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Effect of 1·2 mmol/l calcium, triamcinolone acetonide, and retinoids on low-calcium regulated keratinocyte differentiation

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SUMMARY

Neonatal mouse keratinocytes cultured in low calcium (0·01 mmol/l) show rapid growth and little stratification when compared with cells grown in normal 1·2 mmol/l calcium. The effect of low calcium on the amount and synthesis of specific differentiation proteins was studied; additionally, the effect of 10^{-8} mol/l triamcinolone acetonide, and 6 μg/ml of retinoic acid and of etretinate (Ro 10-9359) on low-calcium regulated keratinocyte hyperproliferation and differentiation was determined. Low-calcium regulated keratinocytes contained less non-covalently cross-linked and disulphide cross-linked keratins, less cell envelopes, much greater amounts of SDS-soluble viable cell proteins, and slightly more keratohyaline granule-related proteins than normal-calcium regulated keratinocytes. A 24 h switching time to 1·2 mmol/l calcium medium did not affect the amounts or synthesis of these proteins. Both retinoids and triamcinolone acetonide inhibited by approximately 50% the proliferation of the low-calcium regulated keratinocytes. Growth of low-calcium cells in these drugs for 9 days increased the amounts of both keratins and cell envelope proteins in the cultures. We concluded that calcium-dependent processes can regulate epidermal keratinocyte proliferation and differentiation. Our studies suggest that these calcium-regulated events may occur via changes in calcium-dependent proteins.

Epidermal keratinocytes grown *in vitro* form multi-layered, stratifying cultures that proliferate and differentiate (Marcelo *et al.*, 1978). The processes that control the formation of new basal cells and the differentiation of the keratinocytes are regulated by a number of mediators. Among these are cyclic AMP (Marcelo, 1979; Tong & Marcelo, 1983), retinoids (Marcelo & Madison, 1984) and glucocorticoids (Marcelo & Tomich, 1983).

In vitro neonatal mouse keratinocyte proliferation and differentiation is also regulated by the concentration of calcium ions in the growth medium. Hennings *et al.* (1980) and Hennings and Holbrook (1983) have demonstrated that keratinocytes grown in low-calcium medium

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(0·01–0·06 mmol/l) proliferate rapidly, do not stratify and form no intracellular desmosomal connections. Increasing the calcium levels to the normal 1·2 mmol/l level causes the rapid formation of desmosomes (1–2 h) and the eventual stratification, keratinization and cornification of the cells (Hennings & Holbrook, 1983).

We recently described a technique for quantitating the synthesis and processing of epidermal differentiation proteins (Marcelo & Tong, 1983). This type of analysis detects cornified cell formation, the presence of keratohyaline granule related proteins, and the amounts of non-covalently and disulphide cross-linked keratins. Using this technique we investigated the effect of a low-calcium to normal-calcium 24 h switch on the synthesis and processing of the epidermal differentiation proteins, and the effect of two retinoids and the glucocorticoid, triamcinolone acetonide, on the differentiation of low-calcium regulated keratinocytes.

METHODS

Growth of epidermal keratinocytes. The basal cells were prepared as previously described (Marcelo *et al.*, 1978). Low-calcium regulated (LCR) keratinocytes were grown as described by Hennings *et al.* (1980). The cells were grown at 32°C and were fed on alternate days, or daily (the LCR keratinocytes).

Analysis of differentiation. The cultures were serially extracted with four buffers to yield six fractions (Tong & Marcelo, 1983). These consisted of two keratohyaline granule (KG)-related protein fractions, an SDS-soluble cell protein fraction, a non-covalently bound and a disulphide cross-linked keratin fraction, and the residual cornified cell envelope fraction. These proteins were analysed by Lowry assay and by SDS slab gel polyacrylamide electrophoresis (SDS–PAGE) (Marcelo & Tong, 1983).

Analysis of DNA and protein synthesis. Keratinocyte cultures were pulse labelled for 6 h with 1 μCi/ml of [^{3}H]thymidine and processed as previously described (Marcelo *et al.*, 1983). Seven-day-old normal and LCR keratinocytes were pulse labelled for 4 h with 10 μCi/ml of [^{3}H]leucine and cold-chased for 24 h in the appropriate medium (Tong & Marcelo, 1983).

