemistry was performed as described previously (31). Ultrastructure of the tissue prepared with this method is identical to that obtained with the conventional electron microscopy technique and calcium is visualized as electron-dense precipitates. Skin samples were fixed overnight at 4°C in 3% glutaraldehyde, 0.5% paraformaldehyde 90 mM potassium oxalate, 1.9% sucrose solution adjusted to pH 7.4 with KOH. Fixed samples were rinsed in 90 mM potassium oxalate in 1.9% sucrose, pH 7.4, post-fixed in 1% osmium tetroxide and 2% potassium pyroantimonate for 2 h at room temperature. The ultrathin sections for electron microscopy were prepared using standard procedures (12, 31) and examined with the JEOL 1200 EX electron microscope at 80 kV and magnification ×5,000–17,000. Calcium deposits were evaluated semi-quantitatively in 20 sections from each biopsy, as described in the legend to Table I. The method was specific for calcium, since treatment with 3 mmol/l EGTA (31, 32) prevented the formation of calcium precipitates.

To measure the possible effects of the topical treatment on the skin barrier function, the transepidermal water loss was measured with the Evaporimeter (ServoMed, Stockholm, Sweden) (33). The measurements were performed in quadruplicate 2 h after the topical application of the solutions in the groups treated for 1, 2, 3, 5, and 14 days with KH 1060 or the vehicle.

RESULTS

Compared with the control skin, the epidermis of the animals treated with KH 1060 showed features of hyperkeratosis and epidermal hyperplasia, identical to those described previously (12). Epidermal hyperplasia was first evident after a 3-day treatment with KH 1060. The distribution of calcium deposits in the control, vehicle-treated skin followed the pattern described by Menon et al. (32). Calcium precipitates were rarely observed in the basal keratinocytes, and found primarily in the cytoplasm and mitochondria of these cells (Fig. 1). In the granular cell layer and also in a few upper spinous cells calcium deposits were abundant and homed to the peripheral cytoplasm and the intercellular spaces (Fig. 2). Intracellular calcium deposits were larger than those seen in the intercellular space (Fig. 2A versus 2B).

Only a few calcium precipitates were observed in the epidermis of the skin treated with KH 1060. Staining of the basal cell layer was unchanged (not shown), but a dramatic decrease in the number of calcium precipitates was seen in the suprabasal epidermal cell layers (Fig. 3 versus Fig. 2). The spinous and granular keratinocytes and the intercellular spaces in these layers were virtually devoid of the deposits. Depletion of calcium by KH 1060 was seen after a single topical treatment.

