PROTEIN KINASE C: REGULATION, STRUCTURE, FUNCTION AND ROLE IN HUMAN DISEASE

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Poster Sessions

Signal Transduction: Structure, Function and Regulation of Protein Kinase C (Joint)

QZ 001 REGULATION OF PROTEIN KINASE C BY LIPIDS, Robert M. Bell and Andrew Quest, Department of Molecular Cancer Biology, Duke University Medical Center, Durham, NC 27710.

Protein kinase C (PKC) isozymes are physiologically regulated by diacylglycerol (DAG) second messengers and other lipids by mechanisms that are not understood at the molecular level. We have determined the minimal segment within the Cys2 region of protein kinase C-gamma capable of phorbol-ester binding by deletion analysis. This region contains the typical motif present in all PKC family members consisting of six cysteines and two histidines in the pattern $H\text{-}X_{12}\text{-}C\text{-}H_{10\text{-}14}\text{-}C\text{-}X_2\text{-}C\text{-}X_4\text{-}H\text{-}X_2\text{-}C\text{-}X_7$ where X are non conserved residues. The Cys2 region of PKC-gamma (residues 92-173) expressed as a glutathione-S-transferase fusion protein bound phorbol-esters with high affinity and contained 2 moles of zinc. The mechanism of regulation was further probed by studying phorbol-ester binding of GST constructs containing either individual cysteine-rich regions (Cys1 or Cys2) or combinations thereof together with the pseudosubstrate region and calcium-dependent lipid binding domain (CaLB). Affinity constants for phorbol-ester binding, together with phosphatidylserine and divalent cation dependencies revealed interactions between these regions which suggest that a single high affinity phorbol-ester binding site within Cys1 is important for activation. The role of a homologous cys-rich regions within raf-1 kinase was also investigated. Supported by GM38737.

QZ 002 TRANSMEMBRANE SIGNALING BY HYDROLYSIS OF PHOSPHOLIPIDS AND ACTIVATION OF PROTEIN KINASE C, Yasutomi Nishizuka[1,3], Yoshinori Asaoka[3], Shun-ichi Nakamura[1], Kouji Ogita[1], Ushio Kikkawa[3], Naoaki Saito[2], and Chikako Tanaka[2], Departments of [1]Biochemistry and [2]Pharmacology, School of Medicine, and [3]Biosignal Research Center, Kobe University, Kobe, Japan.

Upon stimulation of cells some membrane phospholipids are degraded to produce several lipid mediators in various intracellular compartments for transducing information from extracellular signals across the membrane. Many of the signals, that induce inositol phospholipid (PI) hydrolysis, are likely to cause more sustained hydrolysis of choline phospholipid (PC) by the action of phospholipases A_2 and D, yielding cis-unsaturated fatty acids including arachidonic acid, lysoPC, phosphatidic acid and diacylglycerol (DAG), all of which play roles in subsequent cellular responses.

Several fatty acids such as oleic, linoleic and linolenic acids greatly enhance DAG-dependent activation of protein kinase C (PKC) both in cell-free enzymatic systems and in intact cells such as platelets, without a large increase of Ca^{2+} concentration. A membrane-permeant DAG is essential for this action of fatty acids. LysoPC, the other part of the PC molecule, also potentiates cellular responses, particularly those in long-term such as activation of human resting T-lymphocytes and differentiation of HL-60 cells to macrophages. Kinetic analysis suggests that this lysoPC action appears to interact with the PKC pathway. The biochemical mechanism of this signal-induced activation of phospholipase A_2 is not fully clarified, but intracellular Ca^{2+} at the 10^{-7}M range is critical for the regulation of phospholipase A_2.

Although the hydrolysis of PI initiates a variety of cellular responses, the formation of the second messengers from PI is normally transient. However, sustained activation of PKC is a prerequisite essential to long-term cellular responses. Phospholipase D activation may take part in the prolonged elevation of DAG, that is needed for this PKC activation. Possible mechanisms of activation of the phospholipases mentioned above will be discussed.

Several isoforms of PKC defined thus far show subtly different enzymological properties, specific tissue expression and intracellular localization. Notably, the members of the PKC family are activated differently by various combinations of Ca^{2+}, DAG, free fatty acids, lysoPC and other lipid mediators, in addition to phosphatidylserine. The patterns of activation of these isoforms may vary in the extent, duration and intracellular compartment. Recently, biochemical mechanism of the signal pathway involving PKC eventually leading to the activation of MAP-kinases begins to emerge. MAP-kinases may also play pleiotropic roles in the control of nuclear events as well as the regulation of various membrane functions. Potential roles of the members of the PKC family will be summarized briefly.

QZ 003THE REGULATION AND FUNCTIONS OF PROTEIN KINASE C GENE PRODUCTS, Peter J Parker, Frederic Bornancin, Sylvie Cazaubon, Lodewijk Dekker, Susan Kiley, Andree R. Olivier and Richard Whelan. Imperial Cancer Research Fund, Lincolns Inn Fields, London WC2A 3PX, UK.

Protein Kinase C (PKC) represents a gene family of structurally related protein kinase isotypes. These proteins are phospholipid-dependent protein kinases and except for the atypical PKCs are diacylglycerol (DAG) dependent and thus exist in a latent form in cells prior to DAG (second messenger) production. The latency of these holoenzymes is effected through an inhibitory domain - the pseudosubstrate site - which occupies the catalytic site prior to DAG binding. Interestingly these pseudosubstrate sites play a role in vitro in determining substrate specificity. Recent work on PKC-α has demonstrated that prior to accumulation of the characteristic phospholipid/DAG dependent 80 KDa PKC-α, the primary translation product (± 76 KDa) undergoes multiple phosphorylation events. In particular one set of phosphorylations has been mapped to a particular loop in the kinase domain which contains a cluster of three threonine residues. Mutations at individual sites has further refined the mapping and threonine 497 is shown to play the most critical role. The phosphorylation appears to be carried out by a distinct protein kinase and is essential for PKC-α activity. The extent to which this permissive phosphorylation(s) is regulated is as yet unclear. However similar regulation of other PKCs is evident. In determining the function of PKCs in intact cell contexts, it would be useful to have dominant negative contructs and a mutant based upon the permissive phosphorylation site has such properties. This will be described in the context of PKC function, alongside more conventional approaches to the elucidation of PKC isotypes in growth and differentiation responses.

Signal Transduction: Structure, Function and Regulation of Phospholipases (Joint)

QZ 004 PHOSPHOLIPASE A_2 REGULATION AND SIGNAL TRANSDUCTION, Edward A. DENNIS, Department of Chemistry, University of California, at San Diego, La Jolla, California 92093-0601, U.S.A.

Phospholipase A_2 (PLA_2) constitutes a very diverse family of enzymes with regard to sequence, structure, regulation, localization and Ca^{2+} role. Our current knowledge about the structure, function, and regulation of the most well-characterized PLA_2's will be summarized. Particular attention will be paid to the role of PLA_2 in signal transduction processes (1).

Several forms of PLA_2 occur in individual cell types and tissues including the macrophage-like cell line $P388D_1$ (2). These include a group II secretory or extracellular $sPLA_2$, a cytosolic high molecular-weight $cPLA_2$, and a Ca^{2+}-independent $iPLA_2$. Studies in our laboratory on each of these enzymes will be described and reviewed with particular focus on structure/function correlates. Then studies in the macrophage-like cell line $P388D_1$ will be reviewed which aim at elucidating the receptor activation of PLA_2 by PAF in LPS primed cells (3) and the role of intracellular Ca^{2+} mobilization (4). Special emphasis will be placed on antisense RNA technology to block the expression of PLA_2 in $P388D_1$ cells using phosphorothioate oligonucleotides. In control cells, priming by LPS and activation by PAF leads to enhanced PGE_2 production. In antisense-treated cells, $sPLA_2$ expression as well as PGE_2 production and arachidonic acid release are dramatically reduced (5). These studies demonstrate a role for $sPLA_2$ in PGE_2 generation by these cells. Possible roles for the other PLA_2's present in these cells will also be considered.

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QZ 005PHOSPHOLIPASE D, Mordechai Liscovitch, Department of Hormone Research, the Weizmann Institute of Science, Rehovot 76100, Israel

Phospholipase D (PLD) catalyzes phospholipid hydrolysis to phosphatidic acid and a water-soluble headgroup. Recent evidence indicates that PLD is rapidly activated by a great many extracellular signal molecules in a wide variety of cell types. PLD activation involves distinct, agonist-specific mechanisms, including G proteins, protein kinases C, Ca^{2+} and tyrosine kinases. The signal-dependent activation of PLD in eukaryotic cells has implicated phosphatidic acid, its natural lipid product, as a novel second messenger. However, the intracellualr molecules targeted by phosphatidic acid are largely unknown, although the number of interesting candidates is ever increasing. Similarly, indirect evidence suggests the existence of multiple PLDs but there is little information on the biochemical and molecular properties of signal-activated PLD(s). The activation of PLD is an element in a cascade of multiple signal-activated phospholipases that generate multiple lipid-derived messengers. The actions of PLD and phosphatidic acid in control of cell function and the crosstalk between PLD and other signal-activated phospholipases will be discussed.

PKC-Substrates and Localization

QZ 006CLONING OF AN INTRACELLULAR RECEPTOR FOR PROTEIN KINASE C; A HOMOLOG OF THE β SUBUNIT OF G PROTEINS,
Dorit Ron*, Che-Hong Chen*, Jeremy Caldwell*, Lee Jamieson†, Elisha Orr†, and Daria Mochly-Rosen*, *Department of Molecular Pharmacology, Stanford University School of Medicine, Stanford, and †Department of Genetics, University of Leicester, Leicester, UK.

Protein kinase C (PKC) translocates from the soluble to the cell particulate fraction on activation. Intracellular receptors that bind activated PKC in the particulate fraction have been implicated by a number of studies (1, 2). We have previously identified 30-36 kDa proteins in the particulate fraction of heart and brain that bind activated PKC in a specific and saturable manner (3). These proteins were termed receptors for activated C-kinase or RACKs. In the following study, we describe the cloning of a cDNA encoding a 36 kDa protein (RACK1) that fulfills the criteria for RACKs. (i) RACK1 bound PKC in the presence of PKC activators, but not in their absence. (ii) PKC binding to the recombinant RACK1 was not inhibited by a pseudo-substrate peptide or by a substrate peptide derived from the pseudosubstrate sequence, indicating that the binding did not reflect simply PKC association with its substrate. Moreover, phosphorylation of histone H1 by PKC was not inhibited by RACK1; rather, an increase of H1 phosphorylation occurred when co-incubated with RACK1. (iii) Binding of PKC to RACK1 was saturable and specific; two other protein kinases did not bind to RACK1. (iv) RACK1 contains two short sequences homologous to a PKC binding sequence previously identified in annexin I and in the brain PKC inhibitor, KCIP (4, 5). Peptides derived from these sequences inhibited PKC binding to RACK1. Noteworthy, RACK1 is a homolog of the β subunit of G proteins, recently implicated in membranal anchorage of the β-adrenergic receptor kinase, βARK (6)
Finally, aligning the RACK1 amino acid sequence, revealed homologous sequences between PKC isozymes and RACK1. In three isozymes, these sequences fall within the C2 region, previously found to contain at least part of the RACKs binding site on PKC (7). An intriguing possibility is that these RACK1-like sequences in PKC are "pseudo-RACK" binding sites. PKC contains a pseudosubstrate sequence in the regulatory domain of PKC, that binds to the substrate-site and maintains the enzyme in the inactive form (8). Similarly, the pseudo-RACK binding site on PKC may associate with the RACK-binding site in the inactive enzyme. Activation of PKC may then lead to dissociation of these intramolecular interactions, making the RACK-binding site on PKC available for binding to RACKs. The model predicts that pseudo-RACK peptides as well as peptides derived from the PKC-binding site on RACK1 should interfere with PKC binding to RACK1, *in vitro*, and with PKC-mediated function, *in vivo*. Studies examining these predictions will be discussed. Taken together, our data suggest a role for RACK1 in PKC-mediated signaling.

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QZ 007 REGULATION OF THE STRUCTURE AND FUNCTION OF PROTEIN KINASE C BY LIPID

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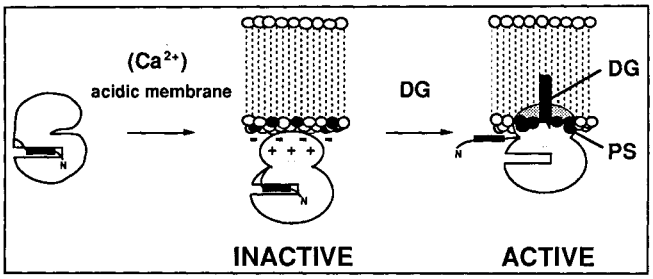
Protein kinase C isozymes are regulated by the lipid second messenger, diacylglycerol, and the aminophospholipid, phosphatidylserine. This contribution presents evidence that the regulation is allosteric: these two lipids, in concert, alter enzyme function by altering protein structure. We have found that Ca^{2+}-dependent and Ca^{2+}-independent isozymes of protein kinase C interact with membranes by cooperatively binding multiple molecules of phosphatidylserine. This specific, high-affinity interaction requires diacylglycerol: the second messenger increases the affinity of protein kinase C for phosphatidylserine by over two-orders of magnitude, renders the binding relatively insensitive to increasing ionic strength, and increases the catalytic efficiency of the enzyme. The diacylglycerol-dependent interaction with phosphatidylserine is accompanied by a conformational change that exposes the enzyme's intramolecular pseudosubstrate domain. Specfically, activation exposes Arg^{19} of the pseudosubstrate of βII protein kinase C to proteolysis. Molecular modeling of protein kinase C's catalytic domain, based on the structure of the cAMP-dependent kinase's catalytic domain, indicates that Arg^{19} is shielded by a cluster of acidic residues when the pseudosubstrate occupies the substrate-binding site. Thus, biochemical data and structural modeling indicate a mechanism of intrapeptide regulation of protein kinase C by phosphatidylserine: high affinity binding to this lipid, induced by diacylglycerol, results in a conformational change that breaks the interaction between the intramolecular pseudosubstrate and catalytic core. This intrapeptide mechanism of regulation may be common to all isozymes of protein kinase C because we have found that a member of the new protein kinase Cs (ε) and atypical protein kinase Cs (ζ) also undergo phosphatidylserine/diacylglycerol-dependent changes in conformation.



PKC-Molecular Biology and Genetics

QZ 008 EXPRESSION AND CHARACTERIZATION OF THE HUMAN PROTEIN KINASE C FAMILY MEMBERS, Nancy R. Rankl, John W. Rice, Thomas M. Gurganus, James L. Barbee, Cindy Moomaw, Carson R. Loomis, and David J. Burns, Sphinx Pharmaceuticals Corporation, Durham, NC 27717.

Transmission of extracellular signals across biological membranes results in the generation of lipid metabolites which in turn influence specific cellular events. The target of many of these lipid messengers is the enzyme, protein kinase C (PKC), whose function is to propagate the extracellular signals by phosphorylating other proteins. The diversity of cellular processes attributed to this enzyme may be due to the identification of PKC as a large family of structurally related proteins. At least eleven members of the PKC family have been cloned to date from a number of different mammalian species. It is thought that the structural differences among the PKC subtypes translate into specific functional roles for each member. Current research efforts are focused on the large-scale production of the individual human PKC subtypes in the baculovirus-insect cell expression system (BEVS). The BEVS system has been extremely useful for the characterization of the biochemical properties of the PKC family members, and for high through-put screening which has permitted the identification of potent and isozyme-selective inhibitors. However, this system has certain limitations with respect to the production of functionally active PKC. Although large quantities of recombinant protein could be produced, a significant portion of enzyme (sometimes >95%) was catalytically inactive. This problem was most critical for the Ca^{2+}-independent family members, such as PKC-δ. Thus, several different approaches were attempted to maximize the production of catalytically active enzyme in this system: 1) optimization of growth and infection conditions for the insect cells; 2) expression of the enzymes in a variety of insect cell lines; and 3) the use of different baculovirus transfer vectors. Vector selection turned out to be the key factor in the production of active PKC. Importantly, insights are emerging which define the role(s) of individual PKC family members in specific cellular and physiological processes. We are also investigating the role(s) of PKC family members in different cells using isozyme-selective PKC antibodies.

QZ 009 THREE DISTINCT CLASSES OF PROTEIN KINASE C IN GROWTH SIGNAL TRANSDUCTION OF RAT 3Y1 FIBROBLASTS

Shigeo Ohno[1], Keiko Mizuno[1], Kazunori Akimoto[1], Shigeharu Moriya[1], Yasushi Adachi[1], Yoshihiko Ueda[1], Ariko Fujise[1], Masami Hirano[1], Naoya Nishioka[1], Masaki Izawa[1], Yasushi Izumi[1], Shin-ichi Osada[1], Syu-ichi Hirai[1], Kotaro Kimura[2], Chieko Kuwabara[2], Yasuhisa Fukui[2], Andrius Kazlauskas[3], and Koichi Suzuki[2]. [1]Department of Molecular Biology, Yokohama City University School of Medicine, 3-9 Fuku-ura, Kanazawa-ku, Yokohama 236, [2]Tokyo University, 1-1-1, Yayoi, Bunkyo-ku, Tokyo 113, Japan, [3]National Jewish Center for Immunology and Respiratory Medicine, Denver, CO 80206.

Recent molecular cloning as well as biochemical analyses reveal the presence of at least 10 PKC family members that can be divided into three distinct classes, cPKC (α, βI, βII, and γ), nPKC (δ, ε, η, and θ), and aPKC (ζ and λ). Among the three PKC subfamilies, cPKC and nPKC respond to DG and phorbol esters while aPKC do not, indicating that the cellular receptor for phorbol esters includes cPKC and nPKC but not aPKC. This paper addresses the question how and when respective PKC members are activated on growth signal transduction in rat 3Y1 fibroblasts. Quiescent rat 3Y1 fibroblasts respond to several mitogens (EGF, TGFα and LPA, but PDGF very slightly) to start the cell cycle progression. The strategy involves the overexpression of PKC or other members of the signaling system in quiescent 3Y1 cells and stimulation of cells with the above mitogens. The extent of activation of each PKC member is then evaluated by several different criteria including phosphorylation level of each PKC member, phosphorylation of MARCKS, PKC-dependent transcriptional activation of TPA-responsive reporter genes, and MAP kinase activation. Mitogenic stimulation causes an increase in the phosphorylation levels of nPKCδ and ε and also causes the enhancement of mitogen-induced transcriptional activation of TRE (TPA response element) and SRE (serum response element) as demonstrated by the overexpression of the one of nPKC and aPKC. This indicates that the activation of cell surface receptors (EGFR and LPAR) triggers the activation of both the nPKC and aPKC subfamilies. The introduction of PDGF receptor to 3Y1 cells renders them responsive to PDGF as well. The introduction of the PDGF receptor signaling mutants (which possess point mutations in the autophosphorylation sites involved in the interaction with PLCγ, PI3K, or GAP) along with the various PKC members permitted us to determine the signaling molecule involved in the interaction between PDGF receptor and the respective PKC members. This point was further analyzed by experiments based on the overexpression of PI3K (p85 and p110) or PLCγ. It was concluded that the three PKC subfamilies are involved in distinct signaling pathways that are activated by mitogenic stimulation of 3Y1 cells. Structure and expression of a new member of the aPKC family, PKCλ, will also be presented.

Lipid Dependent Pathways in Cellular Activation (Joint)

QZ 010 ROLE OF MAP KINASES IN LIPID SIGNALING, Roger J. Davis, Howard Hughes Medical Institute, Program in Molecular Medicine, Department of Biochemistry & Molecular Biology, 373 Plantation Street, Worcester, MA 01605.

Tyrosine kinase growth factor receptors activate MAP kinase by a complex mechanism involving the SH2/3 protein Grb2, the exchange protein Sos, and Ras. The GTP-bound Ras protein binds to the Raf kinase and initiates a protein kinase cascade that leads to MAP kinase activation. Three MAP kinase kinase kinases have been described (c-Raf, c-Mos, and Mekk) that phosphorylate and activate the MAP kinase kinase Mek. Activated Mek phosphorylates and activates MAP kinase. Subsequently, the activated MAP kinase translocates into the nucleus where many of the physiological targets of the MAP kinase signal transduction pathway are located. These substrates include transcription factors that are regulated by MAP kinase phosphorylation (e.g. p62^{TFC}/*Elk*-1, c-Myc, c-Jun, c-Fos, and C/EBPβ). Thus, the MAP kinase pathway represents a significant mechanism of signal transduction by growth factor receptors from the cell surface to the nucleus that results in the regulation of gene expression.

In the yeast *Saccharomyces cerevisiae*, four MAP kinase homologs have been described: *Mpk*1, *Hog1*, *Fus*3, and *Kss1*. In addition, three MAP kinase homologs have been identified in the rat: *Erk*1, *Erk*2, and *Erk*3. Human MAP kinases that are similar to the rat *Erk* kinases have also been identified by molecular cloning. The human *Erk*1 protein kinase has been shown to be widely expressed as a 44-kDa protein (p44^{mapk}) in many tissues. The human *Erk*2 protein kinase is a 41-kDa protein (p41^{mapk}) that is expressed ubiquitously. In contrast, a human *Erk*3-related protein kinase (p63^{mapk}) has been found to be expressed at a high level only in heart muscle and brain. The genomic loci of these MAP kinase genes are widely distributed within the human genome: p41^{mapk} (*Erk*2) at 22q11.2; p44^{mapk} (*Erk*1) at 16p11.2; and p63^{mapk} (*Erk*3-related) at 18q12-21.

By binding to specific cell surface receptors some "lipid messengers" can activate the MAP kinase signal transduction pathway. Examples of lipid messengers that utilize the MAP kinase pathway will be described. Lipids also have an important role as targets of the MAP kinase signal transduction pathway. One example of a target is provided by the cytosolic phospholipase A2 (cPLA2) which is phosphorylated and activated by MAP kinase. Thus, the MAP kinase signaling pathway leads to the production of "lipid messenger" molecules as a result of increased cPLA2 activity.

QZ 011 ACTIVATION OF Ca^{2+}-SENSITIVE PHOSPHOLIPASE A_2 ($cPLA_2$) BY THROMBIN IN HUMAN PLATELETS Ruth M. Kramer, Edda F. Roberts, Joseph V. Manetta, and Joseph A. Jakubowski
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Platelets respond to the physiological agonist α-thrombin with shape change, aggregation and release of granular contents. Thrombin also evokes the rapid release of arachidonic acid esterified to platelet membrane phospholipids thereby initiating the biosynthesis of thromboxane A_2 (TXA_2). The mobilization of arachidonic acid from thrombin-stimulated platelets can be attributed largely to the action of a phospholipase A_2 (PLA_2). Two types of PLA_2 enzymes have been purified from platelets and structurally identified by cDNA cloning and expression: (1) a 14-kDa PLA_2 that is rapidly secreted by stimulated platelets ($sPLA_2$) and (2) an 85-kDa cytosolic PLA_2 that associates with membranes upon thrombin stimulation ($cPLA_2$). While $sPLA_2$ requires millimolar [Ca^{2+}] for catalytic activity and is nonselective towards fatty acyl chains at the *sn*-2 position of phospholipids, $cPLA_2$ requires submicromolar [Ca^{2+}], as found in stimulated cells, for binding to its membrane phospholipid substrate and preferentially hydrolyzes *sn*-2-arachidonoyl phospholipids. Thrombin-induced generation of TXA_2 by platelets readily occurs in the presence of EGTA ruling out the involvement of newly secreted, surface-associated $sPLA_2$. Using immunochemical analysis we found that $cPLA_2$ is the predominant PLA_2 in human platelets. Upon stimulation of platelets with thrombin $cPLA_2$ is phosphorylated and exhibits enhanced catalytic activity, as well as a change in its electrophoretic and chromatographic properties compared to $cPLA_2$ from control platelets. These changes of $cPLA_2$ are reversed by treatment with phosphatase demonstrating that they are the consequence of thrombin-stimulated phosphorylation. Thrombin-induced phosphorylation and activation of $cPLA_2$ is rapid, dose-dependent and more sensitive to agonist than the generation of TXA_2 suggesting that it may be an early event in the sequence of steps leading to the mobilization and further conversion of arachidonic acid to TXA_2. By comparing the functional properties of $cPLA_2$ from control vs. thrombin-stimulated platelets, we found that while activated $cPLA_2$ exhibits the same Ca^{2+}-requirement and apparent substrate affinity (K_m), its maximal velocity (V_{max}) is increased compared to control $cPLA_2$. We conclude that $cPLA_2$ is likely to play an important role in agonist-induced liberation of arachidonic acid in human platelets and is regulated not only by elevation of cytosolic free [Ca^{2+}], but also by phosphorylation.

QZ 012 SIGNAL TRANSDUCTION BY LYSOPHOSPHATIDIC ACID, A PLATELET-DERIVED BIOACTIVE PHOSPHOLIPID, Wouter H. Moolenaar, Kees Jalink, Peter Hordijk, Emile van Corven, Thomas Eichholtz, Wim van Blitterswijk and Rob van der Bend, The Netherlands Cancer Institute, 1066 CX Amsterdam, The Netherlands.

Lysophosphatidic acid (LPA; 1-acyl-glycerol-3-phosphate) is a platelet-derived serum phospholipid (1) that evokes a wide range of biological responses in numerous cell types. For example, LPA induces platelet aggregation, smooth muscle contraction, neuronal shape changes, *Dictyostelium* chemotaxis, tumor cell invasion and, in fibroblasts, stress fiber formation and stimulation of DNA synthesis (2-4). LPA activates its own G protein-coupled receptor(s) (5,6) to stimulate phospholipase C and inhibit adenylylcyclase (2). Recent findings reveal that classic second messenger systems fail to account for all cellular events activated, particularly rapid cytoskeletal changes and long-term mitogenesis. LPA-induced cytoskeletal changes, including neurite retraction and stress fiber formation, depend on the small GTP-binding protein Rho and are accompanied by activation of the $p60^{src}$ protein tyrosine kinase and enhanced phosphorylation of the $p125^{FAK}$ "focal adhesion kinase" (3,7). LPA-induced mitogenesis occurs predominantly through a novel G_i-$p21^{ras}$-MAP kinase route (8), which functions independently of the Rho-mediated cytoskeletal changes. Genistein and staurosporine block LPA-induced, but not EGF-induced, activation of $p21^{ras}$ and MAP kinase, suggesting that an intermediate tyrosine kinase links the LPA receptor to $p21^{ras}$ activation (7,8). Furthermore, a rise in cAMP abrogates the $p21^{ras}$-MAP kinase pathway at a point downstream of $p21^{ras}$ resulting in inhibition of mitogenesis (9). A model is presented that attempts to provide a molecular basis for these new G protein-mediated signal transduction pathways.