Addition of drugs. Six μg/ml of retinoic acid (RA), etretinate (RO, Ro 10-9359) or 10^{-8} mol/l triamcinolone acetonide (TA) were added to 7-day-old LCR keratinocyte cultures. DMSO (0·05%) was the vehicle control for the retinoids.

RESULTS AND DISCUSSION

Growth properties of low-calcium regulated (LCR) keratinocytes

Fig. 1 is a phase photomicrograph of a LCR keratinocyte (0·01–0·02 mmol/l calcium). The cells form a non-stratifying, non-cornifying monolayer. The keratinocytes show exceptionally wide intracellular spaces. Hennings & Holbrook (1983) report that no intracellular desmosomal complexes occur. Fig. 2 shows the proliferative rate as measured by [^{3}H]thymidine incorporation per μg DNA of normal, LCR and switched (low calcium to 1·2 mmol/l calcium) keratinocytes. The LCR cells proliferate very rapidly, with a labelling index of 50% or more. Switched keratinocytes ceased this rapid proliferative activity and reached normal keratinocyte proliferative rates after 4–5 days.

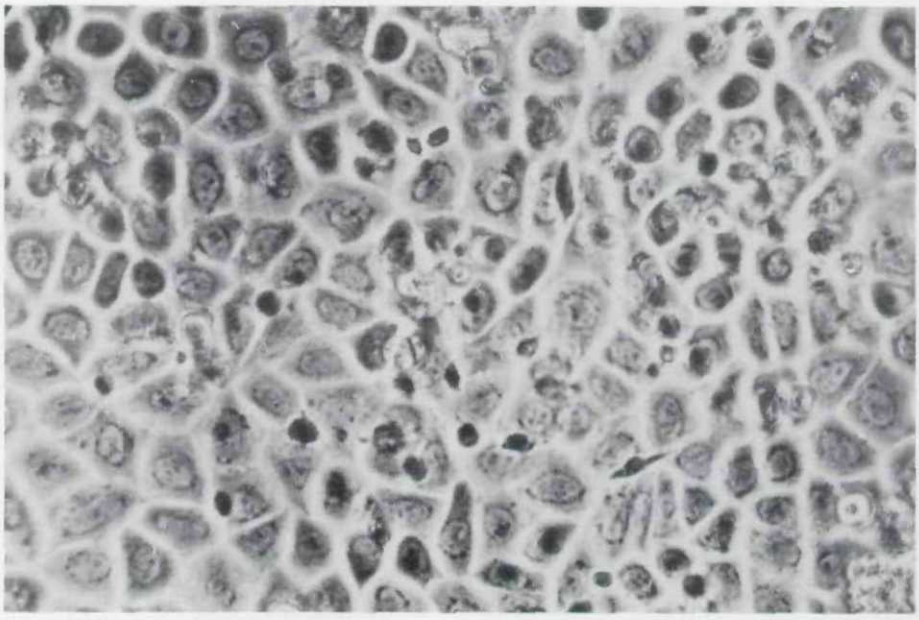

FIGURE 1. Photomicrograph of epidermal keratinocytes grown in 0·01 mmol/l calcium medium. (× 214.)

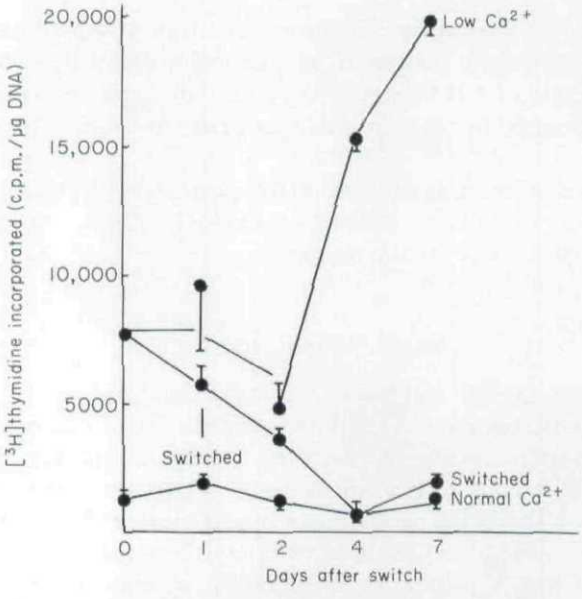

FIGURE 2. [^{3}H]thymidine incorporation of low-calcium and normal-calcium regulated keratinocytes, and of low-calcium keratinocytes switched to normal-calcium growth medium.

