LIPID SECOND MESSENGERS

Organizer: Edward A. Dennis

February 26 - March 4, 1994; Taos, New Mexico

Plenary Sessions *Page*

Poster Sessions

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

Q 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.

Q 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.

Q 003 THE 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)

Q 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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Q 005 PHOSPHOLIPASE 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.

Phospholipases, G-Proteins, Kinases, PAF and Signaling

Q 006 Phosphatidylinositol kinases and signaling, Lewis Cantley, Christopher Carpenter, Rosana Kapeller, Karen Wang, Kimberly Fuchs, Brian Duckworth, Zhou Songyang, Lucia Rameh and Alex Tokar. Harvard Medical School and Beth Israel Hospital, Boston, MA 02115.

Phosphorylated forms of phosphatidylinositol (PtdIns) are involved in a variety of signaling pathways. Distinct subfamilies of enzymes have been found that phosphorylate the D-4 and D-3 positions of PtdIns. Another family of enzymes phosphorylates the 5 position of PtdIns-4-P. At least one enzyme is known to phosphorylate the 4 position of PtdIns-3-P. The lipid products of these enzymes have all been detected in mammalian cells and shown to be acutely affected by the addition of hormones and growth factors. Recently several laboratories have obtained cDNA clones that encode the catalytic subunits of some of the phosphatidylinositol kinases. The proteins predicted from these clones have sequence homology with each other and a distant but significant homology to the catalytic domains of protein kinases. In fact, the phosphoinositide 3-kinase (PI 3-kinase) can phosphorylate its regulatory subunit on Ser and thereby turn off its lipid kinase activity. The structural basis for the complex regulation of this enzyme by protein-tyrosine kinases will be discussed. The lipid products of PI 3-kinase, especially PtdIns-3,4,5-P_3 appear to act as second messengers. Possible targets of these lipids and their role in cellular responses will be discussed.

Acyl Transferases and Phospholipase Receptors

Q 007 ROLE OF PHOSPHOLIPASE A2 RECEPTORS ON CELL SURFACES, Hitoshi Arita, Shionogi Research Laboratories, Shionogi & Co., Ltd. Osaka 553, Japan.

We found a specific and high affinity binding site for mammalian pancreatic group I phospholipase A2(PLA2-I) in a wide variety of mammalian cells and tissues including human origin. Mature form of PLA2-I specifically recognized these binding sites, whereas its inactive zymogen, synovial group II PLA2, snake and bee venom PLA2s exhibited much lesser binding activities. These findings suggested that PLA2-I acts not only as a digestive enzyme for nutrients but also as an extracellular modulator of cellular functions via the specific binding site, i.e., PLA2-I receptor. In fact, our studies have elucidated that the binding of PLA2-I to the receptor elicits a broad spectrum of cellular events including proliferation, eicosanoid production and cell migration. The PLA2-I receptor was purified to homogeneity from bovine corpola lutea and consequently found to consist of a single glycoprotein with an apparent molecular mass of 190 KDa. Based on partial amino acid sequences from a purified bovine PLA2-I receptor, we cloned and sequenced cDNA encoding this receptor protein. The PLA2-I receptor composed of 1463 amino acid residues with canonical sequence motifs for glycosylation and endocytosis in addition to signal and transmembrane sequences, which is consistent with the reported properties of the native receptor. Conclusive evidence for the identity of the protein encoded by the cDNA with native PLA2-I receptor was obtained by transient expression experiment with COS-7 cells. The structure of the PLA2-I receptor was found to be homologous to that of mammalian mannose receptor, although the sequence identities are not so high. For confirming the assignment of transmembrane and cytoplasmic domains, two genetically engineered PLA2-I receptors were transiently expressed in COS-7 cells. One mutant was devoid of the predicted cytoplasmic portion and another was predicted transmembrane + cytoplasmic portions respectively. The results indicated that the latter was secreted into culture medium whereas the former was retained on cell surface. These findings would provide further evidence for PLA2-I as a new type of mediator with some physiological importance. The elucidation of the molecular structure of PLA2-I receptor provides indispensable information for a comprehensive understanding of its function on a molecular basis. Furthermore, recombinant PLA2-I receptor generated by DNA technology would be a powerful tool for further analysis of PLA2-I receptor mediated cellular events.

Lipid Dependent Pathways in Cellular Activation (Joint)

Q 008 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, Fus3*, 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.

Q 009 ACTIVATION OF Ca^{2+}-SENSITIVE PHOSPHOLIPASE A_2 ($cPLA_2$) BY THROMBIN IN HUMAN PLATELETS
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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.

Q 010 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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Q 011 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.

Generation and Role of Arachidonic Acid and Lysophospholipids as Bioactive Lipids in Signaling

Q 012 PAF ACETYLHYDROLASE AS A NOVEL CALCIUM-INDEPENDENT PHOSPHOLIPASE A_2, Hiroyuki Arai[1], Mitsuharu Hattori[1], Hideki Adachi[2], Kenji Hattori[1], Masafumi Tsujimoto[2], and Keizo Inoue[1], [1]Faculty of Pharmaceutical Sciences, The University of Tokyo, Tokyo, Japan and [2]Suntory Institute for Biochemical Research, Osaka, Japan.

Platelet-activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a unique phospholipid that has been shown to possess potent biological activities. Removal of the acetyl moiety at the sn-2 position of the glycerol backbone produces the biologically inactive lysoPAF and this reaction is catalized by a specific enzyme, PAF acetylhydrolase, present in animal tissues and plasma. PAF acetylhydrolase activity in bovine brain soluble fraction was chromatographically separated into three distinct fractions (designated Ia, Ib, and II), all of which exhibited pH optima in the neutral to mild alkaline region and were not affected by EDTA. We have purified one major fraction of the enzyme (i.e. isomer Ib) to near homogeneity using ammonium sulfate fractionation and a series of chromatography. The purified enzyme had a molecular mass of about 100 kDa, as estimated by gel filtration chromatography, and gave three distict bands of 45, 30, 29 kDa, respectively, on SDS-PAGE. These polypeptides exclusively co-migrated with the activity throughout the purification steps. These data suggest that this set of polypeptides corresponds to the subunits of bovine brain PAF acetylhydrolase, isomer Ib. The purified enzyme displayed similar activity against PAF and oxidatively fragmented phosphatidylcholine, but did not hydrolyze phosphatidylcholine or phosphatidylethanolamine with two long chain acyl groups. Diisopropylfluorophosphate (DFP) almost completely inhibited the activity at 0.1 mM. [^{3}H]DFP labeled only the 29 kDa polypeptide, suggesting that this polypeptide possesses an active serine residue(s). The 45 kDa subunit could be dissociated from the 29/30 kDa complex by heparin Sepharose column chromatography; 29/30 kDa complex was recovered in the flow-through fractions, the 45kDa subunit being left associated with the resin. The 29/30 kDa complex still possessed the catalytic activity, suggesting that the 45 kDa subunit is not essential for the activity. The same three isomers were also detected in the boine kidney soluble fraction, although relative ratio of the activity among these isomers were different between brain and kidney. The studies of immunoblotting and active site labeling with [^{3}H]DFP have revealed that the isomer Ia may share the common catalytic subunit (i.e. 29 kDa) with that of the isomer Ib, whereas the isomer II is a distinct enzyme. We have also succeeded in cloning the cDNAs for the subunits of the isomer Ib and found that all three polypeptides do not have significant homology with other mammalian phospholipases. These results indicate that bovine brain PAF acetylhydrolase (isomer Ib) is a novel calcium-independent phospholipase A_2.

Q 013 CALCIUM-INDEPENDENT PHOSPHOLIPASES A_2 AND THE PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL EFFECTS OF THEIR PRODUCTS, Richard W. Gross, Washington University School of Medicine, St. Louis, MO 63110

Recent work has demonstrated a Ca^{2+}-independent, plasmalogen-selective phospholipase A_2 in myocardium, smooth muscle and pancreatic β cells. Purification of the calcium-independent phospholipase A_2 activity from myocardium demonstrated that this activity is catalyzed by a 40kDa polypeptide which is regulated by ligand-modulated protein-protein interactions. Specifically, an 85kDa polypeptide was identified which mediated the ATP-induced activation and stabilization of myocardial calcium-independent phospholipase A_2. This 85kDa polypeptide possessed sequence homology to and chemical, physical, immunologic and chromatographic similarities with phosphofructokinase and specifically interacted with the 40kDa phospholipase A_2 catalytic subunit. Accordingly, these results demonstrate that the protein regulatory element responsible for ATP-mediated activation of calcium-independent phospholipase A_2 activity is an isoform of phosphofructokinase. Through gel filtration chromatography, we demonstrated that the catalytic complex migrated with an apparent molecular weight of 400kDa suggesting that the phospholipase A_2 catalytic complex was comprised of a tetramer of phosphofructokinase and a monomer of the 40kDa catalytic subunit. To gain insight into the biological significance of Ca^{2+}-independent phospholipase A_2 as a mediator of arachidonic acid release during agonist stimulation, we exploited the specificity inherent in the mechanism-based inhibitor, (*E*)-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one (HELSS), which possesses a 1,000-fold selectivity for Ca^{2+}- independent versus calcium-dependent phospholipase A_2. The results demonstrated that Ca^{2+}- independent phospholipase A_2 is responsible for the large majority of vasopressin-stimulated arachidonic acid release in A10 smooth muscle cells. Collectively, these results demonstrate the fundamental relationship between phospholipolysis and glycolysis supporting the notion that calcium-independent phospholipase A_2 activity is responsible for cellular adaptation to a variety of external perturbations both through the release of lipid second messengers as well as through the modulation of membrane physical properties.

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Q 014 REGULATION AND PHOSPHORYLATION OF THE CYTOSOLIC PHOSPHOLIPASE A_2 ($cPLA_2$)

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Arachidonic acid is the rate-limiting precursor in the biosynthesis of two potent classes of inflammatory mediators, prostaglandins and leukotrienes. The production of these two inflammatory mediators is initiated primarily by phospholipase A_2 (PLA_2), which hydrolyzes arachidonic acid from the sn-2 position of membrane phospholipids. Lysophospholipid, the other product of the reaction, can serve as a precursor for platelet-activating factor, another potent inflammatory mediator. Therefore, the activation of an arachidonic acid-selective PLA_2 serves as a key step in initiating an inflammatory response. We have cloned an 85 kDa-cytosolic phospholipase A_2 ($cPLA_2$) (1). $cPLA_2$ is expressed by many cell types and its expression can be induced by inflammatory cytokines, including IL-1 (2). $cPLA_2$ contains both a catalytic domain and a calcium-dependent lipid binding (CaLB) domain with homology to protein kinase C. The CaLB domain mediates the translocation of $cPLA_2$ from the cytosol to the membrane in response to Ca^{2+}, resulting in the stimulation of arachidonic acid release. $cPLA_2$ function is also regulated by phosphorylation. Recently, we have demonstrated that phosphorylation of Ser-505 by MAP kinase is essential for the full activation of $cPLA_2$ (3). We also have evidence suggesting that $cPLA_2$ is phosphorylated at an additional site(s). The site(s) of this phosphorylation and its effect on the function of $cPLA_2$ are currently under investigation.

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Lipids as Effector Molecules

Q 015 ANTI-PHOSPHOLIPID MONOCLONAL ANTIBODY AS A STRUCTURAL TEMPLATE FOR STUDYING LIPID-PROTEIN INTERACTIONS, Keizo Inoue[1], Masato Umeda[1], Koji Igarashi[1], Farooq Reza[1], Shigeru Tokita[1], Akiko Yamaji[1], and Yoshinori Asaoka[2]. [1]Faculty of Pharmaceutical Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113, [2]Biosignal Research Center, Kobe University, Kobe 657.

Although phospholipids in membranes have been shown to contribute to many regulatory processes of biological responses, the molecular mechanisms of modulation of protein function by phospholipids is still not clear. Phospholipids in membrane may play two distinct functions: (1)the bulk phospholipids in the bilayer would provide the working environment for many membrane-bound enzymes through hydrophobic interactions, and (2) a specific lipid would serve as an allosteric effector in modulating enzyme activity. Although these two functions are quite distinct theoretically, it is quite difficult that these two functions are distinguished experimentally, since the "specific" interaction is buried in the "non-specific" hydrophobic interactions.

Our approach to this problem has involved the production of a series of anti-phospholipid monoclonal antibodies (mAb) with binding profiles similar to those of either intracellular or extracellular phospholipid-binding proteins. The mAbs are divided into two groups, "specific" and "polyreactive" mAbs.

"Specific" anti-phospholipid mAbs include those against PS, PC, PIP and PIP2. Analysis of the interaction between PC-specific mAb and various spin-labeled derivatives of PC revealed that the anti-PC mAb could recognize a monomeric form of PC molecule and substantial portion of fatty acyl chain of PC is also involved in the interaction. These "specific" mAbs recognize the precise conformational structure of each phospholipid. For instance, a PS-specific mAb can recognize the stereo-specifc configuration of serine residue in PS molecule. "Polyreactive" mAbs show an extensive cross-reaction with various acid phospholipids, and some of them also react with specified polyanionic substances such as dextran sulfate, sulfatides, double-stranded DNA and a certain kind of proteins such as platelet glycoprotein IIIa. These "poly-reactive" antibodies may recognize a particular geometric distribution of negative charge on a surface of various ligands.

We have undertaken structural and idiotypic analyses of a series of the mAbs which bind to PS. A computer-aided modeling of the variable regions showed that the combining site surface of a "PS-specific" mAb has a deep pocket like structure (cavity type), while that of a "poly-reactive" mAb was a shallow groove-like structure where positively charged amino acids clustered on the surface (groove type). In order to study the structural similarity between the anti-PS mAbs and the cellular PS-binding proteins, we have established a series of anti-idiotypic (anti-Id) mAbs which specifically recognize the combining site structure of either the "specific" or "polyreactive" anti-PS mAb. Both the anti-"specific" and anti-"polyreactive" anti-Id mAbs showed an extensive cross-reaction with PKC and blood coagulation factor V (factor V), both of which require PS for activation. The binding of anti-"specific" anti-Id mAb to PKC and factor V was inhibited specifically by PS, while the binding of anti-"polyreactive" anti-Id mAb was effectively inhibited by both PS and PI. These observations suggest that these enzymes may have two distinct phospholipid binding sites; one is specific for PS and the other is polyreactive with various acidic phospholipids. The PS-specific site may be involved in the allosteric regulation of the enzyme activation and the polyreactive site in the association to the membrane lipids. Our current studies are directed toward identifying these phospholipid-binding sites.

Q 016 INFLAMMATION AT THE VASCULAR WALL: THE ROLE OF PAF AND OXIDIZED PHOSPHOLIPIDS

Stephen M. Prescott[1], Diana M. Stafforini[1], Diane Lorant[2], Thomas M. McIntyre[2], and Guy A. Zimmerman[2]
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The adhesion of leukocytes to the endothelium is one of the earliest steps in inflammation. As such, it is a critical step that must be closely regulated to achieve the physiological goal of suppressing infection, but avoiding the pathological consequence of damage to the vascular wall by activated leukocytes. We have demonstrated that the adhesion of neutrophilic leukocytes (PMN) to EC is a multi-step process that begins with activation of the EC by inflammatory mediators. The first step in this process is the rapid expression of P-selectin from an intracellular storage granule to the surface of the EC. This protein binds to a counter receptor on the PMN through a low affinity site and "*tethers*" the PMN to the EC. At this point in vivo the PMN would be close to the vascular wall or "marginated". However, the PMN is not activated and has not expressed its own adhesion proteins. This response requires a second stimulus from EC. Thrombin, histamine, bradykinin and other inflammatory mediators cause the rapid expression of P-selectin, and also stimulate the EC to synthesize PAF and transfer it to their surface. This phospholipid then binds to its receptor on the tethered PMN and activates it. The activation response includes up-regulation of adhesive proteins on the surface of the PMN which tighten the adhesion to the vascular wall and facilitates emigration from the vasculature. This is a precisely regulated process that is subsequently turned off by re-internalization of the P-selectin and by degradation of the PAF by specific enzyme, the PAF acetylhydrolase. In recent studies, we have shown that pathological stimuli can mimic this highly regulated process. For example, exposure of EC to oxidants causes the dysregulated expression of P-selectin and also generates phospholipids that have been oxidatively fragmented. These compounds are structurally similar to PAF and bind to its receptor and activate the PMN. However, synthesis is not regulated in the same manner as PAF since the compounds are generated by free radical oxidation. In this case, the accumulation of the potent activator is controlled strictly by the rate of hydrolysis, which again is catalyzed by the PAF acetylhydrolase. Thus, the physiological response that recruits PMN involves the potent lipid autacoid PAF. In response to oxidants, related compounds are generated, and in both cases the degradative enzyme, PAF acetylhydrolase may be crucial in regulating inflammation and other responses.

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

Q 017 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.

Q 018 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.

Q 019 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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Q 020 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.

This work was supported by Research Grants 1RO1 GM43880 from the NIH and 3018M from The Council For Tobacco Research.

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Lipid Signals and Human Disease

Q 021 THE BACTERICIDAL/PERMEABILITY INCREASING PROTEIN (BPI) OF NEUTROPHILS AND ITS POSSIBLE ROLE IN SEPTIC SHOCK, Peter Elsbach[1], Jerrold Weiss[1], Yvette Weinrauch[1], Ofer Levy[1], Chean Eng Ooi[1], Martin Doerfler[1], Arnie Horwitz[2], Georgia Theofan[2], Helene Gazzano-Santoro, Karoly Meszaros, J. Brian Parent, and Ada Kung[2], [1]New York University School of Medicine, New York, N.Y. 10016 and [2]XOMA Corporation, Berkeley, CA 94710.

Invading microorganisms elicit prompt, generally effective, host-responses that may, however, become self-destructive, leading to septic shock and death. The nature of the host responses has been studied most toward Gram-negative bacteria (GNB). The principal signal that the host recognizes from GNB is a lipid, the lipid A portion of the envelope lipopolysaccharides (LPS). Several LPS (lipid A) -binding proteins have been shown to be critical determinants of host responses. Best defined to date are three proteins: circulating acute-phase LPS-binding protein (LBP); cell-associated or extracellular CD14; and cell-associated or extracellular BPI, all ca. 55-60 kDa proteins. Whereas LBP amplifies LPS-recognition and responses, apparently via delivery of LPS to cell-associated CD14, BPI inhibits LPS-signalling by competing with LBP. Thus, binding of LPS by both LBP and LPS, based likely on ~45% primary structural similarity, results in opposite effects on LPS activity. BPI is a major constituent of the antimicrobial arsenal of granulocytes and is potently toxic for GNB, but not for Gram-positive bacteria or eukaryotic cells. Under inflammatory conditions BPI is released from the cells and can function, therefore, as both a bactericidal and LPS-neutralizing agent within the phagocyte as well as extracellularly. The N-terminal half of BPI is $\geq$ bioactive as the holo-protein. The recombinant fragment protects animals against endotoxin and GNB and is well-tolerated by human volunteers.

Q 022 SPHINGOMYELINASE, CELL ADHESION AND INFLAMMATION, Thomas M. McIntyre[1], Kamala D. Patel[2], Vijayanand Modur[1], Michael J. Feldhaus[1], Guy A. Zimmerman[1], and Stephen M. Prescott[1], [1]University of Utah, Salt Lake City, UT 84112, [2]Oklahoma Medical Research Foundation, Oklahoma City.

The venom of the brown recluse spider (*Loxosceles reclusa*) can cause an impressive necrotic lesion that is intriguing as it clearly derives from an inappropriate inflammatory reaction, and the active component in the venom is a sphingomyelinase D. This system might, therefore, be a direct and convincing demonstration that sphingomyelinases are in *vivo* biologic response modifiers. Unfortunately the mechanism by which the venom causes such lesions is currently obscure. Polymorphonuclear leukocytes (PMN, neutrophil) are responsible for the tissue destruction as their depletion blocks this. However, human PMN are not directly activated by the venom *ex vivo* and, in fact, are actually inhibited by it. This apparent paradox may be resolved by the observation that the first ultrastructural change after envenomation is selective damage to vascular endothelium, with adhesion of PMN to the capillary wall followed by infiltration into the surrounding tissues. This suggests that an essential component of this inflammatory reaction may be activation of vascular endothelium, with subsequent sequestration and activation of passing PMN by the perturbed endothelial cells. This would parallel events in the normal inflammatory reaction where inflammatory agents like TNF induce regulated endothelial cell-dependent PMN adhesion and activation of these PMN. We therefore determined if *Loxosceles* venom was an endothelial cell agonist, and found that as little as 10 nl of crude venom induced endothelial cells to bind quiescent PMN. Higher doses gave levels of adhesion equivalent to that induced by TNF. Activation was time dependent, and required *de novo* protein synthesis. The relevant adhesion molecule expressed by venom-treated endothelial cells was E-selectin, and these cells also released PMN agonists IL-8 and GM-CSF. However these were differentially expressed, indicating that transcriptional and posttranscriptional regulation for each was variably affected by the venom. Sphingomyelinase activity was the agonist in the venom as DFP blocked its effect, purified toxin was equally effective, and purified recombinant *Corynebacterium pseudotuberculosis* sphingomyelinase D were also effective agonists. Mammalian sphingomyelinase activity is a C type that generates ceramide. Consequently we determined if this type of activity would also activate the inflammatory responses of endothelial cells. We found that *Staphylococcus aureus* sphingomyelinase C, or the water-soluble octanoyl-ceramide analog, also were effective endothelial cell agonists. The nature of the response to these agents was not appreciably different from that of *Loxosceles* venom, and, more importantly, from that of TNF. Given the recent interest in a sphingomyelin cycle as a possible effector mechanism for inflammatory cytokines such as TNF, we conclude that exogenous sphingomyelinase activity mimics and subverts the normal inflammatory response of endothelial cells. We also propose that some, but given the differential induction of rapid response genes, not all of effects of TNF on endothelial cells may proceed through an activated sphingomyelinase activity.

Signal Transduction; Cellular Activation; Regulation

Q 100 POTENTIAL ROLE OF PHOSPHOLIPASE A_2 IN HL-60 CELL DIFFERENTIATION TO MACROPHAGES THROUGH PROTEIN KINASE C ACTIVATION, Yoshinori Asaoka, Kimihisa Yoshida, Hiroshi Yoshida, Yosuke Tsujishita and Yasutomi Nishizuka, Biosignal Research Center, Kobe University, Kobe 657, JAPAN

Phospholipid degradation initiated by receptor stimulation produces various lipid mediators that relay information from extracellular signals across the membrane. The hydrolysis of phosphatidylcholine (PC) by phospholipase D (PLD) as well as that of phosphatidylinositol by phospholipase C (PLC) generates diacylglycerol (DG) that is needed for protein kinase C (PKC) activation. The reaction products of PC hydrolysis by phospholipase A_2 (PLA_2), 2-lysophosphatidylcholine (lysoPC) and cis-unsaturated fatty acids, both serve as enhancer molecules for PKC activation in cell-free enzymatic reactions and in intact cells such as platelets.

In HL-60 cells, a membrane-permeant DG and tumor-promoting phorbol ester induce their differentiation to macrophages. LysoPC and cis-unsaturated fatty acids such as linoleic and linolenic acids significantly potentiate this HL-60 cell differentiation that is induced by either a membrane-permeant DG or phorbol ester, as measured by the expression of CD11b, appearance of phagocytic activity, and morphological changes. Neither lysoPC nor cis-unsaturated fatty acid is active unless a membrane-permeant DG or phorbol ester is present. The biochemical mechanism of this PLA_2 activation is not fully clarified, but kinetic analysis with permeabilized HL-60 cells suggests that intracellular Ca^{2+} at the 10^{-7} M range is critical for the regulation of intracellular PLA_2.

The physiological signals for the differentiation of HL-60 cells to produce macrophages is presently unknown, but various metabolites of membrane phospholipid degradation may take part in the sustained PKC activation, which is a prerequisite for the cellular responses.

Q 101 ROLE OF INTRACELLULAR CA^{2+}, INOSITOL-1,4,5 TRISPHOSPHATE AND ADDITIONAL SIGNALING IN THE PAF STIMULATION OF PGE_2 FORMATION IN $P388D_1$ MACROPHAGE-LIKE CELLS, Reto Asmis*, Clotilde Randriamampita#, Roger Y. Tsien# and Edward A. Dennis*, *Department of Chemistry and #Department of Pharmacology , University of California, San Diego, CA 92093-0601

We have studied the role of inositol-1,4,5-trisphosphate ($(1,4,5)IP_3$) and Ca^{2+} in the stimulation of prostaglandin E_2 (PGE_2) formation by platelet-activating factor (PAF) and bacterial lipopolysaccharides (LPS) in $P388D_1$ macrophage-like cells. Priming of the cells with LPS was required for PAF to stimulate PGE_2 formation, yet LPS affected neither the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) nor the PAF-induced rise in $[Ca^{2+}]_i$. In addition, basal and PAF-stimulated $(1,4,5)IP_3$ levels were not affected by LPS priming. However, the release of $(1,4,5)IP_3$, the Ca^{2+} transient, and the formation of PGE_2 induced by PAF were inhibited in cells pretreated with pertussis toxin. The initial rise in $[Ca^{2+}]_i$, produced by either PAF via the formation of $(1,4,5)IP_3$ or directly by ionomycin, is necessary, but not sufficient for the formation of PGE_2 in LPS-primed $P388D_1$ cells. Furthermore, we demonstrate for the first time that PAF triggers a second signal that is not mediated by a change in $[Ca^{2+}]_i$. However, both signals are required to induce PGE_2 formation.

Q 102 EARLY SIGNAL TRANSDUCTION EVENTS IN THE ULTRAVIOLET A INDUCTION OF THE HUMAN HEME OXYGENASE-1 GENE, Sharmila Basu-Modak, Patrick Luscher and Rex M. Tyrrell, Physical Carcinogenesis Unit, Swiss Institute for Experimental Cancer Research, Epalinges, Lausanne, Switzerland.

Heme oxygenase (HO) is the rate limiting enzyme in the heme degradation pathway and one of the isoenzymes, HO-1, is readily induced by a wide variety of factors including oxidants. In cultured human skin fibroblasts, HO is the major protein induced by UVA and other oxidative stress agents. The induction occurs by activation of the HO-1 gene which results in a transient accumulation of its mRNA and active oxygen species are involved in the induction. It has also been shown in this laboratory that HO-1 induction mediates an adaptive response to oxidative stress in human skin fibroblasts. To gain further insight into the mechanism of induction of this gene, we compared the relative involvement of the hydroxyl radical and singlet oxygen by chemically modulating their cellular concentrations. We find that singlet oxygen is the primary effector in the UVA induction of this gene. The experiments carried out with a more specific generator of singlet oxygen, Rose Bengal, suggest that it is generated at the membrane and therefore probably activates an early step in the signal transduction.

During UVA irradiation of cells at least three processes that include membrane damage by lipid peroxidation, disruption of ion homeostasis and induction of membrane repair occur simultaneously. We are now investigating which of these three processes are involved in the induction of the gene and whether they can act independently or synergistically. Preliminary results show that treating cells with a range of concentrations of arachidonic acid either in medium or in PBS is not sufficient to induce the gene. However, ETYA, an analogue of arachidonic acid induces the gene in the concentration range of 10-50 mM. ETYA blocks arachidonic acid metabolizing enzymes such as lipoxygenases and cycloxygenases by acting as a false substrate. Although, taken together, these results suggest that a step earlier than the arachidonic acid release from membrane phospholipids is involved in gene activation, each potential step will be further investigated in detail in order to develop a consistent model.