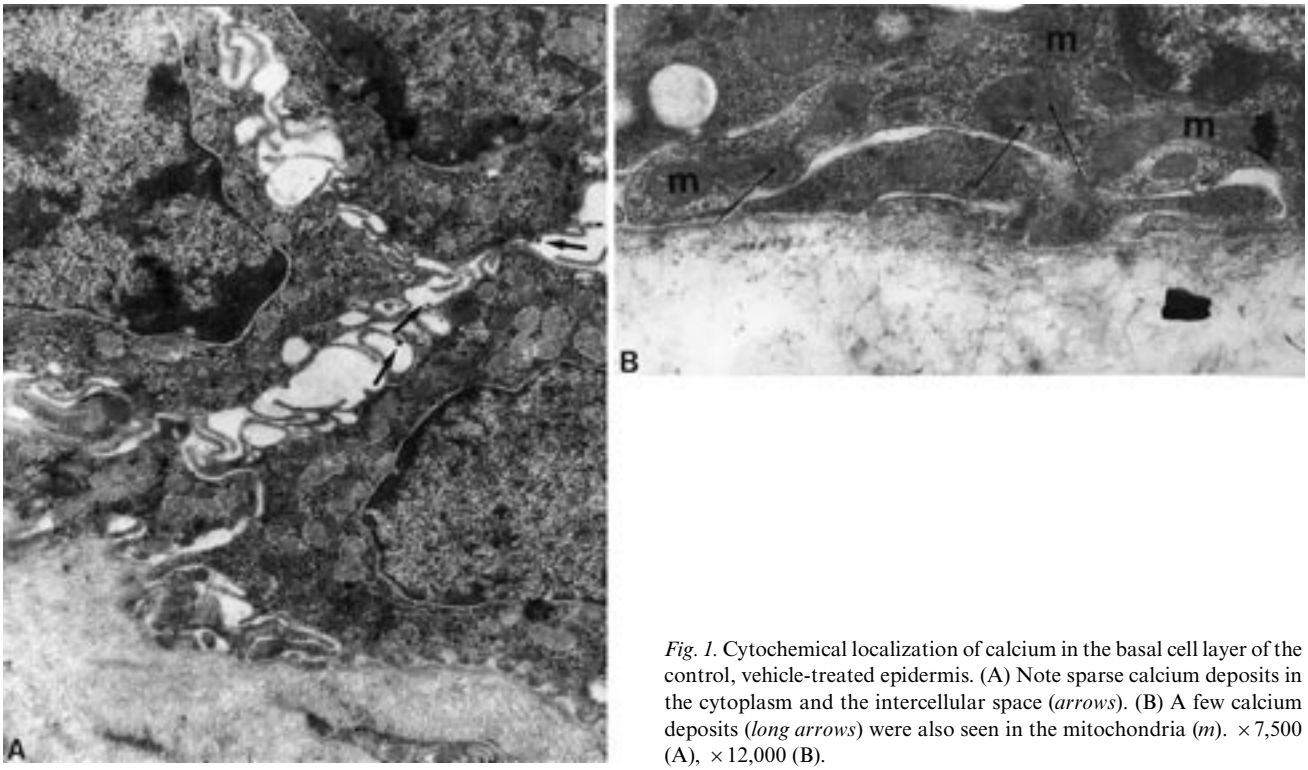

Fig. 1. Cytochemical localization of calcium in the basal cell layer of the control, vehicle-treated epidermis. (A) Note sparse calcium deposits in the cytoplasm and the intercellular space (*arrows*). (B) A few calcium deposits (*long arrows*) were also seen in the mitochondria (*m*). ×7,500 (A), ×12,000 (B).