Effect of low calcium on the percentage and synthesis of specific epidermal keratinocyte proteins
Figure 3 shows pie graphs of the per cent protein/60 mm Petri dish of the proteins in normal calcium, LCR and 24 h switched keratinocytes. SDS–PAGE gels of these fractions are presented in Figure 4. The LCR keratinocytes contained more SDS-soluble viable cell proteins than the normal cultures (Fig. 3). In Figure 4, lane L (low calcium) showed more SDS-soluble protein than the N (normal calcium) lane. An equal portion of a normal or LCR or switched (S) Petri dish was loaded on to the gel, i.e. one-fourth of the protein extracted from a 60 mm dish was run on the gel. In this way the amounts of protein in each type of culture can be compared since 5×10^6 cells were used to initiate all the cultures. The 24 h switch did not alter the amount of protein in the SDS-soluble protein fraction (Figs 3 and 4). KG-related proteins were synthesized by the LCR keratinocytes (Figs 3 and 4) in the same, or slightly greater amount as the normal keratinocyte cultures.

LCR cells synthesized significantly less keratin polypeptides than did the normal-calcium keratinocytes (Fig. 3). This observation is in agreement with the work of Roop *et al.* (1983) who recently reported a marked absence of mRNA coding for keratins in LCR keratinocytes. As seen in Fig. 4 (a. keratins) the LCR keratinocytes contained some non-cross-linked keratin polypeptides. These are probably the keratin filaments reported by Hennings & Holbrook (1983). The 24 h switched cells contained more of these polypeptides, although no increase in the synthesis of these polypeptides was detected using [^{3}H]leucine pulse-labelling (Fig. 5). While large amounts of disulphide cross-linked keratins were found in the normal-calcium keratinocytes (Fig. 4, b. keratins) virtually none were found in LCR cells. Twenty-four hour

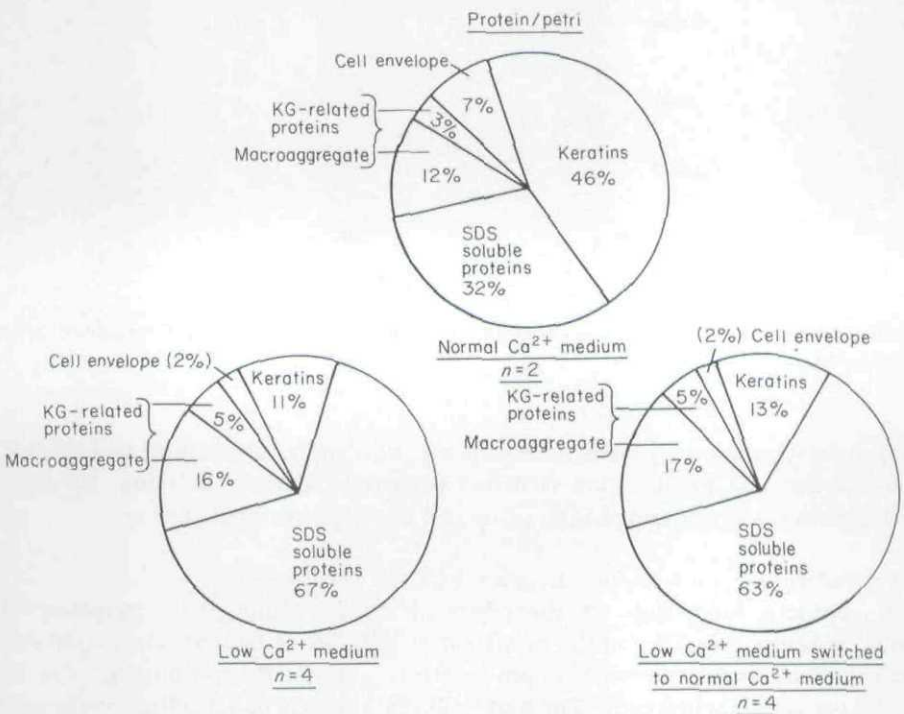

FIGURE 3. Percentage of specific epidermal proteins in normal, low calcium and 24 h switched keratinocytes.