Q 103 THE ROLE OF SPHINGOMYELIN-DERIVED LIPID SECOND MESSENGERS IN THE REGULATION OF IL-8 PRODUCTION BY HUMAN KERATINOCYTES, Marie Chabot-Fletcher and John Breton, Dept. of Pharmacology, SmithKline Beecham Pharmaceuticals, King of Prussia, PA 19406

The proinflammatory cytokines interleukin-1 (IL-1) and tumor necrosis factor (TNF) are known to induce the production of interleukin-8 (IL-8) in a number of cells including human keratinocytes. However, the mechanism underlying this induction is unknown. Recently, the breakdown of sphingomyelin in response to IL-1 and TNF has been reported and a product of this reaction, ceramide, has been suggested to function as a second messenger molecule. Therefore, the role of this signal transduction system in the regulation of IL-8 production in response to stimulation with IL-1 was investigated. The incubation of human keratinocyte cultures with sphingomyelinase was found to induce the production of IL-8 in these cells. Furthermore, both sphingomyelinase and a cell-permeable ceramide potentiated the production of IL-8 in response to stimulation with IL-1. Stimulation of human keratinocytes with 0.1 ng/ml IL-1 resulted in the production of 543 pg/ml IL-8. Co-stimulation with IL-1 and sphingomyelinase (25 mU/ml) or C_2-ceramide (10 μM) resulted in the production of 1616 pg/ml IL-8 and 984 pg/ml IL-8 respectively. These findings indicate that the production of IL-8 by human keratinocytes can be modulated by either exogenous sphingomyelinase or a cell permeable ceramide and further suggest that sphingomyelin turnover may play a role in the regulation of IL-8 production in IL-1-stimulated human keratinocytes.

Q 104 CD28 SIGNAL IN T LYMPHOCYTES IS TRANSDUCED BY SPHINGOMYELIN-CERAMIDE SIGNALING PATHWAY

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Although antigen receptor occupancy with a peptide in association with major histocompatibility complex antigen initiates T cell activation, a second co-stimulatory signal is necessary the optimal proliferation and lymphokine secretion. CD28 is a homodimeric membrane glycoprotein expressed on most of the T cells and the signal generated by the interaction with its natural ligand, B7, has been implicated as a co-stimulatory signal. While the CD28 signal in concord with the TCR signal induces proliferation and lymphokine secretion by T cells, a notable activation event is nonexistent by the CD28 signal alone and very little is known about CD28 signaling pathway. In this presentation we would like to report that CD28 ligation activates sphingomyelinase thereby triggering the sphingomyelin (SM) signaling pathway. Activation of this pathway resulted in the induction of ceramide driven kinases specific for serine and threonine. Importantly the sphingomyelin-ceramide signaling pathway is also known to couple to receptors for interleukin 1 and tumor necrosis factor which are co-stimulatory to T cell activation. The data demonstrate the pivotal role of SM signaling pathway to conduct co-stimulatory signal in T cells.

Q 105 DIFFERENTIAL COUPLING OF ENDOTHELIN RECEPTORS TO PHOSPHOLIPASE D ACTIVATION IN GIRARDI HEART CELLS AND A7r5 CELLS, Catherine L. Cioffi, Research Department, CIBA-GEIGY Corporation, Summit, NJ 07901. Endothelins (ET), a family of vasoactive peptides, interact at two classes of receptors: ET_A (ET-1=ET-2>ET-3) and ET_B (ET-1=ET-2=ET-3). ET_A receptor activation is coupled to phospholipase D (PLD) but no information exists on whether the ET_B receptor subtype is linked to this response. The present study utilizes human Girardi heart (GH) cells, which possess only ET_B receptors, to investigate whether this receptor subtype stimulates the production of phosphatidylethanol (PEt), an indicator of PLD activation, and compares the results to ET_A receptor-stimulated PEt formation in A7r5 smooth muscle cells. ET peptides did not stimulate [^{3}H]PEt formation when added to [^{3}H]oleic acid-labeled GH cells in the presence of 0.5% ethanol but did inhibit cyclic AMP accumulation. In contrast, sodium fluoride and phorbol 12-myristate 13-acetate (PMA) stimulated [^{3}H]PEt accumulation 10-fold over basal levels indicating the involvement of a guanine-nucleotide binding protein and protein kinase C (PKC) in the activation of PLD in GH cells. In A7r5 cells, which possess the ET_A receptor subtype, ET-1 and sarafotoxin (SFX) S_{6b} stimulated the release of [^{3}H]PEt in a time-dependent manner with EC_{50} values of 0.6 ± 0.1 nM and 5 ± 1 nM, respectively, while the ET_B-selective SFX S_{6c} was without effect. Neomycin (1 mM) attenuated ET-1-induced [^{3}H]inositol phosphate formation by 61% and decreased ET-1-stimulated PLD activation by 24% suggesting that the latter response may be partly dependent on inositol lipid hydrolysis. In addition, ET-1-induced [^{3}H]PEt production was additive to that stimulated by PMA and was not significantly effected by inhibitors of PKC. These data suggest that activation of ET_A receptors on A7r5 cells, but not ET_B receptors on GH cells, are coupled to PLD independently of PKC activation.

Q 106 IDENTIFICATION OF THE SERINES REQUIRED FOR PKC- AND cAPK-MEDIATED DESENSITIZATION OF THE β_2-ADRENERGIC RECEPTOR: ADDITIVITY OF COINCIDENT SIGNALS FROM TWO PATHWAYS. Richard B. Clark and Nanyong Yuan, Graduate School of Biomedical Sciences and the Department of Pharmacology, The University of Texas Health Science Center, Houston, Texas 77225-0334

PMA treatment of L cells causes two opposing effects on β_2-adrenergic receptor (β_2AR) stimulation of adenylylcyclase (AC), a desensitization caused by a 4-fold decrease in the EC_{50} of epinephrine (EPI) stimulation, and a 2-4 fold sensitization caused by an increase in the V_{max}. Deletion of the consensus site for PKC ($RRSSK_{263}$) blocks the PKC and cAPK desensitizations, but does not alter the PMA-induced sensitization. Our objectives here were to determine: (i) the precise serines required for the desensitization in response to activation of PKC and cAPK, and (ii) whether coincident signals from the two pathways caused an additive desensitization. Three β_2AR substitution mutants were constructed and stably expressed: S261A, S262A, and S261,262A. Their desensitization in response to treatment with either 100 nM PMA, 50 nM EPI, 1.0 μM PGE_1, or combinations of PMA and either EPI or PGE_1 was determined and compared to the $WT\beta_2AR$. The EC_{50} for EPI stimulation of AC was increased 3.8, 4.1, 2.5 and 7.5 fold following 10 min treatment of the $WT\beta_2AR$ with EPI, PMA, PGE_1, or EPI plus PMA respectively. The double substitution mutant eliminated 90-95% of the desensitization in response to either PMA, PGE_1 or both. While some desensitization in response to 50 nM EPI was retained it could be attributed to homologous desensitization. Neither of the single substitutions altered the PMA-induced desensitization, and the additive effect of PMA in combination with either EPI or PGE_1 was only partially decreased. The cAPK-mediated desensitization was blocked 85% by the S262A mutation, but was unaffected by the S261A mutation. These results demonstrate: (i) a striking additive desensitization following coincident stimulation of the PKC and cAPK pathways; (ii) that S262 is the site phosphorylated by cAPK; and (iii) that either S261 or S262 suffices for the PKC-mediated desensitization.

Q 107 GLUCOSE-INDUCED PHOSPHORYLATION OF CYTOSOLIC PHOSPHOLIPASE A_2 IN NEONATAL RAT ISLETS Stella Clark & Marjorie Dunlop, Department of Medicine, University of Melbourne, Royal Melbourne Hospital, Victoria 3050, **Australia**.

Activation of phospholipase A_2 (PLA_2) may be important in the regulation of insulin release as both the products of PLA_2 action (arachidonic acid and lysophospholipid) and PLA_2, when added exogenously, can promote insulin release. To date the PLA_2 enzymes described in the islet have Ca^{2+} requirements for activation inconsistent with that occurring upon insulin release (submicromolar). However, a recently described high molecular weight, cytosolic PLA_2 ($cPLA_2$) is activated by submicromolar Ca^{2+} and thus could be physiologically relevant in the islet. We have now identified this form of PLA_2 in neonatal rat islets (NRI) with antibodies raised against the cloned protein. Approximately 500 NRI were solubilised and proteins resolved by SDS-PAGE. After transfer to nitrocellulose, $cPLA_2$ was identified by immunoblotting with a specific antibody (Genetics Institute). In intact NRI, high glucose increased PLA_2 activity against an exogenous substrate (1-stearoyl-2-[^{14}C]arachidonyl phosphatidylcholine) from 2.70 ± 0.01 to 41.6 ± 0.4 pmol hydrolysed/10 min. Stimulation of $cPLA_2$ with a variety of agonists results in phosphorylation of the enzyme possibly via a protein kinase C (PKC)-dependent mechanism. We have demonstrated that the phorbol ester PMA (10^{-6}M, 10min) can enhance $cPLA_2$ phosphorylation in ^{32}P-orthophosphate labelled NRI. Most importantly high concentrations of glucose (25mM) also stimulate phosphorylation of $cPLA_2$ approximately 2 fold. At least 1h of glucose pre-exposure was required to observe this effect. If NRI in 25mM glucose were coincubated with 10μg/ml Ro 31-8220, a specific PKC inhibitor, the glucose-dependent increase in phosphorylation was not seen. These studies demonstrate, for the first time, the presence of cytosolic PLA_2, $cPLA_2$, in NRI. PLA_2 activity in these islets is stimulated by high glucose concentrations as is phosphorylation of the $cPLA_2$ protein. Inhibition of PKC isoforms blocked the glucose-induced phosphorylation implicating PKC in the pathway of PLA_2 activation in these cells.

Q 108 EVIDENCE FOR THE ASSOCIATION OF PHOSPHATIDYLINOSITIDE SIGNALLING IN OPIOID INHIBITION OF THYMIDINE INCORPORATION INTO DNA
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Opioid agonists inhibit DNA synthesis in rat fetal brain cell aggregates and in desipramine (DMI)-treated C6 glioma cells. Endogenous opioid peptides have proven to be most potent in aggregates. β-Endorphin decreased thymidine incorporation with an IC_{50} of 0.7 nM acting via μ and κ opioid receptors. Dynorphin B attenuated DNA synthesis (IC_{50} = 0.1 nM) via κ receptors. Neither ε- nor δ-opioid receptors influence proliferation in aggregates under the same experimental conditions. μ and κ receptors appear to act via pertussis toxin-insensitive and sensitive G proteins, resp. Opioids reduced basal and glutamate-stimulated inositol phosphate (IP) accumulation in the aggregates under conditions temporally correlated with DNA synthesis inhibition. Opioids also reversed endothelin-induced DNA synthesis, IP accumulation, Ca^{2+} mobilization in DMI-treated C6 cells. A selective protein kinase C inhibitor, chelerythrine, inhibited DNA synthesis in aggregates that was temporally correlated with μ and κ opioid effects. The effect of μ agonist and chelerythrine was not additive but appears to be consistent with their actions being mediated via a common signalling pathway. κ actions were additive with chelerythrine. Isoproterenol, forskolin, IBMX and dibcAMP also inhibit DNA synthesis but opioids block cAMP production in C6 cells. Collectively, the results suggest that μ and κ agonists display mechanistically different inhibitory neurotrophic actions and the phosphatidylinositide rather than cAMP signalling system may be involved.

Q 109 TREATMENT OF CELLS WITH POLYUNSATURATED FATTY ACIDS ALTERS THE COMPOSITION OF INOSITOL PHOSPHOGLYCERIDES AVAILABLE FOR TRANSMEMBRANE SIGNALLING,
Al C. DeMarco, Richard C. Cantrill, Pat Patterson and David F. Horrobin, EFAMOL Research Institute, P.O.Box 818, Kentville, Nova Scotia, Canada, B4N 4H8
Many hormones stimulate phospholipase C-dependent hydrolysis of membrane inositol phospholipids through a sequence of interactions involving G-proteins and/or changes in the levels of intracellular calcium. Phosphatidylinositol 4,5-bisphosphate is hydrolyzed to produce inositol trisphosphate and diacylglycerol. The addition of polyunsaturated fatty acids (PUFA) to cell culture medium leads to alterations in membrane lipid composition and rates of cell division. In this study we have incubated human promonocytic leukemia cells, U937, with PUFA of both the n-3 and n-6 series and extracted inositolphospho-glyceride species and compared their fatty acid composition. In control cells, saturated, monounsaturated, and polyunsaturated fatty acids (PUFA) are present in phosphatidylinositol in equal amounts whereas saturated and mono-unsaturated fatty acids were increased with respect to polyunsaturated fatty acids in phosphatidylinositol phosphate and phosphatidyl-inositol 4,5-bisphosphate species. Treatment with PUFA led to an alteration in the pattern of fatty acids in inositolphosphoglyceride species. The presence of elongation and desaturation products may alter the precursor pool for second messenger and eicosanoid production and result in the hormone dependent release of species of diacylglycerol with reduced interaction with protein kinase C.

Q 110 ARACHIDONIC ACID METABOLITES ALTER THE Na,K-ATPase RESPONSE TO PROTEIN KINASE C ACTIVATION,
Nicholas A. Delamere and James Parkerson, Department of Ophthalmology and Visual Sciences and Department of Pharmacology and Toxicology, University of Louisville School of Medicine, Louisville, KY 40292

The non-pigmented ciliary epithelium (NPE) is responsible for the secretion of aqueous humor into the eye by a mechanism involving the Na,K-ATPase. In a cell line derived from rabbit NPE, activation of protein kinase C by exogenous phorbol dibutyrate (PDBu) stimulates the Na,K-ATPase as judged by an increase in ouabain-inhibitable ^{86}Rb uptake rate measured 10 min after the addition of PDBu. We now report that indomethacin, a cyclooxygenase inhibitor, changes the size of the Na,K-ATPase response; in the present of 1 μM indomethacin, the Na,K-ATPase stimulation caused by PDBu was increased more than two fold. In control cells (no PDBu), 1 μM indomethacin did not change the ouabain-inhibitable ^{86}Rb uptake rate. One possible explanation for these findings is that cyclooxygenase metabolites of arachidonic acid are generated and inhibit Na,K-ATPase activity in PDBu-treated cells. In parallel studies, we have isolated plasma membranes from ciliary epithelium and demonstrated marked Na,K-ATPase inhibition by the cyclooxygenase metabolite, prostaglandin E_2.
(Supported by grant number EY06915 from the National Eye Institute)

Q 111 THE INCREASED ACTIVITY OF A PHOSPHOINOSITIDE-SPECIFIC PHOSPHOLIPASE A2 LEADS TO THE FORMATION OF NOVEL ACTIVE METABOLITES IN K-RAS TRANSFORMED THYROID CELLS,
Marco Falasca and Daniela Corda, Laboratory of Cellular and Molecular Endocrinology, Istituto di Ricerche Farmacologiche "Mario Negri", Consorzio Mario Negri Sud, 66030 S. Maria Imbaro, Italy.
In cells lines stably (KiKi) or reversibly (Ts) transformed by the k-ras oncogene originated from a normal rat thyroid line (FRTL5 cells), it has recently been demonstrated that k-ras transformation is associated with an increased phospholipase A2 (PLA2) activity which leads to increased production of glycerophosphoinositols (GroPIns) (Valitutti et al., Cell. Sign. 1991, 3, 321). We have investigated whether a PLA2 specific for membrane phosphoinositides is active in thyroid cells and whether the metabolites produced by this enzyme are able to affect cell functions. TLC analysis of lysolipids revealed that lysophosphatidylinositol (LPI) increased by 2-3 fold in k-ras transformed cells (KiKi cells and Ts at the permissive temperature of 33°C) as compared to normal FRTL5 cells or to Ts mantained at 39°C, i.e. at the temperature where p21, the product of the ras oncogene, is inactive. In the same cells, the levels of lysophosphatidylcholine (LPC) and of lysophosphatidylethanolamine (LPE) were not different, suggesting that the PLA2 costitutively active in ras-transformed cells is phosphoinositide-specific. In addition, the LPI levels were analyzed in other cell types transformed by K- or H-ras oncogene (PcKiKi, Pc-Ha, TL5-Ha); in all cases ras expression was associated with a 2-3 fold increase in LPI levels. The biological activity of GroPIns and LPI was then examined. Exogenous addition of GroPIns4P inhibited the adenylyl cyclase activity both in intact FRTL5 cells and in membrane preparations; this regulation seems to occur at the G-protein level. The data are compatible with a role of GroPIns4P as novel endogenous inhibitor of adenylyl cyclase (Iacovelli et al., J. Biol. Chem., 1993, 268, 20402). Exogenous addition of LPI stimulated instead [^{3}H]-thymidine incorporation in both FRTL5 and KiKi cells and acted synergistically with insulin in promoting cell growth. These LPI effects were blocked by the tyrosine kinase inhibitors tyrphostins, but not by staurosporine, suggesting that a tyrosine kinase, rather than PKC, is involved in its action. In addition, LPI (10-20 μM) was found to induce intracellular calcium increase and to inhibit GTPase activating protein (rasGAP) activity (IC50 = 90 μM). In summary, a phosphoinositide-specific PLA2 originates metabolites that are accumulated in ras-transformed cells, and are able to directly affect cell function.
*Supported by AIRC, CNR, (PF-ACRO), AgenSud.

Q 112 INVOLVEMENT OF A NOVEL FORM OF PKC AND TYROSINE KINASE IN THE ACTIVATION OF PLD BY LHRH, Myles Fennell, James Simpson, Rory Mitchell, Neil Thompson* and Lawrie Garland*, MRC Brain Metabolism Unit, 1 George Square, Edinburgh and *Wellcome Research Laboratories, Langley Court, Beckenham, U.K.

The αT3-1 gonadotrope cell line was used to study signalling pathways associated with the LHRH receptor, including phospholipase D (PLD) and the *src* family of tyrosine kinases. [^{3}H]palmitate was used to label membrane phospholipids and cells were incubated with butan-1-ol (30 mM) in order to assay PLD activity as production of [^{3}H]phosphatidylbutanol (PtdBut). PLD activation by LHRH (100 nM) proceeded with a lag of about 5-10 min. After this the rate was approximately constant up to 40 min, which was the longest time point examined. In phorbol 12,13-dibutyrate (PDBu) (1 μM)-stimulated cells, activation of PLD occured after a delay of 10-15 min, but had virtually stopped by 25 min. After a 30 min stimulation the accumulation of PtdBut was approximately 10-fold and 4-fold over basal for LHRH and PDBu respectively. Ionomycin (30 μM) did not activate PLD. Ro 31-8220 (a selective PKC inhibitor) inhibited PLD activity elicited by 30 min incubations with PDBu or LHRH, displaying IC_{50}s of 62 ± 30 nM and 460 ± 180 nM respectively. H7 inhibited PDBu-induced PLD activity in a manner that indicated there are multiple components to the inhibition. LHRH-induced PLD activation was inhibited with low potency by H7 with an IC_{50} of 240 ± 14 μM. Another PKC inhibitor GF 109203X also inhibited the LHRH-stimulated PLD activity with an IC_{50} of 1 ± 0.1 μM. The tyrosine kinase inhibitor lavendustin A inhibited approximately 70% of the PLD response to LHRH with an IC_{50} of 133 ± 17 nM. Lavendustin A (0.1-3 μM) had no effect on the response elicited by PDBu. Another tyrosine kinase inhibitor piceatannol also caused partial inhibition of LHRH-stimulated PLD at concentrations up to 100 μM. In addition, the tyrosine phosphatase inhibitor, pervanadate (1 mM) caused significant activation of PLD. LHRH was also shown by antiphosphotyrosine immunoblots to induce tyrosine phosphorylation of numerous proteins of molecular mass 63 to >170 kDa. A number of these phosphorylations were also elicited by PDBu but not by ionomycin. Phosphorylation of proteins >170 kDa increased continuously over 3-60 min, whereas other phosphorylations were maximal at 10 min. Immunoblotting experiments showed that αT3-1 cells contain *src* and *fyn* (but not *fgr, hck, lyn* or *yes*) tyrosine kinases and preliminary results using an immunoprecipitation/kinase assay have shown that *fyn* is activated by LHRH (100 nM) within 10 min.

Q 113 PC-PLC-INDUCED HYDROLYSIS OF PHOSPHATIDYLCHOLINE ACTIVATES NF-κB AND HIV REPLICATION IN T CELLS AND MACROPHAGES.

B. Fernandez, M. T. Diaz, F. Arenzana-Seisdedos*, I. Dominguez, J.L.Virelizier,* and J. Moscat. Institut Pasteur, Paris, France*. CBM-CSIC, Univ. Autonoma, Madrid, Spain.*

Activation of NF-κB transcription factor in monocytes and lymphocytes can be obtained by stimulation with physiological inducers like TNF, IL1 or , in the case of T lymphocytes, following engagement of the CD3-TCR complex. Additionally, expression of activated p21ras in human cells leads to transactivation of κB-dependent vectors. A characteristic shared by the above cited inducers of NF-κB is their capacity to induce hydrolysis of phosphatidylcholine (PC) by the phospholipase C (PLC). That suggests that, in human monocytes and T lymphocytes, the two types of target cells of HIV infection, phosphodiesterase-mediated hydrolysis of PC has the potential to be a major activation pathway leading to induction of nuclear NF-κB and transactivation of the HIV enhancer. Using cell systems relevant to HIV pathogenesis, including an IL2-dependent T-cell clone, we demonstrate that specific hydrolysis of PC induces nuclear translocation of bona fide NF-κB. This nuclear factor increases HIV enhancer activity and viral replication in chronically infected cells treated with exogenous Bacillus cereus PC-PLC. Interestingly, PI-PLC induced DAG formation, but not NF-κB activation. Furthermore, expression of a Bacillus cereus-PC-PLC gene induces an intense transactivation effect in co-transfected κB-dependent reporter vectors. Additionally, we demonstrate that overexpression in fibroblasts, of a PKCζ isoform, non-inducible by PMA, induces expression of elevated amounts of nuclear NF-κB and transactivates reporter vectors drived by κB enhancers. Finally, we prove that the transactivatory effect induced either by PC-PLC or TNF on κB-directed reporter vectors can be blocked by the expression of a transdominant negative mutated form of PKCζ, carrying a K275W mutation in its catalytic domain.

Q 114 Activation of the *PHO* Regulon Relieves an Essential Requirement for Phospholipase C Activity in *Saccharomyces cerevisiae*. Jeff Flick and Jeremy Thorner, Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720

We have recently cloned and characterized a gene (*PLC1*) encoding a PIP_2-specific PLC from yeast. Disruption of this gene results in a temperature-sensitive (ts) growth phenotype. We have initiated genetic analysis of the essential *PLC1* function by screening a yeast genomic DNA library for dosage suppressors that allow growth at 35°C. To date, two genes have been isolated and characterized that bypass the requirement for Plc1p activity. The first suppressor has been identified as the *PHO81* gene, previously characterized as a positive regulator of the repressible phosphatase (*PHO*) regulon. The *PHO* genes act to scavenge phosphate and are normally derepressed only during growth in limiting phosphate. Increased dosage of the *PHO81* gene leads to constitutive expression of both vacuolar and secreted phosphatases. This finding suggested that constitutive *PHO* gene expression might also compensate for the loss of Plc1p activity in yeast. Two observations support this conclusion: 1) growth on a low phosphate medium, which induces *PHO* gene expression, is able to suppress the ts phenotype of the *plc1* mutant; 2) mutational inactivation of *PHO80*, which encodes a negative regulator of the *PHO* regulon, leads to constitutive *PHO* gene expression, and also suppresses the Plc1p-deficiency since a *pho80 plc1* double mutant is able to grow at 35°C.

Our hypothesis for the mechanism by which *PHO* gene expression is able to suppress the *plc1* mutation assumes that the ts phenotype of the *plc1* mutant results from a defect in mobilization of intracellular Ca^{2+}. Constitutive phosphatase expression may act to mobilize a form of Ca^{2+} which is normally non-exchangeable, perhaps by hydrolysis of the polyphosphate•Ca^{2+} complex ($Ca_n[PO_4]_{2n}$) that is sequestered in the yeast vacuole.

Overexpression of a second gene, designated *SPL2* (suppressor of *plc1*), also suppresses the ts phenotype of a *plc1* mutant. *SPL2* encodes a novel polypeptide of 148 amino acids containing both highly basic and acidic domains but lacking strong homology to any protein currently in a database. Interestingly, the promoter region of *SPL2* contains two copies of the recognition element for the transcriptional activator (Pho4p) of the *PHO* regulon. Therefore it is possible that induction of *SPL2* expression via the phosphate regulatory pathway is responsible for the ability of constitutive *PHO* gene expression to suppress *plc1*. Regulation of *SPL2* expression by phosphate is currently being tested.

Q 115 DIFFERENTIAL DAG SPECIES ACCUMULATION IN RESPONSE TO PURINERGIC AND MUSCARINIC STIMULATION OF LM5 CELLS DOES NOT ACCOUNT FOR THE DIFFERENCES IN THEIR SENSITIZATION OF ADENYLYLCYCLASE, Rekha D. Halligan, Rex D. Wiegand*, Robert E. Anderson*, and Richard B. Clark, GSBS, The University of Texas Health Science Center at Houston, Houston Texas and *The Cullen Eye Institute, Baylor College of Medicine, Houston, Texas.

Recently we found that treatment of LM5 cells with either 4ß-phorbol 12-myristate 13-acetate, or ATP, which activates PLC through P_{2U} purinergic receptors, caused a stable sensitization of adenylylcyclase (AC) activity. In contrast to ATP, stimulation of the M5 muscarinic receptor by carbachol, which has been shown to be a significantly more effective activator of the PLC pathway, caused no sensitization of AC [Mol.Pharmacol.39:539-546 (1991)]. Given the likely involvement of protein kinase C in the sensitization by ATP, we tested the possibility that differences in the amounts and/or types of diacylglycerol (DAG) produced by ATP and carbachol stimulation of the cells could account for the differences in their sensitization of AC. A combined HPLC and gas-liquid chromatography procedure was utilized to quantitate and identify the DAG species, respectively. Treatment of the cells with either ATP or carbachol for 5 min increased the level of 23 species of DAG. Relative to the controls (0.26 ± 0.02 nmol DAG/100 nmol phospholipid), carbachol increased total DAG levels 280% (0.74 ± 0.05 nmol DAG/100 nmol phospholipid) whereas ATP increased levels 180% (0.44 ± 0.01 nmol DAG/100 nmol phospholipid). Neither agonist stimulated the production of new species of DAG or eliminated those found in controls. In either controls or agonist-stimulated cells, ≈ 70% of the total cellular DAG consisted of 18:0-22:6, 16:0-16:1, 18:0-20:4, 18:1-18:1, 16:0-18:1, and 18:0-18:1. Since carbachol was more effective than ATP at stimulating these major species, we conclude that factor(s) other than DAG may contribute to the differences between ATP- and carbachol-induced sensitization of AC. Moreover, our data indicate that the ability of an agonist to increase cellular DAG does not correlate with its ability to stimulate a presumed protein kinase C-mediated process, at least in the absence of compartmentalization.

Q 116 CONVERGENT PATHWAYS FOR THE ACTIVATION OF PHOSPHOLIPASE A_2 IN A MAST CELL LINE: STUDIES WITH PERMEABILIZED RBL-2H3 CELLS, Noriyasu Hirasawa, Francesca Santini and Michael A. Beaven, Laboratory of Chemical Pharmacology, NHLBI, NIH, Bethesda MD 20892

Stimulation of intact RBL-2H3 cells results in rapid induction of phospholipase A_2 (PLA_2) activity and a Ca^{2+}-dependent, protein kinase C-independent release of arachidonic acid (reviewed by Beaven, M.A. and Metzger, H., Immunology Today, 14: 222, 1993). RBL-2H3 cells contained a soluble high molecular weight PLA_2 activity which was retained after permeabilizing cells with streptolysin O. Cells were left unwashed to minimize loss of protein kinase C. Under these conditions, PLA_2 activity, as indicated by release of arachidonic acid from intracellular prelabelled phospholipids, was stimulated by elevation of free Ca^{2+} ($[Ca^{2+}]_i$) to 1 μM or greater (EC_{50}, ~0.8 μM). In the presence of antigen, carbachol (in a subline transfected with the muscarinic m1 receptor) and GTPγS, the requirement for Ca^{2+} was reduced (EC_{50} ~0.2 μM). Release evoked by Ca^{2+} alone was blocked by inhibitors of protein kinase C (Ro31-7549 and staurosporine), whereas the enhancement of release by the other stimulants was not dependent on protein kinase C. Release by all stimulants, including Ca^{2+}, however, was blocked by GDPβS and quercetin. Inhibition was accomplished by concentrations of quercetin (IC_{50} 6 μM) that selectively blocked the activation of MAP kinases in RBL-2H3 cells. These and other data suggested that for all stimulants activation of PLA_2 is dependent on activation of a G protein and MAP kinase(s) but these activations can be achieved by more than one pathway. Interestingly, PLA_2 itself appeared to be fully activated when isolated in soluble form from unstimulated cells and it may, therefore, be negatively regulated in intact unstimulated cells.

