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Phorbol-myristate-acetate, but not Interleukin-1β or Insulin-like Growth Factor-I, Regulates Protein Kinase C Isoenzymes in Human Dermal Papilla Cells

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The in vitro growth of human hair follicles is inhibited by interleukin (IL)-1β and phorbol esters, such as phorbol-myristate-acetate (PMA), but enhanced by insulin-like growth factor (IGF)-I. Although this process is only incompletely understood, the dermal papilla as a pivotal part of the hair follicle is almost certainly involved. Since protein kinase C (PKC) isoenzymes are activated by phorbol esters and are key enzymes in signalling pathways of several hormones, neurotransmitters, and growth factors, we addressed the question whether the action of the above-mentioned hair growth-modulating substances may affect PKC isoenzymes in cultured dermal papilla cells (DPC). By Western blot analysis, protein kinase Cα, -ε, -γ, -ι, -λ, and the RACK1 receptor protein were detected in dermal papilla cell cultures, whereas the isoenzymes δ and μ were expressed only at low levels and protein kinase C-β, -θ, and -ζ were not present. After PMA stimulation, the PKC α, -ε, and -γ were translocated from the cytosol to the membrane fraction and subsequently down-regulated. PKCι was down-regulated but not translocated, and PKC λ and RACK1 were not affected by PMA. Neither IL-1β nor IGF had an effect on PKC or RACK1 expression.

We conclude that cultured DPC express a distinct PKC isoenzyme pattern and that the PMA-induced growth arrest in cultivated hair follicles may be transmitted via protein kinases, whereas the effects of IL-1β or IGF may be transduced via other signal transduction pathways or other cell types. *Key words: hair growth; signal transduction; cell culture; in vitro.*

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The protein kinase C (PKC) enzyme family contains at least 11 isoforms of serine/threonine protein kinases, which transmit signals of various extracellular substances. The PKC isoenymes are grouped into three classes: a) conventional PKC, which are Ca^{2+}-dependent and activated by phorbol esters, b) the novel PKC, which are Ca^{2+}-independent and phorbol ester-activated, and c) atypical, Ca^{2+}- and phorbol ester-independent PKC (1). PKC are ubiquitous but show a distinct cell- or tissue-specific distribution pattern and different physiological functions of the isoforms (2). Apart from the isoform η (3), the activation of PKC is accompanied by a translocation from the cytoplasm to the plasma membrane (4) and a subsequent down-regulation (5–7). In skin and its appendages, PKC isoenzymes are involved in the proliferation and differentiation of keratinocytes (8, 9) and dermal fibroblasts (9). Differences in the PKC distribution pattern are present in various skin diseases, such as psoriasis (10), squamous cell carcinoma, basal cell carcinoma, naevus pigmentosus and seborrhoeic keratosis (11). Recently, a role of PKC in the transduction of phorbol-myristate-acetate (PMA)-induced hair growth arrest has been proposed (12), implying the presence of PKC-dependent signal transduction pathways in hair follicle cells. Provided that the hair cycle is, at least in part, regulated via the dermal papilla, PKC-dependent signal transduction pathways could be involved in the reaction cascade tuning hair growth. Therefore, we investigated the PKC isoenzymes present in DPC and their PKC expression pattern after stimulation with interleukin (IL)-1β, PMA or insulin-like growth factor (IGF)-I. In this way we wanted to clarify whether a distinct PKC isoenzyme in DPC may mediate a specified cell function, as it has been reported from other cell types (13–16).

MATERIAL AND METHODS

Cell culture

Intact single hair follicles were isolated from scalp biopsies, obtained from 6 healthy volunteers after informed consent according to the regulations of the ethics committee.

Dermal papilla cell lines were established from at least 5 pooled dermal papillae per volunteer (17). The dermal papillae were microdissected from isolated human hair follicles (18). Cell cultures were established from the outgrowing cells of the intact papilla, according to Warren et al. (17), with the modification that Amniomax culture medium (Life Technologies, Eggenstein, Germany) was used instead of Chang's medium. Cells were passaged with trypsin/EDTA (Life Technologies, Eggenstein, Germany) and cultivated in Amniomax medium. Cells were checked by light microscopy for morphology and the typical aggregative behaviour after reaching confluency (19) and by immunohistochemistry and Western blotting, using a monoclonal antibody against human α-smooth muscle actin (DAKO, Hamburg, Germany), a marker for low-passage hair papilla cells (20). Cells from the fourth passage were used for the experiments.

