The functional role of the intracellular effectors of the diacylglycerol pathway and new opportunities for their pharmacological modulations

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The Examination takes place at Department of Medical Chemistry, Medical and Health Science Center, University of Debrecen
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INTRODUCTION

Diacylglycerol and C1 Domains

The lipophilic second messenger DAG plays a central role in signaling pathways mediating the cellular effects of various extracellular mediators (e.g. hormones, neurotransmitters, growth factors). Following the activation of either G-protein-coupled receptors or tyrosine kinase receptors these mediators stimulate distinct phospholipase C isoenzymes, which, in turn, generate IP_3 and DAG by hydrolysing PIP_2 of the lipid membrane. After binding to its receptor IP_3 elevates ic. Ca^{2+}-concentration, which triggers a series of Ca-sensitive mechanisms in the cell; DAG exerts its cellular effects through its interaction with a multitude of downstream effectors that possess a specialized recognition motif, the C1 domain. The highly conserved domain was first described in protein kinase C (PKC) enzymes. Interaction between the C1 domain and DAG induces the translocation of PKCs and triggers an intramolecular conformational change, that mediates allosteric activation of kinase function. C1 domains have also been shown to selectively recognize a series of naturally occurring DAG analogs (phorbol esters, bryostatins, indolactams) that have the potential to induce permanent enzyme-activation. Beside classical and novel PKCs, six other protein families have been identified to contain a DAG/ phorbol ester-responsive "typical" C1 domain. The families of protein kinase D, DAG kinase and MRCK possess kinase activity; RasGRP and chimaerins regulate the activation of small GTPases; whereas Munc-13 proteins act as scaffold molecules. Via the C1 domain-driven activation of these downstream effectors, DAG is involved in numerous signaling pathways that control proliferation, differentiation, apoptosis, angiogenesis and drug resistance. These important functions have focused attention on the research of DAG-mediated cellular mechanisms.

The Domain Dtructure of PKC Isoforms and the Mechanism of Activation

Based on their structural and functional properties, members of the PKC family are classified into three subfamilies. "Conventional" PKCs (cPKCs) α, β and γ require both Ca^{2+} and DAG for their activation; whereas "novel" PKCs (nPKC) δ, ε, η and θ require only DAG for complete activation; and "atypical" PKCs (aPKC) ζ and λ/ι are unresponsive to either Ca^{2+} or DAG. All family members share the same basic domain architecture: they contain a C-terminal catalytic subunit (responsible for the kinase activity), and a regulatory subunit at the N-terminus (responsible for controlling the enzyme activity). Second messengers generated by phospholipase C bind to the C1 and the C2 domains of the regulatory subunit, which: *i)* induces the translocation of the given PKC isoform to the plasma membrane and other intracellular membrane structures; and *ii)* triggers a conformational change of the PKC molecule, leading to the release of the pseudosubstrate domain from the catalytic site, thereby relieving the enzyme from autoinhibition, and turning it into an active protein kinase, capable of phosphorylating the appropriate substrate molecule. The functional diversitiy within the PKC family originates from the structural differences of the individual C1 and C2 domains within the regulatory subunits of different PKC isoforms. The conventional isoforms posess Ca^{2+}- sensitive C2 domains and DAG-sensitive C1 domains. Ligand binding to both of these domains is a prerequisite of effective enzyme activation and stable membrane association. In contrast, the C2 domains of novel PKC isoforms are not capable of Ca^{2+}-binding, however their C1 domains display an increased sensitivity for DAG (compared with that of cPKCs); and the high-affinity C1-DAG interaction is sufficient to trigger the allosteric activation and the membrane-anchoring of these isoforms. Atypical PKCs do not contain any C2 domain, and their C1 domain is unresponsive to DAG, therefore these molecules do not respond to either Ca^{2+} or DAG. Phosphorylation by PDK-1 and different protein-protein inetractions have been suggested to regulate the enzymatic activity of aPKCs.

Biological Functions of the PKC System

Although PKC enzymes are ubiquitously expressed in human tissues, each cell type possesses a characteristic PKC expresssion pattern. Members of the PKC family represent key components in a wide range of signaling pathways that regulate such essential biological processes as cell proliferation, differention, apoptosis, and cell migration. Hence altered PKC function can have an important pathogenetic role at multiple stages of tumorigenesis. Early experiments identified PKCs as the cellular transducers of phorbol esters, mediating the tumor-promoting properties of these compounds. Subsequent studies have found a substantial amount of evidence linking PKC to carcinogenesis. PKC enzymes were originally considered pro-mitogenetic kinases, promoting cell proliferation and survival. However, later it became evident that several members of the family can also inhibit cell growth and transmit pro-apoptotic signals. Thus, the individual isoforms differentially (and often oppositely) regulate various important cellular processes, even within the same cell type. One of the best characterized examples of this functional diversity of PKC isoforms is the antagonistic behavior of nPKCε and nPKCδ in the regulation of essential signaling pathways. While nPKCε have been found to promote cell proliferation in most cell types, nPKCδ activation has been identified to exert growht-inhibitory or pro-apoptotic effects in the same cells. In agreement with these findings, increased nPKCε and/or decreased nPKCδ expression have been detected in various types of human malignancies, and, in addition, their levels of expression have been implicated to correlate with tumor grade. Moreover, another level of complication is added to the functional complexity of the PKC system by the experimental finding that a certain isoform can also induce opposite cellular effects in distinct cells. For example, the above mentioned nPKCδ isoform can also promote pro-mitogenic signals in certain cell types (e.g. in breast cancer cells). The functional complexity of the

PKC sytem highlights the importance of the detailed characterization of its isoform-specific regulatory roles in the biology of different cells.

The PKC Sytem in the Skin

Maintainance of the physiological functions of epidermis requires a fine balance between continuous proliferation in the basal layer (renewal of keratinocytes) and terminal differentiation in the upper layers (forming the non-viable cornified layer). Accumulating evidence suggest the pivotal functional role of the PKC system in cuteneous biology. PKC enzymes have been shown to be involved in signaling pathways controlling both proliferation and differentiation of keratinocytes. Studies assessing the specific roles of the individual PKC isoforms have revealed that cPKCα and nPKCδ play a central role in mediating kertinocyte differentiation, whereas nPKCε transmits proliferative signals. Therefore reduced cPKCα/nPKCδ activation and/or enhanced nPKCε activation can both promote the development of neoplastic laesions in the skin by altering the fine balance between cell growth and terminal differentiation. In addition to their central role in keratinocyte biology, PKCs have also been shown to control the cellular functions of different cell types of hair follicles (e.g. inner and outer root sheet, dermal papilla). For example, cPKCα and nPKCδ have been shown to be involved in regulating hair shaft elongation and cycling. Beside cPKCα and nPKCδ several other PKC isoforms have been identified in the above-mentioned cell layers of the hair follicle, which presumably have important regulatory roles in these cells.

The Sebaceous Gland, and its Sebocytes

Although the functional role of the PKC system is well-characterized in epidermal keratinocytes (and hair follicles), intriguingly we lack data on the potential involvement of the enzyme family in the regulation of the biology of

the sebacous gland, the other component of the human pilosebaceous unit. The sebaceous gland is a holocrine gland located in the dermis of the skin, where it is primarily associated with hair follicles (pilosebacous unit). Its main task is the production of sebum (tallow), which is mainly composed of neutral lipids. The secreted sebum covers the surface of the skin, thereby forming a water-repellent protective barrier which ensures impregnation. Recent findings have revealed that functions of the sebocaous gland are not restricted to the formation of a passive cutaneous barrier, as it can greatly contribute to the physiological homeostatic function of the skin via numerous endocrine and paracrine mechanisms. In addition, it also participates in immune responses that help prevent the colonization of the skin by pathogenic bacteria.

In the process of holocrine secretion a portion of the sebocyte pool from the peripherial layer (with high mitotic activity) becomes committed towards terminal differentitation; they start to move towards the center of the acini while accumulating enhanced amounts of lipid droplets in their citoplasm, and eventually disintegrate and release their content into the ducts. The complex network of signal transduction pathways regulating this continuous differentiation program is under the control of a multitude of (neuro)endocrine and paracrine mediators (e.g. androgens, PPAR ligands, growth hormone, insulin, IGF-1, CRH, α-MSH, endocannabinoids, arachidonic acid). The receptors of these mediators have all been identified in sebocytes; it should also be emphasized that the PKC system might be involved in the signaling pathways of these receptors.

