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# Keratinocyte differentiation induced by calcium, phorbol ester or interferon-γ elicits distinct changes in the retinoid signalling pathways.

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***Abbreviations****:* CRABPII= cellular RA-binding protein II; ddRA= 3,4-didehydroretinoic acid; DMSO= dimethyl sulfoxide; IFNγ= interferon-γ; PBS = phosphate-buffered saline; PCR = polymerase chain reaction; PMA= phorbol 12-myristate 13-acetate; RA=all-*trans* retinoic acid; RAR= retinoic acid receptors; ROH= retinol; RXR= retinoid X receptor; TGm1= transglutaminase-1.

## Abstract

**Background:** Retinoids influence keratinocyte proliferation and differentiation via binding to nuclear retinoic acid receptors (RARα, -γ) and retinoid X receptor α (RXRα). The effect of keratinocyte differentiation on expression of nuclear retinoid receptors and on the conversion of retinol into retinoic acid has not been examined earlier in depth.

**Objectives:** Our aim was to examine the expression of retinoid receptors and a retinoid-regulated gene *CRABPII*, as well as the metabolism of exogenous [$^3$H]retinol in cultured human keratinocytes induced to differentiate by exposure to either calcium, phorbol 12-myristate 13-acetate (PMA), or interferon-γ (IFNγ).

**Methods:** Normal human keratinocytes were cultured and exposed to differentiation-inducing agents. The mRNA and protein expression of retinoid receptors were examined using real-time PCR and western blot. [$^3$H]Retinol uptake and metabolism was monitored by HPLC with on-line radioactivity detection.

**Results:** In calcium-exposed cells, increased expression of RARγ and RXRα, enhanced metabolism of [$^3$H]retinol to 3,4-didehydro-RA (ddRA), and an induction of CRABPII mRNA and protein was noted. In contrast, treatment with PMA and IFNγ reduced the RARγ and RXRα protein expression (preventable by

the proteasome inhibitor MG132), increased the accumulation of [$^3$H]RA and/or [$^3$H]ddRA in the cells, and changed the CRABPII transcription.

**Conclusions:** Retinoid signalling is profoundly altered upon differentiation of keratinocytes and the effects depend on how cellular differentiation is initiated.

## Introduction

Retinoids have profound influence on normal keratinocytes both *in vitro* and *in vivo*, the best known effect probably being the inhibition of cellular differentiation. The effects of natural vitamin A are mediated by its active metabolite all-*trans*-retinoic acid (RA) [1] and possibly also 3,4-didehydroretinoic acid (ddRA), a substance found exclusively in human keratinocytes and with similar properties to RA [2, 3]. The conversion of retinol (ROH) to RA and ddRA in cultured human keratinocytes is well established [2, 4], and this process is probably linked to the state of differentiation, with production of RA and ddRA occurring primarily in differentiated cells [2, 5].

RA exerts most of its biological effects by binding to and activating nuclear RA receptors (RARα, -β and -γ). A second class of retinoid receptors, RXRs (RXRα, -β and -γ), functions as heterodimerization partner for RARs (for review see[1]). These RAR-RXR heterodimers binds to RA-response elements (RAREs) in the

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*Abbreviations:* PCR, polymerase chain reaction; RA, retinoic acid; ROH, retinol; RAR, retinoic acid receptor; RXR, retinoid X receptor; PMA, phorbol 12-myristate 13-acetate; IFNγ, interferon-γ; CRABPII, cellular retinoic acid-binding protein II; TGase1, transglutaminase 1; PKC, protein kinase C; DMSO, dimethyl sulfoxide; PDTC, pyrrolidine dithiocarbamate.

promoter region of specific genes (for review see [1]), which leads to altered transcription of the adjacent gene. One such gene encodes for cellular RA-binding protein II (CRABPII). The expression of CRABPII is readily inducible by RA and has been used as a marker for retinoid activity in keratinocytes [6, 7]. Several of the retinoid receptors known so far have been recognized in human epidermal keratinocytes [8-11]. In falling order of abundance, the major forms expressed are RXRα, RARγ and RARα [11], thus making RARγ/RXRα the predominant heterodimer. According to some studies, these receptors are expressed mainly by cells in the upper (differentiated) cell layers of normal epidermis [9, 10] suggesting that they are involved in the process of terminal differentiation. Recent studies also indicate that unliganded RARs are important for the formation of lipid-containing lamellar granules in terminally differentiated keratinocytes [12, 13].

Several lines of evidence suggest that a perturbed retinoid signalling in keratinocytes may result in an aberrant phenotype. First, targeted ablation of the retinoid receptor expression will suppress epidermal maturation and barrier function *in vivo* [14, 15] and keratinocyte differentiation *in vitro* [16]. Second, UV irradiation, the major cause of skin cancer and photo-ageing, results in photodecomposition of endogenous epidermal vitamin A [17] as well as reduced amounts of RAR/RXR in keratinocytes *in vivo* [18] and *in vitro* [19, 20]. Third,

squamous cell carcinomas frequently display reduced RARγ expression [21, 22], and the RAR and RXR levels are progressively suppressed during malignant transformation of human epidermis [10]. Fourth, studies of psoriatic epidermis, characterized by abnormal differentiation and hyperproliferation, have disclosed reduced mRNA expression of RARα and RXRα [23] and increased expression of CRABPII [24, 25] that fails to respond to RA treatment [25]. Nonetheless, RA-related drugs (i.e. synthetic retinoids) are successfully used in the treatment of psoriasis and many other disorders where abnormal epidermal differentiation and aberrant retinoid signalling have been incriminated [26].

Retinoids also interfere with signalling pathways other than RAR/RXR, e.g., the activator protein-1 (AP-1) (for review see [1] ). AP-1 has been put forward as a key transcriptional regulator of genes encoding several keratinocyte differentiation proteins, e.g. keratin 1, transglutaminase 1 (Tgm1), loricrin and involucrin (see [27] and references therein), all of which are repressed by RA [28, 29]. In support of this theory, the DNA-binding activity of AP-1 was recently found to increase in cultured keratinocytes induced to differentiate by elevated extracellular calcium [30]. But whether or not retinoids affect the AP-1 pathway is still unknown.

Taken together, retinoid signalling appears crucial for epidermal differentiation under both physiological and pathological conditions. The two major check

points of this system are (i) the rate of formation of active receptor ligands from ROH, and (ii) the expression level of the nuclear receptors. In the present report, we study the metabolism of [$^3$H]ROH to RA and ddRA, the transcriptional activation of *CRABPII*, and the expression of retinoid receptors in relation to keratinocyte differentiation induced by calcium, interferon-γ (IFNγ) or phorbol 12-myristate 13-acetate (PMA). It is well-known that keratinocytes cultured under high calcium conditions will stratify and express physiologic differentiation markers, e.g. transglutaminase 1 and cornified cell envelope precursors [31]. The proinflammatory agents, PMA and IFNγ, are also powerful inducers of keratinocyte differentiation *in vitro*, although their mechanisms of action are different [32-34]. PMA is an activator of the protein kinase C (PKC) – AP-1 signalling pathway and as such a potent inducer of skin tumours, whereas IFNγ elicits a gene response resulting in skin inflammation [35]. The aim of the study was, by using these stimuli and co-addition of various synthetic retinoids and inhibitors of AP-1 activity, to try and correlate the differentiation process to changes in the retinoid signalling pathways of human keratinocytes.