Q 117 *Abstract Withdrawn*

Q 118 PHOSPHATIDYLCHOLINE HYDROLYSIS IN NUCLEI ISOLATED FROM IIC9 FIBROBLASTS Matt B. Jarpe and Daniel M. Raben, Department of Physiology, Johns Hopkins University, Baltimore MD 21205

Several important signal transduction events have recently been shown to take place in the nucleus as well as in the plasma membrane. In particular, the generation of diacylglycerol (DAG) and activation of protein kinase C (PKC) have been identified in cells stimulated with the mitogens IGF-1 and a-thrombin. Using molecular species analysis we recently showed that the induced nuclear DAG in a-thrombin stimulated IIC9 cells are derived from phosphatidylcholine (PC) hydrolysis. Here, we have identified a PC hydrolyzing activity in isolated nuclei and have examined its regulation. Nuclei were isolated from quiescent IIC9 cells or from cells stimulated with a high dose of a-thrombin. [^{3}H-choline]-PC in deoxycholate micelles was added to nuclei and incubated at 37°C for 1 hour and the amount of released water-soluble radioactivity was quantified. We found that nuclei isolated from stimulated cells had a higher PC hydrolyzing activity than those from quiescent cells. We will show data further characterizing this activity, including an analysis of both the water-soluble and organic-soluble products to identify the enzyme or enzymes involved. Since some phospholipases, including PC-hydrolyzing phospholipases, are known to be modulated by GTP-binding proteins, we investigated the effect of factors known to activate these proteins. Both GTPgS and AlF_n, are known to activate heterotrimeric G-proteins. In contrast, it is thought that only GTPgS activates small molecular weight GTP-binding proteins. Both of these agents stimulated PC hydrolysis in isolated nuclei. However, GTPgS proved to be more potent at micromolar concentrations of Mg^{2+} than at milimolar Mg^{2+} concentrations, implicating a small molecular weight GTP binding protein. In view of these data, it is possible that AlF_n is acting as a phosphatase inhibitor. Experiments designed to distinguish between these possibilities will also be presented.

Q 119 DISCREET ARACHIDONIC ACID SIGNALLING PATHWAYS FOR TANNIN VERSUS β-GLUCAN STIMULATED RABBIT ALVEOLAR MACROPHAGES, Michael T. Kennedy, Phil J. Bates, Michael S. Rohrbach, Department of Biochemistry and Molecular Biology, Mayo Foundation, Rochester, MN 55905

Arachidonic acid (AA) plays a major role in inflammatory cells as precursor to several inflammatory mediators. Previously, we observed both tannin and β-glucan to be potent agonists for the release of AA from rabbit alveolar macrophages. Furthermore, tannin inhibited the reacylation of exogneous AA, while treatment with β-glucan had no effect on reacylation, suggesting separate pathways for a similar increase in free AA. Experiments were designed to help discern the contributions of deacylation and reacylation to free AA levels within the cell. In the present study, alveolar macrophages were pretreated with the tyrosine phosphatase inhibitor, sodium orthovanadate, and subjected to stimulation by either condensed tannin or β-glucan. Differential effects on AA release were observed for the two agonists. The release of AA generated by β-glucan stimulation was potentiated over all concentrations of vanadate added, while the tannin-induced AA release was abolished. Furthermore, vanadate reverses the ability of tannin to inhibit reacylation. The effect of vanadate on both tannin-stimulated AA release and inhibition of reacylation was maximal between 5μM-50μM. Additional studies using the phospholipase A probe bis-BODIPY-C_{11}-PC indicated that while the known PLA_2 activator β-glucan generated a positive response for phospholipase A, tannin failed to elicit a response. These findings suggest that the observed increase in AA pool size common to stimulation of rabbit alveolar macrophages by tannin or β-glucan proceeds by two different mechanisms.

Q 120 DIFFERENTIAL GENERATION OF DIGLYCERIDES IN HUMAN AIRWAY EPITHELIAL CELLS. Carole M. Liedtke and Mark Kester. Depts. Pediatrics, Medicine, and Physiology & Biophysics, Case Western Reserve University, Cleveland, OH. 44106.

Induction of Cl secretion in the large airways requires the coordinate activation of an apical Cl channel and NaCl(K) cotransport mechanism. NaCl(K) cotransport plays a critical role by supplying Cl from the blood side of the epithelium for secretion. In human tracheal and nasal epithelium, this transporter is quiescent until stimulated by an α_1-adrenergic (AR) mechanism. To test models for α_1-AR signal transduction, we determined the kinetics of inositol lipid and inositol phosphate metabolism in airway epithelial cells grown in in vitro cell culture. Our studies demonstrate a pertussis toxin-sensitive degradation of phosphatidylinositol bisphosphate (PtdIns(4,5)P_2) and generation of inositol trisphosphate (IP_3) in normal and cystic fibrosis (CF) cells. This suggests the activation of PtdIns(4,5) P_2-sensitive phospholipase C that was investigated next by determining the kinetics of diglyceride (DG) metabolism. Total ^{3}H-DG were generated in a biphasic manner with peak levels observed at < 1 min and at 6-8 min. The early DG peak corresponds to the time course of IP_3 generation and suggests a common source of DG. We extended the ^{3}H-DG flux measurements with studies that assessed ^{3}H-DG mass, as DG-^{14}C-acetate of specific DG molecular species. Our results indicate that ether-linked DG species (alkyl and alkenyl, acyl-glycerol) are proportionally more prevalent 1) in CF compared with non-CF cells and 2) at late incubation times (6 min) compared with initial times (40 sec) relative to ester-linked diacyl-species. The role of ether-linked DG species in human airway cells is not known, nor is the significance of the altered CF response understood. However, the ether-linked DG species are thought to be poor cofactors for PKC, an activator of NaCl(K) cotransport. CML is supported by NIH grant HL-43907 and is an Established Investigator of the American Heart Association. MK is supported by NIH grant AR-40225.

Q 121 PHOSPHATIDIC ACID AND DIACYLGLYCEROL SYNERGIZE FOR ACTIVATION OF NEUTROPHIL NADPH OXIDASE IN A CELL-FREE SYSTEM. Linda C. McPhail, Charles E. McCall, David E. Agwu, and Diane Qualliotine-Mann, Departments of Biochemistry and Medicine, Wake Forest University Medical Center, Winston-Salem, NC 27157

Stimulation of human neutrophils (PMN) by receptor agonists, such as fMet-Leu-Phe, activates phospholipase D, resulting in increased levels of phosphatidic acid (PA). 1,2-Diacylglycerol (DG) levels also increase, due to phospholipase C activation and to the conversion of PA to DG by PA phosphohydrolase. The levels of both PA and DG correlate with activation of the PMN enzyme NADPH oxidase. This enzyme is assembled from both cytosolic and membrane components and can be activated in a cell-free system by anionic amphiphiles, such as arachidonic acid and sodium dodecyl sulfate. We reasoned that PA, as an anionic amphiphile, could mediate cell-free activation of NADPH oxidase. Results showed that PA (either short-chain saturated or long-chain unsaturated) was poorly effective, but activation was markedly enhanced by the presence of DG (either short-chain saturated or long-chain unsaturated). LysoPA could replace PA, but short-chain fatty acid metabolites were inactive. Of the major phospholipid classes, only the acidic phosphatidylserine had activity. Metabolites of DG (mono- and triacylglycerols, short-chain fatty acids) were ineffective. Protein kinase catalytic domain inhibitors (staurosporine, chelerythrine chloride, GF-109203X) reduced NADPH oxidase activation by PA + DG with IC_{50}'s similar to those for inhibition of protein kinase C (PKC). However, phorbol myristate acetate did not substitute for DG. Thus, activation of NADPH oxidase by PA and DG may involve PKC, but DG also has a PKC-independent role in the activation mechanism. These results provide a useful cell-free system for studying physiologically relevant second messenger effects of PA and DG and for examining the role of protein kinases as target(s) for these mediators.

Q 122 PEPTIDE-STIMULATED CYCLIC GMP LEVELS IN THE NERVOUS SYSTEM MEDIATED BY A LIPID MESSENGER AND NOT NITRIC OXIDE. David B. Morton and P. Jeanette Simpson, Arizona Research Labs Division of Neurobiology and Department of Biochemistry, University of Arizona, Tucson, AZ 85721.

We are studying the action of a 62 amino acid neuropeptide on the nervous system of an insect, the tobacco hornworm, *Manduca sexta*. This peptide, eclosion hormone (EH) acts on the nervous system to trigger a stereotyped behavior and is mediated by the action of cyclic GMP.

The ability of EH to elevate cyclic GMP levels in the nervous system requires extracellular calcium, is potentiated by phosphodiesterase inhibitors and is lost when the nervous system is homogenized. These results imply that EH stimulates a soluble guanylate cyclase. Blockers of nitric oxide synthase had no effect on the EH-stimulated cyclic GMP levels. By contrast, various blockers of phospholipase-A_2, phospholipase-C, diacylglycerol (DAG) lipase and lipoxygenase were very effective blockers of the cyclic GMP increase. This suggests that a lipid messenger such as arachidonic acid or a lipoxygenase metabolite is released by the action of EH and stimulates the guanylate cyclase.

Recent experiments have been aimed at elucidating the pathways involved in the generation and identity of the lipid messenger and have shown that EH stimulates a production of both inositol (1,4,5) trisphosphate and DAG prior to the elevation of cyclic GMP.

This work was supported by NIH grant NS29740 and an Alfred P. Sloan Research Fellowship.

Q 123 LYSOPHOSPHATIDIC ACID ENHANCES FIBRONECTIN BINDING TO ADHERENT CELLS, Deane F. Mosher, Qinghong Zhang and William J. Checovich, University of Wisconsin, Departments of Medicine and Biomolecular Chemistry, Madison, WI 53706

We have identified lysophosphatidic acid (LPA) as the factor in serum that enhances binding of ^{125}I-labeled fibronectin by MG63 osteosarcoma cells and human fibroblasts in monolayer cultures (Arterioscler Thromb. 1993). Binding in the presence of LPA was up to threefold over control levels. For osteosarcoma cells, LPA was minimally active at 0.1 to 1 ng/mL (0.2 to 2 nmol/L) and reached maximal activity at 10 to 100 ng/mL (20 to 200 nmol/L). Increased binding was evident within 10 min of treatment of cycloheximide-treated cells with LPA and was due to an increase in the number of fibronectin binding sites. LPA also increased the binding of a fragment containing the 70-kDa amino-terminal region of fibronectin that is primarily responsible for the reversible binding of fibronectin to matrix assembly sites on cell surfaces. The LPA effect was sustained for up to 24 hr. Removal of LPA resulted in prompt return of fibronectin binding to baseline levels. Upon readdition of LPA, cells again bound more fibronectin; i.e., there was no evidence of desensitization. An effect on fibronectin binding was not seen when cells were treated with bradykinin, platelet-activating factor, or a thrombin receptor agonist. Enhanced binding correlated with cell shape change and cytoskeletal rearrangement. These results indicate that LPA uniquely modulates the deposition of extracellular fibronectin matrix via its effects on cells.

Q 124 PHORBOL ESTER, ATP AND GTPγS-DEPENDENT PHOSPHOLIPASE D ACTIVATION IN PERMEABILIZED HL-60 CELLS, Nakamura, S., Inoue, H., Shimooku, K., Akisue, T. Nishizuka, Y. Department of Biochemistry, Kobe University School of Medicine, Kobe 650, Japan

Phospholipase D (PLD) is known to be activated by various agonists including some growth factors and 12-O-tetradecanoylphorbol 13-acetate (TPA). HL-60 cells were first metabolically labeled with [^{14}C]lysophosphatidylcholine, and PLD activity was assayed by measuring the formation of [^{14}C]phosphatidylethanol upon cell stimulation in the presence of ethanol and streptolysin O to permeabilize cell membranes. The PLD activity was normally unable to be activated unless GTPγS was added. In the presence of GTPγS (50 μM), TPA (100 nM) caused a profound stimulation (5-fold) of PLD activity. This stimulatory effect of TPA required both ATP (500 μM) and Mg^{2+} and TPA can be replaced by a membrane-permeant diacylglycerol, suggesting a potential role of protein kinase C (PKC) in this activation of PLD. Addition of a PKC inhibitor, either H-7, staurosporine or calphostin C, abolished the PLD activation. A proteolytically generated, catalytically active fragment of PKC (M-kinase) was also able to activate PLD in an ATP-dependent manner in the absence of TPA. The results are consistent with the notion that two signal pathways involving PKC and G-protein may synergistically regulate PLD. A possible mechanism of PLD activation will be presented with a purified preparation of the enzyme.

Q 125 GENERATION OF PHOSPHATIDIC ACID IN SWISS 3T3 CELLS, Lauren Padmore, C. Aylish Wood and George.K.Radda, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, England.

The generation of phosphatidic acid (PtdA) was studied by observing the incorporation of the fatty acid ^{3}H-palmitate into the lipid species PtdA, phosphatidylinositol (PtdIns) and phosphatidylcholine (PtdCho) in Swiss 3T3 fibroblast cells. Bombesin , vasopressin and phorbol 12-myristate 13-acetate (PMA), were found to enhance the incorporation of ^{3}H-palmitate into PtdA. Bombesin, vasopressin and PMA were also able to stimulate an increase in ^{3}H-palmitate PtdIns levels, however, only vasopressin caused an elevation in the level of ^{3}H-palmitate- PtdCho . Importantly, these data suggest that PtdA can be generated via stimulation of the inositol lipid signalling pathways in response to all three agonists. This effect is likely to be mediated through the activation of protein kinase C. Additionally, only vasopressin stimulates PtdCho turnover in a protein kinase C independent manner leading to an additional source of PtdA. The role of phospholipase D in the generation of PtdA was also investigated. Phospholipase D activity can be monitored by the addition of butanol to stimulated cells. In this assay butanol inhibits the production of PtdA and leads to the preferential production of the non-metabolisable product phosphatidylbutanol. Cells stimulated with bombesin and incubated with butanol showed a decrease in the level of ^{3}H-palmitate-PtdA in the presence of butanol which is consistent with the activation of phospholipase D. A decrease in the level of PtdIns was also observed. These data suggest that PtdA may be generated in the cell via protein kinase C dependent and independent pathways that involve the turnover of inositol lipids and phosphatidylcholine.

Q 126 OXIDANT-INDUCED MODULATION OF PHOSPHOLIPID METABOLISM AND SIGNAL TRANSDUCTION IN PULMONARY ARTERY ENDOTHELIAL CELLS, Jawaharlal M. Patel, Yong D. Li, Alan J. Abeles and Edward R. Block, Department of Medicine, University of Florida College of Medicine and Department of Veterans Affairs Medical Center, Gainesville, FL 32608

Nitrogen dioxide (NO_2), an environmental oxidant, is known to alter the structure and composition of the pulmonary artery endothelial cell (PAEC) plasma membrane lipid bilayer. We sought to determine whether NO_2-induced oxidative injury results in modulation of phospholipid metabolism and production of lipid mediators responsible for activation of signal transduction in PAEC. To examine the effect of NO_2 exposure on PAEC, plasma membrane phospholipase C and phospholipase D activities, cellular diacylglycerol (DAG) production, phosphatidylserine (PS) biosynthesis, protein kinase C (PKC) activity, and expression of cellular protein were evaluated. Confluent monolayers of PAEC were exposed to 5 ppm NO_2 or air (control) in 5% CO_2 for 24 hr at 37°C with or without prelabeling with [^{3}H]glycerol. To examine the effect on PS biosynthesis and protein expression, PAEC monolayers were exposed in medium containing [^{14}C]serine or [^{35}S]methionine, respectively. Exposure to 5 ppm NO_2 significantly increased phospholipase C ($p < 0.001$) and phospholipase D ($p < 0.05$) activities in the plasma membranes of PAEC. Phospholipase C and phospholipase D activities in the plasma membranes of control PAEC were 13 ± 3 and 78 ± 10 pmol/min/mg protein, respectively. Exposure to NO_2 also increased ($p < 0.05$) [^{3}H]glycerol labeling of DAG and incorporation of [^{14}C]serine into total cell and plasma membrane PS fractions of PAEC ($p < 0.05$ for both). Similarly, PKC activity in both cytosolic and membrane fractions was significantly ($p < 0.05$) increased in NO_2-exposed cells compared to controls. Two-dimensional gel electrophoresis of cell homogenates indicated that exposure to NO_2 increased the expression of a number of cellular proteins with 20-80 kDa molecular mass. These results demonstrate that NO_2-induced oxidative injury results in increased PS biosynthesis, activation of phospholipases C and D, production of lipid mediators, activation of PKC, and increased expression of proteins in PAEC.

Q 127 INHIBITION OF PHOSPHOLIPASE A2 BLOCKS THE ABILITY OF CAI, A NOVEL CHEMOTHERAPEUTIC AGENT, TO INDUCE IMMEDIATE EARLY GENE EXPRESSION. Karin D. Rodland, Scott McNeil, and Elise C. Kohn*. Department of Cell Biology & Anatomy, Oregon Health Sciences University, Portland OR 97201 and *Signal Transduction & Prevention Unit, TIMS, Laboratory of Pathology, National Cancer Institute, Bethesda, MD 20892.

CAI or carboxyamido-triazole is an antiproliferative and antimetastatic agent which has been demonstrated to alter calcium homeostasis and arachidonic acid metabolism in CHO cells. VL30 is a transformation- and proliferation-associated immediate early gene which we have used successfully as a model system for interacting signal transduction pathways. The Rat-1 derived TK3R cell contains a triple repeat enhancer element from VL30 linked to the chloramphenicol acetyl transferase reporter gene. Acute treatment with CAI synergistically induces CAT expression from this enhancer when used in conjunction with other agonists such as EGF, TPA, cAMP, and the calcium-releasing agent thapsigargin. Chronic exposure to CAI specifically blocks the ability of thapsigargin, a calcium-elevating agent known to act by inhibiting the sequestration of calcium, to induce TK3R-CAT expression in conjunction with either EGF, TPA or cAMP, while the ability of cAMP to interact synergistically with either EGF or TPA is unaffected. Inhibition of phospholipase A2 activity as a result of treatment with either quinacrine or bromphenacyl-bromide blocks the acute induction of VL30 expression in response to CAI and mimics the effect of chronic CAI on calcium-dependent induction of VL30, while the cAMP-dependent pathway is unaffected. Arachidonic acid release in Rat-1 cells is stimulated by elevation of intracellular calcium, suggesting that the known effects of CAI on calcium homeostasis may influence phospholipase A2 activity and downstream signaling events.

Q 128 THE SYNERGISTIC ACTIVATION OF PRESYNAPTIC PKC BY DIACYLGLYCEROL AND UNSATURATED FATTY ACIDS POTENTIATES GLUTAMATE RELEASE. José Sánchez-Prieto, Inmaculada Herrero, Elena Vázquez and María Teresa Miras-Portugal, Departamento de Bioquímica, Facultad de Veterinaria, Universidad Complutense, Madrid 28040, Spain.
Protein Kinase C, PKC, is present in the brain and relatively abundant in the nerve endings, where the release of neurotransmitters occurs. In isolated nerve terminals, "synaptosomes", the activation of protein kinase C with relatively high concentrations of phorbol esters (100nM) enhances the exocytotic release of the neurotransmitter glutamate a process known to be trigered by a localized entry of Ca^{2+} through voltage-sensitive Ca^{2+}-channels. However, the sensitivity of glutamate release to potentiation by phorbol esters is dramatically increased in the presence of 2μM arachidonic acid, (AA). This facilitatory effect by phorbol esters on glutamate release is also observed in the presence of the agonist of the metabotropic glutamate receptor 1S,3R ACPD but only in the presence of arachidonic acid. The activation of the metabotropic receptor with ACPD is coupled to a rapid and transient generation of diacylglycerol, (DAG), and this response is not altered by the presence of arachidonic acid, indicating that the action of the fatty acid is somewhere down stream of the metabotropic receptor-G protein-effector complex. In phosphorylation experiments of the PKC-substrate MARCKS, we have found a significant increase in the phosphorylation of the protein only with the combination ACPD+AA but not in the presence of either agent alone, suggesting that the role of arachidonic acid is to sensitize PKC to the DAG generated by the metabotropic receptor. Consistent the involvement of the synergistic activation of PKC oleic acid but not stearic is also effective in facilitating the release of glutamate. In conclusion, the activation of the presynaptic PKC that control glutamate exocytosis requires two coincident signals: the agonist ACPD (glutamate), acting on a presynaptic receptor and generating DAG, and arachidonic acid which primes PKC to the generated DAG. When both signals are present PKC inhibits K^+-channels increasing the duration of the action potentials with the subsequent increase in both Ca^{2+}-entry and glutamate release.

Q 129 DIACYGLYCEROL METABOLISM: SIGNAL TERMINATION IN VASCULAR SMOOTH MUSCLE, David L. Severson and Mark W. Lee, MRC Signal Transduction Group, The University of Calgary, Calgary, AB, T2N 4N1, Canada
Diacylglycerols (DAG) derived from phospholipid turnover are intracellular second messengers that activate protein kinase C. Metabolism of DAG will, therefore, be a prerequisite for signal termination. The metabolism of an exogenous long-chain DAG, 1-palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycerol (2-[^{14}C]POG), has been determined with cultured A10 smooth muscle cells that were permeabilized by pretreatment with 340 U/ml *Staphylococcus aureus* α-toxin. Incubation of permeabilized A10 cells with 2-[^{14}C]POG primarily resulted in the incorporation of radioactivity into products of a lipase pathway (monoacylglycerol and fatty acid); very little formation of radiolabelled triacylglycerol or phospholipid was detected. The hydrolysis of exogenous 2-[^{14}C]POG was inhibited by DAG lipase inhibitors, tetrahydrolipstatin and U-57,908. The predominant contribution of the lipase pathway to the metabolism of a long-chain DAG is similar to previous results obtained with the cell permeable DAG analogue, dioctanoylglycerol (diC8). A DAG lipase in a soluble subcellular fraction from bovine aorta has been partially purified by ammonium sulphate precipitation in the presence of 5% Triton X-100, followed by chromatography on DEAE Sephacel, heparin Sepharose and octyl Sepharose in the presence of either CHAPS or Triton X-100 detergents. This procedure resulted in a 155-fold increase in enzyme specific activity, determined from the hydrolysis of the *sn*-1 position of a 1-[^{14}C]-palmitoyl-2-oleoyl-*sn*-glycerol (1-[^{14}C]POG) substrate in the presence of 0.1% Triton X-100. The partially-purified lipase preparation also hydrolysed diC8, and diC8 was a competitive inhibitor of 1-[^{14}C]POG hydrolysis. Monoacylglycerol lipase, DAG kinase and cholesterol esterase activities could not be detected.

Q 130 CYCLIC MEMBRANE LIPID PROPORTION CHANGES INDICATE SIGNALING ACTIVITY DURING SEA URCHIN ACTIVATION AND CLEAVAGE. Mary Lee Sparling, Department of Biology, CSU Northridge, CA, 91330.
Lipid proportion changes in density gradient fractions M1(most dense)-M4 during early development indicate phospholipase activation signaling or resulting membrane or microvilli cycling or changes at cell division. Dramatic relative decrease from unfertized PC, PI is detected at 15min postfert but products of PLipase-A1,-A2, or -C (DAG, LPC, FA, and PA) don't increase as expected; PLC products DG and interconvertable PA show minor increases only in 15min M3, increasing again only after cleavages 1 and 2 when both PC and PI again decrease. DG-TG present in unfert M2 remains until 30m when PA and FA increase. Later between 2 and 4cell there is dramatic decrease in PC and gain of DG-TG and PA in M4. Signal pathway lipids (total of PC, PI, products LPC, DG, FA, PA) decrease in all M1-M4 fractions at 15m and at 1hr 40m (after cleavage 1) indicating production of other lipids by PLD or diacylglycerol phosphotranferase. Activated PLD base exchange converting PE or PC to PS or SPH would explain changes in fert 15m M1 and M4 and A23187-activated 15m M2 and 30m M3, and later increased SPH in M1 and at each cleavage 1,2,4; in M2 at clv 2; in M3 at clv 4; M4 dynamic throughout; or PS in M1 at clv 1-3. High SPH inhibits PLA and PKC-activated PLD, base exchange would cease, no more SPH, then cycle could start again; PLA no longer inhibited, break down PC,PE yielding arachidonic acid (FA) and LPC or LPE which are signals for activation of some PKCs, precursors to other signals or can affect membrane characteristics. Artificially activated (A23187, NH4) eggs show outstanding differences from normal fertilized eggs suggesting that normal phospholipase activities and signal lipid production or membrane cycling resulting or necessary for it is not activated merely by Ca++ release or pH elevation. (NIH-GMS-1R15GM38459-01)

Q 131 cGMP MODULATES THE Ca^{+2} RISE IN RESPONSE TO ATP AND BRADYKININ IN ADRENOMEDULLARY ENDOTHELIAL CELLS. Magdalena Torres, Fernando Rodriguez-Pascual and María Teresa Miras-Portugal. Departamento de Bioquímica, Facultad de Veterinaria, Universidad Complutense, Madrid 28040, Spain.
A number of extracellular signals release Ca^{2+} from intracellular through the generation of the second messenger inositol trisphosphate, IP_3. ATP acting on P_{2y} receptors as well as bradykinin an histamine have been shown to activate the PLC/IP_3 pathway in adrenomedullary endothelial cells. In these cells ATP and bradykinin also activate nitric oxide synthase with the subsequent increase in the cGMP levels. Since cGMP is also able to alter the Ca^{2+} accumulation by internal stores we were interested to study the contribution of cGMP to the mobilization of Ca^{2+} observed in response to bradykinin and ATP. In this study fura-2 was used to monitor changes in intracellular Ca^{2+} concentration evoked by vasoactive substances as well as the effects of both endogenous cGMP and exogenous 8-Br-cGMP in this response. Adrenomedullary endothelial cells, grown to confluence as monolayers, respond to ATP and bradykinin with transient increases in intracellular calcium concentration of 230$\pm$6.9 nM and 85.4$\pm$ 5.4 nM, respectively. The origin of this calcium was intracellular because similar increases were obtained in the absence of extracellular calcium. Preincubation with the permeable analoge of cGMP, (8-Br-cGMP), significantly increased the peak level of the transient rise in $[Ca^{2+}]_i$ evoked by ATP and bradykinin to 130-140% with respect to the control values. Interestingly, the specific inhibitor of the cGMP-dependent protein kinase, H-8, reduced the rise in $[Ca^{2+}]_i$ evoked by ATP. These results suggest that the endogenous cGMP generated upon ATP receptor activation can also contribute to the changes observed in the $[Ca^{2+}]_i$, through the activation of cGMP-dependent protein kinases.

Q 132 CONTROL OF PHOSPHOLIPASE D ACTIVITY IN STIMULATED SWISS 3T3 CELLS BY BOTH PROTEIN KINASE C AND TYROSINE KINASE ACTIVITIES.

Michael J.O. Wakelam, Michael Cross and Celia P. Briscoe, University of Birmingham Institute of Cancer Studies, Birmingham B15 2TT, United Kingdom.

Phospholipase D (PLD) activity is stimulated in cells in response to a range of hormones and growth factors. Most, but not all, of these agonists also stimulate the PLC catalysed hydrolysis of $PtdInsP_2$ thus suggesting a functional link between the two phospholipases. In particular, the activation of PLD by protein kinase C (PKC) stimulation with phorbol esters is a widely reported event, as is the inhibition of agonist-stimulated PLD activity by a range of PKC inhibitors. The use of PKC inhibitors has, however generated conflicting, indeed confusing data. In cell types where the more selective inhibitors (e.g. Ro-312-8220) have been used incomplete inhibition has been found with, for example in bombesin-stimulated Swiss 3T3 cells, a maximum of 70% being achieved. In fMet-Leu-Phe-stimulated neutrophils a range of tyrosine kinase inhibitors inhibited PLD activation whilst PKC inhibition had a minimal effect. This suggests control of the phospholipase by cytosolic tyrosine kinase activity in addition to PKC.