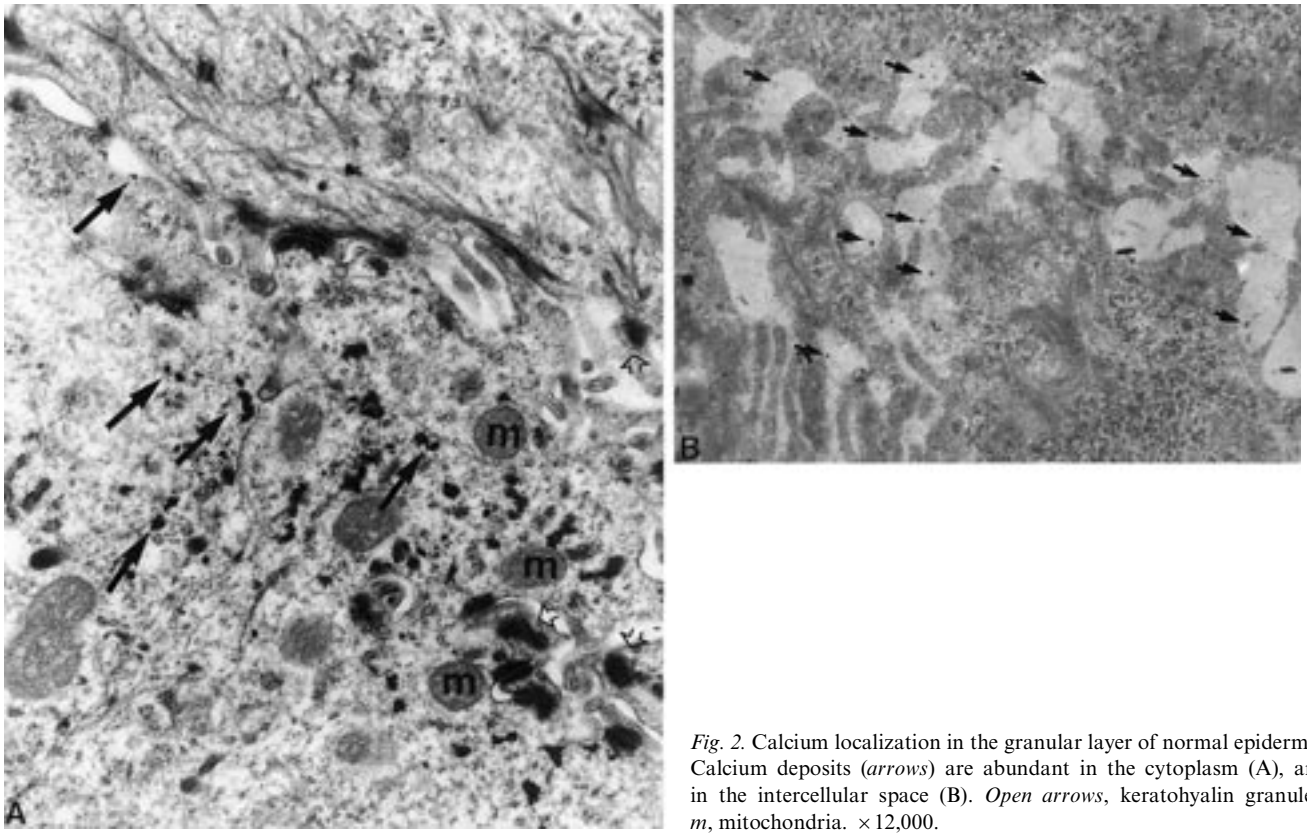

Fig. 2. Calcium localization in the granular layer of normal epidermis. Calcium deposits (*arrows*) are abundant in the cytoplasm (A), and in the intercellular space (B). *Open arrows*, keratohyalin granules; *m*, mitochondria. ×12,000.

The fully developed effect of KH 1060 was observed after 2 days of topical application and persisted throughout the whole treatment period (Table I).

Disruption of the epidermal permeability barrier may affect calcium distribution in the cells (34). To investigate whether KH 1060 could affect the integrity of the barrier we measured the transepidermal water loss following the topical treatment with either KH 1060 or the vehicle. In all cases the transepidermal water loss in mice was minimal (pooled means of 10 animals: 2.3 ± 0.8 [standard deviation] $g \times m^{-2} \times h$ in the KH 1060-treated skin versus 2.4 ± 0.9 $g \times m^{-2} \times h$ in the control, vehicle-treated skin).

DISCUSSION

The technique of ion-capture cytochemistry is well established for investigations of cellular calcium distribution in various tissues, including the epidermis (32, 35, 36). The method is based on the precipitation of calcium by oxalate-pyroantimonate and visualization of the precipitates by electron microscopy. A vertical gradient of calcium in the epidermis has been demonstrated with this approach: the basal cells were shown to contain fewer calcium deposits than the upper keratinocyte layers (32).

The main finding of this study is the disruption of the normal epidermal calcium gradient in mice treated with KH 1060. The most striking observation was a nearly total disappearance of the intracellular and intercellular calcium precipitates in the spinous and granular cell layers. The effects of KH 1060 on calcium distribution were relatively rapid (1–2 days), matching the previously described dynamics of the induction of epidermal proliferation by this compound (11). This observation, together with the well-known involvement of calcium signalling in the processes of cellular growth and differentiation, suggests that changes in calcium gradient by KH 1060 may have functional consequences.

A mechanistic explanation for the disappearance of calcium deposits in the KH 1060-treated skin cannot be given at present. A trivial possibility might be the disruption of the epidermal barrier by KH 1060 (34). This is very unlikely, however, in view of the results showing that KH 1060 did not affect the transepidermal water loss in mice.

It must be remembered that ion capture cytochemistry detects the sequestered, protein-bound calcium rather than the metabolically active-free ionized calcium. Therefore, the decrease in the intracellular and intercellular calcium precipitates may result from the release of bound calcium in keratinocytes and the transmembrane intracellular flux of calcium ions from the extracellular space, respectively. This hypothesis is in accordance with the results of the experiments *in vitro* showing that VD may stimulate calcium entry in keratinocytes (29) and potentate the increase in the intracellular calcium concentration in response to known agonists, such as ATP (30). Thus, it is conceivable that the net result of the topical treatment with KH 1060 *in vivo* is an increase in the cytoplasmic Ca^{2+} concentration in the spinous and granular cells.