Recombinant human IL-1β (Biosource, Camarillo, California, U.S.A.) or recombinant human IGF-I (Biosource, Camarillo, California, U.S.A.) or PMA (Sigma, Deisenhofen, Germany) was added sequentially to the culture medium in the respective wells from stock solutions 18 h or 5 min before the cells were harvested. The final concentrations were: PMA 1μmol/l; IL-1β 10ng/ml; IGF-I 100ng/ml.

Western blot analysis

Cells were snap frozen and stored at −80°C until fractionation. Before use, the cells were scraped from the tissue flasks, using cell scrapers and HEPES buffer (5 mM HEPES, pH 7,4, 1 mM EDTA), and homogenized in a potter elvjehem homogenizer. The homogenate was centrifuged for 30 min at 300,000 g at 4°C, yielding a particulate fraction and the cytosolic supernatant. The protein content was measured according to Bradford (21).

Cell fractions were separated on SDS-minigels with 20 μg protein per lane under reducing conditions (22) and electrophoretically transferred to nitrocellulose sheets (Gelman Sciences, Roßdorf, Germany), using a semi-dry transfer device (23). Blots were blocked in blocking buffer (5% skimmed milk powder in PBS) for 1 h at room temperature

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and incubated with the primary antibodies, diluted in blocking buffer for 2 h at room temperature. Blots were washed three times in 0.5% TWEEN 20 in PBS. The monoclonal anti-PKCα antibody was obtained from Upstate Biotechnology, Lake Placid, New York, USA. All other monoclonal antibodies (anti-PKCβ, -PKCδ, -PKCε, -PKCθ, -PKCγ, -PKCι, -PKCλ, -PKCμ, -PKCζ, and -RACK1[Receptor for activated *C*-kinase]) and the positive controls were from Transduction Laboratories, Lexington, Kentucky, U.S.A. According to the manufacturer's recommendation, the following positive controls were used with the antibodies: human HeLa cell lysates for PKC α, ε, and λ; human Jurkat cell lysates for PKC β, γ, and θ; mouse RSV-3T3 cell lysate for RACK1.

Visualization of the antigens was performed with anti-mouse IgG-conjugated peroxidase, using the ECL system (Amersham, Braunschweig, Germany) and KODAK X-OMAT AR films (Sigma, Deisenhofen, Germany).

RESULTS

Characterization of cultured cells

The cultured cells showed a fibroblast-like morphology and formed clumps after reaching confluency, a feature of this cell type (19). Moreover, a strong expression of the marker protein α-smooth muscle actin (20) was found by immunohistochemistry and immunoblotting in these cell cultures.

PKC isoenzyme and RACK1 distribution pattern

The following PKC isoenzymes were immunochemically detected in dermal papilla primary cell lines: PKCα, PKCε, PKCγ, PKCι, PKCλ, and the RACK1 receptor protein (Table I). Immunostaining for PKCδ and PKCμ was observed but was too weak for a regulation pattern to be ascertained. PKCβ, PKCθ, and PKCζ isoenzymes could not be detected.

Regulation

The conventional and the novel isoenzymes were activated by translocation to the cell membrane after PMA stimulation, whereas the atypical PKCs showed no translocation. Within 5 min after PMA stimulation, the isoenzymes PKCα, PKCε, and PKCγ were translocated from the cytosol to the membrane fraction (Fig. 1). In the control incubations, immunosignals were seen in the cytosol as well as the membrane fraction. The cytosolic immunosignal decreased sharply after PMA stimulation and was very weak or not detectable after 18 h. In the membrane fraction, the immunosignal increased after 5 min of PMA stimulation, but after 18 h of PMA stimulation these PKC isoenzymes were down-regulated.

PKCι was not translocated from the cytosol to the membrane fraction upon PMA stimulation, but a down-regulation was observed after 18 h.

The PKCλ levels were not changed after incubation with PMA.

The RACK1 receptor protein was immunolocalized in the membrane fraction and, to smaller extent, in the cytosol. It was not regulated within 18 h of PMA stimulation.

No effect, in terms of translocation or downregulation of IL-1β or IGF-I, was seen on any PKC isoenzyme or RACK1. In 2 out of 6 cell cultures, IL-1β resulted in a decrease of the immunosignal of PKCλ in the membrane fraction after 18 h without a former translocation. In general, the isoenzyme pattern and the regulation pattern were reproducible between cell cultures from different donors; however, slight individual variations were observed.

DISCUSSION

The PKC isoenzyme family consists of serine/threonine protein kinases, which are involved in a number of physiological processes like growth, secretion and differentiation. Based on different physicochemical properties and on the distinct isoenzyme pattern in different tissues, special biological functions of the single isoenzymes are proposed (16). A distinct isoenzyme pattern of PKC has been described for a number of cell types (24), and our experiments reveal a specific pattern of PKC isoenzymes also present in DPC. In primary cultures of DPC, we found conventional PKC α and γ, the novel PKC ε, and the atypical PKC ι and λ. So far, no data are available for follicular (root sheath) keratinocytes or follicular (connective tissue sheath) fibroblasts, probably due to difficulties in the isolation and cultivation of these cell types.