## Material and methods

*Cell cultures*

Human foreskin epidermal keratinocytes were obtained from Cascade Biologics, Inc. (Portland, OR) and cultured in EpiLife serum-free keratinocyte growth medium (Cascade Biologics, Inc.) with a calcium concentration of 0.06 mM. All incubations were performed with proliferating keratinocytes (approximately 70% confluency) at passage 4–6. For calcium switch experiments, duplicate plates were exposed to 1.5 mM $CaCl_2$ (added from a 1 M stock solution) for different periods of time prior to harvest. In a separate experiment PMA (Sigma-Aldrich, Sweden AB, Stockholm, Sweden) or recombinant human IFNγ (Promega, Madison, WI 53711 USA) was added to keratinocyte cultures for different periods of time (duplicates for each treatment and time) in final concentrations of 100 nM and 200 U/ml, respectively). PMA was used from a dimethyl sulfoxide (DMSO) stock solution and final vehicle concentration did not exceed 0.04%. IFNγ was dissolved in PBS containing bovine serum albumin (0.1 mg/ml). The proteasome inhibitor MG132 (Sigma Aldrich) (in DMSO; final concentration 200 nM) was used in combination with PMA or IFNγ for 16 h. Nuclear cell extracts were prepared as previously described and the protein concentration was determined [25]. Total cell protein extracts were prepared by adding RIPA buffer containing protease inhibitors to cell cultures, transferring the lysates to microcentrifuge

tubes and keeping them on ice (+4$^0$C) for 30 min. The extracts were centrifuged at 15,000×g for 20 min and the supernatant used as total protein extract. Protein concentration was determined by Bio-Rad protein assay. Plates to be used for RNA preparation and real-time PCR were washed twice in PBS and stored at –70°C.

*Metabolism of [$^3$H]ROH and [$^3$H]RA in cultured keratinocytes*

Uptake and metabolism of [$^3$H]ROH in keratinocytes was examined as previously described [4]. In brief, cells were seeded and cultured for 3 or 6 days before the addition of calcium chloride (1.5 mM), PMA (100 nM), or IFNγ (200 U/ml). After 24 h [$^3$H]ROH (1.5 µCi) in 5 µl ethanol/dish was added to duplicate or triplicate cultures for 24 h before harvest. Subsequently, samples were subjected to alkaline hydrolysis and hexane extraction before and after acidification. The organic phases were collected, evaporated to dryness and injected onto an ODS column (150×3 mm i.d.) and eluted with acetonitrile/water/acetic acid (80:20:0.05) at a flow rate of 0.8 ml/min. Retinoids were quantified and related to protein contents of the samples as determined by the biuret technique.

*RNA extraction and reversed transcription (RT)*

Total RNA was isolated from keratinocytes and transcribed into cDNA as previously described [23]. In short, cells were homogenized in Trizol (Invitrogen, Carlsbad, CA) and total RNA was isolated. First-strand cDNA was synthesized from 3 μg of total RNA in a 30-μl reaction mixture using oligo-$d(T)_{15}$ as primer and M-MLV reverse transcriptase (Invitrogen). The cDNA was diluted with 30 μl $dH_2O$ to obtain a concentration of 50 ng cDNA/μl.

*Real-time polymerase chain reaction (PCR)*

The PCR reaction mixture included 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5.0 mM $MgCl_2$, 200 μM of each dNTP, and 1.25 U AmpliTaq Gold DNA polymerase (Applied Biosystems, Stockholm, Sweden). Primers and TaqMan™ fluorogenic probes, the latter labeled with a 5'-reporter (FAM) and a 3'-quencher (TAMRA), were added to a final concentration of 0.4 μM and 0.2 μM, respectively. Primer sequences for RARα, RARγ, RXRα, cyclophilin, and CRABPII have been published previously [23, 25]. Primers and probes for *TGM1* were 5'-TCTTCAAGAACCCCCTTCCC-3' (forward primer), 5'-TCTGTAACCCAGAGCCTTCGA-3' (reverse primer), and 5'-TCACCCTCACCAATGTCGTCTTCCG-3' (probe). Total PCR volume was 25 μl including 1 μl of the RT reaction, which equals 50 ng of total RNA. Duplicate reaction tubes were set up for each sample. The tubes were placed in an ABI

Prism 7700 System programmed as follows: 15 s at 94ºC, 30 s at 60ºC for 40 cycles. Simultaneous amplification of known amounts of a PCR-product generated a standard curve for comparison.

All mRNA values were related to the amount of the reference gene *PPIA*, encoding cyclophilin A, to correct for variations in RNA levels and efficiency in cDNA synthesis.

*Immunoblotting*

Keratinocyte nuclear or total protein extracts were fractionated by SDS-PAGE (12% or 15% acrylamide) and electrotransferred onto Hybond P filters (GE Healthcare Biosciences AB, Uppsala, Sweden). All subsequent washings and dilutions of blocking reagents and antibodies were done in 0.1% (w/v) Tween-20 in PBS (PBS-T). Blocking of non-specific binding was performed in 5% non-fat dried milk overnight at 4°C. The filters were immunoprobed for 1 h with polyclonal subtype-specific retinoid receptor antibodies [RARα (C-20), RARβ (C-19), RARγ (C-19), and RXRα (D-20); Santa Cruz Biotechnologies, Inc., Santa Cruz, CA] or monoclonal anti-CRABPII (5CRA3B3; a generous gift from Dr Rochette-Egly, IGBMC, Strasbourg, France) [36]. All antibodies were diluted 1:8,000. After extensive washing, the filters were incubated for 1 h with horseradish peroxidase-labelled anti-rabbit or anti-mouse IgG diluted 1:50,000 or 1:10,000,

respectively. The complexes were visualized by enhanced chemiluminescence (ECL) on ECL Hyperfilm using ECL Plus™ Western blot detection reagents (all from GE Healthcare Biosciences AB). The films were scanned and equal square areas covering the bands were quantified using ImageQuant software (Molecular Dynamics).

*Statistical analysis*

Data are expressed as mean+/-SD. Data were analyzed for statistical significance with the GraphPad PRISM® software (version 5.02; GraphPad Inc., San Diego, CA) using One-Way ANOVA and Dunnett´s multiple comparison test. A P-value $\leq 0.05$ was considered as the level of significance ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$).

## Results

*Effects of calcium, PMA and IFNγ on keratinocyte morphology*

Stimulation with either calcium, PMA or IFNγ induced similar changes in the culture morphology at the early time-points (up to 6 h); the cells increased in size and became flattened with "diffuse" cell membranes (results not shown). After this time period, the cells assumed distinct morphologies depending on the substance used. Calcium-differentiated cells stratified and assembled into multilayer sheets with subsequent sloughing. PMA, on the other hand, induced a dendritic, melanocyte-like morphology after 16–24 h with detachment of differentiated cells. IFNγ-exposed keratinocytes became even more enlarged with spacious cytoplasm and a "pancake-like" appearance but the cells did not detach to the same extent.

*TGM1 and retinoid receptor expression during onset of differentiation*

By studying time-related changes in the mRNA expression of transglutaminase 1 gene (*TGM1*) and the mRNA and protein levels of the retinoid receptors in cells exposed to high calcium, PMA or IFNγ for up to 48 h, two major observations were made.

