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PROTEIN KINASE C ISOFORMS PLAY DIFFERENTIAL ROLES IN THE REGULATION OF HUMAN SEBOCYTE BIOLOGY

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To: Professor Paul. R. Bergstresser
Editor
Journal of Investigative Dermatology
Editorial Office

December 19, 2011

Dear Professor Bergstresser:

Please find enclosed our revised manuscript (**JID-2011-0692R1**) which we now re-submit for publication to the *Journal of Investigative Dermatology*.

During the revision, we have corrected the manuscript according to the very constructive comments and suggestions of both the Editors and the Reviewers. In the below pages you find our point-by-point answers to the Reviewers. As requested, all changes are underlined in the revised text.

In addition, to show novel experimental data, we have supplemented the manuscript with 3 Supplementary Figures and the corresponding Supplementary data file (these files are also uploaded at the JID website).

We hope that these revisions, along with our point-by-point answers to comments of the Reviewers, are sufficient for considering our work for publication in your distinguished journal.

Yours sincerely,

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Response to the Reviewers

Response to Reviewer 1.

In the paper titled " PROTEIN KINASE C ISOFORMS PLAY DIFFERENTIAL ROLES IN THE REGULATION OF HUMAN SEBOCYTE BIOLOGY" the authors demonstrate differential and antagonistic roles of PKC isoforms in a human sebocyte cell line. The authors show that PKCdelta and PKCzeta are important regulators of lipid synthesis and apoptosis.

Major Revisions:

1. What was the cell viability in Fig 3 after 48 and 72hrs of AA treatment? In figure 5 you demonstrate that there is a 50% decrease in cell viability about 48hrs of AA treatment. So which cells are you measuring PKC levels in if 50% of the cells are dead? Are you just measuring the cells in the population that express less PKCdelta?

--> Very important note, thank you for it. In Figure 5.b., we indeed show that the mitochondrial membrane potential (and early sign of the induction of apoptosis) was significantly decreased following 48-hr AA-treatment. However, we also present that necrosis was not initiated (no change in the intensity of the necrosis marker SYTOX Green) suggesting the lack of sudden changes in the viable cell number.

However, in order to exclude the “PKCdelta-specific selection” mentioned by Reviewer 1, we have performed additional experiments and investigated the effects of prolonged (1-3 days) AA-treatments on the living cell number, using CyQUANT cellular proliferation assay. As now shown in the new Supplementary Figure S2, we found that despite of the induced apoptosis there were no significant changes in the cell count compared to the appropriate vehicle control groups. These results indicate that (i) the early apoptotic processes shown in Figure 5.b. may reduce the cell count only in a longer time scale; (ii) most (if not all) cells were alive at the time when the Western blots were performed; and (iii) hence Western blots show the PKC expression of the whole cell population.

To mention these, we now present in Result section, paragraph 10, new last 2 sentences. In addition, we attach Supplementary data with the corresponding Materials and Methods section and Figure legends.

2. In figures 1, 3, and 4 there are no western blot loading controls such as beta-actin, alpha-tubulin, or GAPDH. I would like to see one of these loading controls included with the isoform western blots.

--> Thank you for the note. Since the goal of the Western blots shown in Figure 1. was only the demonstration of the presence of the PKC isoforms (and not the presentation of the potential changes in their expression level), loading controls could not provide further support for the message of the figure.

However, in the cases of Figures 3. and 4., Reviewer 2 is absolutely right; therefore the following changes were made:

- For Figure 3b, loading controls are now shown in the new Supplementary Figure S1a (with this modification, the originally shown loading control was omitted from Figure 3b). This in now mentioned as the last sentence in paragraph 6 of the Results section. In addition, a new Figure legend is shown in the Supplementary data.
- For Figure 4a, loading controls are now shown in the new Supplementary Figure S1b. This in now mentioned as the last sentence in paragraph 7 of the Results section. In addition, a new Figure legend is shown in the Supplementary data.
- In the modified Figure 4b, we now show the loading controls for those PKCs which were changed upon siRNA transfection; i.e., loading control of cPKCalpha for the upper panel and loading control of aPKCzeta for the lower panel.

Minor Revisions:

3. I would like to see a paragraph in the discussion talking about the prostaglandin pathway in response to the addition of AA. I would like to see discussed whether a COX inhibitor would also decrease the levels of apoptosis in these cells treated with AA.

--> Very good suggestion. We now present a new paragraph 4 to the Discussion section. Please note that 3 additional references are now shown in the Reference list.

4. I would also like to see the paragraph of speculation about applications in human disease removed. Cell culture is notoriously poor at predicting human responses to drugs. If you would like to make this type of speculation I think it would be appropriate that there be some animal testing that demonstrates PKC inhibitors as good drugs to inhibit sebocyte diseases or cancers.

--> As requested, the last paragraph was removed from the Discussion section.

Response to Reviewer 2

Geczy and colleagues investigated the role of PKC family in sebocytes by using human immortalized SZ95 sebocyte cell culture. They examined the expression of PKC family proteins in SZ95 cells with Q-PCR, western and immunostaining, then tested the effects of PKC chemical inhibiters on SZ95 cell lipid synthesis on basal, PMA- and arachidonic acid-induced lipid synthesis and cell survival. Furthermore, isoform-specific knocking down of PKCalpha, delta, zeta was performed to study isoform-specific effects on SZ95 cell lipid synthesis and cell death. They observed that PKCalpha and PKCdelta are required for PMA-induced lipid synthesis, while PKCdelta also involved arachidonic acid-induced lipid synthesis and apoptosis. In contrast, PKCzeta inhibits the basal lipid synthesis and apoptosis. The authors conclude that PKC family play isoform-specific roles in regulation of human sebaceous gland function.

Comments:

The report provides interesting information on the role of PKC in human sebocytes. The manuscript is clearly written with sound data. The study implicates the importance of PKC family proteins in sebocyte biology and provides a potential target pathway for the clinical management of inflammatory SG disorders.

--> Thank you very much for the positive evaluation of our work.

Here are a few specific concerns.

1. The error bars and p value shown in Figure 2-5 appear based on readings from 4 different wells from the same experiment. For transfected cells (transfection is a stochastic process), the results from 3-4 addition independent experiments should be included and analyzed for mean and p value.

--> Very good point. Now we present the mean and p values of the average of 3 independent experiments. In addition, the corresponding figure legends are also changed.

2. The authors need to discuss what the downstream effectors of PKCs in this setting are. Inflammatory cytokines and lipid synthesis pathway enzymes could be examined by Q-PCR as the group has done for other study before. PKCzeta inhibits the fatty acid synthesis in Hela cells through phosphorylates mtF0F1-ATPase β-subunit. Does PKZCzeta play a similar role in the SZ95 cells?

--> Very interesting point. Actually, the Reviewer is “knocking on opened doors”, since we have already started the investigation of how inflammatory cytokines and other agents involved in innate (and/or adaptive) immunity are involved in the action of AA and how the PKC isoforms may influence their expressions. As a new Supplementary Figure S3, we present our preliminary Q-PCR data indicating that 24-hr AA treatment of SZ95 sebocytes markedly alter the expression of certain interleukins, pro-inflammatory agents, anti-microbial peptides, etc. In addition, we

present a new Discussion paragraph (only in the Supplementary Data section) which discuss these findings.

However, according to our opinion, the second part of the note of Reviewer 2 requires a more detailed answer. Using a human hepatoma cell line (HepG2) Guo et al. (J. Lipid Res . 2011. 52: 908–922.) have demonstrated that the aPKCζ-driven phosphorylation of the β-subunit of the mtF0F1-ATP-ase suppresses the activity of this enzyme, which in turn leads to the inhibition of the mitochondrial acyl-CoA:glycerol-sn-3-phosphate acyltransferase 1 (mtGPAT1) enzyme in the cells. Since cellular GPAT enzymes control the first and rate-limiting step of TAG synthesis, the activation of this pathway (by anthocyanin, a naturally occurring plant pigment) has been described to play an important role in the reduction of the de novo lipid biosynthesis in liver cells, thereby reducing high-glucose-induced hepatic steatosis. Therefore it would seem plausible that, by activating the same signaling pathway, aPKCζ would have similar inhibitory effects on sebocyte lipid synthesis as well.

However the following factors needs to be taken into consideration before one decides to dissect the same pathway in sebocytes. As mentioned above, the first committed step of TAG biosynthesis is controlled by GPAT enzymes. To date four mammalian GPAT isoforms have been identified, two of which (GPAT1, GPAT2) localizes to the mitochondrial outer membrane, whereas GPAT3 and GPAT4 reside in the ER. GPAT1 expression levels are highest in liver and adipose tissue, where it plays a key role in determining the fate of FFA molecules, by channeling them toward glycerolipid synthesis (and thereby away from β-oxidation). Thus GPAT1 has a pivotal regulatory role in whole-body lipid metabolism, facilitating pathways that enable energy storage. Apart from these two tissues, mitochondrial GPAT activity is also significant in muscle, brain, lung and kidney. However, other tissues possess only a 10% GPAT activity attributable to the mitochondrial GPAT (GPAT1&2) isoforms (Takeuchi and Reue, Am J Physiol Endocrinol Metab 296: E1195–E1209, 2009). Although the exact GPAT expression pattern of sebocytes is yet to be described, the most important feature of sebocyte lipid synthesis is that it takes place mainly in the smooth ER of the cells, and therefore does not involve mitochondrial enzymes. Throughout their differentiation sebocytes increase their intracellular sER content, thereby enabling the production and accumulation of an increased level of intracellular lipids. (This process probably does not have any significant effect on whole-body lipid metabolism, unlike the ones described in hepatocytes and adipocytes) Ultimately, the cells disintegrate (at the end stage of their maturation) and release their lipid content into the center of the gland (holocrine secretion).

The complex mixture of lipids secreted by the sebaceous gland is called sebum. It consists of a wide variety of lipid molecules. TAG molecules constitute only 30-50% of this lipid mixture, the rest of which consists mainly of FFA, wax esters, squalene and cholesterol (Picardo et al, Dermato-Endocrinology 1:2, 68-71;2009; Smith and Thiboutot, J. Lipid Res. 2008. 49: 271–281.). Since our methods, that analyze the level of sebocyte lipid production, do not distinguish between these individual components, we cannot determine whether or not the changes in overall lipid content of sebocytes are attributable to changes in TAG levels within the cells.