Although caution must be exercised when extrapolating the results of animal experiments, it is tempting to speculate that involvement of Ca^{2+} in VD signalling may be mechanistically important for the action of VD in psoriasis. Ion capture cytochemistry observations have revealed that in the lesional skin in psoriasis an excessive quantity of calcium precipitates is present in the granular cells and in the stratum corneum (35). This may impy that mobilization of calcium from the intracellular

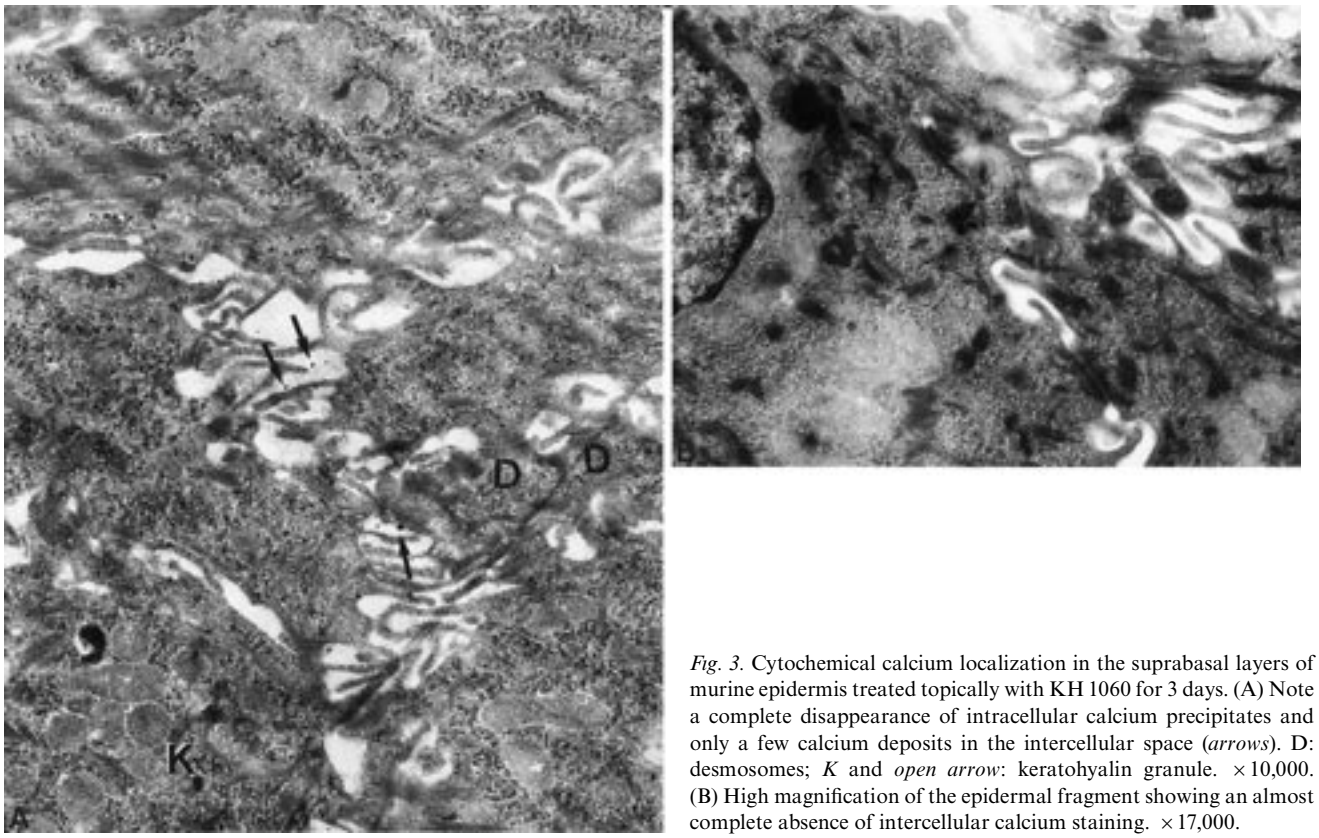

Fig. 3. Cytochemical calcium localization in the suprabasal layers of murine epidermis treated topically with KH 1060 for 3 days. (A) Note a complete disappearance of intracellular calcium precipitates and only a few calcium deposits in the intercellular space (*arrows*). D: desmosomes; *K* and *open arrow*: keratohyalin granule. ×10,000. (B) High magnification of the epidermal fragment showing an almost complete absence of intercellular calcium staining. ×17,000.

stores in these cells is deficient, which in turn may be responsible for the disturbances in epidermal differentiation and maturation. VD may correct these phenomena by stimulating calcium mobilization in the upper epidermal strata. Thus, Ca^{2+} may be an important second messenger in mediating the antipsoriatic activity of VD.