Alterations in the normal differentiation program of sebocytes represent key pathogenetic factors in the etiology of many skin diseases (e.g. seborrhoea, acne vulgaris, rosacea, rhinophyma, sebaceous carcinoma). Among these disorders, doubtless, acne vulgaris has the highest prevalence. One of the key pathogenetic factors of this complex disease is the overproduction of sebaceous lipids (hyperseborrhoea) due to hyperactivity of the sebacous gland and

enhanced terminal differentiation. Chronic local inflammation caused by pathological immune responses is another major contributor to the development of acne. Sebaceous glands have also been implicated to play an important role in these inflammatory processes. A wide array of inflammatory signals can induce the production of various pro-inflammatory mediators (leukotrienes, prostaglandins, interleukins) in sebocytes. One of these inflammatory agents is arachidonic acid, which has also been found to stimulate lipid synthesis of sebocytes, thereby representing a critical link between the two major components of acne pathogenesis: (hyper)seborrhoea and local inflammation.

Since primary isolated sebocytes can only be kept in culture for a very limited amount of time, research on sebaceous gland function has been greatly facilitated by the use of immortalized sebocyte cell lines such as e.g. SEB-1, Seb-E6E7 or SZ95, which serve as an excellent surrogate for modeling normal human sebocyte functions. The use of these cell lines has provided a new insight into sebaceous gland biology and have broadened our understanding of the molecular mechanisms regulating different sebaceous functions.

Signaling Proteins Containing Atypical C1 Domains

Beside signaling molecules containing DAG/phorbol ester sensitive, typical C1 domains (see above), several other protein families have been identified to possess atypical C1 domains that fail to bind ligands. In the case of numerous atypical C1 domains (e. g. that of c-Raf and KSR) the explanation for the absence of their ligand affinity is the presence of a grossly distorted binding cleft geometry. Other atypical C1 domains, however, retain the optimal binding cleft structure, which suggests their potential for ligand binding. From the latter subgroup, six signaling molecules (aPKCζ and λ/ι, RasGRP2, Vav1, 2 and 3) containing such unique atypical C1 domain are currently in the research focus of our laboratory. We have recently described the molecular determinants that account for the absence (or severe reduction) of the ligand sensitivity of aPKCs.

In this study we wished to elucidate the structural components that determine the binding potency of Vav1 C1 for DAG/phorbol esters. The detailed characterization of the molecular factors influencing the ligand recognition of various C1 domains (typical and atypical) could further advance the development of selective ligands targeted to the C1 domains of distinct DAG effector proteins (among them members of the PKC family, as well). These C1 ligands would have the intriguing potential to modify the function of these central signal transducers. In addition, the research on C1 domains facilitates the identification of new intracellular targets for appropriately modified DAG/phorbol ester analogs. Finally, our research broadens our general understanding of the complex network of DAG signaling mechanisms, and helps to develop a better insight into the regulatory factors that govern the specific activation of the individual pathways of the system.

The Biological Function of Vav1

Vav1 is a guanine nucleotide exchange factor for the Rho/Rac family of small GTP-ases, which are important molecular transducers in signaling cascades that regulate cytoskeleton organization, cell cycle progression, gene transcription, adhesion, migration, cell growth and survival. In addition to its activity as an exchange factor, the versatile signal transducer can also regulate various other signaling events in a GEF-independent fashion, functioning as an adapter molecule to facilitate protein-protein interactions. The expression of Vav1 is restricted almost exclusively to normal cells of hemopoetic origin, as opposed to the other two members of the mammalian Vav family, Vav2 and 3, which are more ubiquitously expressed. Via its exchange activity and adapter function, Vav1 participates in signaling pathways that regulate T-cell development and antigene induced T-cell activation. In addition, Vav1 also plays a central role in the regulation of various cellular processes of other members of the immune system (e.g. B-cells, macrophages, Natural Killer

cells). Moreover, recent studies have also described a pathogenetic role of Vav1 in solid tumors of non-hemopoetic origin. Although a mutant Vav1 oncogene have never been detected in human cancer, the ectopic expression of the wild-type molecule could contribute to the develpoment and progression of such malignancies as e.g. neuroblastoma, pancreatic ductal adenocarcinoma and melanoma.

The Domain Structure of Vav1 and the Regulatory Factors of Vav1 Function

Vav1 possesses multiple structural motifs that mediate its versatile functions in cellular signaling. The DH domain is responsible for catalyzing GDP/GTP exchange on substrate molecules. The domains surrounding this catalytic DH domain (CH domain, PH domain, Ac motif, C1 domain) regulate the exchange activity of Vav1. In addition, the structure of Vav proteins also contains an SH2-SH3-SH2 casette at the C-terminus, which is an extraordinary feature within the group of GEF proteins. The structural hallmarks of tyrosine phosphorylation pathways confer a unique role on Vav1, enabling the molecule to participate in a variety of signal transduction pathways as a scaffold protein that facilitates protein-protein interactions. Tyrosine phosphorylation, on a highly conserved tyrosine residue (Tyr ^{174}) within the Ac region, has been reported to be one of the most important regulators of Vav1 activity. Phosphorylation of Tyr^{174} relieves the autoinhibition of the DH domain through an intramolecular conformational change, and enables the direct contact of DH with its substrates, resulting in an increase in GEF activity. In addition several other factors have been shown to regulate exchange activity of Vav1. Among these, C1 domain has also been identified as critical for maintaining efficient exchange activity. Initial studies have hypothesized that this regulatory effect would arise from direct contacts between the C1 domain and the substrate molecules (GTPases), however recent crystallographic studies now provide a different explanation. An intramolecular network of contacts between the C1

and the DH domain helps to stabilize the optimal conformation of a critical helix within the DH domain, which is indispensable for efficient GDP/GTP exchange. Due to the lack of ligand affinity, the C1 domain of Vav1 have been classified as atypical. However the above-mentioned crystallographic studies have revealed a striking structural resemblance between the binding pocket of the potent phorbol ester receptor, nPKCδ and that of Vav1. In addition these studies also highlighted that the solvent accessible cavity of the binding cleft of Vav C1 is located in the vicinity of the above-mentioned intimate intramolecular contacts between the DH and the C1 domains. This finding raises the intriguing potential that appropriately modified ligands (synthetic DAG/phorbol ester analogs) might be able to access this binding pocket, and disrupt the essential intramolecular interction, thereby modifying the enzymatic function of Vav1. In this study, we aimed to characterize the molecular factors that determine the ligand binding affinity of Vav1 C1.

AIMS OF OUR STUDIES

In the first part of our studies we wished to characterize the isoform-specific role of the PKC system in the regulation of signal transduction pathways controlling sebacous gland function. Using a human sebacous gland-derived immortalized sebocyte cell line (SZ95) we wanted to find answers to the following questions:

1. Which PKC isoforms are expressed by human SZ95 sebocytes?
2. Using a pharmacological approach, can we detect a functional role of the PKC system in regulating cell proliferation, differentiation and apoptosis of human SZ95 sebocytes?

3. Using different cell- and molecular biological approaches (e.g. RNA interference), can we identify the isoform-specific regulatory functions of the PKC system, in SZ95 cells?

4. Are certain PKC isoforms involved in the signal transduction pathways of the inflammatory precursor, arachidonic acid?

In the second part of our studies we investigated the molecular determinants affecting the DAG/phorbol ester binding properties of the GEF molecule, Vav1, which possesses an atypical C1 domain. Using ligand binding assays, site-directed mutagenesis and molecular modeling we wished to explain the followings:

1. Can the isolated C1 domain of Vav1 bind DAG/phorbol esters?

2. Based on computer modeling can we identify the central structural factors, which can account for the lack of ligand sensitivity of Vav1?

3. Using site-directed mutagenesis can we specify in detail which amino acids are attributable for the failure of Vav1 to bind ligands?

4. Based on our analyses would it be possible to design modified DAG analogs, selective for the unique structure of the C1 domain of Vav1?

EXPERIMENTAL PROCEDURES

Cell culturing

Immortalized human SZ95 sebocytes (derived from facial sebaceous glands) were cultured in Sebomed basal medium supplemented with fetal bovine serum (FBS), 1mM $CaCl_2$, human epidermal growth factor and antibiotics. Human-prostate-cancer-derived LNCaP cells (used for translocation studies)

were cultured in RPMI-1640 medium supplemented with FBS and 2 mM L-glutamine.