Taken together, we would predict that the mechanism by which aPKCζ activation inhibits mitochondrial GPAT1-driven TAG synthesis in hepatocytes probably does not have a major impact on the sebaceous lipid production of sER-related enzymatic pathways in sebocytes. Therefore, in our future studies we would like to direct our investigative efforts towards other potential downstream mechanisms.

PROTEIN KINASE C ISOFORMS PLAY DIFFERENTIAL ROLES IN THE REGULATION OF HUMAN SEBOCYTE BIOLOGY

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Running title: PKC isoforms regulate human sebocyte biology

Key words: Protein kinase C, isoenzymes, human SZ95 sebocytes, lipid synthesis, apoptosis

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ABSTRACT

Protein kinase C (PKC) isoforms play crucial roles in cutaneous signaling. Interestingly, we lack information about their involvement in human sebaceous gland biology. Therefore, in the current study, we investigated the functions of the PKC system in human immortalized SZ95 sebocytes. Using molecular biological approaches, imaging, and functional assays, we report that SZ95 sebocytes express the conventional cPKCα; the novel nPKCδ, ε, and η; and the atypical aPKCζ. Activation of the PKC system by phorbol 12-myristate 13-acetate (PMA) stimulated lipid synthesis (a hallmark of differentiation), and resulted in translocation and then down-regulation of cPKCα and nPKCδ. In good accord with these findings, the effect of PMA was effectively abrogated by inhibitors and siRNA-mediated "silencing" of cPKCα and nPKCδ. Of further importance, molecular or pharmacological inhibition of nPKCδ also prevented the lipogenic and apoptosis-promoting action of arachidonic acid. Finally, we also found that "knock-down" of the endogenous aPKCζ activity markedly increased basal lipid synthesis and apoptosis, suggesting its constitutive activity in suppressing these processes. Collectively, our findings strongly argue for that certain PKCs play pivotal, isoform-specific, differential, and antagonistic roles in the regulation of human sebaceous gland-derived sebocyte biology.

Key words: protein kinase C (PKC); isoenzymes; human sebaceous gland; human SZ95 sebocytes; lipid synthesis; apoptosis.

INTRODUCTION

Protein kinase C (PKC) comprises a family of serine/threonine kinases (Nishizuka, 1988, 1992) that play crucial roles in the regulation of various biological mechanisms including e.g. cell growth, differentiation, and survival. The PKC family includes the i) calcium- and diacylglycerol (DAG) or phorbol ester-dependent "conventional" isoforms (PKCα, βI, βII, and γ; cPKCs); ii) calcium-independent "novel" nPKCs (PKCδ, ε, η, and θ); and iii) calcium- and phorbol ester-independent "atypical" aPKCs (PKCζ and λ/ι). These isoenzymes possess a characteristic expression pattern in a given tissue and cell type, and differentially regulate numerous cellular processes in an isoenzyme specific fashion (Gould and Newton, 2008; Reyland, 2009; Newton, 2010; Rosse et al, 2010).

Emerging evidence suggest a pivotal role of the PKC system in cutaneous biology (Denning, 2004; Breitkreutz et al, 2007). Indeed, PKC activity has been shown to modulate the proliferation and differentiation of epidermal keratinocytes (Lee et al, 1998; Papp et al, 2003). In addition, isoenzyme-specific roles of PKCs expressed by keratinocytes have also been reported; i.e. cPKCα and nPKCδ were found to be key components in stimulating epidermal differentiation whereas PKCε was shown to promote skin tumor development (Lee et al, 1997; Li et al, 1999a; Jansen et al, 2001; Neill et al, 2003; Papp et al, 2004).

Importantly, PKCs are also involved in controlling hair growth and cycling. Several PKC isoforms (e.g. cPKCα and β, nPKCδ and ε) were shown to be expressed in various compartments (e.g. outer root sheath keratinocytes, dermal papilla) of the hair follicle (Eicheler et al, 1997; Li et al, 2003; Neill et al, 2003). Moreover, levels of certain

isoforms appeared to alter with the hair cycle; for example, expression of cPKCα showed good correlation with increasing hair growth (anagen) in various mouse models (Li et al, 1999b, 2003). Of further importance, modification of PKC activity by either phorbol esters or synthetic inhibitors resulted in profound alterations in human hair shaft elongation as well as dermal papilla fibroblast growth and survival (Harmon et al, 1995; Ferraris et al, 1997).

Intriguingly, we lack data on the functional role of the PKC system in the other component of the human pilosebaceous unit, i.e. the sebaceous gland and its sebocytes. Sebocytes, engaged in holocrine (sebum) secretion, are major site of hormone synthesis and metabolism in the human skin, and possess numerous ionotropic, metabotropic, and nuclear receptor-coupled pathways (Zouboulis et al, 2002; Zouboulis and Bohm, 2004; Alestas et al, 2006; Zhang et al, 2006; Dobrosi et al, 2008; Tóth et al, 2009, 2011) which may all function as potential regulators of PKCs. Hence sebocytes provide a highly instructive research tool for exploring PKC functions, which promises additional sets of information that may complement those obtainable with human hair follicle and with other extrafollicular cell types of the human skin.

Therefore, in the current study, we identified and investigated members of the PKC system in SZ95 sebocytes – a human SG-derived immortalized cell line which possesses striking functional similarities to those of primary human sebocytes (Zouboulis et al, 1999) – and dissected their isoform-specific role in the regulation of sebocyte biology.

RESULTS

Human SZ95 sebocytes possess a characteristic PKC isoform pattern

We first investigated the presence of PKC isoforms in SZ95 sebocytes. Using immunocytochemistry, Western blotting, and quantitative "real-time" PCR, we provide the first evidence that SZ95 cells express cPKCα; nPKCδ, ε, and η; and aPKCζ (**Figure 1a and b**). Importantly, none of the above techniques identified other members of the family (i.e. cPKCβI, βII, and γ; nPKCη and θ; aPKCλ/ι; and the unique PKCμ-PKD) (data not shown). In addition, Q-PCR also revealed that, among the above isoforms, cPKCα, nPKCδ, and aPKCζ exhibited the highest expressions whereas the levels of nPKCε and η were rather low (**Figure 1b**).

Pharmacological modulation of PKC activity alters lipid synthesis but does not influence cell viability of SZ95 sebocytes

One of the main hallmarks of sebocyte differentiation is the synthesis of various lipids, among which neutral lipids represent a major component (Rosenfield, 1989; Thody and Shuster, 1989; Doran et al, 1991, Zouboulis et al, 1998). Therefore, we investigated the effects of PKC-acting agents on the lipid content of cultured SZ95 sebocytes. Nile Red staining-based quantitative FLIPR measurement revealed that treatment with the general PKC activator phorbol 12-myristate 13-acetate (PMA) (up to 48 hrs) significantly stimulated lipid synthesis in a dose-dependent fashion (**Figure 2a**). This effect was equally inhibited by GF109203X (inhibitor of cPKCs and nPKCs), Gö6976 (inhibitor of cPKCs), and Rottlerin (inhibitor of nPKCδ) (**Figure 2b**). Of note, none of the inhibitors alone (i.e. when applied without PMA) affected basal lipid content of the cells (**Figure 2b**). Furthermore, none of the above protocols altered cell viability or induced cell death

of any form (apoptosis, necrosis); namely, the PKC activator or the inhibitors did not significantly modify the viable cell number (MTT-based colorimetric proliferation assay, **Figure 2c**), nor did they induce necrotic (SYTOX-Green accumulation assay) or apoptotic cell death (fluorimetric measurement of the decrease of mitochondrial membrane potential – $DilC_1(5)$ labeling) (**Figure 2d**).

The effect of PMA to stimulate lipid synthesis of SZ95 sebocytes is mediated by cPKCα and nPKCδ

The above results suggested the involvement of multiple PKC isoforms in the action of PMA. However, numerous reports indicate that the above inhibitors may often target other signaling molecules (Gschwendt et al, 1994; Alessi, 1997) which question their specificity and/or selectivity for the PKC isoforms. Therefore, in order to define the specific roles of the PKC isoenzymes that were expressed at the highest levels (i.e. cPKCα, nPKCδ, and aPKCζ) in mediating the lipid synthesis-stimulating effect of PMA, we have carried out a series of cellular imaging and molecular biology experiments.

Activation of a given PKC isoform is accompanied by the translocation of the molecule to another cellular compartment. Therefore, we first investigated the potential alteration in the subcellular localization of PKCs upon short-term (1 hr) PMA treatment. As revealed by immunostaining of PKCs followed by confocal microscopy analysis, 10 nM PMA induced the translocation of cPKCα and nPKCδ. As expected, being a PMA-insensitive isoform, the subcellular localization of aPKCζ was not changed upon the application of the phorbol ester (**Figure 3a**).

Prolonged activation of the PKC isoforms results in the cleavage of the molecule by proteases resulting in a decreased expression of the given PKC (down-regulation). Therefore, we also assessed the effect of long-term (up to 3 days) PMA administration on the levels of the PKCs. Consistent with the above confocal microscopy data, as revealed by Western blot analysis, the phorbol ester markedly down-regulated cPKCα and nPKCδ whereas the level of aPKCζ was not altered (**Figure 3b**). Loading controls are presented in **Supplementary Figure S1a**.

The involvement of cPKCα and nPKCδ in mediating the cellular effects of PMA was further assessed by siRNA technique. Western blot analysis demonstrated that the expressions of "targeted" PKC isoforms were significantly "knocked-down" by the specific siRNA probes at day 2 after transfection and remained suppressed on day 3 as well (**Figure 4a-c**). Specificity and selectivity of the procedure was demonstrated by the followings (i) siRNA-mediated silencing of a given isoforms did not affect the expression of the other two PKCs investigated (**Figure 4a and b**); (ii) scrambled siRNA probes had no effect on the expression of the PKC isoforms (**Figure 4a-c**). Additional loading controls are presented in **Supplementary Figure S1b**.