Compared to the PKC isoenzyme pattern in epidermal keratinocytes (3, 9, 25) and dermal fibroblasts (9), the isoenzymes α, δ, and ε are expressed in all of these cell types, whereas PKCγ is found exclusively and at high levels in DPC. This isoenzyme has been confined primarily to the nervous system, and the strong expression in DPC may be a new marker for this cell type. PKCζ, which is expressed in the keratinocytes and the dermal fibroblasts, is missing in DPC, and PKCβ and θ are not found in either cell type. PKCβ,

Table I. *Regulation of PKC isoenzymes and RACK1 by PMA in dermal papilla primary cell cultures*

The relative intensities of the immunosignals were summarized by use of a semiquantitative scale, ranging from − (no signal) to +++++ (strong signal).

Isotype	Period of PMA stimulation					
	0 h		5 min		18 h	
	Cytosol	Membranes	Cytosol	Membranes	Cytosol	Membranes
PKCα	++	+++	+	++++	−	++
PKCγ	+++	++++	−	++++	−	+
PKCε	+	+++	−	+++	−	++
PKCι	+	+++	++	+	+	+
PKCλ	+	+++	+	++	+	+++
ACK1	+	++	+	++	+	++

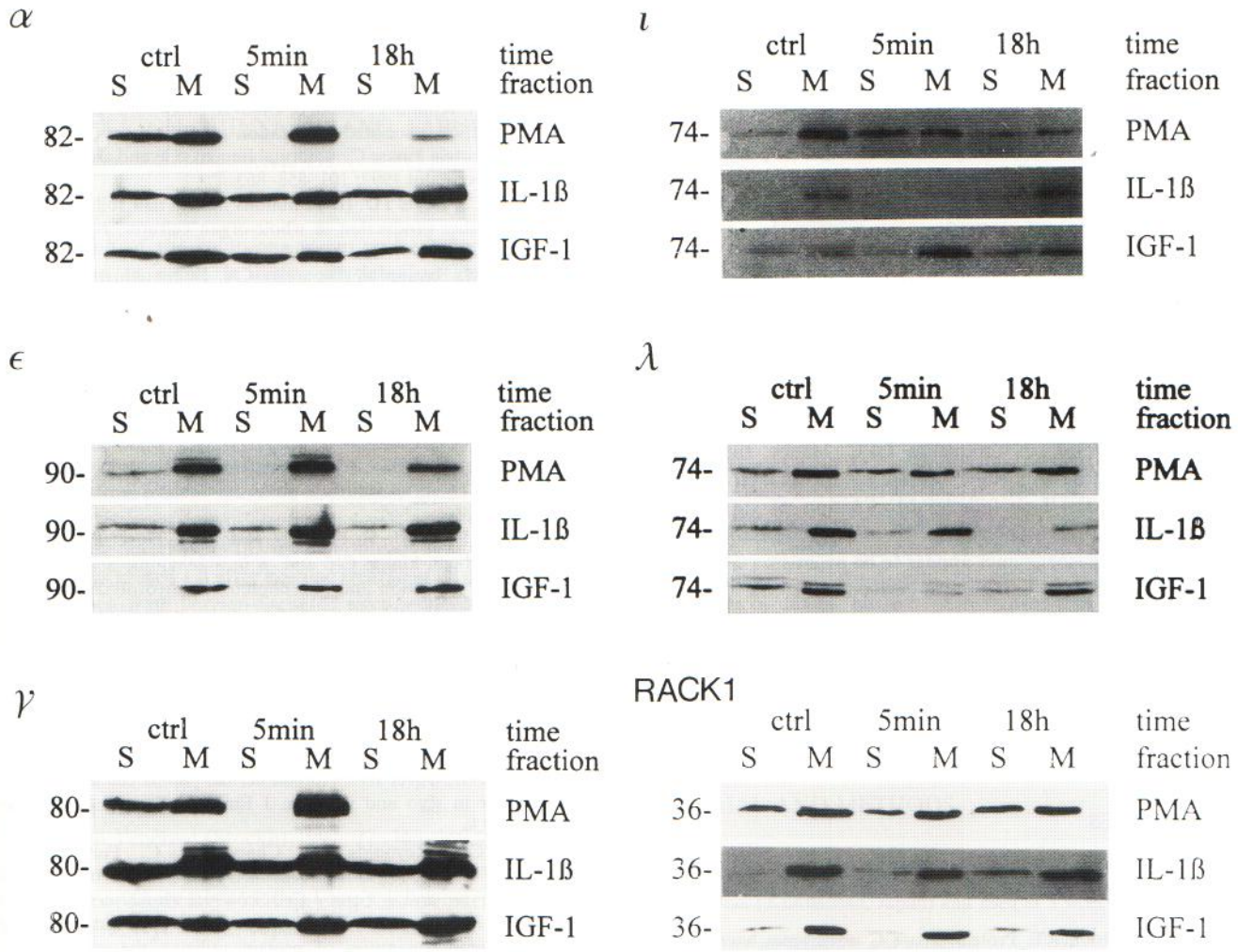

Fig. 1. Western blot analysis of PKC isoenzymes and RACK1 in soluble (*S*) and membrane (*M*) cell fractions from cultured dermal papilla cells in response to PMA, IL-1β, or IGF-I. Translocation and down-regulation of PKC-α, -γ, and -ε in response to PMA, but not IL-1β or IGF-I. No translocation, but down-regulation of PKCι in response to PMA, but not IL-1β or IGF-I. No regulation of PKCλ and RACK1 in response to any of the substances. The apparent molecular size (in kDa) is shown at the left hand side of each blot. The data shown are representative for cells from 6 donors, with the limitation that the down-regulation of PKCλ by IL-1β after 18 h was not observed in all cell cultures and may represent an individual variability of this isoenzyme in these cells.

however, was detected immunohistochemically in the upper epidermal layers and by Northern blotting (10), and its expression may be dependent on the differentiation of the keratinocytes. In response to the phorbol ester PMA, the conventional and the novel PKC isoenzymes showed a biphasic regulation pattern, which is typical for these isoenzymes, whereas the atypical PKC did not respond to PMA. The translocation of PKC isoenzymes from the cytosolic to the membrane compartment of DPC, followed by a down-regulation of the gene expression, is concordant with the regulation pattern described in dermal fibroblasts and keratinocytes by TPA and bryostatin (9). Although PKCι was down-regulated after 18 h of PMA stimulation, it was not initially activated by translocation to the membrane fraction. Since the down-regulation of PKC is a result of an increased proteolytic degradation (7), this regulation pattern of PKCι might be regarded as an inactivation. PKCλ was not activated by translocation to the membrane fraction by PMA; the steady-state level of this isoenzyme was not regulated by PMA.