Western blotting

Cell cultures were harvested in ice-cold lysis buffer, and homogenized by sonication on ice. After determining the protein concentration, SDS-PAGE buffer (containing SDS and β-Mercaptoethanol) was added to the cell lysates, and their protein content was denatured with boiling. Next, equal amount of protein from each sample was subjected to SDS-PAGE, and then transferred to nitrocellulose membranes. After blocking non-specific protein binding sites, membranes were probed with the appropriate primary antibody against the given PKC isoforms or cytochrome-C. Horseradish-peroxidase-conjugated antibody was used as a secondary antibody, and the immunoreactive signals were visualized with chemiluminescence using an Intelligent Dark Box. Where indicated, signal intensity was analyzed with densitometry, using the Image Pro Plus software.

Immunostaining of PKCs, confocal microscopy

The expression of PKC isoforms was determined with immunocyto-chemistry, and the changes in the subcellular localization of PKCs were assessed using confocal microscopy. In the case of studies focusing on subcellular localization sebocyte cultures, at 60-70% confluency, were firts subjected to pharmacological treatments (but no treatment was performed before the analysis of the expression patterns). Cells were fixed and permeabilized, and subsequently incubated with the appropriate anti-PKC primary antibody. Cover slips were then stained with an FITC-conjugated secondary antibody. Ultimately the PKC expression pattern of sebocytes was examined using a fluorescent

microscope, and the changes in the subcellular localization of certain PKC isoforms were detected with the help of a confocal microscopy system.

Quantitative real-time PCR

Total RNA was isolated from cell lysates using TRIzol. A measure of 3 μg of total RNA was then reverse-transcribed into cDNA using AMV reverse transcriptase and oligodT primers. TaqMan primers and probes were applied for subsequent PCR amplification using the TaqMan universal PCR master mix protocol. As internal controls, transcripts of glyceraldehyde 3-phosphate dehydrogenase were determined.

Assessment of intracellular lipid content

For semi-quantitative detection of sebaceous lipids, cells were cultured on glass cover slips. After treatment cells were fixed in paraformaldehyde and the lipid content of their vacuoles was visualized using Oil red O dye. Nuclei were counterstained with Mayer's hematoxylin. Cover slips were mounted in mounting medium, and examined using light microscopy. For a quantitative measurement of the intracytoplasmic lipid content SZ95 cells were plated onto 96-well plates specifically designed for fluorimetric measurements (black-well/clear bottom). Following treatment, cells were incubated in a buffer containing fluorescent Nile Red dye. The fluorescence intensity of the lipid-bound dye was measured on a fluorescence microplate reader (FLIPR). Neutral lipids were detected at 485 nm excitation and 565 nm emission wavelengths. For polar lipids 540 nm excitation and 620 nm emission wavelengths were used.

Assessment of viability

Viable cell number was assessed by measuring the ability of cellular dehydrogenases to convert the tetrazolium salt MTT to formazan. Cells were

plated onto 96-well plates. After treatment, cells were incubated in MTT solution and the amount of formazan crystals (generated within the cells) was determined colorimetrically at 550 nm. The measured absorbance correlates with the viable cell number.

Assessment of apoptosis

Reduced mitochondrial membrane potential serves as an early indicator of the onset of apoptotic processes. SZ95 cells were plated onto 96-well black-well/clear-bottom plates (see above). After treatment, their mitochondrial membrane potential was determined using the MitoProbe $DiIC_1(5)$ Assay Kit. The fluorescence intensity of the accumulated $DiIC_1(5)$ dye (reflecting the level of mitochondrial membrane potential) was measured on FLIPR (see above) using 630 nm excitation, and 670 nm emission wavelengths. Reduced mitochondrial membrane potential was detected in the event of apoptosis induction. Cell cultures treated with CCCP (carbonyl cyanide 3-chlorophenylhydrazone) served as positive controls.

Assessment of cytotoxicity (necrosis)

The cytotoxic effects of the PKC activators/inhibitors were determined by SYTOX Green labeling. Ruptured plasma membranes enable the penetration (and subsequent double-stranded DNA binding) of the fluorescent dye into necrotic cells, whereas viable cells with intact surface membranes display negligible uptake. Therefore increased SYTOX Green fluorescence intensity serves as an excellent indicator of cell necrosis. SZ95 cells were plated onto 96-well black-well/clear-bottom plates (see above). After treatment, cells were incubated with a solution of SYTOX Green. The fluorescence intensity of the accumulated dye was measured on FLIPR (see above), using 490 nm excitation,

and 520 nm emission wavelengths. Cell cultures incubated with lysis buffer served as positive controls.

RNA interference (siRNA)

SZ95 sebocytes were grown until they reached 40–60% confluence. Cells were then transfected with specific double-stranded small interfering RNA (siRNA) oligonucleotide probes against cPKCα, nPKCδ, and aPKCζ, using Lipofectamine RNAiMAX transfection reagent. Cell cultures transfected with siRNA Negative Control Duplexes (scrambled siRNA; not homologous to any known mRNA sequence) served as negative controls. Immunoblotting was performed daily (for 4 days) to follow the changes in the expression level of the given PKC isoform after transfection. The subsequent cellular assays (assessing lipid content, cell viability, apoptosis, and cytotoxicity) were performed at lowest expression levels.

Construction of GST- and GFP-fused C1 domains of PKCδ and Vav1

The wild-type PKCδ C1b domain fused to glutathione-S-transferase (GST) had previously been constructed in our laboratory (PKCδ-C1b-GST). To generate an isolated, recombinant Vav1 C1 domain fused to GST (Vav1-C1-GST) the following steps were performed: First, the appropriate cDNA sequence was PCR amplified, while incorporating EcoRI recognition sites at both terminus. The full-length cDNA clone of Vav1 served as template. In order to create adhesive ends, the DNA fragments of the PCR reaction were digested with EcoRI, in parallel with the bacterial expression vector, pGEX-5X-1, which served as a GST-containing destination. The C1 fragments were finally ligated into the pGEX-5X-1 plasmid using the EcoRI restriction sites. The recombinant Vav1-C1-GST clone constructed in this step, together with the previously constructed PKCδ-C1b-GST clone, served as templates for generating GFP-

fused C1 domains in the next step. The pGEX-5X-1 plasmids containing the recombinant C1 domain sequences from either PKCδ or Vav1 were digested with EcoRI, in order to cleave out the inserts from the vector. The GFP-containing mammalian expression vector, pEGFP-C2, was also digested with EcoRI. Finally, the inserts (the C1 fragments of either PKCδ or Vav1) were ligated into the pEGFP-C2 vector using the EcoRI restriction sites. The DNA sequences of all GST- and GFP-fused C1 domain constructs were confirmed by sequence analysis.

Site-directed mutagenesis

Point mutations of the amino acid residues at positions 9, 10, 11, 22, 24, and 26 of both PKCδ C1b and Vav1 C1 were introduced using the GeneTailor™ Site-Directed Mutagenesis System. To generate the GST-fused C1 domain mutants of PKCδ and Vav1, the above mentioned wild-type C1 constructs (PKCδ and Vav1) in pGEX-5x-1 were used. Single, double, and triple mutations were introduced in one step, whereas quadruple and quintuple mutants were generated in a stepwise fashion using triple mutants as templates. To generate the GFP-fused C1 domain mutants the corresponding GST-fused C1 mutants (PKCδ or Vav1) in pGEX-5X-1 were used as a template. The inserts with the appropriate mutations were cleaved out of the pGEX-5X-1 expression vector, using the EcoRI restriction sites, and finally ligated into the above mentioned pEGFP-C2 vector. The presence of mutations was verified by DNA sequence analysis.

Construction of full-length Vav1 (wild-type, triple- and quintuple mutant) fused to GFP

The GFP-tagged wild-type Vav1 was generated using the pENTR™ Directional TOPO® Cloning Kit. PCR amplification of the appropriate full-

length Vav1 sequence was first carried out, using the cDNA clone of Vav1 as a template. The PCR product was then TOPO-cloned directly (i.e. without a prior digestion step) into a pENTR/D-TOPO vector, which served as an Entry clone. Next, using the LR recombination reaction of the Gateway method, we transferred the appropriate full-length Vav1 sequence from the Entry vector into a mammalian destination vector (pcDNA-DEST53) encoding an N-terminal GFP-tag. The mutants of the full-length Vav1 were generated using the GeneTailor[TM] Site-Directed Mutagenesis System described above for the C1 domain mutants. The DNA sequences of the GFP-tagged full-length constructs were verified by sequence analysis.