Silencing of cPKCα and nPKCδ did not modify the basal lipid synthesis (**Figure 4d**) and did not induce apoptosis/necrosis (**Figure 4e**) in SZ95 sebocytes. However, in good accord with the above data, "knock-down" of either of these isoforms significantly reduced the action of PMA to promote lipid synthesis (**Figure 4d**). Intriguingly, the siRNA-mediated silencing of aPKCζ dramatically increased the basal lipid synthesis of the sebocytes (**Figure 4d**) and, furthermore, suppressed cellular viability and induced

apoptosis (but not necrosis) (**Figure 4e**). Of further importance, elevation of basal lipid content was so marked in the aPKCζ knocked-down cells that PMA was unable to further stimulate lipid accumulation (**Figure 4d**).

Effects of arachidonic acid to stimulate lipid synthesis and induce apoptosis of SZ95 sebocytes are selectively mediated by nPKCδ

The above effects of PMA strikingly resembled the previously described findings with arachidonic acid (AA), one of the most effective inducers of lipid synthesis in SZ95 sebocytes (Wróbel et al, 2003; Alestas et al, 2006; Tóth et al, 2009). Therefore, we wished to dissect whether or not the PKC isoforms are also involved in mediating the cellular effect of AA. As expected, AA induced a marked lipid synthesis in SZ95 sebocytes (**Figure 5a**). This effect was significantly (yet only partially) abrogated by both GF109203X and Rottlerin but not by Gö6976 (**Figure 5a**) suggesting that cPKCα most probably does not participate in mediating the effect of AA. Furthermore, PMA was able to further increase the lipid synthesis in the AA-treated cells (**Figure 5a**).

Our previous studies have also shown that the effect of AA to augment lipid synthesis in SZ95 sebocytes was accompanied by the induction of sebocyte apoptosis (Wróbel et al, 2003; Tóth et al, 2009). Therefore, we investigated the involvement of the PKC system in the AA-induced apoptotic process. As expected, AA induced apoptosis (fluorimetric measurement of mitochondrial membrane potential) but not necrosis (SYTOX-Green accumulation assay) in SZ95 sebocytes (**Figure 5b**). This effect of AA was almost completely prevented by GF109203X and Rottlerin but not by Gö6976, similar to the

actions of these inhibitors on the AA-induced lipid synthesis. Finally, we found that the PKC activator PMA did not modify the effect of AA to promote apoptosis (**Figure 5b**).

Next, using the above cellular and molecular approaches, we further investigated the isoform-selective involvement of certain PKCs in the action of AA. Confocal microscopy analysis revealed that a short-term AA treatment (1 hr) of SZ95 sebocytes selectively translocated nPKCδ whereas it did not modify the subcellular localizations of cPKCα and aPKCζ (**Figure 3a**). Similarly, long-term (1-3 days) administration of AA selectively down-regulated nPKCδ whilst expressions of the other two isoforms were insignificantly changed (**Figure 3b**). In addition, in order to assess the effect of prolonged AA treatment on cell growth, proliferation was additionally measured for 3 days. As shown in **Supplementary Figure S2**, long-term application of AA did not significantly affect the growth rate suggesting that the selective down-regulation of nPKCδ by prolonged AA application was not due to the suppression in cell number or selective suppression of viabilitzy of those SZ95 sebocytes which may express nPKCδ at high levels.

Moreover, the effect of AA to stimulate lipid synthesis and induce apoptosis was significantly suppressed in those SZ95 sebocytes in which the expression of nPKCδ (but, notably, not that of cPKCα) was silenced by siRNA (**Figure 5c and d**). In aPKCζ-silenced sebocytes, AA was able to further stimulate the already highly elevated (basal) lipid synthesis; however, in these cells, the apoptosis was so marked (and hence the mitochondrial membrane potential was so reduced) that the effect of AA to induce further apoptosis was not measurable (**Figure 5c and d**). Nevertheless, these findings

collectively argue for the selective involvement of nPKCδ in mediating the cellular effects of AA.

DISCUSSION

In this study, we report that human sebaceous gland-derived SZ95 sebocytes possess a characteristic PKC isoform pattern (i.e. the highly expressed cPKCα, nPKCδ and aPKCζ; and the poorly expressed nPKCε and η) which is similar to that described on human epidermal keratinocytes (Lee et al, 1997, 1998; Papp et al, 2003). In addition, we provide the first evidence that the highly expressed PKC isoforms play central (yet differential and antagonistic) roles in regulating cellular growth, survival, and lipid synthesis of human sebocytes.

Specifically, we found that the activation of the PKC system by the phorbol ester PMA stimulated lipid synthesis. A series of pharmacological (using various inhibitors) and molecular biology (employing siRNA-mediated “silencing”) studies revealed that this action engaged two isoforms, i.e. cPKCα and nPKCδ. Since these isoenzymes were reported to stimulate differentiation of human epidermal keratinocytes (Lee et al, 1997; Li et al, 1999a; Papp et al, 2004); and, furthermore, since holocrine sebum production is one of the key hallmarks of sebocyte differentiation (Zouboulis et al, 1999, 2002; Alestas et al, 2006), we can conclude that cPKCα and nPKCδ play key roles in promoting differentiation of these cutaneous cell types.

Moreover, nPKCδ was also involved in selectively mediating the lipogenic action of AA, one of the most widely studied inducers of lipid synthesis in SZ95 sebocytes (Wróbel et al, 2003; Alestas et al, 2006; Dobrosi et al, 2008; Tóth et al, 2009), which was previously shown to stimulate skin PKC activity (Reynolds el al, 1993; Lo et al, 1994). Indeed, both the pharmacological and the molecular inhibition of nPKCδ suspended the lipogenic

effect of AA. Of further importance, in SZ95 sebocytes with "silenced" nPKCδ AA was unable to induce apoptosis which suggests the pro-apoptotic role of nPKCδ (in addition to its pro-lipogenic action). Apparently, cPKCα is not involved in mediating the actions of AA since i) the AA-induced lipid synthesis and apoptosis was unaltered on sebocytes with "silenced" cPKCα; and ii) the AA-elevated lipid synthesis (mediated by nPKCδ) was further increased by the co-administration of PMA which (besides stimulating nPKCδ) additionally activates cPKCα.

With respect to the potential link between the prostaglandin pathways and the action of AA, it should be noted that sebocytes were found to produce metabolites of both the lipoxigenase (LOX) and the cyclooxygenase (COX) pathways (Alestas et al, 2006). Moreover, it was also shown that COX inhibitors (indomethacin, diclofenac etc.) enhanced (Iwata et al, 2005) whereas the LOX inhibitor Zileuton dramatically decreased sebaceous lipid production (Zouboulis et al, 2005; Zouboulis, 2009). These data suggest that AA metabolites may play opposite roles in the regulation of the sebaceous lipid synthesis; i.e. LOX products seem to increase, whereas COX products seem to decrease it. Therefore, the facts that increased lipid synthesis and apoptotic processes are usually "walking hand-in-hand" during the sebaceous differentiation, and that COX inhibition enhances lipid production make COX inhibitors very unlikely to decrease AA-induced apoptosis of the sebocytes. (For our preliminary data on the effect of AA on immune profile of SZ95 sebocytes, see **Supplementary Figure S3** and **Supplementary Discussion**).

Interestingly, neither the siRNA-mediated “knock-down”, nor the pharmacological inhibition of cPKCα and nPKCδ resulted in alterations in cellular viability and basal lipid synthesis of the sebocytes. These data implicate that the endogenous activities of the two isoforms are not crucial for regulating basal growth and differentiation of sebocytes. However, results obtained with PMA and AA strongly suggest their central involvement in such conditions when the engagement of various signal transduction mechanisms results in production of PKC-activating intracellular secondary messengers such as e.g. DAG, Ca^{2+}, etc. Since human sebocytes express a wide-array of metabotropic and nuclear receptors and their coupled “lipogenic” signaling pathways (Zouboulis et al, 2002; Zouboulis and Bohm, 2004; Alestas et al, 2006; Zhang et al, 2006; Dobrosi et al, 2008; Tóth et al, 2009, 2011), it is proposed that cPKCα and/or nPKCδ may play a role in their actions. (Evidently, the validity of the above hypothesis should be investigated in the future.)

The siRNA experiments resulted in other intriguing findings. Namely, we found a dramatically increased lipid synthesis and apoptosis rate in cells with “silenced” aPKCζ levels. These results, on the one hand, suggest that – in contrast to the pro-lipogenic actions of cPKCα and nPKCδ, and the pro-apoptotic effect of nPKCδ – aPKCζ rather inhibits lipid production and apoptosis of SZ95 sebocytes. On the other hand, our findings also implicate that the endogenous and, even more importantly, *constitutive* aPKCζ activity is an indispensible regulatory factor for promoting physiological sebocyte proliferation and survival.