*(i) Elevated TGM1 and RARγ expression after calcium-induced differentiation*. Under high (1.5 mM) calcium conditions, we noticed a gradual accumulation of *TGM1*

transcripts up to 8 times higher than in low calcium controls after 48 h (Fig. 1a), thus confirming the onset of differentiation. Parallel to this, there was a slow increase in the mRNA expression of *RARγ* (270% of the control value at 48 h) (Fig. 1c), but no consistent changes in *RARα* and *RXRα* expression (Fig. 1b and d).

The corresponding effects on the nuclear receptor protein levels differed slightly (Fig. 2). RARα was transiently increased by calcium (about 1.4-fold after 16 h) but then fell below control level. Similarly, RARγ and RXRα protein levels gradually increased up to 16 h, then remained at 53 and 31%, respectively, above the low calcium controls at 48 h.

*(ii) Reduced retinoid receptor expression after PMA and IFNγ-induced differentiation which can be overcome by proteasome inhibitors.* While PMA and IFNγ caused an induction of *TGM1* mRNA that was stronger and more rapid than after calcium (Fig. 1a), the retinoid receptors were all reduced by PMA and IFNγ in the early phase of differentiation (Fig. 1b–d). In PMA-exposed keratinocytes, we observed a maximal 60-75% reduction of the retinoid receptors transcripts after 5h, whereas normal *RARγ* and *RXRα* mRNA levels were resumed after 16 h (Fig. 1c and d). The changes in mRNA expression of retinoid receptors were detected already at PMA-concentrations at 1 nM (Fig. 1S). At the protein level, we also detected a reduction of all receptors after exposure to PMA, with RARγ and RXRα levels of only 20% of control value after 24 h (Fig. 3a and c).

IFNγ-exposure rapidly reduced all receptor transcripts to about 30% of control value in early phase (Fig. 1b–d). Only RARα transcripts were normalized after 48 h (Fig. 1b). At the protein level RARγ and RXRα started to fall at 16 h, and after 48 h, a near complete loss of RARγ and RXRα was apparent (Fig. 3b and d). In contrast, RARα protein was not significantly altered during IFNγ-exposure.

In order to examine if the effect of PMA and IFNγ on retinoid receptor protein levels involved proteasomal degradation, we co-incubated keratinocytes with an inhibitor (MG132) of the ubiquitin-proteasome pathway previously implicated in the degradation of retinoid receptors in human keratinocytes [19]. Addition of MG132 for 16 h prevented the loss of RARγ and RXRα proteins induced by PMA and IFNγ alone (Fig. 3e), thus confirming involvement of proteasome degradation in that process.

*[$^3$H]ROH metabolism differs in proliferating versus differentiating keratinocytes*

The metabolism of [$^3$H]ROH to RA, ddROH and ddRA in pre-confluent keratinocytes exposed to calcium, PMA, or IFNγ for 48 h was examined by adding the tracer during the final 24 h. Under high-calcium conditions, [$^3$H]ROH and [$^3$H]ddROH accumulated in the cells to a higher extent than under low-calcium conditions (Fig. 4). Furthermore, formation of [$^3$H]ddRA was readily detected only in differentiating cells, whereas [$^3$H]RA levels were close to the

detection limit in both proliferating and differentiating cells. In contrast, PMA-exposed keratinocytes contained 3 to 7-fold increased amounts of [$^3$H]ROH and [$^3$H]RA compared to untreated cells, and unchanged amounts of the [$^3$H]3,4-didehydro retinoids (Fig. 4). Keratinocytes exposed to IFNγ did not differ from controls in their content of either [$^3$H]ROH or [$^3$H]ddROH, but produced about twice as much of the corresponding retinoic acids (Fig. 4). The effect of IFN-γ was concentration-dependent with changes in [$^3$H]ROH-metabolism detected above 8 U/ml (Fig. 2S).

*CRABPII is induced by calcium and PMA but not by IFNγ*

Calcium-induced differentiation resulted in a time-dependent accumulation of CRABPII transcripts (Fig. 5a). After 24 h, calcium-exposed keratinocytes displayed a 16-fold induction of CRABPII transcripts compared to pre-confluent keratinocytes grown in a low-calcium culture medium. This level appeared to be maximal since it remained constant at 48 h. In total protein extracts, a massive induction of the CRABPII protein was found after 24 h in high-calcium keratinocytes compared to the low level in control cells (Fig. 5a, inset), thus paralleling the effects on CRABPII mRNA.

CRABPII mRNA expression in keratinocytes exposed to PMA and IFNγ is shown (Fig. 5b and 5c, respectively). Most striking was the potent (27-fold) up-

regulation of CRABPII by PMA between 3 and 6 h (Fig. 5b), followed by a gradual decrease. As a result, the CRABPII protein was prominently increased in keratinocytes treated with PMA for 24 h (Fig. 5b, inset). In contrast, IFNγ-exposure caused a reduction of CRABPII, which started at 3 h and persisted throughout the experiment (Fig. 5c). This was reflected at the protein level, where a marked reduction of the CRABPII protein was apparent after 24 h (Fig. 5c, inset).

## Discussion

In this report, we further explore the link between retinoid signalling and the process of squamous differentiation using cultured keratinocytes as an *in vitro* model. We show that differentiation leads to changes in retinoid receptor levels, retinol metabolism, and expression of a retinoid-inducible gene, *CRABPII*. However, the magnitude and direction of all these changes depend on which agent - high calcium, PMA or IFNγ - is used to initiate differentiation. Although all three agents had similar effects in the early phases of differentiation, more prolonged exposure induced characteristic morphologic changes for each agent. The most conspicuous effect was seen for PMA, which induced a dendritic, melanocyte-like appearance of the keratinocytes after 16-24 h. Likewise, the induction pattern of the differentiation-marker *TGM1* differed between the three agents. Calcium-exposure led to a gradual accumulation of *TGM1* transcripts, whereas PMA caused a stronger induction peaking at 16 h, probably reflecting an activation and subsequent desensitization of the PKC/AP-1 signalling pathway. In contrast, IFNγ caused a slow but strong induction of *TGM1* mRNA that peaked after 24 h, in line with growth inhibition preceding the induction of keratinocyte differentiation markers [33].

*Calcium-induced differentiation results in increased retinoid signalling*

The mRNA expression of *RARγ* and protein expression of RARγ and RXRα were increased in keratinocytes induced to differentiate by calcium. On the other hand, *RARα* was reduced after prolonged exposure to calcium. This suggests that RARγ is linked to a differentiated phenotype and RARα to a proliferating one. Our results corroborate previous findings of an increased *RARγ*/*RARα* mRNA ratio in confluent human keratinocytes [37] and induction of *RXRα* in calcium-treated and post-confluent keratinocytes [38]. By extrapolating to the *in vivo* situation, RARα/RXRα heterodimers are likely to predominate in the basal layers of the epidermis while RARγ/RXRα should predominate in the suprabasal layers suggesting separate functions for RARα/γ and RXRα in keratinocyte differentiation [39]. In view of this, our results could have the following implication: The responsiveness of a tissue or cell to a hormonal factor such as RA is largely dependent on the abundance of the corresponding nuclear receptor. Speculatively, a certain subset of genes could be activated at a specific stage of differentiation depending not only on the amount of receptors for signalling but also, more specifically, on available isoforms at that time point. This is supported by a report showing that RA affects different genes in keratinocytes deficient in RARα or RARγ [39]. Further studies are needed to elucidate whether a corresponding mechanism exists also in human normal keratinocytes.