Expression and Purification of GST-tagged C1 Domains from E. coli

The C1 domains of both PKCδ and Vav1 in the pGEX-5X-1 plasmid were transformed into BL21-AI, a chemically competent *E. coli* strain. Transformants were grown at 37°C until the optical density of the bacterial suspension reached 0.6-0.8. Expression of the GST fusion proteins was induced with isopropyl-O-D-thiogalactopyranoside and L-arabinose. Bacterial cells were then subjected to sonication in B-PER Bacterial Protein Extraction Reagent. The expressed GST-tagged C1 proteins were purified using a B-PER GST spin purification kit. Purified fusion proteins were stored in 30% glycerol at -80°C.

Translocation of GFP-labeled proteins

LNCaP cells were plated on special Ibidi dishes. After 48 h in culture, cells were transfected with GFP-tagged recombinant constructs, using Lipofectamine reagent and Plus Reagent. Cellular expression of fluorescent fusion proteins was examined 24 h after transfection on a confocal microscopy system with excitation from a laser tuned to 488 nm and emission collected with a BP 500-530 nm filter. Intracellular translocation of the GFP labeled proteins upon PMA

treatment was detected sequentially after the administration of the drug. Images were acquired every 30 sec for 30 min using a 63x oil immersion objective. To quantify the degree of translocation in response to treatment confocal images were analyzed according to the appropriate algorithm, using Zeiss AIM software.

Radioligand ([^{3}H]PDBu) Binding Assays

To assess the affinity of the different C1 domains to phorbol esters, purified proteins were subjected to an *in vitro* [^{3}H]PDBu binding assay. Increasing amounts of the radioactive ligand were added to the assay mixture containing the receptor (GST-tagged recombinant C1 domains) and phosphatidylserine as a cofactor. Incubation was carried out at 37 °C for 5 min. The receptor-ligand complex was then precipitated with 35% polyethylene glycol, and separated from the rest of the assay mixture with centrifugation. The concentrations of the free ligand (in the supernatant) and that of the receptor-bound fraction (in the pellet) were determined based on their radioactivity by scintillation counting. With the help of the measured concentration values we were able to calculate specific binding at various ligand concentrations. Based on the specific binding values dissociation constants (K_d values) of each receptor-ligand complex could be determined, which represented the affinity of the given C1 domain (receptor) to the phorbol ester ligand. Competitive binding assays (a modified version of the above method) were carried out to assess the affinities of the C1 domains to non-radioctive ligands (DAG-lactones). In this version of binding assay non-radioactive ligands compete for the same binding site with [^{3}H]PDBu, and their affinities can be specified relative to [^{3}H]PDBu. Using this method, inhibitory dissociation constants (K_i values) were determined, which represented the affinity of the given C1 domain (receptor) to the non-radioactive ligands, DAG-lactones.

RESULTS

Pharmacological Modulation of PKC Activity Alters Lipid Synthesis but Does not Influence Cell Viability of SZ95 Sebocytes

Using immunocytochemistry, Western blot and Q-PCR we demonstrated the expression of the following five isoforms of the PKC family in SZ95 cells: cPKCα; nPKCδ, ε, és η; and aPKCζ. (The expression of other members of the enzyme familiy could not be identified by the above techniques.) In addition, Q-PCR also revealed that among the above five isoforms the cPKCα, nPKCδ and aPKCζ exhibited high expression, whereas the expression levels of nPKCε and η were low. Next, we investigated the effect of the activation of the PKC system on cellular processes reflecting terminal differentiation of sebocytes (lipid synthesis, apoptosis). Nile Red staining-based quantitative fluorimetric analysis revealed that treatment with the general PKC activator PMA increased the neutral lipid content of SZ95 sebocytes in a dose-dependent manner. Subsequent experiments using simultaneous treatments with various PKC inhibitors (GF109203X, Gö6976 és Rottlerin) suggested the involvement of multiple PKC isoforms in mediating the cellular effects of PMA, since each of the above inhibitors was able to inhibit the PMA-induced stimulation of lipid synthesis. It should also be noted that none of the inhibitors by itself (i.e. without PMA) affected the lipid content of sebocyte cultures, which suggests that the endogenous PKC activity does not influence basal lipid synthesis of SZ95 cells. In addition, none of the pharmacological modulations of PKC activity altered cell viability or induced cell death of any form (apoptosis or necrosis).

The Ability of PMA to Stimulate Lipid Synthesis of SZ95 Sebocytes Is Mediated by cPKCα and nPKCδ

After the experiments using pharmacological approaches, we next carried out a series of cellular imagaing and molecular biology experiments in order to characterize in detail the isoform-specific roles of PKC isozymes showing highest expression levels in sebocytes. Using confocal microscopy and Western blotting we investigated the occurance of translocation and the down-regulation of a given PKC isoform upon phorbol ester treatment, which can be regarded as a hallmark of the activation of that specific isoform. The results of these experiments clearly demonstrated that upon the application of PMA the α and δ isoforms became activated: i) one hour after treatment with the ligand (10 nM PMA) we were able to detect changes in the subcellular localization of cPKCα and nPKCδ (translocation into cell membrane, nuclear membrane and other intracellular membranes); ii) after long-term treatment with PMA the expression level of the above two isoforms reduced dramatically due to down-regulation (from the second day after treatment). However, as expected, no translocation or down-regulation could be detected for aPKCζ, upon phorbol ester treatment. The involvement of of cPKCα and nPKCδ in mediating the cellular effects of PMA was further investigated by RNA interference technique. After demonstrating (using Western blot) that the isoform-specific siRNA probes effectively and selectively suppress the expression of their "targeted" isoforms, we tested the lipid synthesis of the transfected sebocyte cultures (using Nile Red labeling) under basal conditions and after phorbol ester treatment. Selective "knockdown" of either cPKCα or nPKCδ significantly reduced the cellular effect of PMA to increase the neutral lipid content of the cells. Based on these results, we can conclude that cPKCα and the nPKCδ are both involved the signaling pathways of PMA mediating lipid synthesis of seobcytes. However, silencing the expression of either of these isoforms did not modify the basal lipidsynthesis and the cell viability of SZ95 sebocytes, and it did not induce apoptosis or necrosis either. A further intruiging finding of these experiments is that the siRNA-mediated silencing of aPKCζ (in clear contrast to the findings

obtained with cPKCα and nPKCδ) markedly increased the basal lipid synthesis of sebocytes, and furthermore, suppressed cellular viability and induced apoptosis (but not necrosis). These resuts suggest that the endogenous (and constitutive) activity of aPKCζ is key regulatory factor in signaling pathways inhibiting terminal differentiation (lipid accumulation and apoptosis) and promoting sebocyte proliferation and survival.