~~Our preclinical data that certain PKC isoforms specifically regulate human sebocyte biology may have clinical implications which invite subsequent studies. Namely, it deserves systematic analysis whether selective aPKCζ inhibitors can be employed in the management of sebaceous tumors. Likewise, the data reported here will hopefully encourage one to explore whether the targeted inhibition of cPKCα and/or nPKCδ activity could become a useful treatment strategy for such human dermatoses (e.g. acne vulgaris) which are characterized by pathologically elevated sebum production of the sebaceous glands.~~

MATERIALS AND METHODS

Materials

Phorbol 12-myristate 13-acetate (PMA), GF 109203X and arachidonic acid (AA) were purchased from Sigma-Aldrich (St. Louis, MO). Gö6976 and Rottlerin were from Calbiochem (Nottingham, UK). The primary antibodies against cPKCα, β_I, β_{II}, γ; nPKC ε and η; and aPKCζ and PKD-PKCμ were obtanied from Sigma-Aldrich, and the ones against nPKCδ and θ; and aPKCλ/ι were from Santa Cruz Biotechnology (Santa Cruz, CA)

Cell culturing

Immortalized human SZ95 sebocytes (derived from facial sebaceous glands; Zouboulis et al., 1999) were cultured in Sebomed Basal medium (Biochrom, Berlin, Germany) supplemented with 10% fetal bovine serum (Invitrogen, Paisley, UK), 5 ng/ml human epidermal growth factor (Sigma-Aldrich), 1 mM $CaCl_2$, 50 U/ml penicillin and 50 μg/ml streptomycin (both from Teva, Debrecen, Hungary) (Dobrosi et al, 2008; Tóth et al, 2009)

Western blotting

Immunoblotting was performed to assess the protein expression of the various PKCs in SZ95 cells. Cell lysates were subjected to SDS-PAGE (7.5% gels were loaded with 40 μg protein per lane), transferred to BioBond nitrocellulose membranes (Whatman, Maidstone, UK), and probed with the above mentioned rabbit primary antibodies against distinct PKC isoforms (dilution 1:100-1:1000). Horseradish peroxidase-conjugated rabbit

anti-goat IgG antibody (1:1000, Bio-Rad, Hercules CA, USA) was employed as a secondary antibody, and the immunoreactive signal was visualized by a SuperSignal® West Pico Chemiluminescent Substrate enhanced chemiluminescence kit (Pierce Rockford, IL) using LAS-3000 Intelligent Dark Box (Fuji, Tokyo, Japan). To confirm equal loading, membranes were re-probed with an anti cytochrome-C antibody (Cyt-C, 1:50, Santa Cruz). Where indicated, signal intensity was analyzed with densitometry, using the Image Pro Plus 4.5.0 software (Media Cybernetics, Silver Spring, MD) (Gönczi et al, 2008; Szegedi et al, 2009).

Immunostaining of PKCs, confocal microscopy

Identification and determination of the subcellular localization of PKC isozymes in SZ95 cells were carried out by confocal microscopy following immunolabeling. Cells were fixed in acetone, permeabilized by 0.1% Triton-X-100 (Sigma-Aldrich) and then incubated with the above rabbit anti-PKC primary antibodies for 60 min (dilution 1:100-1:200). Coverslips were then stained with a FITC-conjugated secondary antibody (Vector Laboratories, Burlingame, CA, USA) (dilution 1:200), and examined in a Zeiss LSM confocal microscopy system (Carl Zeiss Inc., Oberkochen, Germany) (Bodó et al, 2005; Dobrosi et al, 2008; Tóth et al, 2009).

Quantitative "real-time" PCR (Q-PCR)

Q-PCR was carried out on an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) using the 5' nuclease assay. Total RNA was isolated from cell lysates using TRIzol (Invitrogen). Three micrograms of total RNA was then reverse-transcribed into cDNA by using 15 U of AMV reverse transcriptase (Promega, Madison,

WI) and 0.025 µg/µl oligodT primers (Promega). TaqMan primers and probes (Applied Biosystems) were applied for subsequent PCR amplification (assay IDs, Hs00176973_m1 for cPKCα, Hs00176998_m1 for cPKCβ, Hs00177010_m1 for cPKCγ, Hs00178914_m1 for nPKCδ, Hs00178455_m1 for nPKCε, Hs00178933_m1 for nPKCη, Hs00234709_m1 for nPKCθ, Hs00177051_m1for aPKCζ, and Hs00702254_s1 for aPKCλ/ι) using the TaqMan universal PCR master mix protocol (Applied Biosystems). As internal controls, transcripts of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were determined (assay ID, Hs99999905_m1 for human) (Dobrosi et al, 2008; Tóth et al, 2009, 2011).

Assessment of viability

Viable cell number was assessed by measuring the ability of cellular dehydrogenases to convert the tetrazolium salt MTT (Sigma-Aldrich) to formazan. Cells were plated onto 96-well plates (Greiner Bio-One, Frickenhausen, Germany) (15,000 cells/well) in quadruplicates. After treatment, cells were incubated with 0.5 mg/ml MTT for 3 hours, and the amount of formazan crystals (generated within the cells) was determined colorimetrically according to our previous reports (Bodó et al, 2005; Kiss et al, 2008; Szegedi et al, 2009).

Assessment of apoptosis

Reduced mitochondrial membrane potential serves as an early indicator of the onset of apoptotic processes. SZ95 cells (15,000 cells/well) were plated onto 96-well black-well/clear-bottom plates (Greiner Bio One) in quadruplicates. After treatment, their mitochondrial membrane potential was determined using MitoProbe™ $DiIC_1(5)$ Assay Kit

(Invitrogen). The fluorescence intensity (reflecting the level of mitochondrial membrane potential) was measured on FlexStation II^{384} Fluorescence Image Plate Reader (FLIPR) (Molecular Devices, San Francisco, CA), as described in our previous reports (Dobrosi et al., 2008; Tóth et al, 2010, 2011).

Assessment of cytotoxicity (necrosis)

The cytotoxic effects of the above PKC-acting reagents were determined by SYTOX Green labeling (Invitrogen). Ruptured plasma membranes enable the penetration (and subsequent nucleic acid binding) of the fluorescent dye into necrotic cells, whereas viable cells with intact surface membranes display negligible SYTOX Green uptake. SZ95 cells were plated onto 96-well black-well/clear-bottom plates (Greiner Bio One). After treatment, supernatants were removed and the cells were incubated with a 1 μM solution of SYTOX Green. The fluorescence intensity of the dye was measured on FLIPR, as described previously (Dobrosi et al., 2008; Tóth et al, 2010, 2011).

Assessment of intracellular lipid content

For a quantitative measurement of the intracytoplasmic lipid content, Nile Red (Sigma-Aldrich) labeling was employed. SZ95 cells (15,000 cells/well) were plated onto 96-well black-well/clear-bottom plates (Greiner Bio One) in quadruplicates. Following treatment, supernatants were removed from wells and cells were incubated in 1 μg/ml Nile Red for 20 min. The fluorescence intensity of the dye (excitation: 485 nm; emission: 565 nm) reflecting the neutral lipid content of intracytoplasmic lipid droplets was measured on FLIPR (Wróbel et al, 2003; Alestas et al, 2006, Dobrosi et al., 2008).

RNA interference (siRNA)

SZ95 sebocytes were seeded on six-well culture plates in medium lacking antibiotics and were grown until reaching 40-60% confluence. Cells were then transfected with specific Stealth diRNAi oligonucleotides (at 40 nM, all from Santa Cruz) against cPKCα (sc-44227), nPKCδ (sc-36253) and aPKCζ (sc-29451) using Lipofectamine RNAiMAX transfection reagent (Invitrogen). siRNA Negative Control Duplexes (scrambled siRNA), were used as controls (sc-37007 and sc-44230; Santa Cruz). Immunoblotting was perfomed daily (for 4 days) to follow the changes in the expression level of the given PKC isoform after transfection. For subsequent cellular assays (assessing lipid content, cell viability, apoptosis, and cytotoxicity) cells were trypsinized 24 h after transfection replated onto 96-well plates and subjected to the above mentioned procedures (Dobrosi et al., 2008; Tóth et al, 2010, 2011).

Statistical analysis

When applicable, data were analyzed using a two-tailed un-paired *t*-test and $P<0.05$ values were regarded as significant differences.

CONFLICT OF INTEREST

The authors declare no competing financial interests.

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FIGURE LEGENDS

Figure 1. *Human SZ95 sebocytes possess a characteristic PKC isoform pattern*

a) Specific immunoreactivity of various PKC isoforms as determined by immunofluorescence (FITC, green fluorescence). NC, negative control obtained after omitting the primary antibody. Nuclei were counterstained with DAPI (blue fluorescence). I**nsets**: Expressions of PKC isoforms were also determined in cell lysates of SZ95 sebocytes by Western blot analysis. **b**) Q-PCR analysis of mRNA transcript expression profile of the PKC isoenzymes. Data (mean±SEM) are expressed as a fraction of the mean value of expression of the housekeeping gene GAPDH (defined as 1). Two additional experiments yielded similar results.

Figure 2. *PMA treatment stimulates lipid synthesis of SZ95 sebocytes*

Cells were treated with vehicle (Control, C); the PKC-activator PMA (at the indicated doses); the PKC inhibitors GF109203X (GF), Gö6986 (Gö), and Rottlerin (Rottl.) at 10 nM; or combinations for 48 hrs. **a, b)** Quantitative measurement of lipids as assessed by Nile Red labeling followed by FLIPR measurement. Neutral lipids indicate *de novo* synthesized intracellular lipids. **c)** Determination of cell viability by colorimetric MTT assay. **d)** Assessment of apoptosis by fluorimetric $DiIC_1(5)$ assay reflecting mitochondrial membrane potential; and of necrosis by fluorimetric SYTOX Green assay reflecting ruptured cell membrane. Data (mean±SEM) are expressed as a percentage of the mean value of the vehicle-treated control group (defined as 100 %, solid line). * marks significant (P<0.05) differences compared to the control group. In panel **b**, # marks significant (P<0.05) differences compared to the PMA-treated group. n=4 in each group. Three-four additional experiments yielded similar results.

Figure 3. *PMA and arachidonic acid differentially translocate and down-regulate certain PKC isoforms in SZ95 sebocytes*

Cells were treated with vehicle (Control), 10 nM PMA, and 50 μM arachidonic acid (AA) for 1 hr (**a**) to assess translocation or for 1-3 days (**b**) to measure down-regulation of PKC isoforms. **a)** Immunofluorescence labeling (FITC, green fluorescence) of PKCs followed by confocal microscopy analysis. Arrows indicate translocation. **b)** Western blot analysis. The amounts of the individual PKC isoforms were quantitated by densitometry (optical density, OD) and expressed as the percentage of the value of immunoreactive bands of daily-matched control group regarded as 100%. Note the translocation and down-regulation of cPKCα and nPKCδ by PMA, and of nPKCδ by AA. Three additional experiments yielded similar results.