The second level of control in retinoid signalling is the formation of active ligands. Although we could not detect any increased RA formation in calcium-differentiated cells, the cells do exhibit increased ddRA formation (see Fig. 4). So far, no unique role for ddRA has been demonstrated in mammalian cells but since its presence appears to be restricted to epidermal cells, we hypothesize that ddRA has as yet unidentified functions in the onset and maintenance of keratinocyte differentiation, thus distinguishing it from RA. Taken together, the increased amounts of RARγ/RXRα receptors and RAR-ligands in calcium-treated versus control cells suggest that differentiated keratinocytes are the primary target cells for retinoid activity in the epidermis. At first sight, this may appear difficult to reconcile with the inhibition of keratinocyte differentiation of RA and ddRA [3], but physiological and pharmacological concentrations of retinoids generate different effects [1].

*Increased CRABPII expression following PMA exposure is partially RAR-mediated and ceases upon receptor degradation*

Using various detection techniques, previous reports have demonstrated the ability of PMA to down-regulate retinoid receptors in keratinocytes *in vivo* [40] and *in vitro* [38, 41]. We expand this knowledge by showing that exposure to PMA results in a time-dependent decrease in retinoid receptor protein and

transcript levels, suggesting that the effect of PMA on retinoid receptor levels is translational or post-translational. This notion is further strengthened by our observations that the protein levels of RARγ and RXRα were more heavily affected than those of RARα, whereas at the mRNA level the situation was reversed. In fact, after 24 h, the mRNA expression of *RARγ* and *RXRα* increased five- and two-fold, respectively, but this was not reflected at the protein level. Similarly, Segaert *et al*. reported PMA-induced loss of RXRα protein but only a transient reduction of mRNA levels in keratinocytes [38].

A recent study has implicated the ubiquitin-proteasome pathway as the major degradation mechanism for retinoid receptors in human keratinocytes [19]. PMA induces proteasomal degradation of many proteins (e.g., STAT3 via PKC-activation [42]). We show here that inhibition of proteasomal protein degradation will counteract PMA-induced loss of retinoid receptor proteins. Thus, it is likely that PMA has a dual effect and induces some (post)transcriptional events as well as activating the ubiquitin-proteasomal degradation pathway. The negative effect of PMA primarily on RARγ and RXRα protein expression is in line with the proposal that highly potent transcriptional inducers are the preferred targets for proteasomal degradation *in vivo* [43]. Whether the changes involve a transcriptional cross-talk between members of the AP-1 complex (i.e. c-fos and

jun) and retinoid receptors is presently unknown but the AP-1 signalling is known *per se* to be altered during keratinocyte differentiation [44].

Interestingly, CRABPII was also rapidly and massively induced following PMA exposure, which could be explained by the substantially increased production of the active ligands RA and ddRA from ROH and thus potentiated retinoid activity. Although the *CRABPII* mRNA induction do not exactly parallel the increase in RA levels, the method used to detect [$^3$H]RA might be too insensitive to reveal RA-levels in the low nM range usually sufficient to activate RARs [3]. The importance of RARs for CRABPII induction is supported by the observed normalization of CRABPII expression after 16–24 h, coinciding with the sharp drop in RARγ and RXRα proteins which together indicate insufficient receptor levels for continued retinoid signalling.

The changes in CRABPII expression could lead to RA-induced alterations of trans-activation of other nuclear receptors. A recent study showed that RA can be delivered to the nucleus by CRABPII or FABP5 [45]. Thus, depending on the cellular CRABPII/FABP5 ratio, RA functions through RAR and is a pro-apoptotic agent in cells with high CRABPII/FABP5 ratio, but it signals through PPARbeta/delta and promotes survival in cells that highly express FABP5. Opposing effects of RA on cell growth thus can emanate from alternate activation of two different nuclear receptors [45].

*Does IFNγ impair retinoid signalling due to reduced RAR-levels?*

Exposure to IFNγ resulted in a severe loss of retinoid receptor proteins and, to a lesser extent, also of mRNA for the receptors. Although the reduction of mRNA precedes the loss of RARγ and RXRα protein, increased protein degradation appears to be involved since a proteasome inhibitor could potently restore the receptor proteins levels. Previous studies have reported that IFNγ increases proteasomal activity [46] but IFNγ also induced a differentiation process resembling an apoptotic pathway [47], which might result in the general loss of proteins. As with PMA, RARα was much less affected than RARγ/RXRα which incriminates proteasomal degradation of the heterodimer due to RA-induced activation [43].

Like calcium and PMA, IFNγ increased the levels of RAR-ligands in exposed keratinocytes. Nevertheless, CRABPII was consistently down-regulated in these cells. IFNγ was previously shown to inhibit RA-mediated induction of CRABPII in breast cancer cells [48]. Although the authors did not explore the mechanism of inhibition, their results are very similar to ours. Furthermore, in a recent study we reported failure of exogenous RA to induce CRABPII in psoriatic skin lesions [25], where IFNγ is markedly increased due to the inflammatory process [35]. One possible explanation is that IFNγ suppresses the retinoid receptor levels. The

near-total loss of RARγ and RXRα proteins after 48 h observed by us would certainly affect the ability of the cells to uphold normal retinoid signalling. However, since the reduction was not significant until after 16 h, while CRABPII transcription was suppressed even at the earliest time-point at 3 h, this explanation does not seem sufficient. Other possible mechanisms include inactivation of receptor transactivation due to reduced heterodimerization by phosphorylation by protein kinases [49] or titration of common co-activators (e.g., p300/CBP) [50, 51].

Our present results contribute to the understanding of epidermal differentiation by showing differentiation-dependent alterations in the retinoid-signalling machinery. Interestingly the expression and degradation of RARγ and RXRα appear to play pivotal roles in this process.

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**REFERENCES**

[1] Fisher G J, Voorhees J J: Molecular mechanisms of retinoid actions in skin. FASEB J. 10:1002-1013, 1996.

[2] Randolph R K, Simon M: Characterization of retinol metabolism in cultured human epidermal keratinocytes. J. Biol. Chem. 268:9198-9205, 1993.

[3] Törmä H, Asselineau D, Andersson E, Martin B, Reiniche P, Chambon P, Shroot B, Darmon M, Vahlquist A: Biologic activities of retinoic acid and 3,4-didehydroretinoic acid in human keratinocytes are similar and correlate with receptor affinities and transactivation properties. J. Invest. Dermatol. 102:49-54, 1994.

[4] Törmä H, Rollman O, Vahlquist A: The vitamin A metabolism and expression of retinoid-binding proteins differ in HaCaT cells and normal human keratinocytes. Arch. Dermatol. Res. 291:339-345, 1999.

[5] Chatellard-Gruaz D, Randolph R K, Hagens G, Saurat J-H, Siegenthaler G: Differentiation of human epidermal keratinocytes is accompanied by increased expression of CRABP-II and increased cellular concentration of retinoic acids: retention of newly synthesized retinoic acids by CRABP-II. J. Lipid Res. 39:1421-1429, 1998.