The Cellular Effects of Arachidonic Acid is Mediated by nPKCδ Activation in SZ95 Sebocytes

In aggreement with previously described findings of the laboratory, our current studies revealed that (among the many endocrine and paracrine mediators capable of regulating sebocyte differentiation) arachidonic acid, an inflammatory precursor, induces a marked lipid synthesis in sebocytes, which resembled (and exceeded) the above-mentioned effect of PMA. Our pharmacological approach suggested that among the three PKC isoforms, exhibiting highest expression in SZ95 sebocytes, nPKCδ might be involved in the signaling pathways mediating the lipogenic effect of AA, since the δ-specific inhibitor Rottlerin (along with GF109203X, inhibitor of classical and novel isoforms) significantly (yet only partially) abrogated the AA-induced lipid accumulation. However, Gö6976, the inhibitor of classical PKC isoforms, was not able to hinder this effect of AA, suggesting that cPKCα most probably does not participate in mediating the lipogenic effect of AA. Beside inducing lipid synthesis in sebocytes, AA have also been described to induce apoptosis in these cells. Using $DilC_1(5)$-assay to detect the reduction in mitochondrial membrane potential (an early indicator of the onset of apoptotic processes), we found that the above-mentioned two inhibitors (Rottlerin, and GF109203X) effectively prevented the AA-induced apoptotic events, as well. To characterize in detail the isoform-specific involvement of certain PKCs in the action of AA, we performed a series of cellular- and molecular biology experiments, analogous to

the previous ones described above for PMA. Confocal microscopy analysis (following short-term AA-treatment) and Western immunoblotting (after long-term AA-application) revealed that the AA-administration selectively translocated and down-regulated the δ isoform, (whereas the subcellular localizations and the expression levels of the other two isoforms, α and δ, were not changed by AA), suggesting selective nPKCδ-activation by the inflammatory mediator. Subsequent experiments using RNA interference further supported the above findings on the central role of nPKCδ in AA signaling. The effect of AA to stimulate lipid synthesis and to induce apoptosis was significantly suppressed in sebocyte cultures with silenced expression of nPKCδ, whereas the siRNA-mediated knock-down of cPKCα had no influence on the cellular effects of AA, and the silencing of aPKCζ by itself already resulted in an enhanced lipid accumulation and marked apoptosis. These results collectively argue for the selective involvement of the δ isoform in the signaling pathways promoting the lipogenic and apoptosis-inducing effect of AA

The Isolated C1 Domain of Vav1 Does Not Bind Phorbol Esters, Despite Conservation of the Appropriate Geometry of the Binding Pocket.

Recent x-ray crystallographic data have revealed a striking structural resemblance between the binding celft of Vav1 C1 and that of PKCδ C1b. Based on these findings, could the C1 domain of Vav1 have the potential to bind DAG/phorbol esters (similarly to other DAG effectors that possess C1 domains with high ligand affinity)? Our computer modelling revealed that phorbol 13-acetate (a prototypic phorbol derivative) is predicted to be able to bind to the Vav1 C1 domain with essentially the same binding mode as in the PKCδ C1b domain, because the pattern of intra-, and intermolecular hydrogen bonding interactions formed by the bound ligand is identical in the two C1 domains. These results suggest that the C1 domain of Vav1 could be capable of accomodating DAG or phorbol ester ligands. However, despite the preserved

geometry of the binding cleft, and the similarities in the binding mode of phorbol-13 acetate between Vav1 and PKCδ C1b (as revealed by the above molecular models), Vav1 has been shown experimentally not to interact with DAG or phorbol esters. Similarly to previous studies on the full-length Vav1, our experiments found no evidence, either, for ligand interaction in the case of the isolated C1 domain of Vav1. On the one hand, the GST-fused C1 domain of Vav1 (expressed and purified from a bacterial system) showed no affinity to the radioactive phorbol ester ligand, [³H]PDBu, *in vitro*. On the other hand, using the GFP-labeled version of Vav1 C1 (expressed in prostate cancer-derived cells, LNCaP) no translocation could be detected upon phorbol ester treatment, *in vivo* (in contrast to findings with PKCδ C1b, which served as a control in this experiment). These results unambiguously suggest that the molecular determinants accounting for the lack of ligand sensitivity are located within the structure of the C1 domain of Vav1, and the failure to bind ligands is not attributable to other structural domains preventing access of ligands by occluding the binding cleft of the C1 domain.

Characterization of the Molecular Factors Accounting for the Reduced Phorbol Ester Sensitivity of Vav1 C1, Using PKCδ C1b Mutants Possessing Vav1-C1-like Mutations

To determine the possible molecular components responsible for the lack of ligand sensitivity of Vav1 C1, we first performed a comprehensive sequence alignment of the atypical C1 domain of Vav1 *versus* all typical C1 domains described in the literature. Our analysis highlighted six unique residues (Glu[9], Glu[10], Thr[11], Arg[22] Thr[24] és Tyr[26]) around the tip of the apparent binding cleft, which are present in the Vav1 C1 structure only. These residues could reduce ligand binding without grossly distorting the appropriate binding cleft geometry, presumably by impeding contacts between the C1 domain and the lipid bilayer, thereby impairing stable membrane association and disrupting the formation of

the ternary C1-ligand-membrane binding complex. A plausible explanation for inhibition of membrane insertion is that five (Glu^9, Glu^{10}, Thr^{11}, Arg^{22} Thr^{24}) out of the above six unique residues could reduce the lipophilicity of the apex of the binding cleft, and the sixth residue (Tyr^{26}) could further weaken the C1 domain-membrane interaction by failing to interact with anionic phospholipid head groups (according to previous structural studies, the ability of positively charged residues (Arg^{26}, Lys^{26}, His^{26}), exposed in the middle third of the C1 domain, to establish such interactions with the acidic phospholipids seems to be another substantial factor stabilizing membrane association). To address this hypothesis, our initial approach was to use the C1b domain of PKCδ as a template. Using site-directed mutagenesis, we first mutated the original residues of PKCδ to the corresponding residues of Vav1-C1 at the above positions, and then determined the ligand sensitivity of the various single- and multiple site mutants using radioactive binding assays (purified GST-fused C1 mutants), and intracellular translocation studies (GFP-labeled C1 mutants; confocal microscopy). Our results with the single-site mutants (M9E, S10E, P11T, W22R, L24T, és K26Y) showed, that the introduction of five (Glu^9, Glu^{10}, Thr^{11}, Thr^{24} és Tyr^{26}) out of six Vav1-unique residues (mentioned above) caused a decrease in the binding affinity of PKCδ C1b for phorbol ester, whereas the presence of Arg^{22} did not change its potency for the ligand. However, the reduction in the ligand sensitivity, detected in all five single site mutants, was only of moderate degree (5-10-fold reduction compared with the wild type construct), as revealed by *in vitro* binding assays. These results argue that none of the five unique residues by itself accounts for a crucial structural distortion that would cause a significant reduction in the binding affinity. However, the combination of five Vav1 C1-like mutations did in deed result in a dramatic decrease in ligand sensitivity. Double- and triple mutations at the apex of both loops of the binding pocket (M9E/S10E és M9E/S10E/P11T at the N-terminal loop and L24T/K26Y at the C terminal loop), reduced the binding potency of the C1b domain for [^{3}H]PDBu by

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a considerable 2-3 orders of magnitude (a range of 250-1000-fold decrease) in the radioactive binding assays.

The ligand affinities of PKCδ C1b constructs incorporating multiple Vav1-like mutations were also analyzed in translocation assays, using a GFP-labeled version of the PKCδ C1b mutants, expressed in LNCaP cells. The results obtained in these *in vivo* experiments further support the previous findings of the *in vitro* binding studies, as the double and triple mutants (mentioned above) showed much weaker translocation responses after phorbol ester treatment, compared with their wild type counterpart (partial redistribution with significantly slower kinetics; complete absence of response at low ligand concentrations). Finally, the combined introduction of all five Vav1 C1-like mutations (M9E/S10E/P11T/L24T/K26Y) into PKCδ C1b completely abolished the ligand sensitivity of the domain, as we were unable to detect any signs of phorbol ester binding with either the radioactive binding assays or the translocation studies. We can conclude that the five unique residues in the binding pocket, although by themselves not causing crucial structural distortion and significant reduction in binding afinity, are together sufficient to account for the complete absence of binding potency of Vav1 C1 for phorbol ligands, presumably by interfering with the processs of effective membrane insertion of the receptor-ligand complex.