Figure 4. *cPKCα and nPKCδ are involved in mediating the lipogenic action of PMA whereas siRNA-mediated "silencing" of aPKCζ induces lipid synthesis and apoptosis in SZ95 sebocytes*

Various siRNA probes against PKC isoforms, as well as a scrambled siRNA probe (Scr. si), were introduced to cells by transfection. To evaluate the efficacy of this intervention, at days 1-4 after transfection, cells were subjected to Western blot analysis. As controls (C), the effect of the transfection reagent was determined. As a house-keeping molecule, expression of Cytochrome-C (Cyt-C) was assessed. **a, b)** Representative Western blot data at days 1-4 (**a**) and at day 2 (**b**) after transfection. **c)** Statistical analysis of Western blot data. Optical density (OD) values of specific immunosignals were determined at day 2 after transfection in 3 independent experiments. Normalized

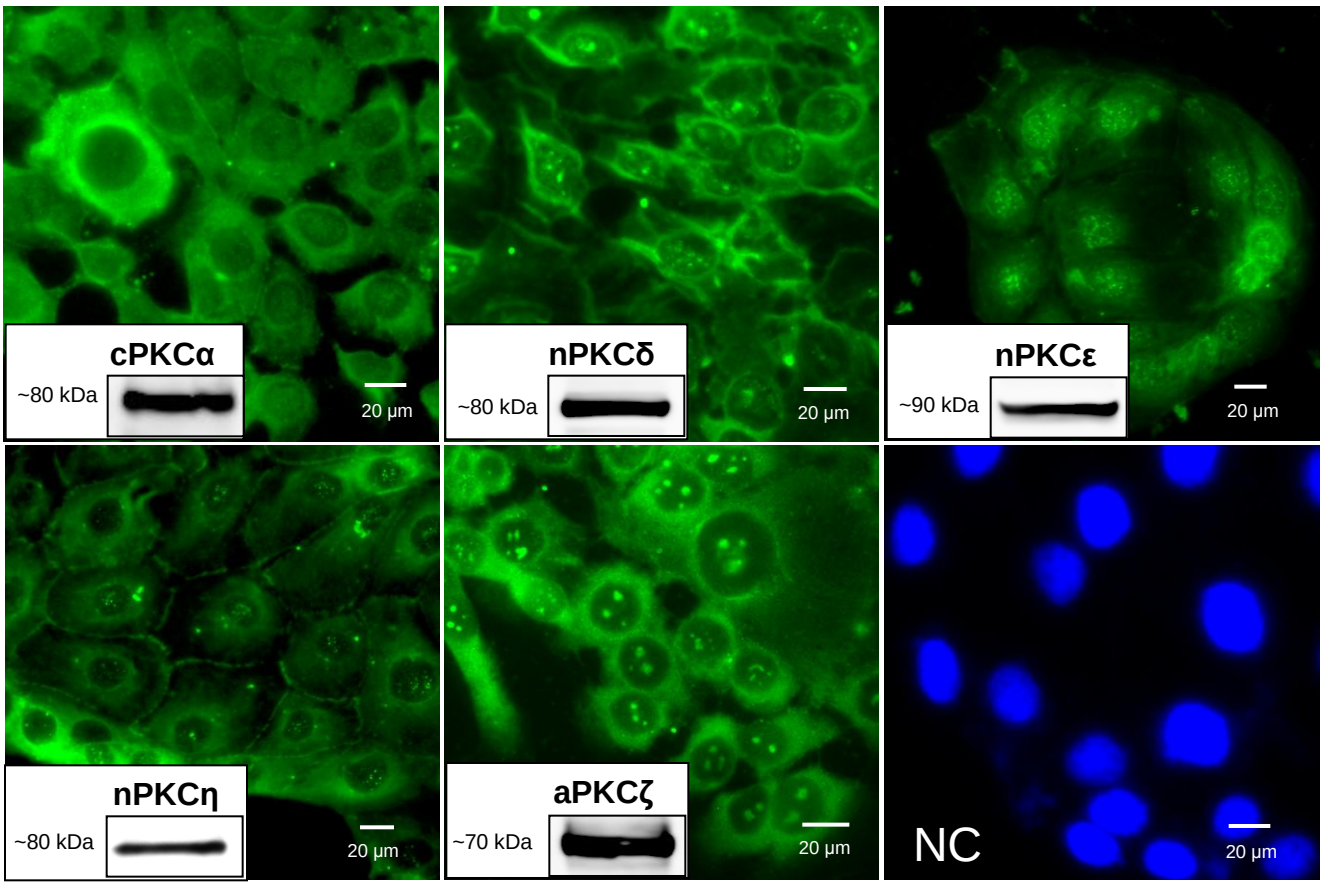
OD values (to Cyt-C) in each group were then averaged and expressed as mean±SEM as the percentage of the averaged values of the control groups regarded as 100%. * marks significant (P<0.05) differences compared to the Scr. si groups. At day 2 after transfection, cells were treated with 10 nM PMA for 48 hrs. **d)** Determination of neutral lipids as assessed by Nile Red labeling followed by FLIPR measurement. **e)** Determination of cell viability by colorimetric MTT assay; of apoptosis by fluorimetric $DiIC_1(5)$ assay; and of necrosis by fluorimetric SYTOX Green assay. Data (mean±SEM) of 3 independent experiments are expressed as a percentage of the mean value of the Scr. si group (defined as 100 %, solid line). * marks significant (P<0.05) differences compared to the Scr. si group whereas # marks significant (P<0.05) differences compared to the PMA-treated group. n=4 in each group. The Scr. si group displayed no significant difference compared to the non-transfected SZ95 sebocyte population subjected to identical treatment protocols (data not shown). ~~Two additional experiments yielded similar results~~.

Figure 5. *The lipogenic and apoptosis-inducing actions of arachidonic acid are mediated by nPKCδ in SZ95 sebocytes*

Control (**a, b**) or siRNA-transfected (**c, d**, 2 days after transfection) SZ95 sebocytes were treated with vehicle (Control, C); 10 nM PMA; the PKC inhibitors GF109203X (GF), Gö6986 (Gö), and Rottlerin (Rottl.) at 10 nM; 50 μM arachidonic acid (AA); or combinations for 48 hrs. **a, c)** Quantitative measurement of neutral lipids as assessed by Nile Red labeling followed by FLIPR measurement. **b, d)** Assessment of apoptosis by fluorimetric $DiIC_1(5)$ assay (**b, d**) and of necrosis by fluorimetric SYTOX Green assay (**b**). Data (mean±SEM) of 3 independent experiments are expressed as a percentage of

the mean value of either the control (**a, b**) or the scrambled siRNA probe (Scr. si; **c, d**) group (defined as 100 %, solid line). * marks significant (P<0.05) differences compared to the control or Scr. si group whereas # marks significant (P<0.05) differences compared to the AA-treated group. n=4 in each group. The Scr. si group displayed no significant difference compared to the non-transfected SZ95 population subjected to identical treatment protocols (data not shown). ~~Two additional experiments yielded similar results.~~

a


cPKCα
~80 kDa
20 μm
nPKCδ
~80 kDa
20 μm
nPKCε
~90 kDa
20 μm
nPKCη
~80 kDa
20 μm
aPKCζ
~70 kDa
20 μm
NC
20 μm

b

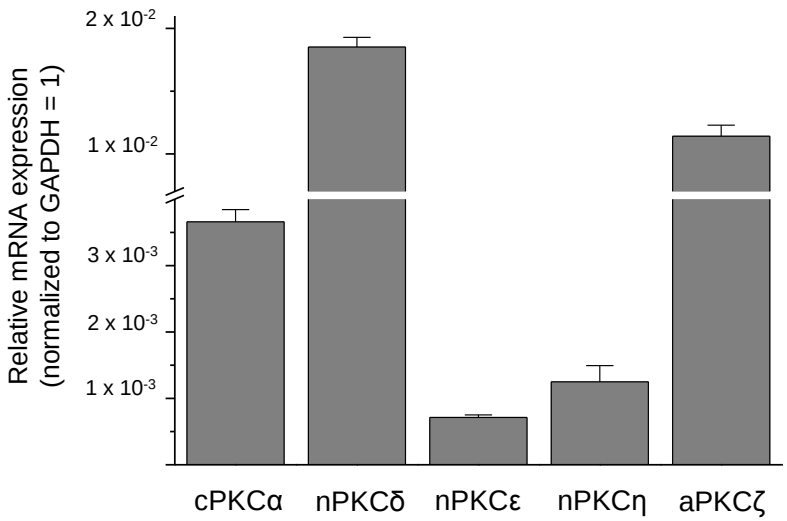

Relative mRNA expression
(normalized to GAPDH = 1)
2 x 10-2
1 x 10-2
3 x 10-3
2 x 10-3
1 x 10-3
cPKCα
nPKCδ
nPKCε
nPKCη
aPKCζ