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QZ 013 INVOLVEMENT OF TRANSACYLASE AND TRANSACETYLASE IN REGULATING THE BIOSYNTHESIS OF ACETYLATED LIPIDS, F. Snyder, T-c. Lee, Y. Uemura, and M-c. Ou, Med. Sci. Div., Oak Ridge Assoc. Univ., Oak Ridge, TN 37831-0117.

Many cells that produce platelet-activating factor (PAF), one of the most potent lipid mediators known, also can form closely related sn-1 acyl and O-alk-1-enyl (plasmalogen) structural analogs of PAF that possess biological activity. The biosynthesis of PAF and its analogs requires the generation of radyllysophospholipids from a membrane precursor via a CoA-independent transacylase/PLA_2 catalyzed reaction sequence which is also important in the trafficking of arachidonate among phospholipids. Acetylation of these lysophospholipids can then be catalyzed by either an acetyl-CoA acetyltransferase or a CoA-independent PAF transacetylase. The closely intertwined cellular network of transacylation and transacetylation reactions appear to provide a common thread of enzymatic interplay that can regulate +/- signalling species of lipids involved in a variety of cellular responses. The transacylase/PLA_2 cycle is directly linked to the biosynthesis of PAF since the lyso-PAF produced is the substrate for acetyltransferase, the enzyme that forms PAF in the remodeling pathway. A specific example of this type of transacylation reaction sequence is the conversion of plasmalogens to lysoplasmalogen via a PLA_2; the lysoplasmalogen formed can then serve as an acceptor molecule for the transfer of arachidonate from alkylacylglycerophosphocholines to generate an arachidonoyl-containing plasmalogen and lyso-PAF. Any of the lyso-diradylglycerophosphoethanolamines (phosphocholines), but not other lyso-phospholipids, can serve as acyl acceptors for the transacylase. The transacylase is not affected by CoA, Ca^{2+}, EGTA, PLA_2 inhibitors, or phenylmethylsulfonyl fluoride (PMSF), whereas deoxycholate and Triton X-100 inhibit the activity. The transacetylase transfers the acetate moiety from PAF to a variety of lyso-phospholipids, fatty alcohols, and sphingosine. Transacetylation appears to play a unique role in the formation of the acyl and plasmalogen (O-alk-1-enyl) analogs of PAF, as well as N-acetylated sphingosine. Like the transacylase, the transacetylase is membrane-bound and has no requirement for CoA, Ca^{2+}, or Mg^{2+}. However, the transacetylase differs from the transacylase in its broad substrate specificity of acceptor molecules, as well as having a reduced activity at lower temperatures and being activated in the presence of PMSF. Moreover, the enzyme activity is not altered when intact HL-60 cells are supplemented with 20:4, differentiated into granulocytes, or treated with calcium ionophore A23187. These differences plus the fact that alkylarachidonoylglycerophosphocholine only partially inhibits the transacetylation rate suggests the transacylase and transacetylase activities represent two distinctly different catalytic proteins. The apparent synergistic role of the transacylase and the transacetylase in generating acetylated lipid products with different biological activities indicate the combined actions of these two enzymes could play an important role in fine-tuning cellular responses elicited by lipid mediators. Supported by DOE DE-AC05-760R00033, NIH HL27109-12 and ACS BE-26X.

PKC-Regulation/Inhibitors

QZ 014 PKC INHIBITORS AS NEW DRUG CANDIDATES, Doriano Fabbro[1], Georges Weissgerber[1], Thomas Meyer[1], Helmut Mett[1], Marcel Müller[1], Josef Pfeilschifter[2], George Thomas[3], Mira Susa[4], Silvia Stabel[5], Barbara Marte[3], Nancy Hynes[3] and Urs Regenass[1], 1:Oncology-Virology, Dept. Pharmaceutical Research, CIBA-GEIGY Basel, Switzerland, 2: Dept. Pharmacology, Biocenter, University of Basel, 3: Friedrich Miescher Institute, CIBA-GEIGY Basel, 4: Inflammation, Bone and Allergy, Dept. Pharmaceutical Research, CIBA-GEIGY Basel, 5: Max Delbrück Laboratory, MPG, Cologne, Germany.

Protein kinase C consists of family of at least 11 related but distinct enzymes which have been classified into the calcium-dependent, conventional cPKCs (α, βI, βII, γ), calcium-independent non-conventional nPKCs (δ, ε, η, θ, μ) and the calcium-independent and phorbol ester unresponsive atypical aPKCs (ζ, λ). Except for the aPKCs, all subtypes bind to and are activated by tumor promoters. According to their differential localization in tissues and cells, their different modes of regulation and substrate-specificity and the findings that more than one subtype of PKC is expressed in a single cell type suggest that each member or at least each class of the PKC family may play discrete roles in the processing of various physiological and pathological responses to extracellular stimuli. This may explain, at least partly, why activation of PKC with tumor promoters elicit pleiotropic cellular responses that can vary widely between cell types. To better understand the functions of the individual PKC subtypes in normal and pathological cellular processes we have analyzed the effects of various activators and potentially selective inhibitors on the individual PKC subtypes in vitro as well as in cells. The subcellular distribution, mode of activation and down regulation as well as the effects of potentially selective PKC inhibitors were analyzed with respect to their effects on the individual PKC subtypes as well as on cellular functions were PKC has been reported to be involved like the tumor promoter and growth factor induced mitogenic signaling through MAP-kinase and S6 kinase activation. Our findings indicate that activation of a particular PKC isoform within a given cell type appear to depend, not only on its intrinsic capacity to associate with membranes, but also on additional cell-type specific factors. These results suggest that the simple scenario of PKC activation and down regulation can be considered, at best, as oversimplified. According to our results, PKC is an attractive molecular target not only for the discovery of potent and effective anti tumor agents but also for other types of pharmaceutical intervention. Selective inhibitors for PKC or for the individual subtypes will be of some value in dissecting the complexity of mitogenic signaling.

QZ 015 THE DESIGN AND *IN VITRO* AND *IN VIVO* BIOLOGICAL PROPERTIES OF POTENT AND SELECTIVE PROTEIN KINASE C INHIBITORS, **J S Nixon, A Birchall, J Bishop, D Bradshaw, L H Elliott, A Greenham, V M Gibson, T J Hallam, W Harris, J Hartley, C H Hill, A Hutchings, G Lawton, A G Lamont, E J Lewis, E J Murray, N Lad, S Orchard and D Williams.**

The structural lead provided by staurosporine, a non-selective protein kinase inhibitor was used as a basis for the design of substituted bis-indolylmaleimides with significantly improved selectivity for protein kinase C (PKC) over other protein kinases [1].

These agents antagonise cellular processes triggered by phorbol esters and are useful for clarifying the role of PKC in mediating functional responses in cells[2]. They inhibit antigen-driven T-cell proliferation *in vitro*, possibly in part, by blocking activation of the transcriptional control protein NF-KB, necessary for IL2 gene transcription.

One of these bis-indolylmaleimides, Ro 32-0432 shows oral anti-inflammatory activity in T-cell mediated models of hind paw inflammation[3] and of experimental autoimmune encephalomyelitis in rodents. The results presented here suggest that selective inhibitors of PKC have therapeutic value in the treatment of T-cell mediated autoimmune diseases such a rheumatoid arthritis and multiple sclerosis.

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PKC and Disease

QZ 016 PROPERTIES AND FUNCTIONS OF PROTEIN KINASE C ISOENZYMES IN NORMAL AND PSORIATIC HUMAN SKIN, Gary J. Fisher[1], Christopher E.M. Griffiths[1], David J. Burns[2], and Carson R. Loomis[2], [1]Department of Dermatology, University of Michigan, Ann Arbor, and [2]Sphinx Pharmaceuticals Corp., Durham.

Human epidermis is a stratified epithelium that undergoes continual self renewal. In normal epidermis, proliferation of keratinocytes, the major cell type, occurs only in the bottom cell layer. As keratinocytes mature, they migrate upward through the epidermis, eventually reaching the uppermost layer, where they undergo terminal differentiation. Psoriasis is a common skin disease characterized by keratinocyte hyperproliferation, accelerated terminal differentiation, and inflammation. Although there is no animal model for psoriasis, many biochemical and histological features of the disease are mimicked by topical application of protein kinase C activators, such as TPA and 1,2-diacylglycerol, on skin. The actions of these agents on skin are blocked by topical application of protein kinase C inhibitors. In addition, the content of 1,2-diacylglycerol, the physiological activator of protein kinase C, is elevated in psoriatic epidermis, and 1,2-diacylglycerol extracted from psoriatic epidermis is capable of activating protein kinase C isoenzymes from skin *in vitro*. These observations have led us to hypothesize that protein kinase C isoenzymes are key molecular mediators of epidermal cellular homeostasis in normal human skin, and that their mis-regulation is a critical feature of the pathophysiology of psoriasis. We have determined that normal human epidermis expresses mRNA and protein for six protein kinase C isoenzymes, PKC-α, PKC-β, PKC-δ, PKC-ε, PKC-ζ, and PKC-η. PKC-γ and PKC-θ are not expressed. In psoriatic epidermis, PKC-α and PKC-β activities and protein levels are significantly reduced (50% and 80%, respectively), while PKC-ζ protein is modestly elevated. These changes in protein kinase C isoenzyme levels occur in the absence of any alterations in their mRNA levels, suggesting post transcriptional modulation. Immunohistochemical analysis has revealed that PKC-α is localized to keratinocytes in the lower epidermal layers, PKC-β is localized predominantly to Langerhans cells (resident epidermal bone marrow-derived antigen presenting cells), PKC-δ is restricted to keratinocytes in the bottom proliferative cell layer, and PKC-η is restricted to keratinocytes in the upper layer undergoing terminal differentiation. This differential localization of protein kinase C isoenzymes within human epidermis, indicates that protein kinase C isoenzyme expression is regulated as a function of keratinocyte maturation, and is consistent with the concept that protein kinase C isoenzymes in skin possess functional specificity.

QZ 017THE ROLE OF PROTEIN KINASE C IN MULTIDRUG RESISTANCE, Robert I. Glazer and Shakeel Ahmad, Department of Pharmacology, Georgetown University Medical Center, Washington, D.C. 20007.

Cross-resistance to structurally unrelated natural product anticancer drugs or multidrug resistance (MDR), is associated with over-expression of a plasma membrane ATP-dependent drug transporter, P-glycoprotein, the product of the *MDR*1 gene. Increased expression of protein kinase C (PKC) often accompanies MDR and may be essential for the optimal functional expression of the MDR phenotype. PKC may serve multiple roles in MDR which may include transcriptional activation through phosphorylation of *trans*-acting factors that are involved in the expression of MDR-related genes or post-translational phosphorylation of P-glycoprotein to enhance its drug efflux activity. To examine the latter mechanism, MCF-7 breast carcinoma cells stably transfected with the *MDR*1 cDNA (BC-19 cells) were transfected with protein kinase Cα (PKCα), the PKC isoform that is elevated 30-fold in MCF-7 cells selected for resistance by continuous exposure to doxorubicin (MCF-7/ADR). BC-19 cells expressing endogenous levels of PKCα were 10- to 20-fold less resistant to doxorubicin and vinblastine than MCF-7/ADR cells, eventhough P-glycoprotein levels were similar. However, BC-19 cells stably transfected with PKCα (BC-19/PKCα) showed a 30-fold increase in PKCα activity and were several fold more resistant to doxorubicin and vinblastine than parental BC-19 cells. In contrast, wild-type MCF-7 cells transfected with PKCα did not exhibit any change in drug sensitivity. Increased resistance in BC-19/PKCα cells was associated with increased phosphorylation of P-glycoprotein and decreased drug accumulation, and the PKC activator, phorbol dibutyrate, further increased resistance to doxorubicin and stimulated P-glycoprotein phosphorylation. Transfection of BC-19 cells with the γ isoform of PKC that is normally not expressed in MCF-7 cells did not increase MDR. However, transfection of MCF-7/ADR cells with the antisense construct of PKCα led to reduced PKC activity and increased sensitivity to doxorubicin. These results demonstrate that PKC serves as an important post-translational modulator of P-glycoprotein and that its effect is isoform specific. Understanding the role that phosphorylation plays in MDR should help to define the mechanisms related to the onset as well as the regulation of drug resistance in normal and malignant cells.

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QZ 018 ROLE OF PROTEIN KINASE C (PKC) AND ITS ISOFORM βII IN THE DEVELOPMENT OF VASCULAR COMPLICATIONS OF DIABETES MELLITUS. George L. King, Toyoshi Inoguchi, Teruo Shiba, Xia Pu, Makoto Kunisaki, and Sven-Erik Bursell. Joslin Diabetes Center and Harvard Medical School, Boston, MA 02215

Vascular abnormalities in diabetes are characterized by increased permeability, basement membrane thickening, enhanced vascular tone and alteration of vascular cell growth. Since the activation of PKC has been reported to affect all of these physiological parameters, the levels of diacylglycerol (DAG) and PKC have been actively characterized in the vascular tissue of diabetic animals. Results have shown that either in cultured vascular cells or in vascular tissue such as retina, aorta, heart, renal glomeruli and granulation tissue, the PKC and DAG levels are increased by hyperglycemic conditions. Both endothelial cells and smooth muscle cells also responded to elevated levels of glucose with increased DAG levels and PKC activity in the membraneous pool. The increase in DAG induced by elevated level of glucose is due to *de novo* synthesis of DAG rather than due to phospho-inositol turnover containing mainly palmitate-labeled DAG. Identification of PKC isoforms have shown that in diabetes βII isoform is predominantly increased in the heart and aorta and in cultured vascular cells. In the retina, however, there appears to be a general increase of all the isoforms of PKC. To identify the consequences of PKC activation intracellularly, we have found that the activation of PKC in smooth muscles cells will increase the phosphorylation level of MARCKS protein. Physiologically the activation of PKC has been correlated in diabetic rats with a decrease in retinal blood flow which can be normalized by a PKC inhibitor and mimicked in normal rats by a PKC activator. The increase in PKC activities is correlated with increases in c-fos activation in the heart of diabetic rats. Studies in vascular tissues and cultured cells have shown that elevated levels of glucose can increase DAG levels via *de novo* synthesis pathway with activation of βII isoform in the membraneous fraction. The consequences of PKC activation leads to an increased phosphorylation of MARCKS protein as well as expression of c-fos and may be responsible for the abnormality in retinal blood flow in diabetic rats.

Role of Sphingolipids in Cellular Regulation and Control of Protein Phosphorylation (Joint)

QZ 019 SPHINGOLIPID-DERIVED SECOND MESSENGERS: TUMOR SUPPRESSOR LIPIDS, Yusuf A. Hannun and Lina M. Obeid, Duke University Medical Center, Durham, NC 27710.

Although membrane phosphoglycerolipids are now recognized to play important roles in transmembrane signal transduction, a similar role for membrane sphingolipids has not been well characterized. Recent investigations have resulted in identification of a sphingomyelin cycle of cell regulation whereby the action of extracellular agents such as tumor necrosis factor α (TNFα) results in activation of a neutral sphingomyelinase and the generation of ceramide. In turn, ceramide may serve as a second messenger/intracellular regulator. In cells, ceramide has been shown to down regulate the c-myc proto-oncogene, regulate the nuclear factor kappa B, modulate protein phosphorylation and the release of prostaglandins. Importantly, ceramide appears to mediate the effects of TNFα and other cytokines on cell growth, differentiation, and programmed cell death. A ceramide-activated protein phosphatase has been identified as an in vitro target for the action of ceramide with ongoing results implicating this phosphatase in mediating the cellular activities of ceramide. Thus, ceramide is emerging as a prototypic sphingolipid-derived second messengers with a role in mediating antiproliferative and apoptotic signals.

QZ 020 THE ROLE OF CERAMIDE-ACTIVATED PROTEIN KINASE IN SIGNAL TRANSDUCTION THROUGH THE SPHINGOMYELIN PATHWAY, Richard Kolesnick, Memorial Sloan-Kettering Cancer Center, New York.

Recent investigations have identified a new signal transduction pathway, termed the sphingomyelin pathway, which may mediate the action of tumor necrosis factor (TNF)-α and interleukin-1 (IL-1)β. Sphingomyelin is preferentially concentrated in the outer leaflet of the plasma membrane of all mammalian cells and can be rapidly metabolized to initiate a cascade of events that couples cell surface receptors to nuclear activation. In this cascade, stimulation of the TNF or IL-1 receptors initiates, within seconds, hydrolysis of sphingomyelin to ceramide by a neutral sphingomyelinase in the plasma membrane. Ceramide, then acts as a second messenger, stimulating a membrane-bound serine/threonine kinase termed ceramide-activated protein kinase (CAPK), to propagate the signal. CAPK is a Mg^{2+}-dependent, exclusively membrane-bound activity which phosphorylates a peptide derived from the sequence around Thr669 of the epidermal growth factor receptor. This kinase recognizes substrates containing the minimal motif X-Ser/Thr-Pro-X where the phosphoaceptor site is followed on the carboxyl terminus by a proline residue. Hence, CAPK appears to belong to the class of proline-directed protein kinases which includes mitogen-activated protein kinase (mapk) [also known as extracellular signal-regulated protein kinase or ERK] and cdc2 kinase(s). We have partially purified and characterized CAPK as a 97 kDa autophosphorylating protein that is enhanced 5-10 fold by ceramide or TNF stimulation.

The evidence that this pathway mediates TNF and IL-1 signal transduction is three-fold; firstly the pathway is activated within seconds of stimulation of the TNF and IL-1 receptors; secondly, cell-permeable ceramide analogs can bypass receptor activation and directly mimic the effect of these cytokines; and thirdly, these events can be reconstituted in a cell-free system indicating that this cascade is tightly coupled to the receptors.

Recent studies have elucidated some of the downstream elements involved in signaling through this pathway. Evidence was provided that the sphingomyelin pathway mediates TNF activation of $p42^{mapk}$ in human leukemia (HL-60) cells within 10-30 sec of cellular stimulation with either TNF, ceramide analogs or addition of exogenous sphingomyelinase to elevate endogenous ceramide levels. Within 30-60 sec of activation of the sphingomyelin pathway, nuclear translocation of NF-κB is detected. Activation of the sphingomyelin pathway also mimicked the effect of TNF to induce replication of the human immunodeficiency virus (HIV)-1, an event which is purportedly mediated by nuclear translocation of NF-κB. The sphingomyelin pathway also plays a role in programmed cell death or apoptosis. In cell lines in which TNF induces apoptosis, exogenous addition of a ceramide analog or sphingomyelinase mimicked this effect of TNF. Direct elevation of the levels of other potential lipid second messengers or addition of the respective phospholipases did not mimic the effects of TNF on NF-κB activation, replication of the HIV virus or apoptosis. Hence, these effects of TNF appear specific for the sphingomyelin pathway.

QZ 021 SPHINGOLIPIDS, TOXINS AND CELL REGULATION, Alfred H. Merrill, Jr.[1], Joseph J. Schroeder[1], Elizabeth R. Smith[1], Elaine Wang[1] & Ronald T. Riley[2], [1]Biochemistry Department, Rollins Research Center, Emory School of Medicine, Atlanta GA 30322-3050, [2]Toxicology and Mycotoxins Research Unit, U.S. Department of Agriculture, Agriculture Research Service, Athens GA 30613

Since the discovery that sphingosine, sphinganine, and other long-chain (sphingoid) bases are potent inhibitors of protein kinase C (1), a number of other cellular targets have been found to be inhibited (e.g., phosphatidic acid phosphatase and the Na^+,K^+ ATPase) or activated (e.g., a phosphatidylethanolamine-selective phospholipase C and a number of "sphingosine" activated protein kinases) by this class of compounds (for a recent review, see ref. 2). Nonetheless, it has remained unclear whether any natural agents increase (or decrease) the cellular amounts of endogenous long-chain bases and affect these systems. We have recently discovered that a group of mycotoxins termed "fumonisins" inhibit ceramide synthase and cause large increases in sphinganine, and sometimes sphingosine (3, 4). Fumonisins cause a number of agricultural diseases and have been associated with human esophageal cancer. Fumonisins are produced by *Fusarium moniliforme* and a number of other fungi that grow on corn and other foods, and are of concern because they are neurotoxic, nephrotoxic, hepatotoxic and hepatocarcinogenic. Recent findings (5) strongly indicate that the carcinogenicity, and perhaps the toxicity, of fumonisins may be due to the accumulation of free long-chain bases *in vivo*.

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QZ 022 SPHINGOSINE-1-PHOSPHATE, A SECOND MESSENGER, INVOLVED IN CELL GROWTH REGULATION. Sarah Spiegel and Ana Olivera. Department of Biochemistry and Molecular Biology, Georgetown University Medical Center, Washington, DC 20007

Growth signaling networks utilizing glycerophospholipid metabolites as second messengers have been well characterized. Less is known of the second messengers derived from another major class of membrane lipids - sphingolipids. An exciting link between sphingolipids and cellular proliferation has emerged from the discovery that sphingolipid metabolites, sphingosine and sphingosine-1-phosphate, stimulate growth of quiescent Swiss 3T3 fibroblasts via a protein kinase C-independent pathway (1). Sphingosine-1-phosphate is rapidly produced from sphingosine and may mediate its biological effects. Sphingosine-1-phosphate triggers dual signal transduction pathways of calcium mobilization and activation of phospholipase D (1,2), prominent events in the control of cellular proliferation. Recently we found that sphingosine-1-phosphate levels are low in quiescent cultures of Swiss 3T3 fibroblasts and are rapidly and transiently increased in response to the potent mitogens, platelet-derived growth factor (PDGF) and fetal calf serum (3). These growth promoting agents also transiently activated cytosolic sphingosine-kinase activity, which catalyzes the phosphorylation of sphingosine to sphingosine-1-phosphate. In contrast, epidermal growth factor (EGF) did not induce significant changes in sphingosine-1-phosphate levels nor did it activate the kinase. Furthermore, DL-*threo*-dihydrosphingosine, a known competitive inhibitor of sphingosine kinase, markedly reduced DNA synthesis induced by PDGF and serum but did not abrogate cellular proliferation induced by EGF. These findings suggest that activation of sphingosine kinase and consequent formation of sphingosine-1-phosphate may have important biological roles in the signal transduction pathways activated by PDGF (3).

Our data provide the first clues to the identity of the potential missing link between the plasma membrane (where the growth factor receptors lie) and the intracellular calcium stores - sphingosine-1-phosphate. Sphingosine-1-phosphate, has appropriate properties that make it a suitable candidate to function as an intracellular messenger: it elicits diverse cellular responses; turnover is extremely rapid; levels in cells are low and increase rapidly and transiently in response to FCS and PDGF; it mobilizes calcium from internal stores via an inositol trisphosphate-independent pathway (4); finally, sphingosine-1-phosphate could also act in a positive feedback loop to amplify the cascade of events following receptor stimulation via its effect on phosphatidic acid levels, which links growth factor signaling to cellular *ras* activity.

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PKC-Function and Disease

QZ 023 ROLE OF PROTEIN KINASE C IN CELLULAR PROLIFERATION, DIFFERENTIATION AND CELL CYCLE CONTROL
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The protein kinase C enzyme family is involved in a wide variety of cellular functions including proliferation and differentiation. The existence of multiple PKC isotypes that differ in their enzymatic activities, co-factor requirements, and patterns of expression suggest functional diversity among PKC family members. My laboratory has been concerned with 1) determining the role of individual PKC isotypes in human leukemic cell proliferation and differentiation and 2) understanding the underlying molecular mechanisms responsible for isotype-specific functions. We have used human erythroleukemia (K562) cells to address these questions because they express multiple PKC isotypes (α, β_{II} and ζ PKC), and can be induced to undergo lineage specific differentiation. Using molecular and immunological approaches, we have found that α (and possibly ζ) PKC plays an important role in PMA-induced megakaryocytic differentiation. Overexpression of α PKC leads to a gene dose-dependent cytostatic effect and increased sensitivity to PMA-induced differentiation. In contrast, β_{II} PKC levels correlate with the proliferative capacity of these cells. Overexpression of β_{II} PKC leads to relative resistance to PMA-induced differentiation and cytostasis whereas inhibition of β_{II} PKC expression using β_{II} PKC-specific antisense oligonucleotides blocks proliferation. Taken together, these results demonstrate that β_{II} PKC plays a requisite role in leukemic cell proliferation. To begin to identify important molecular targets for these isotype-specific responses, we assessed the intracellular localization and activation pattern of α and β_{II} PKC in response to proliferative and differentiating agents. We find that a portion of the cellular β_{II} PKC is selectively translocated and activated at the nucleus in response to proliferative stimuli suggesting that the nucleus is a direct target for β_{II} PKC-mediated proliferative signalling. At the nucleus, β_{II} PKC mediates direct phosphorylation of the major nuclear envelope protein lamin B both in whole cells and *in-vitro*. We have identified the sites of β_{II} PKC-mediated lamin B phosphorylation and shown that phosphorylation of these sites by β_{II} PKC leads to lamin solubilization consistent with mitotic nuclear envelope breakdown *in-vitro*. Preliminary studies using synchronized cell populations indicate that nuclear β_{II} PKC translocation is regulated in a cell cycle-dependent fashion suggesting a role for nuclear β_{II} PKC in specific cell cycle events. Recent studies demonstrate the presence of nuclear envelope components other than lamin B which confer isotype-specific translocation and substrate specificity toward lamin B. In conclusion, our data provide direct evidence that different PKC isotypes are involved in divergent responses within the intact cell and suggest that nuclear β_{II} PKC activation stimulates cellular proliferation, at least in part, through control of cell cycle events.