The Introduction of PKCδ-C1b-like Mutations Transformed the C1 domain of Vav1 Into a Phorbol-binding Receptor

In case the introduction of Vav1 C1-like residues into the binding cleft of PKCδ C1b dramatically reduces its binding potency for phorbol esters, can we expect that (in the opposite case) PKCδ C1b-like "back"-mutations (analogous to previous "forward" mutations) would confer high binding potency on the non-ligand-responsive WT Vav1 C1? To address this question, we introduced PKCδ C1b-like back-mutations at the above mentioned five key positions (Glu[9], Glu[10],

Thr[11], Thr[24] és Tyr[26]) into the binding pocket of Vav1 C1, and, similarly to PKCδ C1b mutants, evaluated the ligand sensitivity of the generated mutants using radioactive binding assays (purified GST-fused Vav1 C1 mutants) and translocation studies (GFP-labeled Vav1 C1 mutants, confocal microscopy). We found that in the presence of the five PKCδ C1b-like residues the C1 domain of Vav1 (which showed no detectable ligand binding in the absence of these mutations) acquired a strikingly high binding potency for phorbol esters. The dissociation constant of the quintuple Vav1 C1 mutant (Vav1 C1 E9M/E10S/T11P/T24L/Y26K), as revealed by binding assay, was almost identical to that of the WT PKCδ C1b. In addition it also displayed a quick and complete translocation response even at low PMA concentrations, reflecting high phorbol ester sensitivity, *in vivo*, comparable with that obtained with PKCδ. These results confirm that the geometry of the binding pocket of Vav1 C1 is conserved and structurally compatible with ligand binding, but the presence these five unique residues at the rim of the binding pocket hinder the association of the C1 domain with the cellular membrane, thereby weakening the molecular interactions responsible for stabilizing the ternary binding complex, which in turn reduces the affinity of Vav1 C1 for DAG/phorbol esters. Another important finding of our studies is that replacing the unique residues of Vav1 at positions 9-11, 24 and 26 (of its C1 domain) with PKCδ C1b-like amino acids conferred phorbol ester sensitivity on the full-length Vav1 protein as well, as the full-length quintuple Vav1 back-mutant (Vav1 E9M/E10S/T11P/T24L/Y26K) displayed intracellular translocation upon PMA treatment (in contrast to the wild type version of the full-length Vav1). Hence the binding pocket within the C1 domain of Vav1 is accessible to ligands in the context of the full-length protein as well, and is not occluded by other structural domains. To elucidate whether all five unique residues contribute uniformly to the lack of binding potency in the WT Vav1 C1(as suggested by the results obtained with the single site mutants of PKCδ C1b), we next generated

quadruple Vav1 C1 mutants ((-E9M, -E10S, -T11P -T24L -Y26K; the antecedent negative sign refers to the missing mutation compared with the quintuple Vav1 mutant) and analyzed the individual contribution of each of the five residues to the absence of ligand binding. (In this set of experiments the quadruple back-mutants of Vav1 can be considered analogous to the single site mutants of PKCδ C1b of our previous experiment since all of them possessed one residue less than the most potent binding cleft geometry.) The results with the quadruple Vav1 C1 mutants suggest that in the backbone structure of Vav1 C1 Glu^9 and Thr^{11} affect ligand binding more significantly than do the other three unique amino acids (Glu^{10}, Thr^{24} és Tyr^{26}), as opposed to that in the context of PKCδ C1b. In contrast to the findings observed with the single site mutants of PKCδ C1b, the presence of Glu^9 and Thr^{11} caused a more severe reduction in the binding potency of the given quadruple Vav1 C1 back-mutant (compared with the quintuple Vav1 C1 mutant) than did the other three quadruple mutants possessing Vav1 C1-like residues at positions 10, 24 and 26.

The correlation between molecular lipophilicity and phorbol ester sensitivity

A feasible interpretation of the effect of the identified crucial residues (Glu^9, Glu^{10}, Thr^{11}, Thr^{24} és Tyr^{26}) on the ligand sensitivity of Vav1 C1 was that these amino acids together significantly hinder the succesful insertion of the apex of the binding cleft into the membrane, due to their inability to facilitate intermolecular interactions with the lipid bilayer. Since hidrophobic interactions represent a substantial factor for lipid association, we built computer model structures for assessing the differences in lipophilicity at the tip of the binding clefts between various C1 domain (PKCδ C1b and Vav1 C1) mutants. The term "molecular lipophilicity potential" was introduced to quantify the lipophilicity of these binding pockets, and each of our C1 domain mutants were investigated using a uniform algorithm for calculations. The results of these calculations clearly demonstrate that there exists a strong (although not a perfectly linear)

relationship between the MLP values of the C1 domain mutants and their ligand sensitivity. At one end of the spectrum one can find C1 domains with high lipophilicity potential on the binding site surface (e.g. WT PKCδ C1b and quintuple Vav1 C1 mutant), which therefore also display high potency for phorbol esters. At the other end of the spectrum binding pockets of C1 domains (e.g. WT Vav1 C1 and quintuple PKCδ mutant) possess low lipophilicity potential, and, as a result, undetectable binding affinity. Between these two extremities there is a wide range of C1 domain mutants with intermediate binding site lipophilicity, which, as a results, show weakened (to a variable degree) but still detectable ligand affinity. Our modeling results also argue that none of the five crucial amino acids in the Vav1 C1structrure is by itself capable of causing a dramatic decrease in surface lipophilicity. The cumulative effect of these residues, however, is sufficient to significantly reduce the lipophilicity at the tip of the binding pocket of Vav1 C1, thereby impeding hidrophobic interactions between the C1 domain and the lipid bilayer, which in turn significantly hinders the stable insertion of the receptor-ligand complex into the membrane and reduces ligand sensitivity.

Initial steps of the development of novel DAG analogs targetting the specific structural features of Vav1 C1

Our computer models depicting the geometrical structure of the C1 domain of Vav1 in the context of the neighbouring DH and PH domains clearly demonstrated that the C-terminal loop making up the bindig cleft of Vav1 C1 is located in close proximity to the critical α6 helix of the DH domain. Therefore a prediction is that an appropriately bulky ligand binding to the C1 domain should disrupt interactions with the DH domain. Since these intermolecular interactions play a substantial role in stabilizing the appropriate conformation of the critical α helix within the DH domain (which is essential for optimal exchange activity), such a ligand might be expected to inhibit the enzymatic function of Vav1. In

the next experiment we wished to explore whether appropriately modified DAG analogs, targetting the unique structure of Vav1 C1 could access its binding pocket and thereby function as selective Vav1 inhibitors. Since the above results of site directed mutagenesis and modeling clearly suggest that eliminating the charged Glu residues (at position 9 and 10) of the C1 domain is neccessary (although not sufficient) for restoring the binding affinity of Vav1, we first tested synthetic DAG derivatives (DAG-lactones) bearing a positively charged side chain. We theorized that the positive charges carried by these ligands might be able to "cancel out" the negative charges of the Glu residues, thereby increasing the lipophilicity of the apex of the binding pocket, which in turn could facilitate the insertion of the C1 domain to the membrane bilayer. To test this theory we used a series of DAG lactones possessing positively charged guanidinium (**6c**) or pyridinium (**8a-f**) moieties on their side chains. To test the relative selectivity of these compounds for the unique structure of Vav1 C1 we performed in vitro competition assays, using the double and the triple mutants of PKCδ C1b (M9E/S10E and M9E/S10E/P11T) as a surrogate. (Since this method is based on competition of ligands for a relatively potent binding pocket, the WT Vav1 could not be investigated directly in this assay.) Our studies identified two compounds (DAG-lactones **6c** and **8f**), which showed a significantly smaller reduction of affinity for the double and triple mutants compared with the decrease obtained with the prototypic phorbol ester [3H]PDBu for the same mutants. (Compared with their affinities for the WT PKCδ C1b domain, compound **6c** displayed a 12-fold and 17-fold, whereas compound **8f** showed a 5-fold and 5.5-fold reduction of affinity for the double and triple mutants, respectively; as opposed to the 261-fold and 968-fold reduction measured for [3H]PDBu). Based on these results we can conclude that the relative binding affinities of these two charged for the C1 domains carrying Vav1-specific structural features significantly exceeds that of [3H]PDBu for the same structure. In addition, our molecular models clearly demontrate that the Glu[10] in the N-

terminal loop of the binding pocket can easily form a salt bridge to both the charged nitrogen in the pyridinium group of DAG-lactone **8f** and the guanidium group of DAG lactone **6c**. These results suggest that DAG -lactones carrying appropriate side chains are in principle structurally capable of forming ion-pair intractions with the charged residues in the C1 domain binding site. While these compounds represent only an early stage in the design of ligands targeting the special structure of the C1 domain of Vav1, they support the concept that appropriate interaction with the unique negatively charged residues at positions 9 and 10 may represent one element in the strategy.