Figure 1

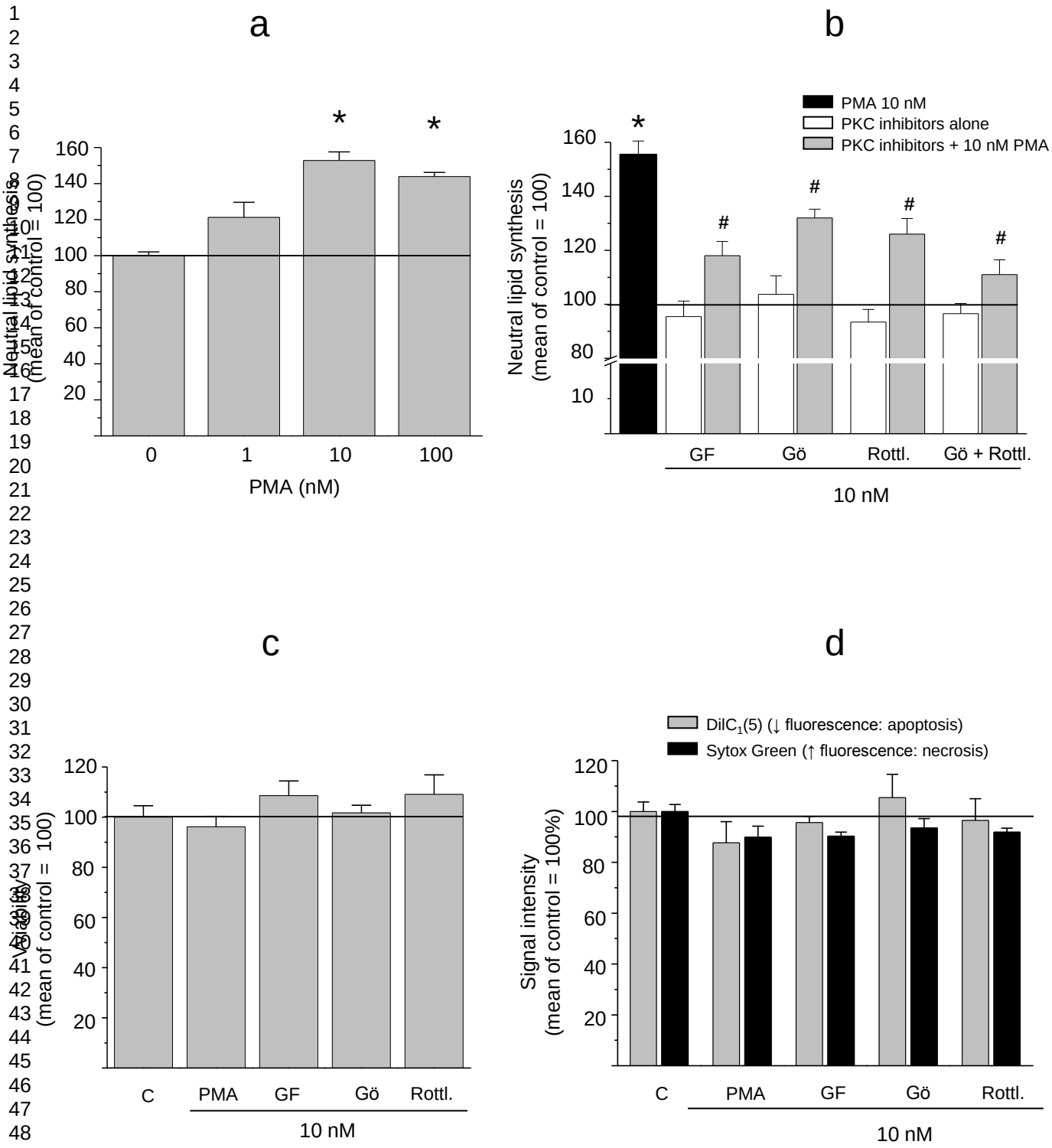
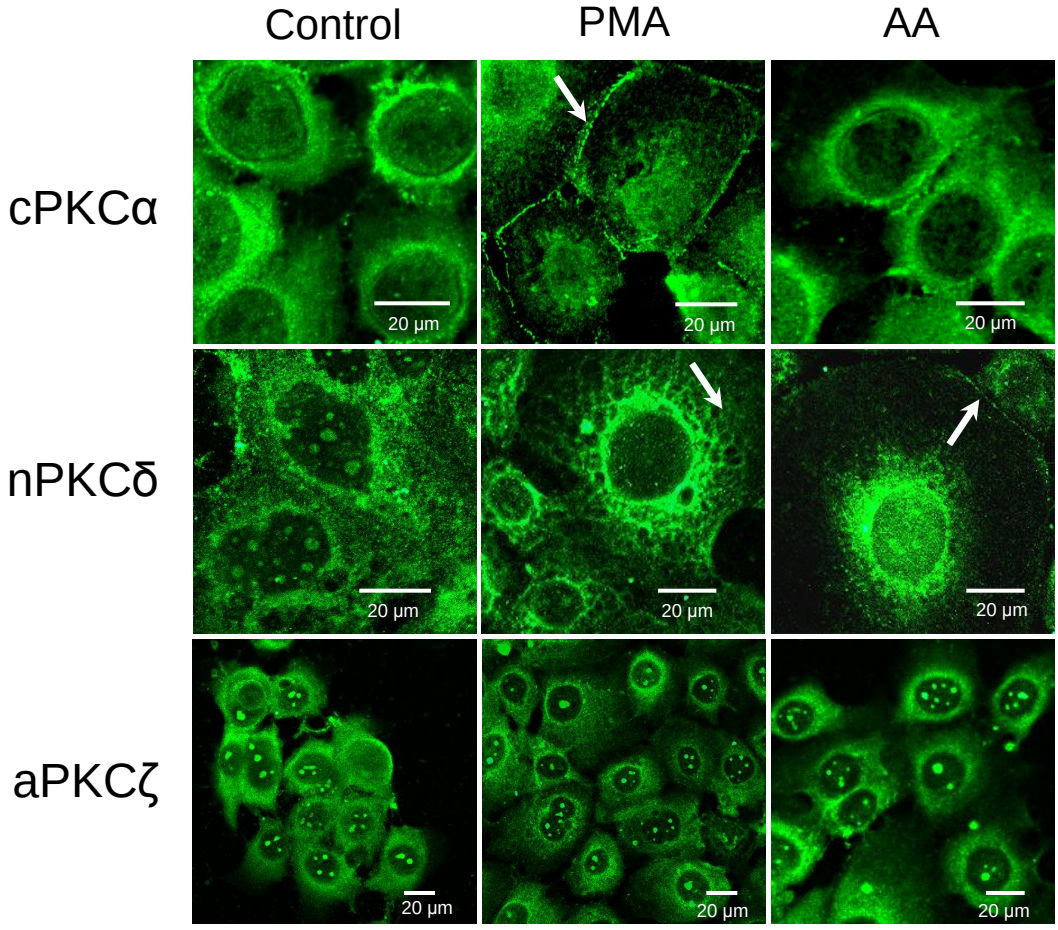

a
b
c
d
Neutral lipid synthesis
(mean of control = 100)
PMA (nM)
0
1
10
100
PMA 10 nM
PKC inhibitors alone
PKC inhibitors + 10 nM PMA
GF
Gö
Rottl.
Gö + Rottl.
10 nM
Viability
(mean of control = 100)
C
PMA
Signal intensity
(mean of control = 100%)
DilC1(5) (↓ fluorescence: apoptosis)
Sytox Green (↑ fluorescence: necrosis)

Figure 2

a


Control
PMA
AA
cPKCα
nPKCδ
aPKCζ
20 μm

b

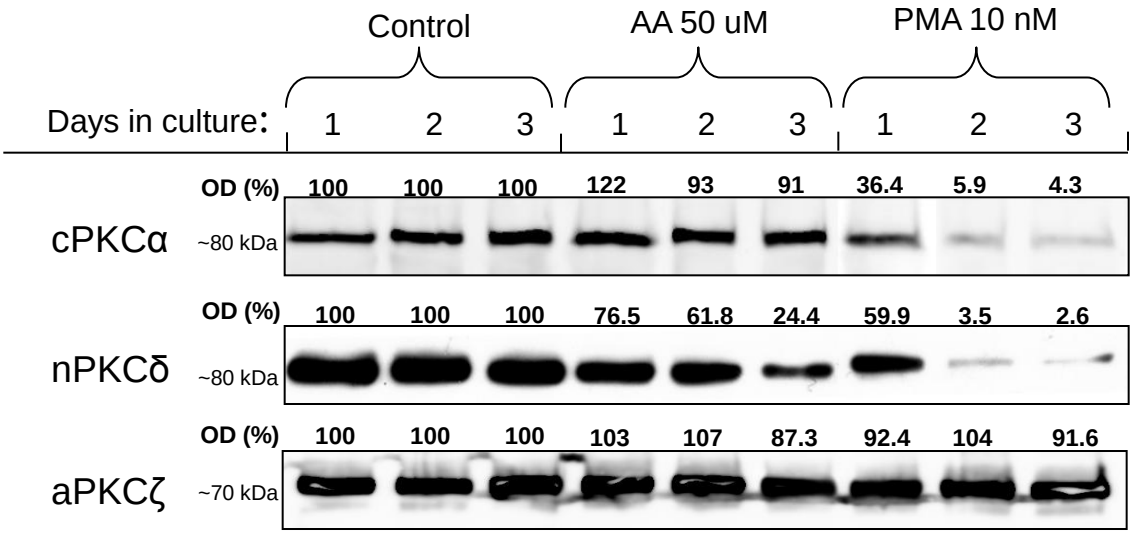

Control
AA 50 uM
PMA 10 nM
Days in culture: 1 2 3 1 2 3 1 2 3
OD (%) 100 100 100 122 93 91 36.4 5.9 4.3
cPKCα
~80 kDa
OD (%) 100 100 100 76.5 61.8 24.4 59.9 3.5 2.6
nPKCδ
~80 kDa
OD (%) 100 100 100 103 107 87.3 92.4 104 91.6
aPKCζ
~70 kDa