[6] Zouboulis C C, Seltmann H, Sass J O, Ruhl R, Plum C, Hettmannsperger U, Blume-Peytavi U, Nau H, Orfanos C E: Retinoid signaling by all-*trans*

retinoic acid and all-*trans* retinoyl-β-D-glucuronide is attenuated by simultaneous exposure of human keratinocytes to retinol. J. Invest. Dermatol. 112:157-164, 1999.

[7] Pavez Loriè E, Cools M, Borgers M, Wouters L, Shroot B, Hagforsen E, Törmä H, Vahlquist A: Topical treatment with CYP26 inhibitor talarozole (R115866) dose dependently alters the expression of retinoid-regulated genes in normal human epidermis. Br. J. Dermatol. 160:26-36, 2009.

[8] Törmä H, Rollman O, Vahlquist A: Detection of mRNA transcripts for retinoic acid, vitamin $D_3$, and thyroid hormone (c-*erb*-A) nuclear receptors in human skin using reverse transcription and polymerase chain reaction. Acta Derm. Venereol. 73:102-107, 1993.

[9] Reichrath J, Mittmann M, Kamradt J, Muller S M: Expression of retinoid-X receptors (–α,–β,–γ) and retinoic acid receptors (–α,–β,–γ) in normal human skin: an immunohistological evaluation. Histochem. J. 29:127-133, 1997.

[10] Xu X C, Wong W Y, Goldberg L, Baer S C, Wolf J E, Ramsdell W M, Alberts D S, Lippman S M, Lotan R: Progressive decreases in nuclear retinoid receptors during skin squamous carcinogenesis. Cancer Res. 61:4306-4310., 2001.

[11] Fisher G, Talwar H, Xiao J-H, Datta S, Reddy A, Gaub M-P, Rochette-Egly C, Chambon P, Voorhees J: Immunological identification and functional

quantitation of retinoic acid and retinoid X receptor proteins in human skin. J. Biol. Chem. 269:20629-20635, 1994.

[12] Calleja C, Messaddeq N, Chapellier B, *et al.*: Genetic and pharmacological evidence that a retinoic acid cannot be the RXR-activating ligand in mouse epidermis keratinocytes. Genes Dev. 20:1525-1538, 2006.

[13] Ghyselinck N, Chambon P: Animal Models for Retinoid Receptor Research; Implications for Epidermal Homeostasis, Skin Barrier Function, Wound Healing and Atopic Dermatitis. In Retinoids and Carotenoids in Dermatology, Edited by Vahlquist A, Duvic M. Informa Healthcare USA Inc., New York, 2007, pp. 27-54.

[14] Imakado S, Bickenbach J R, Bundman D S, *et al.*: Targeting expression of a dominant-negative retinoic acid receptor mutant in the epidermis of transgenic mice results in loss of barrier function. Genes Dev. 9:317-329, 1995.

[15] Li M, Chiba H, Warot X, Messaddeq N, Gerard C, Chambon P, Metzger D: RXRα ablation in skin keratinocytes results in alopecia and epidermal alterations. Development 128:675-688, 2001.

[16] Aneskievich B J, Fuchs E: The A/B domain of truncated retinoic acid receptors can block differentiation and promote features of malignancy. J. Cell Sci. 108:195-205, 1995.

[17] Berne B, Nilsson M, Vahlquist A: UV irradiation and cutaneous vitamin A: An experimental study in rabbit and human skin. J. Invest. Dermatol. 83:401-404, 1984.

[18] Wang Z, Boudjelal M, Kang S, Voorhees J J, Fisher G J: Ultraviolet irradiation of human skin causes functional vitamin A deficiency, preventable by all-trans retinoic acid pre-treatment. Nat. Med. 5:418-422, 1999.

[19] Boudjelal M, Wang Z, Voorhees J J, Fisher G J: Ubiquitin/proteasome pathway regulates levels of retinoic acid receptor γ and retinoid X receptor α in human keratinocytes. Cancer Res. 60:2247-2252, 2000.

[20] Andersson E, Rosdahl I, Törmä H, Vahlquist A: Differential effects of UV irradiation on nuclear retinoid receptor levels in cultured keratinocytes and melanocytes. Exp. Dermatol. 12:563-571, 2003.

[21] Darwiche N, Celli G, Tennenbaum T, Glick A B, Yuspa S H, De Luca L M: Mouse skin tumor progression results in differential expression of retinoic acid and retinoid X receptors. Cancer Res. 55:2774-2782, 1995.

[22] Finzi E, Blake M J, Celano P, Skouge J, Diwan R: Cellular localization of retinoic acid receptor-gamma expression in normal and neoplastic skin. Am. J. Pathol. 140:1463-1471, 1992.

[23] Törmä H, Karlsson T, Michaëlsson G, Rollman O, Vahlquist A: Decreased mRNA levels of retinoic acid receptor α, retinoid X receptor α and thyroid hormone receptor α in lesional psoriatic skin. Acta Derm. Venereol. 80:4-9, 2000.

[24] Siegenthaler G, Tomatis I, Didierjean L, Jaconi S, Saurat J-H: Overexpression of cellular retinoic acid binding protein type II (CRABP-II) and down-regulation of CRABP-I in psoriatic skin. Dermatology 185:251-256, 1992.

[25] Karlsson T, Virtanen M, Sirsjö A, Rollman O, Vahlquist A, Törmä H: Topical retinoic acid alters the expression of cellular retinoic acid-binding protein-I and cellular retinoic acid-binding protein-II in non-lesional but not lesional psoriatic skin. Exp. Dermatol. 11:143-152, 2002.

[26] Peck G L, DiGiovanna J J: Synthetic retinoids in dermatology. In The retinoids : biology, chemistry, and medicine, Edited by Sporn M B, Roberts A B, Goodman D S. Raven Press, New York, 1994, pp. 631-658.

[27] Eckert R L, Crish J F, Banks E B, Welter J F: The epidermis: genes on - genes off. J. Invest. Dermatol. 109:501-509, 1997.

[28] Brown L J, Geesin J C, Rothnagel J A, Roop D R, Gordon J S: Retinoic acid suppression of loricrin expression in reconstituted human skin cultured at the liquid-air interface. J. Invest. Dermatol. 102:886-890, 1994.

[29] Asselineau D, Bernard B A, Bailly C, Darmon M: Retinoic acid improves epidermal morphogenesis. Dev. Biol. 133:322-335, 1989.

[30] Johansen C, Iversen L, Ryborg A, Kragballe K: 1α,25-dihydroxyvitamin D3 induced differentiation of cultured human keratinocytes is accompanied by a PKC-independent regulation of AP-1 DNA binding activity. J. Invest. Dermatol. 114:1174-1179, 2000.

[31] Bikle D D, Gee E, Pillai S: Regulation of keratinocyte growth, differentiation, and vitamin-D metabolism by analogs of 1,25-dihydroxyvitamin D. J. Invest. Dermatol. 101:713-718, 1993.

[32] Dlugosz A A, Yuspa S H: Coordinate changes in gene expression which mark the spinous to granular cell transition in epidermis are regulated by protein kinase C. J. Cell Biol. 120:217-225, 1993.