DISCUSSION

One of the most important effectors of the intracellular second messenger, DAG, is the family of protein kinase C. Members of the enzyme family differentially (and often antagonistically) regulate signaling pathways of various cell-types that control such substantial biological processes as cell proliferation, differentiation and apoptosis. The detailed characterization of specific functions of distinct isoforms is undoubtedly essential for future therapeutic purposes. A large number of studies argue for the central role of the PKC system in the biological processes of the skin. Experiments on epidermal keratinocytes (including previous findings of our laboratory) highlight the functional antagonism of certain isoforms in the regulation of cell growth and differentiation (cPKCα and nPKCδ mediate terminal differentitation and apoptotic processes, whereas nPKCε and cPKCβ transmit proliferative signals). To further advance our understanding of the role of the PKC system in cutaneous biology our current study describes the functional involvement of PKCs in the cellular processess of sebaceous gland derived SZ95 sebocytes, highlighting the characteristic isoform-antagonism found in this cell type as

well. After the identfication of the specific isoform pattern (i.e. highly expressed cPKCα, nPKCδ and aPKCζ and poorly expressed nPKCε and η) of sebocytes (as revealed by Western blotting, Q-PCR and immunocytochmistry), we showed that the activation of α and δ isoforms mediates terminal differentiation, whereas aPKCζ is involved in signaling pathways promoting cell viability. First we described that the activation of the PKC system by phorbol ester stimulated the lipid synthesis of sebocytes. Next, using a pharmacological approach, as well as different cellular- and molecular biology studies, we highlighted the involvement of cPKCα and nPKCδ in the cellular effects of phorbol ester stimulation. As increased lipid accumulation during holocrine sebum production is one of the key hallmarks of sebocyte differentiation, these findings are in agreement with previous results, describing the central role of cPKCα and nPKCδ in promoting differentiation of various cell types of the skin.

However, neither the siRNA-mediated "knockdown" nor the pharmacological inhibition of cPKCα and nPKCδ resulted in alterations in basal lipid synthesis (and cellular viability) of sebocytes, arguing that the endogenous activities of these two isoforms are not crucial for regulating differentiation (and basal growth) of sebocytes. Nevertheless PKC-activity might be involved in mediating the cellular effects of numerous endocrine and paracrine mediators. Beside androgens, PPAR ligands and certain (neuro)endocrine mediators (CRH, ACTH, GH, insulin) various paracrine mediators have been described to stimulate lipid synthesis of sebocytes (similarly to PMA). Among the paracrine lipidmediators endocannabinoids have previously been shown (by our laboratory) to influence lipid synthesis and apoptotic processes of sebocytes via the engagement of a specific cannabinoid receptor, (CB2), expressed by sebocytes. Another paracrine lipidmediator, arachidonic acid, (which is structurally related to endocannabinoids) have previously been described by several laboratories to stimulate the activity of PKC enzymes. In addition the inflammatory precursor (generated by phospholipase A_2 from membrane lipids)

have also been shown to stimulate lipid accumulation, cell differentiation and apoptotic processes of sebocytes. Moreover, AA has also been implicated in sebocytes to induce the production of various inflammatory mediators generated by the cyclooxygenase and lipoxygenase pathways (LTB$_4$, PGE$_2$) as well as the release of certain pro-inflammatory citokines (IL-6, IL-8). These results suggest that cellular signaling pathways induced by AA (and its derivatives) can have a substantial role in certain skin diseases associated with altered sebocyte function (e.g. seborrhoea, acne etc.), resulting in enhanced lipid synthesis, increased sebocyte turnover and elevated levels of sebocyte-derived inflammatory mediators. Our experiments revealed that among the three isoforms, exhibiting highest expression in sebocytes, nPKCδ is a key component in mediating the cellular effects of AA, because: *i)* nPKCδ was shown to be selectively activated by AA treatment (as revealed by the assessment of translocation and down-regulation); and *ii)* only in the case of nPKCδ-silencing could we detect the suppression of the lipogenic and apoptosis-inducing effects of AA (as opposed to silencing of the other isoforms, which had no such effect). Moreover, our findings also revealed that (in contrast to α and δ isoforms) aPKCζ presumably inhibits the induction of lipid synthesis (terminal differentiation) and apoptotic processes of SZ95 cells, thereby promoting cell survival and (physiological) proliferation of sebocytes. Through the characterization of the isoform-specific signaling pathways we have gained insight into the complex regulatory mechanism of the PKC system in a new cell type, the sebocytes; this could have important therapeutic implications in the future for sebocyte-derived diseases.

Altered sebocyte functions (e.g.: sebaceous hyperplasia, sebum overproduction, hyperproliferation) represent key pathogenetic factors in the etiology of several skin diseases (e.g,: seborrhoea, acne, rosacea, rhynophyma, sebaceous carcinoma). The most prevalent (by far) of the above diseases is *acne*, which has a fairly complex pathogenesis. Previous studies unambigouly suggest the pivotal pathogenetic role of AA in mediating the stimulation of lipid

synthesis, the induction of apoptosis and the secretion of elevated levels of inflammatory mediators in sebocytes. Our current findings demonstrate that nPKCδ participates in the signal transduction of this key mediator, whereas cPKCα might be involved in other signaling mechanisms resulting in increased lipid accumulation. In clear contrast, aPKCζ-activity could effectively hinder cellular processes inducing terminal differentiation. The therapeutical implications of these findings are straightforward. "Anti-acne" therapy would require the suppression of sebum overproduction, which could theoratically be achieved either by the selective inhibition of nPKCδ-activity or by increasing the activity of the antagonistic isoform, aPKCζ (using selective ζ agonists). In contrast, the neoplastic overgrowth of sebocytes would require the inhibition of aPKCζ activity and/or the activation of PKCδ/α function, because both of these approaches could induce the terminal differentiation of the cells and decrease their proliferative potential, thereby suppressing tumor growth.

To modulate the kinase activity of PKC enzymes different pharmacological strategies have been described: *i)* inhibition of the catalytic domain by kinase inhibitors (e.g. staurosporin); modulation of the enzymatic activity through the C1 domain (e.g.: phorbol esters); *iii)* silencing the expression of certain PKC isoforms using selective siRNA oligonucleotides (e.g.:aprinocarsen). The first and the third approaches cause enzyme-inhibition, whereas the second approach results in the activation of PKCs. However, taking into account the functional antagonism of different PKC isoforms, it is evident that the activation of an antagonistic isoform may achieve the same end result as the inhibition of the complementary isoform. The existence of multiple signaling proteins with characteristic C1 domains, markedly increases the challenges for designing selective compounds capable of modulating PKC activity through interaction with the C1 domain. In addition to the PKC family (which was the first described DAG-effector family) six other families of signaling proteins have been recognized with C1 domains responsive to DAG/phorbol esters. Although

the challenges of selective drug design increase with the larger number of C1 domain-containing proteins, it should be ephasized that the identification of such molecules also offers new potential targets for selective C1 domain ligands. Since (similarly to PKCs) these signaling proteins control such essential cellular processes as proliferation, differentiation, apoptosis and neurotransmission, the modulation of their activity promises great therapeutic opportunities.

The research focusing on the design of selective C1 domain-ligands follows two main strategic approaches. One approach is directed at the detailed characterization of the structure-activity relationships of different ligands. This strategy attempts to dissect the molecular determinants (e.g. the chemical structure, location and size of moieties of side-chains) affecting the affinity and selectivity of a specific ligand molecule. DAG-lactones provide a powerful synthetic platform for manipulating ligand structure. These cyclic DAG-derivatives are relatively easy to synthesize and with optimization can reach high affinities towards C1 domains. The incorporation of an almost infinite combination of possible side chain structures (using combinatorial chemistry) offers great opportunity to test various determinants of binding affinity.

A complementary approach to further advance the development of selective ligands targeted to the C1 domains is the characterization of the structural factors influencing the ligand recognition of the binding pocket of the receptor module, the C1 domain. These studies of our laboratory have focused attention on a special group of C1 domain-containing proteins, that possess a unique non-sensitive (atypical) C1 domain structure. These atypical C1 domains (e.g. in aPKCζ and ι; RasGRP2; Vav 1, 2, 3) are special in the sense that their binding pocket does not show a distorted structure (as seen with e.g. c-raf) and retain a geometry apparently compatible with high ligand sensitivity. The identification of factors responsible for their reduced/absent ligand affinity can greatly facilitate the generation of selective, high affinity C1 domain-ligands. Based on the intriguing conclusions of this series of experiments we might in the future be

capable of designing C1 domain-ligands that: *i)* are capable of differentiating between highly homologuous typical C1 domains, and/or *ii)* target atypical C1 domain structures, non-responsive to conventional C1 domain ligands (e.g. phorbol esters).