QZ 024 PKCs AND PKC BINDING PROTEINS/SUBSTRATES IN TUMOR PROGRESSION. Susan Jaken, The W. Alton Jones Cell Science Center, Lake Placid, NY.

We have studied the expression and localization of PKCs and several PKC binding proteins/substrates in normal and increasingly transformed fibroblast cell lines. Our results indicate that changes in PKC expression and subcellular location as well as changes in PKC binding proteins/substrate expression accompany transformation and may all contribute to disruption of normal PKC signalling pathways. In normal REF52 cells (REF A cells), α-PKC is concentrated in focal contacts and colocalizes with talin and vinculin. α-PKC message and protein levels are similar in SV40-transformed REF52 cells (REF B); however, localization to focal contacts is decreased. In fact, in temperature-sensitive SV40 cell lines, α-PKC accumulates in focal contacts within 6 h of shifting from the permissive to the non-permissive temperature for functional T antigen expression. The SV40-transformed REF B cells have <1% cloning efficiency in soft agar; however, clones can be selected for anchorage-independent growth (REF Cs). Several REF C clones express 4- to 5-fold more δ-PKC than REF A and B cell lines. In contrast, α- and ϵ-PKC levels are comparable. To test the hypothesis that δ-PKC activity was causal in progression to anchorage-independent growth, we have expressed catalytically inactive PKC constructs as potential isozyme-specific dominant negative inhibitors. The use of inactive PKCs as dominant negative inhibitors was predicted from studies in which we determined that PKCs form phosphatidylserine-dependent interactions with PKC binding proteins/substrates *in vitro*. If these interactions also occur *in vivo*, appropriately designed inactive PKCs should compete with endogenous PKCs for binding to these proteins, and hence, their phosphorylation. Expression of inactive δ-PKC constructs in REF Cs inhibited growth in soft agar which supports the hypothesis that increased δ-PKC activity may drive progression to anchorage-independent growth in these cells. In contrast, expression of inactive α-PKC constructs potentiated growth in soft agar, suggesting that dominant negative effects are isozyme specific. We have cloned 10 PKC binding proteins/substrates using an interaction cloning strategy and have prepared antisera to expressed bacterial fusion proteins. Most binding proteins/substrates are down modulated in REF B and REF C cells. Thus, changes in expression of binding proteins/substrates may be another factor in altered PKC signaling in transformed cells.

Signal Transduction: Structure, Function and Regulation of Protein Kinase C (Joint); Signal Transduction: Structure, Function and Regulation of Phospholipases (Joint)

QZ 100 PROTEIN KINASE C: A MOLECULAR TARGET FOR LONG-TERM PSYCHOTROPIC DRUG ACTION ?

Guang Chen, Husseini K. Manji, David B. Hawver, Clinton B. Wright and William Z. Potter, Section on Clinical Pharmacol., Experimental Therapeutics Branch, NIMH, NIH, Bethesda, MD 20892

Despite extensive research, the molecular mechanism(s) underlying the long-term therapeutic effects of mood-stabilizing drugs as diverse as lithium (a monovalent cation) and Valproic acid (VPA, an 8-C branched-chain fatty acid) remains to be elucidated. PKC isozymes play a pivotal role in the intracellular "cross-talk" between neurotransmitter systems in the central nervous sytem and, as such, represent attractive putative targets for the therapeutic effects of these drugs. We have investigated the effects of lithium on the amount and distribution of PKC isozymes in rat brain. Chronic (4-5 week) but not acute lithium treatment resulted in a significant reduction in membrane-associated [^{3}H]PDBu binding in several hippocampal structures; isozyme-selective antibodies revealed that this decrease in [^{3}H]PDBu binding was due almost exclusively to reductions in the levels of PKC α, without affecting PKC β or γ. We have also investigated the effects of VPA (a clinically effective anticonvulsant with demonstrated mood-stabilizing properties) on PKC. Exposure of rat C6 glioma cells to therapeutic plasma levels (0.35-0.7 mM) of VPA for 6 days did not affect total cell number or total protein content, but significantly reduced protein kinase C (PKC) activity in membrane (-52 %) and cytosol (-35 %) fractions. The VPA exposure induced decreases in the levels of PKC α and ϵ isozymes, but not PKC δ and ζ. The VPA effects on PKCs were time-dependent. Acute VPA did not displace 3H-PDBu binding and did not change activity of partially-purified PKC in the presence of Ca^{++} and DAG or PS. The mechanisms by which these structurally distinct mood-stabilizing drugs produce these isozyme-specific effects on PKC are unknown and currently under investigation.

QZ 101 DISTINCT RESPONSES OF PROTEIN KINASE C ISOZYMES TO c-erbB-2 ACTIVATION IN BREAST CANCER CELL LINE, Marie-Helene Disatnik[1], Carlos Arteaga[2] and Daria Mochly-Rosen[1], Department of Molecular Pharmacology, Stanford University, School of Medicine, Stanford, CA 94305[1], Department of Medicine, Vanderbilt University, School of Medicine, Nashville, TN 37232[2]

The human homolog of the rat *neu* oncogene encodes a 185 kDa protein (p185) also called erbB-2, that is closely related to the 170 kDa epidermal growth factor receptor. Activation of erbB-2 results in the generation of signals leading to cell proliferation. Since protein kinase C (PKC) is a key enzyme in signal transduction regulating cell growth, we determined whether stimulation of the receptor erbB-2 caused activation of PKC isozymes. Using the human breast cancer cell line, SKBR-3 cells which overexpress the erbB-2 receptor, we demonstrated differential activation of PKC isozymes in response to stimulation of erbB-2 by a specific antibody, TAb 250. We found, by Western blot analysis, that an activation of the erbB-2 receptor by TAb 250 (10μg/ml) caused the translocation of ζPKC from the soluble to the cell particulate fraction, an increase of εPKC in the cytosol accompanied by a corresponding decrease in the amount of εPKC immunorcactivity in the particulate fraction, and a decrease and subsequent down-regulation of βPKC from the cytosolic fraction. α and δPKC isozymes (present in SKBR-3 cells) did not respond to exposure to TAb250. The distribution of PKC isozymes in the cells treated with TAB 263, an anti-erbB-2 antibody devoid of agonist activity, was not different from their distribution in IgG$_1$-treated cells. Immunocytochemical studies also demonstrated TAb 250-induced activation of PKC isozymes, with each activated isozyme localized to a specific subcellular site. β_1PKC was localized at the perinuclear area of the cells and translocated to the cytosol and nucleoplasm on TAb 250 treatment. δPKC appeared cytosolic on activation but some localized to nuclear structures as well. ε and ζPKC were in the nucleus in non-stimulated cells and translocated to the perinuclear membrane. These results indicate that PKC isozymes are part of the signalling mechanism dowstream of erbB-2.

QZ 102 SHORT CHAIN PHOSPHATIDYLCHOLINE FORMS SEVERAL DISTINCT COMPLEXES WITH Gd^{3+} AS A FUNCTION OF LIPID CONCENTRATION. Goger, M.J., Sando, J.J., Resnick, M.S. and Grisham, C.M. Depts. of Chemistry and Pharmacology, Univ. of Virginia, Charlottesville, VA 22908

Recent water proton relaxation rate (PRR) NMR experiments (Maurer et al., Biochemistry 31:7714, 1992) provided evidence for a set of multiple Gd^{3+} or Ca^{2+} sites on Protein Kinase C (PKC) α and PKCβ when examined in the presence of short chain phosphatidylcholine (PC7) at critical micelle concentrations (cmc). In energy transfer experiments in which two populations of PC7 were labeled with different probes and then mixed, energy transfer was maximal near the cmc but addition of Ca^{2+} decreased the energy transfer and addition of PKCα caused a further decrease. These results suggest that the metal can interact with the non-acidic lipid. PRR experiments with 50 μM Gd^{3+} revealed an increase in enhancement as PC7 concentrations were increased beyond 2 mM. This Gd^{3+}-binding species was not due to a contaminant as shown by a) lack of observable contaminants on thin layer chromatography, b) failure of the heptanoic acid (the most likely contaminant from water hydrolysis) to exhibit similar enhancements, and c) a similar increase in Gd^{3+} enhancement with PC6 and PC8 that correlated with the cmc of each short chain lipid. These results suggest the formation of a Gd^{3+}-lipid complex with a slow tumbling rate (ie., a large complex) at lipid concentrations at and above the cmc. When PRR experiments were conducted at 10 μM Gd^{3+}, a decrease in enhancement (below 1) was observed at concentrations of lipid below the cmc and sometimes evidence for two low enhancement components was seen, consistent with formation of Gd^{3+}-lipid complexes containing few lipid molecules (ie., small complexes with fast tumbling rates). One or more of these lipid-metal structures may be important for binding and activation of PKC. [supported by NIH grant GM31184.]

QZ 103 REGULATION OF PROTEINKINASE C ACTIVITY BY NERVE GROWTH FACTOR, Cornelia Hertel, Robert Schubenel, Philipp Kahle, Martine Mangold, Toshikazu Kuwahara, Pharma Division, Preclinical Research, F. Hoffmann LaRoche AG, 4002 Basel, Switzerland

Nerve growth factor (NGF) induces differentiation of rat pheochromocytoma PC12 cells by activating a tyrosine phosphorylation cascade. Phosphorylation of several intracellular proteins results in activation of immediate early genes, such as cfos as well as activation of late genes. The observed increased activity of proteinkinase C is shown to be due to gene induction. The increase in activity can be prevented by addition of cycloheximide.
H7, an isoquinoline derivative described to inhibit serine/threonine kinase, particularly proteinkinase C. Long term treatment of PC12 cells with subtoxic concentrations of H7 does not result in a downregulation of proteinkinaseC, but rather induces activity above the base line activity. This induction is also protein synthesis dependent. The effects of H7 and NGF are additive, suggesting independent pathways for induction of proteinkinase C activity for H7 and NGF. These pathways are further investigated.

QZ 104 PMA-INDUCED SENSITIZATION OF ADENYLYL CYCLASE IS NOT ASSOCIATED WITH PHOSPHORYLATION OF $G_{i\alpha}$, Bridgette G. January and Richard B. Clark, Graduate School of Biomedical Sciences, The University of Texas Health Science Center, Houston, Texas, 77225-0334

Numerous studies have shown that pretreatment of various cell types with 4ß-phorbol 12-myristate 13-acetate (PMA), which has structural similarities to the endogenous protein kinase C activator diacylglycerol, causes a sensitization as well as a desensitization of hormone-stimulated adenylyl cyclase activity. There is very strong evidence that PMA-induced desensitization is caused by protein kinase C-mediated receptor phosphorylation. On the other hand, the mechanism of PMA-induced sensitization remains controversial. In this study, we have examined the proposal that PMA treatment of L cells induces phosphorylation and inactivation of an α subunit of G_i, a heterotrimeric GTP-binding protein that inhibits adenylyl cyclase. The objectives were to identify which $G_{i\alpha}$ subunits were present in mouse L cells, examine the effect of PMA treatment on hormone-stimulated adenylyl cyclase activity and pertussis toxin catalyzed ADP-ribosylation, and determine if $G_{i\alpha}$ was phosphorylated as a result of PMA treatment. $G_{i\alpha}$ subunits, α_{i1}, α_{i2}, and α_{i3}, were identified by Western blots using $G_{i\alpha}$-specific antisera, and urea gradient SDS-gel electrophoresis of $G_{i\alpha}$'s [^{32}P]ADP-ribosylated by pertussis toxin. In response to PMA treatment, hormone stimulated adenylyl cyclase activity showed a 2-4 fold increase in the V_{max}. Also, PMA had no effect on the extent of pertussis toxin catalyzed ADP-ribosylation of $G_{i\alpha}$. G_i phosphorylation was examined in ^{32}P-labeled, PMA treated L cells using an immunoprecipitation protocol employing various $G_{i\alpha}$-specific antibodies. No phosphoprotein that could be identified as $G_{i\alpha}$ was precipitated. These data suggest that although PMA treatment of L cells results in sensitization of hormone-stimulated adenylyl cyclase activity, it does not alter the ability of Gi to act as a substrate for ADP-ribosylation by pertussis toxin, and it does not induce phosphorylation of a $G_{i\alpha}$ subunit that can be detected by immunoprecipitation.

QZ 105 CHARACTERISATION OF A NOVEL ATYPICAL PKC AND ITS INVOLVEMENT IN GROWTH CONTROL; Fanz-Josef Johannes, Jürgen Prestle, Gisela Link, Sabine Dieterich, Petra Oberhagemann and Klaus Pfizenmaier, Institute of Cell Biology and Immunology, University of Stuttgart, 70569 Stuttgart, Germany.

We have isolated the cDNA of a novel human atypical Protein Kinase C gene. The deduced protein sequence shows strong homologies to conserved protein kinase domain and to the duplex zinc-finger like cysteine rich motivs of members of the PKC family. Lack of the C_2 domain of the Ca^{2+} dependent PKCs and deficiency in phorbol ester binding as well as a unique N-terminal sequence with a potential signal peptide and transmembrane domain suggest that PKCμ is a novel member of the subgroup of atypical PKCs. An open reading frame coding for 912 amino acids directs an in vitro translation product with an apparent M_r of 115 kDa. Immunoprecipitation of PKCμ with polyclonal mouse or rabbit antiserum, generated against different epitopes revealed the same apparent molecular weight. In vitro kinase assays with the overexpressed PKCμ cDNA showed autophosphorylation, and in NRK cells, predominant phoshorylation of a 30 kDa protein at serine residues. Southern analysis revealed that PKCμ is a single copy gene located on human chromosome 21. PKCμ overexpressing transfectants showed an increased growth rate indicating an involvement in growth control. There is constitutive low level expression of the human PKCμ gene in normal tissues with a single transcript of 3.8 kb and an overexpresssion in selected tumor cell lines. These data suggest a role of PKCμ in signal transduction pathways related to growth control.

QZ 106 ABNORMAL PROTEIN KINASE C ALPHA IN HUMAN SMALL CELL LUNG CARCINOMA NCI-H345 CELLS. C.L.A.Jones, E.C. Dempsey, & M.A. Kane, Denver VAMC & Univ. Colo. HSC, Denver, CO 80220

Protein kinase C (PKC) activation by bombesin-like peptides (BLPs) in Swiss 3T3 cells led us to investigate the possible role of PKC in the autocrine growth system involving BLPs in human small cell lung carcinoma (SCLC) NCI-H345 cells. By Western blot analysis, we have identified five PKC isoenzymes in NCI-H345 cells, alpha, beta, delta, zeta and eta. This pattern is different from that expressed in Swiss 3T3 cells. A smaller than expected PKC alpha (57 kDa instead of 80 kDa) was observed in NCI-H345 cells. We hypothesized that this was a constitutively active fragment containing the catalytic subunit, and that the mechanism of production was either a genetic mutation or abnormal posttranslational modification. PCR screening using nested primers for human PKC alpha has yielded products of predicted size within the 5′ (regulatory domain) end of the message. These PCR products were the same size as those obtained with Swiss 3T3 cells and the nonmalignant human bronchial epithelial line BEAS B2B. Treatment of NCI-H345 cells with calpain inhibitors decreased the amount of the 57 kDa PKC alpha band on Western blot. Aberrant posttranslational processing of PKC alpha in NCI-H345 cells may yield an abnormal isoenzyme which contributes to the malignant growth properties.

QZ 107 REGULATION OF PROTEIN KINASE C IN *DICTYOSTELIUM DISCOIDEUM* SIGNAL TRANSDUCTION, Menno L.W. Knetsch, Ewa B. Snaar-Jagalska, Departement of Cell Biology, IMP, State University of Leiden, P.O. Box 9515, 2333 AL, Leiden, The Netherlands.

The eucaryotic micro-organism *Dictyostelium discoideum* is used as a model for signal transduction. Despite the evolutionary distance, the signal transduction pathways of *Dictyostelium* show many similarities with those of vertebrates.

Extracellular cAMP is considered as a hormone which regulates chemotaxis, cellaggregation and differentiation. The cAMP signal is detected by cell surface receptors and transduced via G-proteins to the different second messenger systems.

Upon stimulation of cells by cAMP a Protein Kinase C activity is translocated from the soluble to the particulate fraction. This activation induced translocation is transient and peaks at 15-30 seconds.

The Protein Kinase C response in different signalling mutants has been studied. Results show that Protein Kinase C activation is dependent on extracellular cAMP and the $G\alpha_2$ subunit.

In cells overexpressing a *ras* protein in the GTP-bound form, $Gly_{12} \rightarrow Thr_{12}$, the Protein Kinase C is constitutively active. Furthermore we have been able to show Protein Kinase C activity in the nucleus.

QZ 108 ROLE OF ZINC IONS IN REGULATION OF PROTEIN KINASE C IN PLATELETS CELLULAR SIGNALING

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We demonstrated earlier that zinc ions and a zinc ionophore, pyrithione, potentiated the effect of ADP on blood platelet aggregation, release reaction and fibrinogen receptor exposure, while zinc ions chelator, N,N,N',N'-tetrakis(2-pyridylmethyl) ethylenediamine (TPEN) blocked these reactions. Zn^{2+} alone or TPEN did not affect either formation of thromboxane A2 or intracellular calcium mobilization in platelets. However we found that ADP and Zn^{2+} exert a cooperative effect on the phosphorylation of P-47 protein (pleckstrin), a substrate for protein kinase C in platelets. This reaction was inhibited by TPEN and by compound Ro31, specific inhibitor of the regulatory domain of protein kinase C, but not by genistein, inhibitor of tyrosine kinase. P-47 protein phosphorylation in platelets activated by ADP was also promoted by Zn^{2+} ionophore, pyrithione.

	^{32}P-P47 (cpm)			
	Buffer	TPEN 10 μM	Ro31 25 μM	Genistein 25 μg/ml
Control buffer	102	89	70	110
ADP (30 μM)	140	96	110	117
Zn^{2+} (50 μM)	180	80	79	ND
ADP(30μM)+Zn^{2+}(50μM)	240	160	109	228
ADP(30μM)+Pyr(5 μM)	480	120	148	ND
Thrombin(0.5 U/ml)	1850	1280	420	ND

It is conceivable that intracellular zinc ions may act as mediator (second messenger) of ADP induced fibrinogen receptors exposure and platelet aggregation. This effect may be due to enhanced activation of protein kinase C either by interacting directly with protein kinase C or affecting other protein kinases or phosphatases.

The mechanism how Zn^{2+} affects protein kinase C in platelets may be of clinical interest since zinc deficiency has been linked to a bleeding tendency and impaired wound healing in several disease states.

QZ 109 PROTEIN KINASE C-DEPENDENT O_2^- PRODUCTION IS REQUIRED FOR MONOCYTE-MEDIATED LDL OXIDATION,

Qing Li and Martha K. Cathcart, Department of Cell Biology, Reasearch Institute, Cleveland Clinic Foundation, Cleveland, OH 44195

Our previous studies have shown that release of O_2^- by activated human monocytes is a requisite step in oxidizing normal LDL and transforming it to a cytotoxin. We have also found that intracellular Ca^{2+} levels and PKC activity are integrally involved in LDL lipid oxidation by activated human monocytes. To further assess the involvement of the PKC pathway and its participation in monocyte-mediated LDL lipid oxidation, we investigated PKC signaling by using selective PKC inhibitors. Opsonized zymosan (ZOP) is used in our studies as a monocyte activator. Our data indicate that $PKC_{\beta II}$ is one of the few PKC isoenzymes that is induced in ZOP-activated monocytes. PKC inhibitors, H-7 (1 to 100 μM) and GF109203X (0.1 to 10 μM), caused a dose-dependent inhibition of both O_2^- production and LDL lipid oxidation by activated human monocytes. Furthermore, these inhibitory effects of PKC inhibitors on LDL lipid oxidation were reversed by adding the O_2^- generating system xanthine oxidase/hypoxanthine. To rule out the non-specific action of these inhibitors on PKA, we used two other inhibitors that selectively inhibit PKA, HA1004 and H89. Neither O_2^- production nor LDL lipid oxidation were inhibited by these PKA inhibitors. Our data suggest that PKC activity is required for LDL lipid oxidation by activated human monocytes; and one critical pathway regulated by PKC activity appears to be the production of O_2^-.

QZ 110 EFFECTS OF THE EXPRESSION OF PKC ISOFORMS IN 3T3 CELLS ON THEIR GROWTH, MORPHOLOGY AND RESPONSE TO GROWTH FACTORS.

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Swiss/3T3 and NIH/3T3 cells stably overexpressing different protein kinase C (PKC) isoforms were generated. Swiss/3T3 cells overexpressing PKCα were previously described by us. In these cells we have demonstrated reduced expression of the epidermal growth factor (EGF) receptor molecules (Eldar et al. (1990) J. Biol. Chem. 265, 13290-13296). Here we show that the expression of PDGF-α receptors, but not of PDGF-β receptors, was specifically decreased in PKCα–overexpressing cells; both PDGF-α receptor mRNA transcript and protein were significantly diminished. This was further reflected in the ability of these cells to respond to short and long-term signals of PDGF. In response to PDGF-AA (binding only to PDGF-α receptors) we have demonstrated reduced tyrosine autophosphorylation as well as decreased cell growth. A similar decrease in PDGF-α receptors was also demonstrated in parental Swiss/3T3 cells treated with phorbol esters (PMA). Hence, activation of PKCα by PMA or changes in its cellular levels caused suppression of the expression of a specific set of cell surface receptors, including EGF and PDGF-α receptors, but did not alter the expression of PDGF-β or FGF receptors.

Overexpression of PKCα in Swiss/3T3 cells had no effect on their growth and morphology. In contrast, the expression of the PKC-L/η isoform in NIH/3T3 cells induced specific changes in morphology and caused the cells to grow slower. Our studies indicate that PKCη plays a role in the regulation of nuclear processes. Its effects on the expression and phosphorylation of nuclear factors will be discussed.

QZ 111 ACTIVATION OF BETA-ISOZYME OF PROTEIN KINASE C IS NECESSARY AND SUFFICIENT FOR PHORBOL ESTER-INDUCED DIFFERENTIATION OF HL-60 PROMYELOCYTES: STUDIES WITH PKCβ DEFECTIVE PET-MUTANT.

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Tetradecyl phorbol acetate (TPA) and other agents which activate protein kinase C (PKC) induce growth arrest and differentiation of a number of leukemic cell lines including HL-60 human promyelocytic leukemia. Elucidating this process at a molecular level will contribute to our understanding of granulopoiesis and leukemogenesis. PKC is now known to consist of a family of isozymes, of which PKCα, βιι and δ are known to be both present in HL-60 cells and activated by TPA. We investigated the involvement of PKC isotypes in phorbol ester-induced differentiation using our phorbol ester tolerant "PET" mutant of HL-60, which (in contrast to wild type "S" HL-60 cells) do not growth-arrest, become adherent or undergo apoptosis when exposed to TPA (Brit J Haematol, **68** 291-302, 1988). Using ribonuclease protection assays, Northern blots and RT-PCR, we find that proliferating PET-cells markedly underexpress mRNA for PKCβιι in comparison to S-cells, but do express normal amounts of PKCα, and δ. PKCβι appears not to be expressed by either cell. To test the ability of PKCβιι to induce differentiation, we used the PKCβ-selective activator, 12-deoxy-phorbol-13-phenylacetate-20-acetate (DOPPA), which we found to induce growth arrest, adherence, surface expression of CD11a, and apoptosis in S-cells, but not in PET-cells. We also found that the expression of PKCβιι in PET-cells can be restored by exposing them to dihydroxyvitamin D3, and that this treatment restores the ability of subsequently added DOPPA or TPA to induce immediate cell adherence and growth-arrest of PET-cells. These data lead us to conclude that activation of PKCβιι has a unique role in mediating phorbol ester-induced growth arrest and adherence in these myeloid cells, activation of this isozyme being both necessary and sufficient for this pharmacological response. In contrast, activation of PKCα or PKCδ is insufficient. These results also suggest that certain leukemia's might be treatable by a combination of dihydroxyvitamin D3 and a βιι-selective activator of PKC.

QZ 112 PHORBOL ESTER-INDUCED MYELOID DIFFERENTIATION IS MEDIATED ONLY BY PROTEIN KINASE C-α AND -δ, Harald Mischak‡¶, Jacalyn H. Pierce†, JoAnne Goodnight‡, Marcelo G. Kazanietz§, Peter M. Blumberg§ and J. Frederic Mushinski‡..‡ Laboratory of Genetics, § Laboratory of Cellular Carcinogenesis and Tumor Promotion and †Laboratory of Cellular and Molecular Biology of the National Cancer Institute, Bethesda MD 20892,¶ present address: Institute forClinical Molecular Biology and Tumor Genetics, GSF, Marchioninistrasse 25, D-8000 Munich 70, Germany

It is generally accepted that the multiple, similar protein kinase C (PKC) isozymes are responsible for different, specialized physiological processes, but evidence that directly assigns specific functions to specific isozymes is scarce. To test whether specific PKC isozymes are involved in myeloid differentiation, we have studied the effect of overexpression of PKC –α, –β, –γ, –δ, –ε, –ζ and –η in 32D, a mouse myeloid progenitor cell line that does not differentiate in response to 12-O-tetradecanoylphorbol-13-acetate (TPA). No significant morphological or phenotypical changes could be observed in unstimulated cells that overexpress any of these isozymes. However, the cell lines that overexpressed PKC–α or –δ had acquired the ability to become mature macrophages 2 - 6 h after TPA stimulation. Differentiation is dependent on continuous presence of PKC activity and in the absence of TPA, the cells will dedifferentiate. The overexpression of PKC–β, –γ, –ε, –ζ or –η, in contrast, did not permit TPA-induced differentiation. Using the different PKC overexpressing cell lines, we are now atempting to identify the substrates of PKC–α and –δ, that are involved in myeloid diferentiation. Our results indicate that only these two members of the PKC gene family can participate in TPA-induced myeloid differentiation.