Bombesin stimulated protein tyrosine kinase activity in Swiss 3T3 cells with a range of phosphorylated proteins being rapidly detected by Western blotting . The tyrosine phosphorylation could be inhibited by various tyrosine kinase inhibitors, in particular Genistein. This tyrosine kinase inhibitor only had a small inhibitory effect upon bombesin-stimulated PLD activity, however when added to the cells together with Ro-31-8220 complete inhibition of stimulated PLD activity was observed. Thus activation of PLD is under the control of both PKC and tyrosine kinases with the relative importance of each kinase being both agonist and cell type dependent. Whether these results suggest that PLD is directly activated by each of these kinases or that a phospholipase regulatory protein, which is under multi-factorial control exists, remains to be elucidated.

Q 133 G PROTEIN COUPLING TO THE THROMBIN RECEPTOR IN CCL39 FIBROBLASTS, John R. Williamsom, Li-Jun Yang and George Baffy, Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, PA 19104

The specific involvement of G proteins in thrombin receptor-mediated signal transduction processes has been studied using Chinese hamster lung fibroblasts (CCL39 cells) activated by the synthetic hexapeptide SFLLRN. This peptide elicits cellular responses in CCL39 cells that are similar to those induced by α-thrombin. SFLLRN-induced Ca^{2+} mobilization was measured in single CCL39 cells by fura-2 fluorometry, while DNA synthesis induced by SFLLRN was also assessed at the single cell level by measuring bromodeoxyuridine (BrdU) incorporation into DNA by immunocytochemistry. Immunoblots performed with antibodies directed against the COOH terminus of G protein α subunits revealed that α_q, α_i and α_o were all present in approximately equal amounts in CCL39 cells. Microinjection of anti-α_q and anti-α_o antibodies separately into CCL39 cells, 40 to 60 min before stimulation with 50 μM SFLLRN, caused a partial inhibition of the SFLLRN-induced Ca^{2+} response. In these cells microinjected with anti-α_q- and anti-α_o-antibodies, the lag time between exposure of the cells to SFLLRN and the onset of Ca^{2+} mobilization was significantly longer (20±4 s and 24±11 s, respectively) than in control cells microinjected with preimmune serum (9±1 s). Moreover, the peak height of the Ca^{2+} response to SFLLRN was decreased by 36% and 73%, respectively in cells microinjected with anti-α_q- and anti-α_o-antibodies. SFLLRN-induced DNA synthesis in growth-arrested CCL39 cells was also inhibited (44-78%) by prior microinjection of anti-α_q or anti-α_o antibodies. Anti-α_i antibodies had no effect on the SFLLRN-induced Ca^{2+} response or on DNA synthesis. These results provide direct evidence that thrombin receptor-mediated Ca^{2+} mobilization and mitogenesis are coupled in CCL39 cells to two different types of G proteins, namely G_q and G_o.

Q 134 THE EFFECT OF PHOSPHATIDIC ACID ON THE PROLIFERATION OF SWISS 3T3 CELLS.

C.Aylish Wood, Lauren Padmore and George K.Radda, Department of Biochemistry, University of Oxford, South Parks Road, Oxford, OX1 3QU. U.K.

Swiss 3T3 cells, pulse labelled with ^{3}H-palmitate, when stimulated with bombesin, vasopressin and phorbol 12-myristate 13-acetate (PMA) gave rise to ^{3}H-palmitate-phosphatidic acid (PtdA). The pathways for generation of PtdA involve the turnover of both inositol lipids and phosphatidylcholine. In order to investigate whether PtdA acts as a putative second messenger we have investigated the effect of PtdA-dipalmitate on the proliferation of Swiss 3T3 cells. Proliferation was assayed by measuring the uptake of ^{3}H-thymidine into the acid precipitable material of Swiss 3T3 cells. As a comparison the effect of PMA on proliferation was also studied. PMA (10 nM) caused a 7-fold increase in ^{3}H-thymidine incorporation; PtdA-dipalmitate (5 ng/ml) caused a 1.5-fold increase in ^{3}H-thymidine incorporation. When PMA (10 nM) and PtdA-dipalmitate (5 ng/ml) were added together ^{3}H-thymidine incorporation was increased to 10-fold. Down regulation of protein kinase C (PKC) by treatment of the cells for 48 hrs with 400 nM PMA resulted in the abolition of both the PMA and PtdA-dipalmitate induced elevations of ^{3}H-thymidine incorporation. These data suggest that PtdA can evoke a small increase in proliferation that is mediated through PKC dependent pathways. Additionally the observation that PMA and PtdA can both increase proliferation may be important in assessing the relative contributions of phospholipase D and phospholipase C derived second messengers to the proliferative effect of mitogens.

Q 135 ROLES OF SH2/SH3 DOMAINS OF PLC-γ1 IN EGF-INDUCED ACTIVATION AND TRANSLOCATION OF PLC-γ1 TO THE CYTOSKELETON IN HEPATOCYTES, Li-Jun Yang and John R. Williamson, Dept. of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, PA 19104

The signal pathway for activation of phospholipase C-γ1 (PLC-γ1) in hepatocytes by epidermal growth factor (EGF) is unique because it requires a pertussis toxin (PT)-sensitive Gi-protein, as we previously described. However, the details of the relationship between the structure and function of PLC-γ1 are still unknown. In this study, we have examined the relationship between EGF-induced tyrosine phosphorylation of PLC-γ1 and its translocation to the cytoskeleton (CSK) in rat hepatocytes. We found that translocation of PLC-γ1 was specific for EGF stimulation not for insulin or vasopressin. EGF caused a transient increase of PLC activity in the CSK which could be abolished by immunoprecipitating PLC-γ1. Tyrosine phosphorylated PLC-γ1 was seen only in the CSK fraction and was prevented by genistein, suggesting that tyrosine phosphorylation is required for PLC-γ1 translocation. Interestingly, actin was coimmunoprecipitated with PLC-γ1 from the CSK but not from the soluble fraction in response to EGF stimulation. EGF-induced association of PLC-γ1 with the CSK was not inhibited by PT, but Giα was translocated in an EGF-dependent manner, suggesting that the interaction of PLC-γ1 with Giα is downstream from both PLC-γ1 tyrosine phos-phorylation and its binding to the CSK. We have also investigated the molecular mechanism underlying PLC-γ1 translocation and association with the CSK and Gi-protein using the SH2 and SH3 domains of PLC-γ fusion proteins. We found that several tyrosine phosphorylated proteins were brought down in an EGF-dependent manner by SH2 domains of PLC-γ1 from the CSK. The possible roles of the SH3 domain of PLC-γ1 in its translocation to the CSK and association with Gi-protein are in progress. Supported by NIH grants DK-15120 and DK07314.

Inositol Lipid Kinases; G-Proteins; Acyl Transferases Phosphatases; Phospholipid Turnovers

Q 200 GENERATION OF DIGLYCERIDE (DG) SPECIES BY CULTURED HUMAN TRACHEAL EPITHELIAL (HTE) CELLS INCUBATED WITH 15-HETE. Stephen E. Alpert, Ronald W. Walenga and Mark Kester, Departments of Pediatrics and Medicine, Case Western Reserve University, Cleveland, OH 44106
Primary cultured HTE cells selectively incorporate their predominant lipoxygenase product, 15-HETE, into the sn-2 position of phosphatidylinositol (PI) (Alpert and Walenga. Am J Respir Cell Mol Biol 1993;8:273). Here we assessed whether 15-HETE-PI can serve as a substrate for phospholipase C (PLC) to generate monohydroxy-substituted DG species. HTE cells appear to express a receptor for platelet-activating factor (PAF) (Carl and Walenga. Am Rev Respir Dis 1993;147:A449) which may be coupled to PLC and/or PLA_2 as in other cells. Thus, HTE monolayers incubated with 0.25-1.0 μM ^{3}H- or ^{14}C-15-HETE for 1-2 hours were stimulated with PAF (10^{-6} to 10^{-8}M) for 2-10 minutes. The distribution of radiolabel within individual phospholipids and in monoglycerides (MG), DG and triglycerides (TG) was then assessed by separate thin layer chromatography (TLC) systems. PAF mobilized radiolabel specifically from PI in a dose-dependent manner, but release of free 15-HETE was not detected, suggesting a PLC- rather than a PLA_2-mediated PAF stimulation mechanism. At the limits of TLC resolution, redistribution of ^{14}C-radiolabel into MG, DG or TG species was not readily apparent. The putative monohydroxy-substituted DG species migrated slower than authentic 1,2- and 1,3-diacylglycerol standards, consistent with the inclusion of a more polar hydroxyl group of 15-HETE. *In vitro* incubation of isolated ^{14}C-15-HETE-PI with PLC (*B. cerius*) generated a similar pattern of radiolabeled products. If these monohydroxy-substituted DG species have an altered interaction with protein kinase C, or modulate the interaction of other DG species with that enzyme, 15-HETE might thereby modulate HTE cell signal transduction processes.

Q 201 **Phosphatidylinositol 3-kinase catalysis at a lipid/water interface.** Barnett, S.F., Miles L., Stirdivant S., Ahern J., Oliff A. and Heimbrook D. Department of Cancer Research, Merck Research Laboratories, West Point, PA 19486.

Phosphatidylinositol 3-kinase (PI3K) is a heterodimer composed of an 85 kda SH2 domain containing regulatory subunit and a 110 kDa catalytic subunit. PI3K is implicated in src, abl and middle t oncogenic transformation and tyrosine kinase receptor (TKR) mediated mitogenic signaling. The evidence for PI3K involvement in these processes is correlative. PI3K binds to specific tyrosine phosphorylated proteins through SH2 domains and PI3K products are elevated following growth factor stimulation of TKRs and transformation. Our primary interest is in determining if PI3K enzyme activity is necessary for oncogenic transformation and, ultimately, if PI3K is a suitable target for therapeutic intervention. One way to do this is to identify potent, specific and cell permeable small molecule inhibitors of PI3K. Towards this end, we developed a well-characterized assay in which interfacial K_m and K_I values could be determined. Enzymes which work at lipid/water interfaces present special kinetic problems. These enzymes have a binding constant for the interface which is separate and distinct from the interfacial catalytic rate constants. Any added compound or altered reaction condition can effect this binding constant and make interpretation of kinetic experiments impossible. We characterized a system in which PI3K is tightly bound to the lipid/water interface using a previously described approach (Berg et al. *Biochemistry* **1991**, 30, 7283-7297). When PI3K is tightly bound to the interface, the rate of product formation plateaus even though only a small fraction of the lipid substrate has been utilized. Product formation resumes following vesicle fusion and substrate replenishment. If PI3K/vesicle ratios are kept low enough, initial velocities do not increase with increasing vesicle concentration. Initial velocities do increase when the interfacial concentration of substrate increases. The maximum amount of substrate converted is greater when using large vesicles. We have used this system to investigate the effect of phospholipids, detergents and small organic molecules on PI3K activity.

Q 202 ADP-RIBOSYLATION FACTOR, A SMALL GTP-DEPENDENT REGULATORY PROTEIN, STIMULATES PHOSPHOLIPASE D ACTIVITY IN CELL-FREE ASSAYS, H. Alex Brown and Paul C. Sternweis, Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, TX 75235-9041.
The hydrolysis of phosphatidylcholine by phospholipase D (PLD) results in the production of phosphatidic acid and choline. An assay which uses an exogenous substrate was developed to measure this activity in either membranes or a solubilized and chromatographically enriched preparation. The PLD activity was extracted from HL-60 promyelocytic leukemia cells and may be extracted with either 1M NaCl or 1% octyl glucoside. Optimal activity is obtained with vesicles containing phosphatidylethanolamine, phosphatidylcholine, and phosphatidylinositol-4,5-bisphosphate. A cytosolic factor markedly enhanced PLD activity in membranes and was essential for GTPγS-dependent stimulation of an enriched preparation of PLD. The factor was purified to homogeneity from cytosol of bovine brain and identified as a member of the ARF subfamily of small GTP-dependent regulatory proteins. ARF proteins were originally identified as factors for facilitating covalent modification of Gsα proteins by cholera toxin and have been implicated more recently as factors involved in vesicle trafficking. The current finding suggests that PLD activity plays a prominent role in the action of ARF and that ARF may be a key component in the generation of second messengers via phospholipase D.

Q 203 REGULATION OF PHOSPHATIDATE PHOSPHATASE ACTIVITY BY ADENOSINE AND CYTIDINE NUCLEOTIDES, George M. Carman and Wen-I Wu, Department of Food Science, Rutgers University, New Brunswick, N.J. 08903

Phosphatidate (PA) phosphatase catalyzes the dephosphorylation of PA yielding diacylglycerol and P_i. The diacylglycerol derived from this reaction is used for the synthesis of triacylglycerol and the phospholipids phosphatidylethanolamine and phosphatidylcholine. In addition to its role in lipid synthesis, PA phosphatase plays a role in the phosphatidylcholine signaling pathway. The activity of the enzyme controls PA (a mitogen) and diacylglycerol (activator of protein kinase C) levels. Using purified PA phosphatase from the yeast *Saccharomyces cerevisiae*, we examined the effects of adenosine and cytidine nucleotides on PA phosphatase activity. The di- and triphosphorylated derivatives of adenosine and cytidine inhibited PA phosphatase activity in a dose-dependent manner. However, the monophosphorylated derivatives of adenosine and cytidine had no effect on activity. The most potent inhibitors of PA phosphatase activity were ATP and CTP. A detailed kinetic analysis was performed using Triton X-100/PA-mixed micelles to determine the mechanism of PA phosphatase inhibition by ATP and CTP. The PA phosphatase dependence on PA was cooperative (Hill numbers of 2) in the absence and presence of ATP and CTP. Appropriate replots of the data showed that ATP and CTP were competitive inhibitors with respect to PA. However, the competitive inhibition of the enzyme was not linear indicating a complex mechanism of inhibition by ATP and CTP. The inhibition of PA phosphatase activity by ATP and CTP was overcome by increasing concentrations of magnesium ions. PA phosphatase dependence on magnesium ions followed saturation kinetics in the absence and presence of ATP and CTP. ATP and CTP were nonlinear competitive inhibitors of PA phosphatase activity with respect to magnesium ions. The apparent inhibitor constants for ATP and CTP were in the cellular range for these nucleotides. Thus the inhibition of PA phosphatase may be regulated *in vivo* by ATP and CTP.

Q 204 DIACYLGLYCEROL AND PHOSPHATIDIC ACID METABOLISM IN A MURINE STEM CELL RESPONSIVE TO ERYTHROPOIETIN

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The metabolic source and fate of cellular 1, 2 diacylglycerol (DG) and phosphatidic acid (PA) in murine hematopoietic stem cells responsive to erythropoietin (EPO) has been analyzed. B6USt. Ep cells were found to exhibit both early and late changes in DG and PA accumulation as measured by high performance liquid chromatography analysis (HPLC), followed by refractive index detection and thin layer chromatography (TLC). DG levels peaked at 5 seconds with a 28.1% increase compared to control levels (17.3 to 22.2 pmoles/10^6 cells) with a later peak at 30 minutes (84.2% increase of 17.3 to 31.9 pmoles). These changes were concentration-dependent from 0.025 to 10 units/ml EPO. In addition PA increased 652.3% compared to control (8.6 to 64.7 ug/10^6 cells) with an early peak at 20 sec. as measured by HPLC. G protein regulation was investigated by studying the effects of the non-hydrolyzable GTP analogue guanosine 5'-0 (3-thiotriphosphate; GTPgamma S on PA synthe-sis. The addition of GTPgamma S (10 uM) increased PA from 6.3 ug to 48.6 ug per million cells. In the presence of EPO and GTPgamma S, PA levels were 64.8 ug. An antagonist of G proteins, GDPBS had no effect on control levels of PA (5.9 ug/10^6 cells) but blocked EPO's effect on PA (30.0 ug/10^6 cells). Thus EPO stimulates generation of both lipid second messengers DG and PA. Some of these responses are rapid and transient, while others are slower and sustained. Our data demonstrate DG kinetics to be biphasic as observed with high concentration of EPO or mono-phasic as observed with low concentrations of EPO or high concentrations of EPO added to cultures which had been pretreated with chymotrypsin. The PA accumulation preceeding that of DG suggests PA was not derived from DG. This is confirmed by the stimulation of PA (without ATP) of GTP gamma S, effectively excluding phosphorylation of DG by DG kinase in the formation of PA, and possibly involving a G protein coupled to phospholipase D.

Q 205 SUBCELLULAR LOCALISATION OF THE CoA INDEPENDENT TRANSACYLASE AND OF NEWLY SYNTHESIZED ETHER PHOSPHOLIPIDS IN HUMAN NEUTROPHILS. O. Colard(a), M. Breton(a), G. Ribbes(b), M. Record(b), A. Cane(a), G. Bereziat(a), H. Chap(b). (a), CNRS URA 1283, CHU Saint Antoine, Paris, France and (b), INSERM U 326, CHR Purpan, Toulouse, France.

Transacylases are involved in two major cellular processes of lipid metabolism : first, in arachidonate movement between phospholipids (from choline to ethanolamine phospholipids and from diacyl to ether phospolipids) thus contributing to the control of eicosanoids production, and second in PAF production, by providing the lyso PAF in the remodeling pathway. In this paper, we investigated the subcellular localisation of transacylases and the newly synthesized ether phospholipids, using a fractionation procedure which allows the separation of plasma membranes from endoplasmic reticulum and from the bulk of granules, after nitrogen cavitation of neutrophils.

The CoA-independent transacylase as measured by in vitro [3H]alkyl-lyso-GPC acylation was only located in the endoplasmic reticulum in resting as well as in stimulated cells. However, at 15 min following cell labeling with [3H]alkyl lyso-GPC, one third of the [3H]alkyl-arachidonyl-GPC was recovered in the plasma membrane indicating a rapid redistribution of the compound from its site of synthesis.

We also observed that every enzyme involved in arachidonate incorporation into phospholipids (ATP dependent acyltransferase and CoA-dependent as well as independent transacylases) was located in the endoplasmic reticulum. Following one hour chase with [3H]arachidonate (AA), the labeling of phospholipids indicated that the redistribution to the plasma membrane was identical for every subclass of ethanolamine phospholipid. In contrast, the ratio of alkyl-[3H]AA-GPC over acyl [3H]AA-GPC was higher in the endoplasmic reticulum than in the plasma membrane.

These results are consistent with the production of alkyl-lyso GPC by transacylase leading to PAF in the endoplasmic reticulum. The rapid redistribution also allows phospholipids to be substrate for phospholipases acting in the plasma membrane.

Q 206 MAPPING THE SITES OF $p34^{cdc2}$ KINASE AND CASEIN KINASE II PHOSPHORYLATION ON CTP: PHOSPHOCHOLINE CYTIDYLYLTRANSFERASE. R. B. Cornell, G. B. Kalmar, R.J. Kay*, S.L. Pelech', & J. S. Sanghera'. Institute of Molecular Biology, Simon Fraser University; & Departments of Medical Genetics* and Medicine', University of British Columbia, Vancouver, B.C., Canada.

Cytidylyltransferase (CT) is a key regulatory enzyme involved in the control of the phosphatidylcholine metabolic cycle. It is regulated by translocation between an inactive soluble form, stabilized by phosphorylation, and an active membrane-bound form, stabilized by anionic lipids or diacylglycerol. Wild-type rat liver CT and five CT mutants lacking segments of the C-terminal domain were over-expressed 50-fold in COS cells. Four of the mutants were truncations after residues 228, 312, 338, and 349 (wild-type has 367 residues). The fifth mutant was a substitution of ser 362 for ala. The specific activity of the Δ228 mutant was decreased 80-90% and was not influenced by lipids, but the specific activity of the other mutants was the same as wild-type, and was lipid dependent. *In vitro* phosphorylation of COS cell extracts by endogenous kinases showed that CT was a major phosphorylated protein. Although wild-type CT was phosphorylated by the endogenous kinases, none of the mutants were phosphorylated, including the ser362 →ala mutant. The endogenous kinases actively phosphorylated casein, were inhibited by heparin, and could be removed from the extract by heparin-Sepharose chromatography. These findings suggested that casein kinase II was the major endogenous kinase activity. *In vitro* phosphorylation of CT catalyzed by sea star $p34^{cdc2}$ kinase retarded the electrophoretic mobility of ~50% of the CT protein. The CT activity was inhibited by cdc2 catalyzed phosphorylation when the assay was carried out in the absence of lipids. In the presence of 0.1 mM PC-oleic acid (1/1) vesicles there was no inhibition of CT activity by the kinase. Phosphorylation by casein kinase II did not affect CT activity. Casein kinase II phosphorylated only the wild-type CT. Substitution of ser 362 →ala resulted in loss of phosphorylation by casein kinase II, implicating this site as the casein kinase II site on CT. Ser 362→ala, Δ349, and Δ 338 mutants were substrates for $p34^{cdc2}$ kinase, but Δ 312 and Δ228 were not. The latter two mutants are missing a set of three ser-pro rich motifs, implicating these as the cdc2 sites on CT.

Q 207 TRYOSINE KINASE MEDIATED MACROPHAGE ACTIVATION: EFFECTS OF PERVANADATE AND ZYMOSAN ON MOUSE PERITONEAL MACROPHAGES,

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Cells of the monocyte/macrophage (MΦ) lineage are able to respond to a variety of stimuli with the extracellular release of products that can modify the activities of other cells in their proximity. As such, the MΦ plays a role central to host defense and contributes to various aspects of the inflammatory response. We demonstrate that the application of pervanadate to resident peritoneal mouse macrophages engenders the cellular response and release of products similar to those stimulated by inflammatory substances such as zymosan. Pervanadate activated phospholipase D, quantified with the generation of phosphatidylethanol, phospholipase C, quantified by the generation of inositol phosphates, and phospholipase A_2 as evidenced by the release of arachidonic acid and its oxygenated metabolites, prostaglandin E_2 and leukotriene C_4. In addition, pervanadate stimulated lysosomal enzyme release but had minimal effects on the release of Tumor Necrosis Factor. The responses stimulated by pervanadate were paralleled by the intracellular accumulation of proteins phosphorylated on tyrosine. A similar accumulation of phosphotyrosyl proteins was seen with zymosan stimulation. In both cases, this was due to the activation of phosphorylation rather than inhibition of phosphotyrosine phosphatases. These data suggest that the activation of tyrosine kinases is a critical step in the activation of the MΦ

Q 208 SHORT-TERM REGULATION OF NUCLEOSIDE TRANSPORT IN CHROMAFFIN CELLS. INVOLVEMENT OF PROTEIN KINASES A AND C. Delicado, E.G., Sen, R.P., Casillas, T. and Miras-Portugal, M.T. Departamento de Bioquímica. Facultad de Veterinaria. Universidad Complutense. 28040 Madrid. Spain.

ATP is co-estored with several neurotransmitters such as acetylcholine, catecholamines in the neurosecretory vesicles and is released to the extracellular space during the exocytotic process. This nucleotide is degraded to adenosine by the action of ectonucleotidases. Extracellular adenosine is transported into the cells and then incorporated into the nucleotide pools. Adrenal chromaffin cells have been proved to be a good model to study the exocytotic process. The transport of adenosine has been extensively characterized in these neural cells. The nucleoside transporter present a high affinity (K_m = 1 μM) for adenosine and is highly sensitive to NBTI inhibition. Secretagogues, such as carbachol and nicotine, which are known to activate the protein kinase C (PKC) in these cells, inhibited the adenosine transport. This inhibition corresponding with a decrease in the transport capacity (V_{max}) without causing apparent changes in the transporter affinity. The activation of protein kinase C with phorbol esters also inhibited the adenosine transport. It has been also demonstrated that bradykinin and P_{2y} agonists acting through purinergic receptors were also able to modulate the adenosine transport. This inhibition being also due to the activation of PKC. In the same model, the activation of protein kinase A by forskolin or chlorophenyl cyclic AMP decreased the adenosine transport. The inhibition of adenosine transport by the activation of the protein kinases A and C runs in parallel with a decrease in the number of adenosine transporters present in the plasma membrane of chromaffin cells. These results suggest the possibility of a molecular modification of the transporter. The treatment of plasma membrane preparation from chromaffin cells with purified protein kinase A or protein kinase C significantly decreased the number of nucleoside transporters (50%). These results support the hypothesis that a chemical modification of transporters occurs by phosphorylation through these kinases.

Q 209 THE CHARACTERISATION OF THE STEAROYL, ARACHIDONYL SELECTIVE DIACYLGLYCEROL KINASE IN THE PARTICULATE FRACTION OF PORCINE TESTES. MATTHEW HODGKIN, SANDRA GARDNER* AND MICHAEL WAKELAM.
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Diacylglycerol (DAG) is a phospholipid-derived second messenger whose intracellular concentration increases rapidly in response to a variety of agonists. The production of DAG is both rapid and sustained and, in certain cases, may remain above unstimulated levels for up to 2 hours. However chemical analysis of DAG structures, produced in cells in response to a single agonist, has shown that there are several molecular species of DAG. By comparing the fatty acid composition of these species with parent phospholipids, it is clear that the total intracellular DAG may be derived from several sources including receptor mediated hydrolysis of both PtdIns and PtdCho. It is these DAG species that charaterisitcally contain unsaturated fatty acids at the two position. Since it has been shown that the signalling function of DAG is to activate protein kinase C the understanding of the regulation of the metabolism of this membrane bound messenger is important.

A major route by which membrane bound DAG is removed from the cell is through enzyme catalysed phosphorylation to phosphatidic acid. This reaction could be catalysed by a cytosolic 80kDa enzyme that has been purified and cloned. However, in a tissue such as testes, cytosolic DAG kinase activity is less than 10% of membrane activity. Thus, here we report the characterisation of the membrane bound and predominant DAG kinase from the particulate fraction of porcine testes. We show that this enzyme is highly selective for 1-stearoyl, 2-arachidonyl glycerol (SAG) and further, preliminary results indicate that it is the unsaturated nature of fatty acid at the two position of the DAG species that may direct subsequent phosphorylation. These DAG species are derived from PtdIns and PtdCho. The enzyme can be partially purified approximately 10 fold by hydroxylapatite chromatography and this preparation exhibits Michaelis Menton kinetics with respect to both SAG and MgATP, with Kms of approximately 0.5 and 0.4 mM respectively.

Q 210 HERBIMYCIN-A BLOCKS THE THROMBIN STIMULATED CYTOSKELETAL ENZYME ACTIVITY OF $pp60^{c\text{-}src}$ AND PHOSPHATIDYLINOSITOL 3-KINASE, WITHOUT AFFECTING PLATELET SHAPE CHANGE, AGGREGATION AND RELEASE. Shaun P. Jackson,* Simone M. Schoenwaelder* and Christina A. Mitchell. Dept. of Medicine, Monash Medical School, Melbourne, Australia, 3128. * Should be considered equal first authors

Thrombin stimulation of platelets activates intracellular signaling pathways capable of inducing rapid cytoskeletal reorganization culminating in platelet shape change, aggregation and release. The cytoskeletal translocation and activation of $pp60^{c\text{-}src}$ and phosphatidylinositol 3-kinase (PtdIns 3-kinase) may represent an important pathway for the initiation of these platelet responses. Incubation of washed platelets for 24 hours with the specific tyrosine kinase inhibitor Herbimycin-A (5 μM), resulted in reduced thrombin-stimulated tyrosine phosphorylation of multiple platelet proteins, as determined by Western Blot analysis using antiphosphotyrosine antibodies. Furthermore, the thrombin-stimulated cytoskeletal enzyme activity of both PtdIns 3-kinase and $pp60^{c\text{-}src}$ was abolished by Herbimycin-A. Despite the absence of cytoskeletal PtdIns 3-kinase and $pp60^{c\text{-}src}$ enzyme activity, platelet shape change, aggregation and serotonin release was unaltered following platelet stimulation with thrombin (0.05-1 unit/ml). Herbimycin-A treated platelets also demonstrated normal platelet aggregation in response to collagen, ionophore A23187 and ristocetin. Our studies indicate that tyrosine phosphorylation is required for the thrombin-stimulated increase in cytoskeletal PtdIns 3-kinase enzyme activity. However, this increase in cytoskeletal PtdIns 3-kinase and $pp60^{c\text{-}src}$ activity is not essential for platelet shape change, aggregation and release.