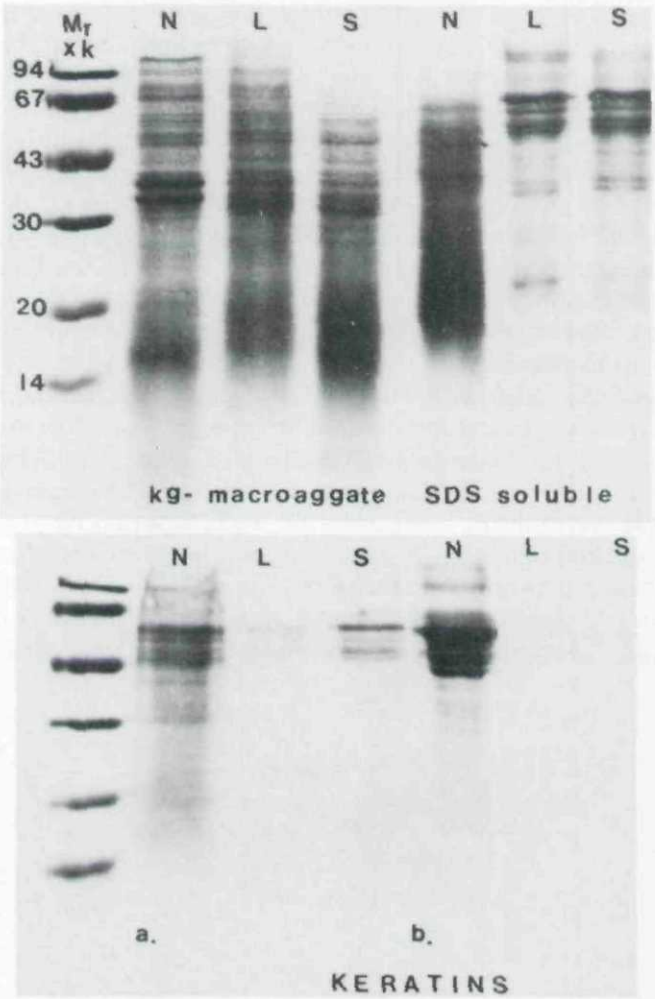

FIGURE 4. Coomassie blue stained gels of normal-calcium (N) and low-calcium (L) regulated, and 24 h switched (S) keratinocyte proteins.

switching did not induce disulphide cross-linking. This analysis technique would have detected this post-translational modification if it had occurred (Marcelo & Tong, 1983). LCR and switched keratinocytes contained little or no cell envelope material (Fig. 3).

Effect of retinoids and triamcinolone acetonide on LCR keratinocytes

Figure 6 shows a time-study of the effect of retinoic acid (RA), etretinate (RO) and triamcinolone acetonide (TA) on the proliferation of LCR cells. The data is presented as per cent control. The control was 100% = cpm/μg DNA in the LCR keratinocytes. The values over the bars are per cent labelled cells. The 0·05% DMSO vehicle had no effect on the proliferative rate of the LCR cells. After 10 and 13 days, both retinoids decreased the proliferative rate of the LCR cells by 50%. This rate was still greater than that of normal-calcium regulated