REFERENCES

1. Hosomi J, Hosoi J, Abe E, Suda T, Kuroki T. Regulation of terminal differentiation of cultured mouse epidermal cells by 1,25-dihydroxyvitamin D_3. Endocrinology 1983; 113: 1950–1957.
2. Smith EL, Walworth NC, Holick MF. Effects of 1,25-dihydroxyvitamin D_3 on the morphologic and biochemical differentiation of cultured human keratinocytes grown in serum-free conditions. J Invest Dermatol 1986; 86: 709–714.
3. Kragballe K, Wildfang IL. Calcipotriol (MC 903), a novel vitamin D_3 analogue stimulates terminal differentiation and inhibits proliferation of cultured human keratinocytes. Arch Dermatol Res 1990; 282: 164–167.
4. Kragballe K. Treatment of psoriasis with calcipotriol and other vitamin D analogues. J Am Acad Dermatol 1992; 27: 1001–1008.
5. Kragballe K, Gjertsen BT, De Hoop D, Karlsmark T, van de Kerkhof PC, Larkö O, et al. Double-blind, right/left comparison of calcipotriol and betamethasone valerate in treatment of psoriasis vulgaris. Lancet 1991; 337: 193–196.
6. Perez A, Raab R, Chen TC, Turner A, Holick MF. Safety and efficacy of oral calcitriol (1,25-dihydroxyvitamin D_3) for the treatment of psoriasis. Br J Dermatol 1996; 134: 1070–1078.
7. Gniadecki R, Gniadecka M, Serup J. Inhibition of glucocorticoid-induced epidermal and dermal atrophy with KH 1060 – a potent 20-epi analogue of 1,25-dihydroxyvitamin D_3. Br J Pharmacol 1994; 113: 439–444.
8. Lützow Holm C, De Angelis P, Grosvik H, Clausen OP. 1,25-Dihydroxyvitamin D_3 and the vitamin D analogue KH1060 induce hyperproliferation in normal mouse epidermis. A BrdUrd/DNA flow cytometric study. Exp Dermatol 1993; 2: 113–120.
9. Gniadecki R. Stimulation *versus* inhibition of keratinocyte growth by 1,25-dihydroxyvitamin D_3: dependence on cell culture conditions. J Invest Dermatol 1996; 106: 510–516.
10. Gniadecki R, Gniadecka M, Serup J. The effects of KH 1060, a potent 20-epi analogue of the vitamin D_3 hormone, on hairless mouse skin *in vivo*. Br J Dermatol 1995; 132: 841–852.
11. Gniadecki R, Serup J. Stimulation of epidermal proliferation in mice with 1,25-dihydroxyvitamin D_3 and receptor-active 20-epi analogues of 1,25-dihydroxyvitamin D_3. Biochem Pharmacol 1995; 49: 621–624.
12. Kobayasi T, Gniadecki R, Serup J. Ultrastructure of murine epidermis treated with the vitamin D_3 analogue KH–1060. Acta Derm Venereol (Stockh) 1995; 75: 222–227.
13. Gniadecki R. A vitamin D analogue KH 1060 activates the protein kinase C–c-*fos* signalling pathway to stimulate epidermal proliferation in murine skin. J Endocrinol 1994; 143: 521–525.
14. Yada Y, Ozeki T, Meguro S, Mori S, Nozawa Y. Signal transduction in the onset of terminal keratinocyte differentiation induced by 1,25-dihydroxyvitamin D_3: role of protein kinase C translocation. Biochem Biophys Res Commun 1989; 163: 1517–1522.
15. Bellido T, Morelli S, Fernandez LM, Boland R. Evidence for the participation of protein kinase C and 3′,5′-cyclic AMP-dependent protein kinase in the stimulation of muscle cell proliferation by 1,25-dihydroxy-vitamin D_3. Mol Cell Endocrinol 1993; 90: 231–238.
16. van Leeuwen JPTM, Birkenhäger JC, van den Bemd G-JCM, Buurman CJ, Staal A, Bos MP, Pols HAP. Evidence for the functional involvement of protein kinase C in the action of 1,25-dihydroxyvitamin D_3 in bone. J Biol Chem 1992; 267: 12562–12569.