QZ 113 EVIDENCE FOR A NOVEL H7-RESISTANT SPECIES OF PROTEIN KINASE C IN PITUITARY GLAND THAT LEADS TO ACTIVATION OF MAP KINASE, Rory Mitchell, Angela Ison, Pauline Sim, James Simpson, Melanie Johnson, Roger Clegg* and Kevin Connor*, MRC Brain Metabolism Unit, 1 George Square, Edinburgh and *Hannah Research Institute, Ayr, U.K.

Models of the role of PKC in stimulus-secretion coupling, in the activation of phospholipases A_2 and D and in the regulation of Ca^{2+} channels in the anterior pituitary gland have pointed to the presence of a form of PKC which is resistant to H7 but sensitive to GF 109203X and Ro 31-8220. Cytosolic PKCs from anterior pituitary tissue were partially purified on DEAE cellulose followed by fractionation on hydroxylapatite, revealing an unusual late-eluting component of activity absent from a range of other tissues tested. This fraction contained a phorbol-activated, PS-dependent histone IIIs- or GS peptide-kinase activity which was Ca^{2+}-independent and resistant to H7 but not to Ro 31-8220. All other eluting activity was H7-sensitive. Immunoreactivity for PKC α, β_1, (no γ), δ, ε, (no η) and 81 kDa ζ eluted at different positions from the H7-resistant species. Only 88 kDa ζ immunoreactivity was present in the same fraction. Although all bands were blocked in the presence of the relevant peptide antigens, the identity of the high MWt ζ product is uncertain. [^{32}P]auto-phosphorylation experiments revealed an unusual high MWt product (>130 kDa) predominantly in the H7-resistant fraction from pituitary but not midbrain control, whereas other fractions all produced autophosphorylation signals clustered around 90 - 95 kDa. The H7-resistant PKC may be a novel species, one of the newly-described species such as θ, λ or μ or a modified form of a known species such as ζ. In the α T3-1 gonadotroph cell line, stimulation of the phosphoinositide-hydrolysing LHRH receptor evokes activation of MAP kinase as measured by phosphorylation of a selective peptide substrate in an assay incorporating immunoprecipitation with an anti-ERK1/2 reagent. This activation is unaffected by tyrosine kinase inhibitors such as lavendustin A yet is sensitive to PKC inhibitors such as GF 109203X but not H7. This suggests that one of the cellular roles of this H7-resistant PKC is in the G protein receptor-induced cascade of MAP kinase activation involving either MEKK- or perhaps raf-mediated pathways.

QZ 114 LYSOPHOSPHATIDIC ACID ACTIVATES NOVEL PROTEIN KINASE C δ AND ε IN A G-PROTEIN-DEPENDENT MANNER IN RAT 3Y1 FIBROBLASTS, Keiko Mizuno, Yasushi Adachi, Syu-ichi Hirai, Shin-ichi Osada, and Shigeo Ohno Department of Molecular Biology, Yokohama City University School of Medicine, 3-9 Fuku-ura, Kanazawa-ku, Yokohama 236, JAPAN

We have previously demonstrated that protein kinase C (PKC) isozymes in quiescent rat fibroblast 3Y1 cells, conventional PKC (cPKC) α and novel PKC (nPKC) δ and ε, were activated and their phosphorylation was increased by a tumor promoting phorbol ester, TPA, while activation and phosphorylation of only nPKCδ and ε was observed when the cells were treated with serum or EGF. These results indicate that there are different regulatory mechanisms between nPKC isozymes and cPKCα.

In the present study, we show that lysophosphatidic acid (LPA), one of the metabolites of membrane phospholipids, activates endogenous and overexpressed nPKCδ and ε in 3Y1 cells and induces their phosphorylation in the similar manner to serum. LPA has been reported to stimulate growth and motility of a variety of cells, where involvement of specific cell surface receptors coupled to G-protein has been proposed. Therefore we also demonstrate that LPA activates mitogen-activated protein kinases (MAPK) and stimulates DNA synthesis in 3Y1 cells. The activation of nPKC isozymes and MAPK by LPA were, at least in part, sensitive to pertussis toxin. These results suggest that the activation of nPKC by LPA is mediated by a specific cell-surface receptor coupled to pertussis toxin-sensitive G-protein. It might be possible that nPKC activation may lead to DNA synthesis through MAPK pathway.

QZ 115 EVIDENCE FOR A STRUCTURAL PHOSPHORYLATION OF PROTEIN KINASE C Jeffrey W. Orr and Alexandra C. Newton, Department of Chemistry, Indiana University, Bloomington, Indiana 47405

Phosphorylation of Thr 197 in the cAMP-dependent protein kinase is necessary for structural stability of the enzyme[1]. Increasing evidence has implicated a post-translational phosphorylation in the production of a catalytically active protein kinase C[2,3]. Modeling of the catalytic core of protein kinase C, based on the crystal structure of the cAMP-dependent protein kinase, reveals that Thr 500 occupies the same position in protein kinase C bII as the phosphorylated Thr in the cAMP-dependent protein kinase. Phosphorylation at Thr 500 would provide a favorable electrostatic interaction with Lys 489 and Arg 465 near the catalytic core. To test whether phosphorylation at this site results in stabilization of the enzyme through an electrostatic interaction, we made mutants in which this Thr was replaced with acidic residues or non-phosphorylatable residues. Expression in COS cells revealed that the presence of an acidic residue (Glu) at position 500 was sufficient to produce a stable, catalytically active protein kinase C. This result is consistent with phosphorylation at Thr 500 of protein kinase C bII playing a structural role analogous to that of Thr 197 in the cAMP-dependent protein kinase.

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3. Cazaubon, S., and Parker, P. J. (1993) *J. Biol. Chem.* 268, 17559-17563.

QZ 116 ISOFORM EXPRESSION AND GROWTH REGULATING EFFECT OF PROTEIN KINASE C IN THE DEVELOPING KIDNEY, Eva Östlund, Carlos Mendez, Anita Aperia, Dept of Woman and Child Health, Pediatric Unit, St. Göran's Children's Hospital, Karolinska Institutet, Stockholm, Sweden

PKC plays a central role for the regulation of renal function. The enzyme Na^+,K^+-ATPase, which is of vital importance for the reabsorption of Na^+ in all tubular segments, is phosphorylated and inactivated by PKC (Proc Natl Acad Sci 1991, 88, 11359-11362). In intact tubular segments Na^+,K^+-ATPase activity is inhibited by phorbolesters, DAG and dopamine via the PKC pathway (Am J Physiol 1989,256, F370-F373). This effect is developmentally regulated (Pediatr Res 1991,30(2), 131-134). We have now studied the role of PKC for growth regulation of renal tubular cells. Calphostin C, which selectively inhibits PKC by competing at the binding site for diacylglycerol and phorbolesters, dose dependently inhibited growth of rat proximal tubular (PT) cells in primary cell culture. At 100 nM half-maximal inhibition (35.7±13.4%, $p<0.05$) was observed in infant PT cells. In adult PT cells this dose had a sigificantly less pronounced effect (12.0±2.8%, $p<0.05$). Using Northern blot techniques, four PKC isoforms were identified in the kidney and quantitated with dot blots.Transcripts for the classical, Ca^{2+}-dependent, diacylglycerol-activated isoform α, the new, Ca^{2+}-independent isoform δ and the atypical isoform ζ were abundantly expressed in renal tissue. The nPKCε was very weakly expressed. βI, βII and γ could not be detected. There was a tissue-specific and developmentally dependent expression of both the nPKCδ and aPKCζ.The nPKCδ and aPKCζ were significantly higher expressed in the infant medulla compared to cortex.The nPKCδ did also display a developmental increase with a 3-fold higher expression in the adult cortex. mRNA expression for the aPKCζ showed a 2-fold increase from infant to adult life in both cortex and medulla.

In conclusion: Calphostin C inhibits growth of renal epithelial cells with a more pronounced effect in immature cells.This might imply that the growth regulating effect of the diacylglycerol activated PKC isoforms cPKCα and nPKCδ undergoes postnatal changes in the kidney.Tissue- and developmentally dependent expression of both nPKCδ and aPKCζ suggests a specific role for these isoforms for the functional regulation of the mature kidney.

QZ 117 BIOCHEMICAL PROPERTIES OF A YEAST PROTEIN KINASE C HOMOLOGUE.

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The *S. cerevisiae PKC1* gene encodes a protein of approximately 130 kDa. which shows considerable homology to mammalian PKC's. PKC1p appears to play a role in cell wall biogenesis or repair. Many putative downstream effectors have been elucidated but there is as yet no evidence as to what the upstream activators of this kinase might be. The *PKC1* gene product is a phosphoprotein *in vivo* and is found mostly associated with a large pelletable complex. In order to purify and study the protein in more detail we have tagged PKC1p with the IgG binding domain of *S. aureus* protein A and fractionated yeast extracts overexpressing this tagged construct using IgG sepharose. Enzymatic activity is followed using a modified pseudosubstrate peptide.

Using PKC1p purified in this way we have shown that the enzyme autophosphorylates on ser/thr and is strongly inhibited by staurosporine and peptides corresponding to the pseudosubstrate site. Results of further biochemical characterisation of this protein will be described.

QZ 118 PROTEIN KINASE C- MEDIATED MAP KINASE ACTIVATION IS INVOLVED IN PMA-INDUCED BUT NOT FMLP-INDUCED PHOSPHOLIPASE D ACTIVATION IN HUMAN NEUTROPHIL .

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Phospholipase D (PLD) activation by fMet-Leu-Phe(fMLP) and phorbol ester (PMA) was refered as short-term and long term activation respectively, since fMLP induced maximum PLD activity at 45-60 sec, whereas PMA started to trigger the PLD after a lag of 2 min.The involvement of protein kinase C (PKC) has been investigated using an inhibitor belonging to the bisindolmaleimid family (GF 109203X).

We have devised an in-vitro assay for PLD measurement and observed that the plasma-membrane associated PLD required a cytosolic co-factor for full activity. Since PKC is known to activate downstream MAP kinases (MAPK),we have postulated that the MAPK could represent this factor, and have investigated their activation in relationship with PKC and PLD.

When fMLP was used as agonist, the $p40^{mpk}$ and $p42^{mpk}$ were phosphorylated. The PKC inhibitor was ineffective towards both the PLD activation and the phosphorylation of the two MAPK isoforms. When cells were activated with PMA, only the p40 isoform was activated. Cell treatment with the GF109203X inhibited both PLD and MAP kinase activation.

Altogether our results suggested different pathways for short-term and long-term PLD activation, discriminated by the involvement of PKC-mediated MAPK phosphorylation. Our results demonstrated differential regulation of MAPK which might depend upon the isoforms of PKC activated by the agonist. In that respect, calcium chelation fully inhibited the fMLP-induced PLD activation, whereas it partially inhibited the activity triggered by PMA.

This work has been supported by a grant fom BAYER-PHARMA. H.TRONCHERE is a recipient of an allowance from that company.

QZ 119 PLATELET DEPENDENT PHOSPHORYLATION OF COAGULATION FACTOR Va BY PKC-LIKE AND CASEIN KINASE II-LIKE KINASES, Matthew D. Rand, Michael Kalafatis and Kenneth G. Mann. Department of Biochemistry, University of Vermont, Burlington, VT 05405.

Coagulation factor V (FV) is a M_r=330,000 procofactor which is activated by thrombin cleavage to factor Va (FVa), a heterodimer of a M_r=105,000 heavy chain and a M_r=74,000 light chain associated by Ca^{2+}. FVa acts as a receptor and cofactor for the enzyme factor Xa (FXa) in the *prothrombinase* complex making the complex 300,000-fold more efficient than FXa alone in generating thrombin and thus is required for hemostasis. Platelet stimulation by thrombin results in secretion and activation of FV and provides the physiologic surface for formation of *prothrombinase* and the propagation of coagulation. The FV circulating in plasma is synthesized by the liver whereas FV stored in the secretory α-granules of platelets is synthesized by megakaryocytes. Platelet FV, representing 20% of the total FV in blood, is essential as demonstrated by patients with hemorrhagic disease attributed to deficient platelet FV despite normal plasma FV. We have identified two platelet kinase activities that phosphorylate FVa on the light and heavy chain independently. Light chain phosphorylation occurs exclusively with secreted platelet FVa at a minimum of two serine residues and is inhibited with H-7 and staurosporine suggesting PKC is involved. Purified PKC from rat brain phosphorylates plasma derived FVa on the light chain and a 2-dimensional tryptic map shows two major spots and four minor spots. Light chain phosphorylation does not alter FVa activity in *prothrombinase* however, FVa light chain is the major secreted platelet phosphoprotein containing 50% of the protein associated phosphate. Platelet dependent heavy chain phosphorylation is only observed with plasma derived FVa and occurs with or without platelet stimulation. Casein kinase II phosphorylates the heavy chain similar to platelets and the site is localized to the COOH terminus by cleavage with the enzyme activated protein C. We have previously shown heavy chain phosphorylation by casein kinase II accelerates FVa inactivation by activated protein C. These phosphorylation events identify a possible mechanism for platelet kinases in regulation of coagulation.

QZ 120 A RAPID, PHENOTYPIC SCREEN TO DISSECT PROTEIN KINASE C STRUCTURE-FUNCTION AND DRUG INTERACTION IN YEAST, Heimo Riedel and Hui-Ling Shieh, Section on Molecular Biology, Joslin Diabetes Center and Harvard Medical School, Boston, MA 02215

The molecular dissection of PKC action has been based in part on complex functional assays such as the mouse skin model for the testing of tumor promoter activity of phorbol esters and related compounds. To overcome the limitations imposed by the complexity of such assays we have developed the yeast *Saccharomyces cerevisiae* as an alternative, rapid and simple model system. We have established the conditions for efficient expression of mammalian PKC isoforms, confirmed their proper biochemical characteristics and investigated the specific physiological consequences. We have defined a specific yeast phenotype, an increase in the cell doubling time which is proportional to the level of mammalian PKC enzymatic activity. We have exploited this phenotype to create and functionally evaluate a number of random PKC cDNA mutations which have contributed to our understanding of the structural requirements of the PKC amino and carboxyl terminus in ligand-regulated PKC function.

We have established an *in vivo* assay that allows rapid evaluation of the interaction between PKC isoforms and drugs based on the resulting modulation of the yeast doubling time. With this assay we have defined differential interactions in PKC activation of the phorbol ester PMA, the diterpene mezerein, and the indole alkaloid indolactam V with two cysteine-rich regions in the conserved PKC region C1. We propose this assay as a rapid and general screen 1.) for the PKC activating or possibly inhibitory potential of drug candidates and 2.) to identify the PKC domains involved in these interactions with the help of PKC mutants.

QZ 121 MUTATIONAL ANALYSIS OF THE PKC-γ AND PKC-ζ ZINC FINGER DOMAINS.

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The regulatory domain of PKC carries a characteristic $(Cys)_6(His)_2$ motif which also occurs in other proteins (*c-raf*, DAG kinase, n-chimaerin, *vav*, *tpa*-1, *unc*-13). This structure in PKC-βI has been shown to tightly bind two zinc ions through either two $(Cys)_3(His)_1$ coordination spheres or by one $(Cys)_4$ and one $(Cys)_2(His)_2$ coordination sphere (1). While this structure is involved in phorbol ester binding by classic PKCs (α, β, γ), by novel PKCs (δ, ε, η) and by n-chimaerin, the corresponding structure in PKC-ζ or *c-raf* does not confer phorbol ester binding.

By exchanging individual amino acids and regions between the zinc finger structures of PKC-γ and PKC-ζ we investigated the structural requirements for phorbol ester binding by this motif.

Replacement of the highly conserved Asn^{137} in PKC-γ by Asp and replacement of Cys^{150} by Ser did not affect phorbol ester binding, whereas most of the other mutations within this region led to a loss of binding. Introduction of the highly conserved amino acids Pro^{111} and Asn^{137} of phorbol ester binding fingers into the zinc finger of PKC-ζ did not confer phorbol ester binding activity to PKC-ζ. Replacement of whole regions of the PKC-ζ finger by corresponding regions of PKC-γ could not convert PKC-ζ into a phorbol ester binding protein.

(1) Hubbard, S.R.; Bishop, W.R.; Kirschmeier, P.; George, S.J.; Cramer, S.P. and Hendrickson, W.A.: Identification and characterization of zinc binding sites in protein kinase C. *Science* **254**: 1776-1779 (1991)

QZ 122 DIABETES INDUCES SELECTIVE ALTERATIONS IN THE EXPRESSION OF PROTEIN KINASE C ISOFORMS IN HEPATOCYTES. Gary Sweeney, Edward S. Tobias, Eric Y. Tang and Miles D. Houslay. Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K.

Analysis of membrane and cytosol fractions from hepatocytes of both normal and streptozotocin-induced diabetic animals failed to identify immunoreactive species consistent with the presence of the β–I, γ, δ and η isoforms of protein kinase C (PKC). However, immunoreactive species of ~84kDa in size were noted with antisera specific for α, β–II, ε and ζ. In addition, a species migrating with an apparent size of ~94kDa was also detected in cytosol fractions using an antiserum specific for PKC-α. Each of these species was specifically displaced when the PKC-isoform specific peptide was included in the immunodection system. Induction of diabetes using streptozotocin invoked selective alterations in the expression of PKC isoforms which were reversed upon insulin therapy. In the cytosol fraction, marked increases of ~3-fold occurred in levels of the β–II isoform and the ~94kDa (upper) form of PKC-α, with no apparent/little change in the levels of the ~84kDa (lower) form of PKC-α and those of PKC-ζ. Diabetes induction also appeared to have elicited the translocation of PKC-β–II and the ~84kDa (lower) form of PKC-α to the membrane fraction where immunoreactivity for these species was now apparent. The levels of PKC-ε, which was noted only in membrane fractions, were also increased upon induction of diabetes. It is suggested that the selective alterations in the expression of PKC isoforms occurring upon streptozotocin-induced diabetes may lead to altered cellular functioning and underly defects in inhibitory G-protein functioning and insulin action which characterise this animal model of diabetes.

QZ 123 INHIBITION OF DOWN-REGULATION OF PROTEIN KINASE C δ BY BRYOSTATIN 1 CORRELATES WITH INHIBITION OF PHORBOL 12-MYRISTATE 13-ACETATE (PMA) INDUCED DIFFERENTIATION IN MOUSE KERATINOCYTES, Zoltan Szallasi, Colin B. Smith, Mitchell F. Denning, Andzrej A. Dlugosz, Stuart H. Yuspa, George R. Pettit*, and Peter M. Blumberg. National Cancer Inst., Bethesda, MD, and *Cancer Research Inst., Arizona State Univ., Tempe, AZ.

Both bryostatin 1 and PMA are potent protein kinase C (PKC) activators although in mouse keratinocytes bryostatin 1 induces only a subset of the responses to PMA and blocks those responses which it does not induce, including differentiation. To explore the mechanism for the differential effects of bryostatin 1 and PMA, we analyzed in detail the translocation and down-regulation of the PKC isozymes present in keratinocytes (α, δ, ε, η and ζ). We find that bryostatin 1 and PMA regulate these isozymes in quantitatively and qualitatively different fashions. PMA was more potent for translocating PKCδ and ε than PKCα. Bryostatin 1 showed a generally similar pattern but was 1-2 orders of magnitude more potent than PMA for translocating all three isozymes - PKCα, δ, and ε. Following translocation, PKCα and δ were fully down-regulated by PMA, whereas PKCε in the particulate fraction was not. Bryostatin 1 readily down-regulated PKCα, failed to down-regulate PKCε in the particulate fraction, and showed a biphasic dose response curve for down-regulating PKCδ. Low doses (0.1-1 nM) fully down-regulated PKCδ; high doses (100nM-1 μM) depleted PKCδ from the soluble fraction but failed to induce down-regulation of PKCδ in the particulate fraction. These high doses of bryostatin 1 inhibited down-regulation of PKCδ by 1 μM PMA. Furthermore, similar to the PMA-induced differentiation, the inhibition of epidermal growth factor binding by PMA was blocked by bryostatin 1. The dose-response curves for the inhibitory effects of bryostatin 1 on down-regulation of PKCδ and on the two biological responses showed excellent correlation. The membrane bound PKCδ protected from down-regulation by higher doses of bryostatin showed PKC activity. These results suggest that the differential regulation of PKC isozymes plays an important role in the differentiation process and focuses particular attention on PKCδ.

QZ 124 RESTORATION OF SUSCEPTIBILITY TO DIFFERENTIATION INDUCTION BY PMA IN A PMA-RESISTANT HL-60 CELL VARIANT BY TRANSFECTION WITH PKC β cDNA, Debra A. Tonetti, Cynthia Henning-Chubb, Douglas T. Yamanishi, Eliezer Huberman, Center for Mechanistic Biology and Biotechnology, Argonne National Laboratory, Argonne, IL 60439 and Department of Medicine, Division of Hematology/Oncology, University of California, Irvine, CA 92717

To identify the protein kinase C (PKC) isozymes that participate in the signal transduction pathway that leads to phorbol-ester-induced differentiation in HL-60 cells, we examined HL-60 variants that are either susceptible or resistant to such differentiation. The phorbol 12-myristate 13-acetate (PMA)-resistant cell variants exhibit reduced levels of PKC β steady-state RNA and protein levels as determined by Northern and Western blot analyses (Tonetti *et al., Cell Growth & Differ.*, **3**, 739-745, 1992). To substantiate the relationship between the PKC β deficiency and resistance to PMA-induced differentiation, we transfected into the resistant cells expression vectors containing cloned PKC βI or βII cDNAs. Correction of the PKC β deficit using this approach resulted in restoration of PMA-induced differentiation, as characterized by cell attachment, growth inhibition, reactivity with the OKM1 monoclonal antibody, and the ability to perform phagocytosis. These results indicate that PKC β is required for PMA-induced differentiation of HL-60 cells. Work supported by the U.S. Department of Energy, Office of Health and Environmental Research, under contract W-31-109-ENG-38.

QZ 125 **ROLE OF PROTEIN KINASE C IN RAS-MEDIATED FOS EXPRESSION.** Florian Uberall, Sonja Kampfer, Christian Schubert, Wolfgang Doppler and Hans H. Grunicke, Institute of Medical Chemistry and Biochemistry, Fritz Preglstrasse 3, A-6020 Innsbruck, AUSTRIA.

The mechanism by which transforming Ha-ras induces c-fos expression in HC11 mouse mammary epithelial cells was investigated with regard to controversial data concerning the role of protein kinase C (PKC) and the required promoter elements of the fos gene. HC11 cells carrying a glucocorticoid- inducible Ha-ras (val^{12}) construct were transfected with a chloramphenicol acetyltransferase (CAT) reporter gene under the control of a human fos promoter which includes the serum response element (SRE), the adjacent c-fos AP-1 site (FAP) and the cAMP response element (CRE). Induction of the Ha-ras gene by dexamethasone lead to a transactivation of expression of the transfected fos promoter construct which was inhibited by the PKC inhibitor BM41440 and abrogated in PKC-"depleted" cells. A similar transactivation was observed when the fos promoter construct was cotransfected with a constitutively active ras expression vector. Again, this effect was depressed by the PKC inhibitor and abolished in PKC-"depleted" cells. "PKC-depletion" was achieved by long-term exposure to TPA. This procedure was shown to deplete cells of PKC α and to significantly reduce PKC ε. Long-term exposure to bryostatin 1 selectively depletes PKC α. Depletion of PKC α by bryostatin 1 does not reduce the transcriptional activation of the SRE-FAP-TKCAT construct by Ha-ras. However, further support for the assumption that transforming Ha-ras does not employ a c-type PKC comes from observations with the staurosporine derivative CGP41251. 30-300 nM of CGP41251 corresponding for the IC_{50} values of HC11 cPKC isoforms do not affect transforming Ha-ras-mediated SRE-FAP-TKCAT induction. In order to delineate the promoter elements mediating the transcriptional activation, constructs which lack the FAP and the CRE sites but contain an intact SRE were cotransfected with the ras construct. Elimination of the FAP and CRE sequences did not affect the transcriptional activation by Ha-ras (val^{12}). It is concluded that in HC11 cells, transforming Ha-ras activates c-fos expression in a PKC-dependent manner presumably implying PKC ε and that the SRE is sufficient to mediate transcriptional activation.

QZ 126 DOPAMINE RECEPTOR STIMULATION DECREASES CYTOSOLIC GAMMA PKC IMMUNOREACTIVITY IN RAT HIPPOCAMPAL SLICES, Karin A. Yurko-Mauro and Eitan Friedman, Department of Pharmacology, Medical College of Pennsylvania, Philadelphia, PA 19129

Dopamine receptor activation has been shown to stimulate the hydrolysis of phosphoinositide in hippocampal brain slices (Undie and Friedman, 1990). This brain area is rich in PKC isozymes (γ, α and ß). In the present communications we investigated in hippocampal slices the effect of dopamine receptor stimulation on membrane and cytosolic PKC as determined by immunoblotting. Nanomolar concentrations of dopamine induced significant decreases in cytosolic γ PKC without causing changes in membrane enzyme levels. Dopamine receptor stimulation did not effect α PKC immunoreactivity in the hippocampus. The effects of dopamine appear to be mediated via a D1-like dopamine receptor since the effect was blocked by the D1 antagonist, SCH23390, but not by the D2 antagonist, sulpiride. The partial D1 agonist, SKF38393, produced similar but less effective changes in γ PKC immunoreactivity compared to dopamine. The dopamine-induced decrease in hippocampal γ PKC was inhibited by the calpain inhibitor peptide (N-Acetyl-leu-leu-norleucinal), suggesting that dopamine, by stimulating a D1-like receptor may be activating Ca^{++}-dependent proteolysis of hippocampal γ PKC.

This work was supported in part by USPHS Grant #NS29514.