The function of activated PKC enzymes depends on the binding to the appropriate membrane receptor RACK1 (26), which is therefore a putative regulation site. Since none of our stimulatory treatments of DPC could change RACK-1 expression, our data suggest that PKC action in papilla cells is not regulated at the receptor level.

Harmon & Nevis (12) reported inhibition of hair growth in vitro after PMA stimulation. Our results suggest that down-regulation of PKC α, ε, and γ isoenzymes may be involved in this process.

IL-1β has been shown to be a potent inhibitor of hair growth in vitro. We have recently shown that this phenomenon is mediated via cyclic AMP as an intracellular second messenger (27). The results of the present study are in line with this mechanism, because IL-1β had no effect on PKC isoenzyme expression in DPC. However, PKCλ was down-regulated, but not translocated, after 18 h of IL-1β in cells from 2 of 6 donors. Individual variations, which are often observed in biological systems, may account for this differential response.

In contrast to IL-1β, IGF-I is an enhancer of hair growth in vitro (28). Although IGF-I has been reported to use PKC

isoenzymes for signal transduction (29), IGF-I has in this regard no effect in DPC. Therefore, IGF-I intracellular signalling in hair follicles is either independent of PKC or involves hair follicle cells different from DPC. Like many other cultured cells, DPC change some of their biological properties with increasing passage numbers. In DPC, changes of physiological parameters, such as the hair follicle-inducing potential, are described for passages higher than ten (30). Addressing this question, we used only cells from passage number 4, and all experiments were performed with cells from the same passage. The ability to form clumps and the expression of α-smooth muscle actin in our cultured DPC indicate that they are comparable with the DPC described in the literature.

In conclusion, we have shown that a specific pattern of PKC isoenzymes is present in cultivated DPC and that PKC α, ε, and γ are activated by PMA treatment. In the skin, the expression of PKCγ is unique to DPC. Hence, these PKC isoenzymes may be important signal-transducing factors within the papilla of the hair follicle. Modulation of single PKC isoenzymes may be a future tool to manipulate hair growth. Therefore, these isoenzymes appear to be promising candidates for further studies in hair follicle-derived cell types.

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