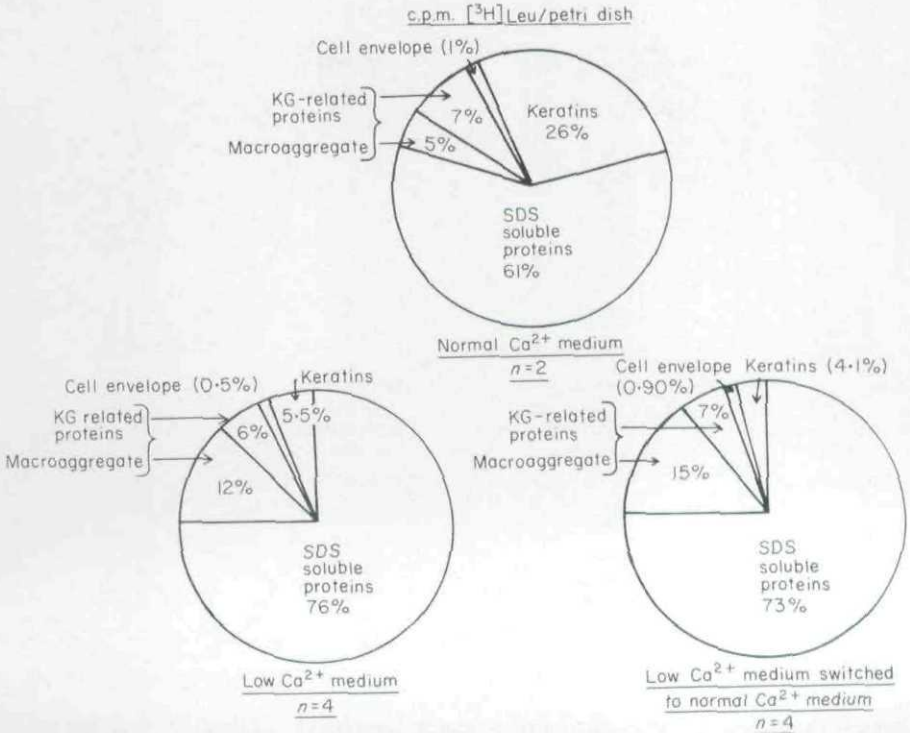

FIGURE 5. Percentage of [^{3}H]leucine incorporation into specific epidermal proteins by normal-calcium and low-calcium regulated, and 24 h switched keratinocytes.

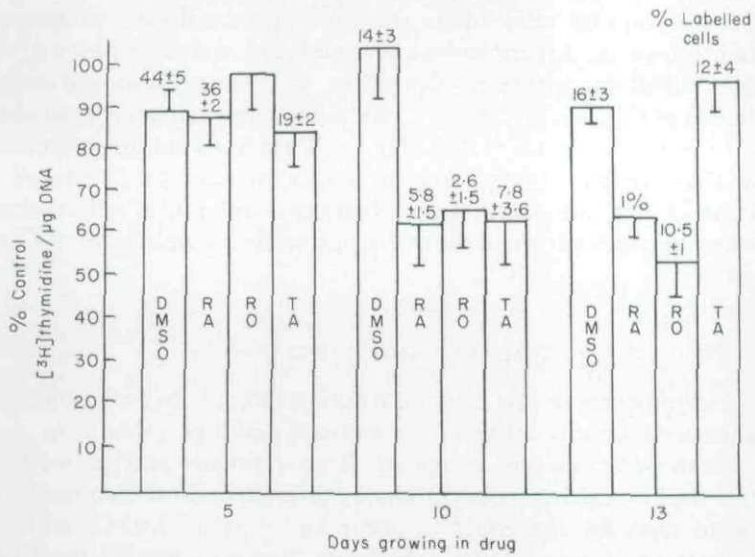

FIGURE 6. [^{3}H]thymidine incorporation by low-calcium regulated keratinocytes growing in 6 µg/ml of retinoic acid (RA) and of Ro 10-9359 (etretinate; RO) and 10^{-8} mol/l triamcinolone acetonide (TA). The values over the bars are the per cent labelled cells for each time point.