PKC-Substrates and Localization;
PKC-Molecular Biology and Genetics

QZ 200 THE LOCALISATION OF FLUORESCENTLY LABELED PROTEIN KINASE C IN LIVING CELLS BY DIGITAL IMAGING MICROSCOPY. Philippe I.H. Bastiaens, Jan-Willem Borst, Antonie J.W.G. Visser and Thomas M. Jovin, Department of Molecular Biology, Max Planck Institute for Biophysical Chemistry, D3400 Göttingen, Germany. Department of Biochemistry, Agricultural University, 6703HA Wageningen, The Netherlands.
In order to visualize the distribution of protein kinase C (PKC) in living cells the enzyme isolated from rat brain was covalently labeled with the fluorescent succinimidyl ester Cy-3. The labeled PKC (CyPKC) retains full activity upon covalent attachment of one succinimidyl ester per protein and displays a fluorescent band on SDS-PAGE corresponding to a particle with the molecular weight of PKC. The CyPKC was microinjected into living 3T3 mouse fybroblasts and SV40 transformed 3T3 cells. The distribution of the protein was studied in both living and fixed cells by confocal microscopy and fluorescent lifetime imaging microscopy. The images show that cytosolic membranes and nuclei of cells are predominantly stained. The data imply that PKC has a role in signal transduction cascades not only at the plasma membrane interface but also within the nucleus.

QZ 201 ALTERATIONS IN PROTEIN KINASE C ISOZYME SUBCELLULAR DISTRIBUTION CORRELATE WITH CELL GROWTH CESSATION AND MATURE FUNCTION IN INTESTINAL EPITHELIUM IN SITU. Jennifer D. Black and Marian L. Saxon. Department of Experimental Therapeutics, Roswell Park Cancer Institute, Buffalo, NY 14263.
The mechanisms underlying control of cell growth and differentiation in epithelial tissues are poorly understood. Using the rat intestinal epithelium as a model system, we have examined the role(s) of PKC isozymes in epithelial cell renewal *in situ*. Immunofluorescence and Western blot analysis using a panel of isozyme-specific antibodies revealed that PKC α, βII, δ, ε, and ζ are expressed in all intestinal epithelial cells *in situ* and exhibit distinct subcellular distribution patterns along the crypt-villus unit. The combined morphological and biochemical approach used permitted analysis of specific PKC isozyme activation status at the individual cell level. These studies showed that dramatic changes in membrane association and level of expression of PKC α, βII, δ and ζ occur as cells cease division in the mid-crypt region and begin differentiation. Additional changes are observed with acquisition of mature function. Individual PKC isozymes exhibit different localization patterns within the same cell. These studies represent the first demonstration of naturally occurring changes in PKC isozyme subcellular distribution in a single cell type, within the context of a tissue. Changes occur at specific transition points associated with cell growth cessation and mature function. Direct activation of PKC in an immature intestinal crypt cell line results in growth inhibition and coincident translocation of PKC α from the cytosolic to the particulate subcellular fraction, paralleling observations made *in situ* and providing further support for a role for intestinal PKC isozymes in post-mitotic events. In addition to membrane interactions, tight association of individual isozymes with cytoskeletal elements suggests participation of PKC in control of the structural organization of the enterocyte. Taken together, the results presented indicate an involvement of PKC isoforms in signalling events related to growth cessation, differentiation and function of intestinal epithelial cells *in situ*. Supported by NSF grant DCB-8917424 and NIH grant CA 16056.

QZ 202 IDENTIFICATION OF SEQUENCE SPECIFIC INTERACTIONS OF PROTEIN KINASE C ISOENZYMES WITH THE ACTIN CYTOSKELETON, Gerard C. Blobe, Doriano Fabbro, Sylvia Stabel and Yusuf A. Hannun, Departments of Medicine and Cell Biology, Duke University Medical Center, Durham, NC 27710; CIBA-GEIGY, Basel, Switzerland and Max-Planck-Gesellschaft, Koln, Germany.
Protein Kinase C βI and βII differ only over the last 50-52 aa at their carboxy terminus. Although PKC βI and βII exhibit identical catalytic properties with most in vitro substrates, PKC βII can selectively phosphorylate actin in vitro. In the human T-cell leukemia cell line, MOLT-4, activation with PMA (100 nM) causes rearrangement of the actin cytoskeleton and specific translocation of PKC βII to the cytoskeleton where interaction with actin occurs. PKC βI was unable to translocate to the cytoskeleton or interact with actin. Using the F-actin co-sedimentation assay with baculovirus expressed, partially purified PKC isoenzymes, PKC βII was able to bind F-actin while PKC βI was not. The interaction of PKC βII required 1 mM Mg^{2+} and could be competed off with peptides based on the divergent region of PKC βII, but not by peptides based on PKC βI specific sequence. The divergent region of PKC ßII contains a potential actin binding site (ABS) highly homologous to the ABS of Troponin I. Analysis of PKC isoenzyme sequences revealed that, along with PKC βII, PKC α, γ and ζ also contained this ABS. In agreement with this, these isoenzymes also specifically bound F-actin. In contrast, PKC δ and ε along with PKC βI did not contain this sequence and did not bind F-actin specifically. Thus, PKC isoenzymes diverge in their ability to bind to and interact with actin in a sequence specific manner. These findings establish a direct relationship between PKC and the actin based cytoskeleton and elucidate isoenzyme specific function at the molecular level.

QZ 203 CLONING AND CHARACTERIZATION OF THE GENE AND TWO MESSAGES OF HUMAN PKC-α. Russell T. Boggs, Kathy A. McGraw, Robert McKay, and Nicholas M. Dean. Dept. of Molecular Pharmacology, Isis Pharmaceuticals, Carlsbad, CA. 92008.

Two transcripts of Protein kinase C-α (PKC-α) (4 kb and 8.5 kb) are seen on Northern blots derived from both human and mouse cells. The full length 4 kb transcript from the mouse has been cloned and sequenced (GenBank accession number m25811), but in the case of the human gene, only the open reading frame and small amounts of 5' and 3' untranslated regions are known (GenBank accession number x52479). One would expect approximately 1 kb of additional sequence to be present in the shorter message. In order to acquire novel sequences for use in designing antisense oligonuleotide drugs, we obtained further sequences from both messages, including some which are unique to the long message. The work was performed using the techniques of 3' R.A.C.E. (Frohman, *et al.* (1988) P.N.A.S. 85: 8998) and Inverse PCR (Ochman, et al. (1988) *Genetics* 120: 621) applied to RNA derived from human A549 cells. We have also isolated bacteriophage clones corresponding to the gene of PKC-α.

QZ 204 PROTEIN KINASE C ISOFORMS HAVE DISTINCT NUCLEAR LOCALIZATIONS IN NEUROBLASTOMA x GLIOMA HYBRID CELLS. Klaus Buchner[§], Roland Beckmann[§], Carsten Lindschau*, Hermann Haller*, Ferdinand Hucho[§], [§]Institute for Biochemistry, Free University Berlin, 14195 Berlin and *Max-Delbrück-Center for Molecular Medicine, 13122 Berlin-Buch, Germany

The role of the different protein kinase C (PKC) isoforms and their specific regulation and localization is not yet understood. Especially in neuronal cells, different PKC isoforms seem to be involved in different cellular processes.

We used neuroblastoma x glioma NG 108-15 cells to determine the specific localization of PKC isoforms by confocal laser scanning fluorescence microscopy.

We found by Western blotting that PKCα, PKCδ and PKCε are the predominant isoforms in these cells. In contrast to other cell lines, the PKC α, δ and ε are not down-regulated during differentiation.

Immunofluorescence microscopy revealed that in undifferentiated cells PKCα is located either in the nucleus excluding nucleoli, or in the cytoplasm. In differentiated cells PKCα was almost exclusively detected in the cytoplasm. Stimulation of the cells with phorbol ester resulted in the translocation to the plasma membrane.

PKCδ was found in the nucleoli and particularly in the neurites. Stimulation with phorbol ester did not cause a change of localization in undifferentiated or in differentiated cells.

PKCε is co-localized with the nuclear pore complexes at the nuclear envelope. In differentiated cells both a strong signal at the nuclear envelope and a translocation to the plasma membrane was observed upon phorbol ester stimulation.

QZ 205 THE η (eta) ISOFORM OF PROTEIN KINASE C (nPKCη) IS LOCALIZED ON ROUGH ENDOPLASMIC RETICULUM. Kazuhiro Chida[1], Hiroshi Sagara[2], Yasutoshi Suzuki[1], Akiko Murakami[1], Shin-ichi Osada[3], Shigeo Ohno[3], Kazushige Hirosawa[2], and Toshio Kuroki[1] ([1]Department of Cancer Cell Research, [2]Department of Fine Morphology, Institute of Medical Science, University of Tokyo, Tokyo 108, Japan, [3]Department of Molecular Biology, Yokohama City University School of Medicine, Yokohama 236, Japan)

The η isoform of protein kinase C (nPKCη), isolated from a cDNA library of mouse skin, has a unique tissue and cellular distributions; it is predominantly expressed in epithelia of the skin, digestive and respiratory tracts in close association with epithelial differentiation. We report here that this isoform is localized on rough endoplasmic reticulum (rER) in transiently expressing COS1 cells and constitutively expressing mouse keratinocyte line BALB/MK-2, human normal keratinocytes and epidermoid carcinoma cell line A431. By using polyclonal antibodies raised against peptides of the diverse D1 and D2/D3 regions, we found that immunofluorescent signals were most strong in the cytoplasm around the nucleus and became weaker toward the peripheral cytoplasm. In immunoelectron microscopic examination, the antibody-specific electron dense signals were detected on rER and the outer nuclear membrane that is extension of rER. However, no signal was detected in the nucleus, inner nuclear membrane, smooth ER, Golgi apparatus, mitochondria, peroxisomes or plasma membrane. Treatment of the cells *in situ* with saponin or non-ionic detergent NP-40 suggested that the association of nPKCη with intracellular membrane structure. By immunoblotting, a distinct single band with M_r 80,000 was detected in the whole human keratinocyte lysate, rough microsome and crude nuclear fractions, all of which contain outer nuclear membrane and/or rER, keeping with the localization of nPKCη on rER. The unique localization of nPKCη may be linked with its activation mechanism and substrate specificity.

QZ 206 CHRONIC MORPHINE ALTERS PROTEIN KINASE C-δ IMMUNOREACTIVITY IN RAT BRAIN: LACK OF EVIDENCE FOR NMDA RECEPTOR INVOLVEMENT. Meredith M. Garcia and Richard E. Harlan, Departments of Otolaryngology and Anatomy, Tulane University School of Medicine, New Orleans, LA 70112.

The protein kinases C (PKC) are a family of serine-threonine kinases, all of which require a lipid cofactor for activation and some of which are also calcium-dependent. At least 10 different isoforms of PKC exist, many of which are highly expressed in brain; among these is the δ isoform (PKC-δ), which is member of the calcium-independent subgroup of PKC. As this isoform has a highly restricted distribution in rat brain, including the thalamic nuclei which express the μ opiate receptor (Garcia et al., 1993), we studied the effect of chronic morphine treatment on PKC-δ immunoreactivity (ir) in rat brain. Male Sprague-Dawley rats (5 per group) were injected with vehicle or morphine (10 mg/kg), daily for 5 days and sacrificed on day 6; this treatment paradigm has been shown to induce both morphine tolerance and dependence. Following chronic morphine administration, there were significant decreases in the intensity of immunostaining in cell bodies of the lateral posterior, mediodorsal, and ventral posteromedial thalamic nuclei; no changes were detected in several other thalamic nuclei which lack μ receptors. Conversely, chronic morphine administration induced increased PKC-δ-ir in certain axonal projections to cortex, especially from the anterior nuclear group, and in axons of layer 6 of frontal cortex. Interestingly, cells in layer 6 project preferentially to the patch compartment of striatum, where we have found an increase in calbindin D28k in response to chronic morphine (Garcia and Harlan, 1993), suggesting a thalamocortical-corticostriatal pathway which may be activated by μ opiates. Thalamus is a major relay station in the pain pathway and is also enriched in the NMDA subtype of excitatory amino acid receptors. Some recent studies have shown that the NMDA receptor antagonist MK-801 inhibits the development of tolerance to morphine analgesia (Trujillo and Akil, 1991), while others have shown that μ opiate agonists may modulate NMDA receptor activity through activation of PKC (Chen and Huang, 1991). To determine if changes induced in PKC-δ-ir by morphine involved the NMDA receptor, we treated rats twice daily for 5 days with MK-801 (0.2 mg/kg)+vehicle or MK-801 +morphine (10mg/kg). PKC-δ-ir in thalamic nuclei of theMK-801+morphine brains was not different from that seen in brains of rats treated with morphine alone; MK-801 alone had no effect on PKC-δ-ir. This suggests an NMDA receptor-independent mechanism for morphine's effects in thalamus. (Supported by DA05411 [MG] and DA06194 [RH])

QZ 207 EXPRESSION AND CHARACTERIZATION OF WILD-TYPE AND MUTANT HUMAN LAMIN B IN THE BACULOVIRUS INSECT CELL SYSTEM. VALERIE L. GOSS[1], CARL A. STRATTON[1], BARBARA A. HOCEVAR[1], DAVID J. BURNS[2] AND ALAN P. FIELDS[1] [1]Department of Pharmacology, Case Western Reserve University School of Medicine, Cleveland, OH and [2]Sphinx Pharmaceutical Corp., Durham, NC.

Multi-site phosphorylation of the nuclear lamins is thought to regulate the process of mitotic nuclear envelope breakdown (NEBD). However, the phosphorylation sites that mediate NEBD are still in question. Two potential human mitotic lamin kinases, βII PKC and $p34^{cdc2}$/cyclin B kinase, have previously been identified. βII PKC-mediated phosphorylation is confined to two sites, $Ser^{395}$and Ser^{405}, within the carboxy-terminal domain of lamin B. On the other hand, sequence analysis reveals the presence of two potential phosphorylation sites for $p34^{cdc2}$/cyclin B, Ser^{23} and Ser^{393}. We have utilized site-directed mutagenesis of the human lamin B_1 cDNA to convert each of the proposed serine phosphorylation sites to alanines. Recombinant baculoviruses encoding wild-type or mutant lamin B have been produced which allow for high level expression of these proteins in Sf9 insect cells. The recombinant proteins migrate with the same apparent molecular weight (67 kDa) and isoelectric point (6.0) as native lamin B. Furthermore, recombinant lamin B is isoprenylated normally, exhibits the same fractionation and solubility properties as native lamin B and is recognized by a specific mouse anti-lamin B monoclonal antibody. *In vitro* phosphorylation of wild type and mutant lamin B confirmed Ser^{395} and Ser^{405} as βII PKC phosphorylation sites and identify Ser^{23} as the sole site for human $p34^{cdc2}$/cyclin B kinase phosphorylation. A second potential $p34^{cdc2}$ kinase site, Ser^{393}, is phosphorylated by sea star $p34^{cdc2}$ kinase but not the human enzyme demonstrating that these enzymes have different specificities. We are currently using these site-directed mutants to determine the role of individual phosphorylation sites and these two lamin kinases in mitotic NEBD and reassembly in intact cells.

QZ 208PKC SUBSTRATES: DIFFERENTIAL PHOSPHORYLATION BY PKC ISOENZYMES,
Michael Gschwendt, Kirsten Kielbassa, Walter Kittstein and Friedrich Marks, German Cancer Research Center, Heidelberg, Germany

Several PKC substrate proteins, such as GAP, cdc2 kinase and partially purified proteins from murine epidermis and porcine spleen, were studied with respect to their phosphorylation by the Ca^{2+}-responsive cPKC (α,β,γ) and the Ca^{2+}-unresponsive PKCδ. Some of these substrates were found to be phosphorylated preferentially by one of the two types of isoenzymes (either cPKC or PKCδ). The details of this differential phosphorylation will be discussed. At least one of these substrates appears to be phosphorylated by cPKC and PKCδ at different sites. Other substrates, as for instance GAP, are phosphorylated by cPKC and PKCδ nearly equally well. GAP not only serves as a substrate for PKC but also forms a complex with the enzyme, which is more stable than ordinary enzyme/substrate complexes. The properties and functions of this putative signalling complex will be discussed.

QZ 209DIFFERENT SPATIAL DISTRIBUTION OF PKC-α AND -β IN VSMC BY PDGF AND AII
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Proteinkinase C is an important intracellular second messenger for growth factors which is translocated from a cytosolic compartment to cell membranes upon cell stimulation. Using confocal microscopy we investigated the spatial distribution of PKC after stimulation of cultured VSMC with PDGF and angiotensin II. Monoclonal antibodies for PKC-α, -β and -γ were used. PKC translocation was also assessed by Western blot analysis after cell fractionation.
Immunoreactivity for PKC-α and -β was detectable in VSMC. PKC was evenly distributed in the cytosol, while PKC formed coarse granules in the perinuclear region. Using Western blot we observed a shift of the immunoreactivity of both PKC isoforms from the cytosolic to the membrane fraction after exposure of VSMC to AII (10-7M) or PDGF (100 ng/ml) (6 and 12 min). Confocal microscopy revealed that AII induced a rapid assembly of PKC-α along cytosolic fibers (6 min) followed by a translocation of the isoform towards the perinuclear region (12 min) and into the nucleus. PDGF also increased immunoreactivity for PKC-α in the nucleus but no fibrillar distribution in the cytosol was observed. PKC-β was rapidly translocated by AII to the perinuclear region (6 min) followed by a bright staining of the nucleus (12 min). Similar nuclear staining for PKC-β was observed after PDGF (12 min).
Thus PKC isoforms are differently distributed in VSMC. AII induces a rapid assocation of PKC-α with cytoskeletal structures followed by translocation into the nucleus, while PDGF increases mostly nuclear PKC-a. Translocation of PKC into the nucleus by growth-promoting factors may be important for the induction of VSMC growth.

QZ 210 PROTEIN KINASE C β ISOFORMS REVEAL A FUNDAMENTAL ORGANIZATION OF THE RAT FOREBRAIN
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The mammalian forebrain can be organized into three major hierarchical systems. The first of these consists of pyramidal and non-pyramidal neurons of the cerebral cortex, which project to subcortical neurons. The second system consists of medium spiny neurons in the striatum and related subcortical areas, which receive cortical input. The third system consists of magnocellular neurons in the forebrain and midbrain, which receive input from the striatal neurons and project back to the cerebral cortex, either directly or indirectly (through the thalamus). Using immunocytochemistry with antibodies specific for the βI and βII protein kinase C (PKC) isoforms (Wetsel et al., 1992), we have found that the antibody to the βII isoform intensely labels essentially all cells within the second system in forebrain, i.e., the striatum and related structures, including their projections to the magnocellular neurons of the globus pallidus and substantia nigra. The antibody to the βI isoform intensely labels all magnocellular neurons of the third system, including neurons in the diagonal bands of Broca, ventral pallidum, substantia innominata, globus pallidus and substantia nigra pars reticulata. It is significant that these magnocellular neurons, in general, are born before the medium spiny neurons, suggesting that the βI isoform is found in neurons that are phylogenitically and ontogenetically older than the neurons that express the βII isoform. Since these two isoforms are generated by differential splicing of the same primary transcript, it is likely that specific mechanisms have evolved to generate the βI variant in older, magnocellular neurons, and the βII variant in newer striatal neurons. These data suggest that the β isoforms of PKC may participate in the fundamental mechanisms of neural organization during development of the forebrain. (DA06194 [RH] &DA054ll [MG])

QZ 211ENDOTHELIAL PROLIFERATION AND THE PROTEIN KINASE C GENE FAMILY,
Elizabeth O. Harrington, K. Craig Kent, James D. Chang, Laura J. Collins, Sonja M^{c}Grail, J. Anthony Ware, Cardiovascular Division, Beth Israel Hospital, Harvard Medical School, Boston, MA 02215

Many developmental, physiological, and pathological states are associated with vascular endothelial cell proliferation. Several mitogens and cytokines that induce endothelial cell proliferation have been identified; however, the intracellular signalling events that lead to endothelial proliferation are not understood. Accordingly, we determined whether protein kinase C (PKC) was required for proliferation of endothelial cells (EC) and characterized the distribution of PKC isoenzymes. Addition of phorbol ester (10 nM PMA) significantly increased proliferation of human EC by 72 hours after treatment, with maximal proliferation (180±55%) induced by 1 μM PMA. The ability of the endothelial mitogen fibroblast growth factor to stimulate human EC proliferation was inhibited by the known PKC inhibitors H-7 or chelerythine, and by prolonged treatment of human EC with PMA. Immunoblotting with anti-PKC antibodies revealed no α or β isoenzymes in human EC, but these isoenzymes were present in vascular smooth muscle cells or bovine aortic endothelial cells. Northern blotting analysis revealed the presence of mRNA encoding PKC η, ζ, and θ in serum starved primary human EC, but PKC α and δ mRNAs were not expressed. RNA extracted from primary human EC or two cell lines of endothelial cell origin was reverse transcribed and the cDNA was amplified using nested degenerate oligonucleotides based on sequences conserved in all PKC isoenzymes; no product corresponding to "conventional" PKCs was identified. Thus, the important role of PKC in endothelial proliferation appears to be mediated entirely by "novel", non-calcium containing PKCs; furthermore, EC are potentially valuable models to examine the selective function of the conventional PKCs.

QZ 212 INTRACELLULAR RECEPTORS OF PROTEIN KINASE C IN THE YEAST *SACCHAROMYCES CEREVISIAE*

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Protein kinase C (PKC) activity purified from *Saccharomyces cerevisiae* displays properties similar to those of the mammalian novel and classical PKC isozymes [1][2]. A yeast gene, *PKC1*, homologous to the mammalian isozymes has been isolated through the screening of yeast libraries with mammalian cDNA clones [3]. Pkc1p is thought to participate in maintaining the integrity of the cell envelope. Several different *PKC* genes have recently been isolated in our laboratory, and the activity of one gene product, Pkc2p, has been functionally implicated in amino acid metabolism (Simon *et al.*, submitted).

Different mammalian PKC isozymes have been shown to translocate into a variety of distinct cellular compartments upon activation. It has been proposed that this translocation reflects the binding of PKC to intracellular Receptors of Activated C Kinase (RACKs) [4]. Such receptors were subsequently purified from mammalian cells and the corresponding gene for one of these (*RACK1*) cloned and characterised (Mochly-Rosen *et al.*, submitted). The binding of PKC to RACKs has been found to be specific, concentration-dependent and saturable.

We have recently confirmed the existence of RACKs in yeast. An immunological screen of a yeast expression library produced two RACK clones, isolated by their ability to bind mammalian PKC. Sequence analysis shows that one of the cloned genes encodes a protein involved in the response to nitrogen starvation, whilst the other gene shows no homology to any known sequences. The biochemical analysis of the yeast RACKs should provide an insight into the ways by which PKC interacts with its receptors.

1. Ogita, K., *et al.*, (1990) Proc. Natl. Acad. Sci. USA. **87**: 5011-5015.
2. Simon, A.J., *et al.*, (1991) Proc. R. Soc. Lond. B. **243**: 165-171.
3. Levin, D.E., *et al.*, (1990) Cell. **62**: 213-224.
4. Mochly-Rosen, D., *et al.*, (1991) Proc. Natl. Acad. Sci. USA. **88**: 3997-4000.

QZ 213 DIFFERENT SUBCELLULAR LOCALIZATION OF PKC ISOZYMES IN U937 CELLS SUGGESTS UNIQUE FUNCTIONS DURING TPA-INDUCED DIFFERENTIATION,

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ICRF U937 human promonocytic leukemia cells express at least four PKC isozymes: β_1, β_2, ε and ζ. Indirect immunocytofluorescence using affinity-purified PKC-specific antibodies shows distinct subcellular localization and translocation patterns for the four isozymes. PKCs β_1 and ε are found in the cytoplasm and in membrane-bound compartments of the resting cell, whereas PKC-ζ is found exclusively in the cytoplasm. Membrane-bound PKC-β_1 is associated with performed β_2-integrin vessicles and PKC-β_2 is associated with microtubules (MT). Acute TPA-treatment causes translocation of all PKC isozymes except ζ and prolonged TPA-treatment down-modulates cytoplasmic non-ζ PKCs, but not vessicle-associated PKC-β_1. In differentiated U937 cells, growth-arrested in G_2, PKC-ζ is found in the Triton-insoluble nuclear cytoskeleton (CSK). TPA-differentiation resistant U937 clones were derived to further explore the role of individual PKC isozymes in macrophage differentiation. These cells differ from parent U937 cells in several respects: TPA does not induce β_2-integrin surface expression; PKC-β_2 decoration of MT is markedly decreased; MT depolymerization response to TPA is lost; and integrin vessicles do not translocate in response to TPA. Overlay analysis of PKC-β_2 binding proteins showed that several MT-associated proteins are down-modulated in the resistant cells, suggesting a loss of PKC-β_2 substrates that regulate MT reorganization may result in the phenotype observed. Our results implicate both PKC-β_1 and -β_2 in the regulation of β_2-integrin expression and PKC-ζ in nuclear CSK events during late G_2/M transition.

QZ 214 IDENTIFICATION AND SUBCELLULAR CHARACTERIZATION OF PROTEIN KINASE C (PKC) ISOFORMS IN A GLUCOSE-SENSITIVE INSULINOMA,

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Protein kinase C (PKC) represents a growing family of serine/threonine kinases which include both Ca^{2+}-dependent and Ca^{2+}-independent members. To further evaluate the specific expression of PKC isoforms in insulin-secreting beta-cells isolated cells from a glucose-sensitive rat insulinoma were fractionated into cytosolic, crude membrane, and cytoskeletal/nucleoskeletal fractions. Protein samples (40-100 micrograms from each fraction) were resolved with sodium-dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transblotted to nylon membranes. The blots were then analyzed with antibodies specific for the a, β, γ, ε, ζ, and δ isoforms. In addition, comparisons were also made with whole isolated rat islets. Expression of all but the γ isoform was detected in the insulinoma-derived cells. Expression of the α, β and ε isoforms was confined predominantly to the cytosolic fractions. Interestingly, the δ isoform was detected in all three of the subcellular fractions while the ζ isoform was present in approximately equal amounts in both the cytosolic and crude membrane fractions. The δ isoform could be eluted from the cytoskeletal/nucleoskeletal fraction with 1% Triton X-100®. All of the isoforms detected in the insulinoma-derived beta-cells were also detected in whole isolated rat islets. It is concluded that beta-cells express numerous isoforms of PKC including both Ca^{2+}-dependent and Ca^{2+}-independent forms which may be important in the various signal transduction processes of insulin secretion, growth, insulin biosynthesis, and insulin-gene expression.

QZ 215 EXPRESSION OF PKC ISOZYMES IN HUMAN T CELLS.