17. Khare S, Wilson DM, Tien X-Y, Dudeja PK, Wali RK, Sitrin MD, Brasitus TA. 1,25-dihydroxycholecalciferol rapidly activates rat colonic particulate guanylate cyclase via a protein kinase C-dependent mechanism. Endocrinology 1993; 133: 2213–2219.
18. Slater SJ, Kelly MB, Taddeo FJ, Larkin JD, Yeager MD, McLane JA, et al. Direct activation of protein kinase C by 1,25-dihydroxyvitamin D_3. J Biol Chem 1995; 270: 6639–6643.
19. Bissonnette M, Wali RK, Hartmann SC, Niedziela SM, Roy HK, Tien X-Y, et al. 1,25-dihydroxyvitamin D_3 and 12-*O*-tetradecanoyl phorbol 13-acetate cause differential activation of Ca^{2+}-dependent and Ca^{2+}-independent isoforms of protein kinase C in rat colonocytes. J Clin Invest 1995; 95: 2215–2221.
20. Massheimer V, de Boland AR. Modulation of 1,25-dihydroxyvitamin D_3-dependent Ca^{2+} uptake in skeletal muscle by protein kinase C. Biochem J 1992; 281: 349–352.
21. Norman AW, Nemere I, Bishop JE, Lowe KE, Maiyar AC, Collins ED, et al. 1,25$(OH)_2$-vitamin D_3, a steroid hormone that produces biologic effects via both genomic and nongenomic pathways. J Steroid Biochem Molec Biol 1992; 41: 231–240.
22. Clapham DE. Calcium signaling. Cell 1995; 80: 259–268.
23. Bikle DD, Ratnam A, Mauro T, Harris J, Pillai S. Changes in calcium responsiveness and handling during keratinocyte differentiation. Potential role of the calcium receptor. J Clin Invest 1996; 97: 1085–1093.
24. Bikle DD, Pillai S. Vitamin D, calcium, and epidermal differentiation. Endocr Rev 1993; 14: 3–19.
25. Morelli S, de Boland AR, Boland RL. Generation of inositol phosphates, diacylglycerol and calcium fluxes in myoblasts treated with 1,25-dihydroxyvitamin D_3. Biochem J 1993; 289: 675–679.
26. Sergeev IN, Rhoten WB. 1,25-dihydroxyvitamin D_3 evokes oscillations of intracellular calcium in a pancreatic β-cell line. Endocrinology 1995; 136: 2852–2861.
27. Lieberherr M. Effects of vitamin D_3 metabolites on cytosolic free calcium in confluent mouse osteoblasts. J Biol Chem 1987; 262: 13168–13173.
28. Massheimer V, Boland R, de Boland AR. Rapid 1,25$(OH)_2$-vitamin D_3 stimulation of calcium uptake by rat intestinal cells involves a dihydropyridine-sensitive cAMP-dependent pathway. Cell Signal 1994; 6: 299–304.
29. Jones KT, Sharpe GR. Intracellular free calcium and growth changes in single human keratinocytes in response to vitamin D and five 20-epi-analogues. Arch Dermatol Res 1994; 286: 123–129.
30. Pillai S, Bikle DD, Su M-J, Ratnam A, Abe J. 1,25-dihydroxyvitamin D_3 upregulates the phosphatidylinositol signalling pathway in human keratinocytes by increasing phospholipase C levels. J Clin Invest 1995; 96: 602–609.
31. Gajkowska B, Zareba-Kowalska A. Cytochemical calcium localization in hypothalamo-neurohypophysial system of rats after ischemia. Preliminary observations. J Hirnforsch 1989; 6: 747–755.
32. Menon GK, Grayson S, Elias PM. Ionic calcium reservoirs in mammalian epidermis: ultrastructural localization by ion-capture cytochemistry. J Invest Dermatol 1985; 84: 508–512.
33. Pinnagoda J, Tupker RA. Measurement of the transepidermal water loss. In: Serup J, Jemec GBE, eds. Non-invasive methods and the skin. Boca Raton, FL: CRC Press, 1995: 173–178.
34. Menon GK, Elias PM, Feingold KR. Integrity of the permeability barrier is crucial for maintenance of the epidermal calcium gradient. Br J Dermatol 1994; 130: 139–147.
35. Menon GK, Elias PM. Ultrastructural localization of calcium in psoriatic and normal human epidermis. Arch Dermatol 1991; 127: 57–63.
36. Pillai S, Menon GK, Bikle DD, Elias PM. Localization and quantitation of calcium pools and calcium binding sites in cultured human keratinocytes. J Cell Physiol 1993; 154: 101–112.