From the above-mentioned unique group of proteins the Dissertation describes the ligand affinity of Vav1, a member of the Vav family of guanine nucleotide exchange factors. Our findings clarify the molecular basis for the absence of its binding potency for DAG/phorbol esters. We identified four hidrophilic residues in the "upper third" of the binding pocket that impair the membrane association of the receptor-ligand complex by reducing the lipophilicity of the apex of the cleft. In addtition, we identified another residue (Tyr^{26}), under the apical region, which further contributes to the inhibition of binding by its inability to interact with anionic phospholipid head groups of the lipid bilayer (as opposed to basic residues found in this annular region below the top third of typical C1 domains). Since interacton with the lipid membrane is a critical requirement for high affinity binding, it is evident that all factors that hinder the formation of the ternary C1 domain-ligand-membrane complex will reduce the ligand sensitivity of the given C1 domain. Similarly to Vav1, we were able to partially determine the structural factors that acount for the dramatically reduced ligand sensitivities of the other two members of the Vav family, along with that of RasGRP2. Although the exact mechanism for the lack of their binding potencies remain to be elucidated, our results suggest that the C1 domain of these signaling proteins (together with Vav1, aPKCζ és az aPKCι) belong to a new subclass of atypical C1 domains. Members of this subclass can clearly be distinguished from proteins containing atypical C1 domains with a structurally distorted binding pocket, as these molecules retain a compatible binding cleft geometry, which is potentially accessible for ligands. The investigation of the growing number of signal-proteins classified into this subgroup could provide invaluable information on the C1 domain structure-

activity relationship, which in turn could greatly facilitate the design of new pharmacological compounds capable of modulating the function of C1 domain-containing signaling molecules. In addition, the compatible binding cleft geometries of these atypical C1 domains represent new therapeutic targets for DAG/phorbol ester derivatives designed to interact with C1 domains. These C1 ligands would in turn have the intriguing potential to pharmacologically modulate the enzymatic activity of such proteins, provided that their C1 domains display a regulatory function in the enzymatic activity.

The C1 domain has previously been demonstrated to play a critical role in the enzymatic function of Vav1. In addition, recent crystallographic studies have revealed a complex network of intramolecular contacts between the C1 (and the PH) domain and the DH domain, which are essential to stabilize the DH domain in a conformation that facilitates maximal exchange ativity. Since several residues (Leu[20], Arg[22], Phe[25]), located at (or in close proximity) of the rim of the binding pocket, have been shown to form very significant intramolecular interactions with the α6 helix of DH domain, a plausible hypothesis is that ligand binding to the cleft might induce a conformational change that could disrupt these intimate DH-C1 connections. As a result, the optimal conformation of the DH domain would no longer be stabilized by these interaction, which would eventually reduce the exchange activity of Vav1. (This hypothesis is supported by computer modeling, which demonstrates that docking of phorbol 13-acetate into the binding cleft would cause a steric clash between certain residues of the α3 and α6 helices of the DH domain and certain parts of the ligand. Thus stable binding of phorbol 13-acetate would lead to a conformational change of the DH domain.) Therefore, if we were able to generate synthetic ligands with high affinity to the C1 domain of Vav1, a strong prediction is that these compounds would function as inhibitors of Vav1 activity (by disrupting the above-mentioned DH-C1 interactions).

To begin to explore this concept, we tested a series of DAG-lactone derivatives that contained a positively charged side chain. We hypothesized that these positive charges might be able to neutralize the negative charges of the Glu residues located at positions 9 and 10 in the N-terminal loop of Vav1 C1. As a results membrane insertion of the C1-ligand complex could be facilitated by these positive charges. These experiments identified two DAG-lactone molecules (**6c** and **8f**), which, compared with the prototypic phorbol ester, PDBU, exhibited markedly increased relative selectivity towards C1 domains with Vav1-specific structure (which was represented by double and triple mutants of PKCδ C1b in this experiment). Our computer models revealed that the above DAG derivatives do have the potential to form ion-pair interactions with the anionic Glu residues of Vav1 C1. Via these inteactions the lipophilicity of the apical surface of the binding cleft could be increased, which would enable the stable membrane association of the receptor-ligand complex. It should be emphasized, however, that these compounds represent only an initial step in the drug design aimed at generating selective Vav1 C1 ligands.

According to previous studies, it became evident that Vav1 represents a therapeutic target rich in potential applications. Vav1 is an essential regulator of the T-cell maturation and T-cell activation. In addition, it also plays a substantial role in signaling pathways controlling B-cell activation, killing function of Natural Killer cells, and the migration and phagocytotic activity of macrophages. These characteristics indicate that drugs that inhibit Vav1 function may prove to be powerful therapeutic agents for immunosuppressive therapy, which is an essential component of organ transplantation. In addition, Vav1 have also been implicated to have a pathogenetic role in human tumors. The ectopic expression of Vav1 have been identified in several malignancies including e.g. melanoma, pancreatic carcinoma etc,; these findings suggest that Vav1 activity could mediate tumorigenesis and tumor progression. Through the induction of cytoskeleton rearrangements (by activating Rho/Rac1 GTP-ases)

Vav1 can promote invasion of neoplastic cells, thereby increasing their metastatic potential. Based on these findings, the inhibition of Vav1 could be a promising target for cancer therapy. Moreover, recent studies have revealed that Vav1 and Vav3 can also play an important regulatory role in platelet biology.Several platelet agonists (e.g. thrombin, thrombopioetine, collagene, etc.) have been found to induce signaling pathways that involve Vav1/Vav3 activation. In addition, Vav1/Vav3 activation has recently been identified to participate in a signaling pathway (inside platelets) that links hyperlipidaemia to thrombus formation. In hyperlipidemia, circulating oxidized LDL molecules can bind to CD36 receptors on the surface of platelets, which renders them more responsive to activation by agonists. Vav1 and Vav3 have been shown to act as downstream effectors of CD36 signaling in platelets. Thus, inhibition of Vav1 activity could represent a novel approach for antiplatelet therapy.Since hyperlipidaemia and associated atherosclerosis are key components in the pathogenesis of cardiovascular diseases, inhibiting prothrombotic factors has a substantial therapeutic significance in the battle against these disease states, which undoubtedly have the highest mortality and morbidity rates in the developed world.

SUMMARY

In the first part of the Dissertation, we demonstrate the pivotal, isoform-specific, differential and antagonistic role of protein kinase C (PKC), one the most important family of DAG-effector molecules, in the regulation of sebaceous gland biology. We report that the phorbol-ester-(PMA)-driven activation of the PKC system stimulates the lipid synthesis (hallmark of terminal differentiation of sebocytes) of human sebocytes. Our studies revealed that, among the five isoforms (cPKCα, nPKCδ, ε, η and aPKCζ) expressed in sebocytes, the activation of cPKCα and nPKCδ participates in mediating the lipogenic effects of the PKC activator. Of further importance, nPKCδ was also found to play a major role in the cellular transduction of the lipogenic and apoptosis-inducing effects of arachidonic acid (a well-known inflammatory precursor). Finally, endogenous aPKCζ activity was shown to constitutively suppress basal lipid synthesis and apoptosis. In the second part of our studies, we characterized the ligand binding properties of Vav1. The DAG recognition motif, C1 domain, plays a key role in the regulation of Vav activity. However, it is classified as atypical (non-phorbol-responsive), despite retaining a binding pocket geometry homologous to that of the typical (phorbol-responsive) C1 domains of PKCs. Using site-directed mutagenesis, we clarified the basis for its failure to bind ligands and identified five crucial residues (Glu[9], Glu[10], Thr[11], Thr[24] and Tyr[26]) along the rim of the binding pocket, which weaken binding potency in a cumulative fashion. With the help of computer modeling, we predicted that these unique residues in Vav1 decrease the hydrophobicity of the rim of the binding cleft, impairing membrane association and thereby preventing formation of the ternary C1-ligand-membrane binding complex. Initial design of DAG-lactones to exploit these Vav1-unique residues showed enhanced selectivity for C1 domains incorporating these residues, suggesting a strategy for the development of ligands targeting Vav1 activity.

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List of publications related to the dissertation

1. **Géczy, T.**, Oláh, A., Tóth, I.B., Czifra, G., Szöllősi, A., Szabó, T., Zouboulis, C.C., Paus, R., Bíró, T.:
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