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Protein kinases C (PKC) constitute a family of kinases involved in numerous cellular events including cell proliferation and differentiation in response to soluble factors (hormones, cytokines etc.). In T cells PKC are suspected to mediate antigen induced signals. Tissular distribution as well as intracellular localisation varies from one PKC isozyme to another. We have examined here the expression of PKC isozymes in freshly prepared human T cells from both peripheral blood and thymus. We found that PKCβ was highly expressed in both CD4+ and CD8+ sub-types and the double positive CD4/CD8 thymocytes, whereas the α isozyme was detected mainly in the CD4+ T cells, was under-expressed in CD8+ cells and was not detectable in double positive CD4/CD8 thymocytes. These preliminary results might reflect a functional correlation between PKCα and the intrathymic T cell development.

QZ 216 PLASMA MEMBRANE Ca^{2+}-ATPase FROM CEREBELLUM IS A SUBSTRATE FOR PROTEIN KINASE C

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The plasma membrane Ca^{2+}-ATPase purified from rat cerebellum synaptosomal membranes was phosphorylated by protein kinase C (from rat brain) at 500 μM free calcium in the presence of different activators, including 12-myristate-13 acetate phorbol (PMA), 1,2-dioctanoyl-sn-glycerol (DAG), and phosphatidylserine (PS) alone and combined. The highest level of the protein kinase C catalyzed ^{32}P incorporation into the Ca^{2+}-ATPase was observed with the PMA-activated kinase resulting in an increase of the Ca^{2+}-ATPase activity by 30-40%. When calmodulin, a Ca^{2+}-ATPase modulator, was included in the assay, PS enhanced the PMA dependent phosphorylation by protein kinase C five times. Additionally, in the presence of calmodulin the DAG/PS-activated protein kinase C was able to phosphorylate the Ca^{2+}-ATPase to the same high level.

Our studies indicate that the Ca^{2+}-ATPase is a substrate for protein kinase C in vitro. Protein kinase C-dependent phosphorylation could be a regulatory mechanism of the plasma membrane Ca^{2+}-ATPase in cerebellum. Calmodulin is most probably needed for DAG to be an activator in this process. It is plausible that, depending on the availability of calmodulin to the Ca^{2+}-ATPase, protein kinase C would respond to different activators present in its environment.

QZ 217 PROTEIN KINASE C LOCALIZATION AND PHOSPHATIDYLCHOLINE HYDROLYSIS IS STIMULATED IN THE NUCLEUS OF α-THROMBIN-TREATED IIC9 CELLS.

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An understanding of how signals originating at the plasma membrane are communicated to the nucleus is an important biological question. We have previously shown (*J. Biol. Chem.* **266**, 3215) that α-thrombin treatment of IIC9 cells results in increased levels of cellular 1,2-diacylglycerol (DAG) and activation of protein kinase C (PKC). We show here that changes in nuclear PKC and nuclear DAG are also induced following α-thrombin treatment. IIC9 cells were treated with 500 ng/ml α-thrombin and nuclei were then isolated. PKC α, but not PKC ε or ζ, was present in the nuclei of cells treated with either phorbol 12,13-myristate (PMA) or α-thrombin. The rise in nuclear PKC levels occurred rapidly and reached a maximum at 30-60 sec, which was followed by a decline back to the control level over the next 15 min. In addition, α-thrombin treatment resulted in an immediate rise in DAG mass levels in the nuclear fractions. The potential source of the induced nuclear diglycerides was identified by carrying out molecular species profiles of both the induced diglycerides and nuclear phospholipids by capillary gas chromatography. The molecular species profiles of the nuclear diglycerides genereated resembled the species profiles of phosphatidylcholine, and not phosphoinositides species, at all times. Our results suggest that increases in both nuclear DAG levels and PKC activity following α-thrombin treatment may play a role in mediating thrombin-induced nuclear responses such as changes in gene expression and cellular proliferation.

QZ 218 INTRACELLULAR LOCALIZATION AND BIOLOGICAL EFFECTS OF DIFFERENT DOMAIN FRAGMENTS OF PROTEIN KINASE C-EPSILON OVEREXPRESSED IN NIH 3T3 CELLS,

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Protein kinase C ε (PKCε) has been reported to exhibit oncogenic potential. To better define the interrelationship between its biological effects and its domain structure, experiments were carried out to transfect fragments of PKCε into NIH 3T3 cells. A new metallothionein promoter driven ε-epitope tagging vector, the εMTH, was used to overexpress ε-tagged PKCε peptide fragments designed to represent various domains of PKCε. The overexpressed proteins each contain the ε-tag peptide at the C-terminus to allow ready detection with an antibody specific for the ε-peptide tag. Transfection of NIH 3T3 cells with the tagging vector constructs resulted in the Zn-inducible overexpression of ε-tagged fragments including the whole N-terminal regulatory fragment (ε1), a pseudosubstrate-Zn finger fragment (ε2), a Zn finger fragment (ε3), a Zn finger-hinge region fragment (ε7), and a catalytic domain fragment (ε4). The different overexpressed PKCε fragments exhibited differential effects on cell growth. The various PKCε fragments also exhibited different patterns of subcellular localization as determined by immunocytochemistry, and by cell fractionation combined with Western blot analysis. Taken together, the results suggest 1/ that the different fragments of PKCε contain domains which drive localization to different subcellular compartments and indicate 2/ that PKCε fragments lacking phosphotransferase activity nonetheless may exhibit biological activity.

QZ 219 IN VIVO MUTAGENESIS OF THE MOUSE PKC-β GENE BY HOMOLOGOUS RECOMBINATION.

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In order to explore the physiological role of PKC-β in the organism we set out to mutate the PKC-β gene in the mouse by homologous recombination. From a mouse genomic DNA library (strain 129) several phages were isolated spanning a region of about 30 kb of the mouse PKC-β gene covering the promoter region and the first and second exon. From these phages two types of mutagenesis vectors were constructed which both are designed to generate a null mutation at the PKC-β locus:

1) the first two exons of the PKC-β gene were replaced by the Neomycin resistance gene,

2) the second PKC-β exon was fused to the bacterial lacZ gene in order to monitor PKC-β gene expression in the targeted mouse.

The type 2 vector was introduced into mouse embryonic stem (ES) cells by electroporation and homologous recombination events were detected at a frequency of 1/30 G418-resistant colonies.

Several of these targeted ES cell lines are currently being injected into blastocysts of strain C57 Bl/6 mice in order to generate PKC-β deficient mice. In addition, expression of β-galactosidase (lacZ) activity in these mice is governed by the PKC-β promoter and will allow to monitor the expression of PKC-β during development and in the adult.

QZ 220 PROTEIN KINASE C INVOLVEMENT IN PMA- AND TNFα-INDUCED ICAM-1 AND E-SELECTIN EXPRESSION. INHIBITION BY TWO ISOENZYME SPECIFIC PKC INHIBITORS, GÖ6850 AND GÖ6976

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Previous studies have shown that PKC is involved in ICAM-1 and E-Selectin expression induced by PMA. In contrast, most studies have shown that TNFα-induced adhesion ligand expression is not PKC dependent. Studies of PKC involvement in these systems have been hindered by the lack of selective PKC inhibitors and isoenzyme-specific inhibitors. The bisindolylmaleimides are a recently described class of potent, selective PKC inhibitors. Two compounds from this class, Gö6850 and Gö6976, differ in activity against Ca^{++} independent PKC isoenzymes. Gö6850 is active against both calcium-dependent and independent isoenzymes (α, ß1, δ, ε, and ζ). Gö6976 is a selective inhibitor of the calcium dependent isoenzymes (α and β1). Both of the inhibitors act at the ATP binding site. (J. Biol Chem 268:9194, 1993). We tested these inhibitors against PMA and TNFα-induced adhesion ligand expression and neutrophil adhesion to activated endothelium. Both inhibitors were less effective (IC_{50}s > 4 μM) against TNFα-induced adhesion ligand expression and neutrophil adhesion which is consistent with their relatively weak inhibition of PKCζ. Gö6850 and Gö6976 were very effective against PMA-induced ICAM-1 and E-Selectin expression. Gö6850 was equipotent against both ligands (IC_{50}s = 37 and 57 nM, respectively). Gö6976 was more active against ICAM-1 than E-Selectin (IC_{50}s = 7.1 and 139 nM, respectively). Gö6850 and Gö6976 also inhibited neutrophil adhesion to PMA-stimulated endothelium (IC_{50}s = 535 and 251 nM). These results suggest that calcium-dependent isoenzymes are involved in the PMA-induced adhesion ligand expression by activated endothelial cells. Gö6850 and Gö6976 are useful probes for the evaluation of PKC and PKC isoenzyme-related cellular events.

QZ 221 PKC-δ ASSOCIATES WITH VIMENTIN IN DIFFEREN TIATED HL60 CELLS

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Protein kinase C is a family of isoenzymes that regulate a wide range of cell functions including, cell morphology and cell adhesion. Immunocytochemical studies have revealed distinct patterns of distribution for the different PKC isozymes, this pattern of distribution can alter upon activation of PKC. Thus, PKC isoenzymes have cell-specific localisation and may phosphorylate different substrates to regulate cell functions.In this study we use an isoenzyme specific antibody to determine the distribution of PKC-δ in proliferating HL60 cells and cells induced to differentiate with TPA. Treatment with 10nM TPA induced growth arrest, expression of monocyte specific enzymes and attachment of cells to tissue culture plates. In the proliferating, unattached cells PKC-δ was expressed at a low level and was associated predominantly with nucleolar structures. This distribution may reflect the involvement of PKC-δ in the regulation of nuclear function, particularly protein synthesis. Several nuclear proteins have been shown to be substrates for PKC in vitro, including RNA polymerase. Following 48h treatment with TPA the expression of PKC-δ was increased and the enzyme was predominantly in the cytoplasm. PKC-δ staining was filamentous and reminiscent of intermediate filament (IF) staining. Immunocytochemistry, including dual labelling experiments, revealed that PKC-δ co-localised with vimentin. Vimentin expression increased in differentiated cells, reflecting its role in cell attachment. The significance of these findings is not known, it may be that PKC-δ associates with vimentin following activation and autophosphorylation. Vimentin may be a substrate for PKC-δ, it is a substrate for PKC in vitro and IF assembly is controlled by phosphorylation.

QZ 222 INCREASED TURNOVER OF PHOSPHATIDYCHOLINE IN RESPONSE TO PHORBOL ESTER IS CORRELATED WITH EXPRESSION OF PROTEIN KINASE C-ALPHA AND MARCKS IN FOUR NEURAL CELL LINES, Sherry C. Morash, Sergio Rosé, David M. Byers, F.B. St.C. Palmer and Harold W. Cook, Atlantic Research Centre, Departments of Pediatrics and Biochemistry, Dalhousie University, Halifax, Nova Scotia, Canada B3H 4H7

We previously demonstrated a differential response of four cell lines of neural origin (C6 rat glioma, N1E-115 mouse neuroblastoma, and HTB-10 and HTB-11 human neuroblastomas) to β-12-O-tetradecanoylphorbol-13-acetate (TPA) stimulation of phosphatidylcholine (PtdCho) turnover. In C6 and HTB-11 cells, incorporation of [^{3}H]choline into PtdCho was stimulated 2-4 fold by 50-200 nM TPA. Minimal stimulation was observed in N1E-115 and HTB-10 cells with TPA but turnover could be stimulated by other agents. Since protein kinase C (PKC) activity and the ability to bind phorbol ester were present in all cell lines, we postulated that the differential response to TPA reflected the presence of one or more protein kinase C isozyme(s). Expression of mRNA for the alpha, beta, gamma and zeta isoforms of protein kinase C was examined in the cell lines using Northern analysis and S1 nuclease protection assays. Under standard incubation conditions, alpha isoform predominated in C6 and HTB-11 cells and was present in low amounts in N1E-115 and HTB-10 cells. Beta isoform was detected only in HTB-10 and C6 cells. Zeta isoform was present in all cell lines while no mRNA for gamma isoform was detected. Amounts of mRNA for PKC isoforms varied with pretreatment of cells with phorbol ester. The PKC substrate, MARCKS, was expressed at much higher levels in C6 and HTB-11 cells than in N1E-115 and HTB-10 cells. Based on these observations we postulate that increased PtdCho turnover and phosphorylation and translocation of MARCKS in response to TPA in C6 and HTB-11 cells is a result of activation of the alpha isozyme of PKC. (Supported by Medical Research Council of Canada, PG-16 and the Network of Centres of Excellence for Neural Regeneration and Functional Recovery)

QZ 223PRESENCE OF A βII PROTEIN KINASE C-SPECIFIC NUCLEAR ACTIVATION FACTOR IN HL60 CELL NUCLEAR ENVELOPES NICOLE R. MURRAY, DAVID J. BURNS AND ALAN P. FIELDS Department of Pharmacology, Case Western Reserve University School of Medicine Cleveland, OH and Sphinx Pharmaceuticals Corp. Durham, NC

Protein kinase C (PKC) is activated at the nucleus in response to many mitogenic stimuli. In human leukemic cells,which express α and βII PKC, nuclear PKC corresponds to the βII PKC isotype. At the nucleus, βII PKC directly phosphorylates the major nuclear envelope protein lamin B on two sites, Ser^{395} and Ser^{405}. Phosphorylation of these sites leads to solubilization of interphase nuclear envelope lamin B indicative of mitotic nuclear envelope breakdown *in-vitro*. We have begun to investigate the molecular basis for βII PKC-specific nuclear translocation and lamin B phosphorylation using an *in-vitro* reconstitution system consisting of isolated nuclear envelopes and recombinant human α and βII PKC. We find that βII PKC phosphorylates nuclear envelope lamin B 20-30 times better than does α PKC when normalized to histone kinase activity. In contrast, α and βII PKC phosphorylate soluble lamin B with equal efficiency suggesting that a component(s) of the nuclear envelope other than lamin B confers βII PKC specific lamin B phosphorylation. Extraction of nuclear envelopes with non-ionic detergent abolishes βII PKC-specific lamin B phosphorylation indicating that a nuclear membrane-associated component(s) facilitates βII PKC-specific nuclear translocation and lamin B phosphorylation. We are currently determining the biochemical properties of this detergent-soluble component and assessing its potential role as a βII PKC-specific nuclear membrane activation factor.

QZ 224 INTERFERONα INDUCES PKCε GENE EXPRESSION AS WELL AS A NOVEL PKCε-RELATED TRANSCRIPT.

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Protein kinases play key roles in the interferon-alpha (IFNα)-induced gene expression and biological activity in various IFN-responsive human cell lines. We report that IFNα increased the 7 kb transcript for the ε isotype of protein kinase C (PKCε) and the cellular content of PKCε at 24 and 48 hr after IFNα addition (a 2-fold and 6-fold increase, respectively) in the highly IFN-responsive Daudi lymphoblastoid cell line. Furthermore, IFNα markedly induced a 4.7 kb transcript which hybridized to a PKCε-specific, but not to a PKCη-specific, cDNA probe. PKCη protein was not expressed in control or IFNα-treated Daudi cells, although the 82 kDa PKCη protein was detected in rat lung tissue. The induction of the 4.7 kb PKCε-related mRNA by IFNα had the following properties reported for the classical IFNα-stimulated genes (ISGs): rapid kinetics of induction, high maintained levels in IFNα-sensitive but not in IFNα-resistant cell lines, protein synthesis-independent induction and high sensitivity to inhibitors of protein tyrosine kinase activity. These results show that the regulation of gene expression by IFNα include not only the classical IFNα- stimulated genes, but also the coordinated regulation of two PKCε-related transcripts, which appeared to be highly relevant to the biological actions of IFNα.

QZ 225 PKC ISOZYMES DIFFERENTIALLY REGULATE PROMOTERS CONTAINING PEA-3 / AP-1 MOTIFS , Anne E. Reifel-Miller, Doreen M. Conarty, Kathleen M. Valasek, Kimberly A. Birch and David J. Burns*. Lilly Research Laboratories, Lilly Corporate Center, Indianapolis, IN. 46285 and *Sphinx Pharmaceuticals, Quadrangle Drive, Chapel Hill, NC 27514

To investigate regulation of promoters containing PEA-3 / AP-1 motifs by protein kinase C (PKC) isozymes, cotransfections were performed in human dermal fibroblasts (HDF) with either the human collagenase promoter linked to the chloramphenicol acetyltransferase (CAT) gene (Angel et al., MCB 7:2256, 1987) or the porcine urokinase plasminogen activator (uPA) promoter linked to the CAT gene (Cassady et al., NAR 19:6839, 1991) and a plasmid expressing an individual PKC isozyme. Using this experimental design PKC alpha, beta I, beta II, gamma, delta, epsilon, zeta and eta were analyzed for their ability to trans-activate the collagenase and uPA promoters in both the presence and absence of exogenous stimulation. Our results demonstrate that the collagenase promoter was trans-activated eight-fold by epsilon, six-fold by eta and weakly by delta in the absence of PDBu. The other isozymes had no effect on the promoter. In the presence of exogenous stimulation (10 nM PDBu), the trans-activation profile was nearly identical to that seen in the absence of PDBu. When this same experimental design was used to investigate trans-activation of the uPA promoter, a very different profile was seen. In the absence of PDBu stimulation, all isozymes trans-activated the promoter two- to four-fold, except beta I which appeared to have no effect on the promoter. In the presence of PDBu stimulation, the results were nearly identical to those seen in its absence with the exception of epsilon which trans-activated the promoter seven-fold. Our results demonstrate that the PKC isozymes differentially regulate promoters containing PEA-3 / AP-1 sites and that the collagenase promoter is regulated by specific isozymes. The molecular mechanism for isozyme-specific regulation will be presented.

QZ 226 INTERACTION BETWEEN PKC AND ITS INTRACELLULAR RECEPTOR RACK1 - ROLE OF HOMOLOGOUS SEQUENCES, Dorit Ron and Daria Mochly-Rosen, Department of Molecular Pharmacology, Stanford University School of Medicine, Stanford, CA 94305-5332

Using rat brain cDNA library, we have cloned a 36 kDa protein that fulfills the criteria for a PKC anchoring protein or RACK (Receptor for Activated C-Kinase). The recombinant protein termed RACK1 bound PKC in a dose-dependent manner and binding was specific for PKC. RACK1 was not phosphorylated by PKC nor did it cause inhibition of Histone-III phosphorylation by PKC. Rather, RACK1 increased Histone-III phosphorylation indicating its ability to stabilize the active conformation of PKC. Protein data bank search of RACK1 amino acid sequence, revealed its homology to the beta subunit of the G protein and other G-beta-like proteins, all of which have between two to eight WD40 repeating elements. Furthermore, RACK1 and PKC share short sequences of homology. The RACK1-like sequences were found in PKC isozymes α,β,γ,δ,ε,ζ. For α,β,γPKC isozymes, the RACK1-like sequences were located within the C2 region in the regulatory domain. These sequences aligned with regions in four out of the seven WD-40 repeats in RACK1. δPKC on the other hand, has RACK1-like sequences in the C1 and V3 of the regulatory domain and also in the catalytic domain. Only one of these sequences is shared with α,β,γPKC isozymes. εPKC contains a unique sequence in the V3 region homologous to sequences in three of the RACK1 WD-40 repeats and ζPKC isozyme contains a sequence in the V1 region that aligns with sequences in five WD-40 repeats.

One explanation for the presence of homologous sequence in both the ligand (PKC) and its receptor (RACK1) is, that similar to the pseudosubstrate autoinhibitory sequence, PKC also contains a pseudo-RACK binding site(s). When PKC is in its inactive conformation, the pseudo-RACK site interacts with the RACK binding site on PKC. Only when PKC is activated, the RACK-binding site is exposed. This mechanism can facilitate the regulation of PKC binding to its receptor. To study the role of these homologous sequences, peptides from both RACK1 and the different PKC isozymes were synthesized. The ability of these peptides to bind either RACK1 or PKC and to affect PKC binding to RACK1 *in vitro* and PKC-mediated functions *in vivo* will be described.

QZ 227 THE ISOLATION AND PARTIAL CHARACTERISATION OF *PKC2*, *PKC3* AND *PKC4*, THREE ADDITIONAL MEMBERS OF THE *SACCHAROMYCES CEREVISIAE* PKC FAMILY.

Stephen P. Saville, Amos J. Simon, and Elisha Orr, Dept of Genetics, Leicester University, Leicester, England, U. K.

The existence of PKC in the yeast *Saccharomyces cerevisiae* has only recently been demonstrated following the cloning of a gene, *PKC1*, bearing homology to the mammalian enzyme[1]. DNA probes derived from *PKC1* failed to hybridise, even at low stringency, to other yeast sequences suggesting that this was the only PKC gene in yeast. Shortly afterwards, both Ogita *et al*[2]., and ourselves[3] demonstrated the existence of PKC-like activities in yeast. While the activity described by the former group was markedly different from the mammalian enzyme, that purified by ourselves was remarkably similar and specifically cross-reacted with a monoclonal anti-rat brain PKC antibody.

Using this antibody, and others cross-reacting with partially purified yeast PKC, we screened yeast expression libraries and obtained 18 positive clones. These were subdivided into 6 different groups on the basis of restriction mapping, Southern hybridisation and partial sequencing. We report here the isolation of three new genes *PKC2*, *PKC3* and *PKC4*. The *PKC2* gene is approximately 3kb long and is homologous to metazoan PKCs . It lacks the conserved C2 region and therefore seems to encode an isozyme belonging to the novel nPKC group; this idea is reinforced by the presence, in the PKC2 protein, of a motif bearing very high homology to the peptide used to raise the δ-specific antibodies.

A disruption in the *PKC2* gene, removing the catalytic domain of the protein is not lethal on rich media. Growth of *pkc2* mutants is impaired, however, on minimal media containing reduced amounts of amino acids, implicating the Pkc2p in the cellular response to amino acid starvation. Analysis of the *PKC3* and *PKC4* genes demonstrates that they are novel yeast genes and not allelic. Preliminary sequence data indicate that the *PKC3* is likely to be a member of the nPKC group.

1. Levin *et al.* (90) Cell 62 pp213-224
2. Ogita *et al.* (90) Proc. Natl. Acad. Sci. USA 87 pp5011-5015
3. Simon *et al.* (91) Proc. R. Soc. Lond. B 243 pp165-171

QZ 228 PROTEIN KINASE C IS REQUIRED FOR LIGHT ADAPTATION IN DROSOPHILA PHOTORECEPTORS, Z. Selinger, R.C. Hardie*, A. Peretz, E. Suss-Toby, A. Rom-Glas, S.A. Bishop* and B. Minke, Departments of Biological Chemistry and physiology, The Hebrew University, Jerusalem, 91904 Israel, *Department of Zoology, Cambridge University, UK

An excellent opportunity to investigate the functions of PKC has been provided by identification of a Drosophila eye-specific PKC and a null mutant (inaC^{P209}) lacking the eye-PKC enzyme[1]. Invertebrate photoreceptors respond to single photons with the so-called quantum bumps. Several lines of evidence suggest that quantum bumps are generated by quanta of Ca^{++}, released from intracellular stores. Light adaptation enables photoreceptors to adjust their sensitivity over the enormous range of ambient lights. While the detailed mechanism of adaptation is unknown, it is a negative feedback process mediated by a rise in cytosolic Ca^{++} and results in a decrease in bump size. Rigorous analysis[2] of adaptation by intracellular recordings, measuring the shift of the intensity/response function to higher light intensities, caused by background lights, revealed that adaptation is severly reduced in inaC, indicating that PKC is required for adaptation. Further analysis by whole cell recordings showed the following pleiotropic manifestations of the inaC phenotype; a response inactivation to baseline by continuous intense illumination b decreased sensitivity to strong light c supersensitivity to dim light d response inactivation by dim light upon removal of external Ca^{++} e slow termination of the response to short pulse of light. Analysis of single photon events in inaC photoreceptors revealed that in the absence of PKC, individual bumps fail to terminate normally. This primary defect can account for all the seemingly paradoxical manifestations of the inaC phenotype, and suggests a particular site of PKC's action.

1. Smith, D.P. et al., Science 254:1478 (1991)
2. Hardie, R.C. et al., Nature 363:634 (1993)

QZ 229 MARCKS DEPLETION BLOCKS PROGESTERONE-STIMULATED MATURATION IN OOCYTES. Stephen K. Sullivan, Department of Medicine, Columbia University, New York, NY 10032

The myristoylated alanine-rich C kinase substrate (MARCKS) is a major target for phosphorylation by protein kinase C (PKC) which has been implicated in diverse functions including neurosecretion, membrane-cytoskeletal interactions, and mitogenic signaling pathways. In order to design an antisense reagent for functional experiments, a small N-terminal region of Xenopus laevis oocyte MARCKS was cloned by RT-PCR. The amino acid sequence over this region (20 residues) was 95% identical to chicken and 80% identical to mouse and cow sequences, suggesting Xenopus MARCKS is highly conserved. Four adjacent 10mer antisense oligonucleotides (Mix-A) or two irrelevant 17mers (Mix-C) or the same four antisense 10mers except with every third nucleotide changed to its complement (Mix-C') were used for injection (50 ng/oocyte). In 11 experiments on oocytes from 3 frogs assayed 2-6 days following injection, there was no effect of MARCKS depletion on PMA (3 uM) -stimulated cortical granule exocytosis assayed spectrophotometrically as previously described (J Cell Biol 108: 885-92, 1989). By contrast, in 4 experiments on oocytes from 3 frogs assayed 4-8 days following injection, progesterone (2-20 ug/ml) -stimulated maturation (scored by the appearance of a white spot) was frequently observed in oocytes receiving Mix-C, Mix-C', H2O or no injection but was much less prevalent in oocytes receiving Mix-A. For example, in the first experiment maturation was observed in 1/10, 9/10 or 7/8 oocytes that received Mix-A, Mix-C or were non-injected, respectively. These preliminary results suggest a role for MARCKS in the progesterone-stimulated oocyte maturation pathway.

QZ 230 LYMPHOCYTE SUBSETS EXPRESS DIFFERENT AMOUNTS OF PKC ISOFORM PROTEINS. Akito Tsutsumi, Masao Kubo, Takao Koike, John Ransom. Syntex Research, Palo Alto, CA 94304. Hokkaido University, Sapporo, Japan.