Figure 3

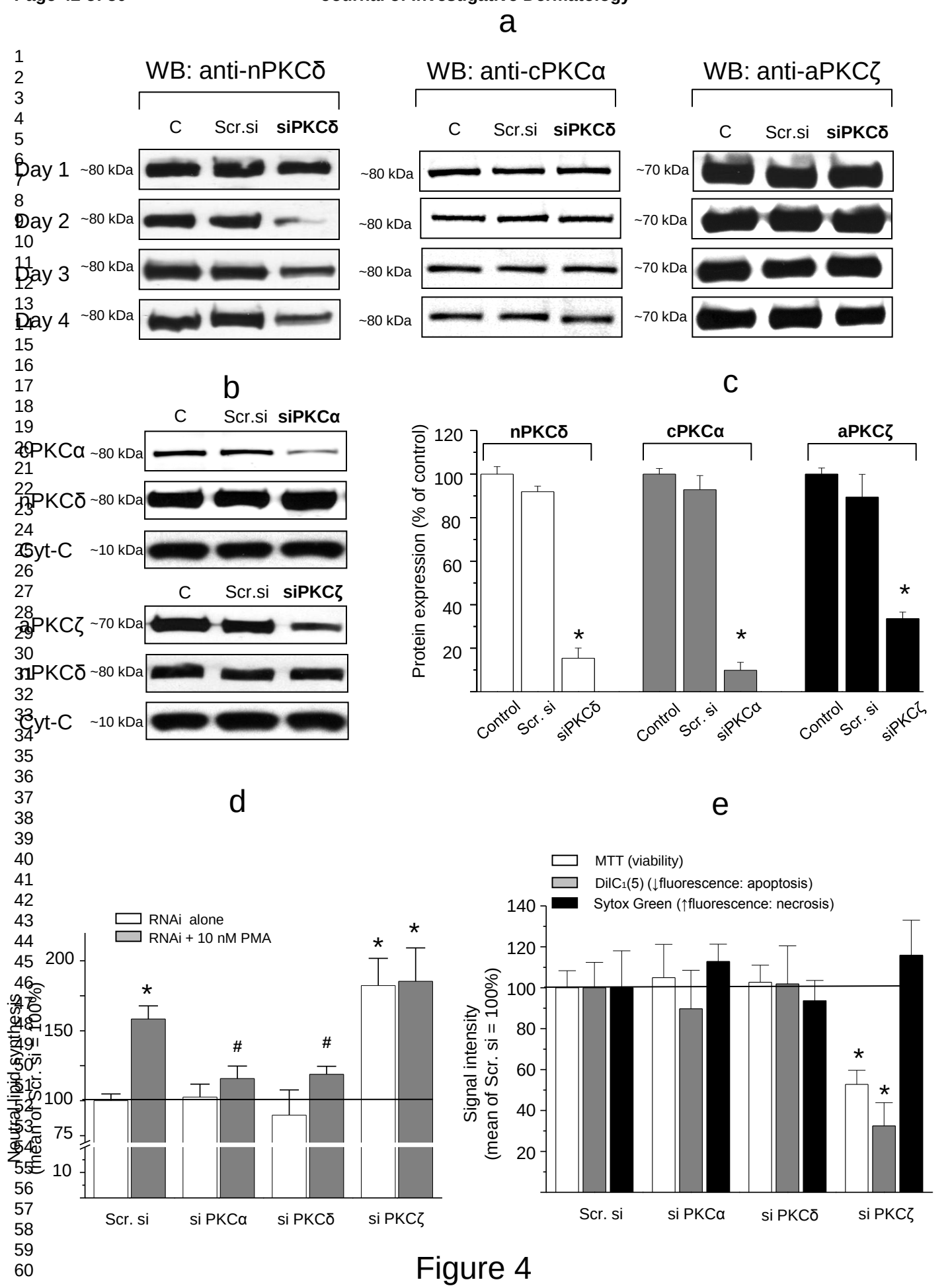

a
WB: anti-nPKCδ
WB: anti-cPKCα
WB: anti-aPKCζ
C
Scr.si
siPKCδ
Day 1
Day 2
Day 3
Day 4
~80 kDa
~70 kDa
b
C
Scr.si
siPKCα
cPKCα
nPKCδ
Cyt-C
~10 kDa
C
Scr.si
siPKCζ
aPKCζ
nPKCδ
Cyt-C
c
nPKCδ
cPKCα
aPKCζ
Protein expression (% of control)
Control
Scr. si
siPKCδ
siPKCα
siPKCζ
d
RNAi alone
RNAi + 10 nM PMA
Neutral lipid synthesis (mean of Scr. si = 100%)
Scr. si
si PKCα
si PKCδ
si PKCζ
e
MTT (viability)
DilC1(5) (↓fluorescence: apoptosis)
Sytox Green (↑fluorescence: necrosis)
Signal intensity (mean of Scr. si = 100%)

Figure 4

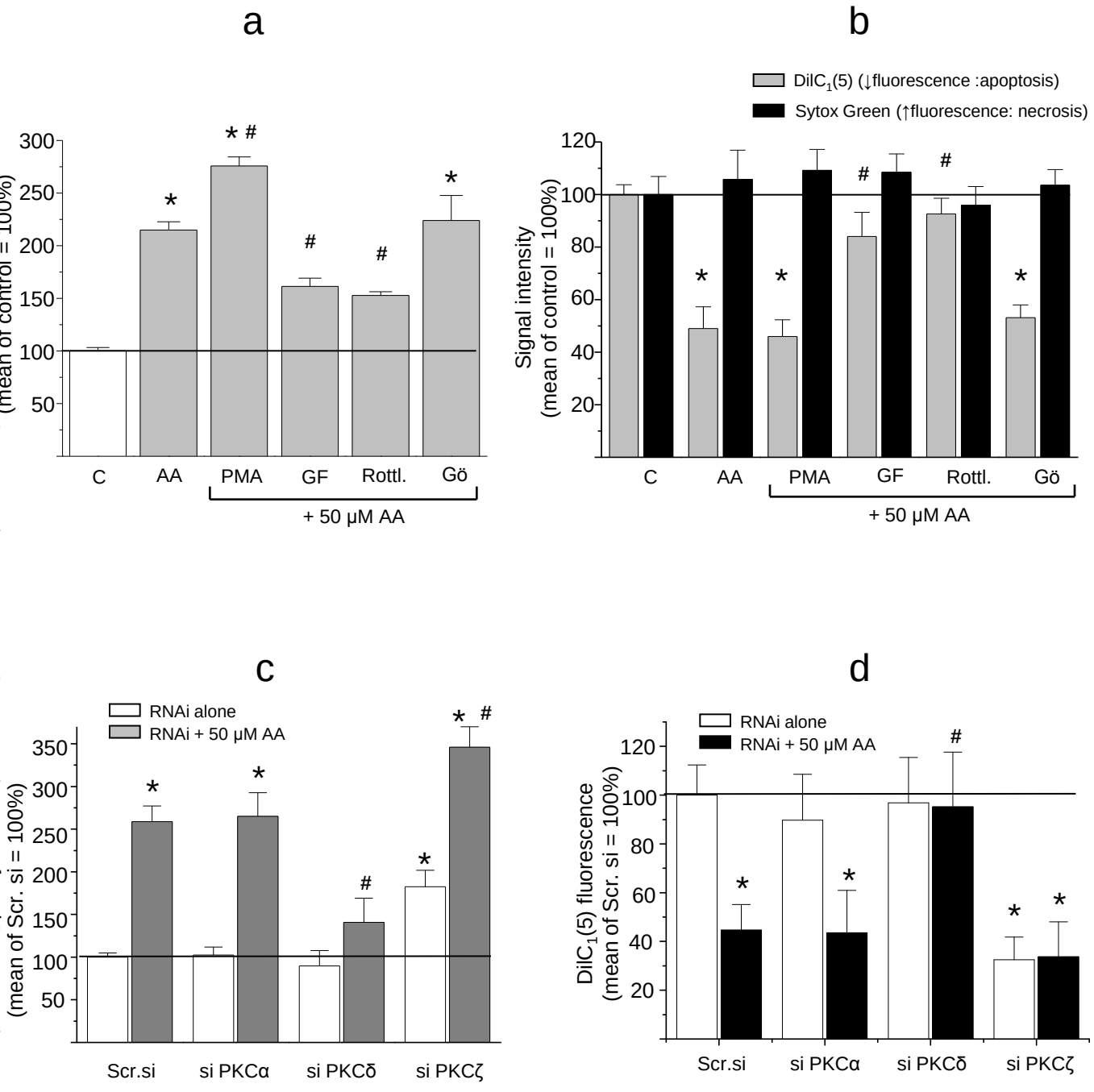

a
Neutral lipid synthesis
(mean of control = 100%)
C
AA
PMA
GF
Rottl.
Gö
+ 50 µM AA
b
DilC1(5) (↓fluorescence :apoptosis)
Sytox Green (↑fluorescence: necrosis)
Signal intensity
(mean of control = 100%)
c
RNAi alone
RNAi + 50 µM AA
Neutral lipid synthesis
(mean of Scr. si = 100%)
Scr.si
si PKCα
si PKCδ
si PKCζ
d
DilC1(5) fluorescence
(mean of Scr. si = 100%)

Figure 5

SUPPLEMENTARY DATA

Supplementary Materials and methods

Quantitative "real-time" PCR (Q-PCR) of immune genes

Q-PCR was carried out on an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) using the 5' nuclease assay. Total RNA was isolated from cell lysates using TRIzol (Invitrogen). Three micrograms of total RNA was then reverse-transcribed into cDNA by using 15 U of AMV reverse transcriptase (Promega, Madison, WI) and 0.025 μg/μl oligodT primers (Promega). TaqMan primers and probes (Applied Biosystems) were applied for subsequent PCR amplification (assay IDs, Hs00174092_m1 for interleukin-1α (IL1α), Hs00174097_m1 for IL1β, Hs00985639_m1 for IL6, Hs00174103_m1 for IL8, Hs00174086_m1 for IL10, Hs01038788_m1 for IL18, Hs00174128_m1 for tumor necrosis factor α (TNFα), Hs00189038_m1 for LL37 cathelicidin (CAMP), Hs00914334_m1 for lactotransferrin (LTF), Hs00608345_m1 for β-defensin-1 (bDEF1) and Hs00173615_m1 for pentraxin-3 (PTX3) using the TaqMan universal PCR master mix protocol (Applied Biosystems). As internal controls, transcripts of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were determined (assay ID, Hs99999905_m1 for human) (Dobrosi et al, 2008; Tóth et al, 2009, 2011).

Assessment of cellular proliferation

The degree of cellular growth (reflecting number of viable cells) was determined by measuring the DNA content of cells using CyQUANT Cell Proliferation Assay Kit (Invitrogen). SZ95 sebocytes (5,000 cells per well) were cultured in 96-well black-well/clear-bottom plates (Greiner Bio-One) in quadruplicates and were treated with 50

μM AA for 24, 48 and 72 hrs. Supernatants were then removed by blotting on paper towels, and the plates were subsequently frozen at -70℃. The plates were then thawed at room temperature, and 200 μl of CyQUANT dye/cell lysis buffer mixture was added to each well. After 5 minutes of incubation, fluorescence was measured at 490 nm excitation and 520 nm emission wavelengths using FLIPR (Molecular Devices).