[33] Saunders N A, Jetten A M: Control of growth regulatory and differentiation-specific genes in human epidermal keratinocytes by interferon-γ. Antagonism by retinoic acid and transforming growth factor $\beta_1$. J. Biol. Chem. 269:2016-2022, 1994.

[34] Younus J, Gilchrest B A: Modulation of mRNA levels during human keratinocyte differentiation. J. Cell. Physiol. 152:232-239, 1992.

[35] Liu Y, Krueger J G, Bowcock A M: Psoriasis: genetic associations and immune system changes. Genes Immun. 8:1-12, 2007.

[36] Gaub M-P, Lutz Y, Ghyselinck N B, Scheuer I, Pfister V, Chambon P, Rochette-Egly C: Nuclear detection of cellular retinoic acid binding proteins I and II with new antibodies. J. Histochem. Cytochem. 46:1103-1111, 1998.

[37] Vollberg T M, Nervi C, George M D, Fujimoto W, Krust A, Jetten A M: Retinoic acid receptors as regulators of human epidermal keratinocyte differentiation. Mol. Endocrinol. 6:667-676, 1992.

[38] Segaert S, Garmyn M, Degreef H, Bouillon R: Suppression of vitamin D receptor and induction of retinoid X receptor a expression during squamous differentiation of cultured keratinocytes. J. Invest. Dermatol. 114:494-501, 2000.

[39] Goyette P, Feng Chen C, Wang W, Seguin F, Lohnes D: Characterization of retinoic acid receptor-deficient keratinocytes. J. Biol. Chem. 275:16497-16505., 2000.

[40] Kumar R, Shoemaker A R, Verma A K: Retinoic acid nuclear receptors and tumor promotion: decreased expression of retinoic acid nuclear receptors by the tumor promoter 12-O-tetradecanoylphorbol-13-acetate. Carcinogenesis 15:701-705, 1994.

[41] Darwiche N, Scita G, Jones C, Rutberg S, Greenwald E, Tennenbaum T, Collins S J, De Luca L M, Yuspa S H: Loss of retinoic acid receptors in mouse skin and skin tumors is associated with activation of the *ras*$^{Ha}$

oncogene and high risk for premalignant progression. Cancer Res. 56:4942-4949, 1996.

[42] Malek R L, Halvorsen S W: Ciliary neurotrophic factor and phorbol ester each decrease selected STAT3 pools in neuroblastoma cells by proteasome-dependent mechanisms. Cytokine 11:192-199, 1999.

[43] Molinari E, Gilman M, Natesan S: Proteasome-mediated degradation of transcriptional activators correlates with activation domain potency in vivo. EMBO J. 18:6439-6447, 1999.

[44] Welter J F, Eckert R L: Differential expression of the fos and jun family members c-fos, fosB, Fra-1, Fra-2, c-jun, junB and junD during human epidermal keratinocyte differentiation. Oncogene 11:2681-2687., 1995.

[45] Schug T T, Berry D C, Shaw N S, Travis S N, Noy N: Opposing effects of retinoic acid on cell growth result from alternate activation of two different nuclear receptors. Cell 129:723-733, 2007.

[46] Aki M, Shimbara N, Takashina M, *et al.*: Interferon-γ induces different subunit organizations and functional diversity of proteasomes. J. Biochem. (Tokyo). 115:257-269, 1994.

[47] Brysk M M, Selvanayagam P, Arany I, Brysk H, Tyring S K, Rajaraman S: Induction of apoptotic nuclei by interferon-gamma and by predesquamin in cultured keratinocytes. J. Interferon Cytokine Res. 15:1029-1035, 1995.

[48] Widschwendter M, Daxenbichler G, Dapunt O, Marth C: Effects of retinoic acid and γ-interferon on expression of retinoic acid receptor and cellular retinoic acid-binding protein in breast cancer cells. Cancer Res. 55:2135-2139, 1995.

[49] Delmotte M-H, Tahayato A, Formstecher P, Lefebvre P: Serine 157, a retinoic acid receptorα residue phosphorylated by protein kinase C in vitro, is involved in RXR:RARα heterodimerization and transcriptional activity. J. Biol. Chem. 274:38225-38231, 1999.

[50] Horvai A E, Xu L, Korzus E, Brard G, Kalafus D, Mullen T M, Rose D W, Rosenfeld M G, Glass C K: Nuclear integration of JAK/STAT and Ras/AP-1 signaling by CBP and p300. Proc. Natl. Acad. Sci. U. S. A. 94:1074-1079, 1997.

[51] Ghosh A K, Yuan W, Mori Y, Chen S, Varga J: Antagonistic regulation of type I collagen gene expression by interferon-γ and transforming growth factor-β. Integration at the level of p300/CBP transcriptional coactivators. J. Biol. Chem. 276:11041-11048, 2001.

**FIGURE LEGENDS**

**Figure 1. The mRNA expression of *TGM1, RARα, RARγ,* and *RXRα* in cultured keratinocytes is differently affected by adding calcium, IFNγ, or PMA to induce differentiation.**

Seventy percent confluent keratinocytes were exposed to calcium (▲) (1.5 mM), phorbol myristate acetate (□) (100 nM), or interferon-γ (●) (200 U/ml) for the indicated time. Total mRNA was extracted and used for real-time quantitative PCR (see *Material & Methods* for details) to determine transcript levels of a) *TGM1*, b) *RARα*, c) *RARγ* and d) *RXRα*. Results are given as percent of the corresponding low calcium/vehicle-treated control (mean ± SD). All values are normalized to the mRNA expression of *PPIA* (a reference gene encoding cyclophilin A). Statistical analysis were performed by ANOVA followed by Bonferroni's (calcium-exposed cells) or Dunnett's (phorbol myristate acetate and IFNγ exposed cells) multiple comparison tests; *=$p<0.05$, **=$p<0.01$ and ***=$p<0.001$.

**Figure 2. Increased protein expression of RARγ and RXRα but not RARα following calcium-induced keratinocyte differentiation.**

Pre-confluent cultured keratinocytes were established in low calcium medium (C) and exposed to low (0.06 mM; L) or high (1.5 mM; H) extracellular calcium

conditions for the indicated time to achieve terminal differentiation. Nuclear proteins were extracted as described under *Material & Methods*. (*a*) Nuclear protein extracts (10 μg) were separated by 12% SDS-PAGE and analyzed by immunoblotting using specific RAR/RXR antibodies diluted 1:8,000. The positions of pre-stained molecular weight standards (New England Biolabs) are indicated in kilodaltons. (*b*) The inset table shows a semi-quantification of the retinoid receptor protein stained bands in *(a)*. Data are expressed as percent of corresponding densitometric values in low calcium controls.

**Figure 3. PMA and IFNγ reduce RARγ and RXRα protein expression in cultured keratinocytes by proteasomal degradation.**

Pre-confluent keratinocytes were established in low-calcium medium (C) and exposed to vehicle (-), PMA (P; 100 nM), or IFNγ (I; 200 U/ml) for the indicated time. Nuclear protein extracts (10 μg) were separated by 12% SDS-PAGE and analyzed by immunoblotting using specific RAR and RXR antibodies diluted 1:8,000. The position of a pre-stained molecular weight standard (New England Biolabs) is indicated. The blotting results after exposure to a) PMA and b) IFNγ are shown. A semi-quantification of the receptor levels is shown in (*c*) and *(d)*. Especially RARγ and RXRα are heavily down-regulated by both agents. e) When keratinocytes were cultured in the presence of 200 nM MG132 (+) for 16 h the

lowering effects of PMA and IFNγ on the receptor protein levels could be abolished.