The protein kinase C (PKC) family consists of at least nine isoforms that exhibit different activation requirements, substrate specificities, and cellular localization profiles in vitro. In this study, the relative amounts of six PKC isoform proteins were assessed in murine thymocytes and spleen T and B lymphocytes by Western blot analysis. PKC alpha was most abundant in spleen T cells. PKC beta was found to be more abundant in spleen B cells and thymocytes than in spleen T cells. PKC delta and epsilon were detectable in spleen B cells but were very low in thymocytes. There were three to four fold more PKC zeta in thymocytes and T cells as compared to B cells. We also studied the effect of PMA treatment on the amount of PKC isoform proteins in murine thymocytes. Unlike the behavior of the other isoforms, PKC zeta was not down regulated after 8 hours of PMA treament, showing that PKC zeta is not regulated like the other isoforms in normal lymphocytes. These heterogeneous expression patterns suggest that certain isoforms may be of greater significance in the activation and development of different lymphocyte populations.

QZ 231 EFFECTS OF PROTEIN KINASE C ON THE PHOSPHORYLATION OF RHODOPSIN IN SITU

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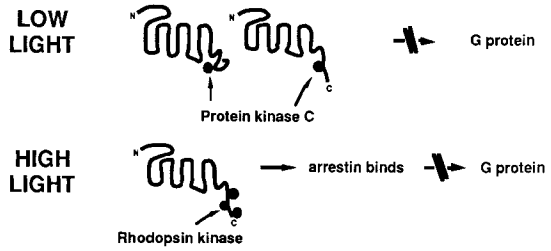
The possibility that protein kinase C is involved in phototransduction by phosphorylating rhodopsin was explored *in situ*. Pre-treatment of intact retinas with phorbol myristate acetate resulted in a light-dependent alteration of the phosphorylation state of rhodopsin. At high light levels, which cause maximal bleaching of rhodopsin, the phosphorylation of rhodopsin decreased. In contrast, phorbol esters caused an increase in rhodopsin phosphorylation in retinas exposed to a brief flash of light that bleaches 10% of the rhodopsin. No phosphorylation was detected in the absence of light. Partial proteolysis by Asp-N showed that phorbol esters alter the phosphorylation of the carboxy terminus of rhodopsin. Phorbol esters had no significant effect on the phosphorylation of other rod outer segment proteins; rhodopsin appears to be the main *in situ* substrate of protein kinase C. Our results indicate that the protein kinase C could function with rhodopsin kinase in fine-tuning the desensitization of the visual receptor. As a working model, we suggest that protein kinase C functions primarily at low light levels, and rhodopsin kinase at high light levels. In this respect, rhodopsin appears similar to the β-adrenergic, muscarinic, and olfactory receptors, each of which is desensitized by two distinct kinases.



Lipid Dependent Pathways in Cellular Activation (Joint); PKC-Regulation/Inhibitors

QZ 300 INHIBITION OF MELANOMA PHOSPHATASE BY RGDS TETRAPEPTIDE, AL-MAHMOOD Salman, LUGASSY Claire, and ESCANDE Jean-Paul, Laboratoire d'Oncologie, Hôpital Tarnier-Cochin, 89 Rue d'Assas, 75006 Paris, France.

Integrin-mediated cell-extracellular matrix adhesion are shown to induce intracellular signals throughout cytoskeleton leading to the phosphorylation of a small number of cellular proteins. This adhesion phenomenon was shown to be inhibited by RGDS tetrapeptide. However the biological significance of this inhibition is not yet understood. Here we show that human melanoma cell-RGDS interaction lead to the inhibition of some melanoma cell phosphatase. This inhibition phenomenon seems to be independent of integrins's cytoplasmic regions-cytoskeleton complex signaling pathways since cytocalasin D has no effect on this phenomenon. RGDS-inhibition of phosphatase is independent of protein biosynthesis and genes transcription since neither cycloheximide nor actinomycine D have effect on this phenomenon. All considering, these findings show besides that RGDS mimic the integrin binding sites at the extracellular matrix, it has also a biological effect in inhibiting certain cellular phosphatases.

QZ 301 **ROLE OF PROTEIN KINASE C IN GROWTH STIMULATION OF PRIMARY COLONIC EPITHELIAL CELLS.** Christina Branting, Joseph Rafter, Department of Medical Nutrition, Karolinska Institute, F60 NOVUM, Huddinge University Hospital, S-141 86 Huddinge, Sweden.

The effects of potential tumor promoters and their mechanisms of action on normal cells are of interest in order to understand the altered growth regulation known to occur during colon carcinogenesis. Because of its proposed central role, the purpose of the present study was to investigate the involvement of protein kinase C (PKC) in the proliferative response of epithelial cells from the normal mouse colon to two potential tumor promoters in the colon, the phorbol ester 12-0-tetradecanoylphorbol-13-acetate (TPA) and the bile acid deoxycholic acid (DCA). Using autoradiographic analysis of 3H-thymidine incorporation, exposure of quiescent cells to TPA (100nM) and DCA (50μM) for 24h stimulated proliferation, yielding 14% and 9% labeled epithelial cells compared to the background level of about 4% labeled cells. The PKC inhibitor bisindolylmaleimide efficiently blocked the proliferative response to TPA and reduced the growth stimulating effect of DCA in a dose dependent manner. Another inhibitor, H-7, did not influence proliferation induced by TPA, but the DCA effect was reduced by about 50%. Attempts to down-regulate PKC by extended treatment with the phorbol ester resulted in a reduction in the proliferative response to DCA. Direct measurement of the intracellular distribution of PKC activity showed that both TPA and DCA caused a time-dependent translocation of the enzyme activity from the soluble to the particulate cell fractions. TPA also caused a time-dependent increase in the expression of c-fos mRNA measured by solution hybridization and c-fos protein measured by immunocytochemistry. No effect of DCA on expression of the gene could be observed under present conditions. These data clearly indicate that PKC is involved in the proliferative response of the cells to the bile acid, however, some discrepancies between mechanisms underlying TPA and DCA induced proliferation are evident. In conclusion the results demonstrate the presence of PKC activity in normal colonic epithelial cells and suggest that bile acids can modulate PKC activity in the tissue.

QZ 302 SELECTIVE INHIBITION OF PKC-β ACTIVITY IN NORMAL GUINEA PIG EPIDERMIS BY 13-HYDROXYOCTADECADIENOIC ACID-CONTAINING DIACYGLYCEROL, Yunhi Cho and Vincent A. Ziboh, Department of Dermatology, University of California, Davis, Davis, CA 95616, USA.

13-hydroxyoctadecadienoic acid (13-HODE), a 15-lipoxygenase metabolite of linoleic acid was shown in our laboratory to inhibit experimentally-induced hyperproliferation in guinea pig skin. To delineate a possible mechanism for this antiproliferative action we explored a possible contribution via the inositol phospholipid turnover and modulation of protein kinase C activity. To test this hypothesis we first demonstrated that 13-HODE is incorporated into epidermal phospholipids, particularly phosphatidyl 4,5-bisphosphate (PtdIns 4,5-P_2). Incubation with epidermal phospholipase C resulted in the release of 13-HODE-containing-diacylglycerol. We next synthesized and tested the possibility that putative 13-HODE-containing-DAG (13HODE-DAG) could exert a modulatory effect on epidermal protein kinase C (PKC) activity which was found to be elevated in the experimentally induced hyperproliferative skin. The effects of the putative 13HODE-DAG were tested on: (i) basal epidermal cytosolic PKC activity, (ii) on diolein-activated PKC activity and (iii) on the two major epidermal PKC-isozymes (PKC-β and PKC-α). Our data on (i) revealed a dose-dependent activation of basal PKC activity by diolein, which is maximal at 4 μM. In contrast, replacement of sn-2 position of diacylglycerol (1-palmitoyl-2-oleylglycerol) with 13-HODE (1-palmitoyl-2-13HODE-glycerol) significantly inhibited basal PKC activity (approximately 45%). In further experiment, putative 13HODE-DAG inhibited diolein-activated PKC activity in a dose-dependent manner and 50% at 10 μM. To determine whether the effects of 13HODE-DAG is selective, we tested its effects on the activities of epidermal PKC-isozymes (β and α). Our data revealed that putative 13HODE-DAG selectively inhibited the activity of PKC-β isozyme, while exerting negligible effect on the PKC-α isozyme. This selective inhibitory effect of putative 13HODE-DAG on PKC-β is novel and suggests that in vivo generation of 13HODE-DAG may be involved in modulating epidermal hyperproliferation.

QZ 303 **INHIBITION OF PKC-α EXPRESSION IN HUMAN A549 CELLS BY ANTISENSE OLIGONUCLEOTIDES INHIBITS INDUCTION OF ICAM MRNA BY PHORBOL ESTERS,** **Nicholas M. Dean, Robert McKay, Tom Condon and C. Frank Bennett. ISIS Pharmaceuticals, 2280 Faraday Ave. Carlsbad, CA 92008.**

Phosphorothioate oligonucleotides targeting multiple diverse sites on human PKC-α mRNA including the AUG translation codon and 3'-untranslated sequences have been identified which inhibit PKC-α expression in A549 cells. This effect was specific for PKC-α (levels of PKC-δ, ε and ζ were unchanged), and very potent (IC_{50} of 200nM in the presence of cationic liposomes). By contrast, 2'-O-methyl phosphorothioate analogs of these oligonucleotides were without effect on PKC-α mRNA levels, suggesting that the reduction in targeted PKC-α is through RNAse H mediated cleavage. One oligonucleotide however, was effective at inhibiting PKC-α protein levels as a 2'-O-methyl phosphorothioate at concentrations 2-3 fold greater than its phosphorothioate/deoxy analog. These results suggest that the ability to serve as an RNAse H substrate, although not required for all oligonucleotides certainly increases their potency. These oligonucleotides have been used to examine the role played by PKC-α in mediating the phorbol ester induced changes in mRNA levels of the cell adhesion molecule ICAM. In A549 cells ICAM mRNA is increased 10-20 fold after 2 hours by treatment of cells with the phorbol ester PMA. When PKC-α protein levels are depleted by oligonucleotide treatment of A549 cells the increase in ICAM expression in response to PMA is greatly reduced, demonstrating that PKC-α plays a major role in this process.

QZ 304 12-DEOXYPHORBOL-13-O-PHENYLACETATE-20-O-ACETATE (DOPPA), A β_1-PKC SELECTIVE ACTIVATOR *IN VITRO*, IS METABOLISED TO 12-DEOXYPHORBOL-13-O-PHENYLACETATE (DOPP), IN HL-60 AND U937 CELL LINES.

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We have recently shown that different members of the phorbol ester family of diterpene ester toxins with differing biological activities are capable of selective activation/non-activation of purified PKC isotypes *in vitro* [1]. In particular, (DOPPA) was a selective β_1-PKC activator and Sapintoxin A a selective δ-PKC non-activator *in vitro*. However, there would appear to be some difficulty in utilising DOPPA as an *in vivo* β_1-PKC agonist since DOPPA can, at high doses, cause the translocation and down-regulation (which are presumed to correlate with activation)of other PKC isotypes in U937 and Swiss 3T3 cells [2].

We have attenpted to address this anomolous situation by considering the possibility that DOPPA could be metabolised *in vivo*. Our initial observation in HL-60 and U937 cell lines indicate that DOPPA is, in fact, metabolised to DOPP [2,3]. This metabolism can be clearly observed by TLC within 4-6 hours of treating cells with DOPPA. As a continuation of this study, we have developed a RP-HPLC analytical technique. This indicates that DOPPA metabolism commences within 30 minutes to 1 hour of treating HL-60 cells with DOPPA; 3 days after cell treatment with DOPPA, the majority of phorbol ester positive material is DOPP.

This may explain why PKC isotypes other than β_1-PKC are translocated and/or down-regulated following treatment of cells with DOPPA, since DOPP is not a selective PKC isotype activator/non-activator *in vitro*. This data points to a complexity in the biological mechanisms of action of the phorbol esters and their interaction with PKC isotypes *in vivo*.

References: [1] Ryves, W J *et al* (1991) *FEBS.Letts.* **288** 5.
[2] Kiley, S C *et al* (1993) *Carcinogenesis*. (Submitted).
[3] Gordge, P C *et al* (1993) *J.Pharm.Pharmacol.* **45** 38P.

QZ 305 SIGNAL TRANSDUCTION IN BASIC FIBROBLAST GROWTH FACTOR-MEDIATED PROTECTION OF ENDOTHELIAL CELLS AGAINST RADIATION-INDUCED APOPTOSIS.

Adriana Haimovitz-Friedman, Naomi A. Balaban, Maureen McLoughlin, Desiree Ehleiter, Joseph Michaeli, Israel Vlodavsky, and Zvi Fuks. Departments of Radiation Oncology (A.H-F.; N.A.B.; M.M.; D.E.; Z.F.) and Medicine (J.M), Memorial Sloan-Kettering Cancer Center, New York, and the Department of Oncology

Basic fibroblast growth factor (bFGF) protects endothelial cells against the lethal effects of ionizing radiation by inhibiting the programmed cell death (apoptosis) induced in endothelial cells by radiation exposure. Basic FGF was found to induce the translocation of cytoplasmic PKCa into the membrane and to activate membranal PKC within 30 seconds of bFGF stimulation in confluent bovine aortic endothelial cells (BAEC) in vitro. To investigate whether PKC is involved in mediating the signaling of the inhibitory effects of bFGF on radiation-induced apoptosis, various kinase inhibitors were evaluated for their ability to abrogate this radioprotective effect. Both the tyrphostin AG213 (22.5 μM), which was found to be a specific inhibitor of bFGF receptor tyrosine kinase, and the PKC inhibitor H-7 (20μM) abrogated the radioprotective effect of bFGF, as was observed following depletion of cellular PKC by overnight preincubation with 200 nM TPA, while the PKA inhibitor HA-1004 did not exhibit a similar property. Further evidence for the involvement of PKC in down-regulating radiation-induced apoptosis in endothelial cells was derived from agarose gel electrophoresis of DNA extracted from irradiated BAEC. Immediate post-radiation treatment of BAEC with TPA (30 ng/ml; 30 minutes) inhibited the apoptotic degradation of DNA induced in BAEC by irradiation, similar to the inhibition observed after immediate post-radiation stimulation with 0.75 ng/ml of recombinant bFGF, and prevented the appearance of the typical DNA ladder of oligonucleosomal fragments, characteristic of apoptosis. Both the bFGF- and TPA-mediated inhibition of apoptosis were abrogated by treatment with the PKC inhibitor H-7 (20μM), indicating the involvement of PKC in down-regulating radiation-induced apoptosis and in the rescue of endothelial cells from these mode of radiation-induced cell death.

QZ 306 A NOVEL ACTIVATION MECHANISM OF nPKCη MEDIATING EPIDERMAL DIFFERENTIATION

Togo Ikuta[1], Kazuhiro Chida[1], Yoshiharu Matsuura[2], Shinichi Osada[3], Shigeo Ohno[3] and Toshio Kuroki[1], [1]Department of Cancer Cell Research, Institute of Medical Science, University of Tokyo, [2]Department of VirologyII, National Institute of Health Japan, [3]Department of Molecular Biology, Yokohama City University School of Medicine

The η isoform of protein kinase C (nPKCη), isolated from a cDNA library of mouse skin, is predominantly expressed in epithelia of the skin, digestive and respiratory tracts in close association with epithelial differentiation. We report here that this isoform is activated by cholesterol sulfate, a membrane component produced by cholesterol sulfotransferase in response to differentiation inducers. Recombinant nPKCη was prepared by the baculovirus expression system and purified by DE52 and Thr-conjugated affinity chromatography. Kinase activity was measured by phosphorylation of myelin basic protein. Among phospholipids and lipids found in skin, cholesterol sulfate was found to activate most efficiently nPKCη at a higher extent than phosphatidylserine (PS) plus phorbol ester, while cholesterol had no activating effect. PS had an additive effect to cholesterol sulfate. Phorbol ester activated nPKCη in the presence of cholesterol sulfate but to a lesser extent than in the presence of PS. Cholesterol sulfate did not activate recombinant cPKCα or nPKCδ. The above observation implies that nPKCη plays a crucial role in the signal transduction pathway of epidermal differentiation.

QZ 307 CYSTEINE-RICH REGIONS AND PHORBOL ESTER BINDING: HIGH AFFINITY INTERACTIONS IN PKC ISOZYMES, N-CHIMAERIN AND UNC-13.

Marcelo G. Kazanietz, Jay D. Bruns, Michael Axeen, Liliana B. Areces and Peter M. Blumberg. Laboratory of Cellular Carcinogenesis and Tumor Promotion, National Cancer Institute, Bethesda, MD 20892.

The phorbol ester binding site in PKC isozymes was mapped at the cysteine-rich regions present in the regulatory domain of the enzymes. [^{3}H]Phorbol 12,13-dibutyrate (PDBu) binds to calcium-dependent isozymes (alpha, beta, and gamma) with a Kd of 0.1-0.3 nM, and to calcium-independent isozymes (delta, epsilon, and eta) with slightly higher affinity (0.6-0.8 nM)(Kazanietz et al., Mol. Pharmacol, 44, 298-307, 1993). We found three patterns of recognition: compounds with higher affinity for calcium-dependent isozymes (phorbol esters, 12-deoxyphorbol esters), compounds with higher affinity for PKC alpha and calcium-independent isozymes (bryostatins) and compounds with similar affinities for all the PKC isozymes (diacylglycerol, indole alkaloids). The cysteine-rich regions of PKC alpha, n-chimaerin, and unc-13 expressed in E. coli as GST-fusion proteins bound [^{3}H]PDBu with a Kd of 1-2 nM. The cysteine-rich region of PKC zeta bound neither [^{3}H]PDBu nor the ultrapotent ligand [^{3}H]bryostatin 1, suggesting that this isozyme lacked even a low affinity interaction. Restoration of the postulated consensus for phorbol ester binding (Ahmed et. al., Biochem. J., 280, 233-241, 1991) in PKC zeta by site-directed mutagenesis (Pro141→Gly) did not restore ligand binding. All of these proteins bind Zn. In conclusion, each cysteine-rich region may have unique high affinity interactions with phorbol esters and related compounds. The differences between PKC isozymes are as great as those between PKC and the non-PKC phorbol ester receptors n-chimaerin and unc-13.

QZ 308 INHIBITION OF PKC-η EXPRESSION BY ANTISENSE OLIGONUCLEOTIDES IN MOUSE AND HUMAN CELL LINES. Robert McKay and Nicholas M. Dean. Isis Pharmaceuticals, 2292 Faraday Ave., Carlsbad Ca. 92008

Protein kinase C (PKC) is a family of lipid-regulated serine/threonine kinases that have been shown to play crucial roles in regulating many cellular functions. Distinct patterns of tissue and subcellular distribution suggests that individual isozymes of PKC may have distinct functions. One of the calcium independent isozymes, PKC-η, is highly expressed in skin and other epithelial tissue. It is our aim to determine the roles played by this isozyme in regulating specific cellular responses. To do this, we have identified an antisense phophorothioate oligonucleotide which potently and specifically inhibits the expression of PKC-η mRNA in human keratinocytes (NHEK) and lung carcinoma (A549) cells. Isis 6445, designed to hybridize against the coding region of the human PKC-η mRNA, reduces mRNA expression by 95%. The reduction of PKC-η mRNA was specific (levels of PKC-α and ζ were unchanged) and had an IC_{50} of 100 nM in A549 cells and <100 nM in NHEK. A similar oligo designed to murine PKC-η potently reduced the PKC-η mRNA in murine bEnd.3 cells. We are currently investigating whether PKC-η is involved in regulating keratinocyte proliferation and differentiation.

QZ 309 DIFFERENTIAL SENSITIVITY OF HUMAN CANCER CELL LINES TO PROTEIN KINASE C AGONISTS. Ana T. Menendez, Alan Kozikowski, Michele Dougherty, Robert Kramer. Oncology Discovery, American Cyanamid, Pearl River, New York, 10965, and Neurochemistry Research, Mayo Clinic, Jacksonville, Florida, 32224.

Protein kinase C (PKC) lies on the signal transduction pathway utilized by many oncogenes, growth factors and mitogens, as well as being the mediator of the actions of tumor promoters as phorbol esters (TPA). Bryostatin, a partial PKC agonist, is currently undergoing Phase II clinical trials as an anticancer agent. These properties have led us to investigate PKC as a potential new cellular target for the treatment of cancer. We treated 25 human cancer cell lines with several PKC agonists and found a select subset that was selectively inhibited (up to 500 times more sensitive) only by TPA and the indolactam V-type PKC agonists (i.e., teleocidin, lyngbyatoxin, indolactam V [ILV], 7-(8)-hydroxy-1-octanoyl [ILV], and 7-n-hexyl [ILV]) but not bryostatin and staurosporine. These cell lines were: Colo 205 (colon), A549 (lung), HL-60 (leukemia), LNCAP (prostate), and SKBR3 (breast). Short exposure cell growth inhibition experiments and results from PKC downregulation studies support the fact that the anti-proliferative activity was due to the activation and not the down-regulation of PKC by TPA and the indolactams. In addition, TPA sensitive cells had 10-fold more PKC activity than the PKC resistant cells. Qualitative PKC isotyping by Western blot analysis showed that the TPA sensitive cell lines did not have a different PKC isotype profile than the TPA resistant cell lines. Resistant and sensitive cells expressed α, ε and ζ, but not β, δ or γ. The prevailing wisdom at this time is that PKC may be too ubiquitous to be a target for anticancer agents. Our results suggest the contrary, that PKC is potentially a unique target for treating a subset of human tumors. Understanding the mechanism responsible for the TPA sensitivity of some cells will help define and optimize the potential therapeutic utility of targeting the PKC signal transduction pathways.

QZ 310 FLUORESCENCE DETECTION OF PROTEIN KINASE C - LIPID COFACTOR INTERACTION. E.H.W. Pap, P.I.H. Bastiaens, J.W. Borst, A. van Hoek and A.J.W.G. Visser. Departments of Biochemistry and Molecular Physics, Agricultural University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands. The interaction of protein kinase C with lipid cofactors was investigated by a dual approach. First the binding to pyrene labelled diacylglycerol (pyDG) and phosphatidylinositol-4,5-biphosphate ($pyPIP_2$) was quantitatively characterised by monitoring resonance energy transfer with time-resolved fluorescence techniques. The quenching efficiency of the tryptophan fluorescence was determined as function of lipid probe concentration in mixed micelles consisting of polyoxyethylene-9-lauryl ether, PS and various mole fractions of lipid cofactor. At 5 mol% PS, the affinity of PKC for $pyPIP_2$ is higher than that for pyDG. Double labelling experiments suggest that $pyPIP_2$ and DG indicate a reciprocal binding relationship of both cofactors. Secondly, reorientational properties of diphenylhexatriene (DPH) labelled DG in vesicles and in mixed micellar systems were investigated. The order and rotational dynamical properties of DPH-DG in vesicles are considerably different from those of the control phosphatidylcholine analogue DPH-PC. In micelles, the interaction with PKC resulted in a significantly slower decay of the fluorescence anisotropy of DPH-DG. This effect was not seen when DPH-DG was replaced by DPH-PC or when PS was replaced by PC.

QZ 311 EVIDENCE FOR DISTINCT PHORBOL ESTER AND DIACYLGLYCEROL BINDING SITES ON PROTEIN KINASE C. Slater, S.J., Kelly, Mary Beth, Taddeo, F.J., and Stubbs, C.D., Department of Pathology and Cell Biology, Thomas Jefferson University, Philadelphia, PA 19107

The activation of rat brain protein kinase C (PKC) isozymes in the presence of saturating concentrations of both diacylglycerol (DAG) and phorbol ester (TPA), and excess PS, was additive, with respect to the two activators, from which it was hypothesized that PKC contains minimally two non-equivalent activator binding sites. It was found that calcium chelation inhibited DAG activated PKC, while having a negligible effect on the TPA stimulated enzyme, suggesting that interactions of the two activators with PKC are dissimilar. Measurement of the concentration dependence of the additive activation effect in the presence of TPA and DAG indicated negligible effects on the cooperativity of activator binding, and therefore negligible allosteric interaction between the activator binding sites. As shown in a previous study (Slater et al., (1993) *Nature* **364**, 82-84) n-butanol, inhibits TPA activated phosphorylation of histone H-1, catalyzed by PKC. Replacing histone with a peptide substrate removed the inhibition of TPA stimulated PKC activity. By contrast, the alcohol inhibited DAG stimulated activity was still seen, again pointing to differences in DAG and TPA binding, and the possible existence of multiple activator binding sites.

QZ 312 PROTEIN KINASE C INITIALLY INHIBITS THEN STIMULATES THE INDUCTION OF CELL DIVISION.
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Protein kinase C (PKC) may be involved in the induction of cell division since phorbol ester will induce meiotic cell division in Xenopus oocytes (Stith and Maller, Expt. Cell Res. 169:514,1987). In this manner, phorbol esters mimic growth factors such as insulin, insulin-like growth factor 1 and progesterone. These growth factors increase sn-1,2-diacylglycerol (DAG) mass levels 15 min and 2 hrs after addition (cell division begins at 4-6 hrs after growth factor addition) (Stith et al., J. Cell. Physiol. 149:252, 1992). Since the two DAG peaks are about 1000-fold larger than the simultaneous inositol 1,4,5-trisphosphate increase, and since phosphatidylcholine ^{32}P turnover correlates with DAG production, the DAG increase may be due to the breakdown of phosphatidylcholine. Ras p21 also induces cell division in the oocyte, yet it causes a decrease in DAG mass levels. The use of a PKC activator (phosphatidylcholine-specific phospholipase C) and two PKC inhibitors (inhibitor peptide PKC(19-36) and staurosporine) suggest that:
(a) by following a PKC-dependent event (intracellular pH), the activator and inhibitors regulate in vivo PKC activity in the manner suggested.
(b) in the unstimulated cell, PKC has an inhibitory action. Upon addition of a growth factor, an additional synergistic PKC activity arises to promote cell division. The inhibitory and synergistic activities of PKC may be due to different isozymes or different locations of the same isozyme of PKC.
(c) PKC inhibition is relieved after about 2 hrs after growth factor addition producing a net synergistic PKC effect and allowing progression toward cell division. The inhibitor activity of PKC may be why growth factors must be present for long periods before cell division is induced.