Q 211 GLUCOCORTICOID-INDUCED DIACYLGLYCEROL KINASE HOMOLOGUE. Theresa Klauck and Susan Jaken, W. Alton Jones Cell Science Center, Lake Placid, NY 12946.

In the course of studying α-PKC regulation in DDT1 smooth muscle tumor cells, we found that glucocorticoids (TAA) induced a 140 kDa phosphoprotein that cross-reacted with α-PKC specific antibodies. A p140 cDNA clone (2.6 kb) was isolated by immunoscreening a lambda expression library made from TAA-treated DDT1 cells with the α-PKC antibodies. Sequence analysis demonstrated an open reading frame of 1.3 kb which correlated with the size of the bacterially expressed protein (48 kDa). Neither the cDNA nor the deduced amino acid sequences had strong homology to PKC or to any other sequences in GenBank. The cDNA clone hybridized to a DDT1 cell TAA-induced message at 6.6 kb with weaker messages at 4.4 kb and >9.5 kb. Antisera were raised to a C-terminal peptide from the translated sequence and to the bacterially expressed fusion protein. Both recognized a 140 kDa TAA-induced protein in DDT1 cells, indicating that we have isolated a partial p140 cDNA clone.

Further library screening with a more 5′ probe, as well as 5′ RACE, was used to isolate several additional clones which includes some potential splice variants. The additional 5′ regions contain sequences with significant nucleotide and amino acid homology to specific regions of the 80 kDa porcine and human diacylglycerol kinase (DGK). One of these regions is the cysteine-rich domain which is important for diacylglycerol binding and is also conserved in the PKC family. The other two regions of homology are found within the putative DGK catalytic domain and are conserved in DGKs from several species. It is possible that one of the splice variants may express a truncated form of DGK that functions as a negative regulator of normal DGK function. The homology between the 80 kDa porcine DGK and p140 indicates that p140 may be a new form of DGK which is under glucocorticoid regulation.

Q 212 MAMMALIAN PHOSPHATIDYLINOSITOL 3'-KINASE INDUCES A LETHAL PHENOTYPE ON EXPRESSION IN *SCHIZOSACCHAROMYCES POMBE*,

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Phosphatidylinositol (PI) 3'-kinase has been implicated in intracellular signalling via diverse receptor subtypes and is postulated to be a key mediator in events leading to mitogenesis. Mammalian PI 3'-kinase is an heterodimer of 85 kDa and 110 kDa subunits; the 110 kDa subunit (p110) represents the catalytic subunit. A *Saccharomyces cerevisiae VPS34* gene product (Vps34p) has significant homology to p110 and has recently been shown to have PI 3'-kinase activity. In this study, we expressed and characterised p110 and Vps34p in *Schizosaccharomyces pombe* using a inducible *nmt1*-promoter. p110 is shown to be expressed in *S. pombe* as a functional lipid kinase as judged by both the accumulation of 3'-phosphorylated lipids *in vivo* and the extraction of 3'-kinase activity *in vitro*. *In vitro*, extracts from p110 expressing cells were found to phosphorylate PI, PI 4-phosphate and PI 4,5-bisphosphate. *In vivo*, the cells expressing p110 accumulated PI 3-phosphate, PI 3,4-bisphosphate and PI 3,4,5-trisphosphate. Furthermore, expression of p110 was shown to cause cell death and be associated with marked chromosome condensation. It has been shown that introduction of a regulatory subunit of PI 3'-kinase (p85α and p85β) suppresses this lethal phenotype, providing evidence that *in vivo* the regulatory subunit exerts a tonic inhibitory effect on p110 function. In contrast, while the *S. cerevisiae* protein Vps34p can be expressed in *S. pombe* as a functional, extractable PI 3'-kinase, expression of this protein fails to increase substantially 3'-phosphorylated lipids *in vivo* and does not induce a phenotype equivalent to that induced by p110.

Q 213 INTERACTION BETWEEN THE EPIDERMAL GROWTH FACTOR RECEPTOR AND PHOSPHATIDYLINOSITOL 4-PHOSPHATE KINASE. Michael Loubtchenkov, Reinhard Wetzker, Max-Planck Society Research Group `Growth Factors Signal Transduction`, Löbderstrasse 3, D-07743, Jena, Germany

Ligand activation of the epidermal growth factor (EGF) receptor is supposed to cause a pronounced increase of phosphotidylinositol (PI) 4-phosphate 5-kinase activity in several cell lines. Recent results point to an involvement of tyrosine phosphorylation in the activation process. (Payrastre et al., 1990).

In order to elucidate the mechanism of the interaction of the EGF receptor with PI 4-P kinase we investigated an EGF receptor preparation produced by wheat germ agglutinin affinity chromatography. Using exogenous PI 4-P as substrate significant PI 4-P kinase activity could be detected associated with the EGF receptor. Moreover, PI kinase activity could also be detected in this preparation.

Specifity of the PI 4-P kinase interaction with the EGF receptor could be confirmed by coimmunoprecipitation of the enzyme using several anti EGF receptor antibodies. Preincubation of EGF receptor with 20mM phenyl phosphate strongly reduced PI 4-P kinase activity in the immunoprecipitate suggesting an essential role of a SH2 domain in the direct or indirect interaction of both proteins.

Payrastre, B., Plantavid, M., Breton, M., Chambaz, E. & Chap, H. (1990) Biochem. J. 272, 665-670

Q 214 THE ROLE OF DIACYLGLYCEROL KINASE ACTIVATION IN INTERLEUKIN-2-DEPENDENT LYMPHOCYTE PROLIFERATION

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Generation and attenuation of lipid second messengers are key processes in cellular signalling. Interleuquin-2 binding induced the rapid synthesis of phosphatidic acid by activation of diacylglycerol kinase on T lymphocytes. Direct measurements of diacylglycerol phosphorylation in lysates of quiescent and stimulated cells showed that IL-2 stimulated the phosphorylation of diacylglycerol from multiple endogenous and exogenous sources. On a mixed micellar assay no specificity for any diacylglycerol species was detected Subcellular distribution of this activity as well as association and/or activation by tyrosine kinases from the src-family was determined. The phosphatidic acid produced was, in turn, shown to stimulate the accumulation of c-myc mRNA and augment DNA synthesis when added to IL-2 dependent cell lines. These results link previous observations of IL-2 and glycosylphosphatidylinositol-dependent diacylglycerol production to phosphatidic acid accumulation, and suggest that diacylglycerol kinase activation is part of an intricate cascade that utilizes phosphatidic acid as an effector molecule.

Q 215 CHARACTERIZATION OF A PHOSPHATIDYLINOSITOL 4,5 BISPHOSPHATE 5-PHOSPHATASE IN PLATELETS

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Phosphatidylinositol (4,5) bisphosphate (PtdIns (4,5)P_2) is the precursor of several second messenger molecules. In unstimulated cells PtdIns (4,5)P_2 is hydrolyzed by a PtdIns (4,5)P_2 5-phosphatase to form phosphatylinositol 4-phosphate (PtdIns (4) P) which is subsequently recycled to phosphatidylinositol.

PtdIns (4,5)P_2 5-phosphatase activity was detected in platelet cytosolic and particulate fractions. The platelet PtdIns (4,5)P_2 5-phosphatase was dependent on magnesium but not calcium for activity. The elution profile of platelet cytosolic PtdIns (4,5)P_2 5-phosphatase from anion exchange resins exactly matched that of the 75 kDa inositol polyphosphate 5-phosphatase (Ins (1,4,5)P_3 5-phosphatase). The latter is a signal terminating enzyme responsible for the hydrolysis of inositol (1,4,5) trisphosphate (Ins (1,4,5)P_3) to inositol (1,4) bisphosphate. Polyclonal antibodies raised against recombinant 75 kDa Ins (1,4,5)P_3 5-phosphatase specifically immunoprecipitated all PtdIns (4,5)P_2 5-phosphatase activity from both the platelet membrane and cytosolic fractions. Purified 75 kDa Ins (1,4,5)P_3 5-phosphatase hydrolyzed PtdIns (4,5)P_2 forming PtdIns (4)P (Km = 250 uM). By contrast, purified membrane associated 43 kDa Ins (1,4,5)P_3 5-phosphatase did not hydrolyze PtdIns (4,5)P_2.

These results suggest in the unstimulated platelet, recycling of PtdIns (4,5)P_2 to PtdIns (4)P is mediated by the 75 kDa Ins (1,4,5)P_3 5-phosphatase. The enzyme metabolizes both water soluble inositol phosphates and a phosphoinositide, thereby functioning as an important regulator of phosphoinositide signaling.

Q 216 POSSIBLE REGULATION OF HUMAN NEUTROPHIL PHOSPHOLIPASE D BY THE TOPOGRAPHICAL DISTRIBUTION OF ENZYME AND SUBSTRATE.

M.Record(**a**), G.Ribbes(**a**), O.Colard(**b**), M.Breton(**b**), H.Chap(**a**), G.Béréziat(**b**).
(**a**): INSERM 326, CHR Purpan, 31059 Toulouse Cedex, and (**b**): URA CNRS 1283 , Hôpital Saint-Antoine, Paris, 75012.

We have investigated the intracellular distribution of alkylacyl-GPC synthesis by transacylases (TAse) and its subsequent hydrolysis by phospholipase D (PLD) activation. We separated the plasma membrane from the endoplasmic reticulum after cell lysis cavitation followed by self-generated Percoll gradients.

The CoA-independant TAse responsible for the conversion of lyso-PAF into alkylacyl-GPC was located only in the endoplasmic reticulum. Instead, the PLD activity hydrolysing the alkylacyl-GPC was detected only in the plasma membrane.

When cells were incubated with (^{3}H)alky-lyso-GPC for 15 min, 60 % of the compound was converted into (^{3}H)alkylacyl-GPC, and about one third of the latter phospholipid was recovered in the plasma membrane. This result indicated a rapid redistribution of the newly-synthesized phospholipid. In that respect, we demonstrated earlier that human neutrophil contained an active phosphatidylcholine transfer protein (PCTP) (G.Ribbes et al., J.Lipid Med., 1991,4, 251-254).

Instead phosphatidic acids (PA) formed in the plasma membrane upon phospholipase D activation were not redistributed, whereas PA-derived diglycerides were recovered both in the plasma membrane and the endoplasmic reticulum.

Using our in-vitro assay for PLD activity we observed that the membraneous enzyme needed a cytosolic factor for full activity. The PCTP could represent one of these factors by governing the supply of substrate.

The work related to PLD has been supported by a grant from BAYER-PHARMA

Q 217 INCREASED ACTIVITY OF PHOSPHATIDYLINOSITOL 4-KINASE (EC 2.7.1.67) IS ASSOCIATED WITH NEOPLASTIC PROLIFERATION, Maria Teresa Rizzo and George Weber, Division of Hematology, University of Parma, Parma, Italy, Walther Oncology Center and Laboratory for Experimental Oncology, Indiana University School of Medicine, Indianapolis , IN 46202

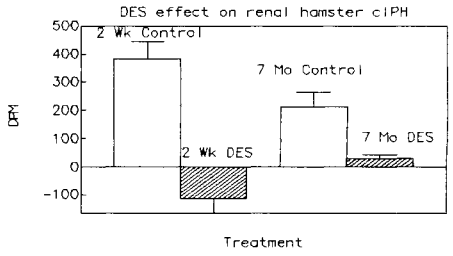
Incorporation of ^{32}Pi into phosphatidylinositol (PI) to generate phosphatidylinositol 4-monophosphate (PI4-P) was assayed in the particulate cell fraction obtained from normal and neoplastic rat tissues. When PI4-kinase was assayed in the particulate cell fraction of normal liver and in the rapidly growing hepatoma 3924A, the majority of the enzyme activity was detected in the membrane-bound fraction. Enzyme activity, assayed in the presence of Triton X-100, was linear with time throughout 2 min and was proportional with protein concentration over a range of 0.1 to 1.0 mg. The apparent Km's of the enzyme for ATP, Mg, and PI in normal liver were 0.03 mM, 10 mM, and 0.2 mM, respectively. The affinity of the enzyme for ATP, Mg, and PI in hepatoma 3924 A yielded apparent Km's of 0.01 mM, 6.2 mM and 0.1 mM, respectively. In rat hepatomas of slow and medium growth rates, PI-4 kinase activity was increased 5.3 to 7.6-fold whereas in hepatoma 3924A PI-4 kinase activity was 28.5-fold over values of normal liver. In rat sarcoma PI4-kinase activity was increased 12.6-fold compared to that of normal liver. To further clarify the linkage between PI4-kinase and neoplastic proliferation, enzyme activity was determined in proliferative non-malignant tissues. In rat organs with different cell renewal, PI4-kinase was higher in organs with high growth rate: activity in thymus, bone marrow, spleen and testis was increased 8.4, 7.6, 5.8, and 5.1-fold, respectively as compared to values of normal liver. In contrast, in organs with low cell renewal PI4-kinase activity ranged from 0.4 to 3.6-fold that of control liver. In rapidly growing differentiating (6-days-old) and 24-h regenerating livers, activities were 3.4 and 3.1-fold higher than in adult liver. Thus, increased PI4-kinase activity in tumors appears to be a characteristic of neoplastic proliferation.

Q 218 BIOCHEMICAL AND GENETIC ANALYSIS OF THE FUNCTION OF AN ESSENTIAL PHOSPHATIDYLINOSITOL 4-KINASE (*PIK1* GENE PRODUCT) FROM THE YEAST *SACCHAROMYCES CEREVISIAE*. Elisabeth A. Schnieders and Jeremy Thorner, Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720.

To investigate the contribution of PtdIns[4,5]P_2 production to the physiology of a eukaryotic cell, our group purified a high MW soluble PtdIns 4-kinase from the yeast *Saccharomyces cerevisiae* [Flanagan & Thorner (1992) *J. Biol. Chem.* **267**: 24117-24125] and then isolated the gene, *PIK1*, encoding this enzyme [Flanagan, Schnieders, Emerick, Kunisawa, Admon & Thorner (1993) *Science*, in press]. We found that *PIK1* function is essential for cell viability. To identify other genes that may be downstream components of the pathway that requires the PtdIns[4,5]P_2 generated by the action of Pik1p, we have constructed a yeast strain that relies on the expression of *PIK1* from the galactose-inducible, glucose-repressible *GAL1* promoter. We are using this conditional strain to select both chromosomal mutations and dosage suppressors that permit cell growth in the absence of *PIK1* expression. The nature of these bypass suppressors will be presented. To determine its subcellular location, state of post-translational modification, and ability to associate with other cellular proteins, we are raising a variety of antibodies directed against full-length Pik1p or against various discrete sub-domains of the polypeptide. Information on the biochemical and cell biological properties of Pik1p will also be presented.

Q 219 INHIBITION OF CYCLIC INOSITOL PHOSPHOHYDROLASE ACTIVITY BY DIETHYLSTILBESTROL, **M. Chandra Sekar, Vijaya Sambandam and Deodutta Roy*, Department of Pathology and * Department of Environmental Health Sciences, University of Alabama at Birmingham, Birmingham, Al 35294**

Cyclic inositol phosphohydrolase (cIPH) hydrolyzes the cyclic phosphodiester bond of cyclic inositol mono phosphate to yield inositol 1-phosphate. Cyclic inositol monophosphate has been recently implicated in cell growth and proliferation. We have developed a non-radioactive method for cIPH activity measurement, where inositol released following hydrolysis of cyclic inositol monophosphate by the enzyme, is separated from the substrate by Dowex formate resin. Inositol levels are subsequently determined in a 96-well plate by colormetric method developed by Dolhofer and Wieland, J. Clin. Chem. Clin. Biochem. (1987) 25:733-736. Diethylstilbestrol implantation in hamster leads to a specific development of renal tumor. We have found that like rat, cIPH activity in hamster kidney is particulate. Comparison of cIPH activity in control versus DES implanted pellet (a single 25 mg dose) indicate a significant inhibition of cIPH activity within two weeks.



Q 220 *Abstract Withdrawn*

Q 221 CLONING, BACULOVIRUS EXPRESSION AND CHARACTERIZATION OF A HUMAN PHOSPHATIDYLINOSITOL 3-KINASE p110 SUBUNIT,

Steven M. Stirdivant, Janet D. Ahern, Robert Conroy, Stan F. Barnett, Lynette M. Miles, Allen Oliff and David E. Heimbrook. Dept. of Cancer Research, Merck Research Laboratories, West Point, PA. 19486.

The inositol lipid kinase, Phosphatidylinositol 3-Kinase (PI3-Kinase), has been implicated as an essential component of mitogenic signalling by tyrosine kinase growth factor receptors such as PDGFR, Insulin receptor, CSF-1 receptors and others. It also appears to be involved in oncogenic transformation by v-src, v-abl and polyoma middle T. The PI3-Kinase enzyme is a heterodimer consisting of a p85 adapter subunit and a p110 catalytic subunit. Autophosphorylation of growth factor receptor tyrosines, upon ligand binding, generates binding sites for the SH2 domains of the PI3-Kinase p85 subunit. The interaction of PI3-Kinase with the growth factor receptors results in its activation through a mechanism which is, as yet, not clearly defined. This activation results in elevated levels of the PI3-Kinase products PI(3,4)P_2 and PI(3,4,5)P_3. The downstream signaling pathway initiated by these products is unknown. To help define further the role of PI3-Kinase in mitogenic signaling, we have cloned a human p110 gene. The sequence of this p110 clone is highly homologous to the bovine p110 gene (Hiles et al., 1992, *Cell 70:* 419). Coexpression of this human p110 clone with a human p85α clone, in a baculovirus expression system, yields a soluble active enzyme. Expression of the p110 subunit alone yields an insoluble p110. The recombinant holoenzyme has been characterized enzymologically. We have also studied the ability of the putative PI3-Kinase inhibitor wortmannin, to attenuate mitogenic signalling through tyrosine kinase and non-tyrosine kinase growth factor receptors. An attempt has been made to link wortmannin inhibition of mitogenesis to its inactivation of PI3-Kinase *in vivo.*

Q 222 COMPARTMENTALIZATION, ACYL SPECIFICTY AND DIACYLGLYCEROL RECYCLING IN FIBROBLAST PHOSPHOLIPID METABOLISM

Vidar A.T. Thorsen and Holm Holmsen, Department of Biochemistry and Molecular Biology, Univ. of Bergen, Norway.

According to Kennedy's pathway for phospholipid synthesis, glycerol and phosphate should be incorporated in a 1:1 proportion into phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Thus, cells carrying ATP labelld with [^{32}P]P_i and glycerol-3-phosphate (Gro-3-P) labeld with [^{3}H] should be expected to syntesize PC and PE with the same [^{32}P] /[^{3}H] ratio as in glycerol-3-phosphate. Under these conditions human platelet and fibroblasts (C3H T10 ½) incorporated [^{32}P] and [^{3}H] into PC and PE with a [^{32}P] /[^{3}H] ratio much lower than in Gro-3-P, phosphatidic acid (PA) and phosphatidylinositol (PI). This suggests distinct metabolic separation (probably by cellular compartmentation) of the glycerol and choline (or ethanolamine) branches of the *de novo* phospholipid biosynthesis. Similar to our results, George,T.P. *et al.,J.Biol.Chem.*266:12419,1991 have shown that [^{3}H]choline was readily incorporated into PC in glioma cells, while [^{3}H]cholinephosphate and [^{3}H]CMP-P.cho were not incorporated when introduced to the cytoplasma in electropermeabilized cells. The [^{32}P] /[^{3}H] ratio also varied strongly among the molecular species of PC, PE, PI and PA, which could indicate that the enzymes innvolved in in these conversions have some degree of acyl specificity.

Q 223 *IN VITRO* ACTIVATION OF THE PROTEIN KINASE C FAMILY OF ENZYMES BY THE NOVEL LIPIDS PI3,4P_2 AND PI3,4,5P_3.

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Activation of conventional protein kinase C (cPKC) isoenzymes *in vitro* requires calcium, phosphatidylserine (PS) and diacylglycerol (DAG), whereas novel PKC's (nPKC) are dependent only on PS and DAG for activation. The 3'phosphorylated lipids PI3,4P_2 and PI3,4,5P_3, produced as a result of the activation of phosphatidylinositol 3-kinase, have previously been shown to activate PKC ζ *in vitro.* In this study we investigated the activation of other PKC isoenzymes by PI3,4P_2 and PIP_3 with respect to autokinase activity and exogenous substrate phosphorylation. Of all the isoenzymes tested (α, βI, βII, γ, δ, ε, η and ζ) only the calcium-independent, nPKC's were activated in response to the novel lipids in the following order: PKC ε> PKC η> PKC ζ. Little or no effect on the activity of the other PKC's above that stimulated by PS was observed. With respect to autokinase activity, PKCε, η and ζ were stimulated to levels above those produced by PS/DAG. In this assay, PI4P and PI4,5P_2 failed to activate these isoenzymes above PS levels. The activation of PKC ε by PIP_3 in particular occurred at concentrations which may be physiologically relevant. We propose that the activation of the tyrosine kinase signaling pathway leading to 3'phosphoinositide production activates a subset of PKC's.

Q 224 LONG-TERM INHIBITION OF CA^{2+}-DEPENDENT CHLORIDE TRANSPORT BY INS(3,4,5,6)P_4.

A. Traynor-Kaplan, M. Vajanaphanich, C.Schultz*†, M. Wasserman, S.B. Shears**, R.Y. Tsien*†, K.E. Barrett. Dept. of Med. & *Pharm., UCSD, †Howard Hughes Med. Inst., La Jolla, CA. **Lab. of Cell. & Mol.Pharm., NIEHS, Research Triangle Park, NC.

We have shown that pretreatment with carbachol desensitizes Cl^- secretion to stimulation by $[Ca^{2+}]_i$ in T_{84} colonic epithelial cells. A 10 min pretreatment with carbachol blocks subsequent thapsigargin (Tg)-stimulated Cl^- secretion as measured by changes in short circuit current in Ussing Chambers, but not the Tg-stimulated $[Ca^{2+}]_i$ response, suggesting that carbachol generates an intracellular signal which uncouples Cl^- secretion from $[Ca^{2+}]_i$. Recent studies indicated that an isomer of inositol tetrakisphosphate ($InsP_4$) could mediate this phenomenon. Here we identify this isomer as D-Ins(3,4,5,6)P_4. The inhibitory effect of carbachol on Tg-stimulated Cl^- secretion began within 10 min, then continued for more than 90min. ^{3}H-Ins(3,4,5,6)P_4, Ins(1,3,4,6)P_4 and Ins(1,3,4)P_3 levels were persistently elevated throughout this time course, long after ^{3}H-Ins(1,3,4,5)P_4 and Ins(1,4,5)P_3 levels had returned to baseline. Inositol phosphates were differentially metabolized following receptor blockage with atropine. Tg-stimulated Cl^- secretion was still inhibited after cells were pre-stimulated with carbachol for 75min, and then atropine added for an additional 15min. At this point, levels of all inositol phosphates other than ^{3}H-Ins(3,4,5,6)P_4 (6-fold elevation) had returned to baseline. Longer atropine treatment eventually reversed both the inhibition of Cl^- secretion and the increase in ^{3}H-Ins(3,4,5,6)P_4. Identification of ^{3}H-Ins(3,4,5,6)P_4 as the D-isomer was determined by incubating this peak with ^{33}P-Ins(1,4,5,6)P_4 and partially purified Ins(1,4,5,6)P_4 3-kinase. Direct confirmation that Ins(3,4,5,6)P_4 regulates Cl^- transport was sought by introducing it into intact cells using a cell permeant analogue, DL-Bt_2Ins(3,4,5,6)P_4/AM. Addition of 400μM DL-Bt_2Ins(3,4,5,6)P_4/AM, substantially reduced Ca^{2+}-dependent Cl^- transport. Control incubations with equivalent concentrations of P_i/AM or K^+ butyrate were without effect. Thus, Ins(3,4,5,6)P_4 may be a novel, inhibitory, second messenger capable of regulating Cl^- transport.

Q 225 DIACYLGLYCEROL KINASE IN RECEPTOR-STIMULATED CELLS IS REGULATED AND CONVERTS ITS SUBSTRATE IN A TOPOLOGICALLY RESTRICTED FASHION.

Wim J. van Blitterswijk, Rob L. van der Bend, Dick Schaap, John de Widt and Henk Hilkmann; The Netherlands Cancer Institute, Division of Cellular Biochemistry, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

The regulation of diacylglycerol (DG) kinase activity was studied in fibroblasts and Jurkat T cells. We questioned whether enzyme activity only depends on substrate availability or whether it requires receptor stimulation. To this end, we raised DG levels up to 15-fold by treatment of cells with bacterial phosphatidylinositol-specific phospholipase C (PLC). In detergent cell lysates, DG kinase was readily capable of converting this surplus of DG to phosphatidic acid (PA), but in intact cells the enzyme remained inactive. Stimulation of fibroblasts with bradykinin or endothelin and Jurkat cells with anti-CD3 resulted in DG kinase-mediated formation of PA, but its level was unaffected by PLC pretreatment. Likewise, in streptolysin-O permeabilized fibroblasts, where bradykinin stimulation in the presence of [γ^{32}P]ATP and GTP-γS generates [^{32}P]PA exclusively via DG kinase, PLC pretreatment did not affect the amount of [^{32}P]PA formed. We conclude that DG kinase acts on DG generated by receptor stimulation, but not on DG generated by exogenous PLC. We propose a model in which DG kinase physically associates with endogenous PLC. Within this complex, receptor-induced DG would then be transmitted ("channelled") from endogenous PLC to the active site of DG kinase, whereas excess DG generated randomly in the plasma membrane by bacterial PLC is inaccessible to this catalytic site.

We have cloned the cDNA of a 80-86 kDa DG kinase and transfected this in Rat-1 fibroblasts, leading to 20-fold overexpression of the enzyme. However, in the intact cell it remained in a "dormant" state, and did not become activated by agonist stimulation.

Q 226 REGULATION OF PC-BIOSYNTHESIS BY GROWTH FACTORS - EVIDENCE FOR AN INVOLVEMENT OF MAP-KINASES

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Phosphatidylcholine (PC) is synthesized in mammalian cells mainly by the CDP-choline pathway, for which CTP:phosphocholine cytidylyltransferase (EC 2.7.7.15) (CT) is often regulatory [1]. There exist many mechanisms for the regulation of CT activity and reversible phosphorylation seems to play an important role. In HeLa cells most of the enzyme is located in the soluble fraction where it exists in a phosphorylated, inactive form. Activation of CT (e.g. by treatment with oleate or phospholipase C) is accompanied by a translocation process to membranes and dephosphorylation of the enzyme [2]. Little is known about the kinases that phosphorylate CT *in vivo*. In HeLa cells treatment with a selective inhibitor of cAMP-dependent protein kinase (H-89) did not affect the phosphorylation of CT indicating that this kinase is not involved [3]. Since there are many serine-residues followed by proline-residues in the cDNA sequence of CT and since p44/42$^{mapk/erk}$ are known to be activated by growth factors in HeLa cells [4], we investigated the possible role of MAP-kinases in phosphorylating CT *in vivo*. The system of oleate-mediated dephosphorylation of CT was used to investigate the rephosphorylation of CT after removal of oleate. In the presence of growth factors CT becomes more rapidly rephosphorylated. Furthermore, the incorporation of choline into PC under these conditions is reduced by 35%. Treatment of cells simultaneously with oleate and growth factors results in a decrease of choline incorporation into PC by 40% compared to cells treated with oleate alone and CT is slightly more phosphorylated in the presence of growth factors. These results together with the finding that CT is a substrate for p44^{mapk} *in vitro* suggest that MAP-kinases might be involved in the phosphorylation of CT *in vivo*.