**Figure 4. [$^3$H]Retinol uptake and metabolism differ in keratinocytes subjected to calcium, PMA, or IFNγ to induce differentiation.**

Keratinocytes were established in low extracellular calcium (0.09 mM) and when still pre-confluent exposed to 1.5 mM extracellular calcium (open bars), 100 nM PMA (striped bars) or 200 U/ml IFNγ (check bars) for 48 h. [$^3$H]ROH was added for the final 24 hours before harvest. Extraction and HPLC-analysis was performed as described in *Material & Methods*. The graphs show protein-normalized amounts of [$^3$H]ROH, [$^3$H]RA, [$^3$H]ddROH and [$^3$H]ddRA. To facilitate for the reader, interrupted lines and the arrows under the x-axis indicate the metabolic pathway of the tracer. The bars represent mean ± SEM.

**Figure 5. CRABPII expression is up-regulated by calcium (in the late phase) and PMA (in the early phase), but consistently down-regulated by IFNγ-exposure.**

Real-time quantitative PCR was performed to determine the amount of *CRABPII* mRNA in keratinocytes exposed to high calcium *(a)*, PMA *(b)*, or IFNγ *(c)* for the indicated time. Results are given as mean ± SD (n=2).

Inserts show the CRABPII protein expression in keratinocytes exposed to high calcium, PMA, or IFNγ for 24 h. Total protein extracts (5 μg) were separated by 15% SDS-PAGE and analyzed by immunoblotting using a monoclonal CRABPII antibody diluted 1:8,000. Statistical analysis were performed by ANOVA and Bonferroni's multiple comparison test; *=p<0.05, **=p<0.01 and ***=p<0.001.

**Figure 1S. mRNA expression of *PPIA* in cultured keratinocytes exposed to calcium, PMA and IFN-γ.**

Seventy percent confluent keratinocytes were exposed a) 0.09 or 1.5 mM $CaCl_2$ or b) vehicle, 100 nM phorbol myristate acetate or 200 U/ml IFN-γ. Total mRNA was extracted and *PPIA* expression determined by real-time quantitative PCR (see *Material & Methods* for details). Results are given as attomole/25 ng cDNA (mean±SD; n=2).

**Figure 2S. The effect of PMA on mRNA expression of *RARα, RARγ* and *RXRα* in cultured keratinocytes is concentration-dependent.**

Seventy percent confluent keratinocytes were exposed phorbol myristate acetate (1-100 nM) for 24 h. Total mRNA was extracted and used for real-time quantitative PCR (see *Material & Methods* for details) to determine transcript

levels of *RARα, RARγ* and *RXRα*. Results are given as percent of the corresponding low calcium control (mean ± SD). All values are normalized to the mRNA expression of *PPIA*, encoding the reference gene cyclophilin A.

**Figure 3S. The effect of IFNγ on [$^3$H]retinol uptake and metabolism is concentration-dependent.**

Keratinocytes were established in low extracellular calcium (0.09 mM) and when still pre-confluent exposed to 8-800 U/ml IFNγ for 48 h. [$^3$H]ROH was added for the final 24 hours before harvest. Extraction and HPLC-analysis was performed as described in *Material & Methods*. The values of [$^3$H]ROH, [$^3$H]RA, [$^3$H]ddROH and [$^3$H]ddRA are normalized to the protein content and expressed in relation to sham-exposed control cells. The bars represent mean ± SD.

**FIGURES**

Figure 1

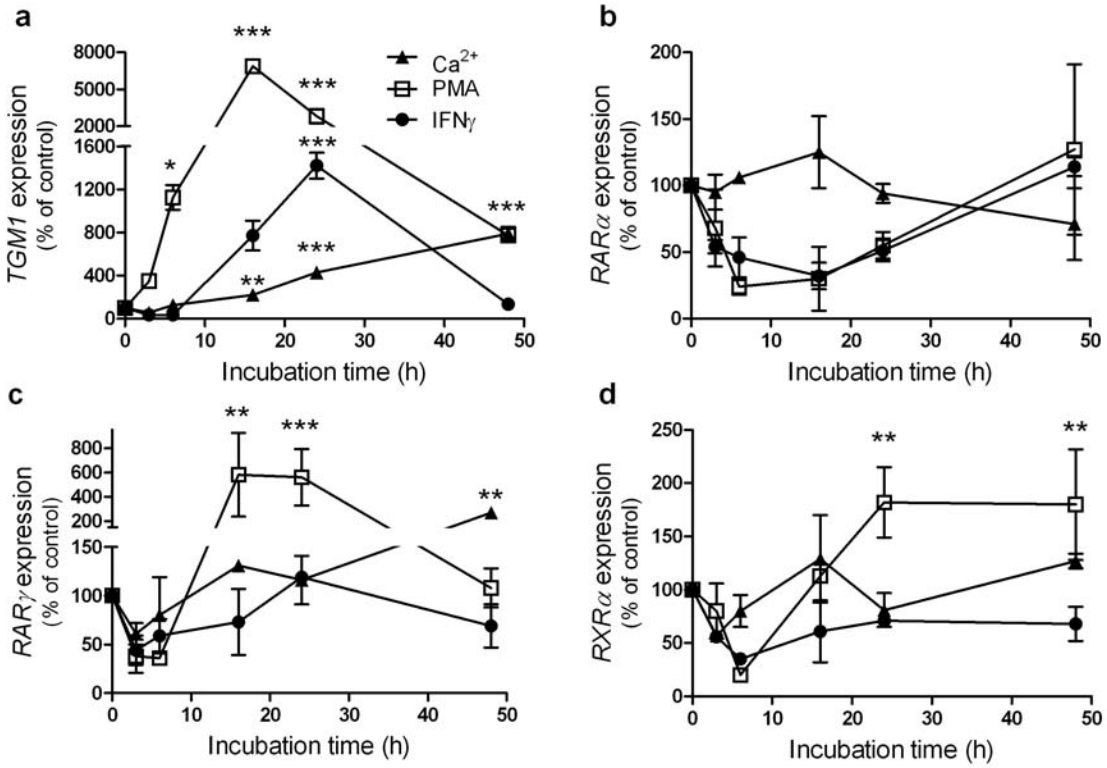
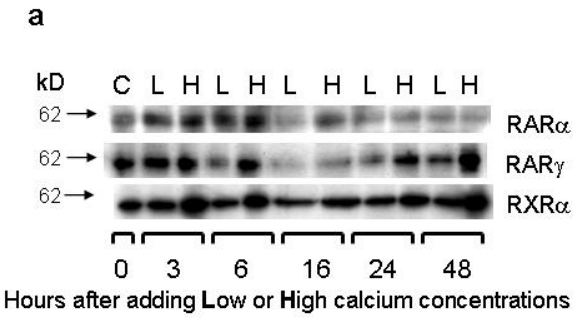