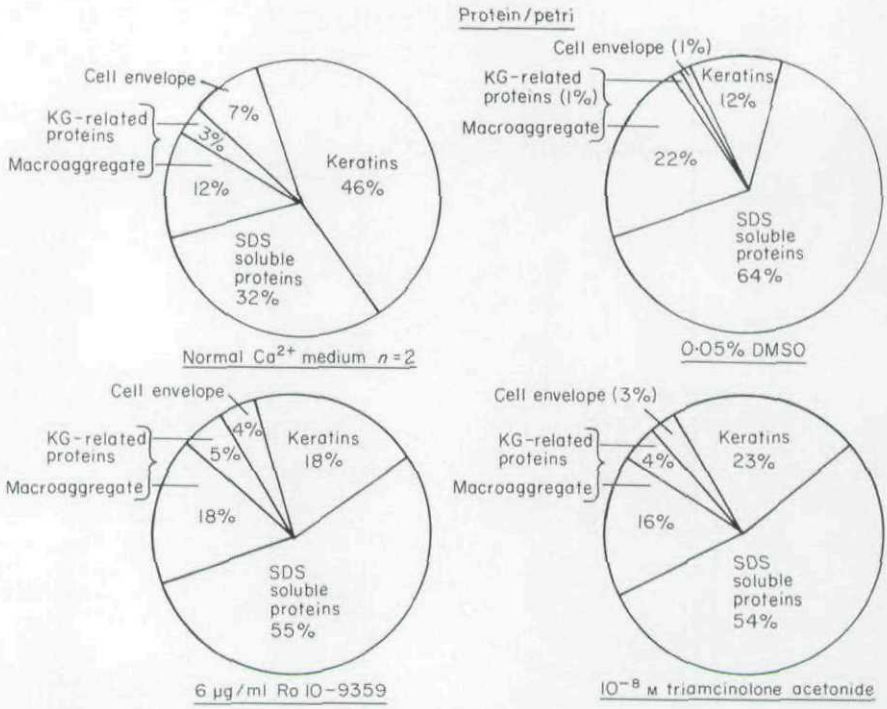

FIGURE 7. Percentage of specific epidermal proteins in low-calcium regulated keratinocytes grown in etretinate (Ro 10-9359) and triamcinolone acetonide.

keratinocytes; thus the retinoids caused a partial reversal of the low-calcium effect. This inhibition of proliferation by retinoids is also seen in normal-calcium grown keratinocytes (Marcelo & Madison, 1984). Triamcinolone acetonide also partially inhibited the low-calcium induced hyperproliferation, although the effect was short lived. Growth of the LCR keratinocytes in RO (18%) and in TA (23%) for 9 days approximately doubled the amount of keratins in the LCR keratinocytes (12%) (Fig. 7). Less SDS-soluble viable cell protein was synthesized by the etretinate (55%) and the triamcinolone (54%) treated cultures when compared with the LCR keratinocytes (64%). Increased amounts of cell envelope protein were observed in the keratinocytes grown in the two drugs, while the amounts of KG-related proteins were unchanged.

SUMMARY AND CONCLUSIONS

The growth of keratinocytes in low-calcium medium induced hyperproliferation and greatly reduced the amount of keratin polypeptides and cell envelope proteins in these cells, while KG-related proteins were slightly increased. Two retinoids and triamcinolone acetonide partially reversed the low-calcium effect, probably through non-calcium transport mechanisms since it took 8–10 days for the effect to occur and 0·05% DMSO, which facilitates the intracellular movement of extracellular substances, had no effect on the LCR keratinocyte culture.

We conclude from this study that calcium-dependent processes can regulate keratin

polypeptide and cell envelope synthesis and processing. Interestingly, KG-related protein synthesis was not affected, suggesting that the various differentiation pathways can be separately controlled. Since no obvious processing or synthesis of the keratinocyte proteins occurred 24 h after the switch, we propose that the morphological events, and possibly the inhibition of proliferation, are occurring via the activation of calcium-dependent proteins. One such protein is skin calcium-binding protein (Saurat *et al.*, 1981); another is calmodulin (Cheung, 1982) which has been reported to be elevated in a hyperproliferative skin disorder (van de Kerkhof & van Erp, 1983; Fairley, Hogen & Marcelo, 1983) and whose synthesis is induced in low-calcium regulated keratinocytes during the 24 h switching period (Fairley *et al.*, 1983). It is possible that the LCR keratinocyte is an *in vitro* model for hyperproliferative skin diseases, a hypothesis currently being tested in our laboratories.

ACKNOWLEDGMENT

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DISCUSSION SUMMARY

Four points were put under discussion by Drs J.McGuire, B.Shroot, D.A.Chambers, M.Faure and J.H.Saurat.

(1) Relationship between keratin synthesis and calcium in the medium: no qualitative changes were visible.

(2) Effect of retinoids: over a long time period the retinoid will decrease. Delipidized sera was not used.

(3) Calmodulin: calmodulin blockers were cytotoxic. Taking Ca^{2+} away or adding cAMP to the culture had the same effect: hyperproliferation. It is not possible to correlate cAMP with calmodulin.

(4) Intracellular calcium: it was not known from where the Ca^{2+} was coming after activation of the cells. It is possible that fluxes are increased in both directions with the switch. Two steps in this calcium activation: a fast initial step changing the morphology and a hyperproliferation with differentiation of the cultures.