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[2] Wang, Y. et al. (1993) J. Biol. Chem. **268**, 5512-5518
[3] Wieprecht, M. et al. Biochem. J., in press
[4] Chen, R.-H. et al. (1992) Mol. Cell. Biol. **12**, 915-927

Q 227 DEPENDENCE OF PROTEIN KINASE C ACTIVATION BY DIACYLGLYCEROLS AND CALCIUM ON LIPID MEMBRANE STRUCTURE,

Raphael Zidovetzki*, David S. Lester‡, Dan B. Borchardt¶, and Edward M. Goldberg*. Departments of *Biology and ¶Chemistry, University of California, Riverside, CA 92521, and ‡Neural Systems Section, National Institutes of Health, Rockville, MD 20852

The combined effects of five diacylglycerols (DAGs) , diolein (DO), 1-stearoyl,2-arachidonoylglycerol (SAG), 1-oleoyl,2-acetylglycerol (OAG), dioctanoylglycerol (diC_8), and dipalmitin (DP), and Ca^{2+} on the activity of protein kinase C (PK-C) were correlated with their effects on the structure of lipid membranes, composed of a mixture of phosphatidylcholine (PC) and phosphatidylserine (PS). The following DAG- and Ca^{2+}- induced bilayer perturbations were identified. (1). Increased tendency to form non-bilayer lipid phases was induced by DO or SAG and synergistically enhanced by the addition of Ca^{2+}. (2). "Transverse" bilayer perturbation was induced by diC_8. The addition of this DAG caused increased ordering of the phospholipid acyl side chains in the region adjacent to the headgroup, with the concomitant *decrease* of the order toward the bilayer interior. (3). Separation of the PC and PS bilayer components was induced by combinations of relatively high Ca^{2+} and DO, SAG, or OAG. (4). Lateral phase separation of the bilayers on the regions of different fluidities was induced by DP. These physicochemical effects were correlated with the effects of these DAGs and Ca^{2+} on the activity of PK-C. The increased tendency to form non-bilayer lipid phases and the transverse bilayer perturbations correlated with the increased PK-C activity, while the actual *presence* of the non-bilayer lipid phases as well as the separation of the PC and PS components was associated with the *decrease* in the PK-C activity. The lateral phase separation of the bilayer on gel-like and liquid crystalline regions did not have an effect on the activity of the enzyme. These results demonstrate the importance of the physicochemical properties of the membranes in the process of activation of PK-C.

Phospholipases

Q 300 CALCIUM-INDEPENDENT CYTOSOLIC PHOSPHOLIPASE A_2 FROM THE MACROPHAGE-LIKE CELL LINE P388D_1. Lisa Ackermann and Edward Dennis, Department of Chemistry, University of California, San Diego, La Jolla, CA 92093-0601.

Intracellular phospholipase A_2's (PLA_2's) are thought to play a crucial role in the production of prostaglandins, leukotrienes, lysophospholipids and PAF. Much research has been focused on the Ca^{2+}-dependent PLA_2's, and very little is known about their Ca^{2+}-independent counterparts. In the present study we have identified and purified a cytosolic, Ca^{2+}-independent PLA_2 from P388D_1 macrophage-like cells. The purification utilized an ammonium sulfate precipitation followed by sequential column chromatography on Octyl-Sepharose, ATP-agarose, Mono Q FPLC and Hydroxyapatite FPLC. The resulting enzyme was purified over 460,000 fold with a final specific activity of approximately 5 μmol/min-mg and gave a major band near 80 kDa on SDS-PAGE. Triton X-100 increased the PLA_2 activity with optimal activity found at a Triton/phospholipid molar ratio of 4:1. When assayed with mixed micelles, the enzyme did not show a preference for either *sn-2* arachidonic acid or *sn-1* alkyl-ether containing phospholipids. The purified PLA_2 activity was increased approximately 2-5 fold by nucleotide di- and triphosphates, and this activation was sensitive to the presence of Triton X-100.

Q 301 PHOSPHOLIPASE C AND THE INDUCTION OF LIPOSOMAL FUSION THROUGH DIACYLGLYCEROL FORMATION, Alicia Alonso, Gorka Basáñez, José L. Nieva and Félix M. Goñi, Department of Biochemistry and Molecular Biology, University of the Basque Country, Bilbao, Spain.

Certain processes of signal transduction across cell membranes imply the transient modification of their lipid composition. One example is the activation of PI- and PC-specific phospholipase C, that induce the local generation of diacylglycerol (DG). We have shown that, when phospholipase C acts on phospholipid vesicles liposome aggregation occurs, followed by mixing of lipid components and mixing of aqueous contents, thus representing the process of membrane fusion. DG levels regulate the rate and extent of the enzyme-induced fusion process; the effects of externally added DG are different from those of the enzymatically produced molecule. X-ray diffraction studies show that DG, at the proportions that allow membrane fusion, facilitates formation of three-dimensional bicontinuous lipid/water phases, while at inhibitory proportions it causes the formation of phases in which no continuity of the water component occurs. The fusogenic action of phospholipase C is further modulated by the presence of gangliosides and other sphingolipids in the bilayer.

Q 302 USE OF SYNTHETIC SPHINGOMYELIN ANALOGS TO PROBE THE SUBSTRATE BINDING SITE OF MEMBRANE-BOUND, NEUTRAL SPHINGOMYELINASE, Robert Bittman and Zhong-shi Ruan, Department of Chemistry and Biochemistry, Queens College of The City University of New York, Flushing, NY 11367, and Mark D. Lister, Department of Biomolecular Research, Sphinx Pharmaceuticals Corp., Durham, NC 27717

Membrane-bound, neutral pH-optimum sphingomyelinase (N-SMase) may play a key role in a variety of signal transduction processes. To determine which structural features of the sphingomyelin (SM) molecule are required for binding to the active site of N-SMase from rat brain, we synthesized a series of SM analogs and examined their abilities to serve as substrates or inhibitors. N-SMase activity was determined by product analysis on TLC plates. Particular attention was given to the requirements involving the C-1 and C-3 positions of the sphingosine backbone of SM. For variation at the C-1 position, ceramide (Cer) 1-phosphate and Cer-1-phosphoethanol-*N,N*-dimethylamine served as modest substrates (having 9% and 18% of SMase activity, respectively) but failed to compete effectively with the substrate (egg SM) for binding to the active site. Thus, a phosphate group or a $-OP(O)(O^-)O(CH_2)_2NHMe_2^+$ moiety at C-1 of Cer is insufficient for strong binding of the sphingolipid. The hydroxy group of *N*-stearoyl-SM at C-3 was replaced to give the deoxy, *O*-methyl, *O*-ethyl, and *O*-tetrahydropyranyl (OTHP) analogs. The deoxy analog did not bind to N-SMase, indicating that the oxygen of the OH group is required at C-3 for binding. SM analogs with small *O*-alkyl groups at C-3 did bind to the active site and inhibited substrate binding (K_i of 3-OMe-SM, 33 μM; K_i of 3-OEt-SM, 133 μM), indicating that the hydrogen of the OH group at C-3 is not required. However, diminished inhibition of N-SMase by the 3-OEt- and 3-OTHP-SM analogs indicate that binding to the active site is sensitive to steric bulk at the C-3 position.

Q 303 ACTIVATION AND INHIBITION OF TYPE I PHOSPHOLIPASE A_2 BY THIOETHER AMIDE PHOSPHOLIPID ANALOGUES, Scott C. Boegeman and Edward A. Dennis, Dept. of Chemistry, University of California San Diego, La Jolla, CA 92093-0601.

Two phospholipid analogues were synthesized by a modification of a published procedure. [Yu, L. and Dennis, E.A., JACS (1992) 114, 8757.] Both analogues consist of a six carbon thioether at the *sn*-1 position and a six carbon amide at the *sn*-2 position, with the headgroup as either a phosphorylethanolamine (DiC_6SNPE) or a phosphorylcholine (DiC_6SNPC). The inhibition characteristics of DiC_6SNPE and DiC_6SNPC were studied using two short chain lipids, a phosphatidylethanolamine (PE) and a phosphatidylcholine (PC), as substrate. Short chain substrates were chosen to allow the study of enzyme\inhibitor interactions in both monomeric and mixed micellar substrate states. Using PC monomers as substrate and phospholipase A_2 from cobra venom (*Naja naja naja*), DiC_6SNPE had an IC_{50} of 7.6 μM and DiC_6SNPC had an IC_{50} of 91 μM. When the substrate form was switched to mixed micelles by the addition of Triton X-100, the IC_{50} of DiC_6SNPE decreased 15 fold to 0.50 μM, as did the IC_{50} of DiC_6SNPC (5.8 μM). Use of PE as substrate and the DiC_6SNPC as inhibitor resulted in a complex response in which activation was observed at low DiC_6SNPC concentrations, followed by inhibition at much higher DiC_6SNPC concentrations. This behavior was not observed for the DiC_6SNPE inhibitor under any substrate conditions. Using PE substrate, DiC_6SNPE showed typical inhibition curves which yielded a monomeric IC_{50} of 21 μM and a micellar IC_{50} of 6.3 μM. These results extend earlier work from our laboratory using long chain thioether amide phospholipid inhibitors. (NIH GM 20501 and Training Grant 5 T32 CA 09523.)

Q 304 CELL-PERMEABLE CERAMIDES INHIBIT THE STIMULATION OF DNA SYNTHESIS AND PHOSPHOLIPASE D ACTIVITY BY PHOSPHATIDATE AND LYSOPHOSPHATIDATE IN RAT FIBROBLASTS. David N. Brindley, Antonio Gomez-Muñoz, Ashley Martin, Lori O'Brien. Department of Biochemistry and Lipid and Lipoprotein Research Group, 328 Heritage Medical Research Centre, Edmonton, Alberta T6G 2S2 Canada

The interactions of ceramides with phosphatidate and lysophosphatidate in the regulation of signal transduction in rat fibroblasts were examined. C_2-and C_6-ceramides (N-acetylsphingosine and N-hexanoylsphingosine respectively) at 10μM almost completely inhibited the stimulation of thymidine incorporation into DNA that is produced by 50-100 μM phosphatidate, or lysophosphatidate. Sphingosine (10 μM) had the opposite effect to the ceramides on DNA synthesis by stimulating this process. However, C_2- or C_6-ceramides failed to inhibit the synthesis of DNA that had been stimulated by insulin, or serum. The effects of the ceramides in inhibiting growth were not caused by modifying the action of phosphatidate or lysophosphatidate in decreasing cAMP formation. C_2- and C_6-ceramides inhibited the stimulation of phospholipase D activity by phosphatidate, lysophosphatidate, phorbol ester, thrombin and serum in intact fibroblasts. The ceramides also inhibited the stimulation of phospholipase D by phorbol ester, or GTPγS in permeabilized fibroblasts. This demonstrates that the ceramides can modify cell signalling and the generation of phosphatidate and diacylglycerol by agonist stimulation of the phospholipase D pathway. However, this effect on phospholipase D alone is not able to explain the effects on DNA synthesis. Incubation of fibroblasts for 30 to 120 min with C_2- or C_6-ceramides, or sphingomyelinase inhibited the uptake of exogenous phosphatidate, or lysophosphatidate by the fibroblasts by 42 and 53% respectively. However, a greater proportion of the phosphatidate, or lysophosphatidate that was taken up by the fibroblasts was metabolized further when the cells were pretreated with ceramides, or sphingomyelinase. This effect was probably partly accounted for by the increased activity of the N-ethylmaleimide-insensitive phosphatidate phosphohydrolase after incubation with sphingomyelinase, or the ceramides. These results demonstrate that ceramides may produce part of their growth inhibitory effects by blocking the signal transducing effects of phosphatidate and lysophosphatidate.

Q 305 VITAMIN E REGULATES DIACYLGLYCEROL AND PHOSPHATIDIC ACID LEVEL IN THROMBIN-STIMULATED ENDOTHELIAL CELLS, Alvin C. Chan and Khai Tran, Department of Biochemistry, Faculty of Medicine, University of Ottawa, Ottawa, Ontario, Canada K1H 8M5

The present study has examined the role of vitamin E, a natural lipid antioxidant, in the production of diacylglycerol (DAG) and phosphatidic acid (PA) in thrombin-stimulated human endothelial cells. Cells were labelled with [^{3}H]-myristate and the incorporation and distribution of [^{3}H]-myristate into cellular lipids was not affected by vitamin E. However, in response to thrombin stimulation, considerably more PA and less DAG were formed in cells enriched with vitamin E. Time-course studies indicated that vitamin E attenuated the accumulation of sustained DAG levels with a concomitant increase in PA. Direct determination of DAG mass further confirmed that vitamin E suppresses the accumulation of DAG induced by thrombin. In the presence of ethanol, the formation of [^{3}H]-phosphatidylethanol (PEt) in [^{3}H]-myristate labelled cells stimulated by thrombin was unaffected by vitamin E enrichment. DL-propranolol, a PA phosphohydrolase inhibitor, caused an accumulation of PA without affecting DAG formation in either vitamin E-treated and untreated cells. This indicated that the increase in PA and decrease in DAG in vitamin E treated cells was not due to a stimulation of phospholipase D or an inhibition of PA phosphohydrolase. Determination of inositol phosphates formation in response to thrombin showed that the change of DAG levels elicited by vitamin E was independent of phospholipase C-induced hydrolysis of inositol phospholipids. In contrast, analysis of DAG kinase activity showed that vitamin E enrichment enhanced the activity of the enzyme in both basal and thrombin-stimulated cells. Taken together, these data indicated that vitamin E caused an increased conversion of DAG to PA by activating DAG kinase activity without causing any change in the activities of phospholipase D, PA phosphohydrolase or phospholipase C. (Supported by the Medical Research Council of Canada.)

Q 306 EPIDERMAL GROWTH FACTOR COORDINATELY REGULATES THE EXPRESSION OF PROSTAGLANDIN G/H SYNTHASE AND CYTOSOLIC PHOSPHOLIPASE A_2 GENES IN EMBRYONIC MOUSE CELLS, Kenneth P. Chepenik[1], Arturo Diaz[2] and Sergio A.Jimenez[2], Department of [1]Anatomy and [2]Rheumatology Division, Department of Medicine, Jefferson Medical College, Philadelphia, PA 19107.

Epidermal growth factor(EGF) and prostaglandins have been implicated as regulating development of the palate. Indeed, EGF will modulate mobilization and metabolism of arachidonic acid in mouse embryo palate mesenchyme(MEPM) cells. Therefore, we explored the hypothesis that EGF regulates gene expression of specific enzymes involved in the production of prostaglandins. Chronic treatment (>6h) of confluent MEPM cells with EGF (a) increased their ability to metabolize exogenous arachidonic acid to prostaglandin E_2 (PGE_2), and (b) stimulated constitutive expression of activities of PLA_2 and prostaglandin G/H synthase (hereafter called Cox). In parallel, EGF stimulated synthesis of PLA_{2C} and Cox, and increased the steady-state levels of the 3.0kb transcript for the high molecular weight, cytosolic PLA_2 (PLA_{2C}), the 2.8kb transcript for Cox-1, and the 4.4kb transcript for Cox-2. *In vitro* nuclear transcription assays showed parallel increases in the transcription rate of the genes for Cox-1 and PLA_{2C}. EGF had no effect on synthesis of the low molecular weight, group II PLA_2, accumulation of its transcript, or the transcription rate of its gene. Coordinate regulation of activities of PLA_2 and Cox in response to EGF did not parallel the mitogenic effects of EGF on confluent MEPM cells. These data lead to the conclusion that EGF modulates production of signalling lipids by upregulating gene expression of Cox-1 and Cox-2 and the high molecular weight, arachidonyl-specific phospholipase A_{2C}.

Q 307 THE USE OF POLYMERIZED MIXED LIPOSOMES TO STUDY INTERACTIONS OF PHOSPHOLIPASES WITH MEMBRANES, Wonhwa Cho, Shih-Kwang Wu and Rajiv Dua, Department of Chemistry, University of Illinois at Chicago (M/C 111), Chicago, IL 60607-7061

To study interactions of phospholipases (PLA_2, PLC and PLD) with membranes, we have developed a novel kinetic system based on polymerized mixed liposomes (PML) of 1,2-bis[12-(lipoyloxy)-dodecanoyl]-*sn*-glycero-3-phosphocholine (BLPC) (or -phosphoglycerol (BLPG)) and pyrene-containing phospholipids. Large liposomes prepared from BLPC (or BLPG) were readily hydrolyzed by phospholipases before polymerization but they were resistant to the enzymatic hydrolysis after polymerization. When PML were prepared from a mixture of BLPC and 1-hexadecanoyl-2-(1-pyrene-decanoyl)-*sn*-glycero-3-phosphocholine (pyrene-PC) (5 mol %), pyrene-PC (-PE or -PG) molecules were uniformly distributed and selectively hydrolyzed by PLA_2. Thus, in this kinetic system one can unambiguously designate those phospholipids which interact with the active site of PLA_2 and those which interact with the interfacial binding site. Based on these findings, various combinations of polymerized mixed liposomes were prepared and their hydrolysis by different PLA_2s measured. Also, several site-specific mutants of bovine pancreatic PLA_2 were prepared and their actions on PML measured. Kinetic analyses showed the importance of electrostatic interactions in the interfacial binding of PLA_2 and in the ensuing catalytic steps, and identified those residues involved in individual processes. Kinetic studies of PLC and PLD using PML of BLPC and N-(1-pyrenesulfonyl)-1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine revealed the similar mode of interfacial catalysis for these phospholipases. In summary, PML, especially when combined with protein engineering, provide a simple and powerful tool for systematically analyzing the interfacial binding and catalytic processes in the action of phospholipases. PLM can also serve as a convenient and sensitive fluorescence assay system for many phospholipases.

Q 308 REGULATION OF PHOSPHOLIPASE D ACTIVITY IN LACRIMAL GLAND ACINI BY CHOLINERGIC AGONISTS, PHORBOL ESTERS AND IONOMYCIN, Darlene A. Dartt and Driss Zoukhri, Schepens Eye Research Institute and Department of Ophthalmology, Harvard Medical School, Boston MA 02114

In the lacrimal gland, cholinergic agonists stimulate phospholipase C activity to cause protein, electrolyte and water secretion and α_1-adrenergic agonists cause protein secretion by an as yet unknown mechanism. To determine if cholinergic and adrenergic agonists regulate phospholipase D (PLD) activity in lacrimal gland, we took advantage of the unique ability of PLD in the presence of ethanol to catalyze a transphosphatidylation reaction to produce phosphatidylethanol (PEt). PLD also catalyzes the hydrolysis of the terminal phosphodiester bond of phospholipids to produce phosphatidic acid (PA) a potential second messenger. Lacrimal gland acini were labelled for 3 h with [^{14}C]stearic acid followed by a 20 min preincubation in the presence of 2% ethanol. Agonists or antagonists were then added for 20 min. After termination of incubation, total cellular lipids were extracted and analyzed by thin-layer chromatography. Carbachol (1 mM), a muscarinic agonist, induced a two-fold increase in the production of [^{14}C]PEt and [^{14}C]PA. This effect was completely blocked by the muscarinic antagonist atropine (10 μM). [^{14}C]PEt accumulation was also stimulated two-fold by the active phorbol esters, 4β-phorbol 12-myristate 13-acetate and 4β-phorbol 12,13-dibutyrate, at 1 μM and ionomycin (1 μM), a Ca^{2+} ionophore. In contrast to carbachol, neither phorbol esters nor ionomycin stimulated [^{14}C]PA production. Neither [^{14}C]PEt nor [^{14}C]PA production was altered by epinephrine (1 mM), a non-selective adrenergic agonist, or phenylephrine (0.1 and 1 mM), a specific α_1-adrenergic agonist. We conclude that PLD activity, modulated by muscarinic receptors, protein kinase C and Ca^{2+}, but not by adrenergic receptors, is present in the rat lacrimal gland acini. We also conclude that activation of PLD by receptors may occur through a different process than by PKC or Ca^{2+}. Supported by NIH Grant EY06177 and an ARVO/CIBA Vision Research Fellowship Grant.

Q 309 FATTY ACID AND PHOSPHOLIPID SELECTIVITY OF DIFFERENT PHOSPHOLIPASES A_2 USING MAMMALIAN MEMBRANES AS SUBSTRATE, Diez E., Stroup G., Mayer R. Chilton F.H., Winkler J.D. and Fonteh A.N., SmithKline Beecham S.A., Department of Molecular Pharmacology, Tres Cantos, 28760 Madrid, (SPAIN)

Phospholipase A_2 (PLA_2) isoenzymes exhibit different selectivity for the fatty acid hydrolyzed, the nature of the chemical bond at the *sn*-1 position, or the phosphobase moiety at the *sn*-3 position of the phospholipid in artificial substrates such as vesicles or mixed micelles. In order to improve our understanding of their substrate specificity, we have studied three different mammalian PLA_2 (Type I, Type II and 85kDa) enzymes in a complex mammalian membrane system. Using unlabeled membranes, we studied the selectivity of these enzymes for the fatty acid in the *sn*-2 position of the phospholipid, and using [^{3}H]arachidonic acid (AA) labeled membranes we studied the selectivity for different AA-containing glycerophospholipids. In agreement with the mixed vesicle model (Diez et al.(1992) J.Biol. Chem.267, 18342-18348) the 85kDa PLA_2 was very selective in releasing arachidonic acid. In addition, the release of AA from phospholipid subclasses was proportional to their AA content suggesting that this enzyme does not have any preference for the *sn*-1 linkage or the *sn*-3 phosphobase moiety. On the other hand, Type I and Type II PLA_2 were about 3 fold less selective for AA release when compared to the 85kDa PLA_2 . Furthermore, Type I and type II PLA_2 displayed a certain preference for the *sn*-1-acyl subclass of phosphatidylethanolamine versus other phospholipids. All these results imply that PLA_2 enzymes interact differently with phospholipids from natural membranes to release AA. However, this complex system must be used with caution, since complicating factors, such as the presence of transacylase enzymes in natural membranes could confuse interpretation of the results.

Q 310 CHARACTERIZATION OF A REGULATED FORM OF PHOSPHOLIPASE D IN *SACCHAROMYCES CEREVISIAE*, Krishna M. Ella, Joseph Dolan, and Kathryn E. Meier, Departments of Pharmacology and Microbiology/Immunology, Medical University of South Carolina, Charleston, SC 29425

Phospholipase D (PLD) is rapidly activated in mammalian cells in response to a variety of stimuli. In order to further define the role of PLD in cellular signal transduction, we have turned to the lower eucaryote *Saccharomyces cerevisiae*. To examine the expression of this enzyme in yeast, we used an *in vitro* assay for PLD activity that utilizes a fluorescent 1-alkyl-2-acyl-phosphatidylcholine as substrate. This assay has been successfully used to assess the state of activation of PLD in membrane preparations prepared from mammalian cells. Both haploid and diploid yeast cells expressed PLD activity that was localized to the cell membrane. The characteristics of the transphosphatidylation reaction were examined. The effects of alcohol chain length on PLD activity were similar to those previously observed for plant and mammalian forms of the enzyme. Alcohols, in addition to serving as substrates for transphosphatidylation, stimulated PLD activity. Treatment of haploid cells with pheromone had no effect on PLD activity. However, yeast PLD activity was regulated in response to nutritional status. PLD activity increased by 60% when haploid or diploid cells were grown on specific non-fermentable carbon sources, such as acetate. When diploid cells growing in acetate were deprived of nitrogen to induce sporulation, further activation of PLD was observed within 8 minutes. PLD activity remained elevated for 2-3 hours, and then declined to control values. When rich medium was added to the sporulated cells to induce germination, PLD activity decreased. These data demonstrate that *Saccharomyces cerevisiae* expresses a form of PLD that is biochemically related to that present in mammals. The sporulation-induced activation of PLD in yeast suggests that phosphatidic acid may play a role in responses of yeast and mammalian cells to changes in nutritional status.

Q 311 HUMAN ACID SPHINGOMYELINASE: PROCESSING OF THE ASM-PRECURSOR TO TWO DISTINCT CATALYTICALLY ACTIVE PROTEINS, Klaus Ferlinz, Robert Hurwitz, Gabriele Vielhaber and Konrad Sandhoff, Department of Organic Chemistry and Biochemistry, University of Bonn, Germany

Human acid sphingomyelinase (ASM) is a lysosomal glycoprotein which degrades sphingomyelin to phosphocholine and ceramide, an intermediate of several metabolic pathways and also potential second messenger. Deficiency of the enzyme causes the inborn error of sphingomyelin metabolism 'Niemann-Pick disease'. The availability of mono-specific polyclonal anti-ASM antibodies raised against recombinant protein recently permitted studies on biosynthesis and processing of the enzyme. Initial experiments using transfected COS-cells and skin fibroblasts indicated that the ASM-precursor which does not exhibit significant catalytic activity is differentially processed to two distinct molecular forms. The major portion of the precursor is converted to enzymatically active ASM after sorting for the acidic compartments. Additionally, small but markedly variable amounts of an early processing form derived from the same ASM-precursor is already generated inside the endoplasmic reticulum. This early degradation product which also contributes to catalytic activity is obviously lacking oligo-mannosyl phosphate residues and, thus, is presumably not targeted to the lysosomes. Distinct amounts of ASM-precursor as well as the early processed ASM but not the mature lysosomal form were recovered from the medium of cultured cells. A possibly regulated formation of the early processed form may point to a physiological relevance different from that of the lysosomal enzyme.

Q 312 ROLE OF Ca^{++}-INDEPENDENT ACIDIC LUNG PHOSPHOLIPASE A_2 IN DEGRADATION OF INTERNALIZED SURFACTANT PHOSPHATIDYLCHOLINE, Aron B. Fisher, Institute for Environmental Medicine, University of Pennsylvania Medical Center, Philadelphia, PA 19104.

Ca^{++}-independent PLA_2 activity with acidic pH optimum has been identified in lung tissue and localized in part to lamellar bodies, the surfactant secretory organelle of lung type II epithelium. Lung surfactant functions in the airspaces to stabilize alveoli by lowering surface tension at the air-liquid interface. Extracellular surfactant is removed from the alveolar space by epithelial cell re-uptake and degradation of internalized phospholipid with a turnover time in the rat of approximately 10 hr. MJ33, a transition state phospholipid analogue that competitively inhibits lung Ca^{++}-independent PLA_2, decreased degradation of internalized surfactant phospholipid by approximately 30% in the isolated perfused rat lung (Biochem. J. **288:** 407, 1992). We have now shown that lung surfactant protein A (SP-A), the major protein component of lung surfactant, also inhibits lung Ca^{++}-independent PLA_2 but has no effect on the Ca^{++}-dependent, alkaline pH optimum enzyme. Ca^{++}-independent PLA_2 activity of isolated lamellar bodies was inhibited by 50% with 0.25 μg SP-A/μg protein. Lamellar body PLA_2 increased 67% after inactivation of endogenous SP-A. Rat lung type II epithelial cells in primary culture were incubated with radiolabeled dipalmitoylphosphatidylcholine (DPPC) in unilamellar surfactant-like liposomes. After two hours, only 36.3 ± 0.7% (mean ± SE, n=6) of radiolabel remained in DPPC in control cells, but increased to 56.0 ± 0.4% with 5μg/ml SP-A, to 68.9 ± 0.7% with 3mol% MJ33, and to 71.6 ± 0.3% with the combination of inhibitors. These results indicate that lung Ca^{++}-independent PLA_2 plays a major role in the turnover of lung surfactant phospholipids and suggest that SP-A may be a physiologic modulator for activity of this enzyme.

Q 313 POTENT, SPECIFIC, AND CELL-PERMEABLE INHIBITORS of 14-kDa AND 87-kDa PHOSPHOLIPASES A_2: TOOLS FOR DELINEATING THE PATHWAY FOR ARACHIDONATE LIBERATION IN MAMMALIAN CELLS, Michael H. Gelb[1], Rafael Apitz-Castro[2], Ian Street[3], Fulvai Bartoli[2], Hung-Kuei Lin[1], Mahendra K. Jain[4], Timothy Bayburt[1], Farideh Ghomashchi[1], Bao-Zhu Yu[4], Sarah Kuhl[5], William R. Henderson, Jr.[5], Dennis Murphy[6].
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We have designed a number of potent, selective, and cell-permeable inhibitors of 14-kDa and 87-kDa calcium-dependent phospholipases A_2. $AACOCF_3$ is an analogue of arachidonic acid in which the COOH group is replaced by a trifluoromethyl ketone ($COCF_3$). This compound inhibits the mammalian 87-kDa phospholipase A_2 ($cPLA_2$), and the *dissociation* constant for the enzyme-inhibitor-calcium ternary complex is less than 5×10^{-5} mole fraction of inhibitor in the interface. $AACOCF_3$ is about 4-orders of magnitude less potent as an inhibitor of the mammalian type II 14-kDa secreted phospholipase A_2 ($sPLA_2$). Fatty acid amides are tight-binding and selective inhibitors of sPLA2 as are phospholipid analogues containing phosphonate-based transition-state isosteres. $AACOCF_3$ inhibits most of the arachidonate liberation in thrombin-stimulated human platelets with an IC_{50} of 5 mM (mole fraction of about 2 % in platelet membranes). None of the sPLA2 inhibitors cause detectable changes in the level of arachidonate release in platelets. $AACOCF_3$ also blocks arachidonate release in calcium-ionophore-treated rat peritoneal mast cells (IC_{50} 1 mM). A number of analogues of $AACOCF_3$ have been prepared and tested as inhibitors of the $cPLA_2$ *in vitro* and of arachidonate release in living cells. There is a strong correlation between the ability of the analogues to inhibit the purified enzyme and to reduce arachidonate release in platelets and mast cells. Taken together, the data suggest that most of the arachidonate produced in activated platelets and rat peritoneal mast cells is due to the action of $cPLA_2$.