Supplementary Discussion

In our ongoing studies, we are currently investigating how AA treatment affects the expression of immune and inflammatory processes of SZ95 sebocytes. In addition, we are also assessing the involvement of certain PKCs in these processes. Our preliminary Q-PCR data suggest that AA treatment markedly alter expressions of certain interleukins, pro-inflammatory agents, anti-microbial peptides, etc. which are involved in innate (and/or adaptive) immune mechanisms of these cells. Specifically, we found that expressions of the well known pro-inflammatory interleukins (IL6 and IL18) (Gauldi et al, 1992; Tsutsui et al, 2011]) as well as the anti-microbial peptide β-defensin-1 (Prado and da Oca, 2010) and pentraxin-3 (PTX3), a central regulator of the relationship between the innate and adaptive immune system (Deban et al, 2011), were significantly elevated by AA. Evidently, further (ongoing) studies are to define the roles of the PKCs in these processes; yet, our preliminary data imply that the above members of the sebocyte immune response machinery may function as putative AA and PKC downstream targets.

Supplementary References

Deban L, Jaillon S, Garlanda C *et al.* (2001) Pentraxins in innate immunity: lessons from PTX3. *Cell Tissue Res* 343:237–249

Gauldie J, Richards C, Baumann H (1992) IL6 and the acute phase reaction. *Res Immunol* 143(7):755-759

Prado E and de Oca M (2010) Human β-defensin 1: A restless warrior against allergies, infections and cancer. *Int J Biochem Cell B* 42:800–804

Tsutsui H, Mizutani H, Nakanishi K (2011) Contribution of interleukin 18 to the development of infection-associated atopic dermatitis. *Curr Probl Dermatol* 41:93-103

Supplementary Figure Legends

Supplementary Figure S1. *Demonstration of equal protein loading during Western blot experiments*

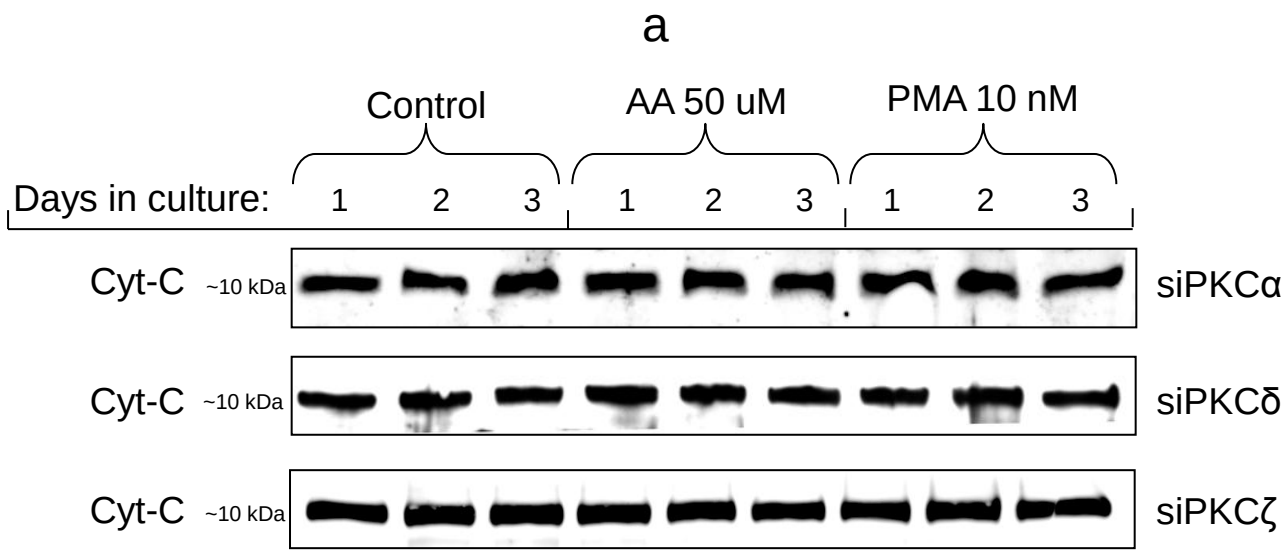
(**a**) Western blot analysis. To assess equal loading, membranes shown at **Figure 3b** were re-probed using an antibody against cytochrome-C (Cyt-C). (**b**) Western blot analysis. To assess equal loading, membranes shown at **Figure 4a** were re-probed using an antibody against cytochrome-C (Cyt-C).

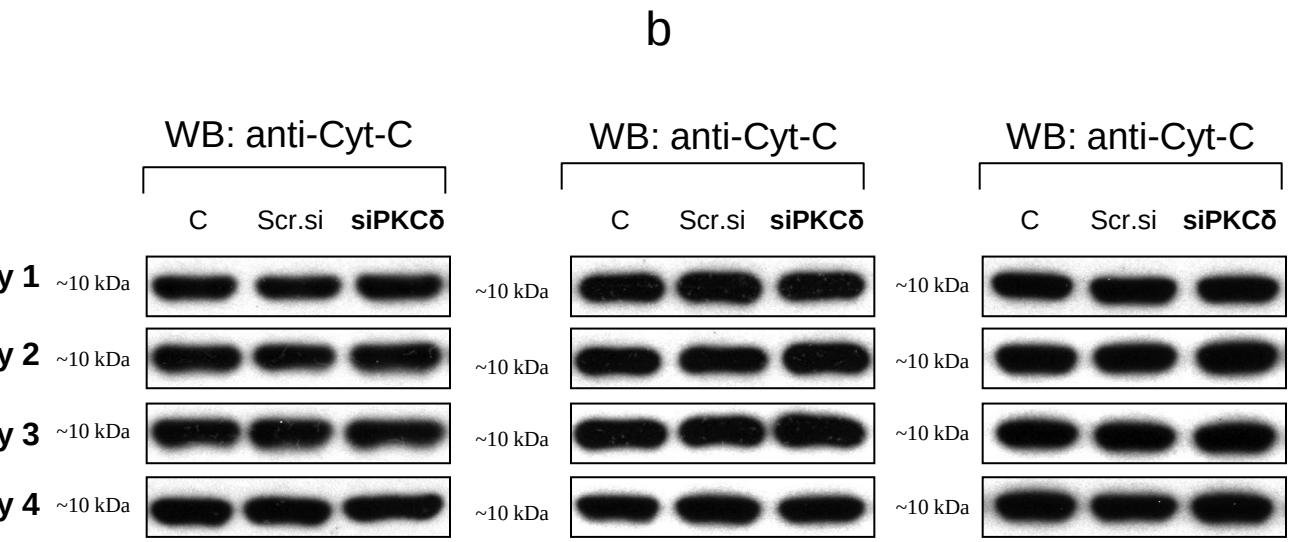
Supplementary Figure S2. *Long-term AA treatment does not influence proliferation of SZ95 sebocytes*

Proliferation (CyQUANT) assay. Results are expressed in the percentage of the 24-hr vehicle control (100%, solid line) as mean±SEM of four independent determinations. Two additional experiments yielded similar results.

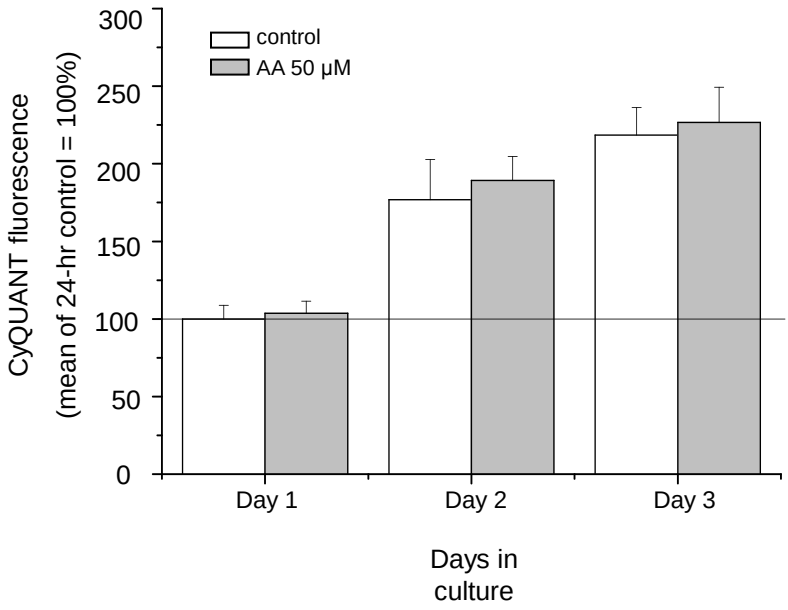
Supplementary Figure S3. *AA regulates expression of genes involved in the innate immunity*

Q-PCR analysis of mRNA transcript expression profile of different selected genes involved in the innate immunity. Data are presented by using ΔΔCT method regarding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) normalized mRNA expressions of the vehicle control as 1 (solid line). Data are expressed as mean±SD of three independent determinations. Two additional experiments yielded similar results. * mark significant (P<0.05) differences compared to the vehicle control.

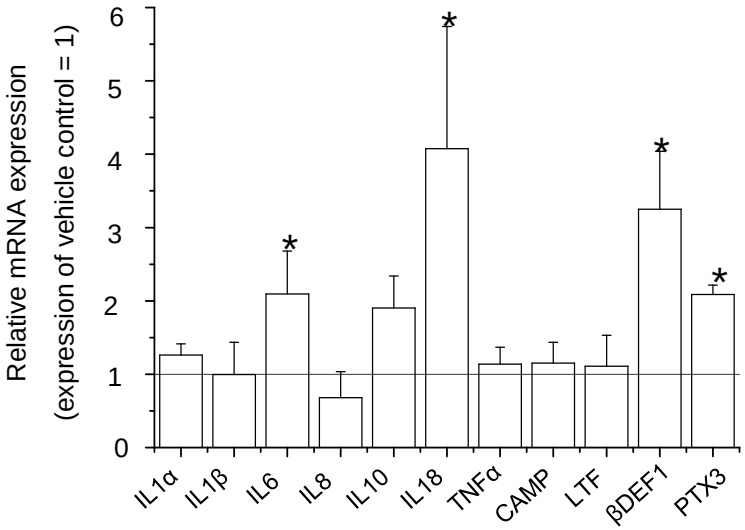




Supplementary Figure S1



Supplementary Figure S2



Supplementary Figure S3