Figure 2




b

| % of control values | | | | | | |
|---|---|---|---|---|---|---|
| 0 h | 3 h | 6 h | 16 h | 24 h | 48 h | |
| 100 | 117 | 118 | 243 | 81 | 76 | RARα |
| 100 | 80 | 148 | 172 | 168 | 153 | RARγ |
| 100 | 155 | 137 | 158 | 147 | 131 | RXRα |

Figure 3

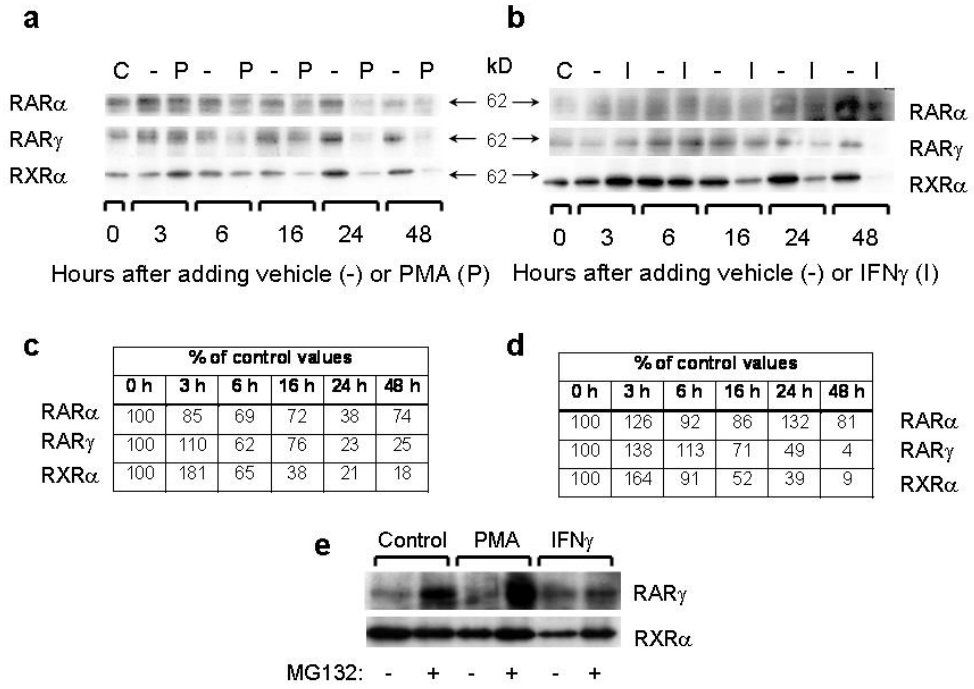



Figure 4

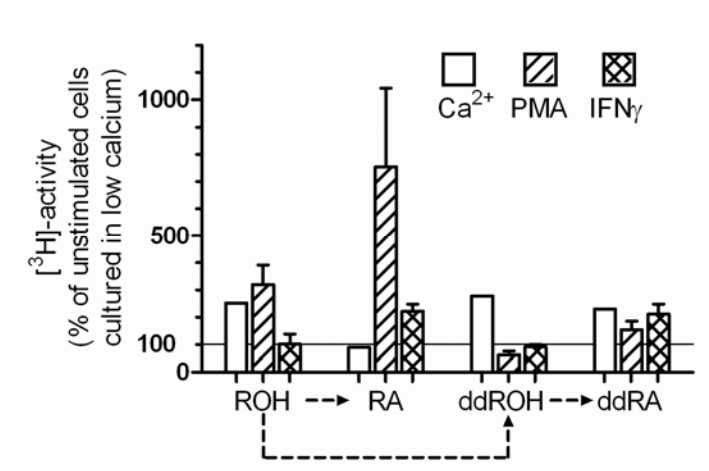


Figure 5

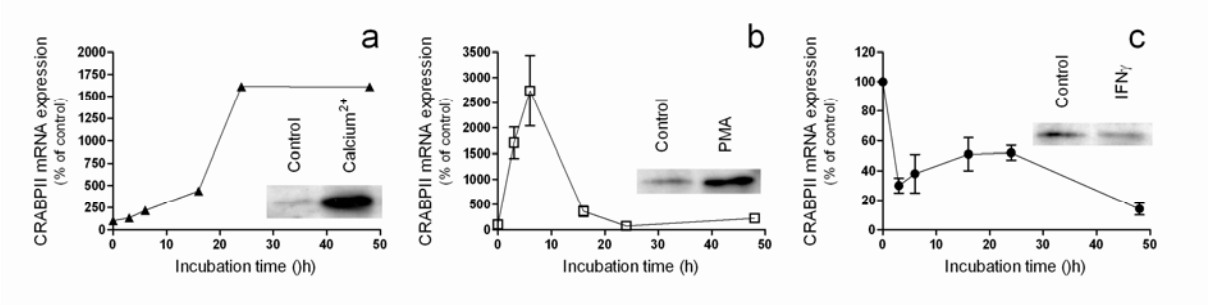



Figure 1S

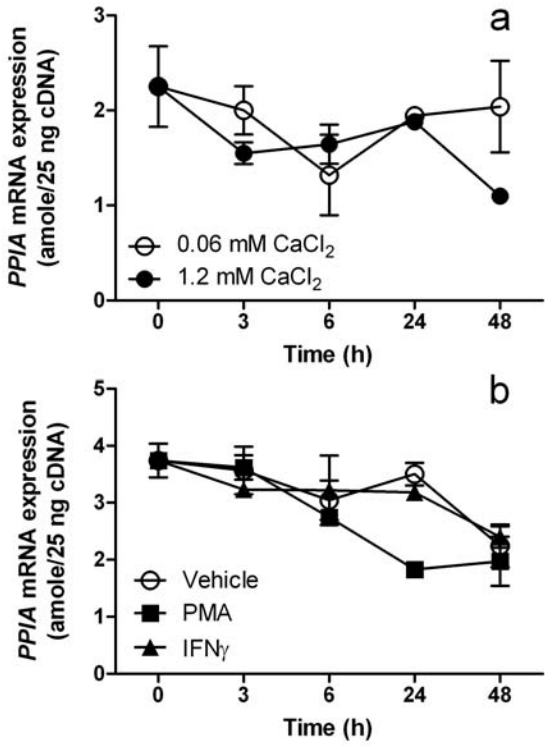


Figure 2S

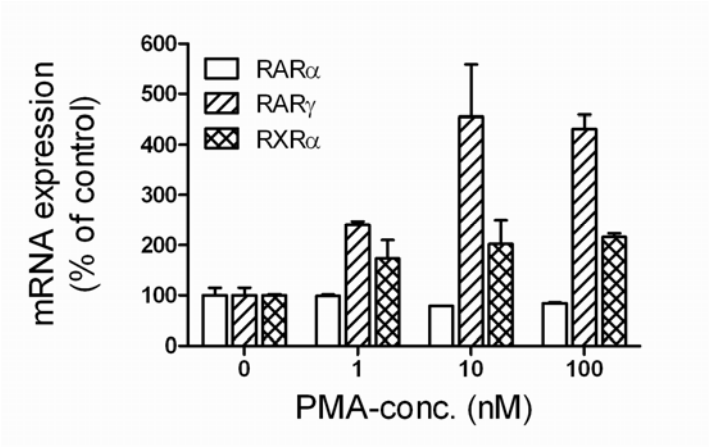



Figure 3S