Q 314 ARACHIDONIC ACID-SELECTIVE PHOSPHOLIPASE A_2 IS CRUCIAL IN THE CYTOTOXIC ACTION OF TUMOR NECROSIS FACTOR

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Tumor necrosis factor (TNF) exerts cytotoxic and cytostatic action against certain tumor cells and is now recognized as a cytokine with multiple biologic functions. To exert its cytotoxic action, TNF must bind to specific receptors on target cells. Although the postreceptor events that lead to the cytotoxic action have not been fully understood yet, certain kinds of inhibitors for phospholipase A_2(PLA_2) are known to reduce or block the cytotoxicity of TNF. Recently, we isolated TNF-resistant sublines from TNF-sensitive cell line L929. The most remarkable features of these TNF-resistant sublines were the defects in TNF-stimulated arachidonate metabolism. Therefore, we first compared the PLA_2 activity in TNF-resistant cells with that in L929 cells. Interestingly, a significant decrease in the activity was observed in one of the TNF-resistant sublines, C12 cells. The enzymological features, such as Ca^{2+} requirement and substrate specificity, suggested that high molecular weight arachidonoyl-selective cytosolic PLA_2 ($cPLA_2$), one of the PLA_2 isozymes, was involved in the TNF action. In practice, Northern hybridization analysis revealed that the expression level of $cPLA_2$ in C12 cells was significantly lower than that of L929 cells. Furthermore, expression of a cloned $cPLA_2$ cDNA in C12 cells increased the sensitivity of cells to the TNF cytotoxicity. These results indicate the crucial role of the high molecular weight $cPLA_2$ in the TNF-induced cytolysis.

Q 315 D-INS(1,4,5)P_3 BINDING OF PHOSPHOLIPASE Cδ,

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Using an Ins(1,4,5)P_3 affinity column, we have recently found that two proteins with a molecular mass of 130 or 85 kDa, isolated from rat brain cytosol were novel Ins(1,4,5)P_3 binding proteins. Partial amino acid sequence determinations revealed that the 85 kDa protein is the δ-isozyme of phospholipase C (PLC). The binding was less specific, compared to that of Ins(1,4,5)P_3 receptor, but the affinity was as high as 5.2 nM for Ins(1,4,5)P_3. Ins(1,4,5)P_3 at concentrations greater than 1 μM strongly inhibited the enzyme activity without changing its dependence on the concentrations of Ca^{2+} and H^+. A $PLC\delta_1$ cDNA clone from spontaneous hypertensive rat was expressed as a fusion protein with glutathione S-transferase, using bacterial expression vector pGEX2T. Bacterial lysate was applied to an Ins(1,4,5)P_3 affinity column and the eluate with a 2M NaCl solution showed a high Ins(1,4,5)P_3 binding activity, thereby demonstrating that $PLC\delta_1$ is capable of binding Ins(1,4,5)P_3. Deletion of 223 residues from the amino terminus of $PLC\delta_1$ abolished the Ins(1,4,5)P_3 binding activity, but deletion of putative catalysis domain, designated as X and Y were little effect on the binding, indicating that the N-terminus of $PLC\delta_1$ is responsible for the binding.

Q 316 DIFFERENCES IN FUNCTIONAL PROPERTIES AND INHIBITOR PROFILES OF GROUPS I, II AND III EXTRACELLULAR PHOSPHOLIPASES A_2, J R S Hoult, Isabel Benito Lobo and Morteza Taherzadeh, Pharmacology Group, King's College, London SW3 6LX, UK.

The stable extracellular 14kDa PLA_2 enzymes are grouped into three types (gps **I**,**II** and '**III**') and are implicated in inflammatory diseases as well as in the offensive action of various venoms. Most reports assume all enzymes to have very similar calcium dependency, susceptibility to PBPB and other properties. Our comparisons of *Naja* venom (gp **I**), bee venom (gp **III**) and recombinant human synovial and rat peritoneal PLA_2 enzymes (both gp **II**) show this assumption to be erroneous.

Unlike *Naja* and the gp **II** enzymes, bee venom PLA_2 was catalytically effective at notional zero calcium (calcium omission) and zero calcium (EDTA chelation); the other enzymes reached near-maximal activity at 1μM in EGTA-regulated Ca^{2+} buffers. When tested over a range of enzyme concentrations, *Naja* and bee venom PLA_2s had reduced activity at pH 5.5 compared to pH 7.5 or pH 9.5; in contrast, pH 9.5 was detrimental to the gp **II** enzymes (using ^{3}H-oleate *E. coli* membranes as substrate). Bee venom enzyme was more active against micellar ^{14}C-PE substrate, whereas *E. coli* membranes were preferred by the other 3 enzymes. PBPB (10^{-6}M-10^{-3}M) did not inhibit bee venom PLA_2 but strongly inhibited the other 3 enzymes. Indomethacin (10^{-5}M-10^{-3}M) strongly inhibited the gp **II** enzymes but not the venom enzymes.

The biochemical profiles of the low MW secretory PLA_2s need to be reassessed, so that pharmaceutical specificity related to their functional differences may be exploited.

Supported in part by a SERC studentship and Biomedical Division, KCL.

Q 317 PHOSPHORYLATION AND CALCIUM SENSITIVITY OF THE 85-KDA HUMAN RECOMBINANT PHOSPHOLIPASE A_2,
Zheng Huang, Khalid Abdullah, Brian Kennedy, Paul Payette, Wanda Cromlish, Ian Street, Michael J. Gresser, Merck Frosst, P.O.Box 1005, Pointe-Claire, Dorval, Quebec, H9R 4P8

The catalytic activities and interface affinities of partially phosphorylated cytosolic phospholipase A_2 were studied. Purified human recombinant $cPLA_2$ from the baculovirus over-expression system was phosphorylated to various degrees on several serine residues. Ser-505 was the major site of phosphorylation and over half of the over-expressed enzyme could be phosphorylated at this site based on the SDS gel-shift assay. Labeling of the Ser-505-Ala mutant $cPLA_2$ with ^{32}P from the over-expression system indicated that phosphorylation also occurred at other serine residues but in relatively lower yields compared with that at Ser-505. Fractionation of the purified wild type $cPLA_2$ with partial phosphorylation at Ser-505 on an ion-exchange column yielded a number of fractions with different ratios of de-phosphor-$cPLA_2$ and phosphor-$cPLA_2$ at Ser-505. No significant changes in catalytic activity were detected for these fractions toward a soluble substrate L-742,595 either in the presence or absence of calcium. The Ser-505-Ala mutant $cPLA_2$, under the same soluble assay condition, was about 60% as active as the wild type $cPLA_2$. When the catalytic activities were measured in the presence of a micelle interface with a lipophilic substrate L-740,490, the wild type $cPLA_2$ fractions had similar activities in the absence of calcium. However, fractions with more phosphor-$cPLA_2$ at Ser-505 had significantly higher catalytic activities than those with less in the presence of calcium. In contrast to the wild type $cPLA_2$, the Ser-505-Ala mutant had significantly decreased calcium sensitivity under the same micelle assay condition and was about 60% as active as the wild type $cPLA_2$ in the absence of calcium. Together, these findings suggest that A): single mutation at Ser-505 to alanine only slightly decreased the catalytic efficiency of the active site; B): phosphorylation of $cPLA_2$ at Ser-505 does not significantly alter its catalytic activity, instead it markedly increases $cPLA_2$'s affinity toward the micellar lipid interface in the presence of calcium.

Q 318 PHOSPHOLIPASE A2 ACTIVITY AND ITS POSSIBLE ROLE IN ROD OUTER SEGMENTS, Michèle Jacob[1], Philip K. Weech[2], Christian Salesse[1], C.R. Photobiophysique[1], Université du Québec à Trois-Rivières, Trois-Rivières, Québec, Canada, Merck Frosst Centre for Therapeutic Research[2], Pte-Claire, Québec, Canada.

The visual transduction process occurs on rod outer segments (ROS). ROS consist of a stack of disks which contain the visual pigment, rhodopsin. Following light absorption, photoexcited rhodopsin binds to and activates a G-protein called transducin. Transducin then dissociates into functional Tα and T$\beta\gamma$ subunits. Tα activates a cGMP-phosphodiesterase (PDE) and the hydrolysis of cGMP by the PDE leads to the closure of $Na+/Ca^{2+}$ cGMP-dependent channels located on the ROS cytoplasmic membrane. As a consequence, the cytoplasmic membrane hyperpolarizes. Jelsema & Axelrod have shown in 1987 (PNAS. 84: 3623) the activation of a phospholipase A2 (PLA2) by T$\beta\gamma$. The biochemical characteristics and the role of this PLA2 are still unknown. Because of the high content of polyunsaturated fatty acids in ROS membrane phospholipids and the high susceptibility of those membranes to peroxidation, PLA2 is thought to play an important role in ROS. Moreover, we found that intact ROS are absolutely required to detect a light-dependent PLA2 activity in ROS preparations. This suggests that 1) the ROS PLA2 could be involved in the phototransduction process or in a mechanism of membrane repair and 2) this PLA2 is a soluble protein or needs some cytosolic soluble factors in order to be activated. We also found that the ROS PLA2 is selective for arachidonoyl- compared to oleoyl-containing phospholipids. Now we want to identify this PLA2 and to test the effect of transducin cofactors (GTP, GTPγS, GDPβS, Mg^{2+}), Ca^{2+}, pH, oxidizing and antioxidizing agents to understand how it is regulated.

Q 319 THE EFFECT OF 1-*O*-OCTADECYL-2-*O*-METHYL-*SN*-GLYCEROL-3-PHOSPHOCHOLINE ON EGF-STIMULATED PHOSPHATIDYL 4,5-BISPHOSPHATE TURNOVER. **Gwenith Jones and Graham Carpenter, Department of Biochemistry, Vanderbilit University School of Medicine, Nashville, TN 32723.**

The alkyl-lysophospholipid, 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycerol-3-phosphocholine (ET-18-OCH_3), is a potent inhibitor of growth for certain tumors and transform cell lines. The exact mechanism by which ET-18-OCH_3 inhibits growth is unknown, nor is it known why some tumors and transformed cell lines are not effected by ET-18-OCH_3. One of the observable effects of ET-18-OCH_3 is inhibition of EGF-stimulated phosphatidyl 4,5-bisphosphate turnover. Incubation of A-431 cells for two days in the presence of 50 μM ET-18-OCH_3 results in 50% inhibition of cell growth. This concentration of ET-18-OCH_3 did not permeabilize the cells as judged by viable staining. The amount of EGF receptor was similar in both the control and ET-18-OCH_3 treated cells. ET-18-OCH_3 did not inhibit tyrosine phosphorylation of the EGF receptor by EGF. ET-18-OCH_3 inhibited the *in vitro* activity of both the tyrosine and non-tyrosine phosphorylated forms of PLC-γ1. The concentration of ET-18-OCH_3 (0.02 mol fraction) that resulted in 50% inhibition of PLC-γ1 activity was similar for both tyrosine and non-tyrosine phosphorylated enzyme. This concentration is 10 fold lower then the half-maximal concentration (0.2 mol fraction) for substrate. At concentrations >0.02 mol fraction, the amount of inhibition by ET-18-OCH_3 is greater for the non-tyrosine phosphorylated form of PLC-γ1. This data suggests that ET-18-OCH_3 inhibition of PLC-γ1 maybe responsible, in part, for its growth inhibitory properties. **This work was supported by NIH Grant CA 43720.**

Q 320CLONING, STRUCTURE AND FUNCTION ANALYSIS OF HUMAN PHOSPHOLIPASE Cδ1, Klim King, Su-Min Liu and Li-Ping Wong, Institute of Biomedical Science, Academia Sinica, Taipei 11529, Taiwan, ROC

Phosphatidyinositide-specific phospholipase C δ1 cDNA was isolated from a human aortic smooth muscle cDNA library. The cloned human cDNA encodes a protein of 776 amino acid and reveals 90% homology to rat enzyme. Southern blot analysis of the human genomic DNA probed with N-terminal, C-terminal coding region or full length cDNA, showed a single copy of gene per genom. Northern blot analysis revealed that PLCδ1 are encoded by a 3.5-kb mRNA and most abundantly expressed in pancreas, liver, lung, skeletal muscle, heart and to a less extent in brain. Using an *Escherichia coli* expression system, the cloned human PLCδ1 was expressed in *E. coli* displayed a protein size of 87 KDa and a specific activity of 0.7 and 1.2 μmole/min/mg crude extract protein to hydrolyse phosphatidylinositol and phosphatidylinositol 4,5 bisphosphate, respectively. Alignment of the deduced amino acid sequences from several cDNA clones encoding phosphatidyl inositide-specific phospholipase (PI-PLC) showed that at least 4 amino acid residues with functional side chain are highly conserved among PI-PLC from human to bacterial enzyme. Site-directed mutagenesis was used to probe the functional roles of these residues in hydrolysis of phosphatidylinositides by human PLCδ1. Conversion of Arg_{338} or of His_{356} to Leu cause a completely loss of the catalytic activity of the mutant enzymes, while conversion of Asp_{368} or of Lys_{434} to Leu reduced the specific activity of the mutant enzymes by a factor of 3. These results suggested that Arg_{338} and His_{356} are essential for the catalysis of phospholipase C.

Q 321 INHIBITION OF IL-1-INDUCED CARTILAGE DEGRADATION AND INFLAMMATION BY THE PHOSPHOLIPASE A_2 INHIBITOR, MANOALIDE

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Cartilage degradation associated with arthritis is believed to result from the production of a variety of inflammatory mediators, including interleukin-1 (IL-1). In view of recent evidence that IL-1 increases phospholipase A_2 (PLA_2) activity and mRNA expression, we tested the antiinflammatory PLA_2 inhibitor, manoalide (MLD), in an IL-1 rabbit model of arthritis. Intraarticular injection of recombinant human interleukin-1 alpha (rIL-1α) in rabbit stifle joints induced a significant accumulation of polymorphonuclear leukocytes (PMNs) and increased levels of soluble glycosoaminoglycans (GAGs) in synovial lavage fluids. These changes were also accompanied by a loss of GAGs from femoral condylar cartilage. Intraarticular injection of MLD inhibited the influx of PMNs (68% at 1mg / joint; n=6) into rIL-1α–treated joints in a dose-dependent manner. MLD (1mg / joint) also inhibited the accumulation of soluble GAGs found in lavage fluids from rIL-1α challenged joints (73%; n=5), and the loss of GAGs from femoral condylar cartilage (66%; n=6). PLA_2 activity in synovial lavage fluids was significantly increased in rIL-1α–treated joints as measured by the hydrolysis of arachidonic acid from radiolabeled *E. coli.* Pretreatment of rIL-1α–challenged joints with MLD significantly inhibited PLA_2 activity. These studies demonstrate that phospholipase A_2 is involved in the pathophysiology of IL-1-induced arthritis in rabbits.

Q 322 SEROTONIN ACTIVATES PHOSPHOLIPASE D IN RAT MESANGIAL CELLS, Doris Kurscheid-Reich, Douglas C.Throckmorton*, and Howard Rasmussen*, Max-Delbrück-Centrum für Molekulare Medizin Berlin-Buch, Germany, * Yale Medical School, New Haven , CT 06510

Serotonin (5-HT), a vasoactiv, inflammatory agent, induces proliferation and a biphasic 1,2-diacylglycerol (DAG) formation in rat mesangial cells. The present study was undertaken to examine the activation of phospholipase D (PLD) by 5-HT in these cells and to determine its contribution to DAG formation and proliferation. The formation of phosphatidylethanol (PEt) from ethanol was used as a measure of PLD activity. 5-HT induces a dose-dependent increase in PEt production with a maximal 15-fold increase being seen at 10^{-6} M. A first peak of PEt production is measured at 30 sec of stimulation, followed by a sustained phase which plateaus at 5 min and lasts at least up to 60 min. Using a different methodological approach we could show that PLD is most activ during the first 5 min, but remains activated at a lower level for up to 60 min. The occurence of a peak of PEt formation, in an experiment where cumulative amounts of the phospholipid are measured, suggests that PEt is subject to metabolic degradation. In fact, after one hour of continued stimulation with 5-HT, in the absence of the continued presence of ethanol, 76% of the PEt, formed during an initial ten minute exposure to 5-HT, is metabolized. This degradation of PEt is inhibited by propranolol, an inhibitor of the phosphatidic acid (PA) phosphohydrolase. Addition of propranolol also increases PA levels, the physiological product of PLD action, and decreases DAG levels, indicating that PA contributes to DAG formation in mesangial cells. Finally, additon of exogenous PLD induces an increase in contents of both PA and DAG. Exogenous PLD also stimulates the proliferation of mesangial cells. Its effect is to increase both the DAG content and the proliferative response to approximately thirty percent of the maximal effect induced by 5-HT. Thus, activation of PLD appears to contribute to both the production of DAG and the proliferative response of these 5-HT-treated cells.

Q 323 PHOSPHOLIPASE Cδ1 FOUND IN MEMBRANE AND CYTOSKELETON IN VASCULAR SMOOTH MUSCLE, Edward F. LaBelle and Hong Gu, Bockus Res. Inst., Graduate Hospital,Philadelphia,PA , 19146.

This study was performed in order to identify the isoforms of phospholipase C (PLC) that are involved in force development and maintenance in vascular smooth muscle. Proteins extracted from rat tail artery were transferred to nitrocellulose filters by Western blotting, and the filters probed with monoclonal antibodies to either PLC γ1, PLC β1, or PLC δ1. Both PLC γ1 and PLC δ1 were detected as single labeled bands in rat tail artery cytosol PLC δ1 was also detected in a crude membrane preparation. The MW of each isoform was the same as we observed using cytosol from calf brain as standards: 145kD for PLC γ1 and 85kD for PLC δ1. No PLC β1 was detected by this procedure in any fraction of rat tail artery. Anti PLC β1 also identified a single protein of MW 150 kD obtained from calf brain cytosol. Treatment of rat tail artery with the contractile agonist norepinephrine failed to induce the translocation of either PLCγ or PLCδ from cytosol into membranes. When a crude low speed pellet fraction of rat tail artery was extracted with Triton X100 (1%) in order to separate membrane proteins from cytoskeleton, PLCδ1 was found in both the Triton x100 supernatant and in the pellet as well, which indicated that PLCδ1 was present in both membrane and cytoskeleton, and might be responsible for force development in this tissue. (Supported by NIH Grant HL 37413).

Q 324 ACTIVATION OF PHOSPHOLIPASE D BY SERUM STIMULATION AND *ras* -INDUCED TRANSFORMATION IN NIH 3T3 CELLS

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Mitogenic stimulation of NIH-3T3 fibroblasts with growth factors or *ras* oncogenes is associated with an increase in the levels of phosphorylcholine and diacylglycerol. Both metabolites could be generated as a result of direct activation of a phosphatidylcholine-specific phospholipase C (PC-PLC) or by a more complex pathway, involving activation of phospholipase D followed by choline kinase and phosphatidic acid-hydrolase. We will present strong evidence indicating that generation of phosphorylcholine and diacylglycerol follow independent mechanisms in both serum-treated and in *ras*-transformed NIH-3T3 cells. No significant activation of a PC-PLC enzyme was observed. Instead, activation of a phosphatidylcholine-specific phospholipase D (PC-PLD) was detected. Moreover, while a 5-fold constitutive activation of the endogenous PLD activity and a 2 fold increase on the levels of phosphatidic acid were observed in *ras*-transformed cells, very small alterations on these parameters were detected at late times after serum stimulation of quiescent cells. Thus, cell proliferation induced by *ras* oncogenes in fibroblasts cells may be functionally linked to activation of a PC-PLD enzyme. The differences found in the activation of this enzyme between *ras*-transformed and normal cells may constitute an important difference in mitogenic signalling between normal and transformed cells.

Q 325 DOES LIPOCORTIN I REGULATE PHOSPHOLIPASE A_2 ACTIVITY DURING STIMULATION OF NADPH OXIDASE IN NEUTROPHILS? Rachel Levy Michaela Kaufman and Raya Dana. Laboratory of Infectious Diseases and Clinical Biochemistry Unit, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel.

Phospholipase A2 (PLA_2) inhibitors suppressed simultaneously, in a dose dependent manner, the activation of NADPH-oxidase and the release of radio labeled arachidonic acid ([3H]AA) stimulated either by 12-myristate 13-acetate (PMA) or opsonized zymosan (OZ) in human neutrophils. The dose-and time- dependent of superoxide production paralleled the release of [3H]AA induced by the aforementioned stimuli, suggesting that PLA_2 activity is needed for the induction and the maintenance of NADPH oxidase activity. The distribution of lipocortin I (LCI) in cytosol and membranes in neutrophils upon stimulation was followed in order to study whether LCI has a role in the regulation of PLA_2 during oxidase stimulation. Immunobloting analysis of LCI revealed marked differences between opsonized zymosan (OZ) and 12-phorbol 13-myristate (PMA) -stimulated neutrophils. LCI was found in cytosol of resting neutrophils. Upon activation with opsonized zymosan LCI temporarily translocates to the plasma membranes. Maximal translocation could be detected at 3 min after stimulation. In contrast, when neutrophils were stimulated with PMA, no translocation of LCI to plasma membranes was detected, suggesting a different role for LCI in cells stimulated by these agonists. Formyl-methionyl-leucyl-phenylalanine (FMLP) showed a similar pattern to that induced by OZ, but to a lesser degree. The results suggest that lipocortin is not involved in the regulation of PLA_2 activity during stimulation of NADPH oxidase in neutrophils. The difference between the effect of the various stimuli on the translocation of LCI to the membranes are attributed to the difference in the rise of intercellular calcium ions concentration $[Ca^{+2}]i$, induced by the different agents.

Q 326 ENDOTOXIN INCREASES MEMBRANE-ASSOCIATED GROUP II PHOSPHOLIPASE A_2 EXPRESSION IN RAT LUNG, Anders G. Ljungman, Christer Tagesson, and Mats Lindahl, Department of Occupational and Environmental Medicine, Faculty of Health Sciences, University of Linköping, Linköping, Sweden.

Phospholipase A_2 (PLA_2) plays an important role in the inflammatory process. This study investigated the expression of group I and II PLA_2, TNF-α and IL-1β in rat lung after *in vivo* or *in vitro* intravenous endotoxin (LPS) injection. Rats were injected with LPS (5mg/kg) and at 2, 4, and 24h the rats were anesthetised, lungs dissected free and perfused clear of blood. The lungs were then prepared for mRNA and PLA_2 activity measurements. Northern blot technique was used to determine mRNA levels. PLA_2 activity in the membrane, microsomal, and soluble fractions were assessed using *E. coli* membranes labeled with ^{14}C - oleic acid. LPS injections resulted in increased group II PLA_2 mRNA levels at 2, 4, and 24h compared to saline - injected controls. The increase was most pronounced at 4h (16 fold increase). Group I PLA_2 mRNA showed no increase. TNF-α and IL-1β mRNA levels were increased at 2 and 4h but were not detected at 24h. The increased mRNA levels of group II PLA_2 corresponded with an increase of PLA_2 activity associated with the membrane fraction, whereas the PLA_2 activity in the soluble fraction was unaffected. Rutin, a flavonol glycoside and inhibitor of group II PLA_2, totally inhibited the increased PLA_2 activity in the membrane fraction but did not affect PLA_2 activity in the soluble fraction. In isolated salt-perfused rat lungs, with LPS added to the perfusate, TNF-α and IL-1β mRNA were detected at 2 and 4h of perfusion but there was no activity increase or induction of PLA_2 mRNA. It is concluded that LPS induces group II PLA_2 mRNA formation and activity in the rat lung *in vivo* but does not affect group I PLA_2.

Q 327 REGULATION OF HUMAN MONOCYTE 85 kDa-PHOSPHOLIPASE A_2 (PLA_2) BY ANTISENSE, Lisa A. Marshall, Amy Roshak, Ganesh Sathe, Department of Inflammation & Respiratory Pharmacology, SmithKline Beecham Pharmaceuticals, King of Prussia, PA 19406

We have previously demonstrated the co-existence of two *sn*-2 acylhydrolase activities which exhibit characteristics of a Type II 14 kDa-PLA_2 and the structurally distinct cytosolic 85 kDa-PLA_2 in human monocyte cell fractions through distinguishing biochemical analysis (Marshall and Roshak, Cell Biol. Biochem., 1993). Incubation of monocytes with lipopolysaccharide (LPS) over 18 hr resulted in a time-dependent increase in prostaglandin E_2 (PGE_2) but not leukotriene C_4. LPS did not induce the extracellular release of PLA_2 activity (8-96 hrs) or alter cell-associated microsomal 14 kDa-like-PLA_2 activity or protein levels. Alternatively, an increase in 85 kDa-PLA_2 protein and activity levels accompanied the up-regulation of cyclooxygenase (COX) II over 18 hrs. Treatment with cycloheximide resulted in a time-dependent decrease in cytosolic 85 kDa-PLA_2 protein and activity ($T_{1/2}$ = 4 hrs) with no change in the microsomal PLA_2 enzyme over 18 hrs. Monocytes were therefore exposed (18 hrs) to an 85 kDa-PLA_2 initiation-site directed antisense thio oligonucleotide which decreased cytosolic PLA_2 activity and protein levels and was associated with a decrease in PGE_2 production. Co-incubation of human monocytes with scalaradial, a 14 kDa-PLA_2 inhibitor, over 18 hrs did not inhibit LPS-induced PGE_2 accumulation. Taken together, these data suggest a role for the cytosolic 85 kDa-PLA_2 in monocyte PGE_2 formation.

Q 328 THE CALCIUM DEPENDENT LIPID BINDING DOMAIN OF CYTOSOLIC PHOSPHOLIPASE A_2 REVERSIBLY ASSOCIATES WITH MEMBRANES IN A PROTEIN INDEPENDENT MANNER, Eric A. Nalefski, Lisa A. Sultzman, Paul Towler, John L. Knopf and James D. Clark. Small Molecule Drug Discovery Group, Genetics Institute, Cambridge, MA, 02140

The 85-kDa cytosolic phospholipase A_2 ($cPLA_2$) preferentially catalyzes the generation of arachidonic acid from the *sn*-2 position of phospholipids. Translocation of $cPLA_2$ to natural membranes is Ca^{2+}-dependent and has been proposed to be mediated by an N-terminal domain of $cPLA_2$, termed the Ca^{2+}-dependent lipid binding (CaLB) domain, that shares considerable sequence similarity with protein kinase C (PKC) and synaptotagmin. A truncated $cPLA_2$ comprising the N-terminal domain, which includes the CaLB domain but lacks the presumptive catalytic domain, translocates to natural membranes in vitro at Ca^{2+} concentrations that cause translocation of full-length $cPLA_2$. A fusion protein containing the N-terminal fragment placed at the C-terminus of the maltose-binding protein (CaLB-MBP) also translocates to natural membranes in a Ca^{2+}-dependent manner, demonstrating that the N-terminal fragment of $cPLA_2$ can confer Ca^{2+}-dependent binding to natural membranes in a position-independent manner. CaLB-MBP binds to synthetic liposomes at Ca^{2+} concentrations that cause translocation of $cPLA_2$ to natural membranes, demonstrating that $cPLA_2$ CaLB domain association with phospholipids does not require other membrane proteins. This translocation is insensitive to the phosphatidy-lserine content of liposomes, demonstrating the striking difference in the phospholipid preference between $cPLA_2$ and other proteins that contain the CaLB domain, including certain isoforms of PKC and synaptotagmin. These results demonstrate that residues within the $cPLA_2$ N-terminal fragment containing the CaLB domain are responsible for the Ca^{2+}-dependent binding of $cPLA_2$ to phospholipids and that this fragment can act in a position-independent manner. This identifies CaLB as a common protein motif found in proteins involved in Ca^{2+}-dependent signal transduction and vesicular transport.