Role of Sphingolipids in Cellular Regulation and Control of Protein Phosphorylation (Joint); PKC-Function and Disease

QZ 400ALTERED PROTEIN KINASE C ACTIVITIES IN CYTOSOL, MYELIN, MICROSOMAL AND SYNAPTOSOMAL FRACTIONS FROM BRAINS OF DYSMYELINATING MOUSE MUTANTS *jp, msd, shi* AND *qk*,
Kamlesh Asotra and Wendy B. Macklin, Department of Psychiatry & Biobehavior, Mental Retardation Research Center, Neuropsychiatry Institute, UCLA Medical Center, Los Angeles, CA 90024
We have recently reported that developmentally regulated PKC enzyme activities modulate the expression of several myelin protein genes in primary cultures of rat brain oligodendrocytes [K.Asotra & W.B. Macklin (1993) J. Neurosci. Res. 34:571-588] and that several PKC isozymes are expressed in enriched oligodendrocytes in a differentiation stage-dependent manner [K. Asotra & W.B. Macklin (1993) Focus 15(4): 94-98; K. Asotra & W.B. Macklin (1993) J. Neurosci. Res., submitted]. In order to examine if PKC has a similar role during normal myelin gene expression and in dysmyelinating diseases *in vivo*, we assayed PKC enzyme activities in 105,000 X g cytosol and sucrose gradient-purified myelin, microsomal and synaptosomal membrane fractions from brains of normal mice and four dysmyelinating mouse mutants. The four mutants were: (i) the autosomal recessive, chromosome 18-linked, dysmyelinating mutant *shiverer (shi)* in which the myelin basic protein (MBP) and MBP mRNAs are absent, (ii) the autosomal recessive *quaking (qk)* mutant in which the expression of the major myelin proteins MBP, proteolipid protein (PLP) and myelin-associated glycoprotein is reduced, (iii) the recessive X-linked, PLP mutant *jimpy (jp)*, and (iv) "myelin synthesis deficient" allele of *jp (msd)* in which PLP is virtually absent. Our results show that PKC activities are increased in cytosolic and synaptosomal fractions of 55-58 day-old *shi* and *qk* but decreased in those from 23 day-old *jp* and *msd* mutant brains. Purified myelin and microsomal fractions from these four dysmyelinating mutants showed reduced PKC activities with *shi* > *qk* > *jp* = *msd*. Thus there was a significant correlation between dysmyelination and loss of PKC activities in myelin and microsomal membranes. Both PLP mutants, mouse models for Pelizaeus-Merzbacher disease in humans, showed approximately 90% loss of PKC activities in all the fractions, compared with normal controls suggesting that PLP plays a crucial role during development to ensure normal expression of PKC activity *in vivo* in other brain regions beside myelin membranes. Our results also suggest that a defect in oligodendrocyte-specific genes such as PLP may determine the fate of synaptosomal markers, e.g., neuron-specific expression of PKC isozymes as well as oligodendrocyte-specific expression of PKC isozymes. Additional results of immunocytochemical and immunoblot studies on the fate of PKC-α, -β, -γ, -δ, -ε and -ζ isozymes in brains of these dysmyelinating mutants will be presented. Funded by NS25304.

QZ 401 ANTICIPATIVE PROTECTION AGAINST CARDIAC ISCHEMIA REPERFUSION INJURY INDUCED BY PKC.
Anirban Banerjee, Max B. Mitchell, Carlo G. Parker, Fabia Gamboni-Robertson, University of Colorado, Denver, CO 80262.
Myocardial preconditioning was originally described as the paradoxical protection conferred by transient ischemic (TI) episodes against a subsequent bout of sustained ischemia (period of oxygen and substrate deprivation) followed by dramatic recovery upon reperfusion. We have reported that the mechanism involves TI stimulated neurotransmitter (norepinephrine) release from adrenergic nerve termini and stimulation of myocardial a_1-adrenoceptors. Improved functional recovery obtained during reperfusion is independent of both protein synthesis and the transient increases in contractile function due to a,ß-adrenergic stimulation. This intriguing and clinically desirable maneuver suggests that mammalian myocardium is potentially able to adapt and resist impending metabolic catastrophe and to resume function during reperfusion with minimal injury. This study evaluates the role of PKC in transducing preconditioning.
METHODS: Myocardial function was assessed as the development of ventricular pressure in isolated buffer perfused rat hearts (Langendorff apparatus). Hearts were equilibrated, subjected to global ischemia (20 min. 37°C) and reperfused (40 min.) to stable recovery. Preconditioning stimuli TI (2 minutes), phenylephrine (PE; a_1-adrenoceptor agonist; 1umole/min) and PKC stimulants SAG (stearoyl,arachidonoyl glycerol; 0.6umole/min) and PdBu (phorbol dibutyrate; 10^{-1} to 10^3 pmol/min) were applied for 2 minutes, 10 minutes before ischemia. PKC blockade with staurosporine (ST) or chelerythrine (CH) was applied before and/or during stimulation. A separate group of hearts were frozen immediately after TI and PE, cryosectioned and stained with antibodies against a, b1, b2, g, d, e, and z PKC isoforms and cy-3 labeled secondary antibody for epifluorescence.
RESULTS: Preconditioning induced by either TI or PE (83±2%; 81±3% $p<0.05$ versus untreated controls:47±3%) was blocked by ST and by CH (NS vs. control). SAG produced preconditioning (74±3%) which could also be blocked by CH. However despite detectable hemodynamics, PdBu did not precondition at any dose. Immunofluorescence microscopy revealed the translocation of PKC d from cytosol to sarcolemma immediately after either TI or PE. TI (but not PE) translocated PKC e into the nucleus.
Of the strategies conceivable against IR injury, we can recognize several, including 1. Decreased calcium overload (diastolic relaxation), 2. Reenergization (glycolytic and mitochondrial ATP production) and 3. Peak contraction (myofilament Ca^{++} sensitization) to have been implemented concomitantly by stimulation of the appropriate PKC isozyme.

QZ 402 PROTEIN KINASE C AND CISPLATIN RESISTANCE, Alakananda Basu, Kelly Burke, Jeffrey Cline and Nagahiro Saijo*, Department of Pharmacology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261 and *National Cancer Center Research Institute, Tokyo, Japan

Protein kinase C (PKC) has been implicated in regulating anticancer drug sensitivity. *cis*-Diamminedichloroplatinum(II) (cDDP) is one of the most important anticancer agents currently available for the treatment of solid tumors. Acquisition of resistance by tumor tissue to cDDP is a major impediment to cancer chemotherapy. We have shown previously that activators of protein kinase C enhance cellular sensitivity to cDDP. In the present study, we have investigated whether PKC signal transduction pathway is affected during development of cellular resistance to cDDP. PKC activity, as determined by its Ca^{2+}-, phospholipid-dependence, was severely compromised in several human tumor cell lines that acquired resistance to cDDP. A 4-fold decrease in PKC activity was observed in cDDP-resistant human small cell lung cancer cell line (H69/CP) compared to drug-sensitive variant (H69). The majority of PKC activity was isolated in the cytosolic fraction. Both cytosolic and particulate PKC activities were decreased in H69/CP cell line. H69 cells expressed PKCα, -β and -ζ isoforms. PKCζ was the major immunoreactive protein detected in H69 cells. We were unable to detect any PKCγ, -δ or -ε isoform. The expression of both PKCα and -β isoforms was reduced significantly in H69/CP cells. There was, however, no change in the expression of PKCζ. We speculate that a decrease in conventional PKC isoforms was associated with cDDP-resistant phenotype.

QZ 403PLATELET-DERIVED GROWTH FACTOR STIMULATES CERAMIDASE ACTIVITY IN MESENCHYMAL CELLS, Mark Kester and Michael Martinez, Departments of Medicine and Physiology/Biophysics, Case Western Reserve University, Cleveland, OH, 44106.

Sphingolipid-derived metabolites have recently been identified as second messengers. We have previously shown that platelet-derived growth factor-AB (PDGF) stimulates an increase in sphingosine and a concomitant decrease in ceramide formation in A7r5 smooth muscle cells (Jacobs, L.S. and Kester M., Am. J. Physiol. 265:C740-C747, 1993). We further demonstrated that L-cycloserine, an inhibitor of sphingolipid synthesis, inhibited PDGF-stimulated proliferation but not PDGF-induced increases in intracellular calcium concentration. We suggested that the mitogenic effects of PDGF in vascular smooth muscle are, in part, mediated by sphingolipid metabolism and that PDGF-induced increases in intracellular calcium are not sufficient for induction of DNA synthesis. To better understand the mechanism of PDGF-stimulated sphingosine formation, we have investigated ceramidase activity *in vitro*. Ceramidase activity was assessed as TLC-separated ^{14}C-oleate released from PDGF-treated (10-50 ng/ml, 1hr.) or untreated rat mesangial cell membrane or cytosolic preparations and exogenous ^{14}C-oleoyl-ceramide substrate. We now report that both PDGF-AB and BB stimulated an alkaline but not an acidic ceramidase activity. Also, PDGF-stimulated membrane-associated but not cytosolic ceramidase activity. The effects of PDGF upon ceramidase activity were relatively specific, as vasoconstrictors (endothelin) or cytokines (interleukin-1) did not stimulate ceramidase activity. A role for phosphorylation of ceramidase is suggested as PDGF-stimulated ceramidase activity was further augmented in the presence of the phosphatase inhibitor, vanadate. We conclude that, in mesenchymal cells, PDGF stimulates ceramidase activity, generating the putative mitogen-sphingosine.

QZ 404 A NOVEL SPINGOLIPID PROTEIN KINASE C INHIBITOR: POTENTIAL FOR DERMATOLOGY. Lee A.A., Kurtz E.S., Bailey S.C., Przekop P.A., Ortega-Nanos M.C., Arshad F., Freed B.S., Hamer R.R.L., Tegeler J.J., Dermatology Div. and Dept. Chemical Research, Hoechst-Roussel Pharm. Inc., Somerville, NJ 08876.

Protein Kinase C (PKC) plays an important role in signal transduction which regulates cellular processes including differentiation, proliferation and inflammation. Therefore, PKC may play a key role in inflammatory and other dermatoses as currently being elucidated by various investigators. This study profiled a potent sphingolipid PKC inhibitor, P-9782 (erythro-2-amino-1[4-(1-dodecynyl)-2-thienyl]-1,3-propanediol). P-9782 inhibited PKC at the regulatory site in an isolated enzyme system (IC_{50}=25μM). This effect was compared to sphingosine and its erythro- and threo-dihydro analogs and catalytic subunit inhibitors. In addition to its in vitro inhibitory PKC activity, P-9782 also demonstrated topical antiinflammatory activity in a murine model (IC_{50}=0.25 mg) in which phorbol ester was utilized to induce a PKC-mediated inflammation. This was substantiated by P-9782 inhibition of the PKC-mediated neutrophil superoxide anion production (IC_{50}=0.72μM). As inflammatory dermatoses can be complicated by hyperproliferation, the antiproliferative activity of P-9782 was assessed by incorporating bromodeoxyuridine into DNA of human proliferating keratinocytes (IC_{50}=3.6 μM). P-9782 demonstrated greater potency than H-7, sphingosine and dihydrosphingosine analogs. This study illustrates that P-9782 is a potent PKC inhibitor which has a potential value as a therapeutic agent for hyperplastic, inflammatory skin disorders.

QZ 405 PKC ISOZYMES THAT MODULATE NGF-INDUCED NEURAL DIFFERENTIATION AND MEDIATE RESPONSES TO ETHANOL IN PC12 CELLS, Robert O. Messing, Bhupinder Hundle, and Reina Roivainen, Gallo Center, Department of Neurology, University of California San Francisco, San Francisco, CA 94110

Ethanol can injure adult and developing nervous systems by disrupting the growth of dendrites and axons (neurites). In several brain regions, ethanol enhances neurite growth, which could alter normal development and remodeling of neural networks, and slow conduction by placing synapses at greater distances from cell somas. We found that chronic ethanol exposure markedly increases NGF-induced neurite outgrowth, expression of Thy-1 glycoprotein, and activation of MAP kinases in PC12 cells. These responses to ethanol are PKC-dependent since they were inhibited by co-treatment with 100 nM PMA, which selectively down-regulated βI, δ, and εPKC. Previously, we found that chronic ethanol exposure increases PKC activity and expression of δ and εPKC isozymes. To investigate whether these isozymes mediate ethanol's enhancement of NGF responses, we stably transfected PC12 cells with full-length cDNAs for mouse εPKC (from J. Knopf, Genetics Institute) or rat δPKC (from P. Parker, Imperial Cancer Research Fund, UK). Cells overexpressing εPKC by 2- to 3-fold demonstrated increased neurite outgrowth in response to NGF. In contrast, there was no increase in the extent of MAP kinase activation in these clones. Studies with clones over-expressing δPKC are in progress. These results suggest that NGF-induced neurite outgrowth and MAP kinase activation are modulated by different PKC isozymes. Enhancement of NGF-induced neurite outgrowth by ethanol may result from increased expression of εPKC.

QZ 406 INDUCTION OF THE PROTEIN KINASE C SIGNAL TRANSDUCTION PATHWAY IS REQUIRED FOR THE BIOSYNTHESIS OF PAPILLOMAVIRUS IN VITRO

Craig Meyers,* and Laimonis A. Laimins†, M. S. Hershey Medical Center, Pennsylvania State University College of Medicine*, Hershey, PA 17033 and Howard Hughes Medical Institute, The University of Chicago†, Chicago, IL 60637

The lack of a reliable in vitro system permissive for the propagation of papillomavirus has hindered the study of the complete vegetative replicative life cycle of papillomaviruses. Recently we reported the development of a cell culture system that allows for the in vitro biosynthesis of papillomavirus. Vegetative viral replication was achieved by the introduction of the phorbol ester, TPA, into the culture medium. The major known activity of TPA is the induction of the protein kinase C (PKC) signal transduction pathway. Use of the following kinds of nonphorbol ester activators of PKC; a teleocidin analog, a synthetic diacylglycerol, and a diacylglycerol kinase inhibitor, demonstrated that the induction of PKC is required for the in vitro biosynthesis of papillomavirus. Inhibitors of PKC were used to show that the PKC signal transduction pathway is necessary in the induction and propagation of papillomavirus. Full expression of the capsid proteins and their assembly into viral particles requires both the induction of PKC and terminally differentiating epithelium.

QZ 407 THE ROLE OF BILE ACIDS AND PROTEIN KINASE C (PKC) ISOFORMS IN COLON CARCINOGENESIS, Judit Pongracz,; John P. Neoptolemos; Janet M. Lord; Department of Immunology , The University of Birmingham, Birmingham B15 2TT, U.K.

The relationship between bile acids and colorectal cancer is not defined at the molecular level. Bile acids (BA) are tumour promoters in animal studies and interact with two second messenger signalling systems, arachidonic acid and PKC. There have been few studies of PKC in human colorectal cancer, but all reported a decrease in PKC. However, there are no data concerning PKC isoenzymes in colorectal cancer. We therefore examined PKC isoenzymes in normal and cancerous colonic mucosa and the ability of bile acids to activate colonic PKC. In 7 paired samples, total PKC was decreased by 61.3±5.2% ($p<0.002$) in cancerous mucosa. Western blotting of tumour extracts showed that PKC α was unchanged, PKC β and ε were decreased (by 37±4.2% and 33±5.1%, respectively, ($p<0.05$) and PKC δ was increased by 71.0±9.8% ($p<0.002$). Normal colonic mucosa contained 4 PKC peaks on hydroxyapatite. Several bile acids activated purified PKC; cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), lithocholic acid (LCA) and ursodeoxycholic acid (UDCA) were the most effective activators of peaks 1 and 2, containing PKC β and ε . (Table1). Since activation causes downregulation of PKC, we propose that reduced levels of PKC β and ε in tumour tissue may result from activation by specific bile acids. (Data are mean±s.d., statistical analysis was by Students t-test for paired data).

TABLE 1.

BA (50 μM)	PEAK1	PEAK2	PEAK3	PEAK4
	PKC activation % of DAG control			
CA	200±25**	171±31*	133±23	300±42**
CDCA	110±13	77±39**	86±11	33±10**
DCA	303±43**	142±12*	85±15	77±12
LCA	350±48***	103±19	14± 3**	78±10
UDCA	540±61***	44±19*	53±16*	189±29*

(* $p<0.05$, ** $p<0.002$, *** $p<0.001$)

QZ 408 EVIDENCE THAT INCREASED PROTEIN KINASE C (PKC) ACTIVITY IN METASTATIC CELLS IS ATTRIBUTABLE TO PKC ISOFORMS OTHER THAN PKCβ1.

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We previously reported that the expression of PKCβ1 mRNA and total PKC activity correlates with tumor cell invasion in the Boyden chamber assay of human gastric cancer cells (SK-GT) derived from the proximal stomach of American patients (JNCI 85:402,1993). In addition we have shown that introduction of the full length PKCβ1 cDNA into non-invasive SK-GT cells induces tumor cell invasion in vitro (AACR 34:71,1993). We examined this relationship further by studying five Japanese gastric cancer cell lines derived from hepatic (MKN-45 and MKN-74) and lymph node metastases (MKN-1, MKN-7, and MKN-28). Similar to that observed for the primary SK-GT cells, MKN-1 was the only metastatic cell line to be both highly invasive in the Boyden chamber assay and express mRNA for PKCβ1, as determined by reverse transcriptase PCR. The expression of mRNA for PKCα, PKCγ and PKCη/L was essentially the same for all MKN and SK-GT cell lines. Total PKC enzyme activity, as measured by ^{32}P-phosphorylation of myelin basic protein, was increased 2-fold in the metastatic MKN-1 cells as compared to the primary invasive SK-GT cells that also express PKCβ1. Furthermore, the PKC activity of hepatic MKN cells which do not express PKCβ1 was increased 30-fold relative to non-invasive SK-GT cells and 6-fold relative to the invasive SK-GT cells. These data confirm that PKCβ1 expression correlates with invasion of gastric cancer cells and suggests that the actual establishment of the metastases at distant sites may depend on the activation and expression of PKC isoforms other than PKCβ1. We continue to examine these cells for the expression of other isoforms in the PKC gene family.

QZ 409THE PROTEIN KINASE C INHIBITOR CGP 41251, A STAUROSPORINE DERIVATIVE WITH ANTITUMOR ACTIVITY, REVERSES MULTIDRUG RESISTANCE. Irene Utz, Susanne Hofer, Urs Regenass*, Hans Grunicke and Johann Hofmann, Inst. of Medical Chemistry and Biochemistry, University of Innsbruck, A-6020 Innsbruck, Austria, *Oncology Research Department, Ciba-Geigy, CH-4002 Basel, Switzerland.

Cancer chemotherapy is limited by cytotoxicity of antitumor drugs to normal tissues and by the development of resistance to these compounds. Several possibilities have been evaluated to overcome resistance to chemotherapy. One mechanism responsible for resistance to several anticancer drugs is multidrug resistance (MDR). Multidrug resistance is frequently associated with expression of a 170 kd membrane protein (P-glycoprotein) and with increased protein kinase C (PKC) activity. It has been shown that protein kinase C is an essential component of signal transduction. Staurosporine is a potent, but unspecific inhibitor of PKC. The staurosporine derivative CGP 41251, an experimental anticancer drug, has been shown to exert selectivity for inhibition of protein kinase C activity and exhibits antitumor activity in vitro and in vivo. Here we show that CGP 41251 is also able to reverse multidrug resistance. After treatment of the multidrug resistant human lymphoblastoid cell line CCRF-VCR1000 with 500 nM Adriamycin, cell proliferation was reduced to 81 % of untreated controls. A combination of 500 nM Adriamycin with a non toxic concentration of 150 nM CGP 41 251 (IC50 for inhibition of cell proliferation 420 nM CGP 41251) inhibits cell proliferation of CCRF-VCR1000 cells to 29 % of untreated controls. In sensitive CCRF-CEM cells no enhancement of the Adriamycin-induced toxicity was observed upon addition of 150 nM CGP 41 251. Strong synergism in inhibition of cell proliferation was also observed after concomitant treatment of KB-8-5-11 cells with CGP 41251 and Vinblastine or Adriamycin. Pretreatment with 50 - 1000 nM CGP 41251 for 30 minutes lead to a dose dependent increase in the intracellular accumulation of rhodamine 123, a substrate of P-glycoprotein. This observation indicates that reversal of multidrug resistance by CGP 41251 is caused by an inhibition of drug efflux. Treatment of multidrug resistant CCRF-VCR1000 cells with CGP 41251 for ten minutes was sufficient to inhibit the efflux of rhodamine 123. Preincubation with CGP 41251 for 12 or 24 hours did not alter MDR1-mRNA levels. A drug with antitumor activity (clinical Phase I trials are in preparation) which also reverses multidrug resistance is of particular interest and may be important for treatment of patients.

QZ 410 ROLE OF PROTEIN KINASE C ISOFORMS IN THE DIFFERENTIATION OF MELANOMA CELLS, Dianne Watters and Peter Parsons, Queensland Cancer Fund Research Unit, Queensland Institute of Medical Research, 300 Herston Rd., Brisbane, Australia 4029.

A line of human melanoma cells, MM96E, was induced to differentiate by a novel differentiating agent, bistratene A. Bistratene A is a polyether toxin from the ascidian *Lissoclinum bistratum* which we have shown to cause partial differentiation of HL-60 human leukemia cells, cytoskeletal rearrangement, accumulation of cells in G2/M, and inhibition of cytokinesis. The mode of action of bistratene A has not been completely established but it is known that it induces protein phosphorylation, does not inhibit phorbol ester binding and is not a phosphatase inhibitor. Bistratene A treatment of MM96E cells results in morphological changes with the elaboration of processes, increased melanin production and increased tyrosinase activity. The mRNA of several pigmentation genes and GAPDH is increased. There is also accumulation of cells at the G2/M boundary although not as extensive as that seen with HL-60 cells.

PKC β has been reported to regulate human melanogenesis by activating tyrosinase, however very little is known about the role of other isoforms in this process. Therefore the role of PKC isoforms in the differentiation of MM96E cells induced by bistratene A was examined by western blot analysis and confocal microscopy using PKC isoform-specific antibodies. By western blotting there was no discernible effect on the distribution of PKC α, ε, γ, or ζ, however there was a reduction of PKC δ in the cytoplasm. Confocal microscopy showed that PKC δ appeared to be associated with the cytoskeleton in untreated cells and confirmed a redistribution of this isoform within 15 min of bistratene A treatment.

Our results show that PKC δ is associated with inhibition of cytokinesis and differentiation of this melanoma cell line by bistratene A.

QZ 411 ALTERATIONS IN THE EXPRESSION OF THE PROTEIN KINASE C ISOTYPES IN HUMAN CELL LINES AND TISSUE BIOPSIES. Douglas T. Yamamishi, Shigeo Ohno[1], James Jakowatz, Matthew Goodman, and Frank L. Meyskens Jr., Clinical Cancer Center, U.C. Irvine, Irvine, CA 92717, and Dept. of Mol. Biol., Yokohama City University School of Medicine, Yokohama, 236, Japan[2].

Our previous studies on the expression of the protein kinase C (PKC) isotypes (α, β, γ, and ε) in human primary melanocytes and metastatic melanoma cell strains had shown that metastatic melanoma cells did not express PKC β_{II} RNA transcripts using Northern blot hybridization analysis. We have extended our PKC studies to include benign, premalignant, and malignant tissue biopsies as well as human tumor cell lines. Expression of the PKC β_{II} RNA transcripts were either decreased or undetectable in tissue biopsies from dysplatic nevi, metastatic melanomas, breast fibroadenomas, and breast tumors. We were also unable to detect the expression of PKC β_{II} RNA transcripts in tumor cell lines from colon, brain, breast, and hemapoietic cells. We have also analyzed the functional role of the PKC isotypes, and activity of the human PKC β promoter. Transfected cell lines could be isolated following transfection of melanoma cells with the selection vector alone or co-transfected with PKC α or β_{II} expression vectors. However, transfection of human melanoma cells with the PKC β_{II} isotype under an inducible promoter induced cell death within two weeks. We have also observed a loss in the activity of the human PKC β promoter in human melanoma cells compared to human melanocytes. These data suggest an alteration in the expression of the PKC β_{II} isotype in the transformation of human primary melanocytes, which may serve as a therapeutic target.

QZ 412 LIPID ACTIVATORS AND SUBCELLULAR DISTRIBUTION OF FETAL BRAIN PROTEIN KINASE C (PKC) ISOZYMES DURING ISCHEMIA IN UTERO, Ephraim Yavin and Shosh Gil. Department of Neurobiology, Weizmann Institute of Science, Rehovot, IL

The adverse consequences of brief occlusion of the placental blood vessels on fetal brain PKC regulation has been studied in 20 days-old fetal rats in the context of phospholipid turnover and the appearance of the endogenous second messengers, arachidonic acid (ArA) and diacyl glycerol (DAG) metabolites. A significant portion of the enzymatic activity estimated by histone III phosphorylation was translocated from the cytosolic (10^5 xg) to the membrane compartment after 5 min occlusion. Enzyme translocation/activation paralleled DAG accumulation and was accompanied by a comparable reduction of poly phosphoinositides as measured by prelabeling of fetal brain lipids with [^{3}H] ArA and [^{14}C] palmitate.

The partition profile of the PKC isospecies was investigated by an immunobloting technique using polyclonal antisera. A great majority of the β1−; β2− and γ-PKC isozymes resided in the 10^5 xg particulate fraction with little or no immunoreactivity detected in the 10^5 xg soluble fraction. There was no measurable translocation of the PKC isospecies after 5 min or more following maternal-fetal blood occlusion. A higher proportion of α-PKC was detected in the soluble fraction. The discrepancy between the enzymatic activity (assessed by histone III) and the immunolocalization of the various isoenzymes point out to the complexity of PKC regulation in various signal transduction processes. The role of the constitutive PKC isozymes and the identification of endogenous phosphorylation substrates and lipid activators during brain ontogeny remains to be established.

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