Q 329 PROTEIN KINASE C-DEPENDENT AND -INDEPENDENT PATHWAYS OF MITOGEN-ACTIVATED PROTEIN KINASE ACTIVATION IN MOUSE PERITONEAL MACROPHAGES BY STIMULI THAT ACTIVATE 85 kDa PHOSPHOLIPASE A_2 Zhihua Qiu and Christina C. Leslie, Department of Pediatrics, National Jewish Center for Immunology and Respiratory Medicine, Denver, CO 80206

In mouse peritoneal macrophages, arachidonic acid (AA) release is induced by diverse agonists including yeast cell walls, zymosan, the protein kinase C (PKC) activator, PMA, the phosphatase inhibitor, okadaic acid, and calcium ionophore, A23187. These agents also induce an increase in activity of the 85 kDa cytosolic phospholipase A_2 (PLA_2) that is due to enhanced serine phoshorylation of the enzyme. Since the 85 kDa PLA_2 has been shown by others to be phosphorylated and activated by MAP kinase in vitro, experiments were carried out to determine if these agonists activate MAP kinase, and to evaluate the involvement of PKC in MAP kinase activation in macrophages. PMA and zymosan maximally stimulated MAP kinase activity against an EGF receptor peptide substrate measured in whole cell lysates by 5 min and 15 min respectively. MAP kinase activation in response to okadaic acid was delayed and was first evident by 30-45 min and maximal by 60-90 min. The increased MAP kinase activity correlated with increases in tyrosine phosphorylation of p44 MAP kinase in PMA stimulated cells, and p44 and p42 MAP kinases in zymosan and okadaic acid-stimulated cells. A23187 did not induce an increase in MAP kinase activity or tyrosine phosphorylation of p42 or p44 indicating that the enhanced activity and increased phosphorylation of the 85 kDa PLA_2 in A23187 stimulated macrophages is not due to MAP kinase. Treatment of the macrophages with the PKC inhibitor, GF109203X was found to completely suppress PMA-induced AA release, partially inhibit (50%) zymosan-induced AA release, but have no effect on okadaic acid or A23187-stimulated release. The inhibitor suppressed PMA- and zymosan-induced MAP kinase and PLA_2 activation, but had no effect in okadaic acid treated cells suggesting that PMA and zymosan stimulation of MAP kinase requires PKC activation, whereas, okadaic acid stimulation of MAP kinase is PKC-independent. The results are consistent with a role for MAP kinase in activation of the 85 kDa PLA_2 in zymosan, PMA and okadaic acid stimulated macrophages.

Q 330 COMPARISON OF ABSOLUTE AND RADIOLABELED ARACHIDONATE RELEASE FROM MACROPHAGES STIMULATED WITH TANNIN AND β-1,3-GLUCAN. Nicholas V.C. Ralston and Michael S. Rohrbach, Thoracic Diseases, Mayo Medical Center, Rochester MN. 55905

While radiolabeled fatty acid incorporation and measurement of its subsequent release is a standard method of studying the response of the alveolar macrophage to agonist stimulation, it has never been verified whether this method provides an accurate reflection of the total arachidonic acid pool activity. In order to determine the validity of studies performed with radiolabeled arachidonic acid probes, we directly compared radiolabel release with the actual release of arachidonate measured via a fluorescent derivatization method. Although significant amounts of arachidonate were released following both tannin and glucan exposure, radiolabeled arachidonate was released to a two to three fold greater extent and more rapidly than the endogenous arachidonate pool it was intended to represent. These differences indicate the recently incorporated arachidonate localizes in a more reactive pool prior to attaining isotopic equilibrium with the total metabolic pool. The phospholipase A activity stimulated by glucan was not specific for release of fatty acid from the 2-acyl position and actually caused greater release of fatty acids from the 1-acyl position. Meanwhile, tannin, which functions through reacylation inhibition, caused a specific release of arachidonate. Although we find radiolabel release is not necessarily reflective of the absolute levels of actual release, it is still a sensitive parallel indication of fatty acid kinetics.

Q 331 PRIMING OF PHOSPHOLIPASE A_2 IN HUMAN NEUTROPHILS INVOLVES TYROSINE KINASE(S). Roger W. Randall, Neil T. Thompson and *Lawrence G. Garland. Biochemical Sciences and *Research Directorate, Wellcome Research Laboratories, Beckenham, Kent, BR3 3BS, U.K.

Normal peripheral neutrophils are poorly responsive to stimulatory agents such as fMet-Leu-Phe. However, if neutrophils are pre-treated with certain cytokines such as Granulocyte-Macrophage-Colony Stimulating Factor (GM-CSF) or tumour necrosis factor alpha (TNF alpha), their responses to subsequent stimulation are greatly enhanced. This phenomenon is termed priming and is associated with increased toxic effects and neutrophil-dependent tissue damage in certain inflammatory conditions (1). We have characterised the priming of human neutrophils by GM-CSF and find that the enhanced fMet-Leu-Phe-stimulated superoxide release in GM-CSF-primed cells is closely correlated with increases in both phospholipase A_2 and phospholipase D activation. Thus, both the concentration of GM-CSF and the time of pre-incubation required for priming are similar for all three responses.

We have used the tyrosine kinase inhibitor ST271 to explore the role of tyrosine kinases in these GM-CSF-primed responses and, as we have previously demonstrated for phospholipase D (2), we now find that tyrosine phosphorylation is also involved in the activation of phospholipase A_2 in human neutrophils. Thus primed phospholipase A_2 and phospholipase D activities are equally sensitive to inhibition by ST271. Inhibition of both phospholipases is partial, with only the primed part of the activity being blocked. These results suggest that the mechanisms of priming of phospholipase A_2 and phospholipase D in human neutrophils are similar and that the process involves tyrosine kinase(s).

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Q 332 IMPORTANCE OF INOSITOL HYDROXYL GROUPS FOR BACTERIAL PHOSPHATIDYLINOSITOL-SPECIFIC PHOSPHOLIPASE C, Mary F. Roberts, Chun Zhou, and Venkat Garigapati, Merkert Chemistry Center, Boston College, 2609 Beacon Street, Chestnut Hill, MA 02167

The importance of inositol hydroxyl groups in phosphatidylinositol has been examined for several phosphatidylinositol-specific phospholipase C enzymes and in greater detail for the PI-PLC from *Bacillus thuringiensis*. Analogs of PI with modifications at the C'2 position (including -OCH_3, -F, and -dihydro) have been synthesized, characterized by NMR (tentative structures proposed on the basis of NOE measurements combined with molecular modeling), and used to investigate the binding of these lipid analogs to PI-PLC. While none of these lipids are substrates (modification or removal of the free -OH group prevents the phosphotransferase reaction which is the first step in the catalytic cleavage of PI by this enzyme), there is a gradation in inhibition potency which can be correlated with the PI analog structure. Other specific hydroxyl positions around the ring have also been modified in an attempt to define PI moieties critical for binding to the enzyme and to produce a specific PI-PLC inhibitor. The same series of modified PI molecules can also be used to determine enzymatic specificity of other enzymes involved in PI cycling.

Q 333 REGULATION OF PHOSPHOLIPASE A2 BY GM-CSF.
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Phospholipase A2 (PLA2) is the rate-limiting enzyme for the production of inflammatory mediators and may be involved in the process of proliferation and differentiation in immature cells. Arachidonate (AA) generated by PLA2 in mature human neutrophils is rapidly metabolized to 5-HETE and leukotrienes by 5-lipoxygenase (5LPO). In order to study the regulation of PLA2 by granulocyte-macrophage colony-stimulating factor (GM-CSF) independently of 5-LPO activity in intact cells, a radiometric assay was devized that measured the release of [3H]AA from intracellular stores (principally phosphatidylinositol and phosphatidyl-choline). Metabolism of AA by 5LPO was inhibited by the 5LPO-activating protein inhibitor, MK886 (Gillard *et al.* Can. J. Physiol. Pharmcol. 67:456), and re-esterification of AA was reduced by binding to extracellular fatty acid-free BSA. Control experiments showed that 400nM MK886 did not inhibit PLA2 activity and 1mg/ml BSA minimally increased the IC50 of MK886 from 12 to 16 nM. All of the AA that was metabolized to leukotrienes in the absence of MK886 was recovered in the extracellular medium as AA in the presence of MK886.

GM-CSF enhanced or 'primed' the release of AA from neutrophils stimulated with calcium ionophore, A23187, by 624% ± 122, (±1SEM, n=10). When neutrophils were stimulated with GM-CSF+A23187, enhanced PLA2 activity above the level stimulated by A23187 alone was detected within one min. Maximal priming of PLA2 activity was complete within 5 min preincubation with GM-CSF (10ng/ml), thus PLA2 priming was rapid compared with maximal priming of the respiratory burst which required 30-45 min.

The sensitivity of both the unprimed and GM-CSF primed PLA2 activity to pharmacological agents was compared. *Bordatella pertussis* toxin, whilst inhibiting the G-protein dependent pathway of chemotactic factor stimulation by 100%, did not inhibit either primed or unprimed PLA2 activity. Both the unprimed and primed response were inhibited by bromophenacyl bromide and the isoquinoline kinase inhibitor H7. In contrast, genestein which inhibited neutrophil tyrosine kinase activity, did not inhibit unprimed PLA2 activity, but completely blocked the priming effect of GM-CSF.

In conclusion, the data show that priming of PLA2 activity by GM-CSF is very rapid, is not dependent on the activity of a pertussis toxin sensitive G-protein, and support the notion that the signal transduction pathway of GM-CSF requires the activity of a tyrosine kinase.

Q 334 Differential calcium-dependent membrane and phospholipid-binding by annexins
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Annexins are a super-gene family of proteins that share the ability to bind to membranes and phospholipids in a calcium-dependent manner[1,2]. They differ from conventional calcium-binding proteins in that they lack the classical EF hand structure exemplified by calmodulin and parvalbumin, instead they have a highly conserved calcium-binding motif formed through the tertiary folding of a 70 amino acid domain characteristic to this group of proteins[3].

There have been many studies on the calcium-dependent interaction of annexins with membranes, results showed that they self-associate on the membrane, although quantitatively, they differ in their effectiveness as membrane aggregators[4]. In this paper, we report the over-expression of annexin VI and annexin V as fusion proteins in *E. Coli*, and the study of their binding characteristics to different phospholipids. We have also investigated the binding properties of the annexins to plasma-membranes prepared from Swiss 3T3 fibroblasts, and those from A431 cells, which do not express annexin VI. The results and implications of the differential binding of annexins to phospholipids and membranes will be discussed.

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Q 335 'PRIMING' OF $cPLA_2$ IN FIBROBLASTS : EFFECT ON ACTIVITY, LOCATION AND PHOSPHORYLATION STATE,
Allison Stewart and Michael J.O. Wakelam, Birmingham Institute for Cancer Studies, University of Birmingham, England, B15 2TT.

fMet-Leu-Phe-stimulated $cPLA_2$ activity in differentiated HL-60 cells (dHL60's), as measured by arachidonic acid release, requires priming of the cells. Substances such as cytochalasin B are widely used as priming agents, however it appears that there is a component of newborn calf serum (NBCS) which can act in a similar manner. When Rat-1 fibroblasts were cultured in 10% NBCS-containing media, as compared to those cultured at 1% NBCS, the onset of lysophosphatidic acid (LPA)-stimulated arachidonate release was most rapid in the 10% serum cultured cells. Western blot analysis using an anti-peptide anti-$cPLA_2$ antibody, suggested only dephosphorylated $cPLA_2$ could be detected in the cytosol in both control and LPA-stimulated conditions, with the phosphorylated form being detected at the membrane. However, less phosphorylated $cPLA_2$ was detected at the membrane in non-stimulated, serum starved cells, than in the corresponding cells in normal cell culture conditions. This suggests $cPLA_2$ 'priming' is due to both phosphorylation and relocation of the enzyme to the membrane.

Q 336 REGULATION OF THE 85 kDa PHOSPHOLIPASE A_2 AND THE EXPRESSION OF PROINFLAMMATORY CYTOKINES IN MACROPHAGES, Ulf Svensson, Roger Sundler and Karin Gäfvert, Department of Medical and Physiological Chemistry, Lund University, P. O. Box 94, S-221 00 Lund, Sweden

There is now considerable evidence that the 85 kDa, intracellular phospholipase A_2 (PLA_2-85) is responsible for the mobilization of the eicosanoid precursor arachidonate in mouse macrophages. Exposure of these cells to certain bacteria cause arachidonate mobilization as well as an increase in the phosphorylation and catalytic activity of PLA_2-85, via a protein-kinase-C-independent pathway. This persistent activation of PLA_2-85 was further promoted by the Ser/Thr protein phosphatase inhibitor okadaic acid, the effect of which was also independent of protein kinase C.
It is well known since long that glucocorticoids inhibit the formation of eicosanoids and that inhibition of arachidonate mobilization accounts for this effect. Treatment of mouse peritoneal macrophages with $\geq 10^{-8}$ M dexamethasone caused a progressive reduction of the mobilization of arachidonate in response to bacteria. Further expriments showed that this was due to (*i*) a reduction in the level of PLA_2-85 down to approximately 35% of control and (*ii*) an impaired signalling to activation of PLA_2-85. However, the latter effect could be overcome by okadaic acid.
Dexamethasone also down-regulated the bacteria-induced expression of TNFα mRNA, but much less so the expression of IL-1β mRNA. Interestingly, okadaic acid alone induced TNFα mRNA, but not IL-1 β mRNA. These results indicate that there are similarities in the intracellular signalling to activation of PLA_2-85 and to the expression of TNFα mRNA.

Q 337 ISOLATION OF THE PROMOTER FOR THE CYTOSOLIC PHOSPHOLIPASE A2 (cPLA2) GENE IN RAT AND HUMAN, Agnes Tay, Peter Maxwell, Ken Hamel and Karl Skorecki, Division of Nephrology, University of Toronto, Canada
To isolate the promoter for rat cPLA2, a rat genomic library was initially screened using a probe derived from the most 5′ end of murine cPLA2 cDNA. Mapping and sequencing of positively hybridizing phage clones revealed small exons flanked by large introns. In view of the large first intron, 5′Rapid Amplification of cDNA Ends (5′ RACE) with rat mesangial cell RNA was performed to obtain the sequence of the non-coding first exon. Screening of the same genomic library witha probe based on the sequence from 5′ RACE yielded one positively hybridizing phage clone, within which the first exon and 5′ flanking sequences were identified. There was marked similarity between mouse, rat and human sequences with conservation of amino acids in the fragments studied. In addition, multiple CA repeats were found in the promoter. To ascertain if CA repeats are also present in the human gene, the polymerase chain reaction (PCR) was used to isolate the human promoter. Sequencing of the 200-bp fragment obtained confirmed similarity to rat promoter, and the presence of 18 CA repeats. PCR-based screening of somatic cell hybrids mapped the human cPLA2 gene to chromosome 1.

Q 338 INSULIN-LIKE GROWTH FACTORS 1 and 2 INHIBIT INTERLEUKIN 1β-STIMULATED ARACHIDONIC ACID RELEASE AND INTERLEUKIN 1β-INDUCED TYPE II PLA2 GENE EXPRESSION IN RABBIT ARTICULAR CHONDROCYTES. G. THOMAS[1], F. BERENBAUM[1], S. POIRAUDEAU[2], M.T. CORVOL[2], G. BEREZIAT[1] and J. MASLIAH[1]. [1]-URA CNRS 1283 CHU St Antoine PARIS (France) [2]-U30 INSERM Hôpital Necker Paris (France).
Chondrocytes have been recently proposed to be the main source of type II PLA_2 secreted in high amounts in synovial fluid during inflammatory processes. Interleukin 1β (IL1β) is a pro-inflammatory cytokine known to induce cartilage destruction and to increase prostanoïd synthesis in chondrocytes. In contrast IGF_s have been shown to increase cartilage synthesis. In primary culture of articular rabbit chondrocytes, we have tested the effect of IL1β and Insulin-like-growth factors (IGF_s) on cytosolic and secreted PLA_2 activities as well as their respective mRNA level, in parrallel with their effect on arachidonic acid (AA) release.
Our results have clearly shown that in chondrocytes a strong dose-dependent-increase of AA release. In contrast, IGF_1 and IGF_2 both reduced the basal and the IL1β-stimulated AA release in these cells. This effect of IGF_s on IL1β stimulation was paralleled with the induction of type II PLA_2, as evidenced by the appearance of its mRNA in northern analysis, and of its enzymatic activity in the culture medium. No secreted PLA_2 activity and no mRNA expression could be detected in non stimulated chondrocytes. The secreted PLA_2 activity was profoundly reduced by IGF_s but the mRNA level was only slightly reduced, suggesting an action at the post-transcriptional level. Non stimulated chondrocytes exhibited an arachidonyl-specific-cytosolic PLA_2 activity and the PLA_2 mRNA could be detected by northern analysis. However, neither IL1β nor IGF_s were able to affect this specific PLA_2 activity and the $cPLA_2$ mRNA level. In contrast with other cell types, $cPLA_2$ seems not to be involved in IL1β-stimulated AA release in rabbit articular chondrocytes. Furthermore a new role for IGF_s, in counteracting inflammatory processes, might be suggested since they inhibit IL1β-stimulated AA release and IL1β-induced type II PLA_2 gene expression. Additional experiments are needed to elucidate the link between AA release and type II PLA_2 secreted by these cells.

Q 339 AN ESSENTIAL ROLE FOR PHOSPHATIDYL-INOSITOL TRANSFER PROTEIN IN INOSITOL LIPID SIGNALLING.

Geraint Thomas, Emer Cunningham, Andrew Ball, Amanda Fensome and Shamshad Cockcroft.

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Using HL60 cells depleted of their cytosol by protracted Streptolysin-O permeabilisation we have demonstrated that cytosolic proteins are reqiured for efficient inositol lipid hydrolysis following G-protein activation (1). Two soluble Phospholipase C's (PLC's) were implicated as the required proteins. One was identified as either PLC-ß2 or -ß3 and could be replaced in a reconstitution system, based on the permeabilised cells, by soluble mammalian PLC-ß1. More interestingly this same reconstitution protocol highlighted a partially purified rat brain PLC that appeared to provide a greater restoration of activity. On further investigation the reconstituting activity could be separated from the PLC and was found to be uniquely associated with a 35KDa protein. Peptide micro-sequencing, western blotting and re-purification with an appropriate assay showed unambiguously that this protein was the well characterised mammalian phosphatidylinositol transfer protein (PI-TP)(2). This finding can be successfully reconciled with the known properties of PI-TP *in vitro* if the substrate initially available to the activated PLC is limited and PI-TP transports PI from the site of synthesis to that of utilisation. This is the most parsimonious interpretation and the role of PI-TP may be more interesting and potentially open to regulation by phosphorylation.

(1) Thomas et al., EMBO J. **10**, pp 2507-2512 (1991).
(2) Thomas et al., Cell **74**, pp 919-928 (1993).

Q 340 THE ROLE OF TYROSINE KINASES IN THE ACTIVATION OF PHOSPHOLIPASE D IN PRIMED HUMAN NEUTROPHILS Neil T. Thompson, Roger W. Randall and *Lawrence G. Garland. Biochemical Sciences and *Research Directorate, Wellcome Research Laboratories, Beckenham, Kent, BR3 3BS UK.

fMet-Leu-Phe activates phospholipase D (PLD) in human neutrophils to a small extent by a mechanism which is not sensitive to tyrosine kinase inhibitors such as ST271. Pre-treatment of the neutrophils for 30 min with GM-CSF or TNFα, which themselves do not activate PLD, greatly potentiates the capacity of fMet-Leu-Phe to activate this phospholipase. This process, termed priming, is associated with potentiation of the cytotoxic effects of neutrophils. fMet-Leu-Phe-stimulated PLD in GM-CSF primed cells is inhibited by ST271, but only that portion of the PLD activity which is dependent on the priming agent is blocked. Thus, tyrosine kinase(s) appear to be particularly important in the enhanced PLD activity in primed cells. The inhibitor ST271 does not need to be present during the priming process but must be present during the action of fMet-Leu-Phe. This suggests that the kinase is not part of the **priming** signal but rather part of the **primed, fMet-Leu-Phe-dependent** signal. This is supported by several observations 1) Pervanadate, an inhibitor of protein tyrosine phosphatases, activates PLD but does not prime the activation of PLD to a subsequent addition of fMet-Leu-Phe. 2) fMet-Leu-Phe stimulates protein tyrosine phosphorylation and this is potentiated in GM-CSF-primed cells.

The fMet-Leu-Phe receptor activates PLD via a pertussis toxin sensitive G-protein (1). This process is mimicked in a receptor-independent manner by NaF which activates heterotrimeric G-proteins directly. NaF-stimulated PLD is also inhibited by ST271. Evidence suggests, therefore, that fMet-Leu-Phe activates PLD via a G-protein dependent tyrosine kinase and that this kinase is responsive to fMet-Leu-Phe only in primed cells. The mechanism of action of the priming agent, GM-CSF, is currently unknown.

1. Agwu et al, (1989) J. Biol. Chem. 264, 1405-1413.

Q 341 DEXAMETHASONE SUPPRESSES GROUP II PHOSPHOLIPASE A_2 IN RAT MESANGIAL CELLS BY TWO DISTINCT MECHANISMS; EVIDENCE AGAINST INVOLVEMENT OF ANNEXINS, Margriet J.B.M. Vervoordeldonk, Casper G. Schalkwijk, Josef Pfeilschifter and Henk van den Bosch, Centre for Biomembranes and Lipid Enzymology, Utrecht University, The Netherlands.

Interleukin-1β (IL-1β) and forskolin have been shown to induce the synthesis and secretion of group II PLA_2 in rat mesangial cells in parallel to the synthesis of PGE_2. These syntheses and secretions of both group II PLA_2 and PGE_2 can be prevented by pre-treatment of the cells with dexamethasone. Western blot analysis implicated that this suppression is caused by direct inhibition of PLA_2 protein expression. However, a contribution of the glucocorticoid-induced anti-PLA_2 proteins, termed annexins, can not be ruled out *a priori*. We observed that under conditions where 14 kDa group II PLA_2 activity- and protein-levels were dramatically affected by cytokines and dexamethasone, the levels of the annexins investigated were not changed. By Western blotting and immunofluorescence studies no evidence was found for dexamethasone-induced translocation of annexins from the cytosol to membranes. Thus, also a possible sequestering of substrates for PLA_2 by annexins could be ruled out and the inhibition of PLA_2 activity after dexamethasone treatment appears to be solely caused by suppression of enzyme synthesis. Thereafter, we examined the effect of dexamethasone on PLA_2 gene-expression in IL-1β- and forskolin-stimulated mesangial cells. Northern blots showed that IL-1β-induced group II PLA_2 mRNA is hardly affected by dexamethasone, whereas the forskolin-induced elevation is completely blocked by this glucocorticosteroid. These results indicate that dexamethasone can suppress both mRNA synthesis and post-transcriptional expression of group II PLA_2 depending on the stimulus used.

Q 342 HIGH MOLECULAR WEIGHT PHOSPHOLIPASE A_2 IN HUMAN NEUTROPHILS: IDENTIFICATION AND STUDIES ON ITS REGULATION, Jonny Wijkander and Robert L. Wykle, Department of Biochemistry, Wake Forest University Medical Center, Winston-Salem, NC 27157

The cytosol of human neutrophils (PMN) was found to contain a phospholipase A_2 (PLA_2) which preferentially hydrolyzed arachidonate-containing phosphatidylcholine. The PLA_2 required submicromolar Ca^{2+} for activity and was active in the neutral to alkaline pH-range with no activity at pH below 5.5. Upon gel chromatography the PLA_2 activity eluted corresponding to a Mr of 70 kDa. These characteristics are in agreement with those shown for the cytosolic 85 kDa PLA_2. A marked improved linearity of hydrolysis with time was seen when either bovine serum albumin (BSA) plus dithiothreitol or reduced BSA was included in the assay mixture.

Stimulation of PMN with either phorbol myristate acetate (PMA) or N-formylmethionyl-leucyl-phenylalanine (FMLP) resulted in a 2-fold increase in the PLA_2 activity. Pretreatment of PMN with staurosporine abolished the PMA-induced activation of PLA_2, while the FMLP response was only marginally affected. Stimulation of PMN with calcium-ionophore A23187 alone, resulted in no persistent activation of the PLA_2, furthermore, it resulted in substantial reduction of the response induced by PMA and FMLP. The A23187-induced reduction of the PMA and FMLP response could, to a large extent, be prevented by pretreatment with cyclosporin A.

Q 343 PROTEIN KINASE C α MEDIATES PHOSHOLIPASE D ACTIVATION BY NUCLEOTIDES AND PHORBOL ESTER IN MADIN-DARBY CANINE KIDNEY CELLS. STIMULATION OF PHOSPHOLIPASE D IS INDEPENDENT OF ACTIVATION OF POLYPHOSPHOINOSITIDES-SPECIFIC PHOSPHOLIPASE C AND PHOSPHOLIPASE A_2 María A.Balboa, Bonnie L. Firestein, Catherine Godson, Kelly S. Bell, and Paul A. Insel. *Department of Pharmacology 0636, University of California at San Diego, La Jolla, CA 92093-0636.*

Protein kinase C (PKC) has been implicated in the activation of phospholipase D (PLD) in a number of systems. By antisense technology, we have "knocked out" α and ß isoforms of PKC to study the role of these isoforms in PLD activation in Madin Darby canine kidney (MDCK) cells. To this end, we have studied PLD activation by phorbol 12-myristete,13-acetate (PMA), ATP, UTP and 2-Methylthio-ATP in cells labelled with [3H]palmitic acid. [^{3}H]PEt production catalized by PLD in the presence of ethanol was time and concentration dependent in PMA- and nucleotide-stimulated cells. In Ca^{2+}-free medium, [^{3}H]PEt accumulation was diminished for all stimuli assayed. Treatment of cells with chelerytrine, an inhibitor of PKC, and phorbol-ester down-regulation of PKC, inhibited [^{3}H]PEt production by both PMA and nucleotides. In cells transfected with antisense PKC_α or both PKC_α and PKC_β, PLD activation was inhibited by both PMA and nucleotides, whereas in cells transfected with antisense PKC_β, PLD activation was similar to that of control cells. Moreover, inhibition of polyphosphoinositide specific PLC (by neomycin) or of release of arachidonic acid and arachidonic acid metabolites (by nordihydroguaiaretic acid or by indomethacin) failed to decrease [^{3}H]PEt accumulation in PMA- and nucleotide-stimulated MDCK-D1 cells. From these data, we conclude that in MDCK-D1 cells PMA and nucleotide receptors utilize PKC_α to regulate PLD activity, and that PLD activation is independent of the activation of polyphosphoinositide specific PLC and PLA_2-mediated release of arachidonic acid or arachidonic acid metabolites.

(NIH, GM 31487 and HL 35018).

Pharmacological/Animal Models for Disease; Inhibitors/Drugs; Phospholipase and Sphingolipid Regulation

Q 400 DIFFERENT PHOSPHOLIPASES, PHOSPHOLIPID SUBSTRATES AND PKC ISOZYMES MEDIATE PHASIC CONTRACTION OF ESOPHAGEAL CIRCULAR MUSCLE AND LOWER ESOPHAGEAL SPHINCTER (LES) TONE. P. Biancani, U. D. Sohn, C. Hillemeier*, K.N. Bitar*, and J. Behar. R.I. Hospital & Brown Univ., Providence RI, *Univ. of Michigan, Ann Arbor, MI

Esophageal circular muscle is normally relaxed, and contracts phasically in response to neural (cholinergic) stimuli. In contrast LES circular muscle maintains spontaneous (myogenic) tone and relaxes in response to neural (non adrenergic-non cholinergic inhibitory) stimuli. We examined intracellular mediators of esophageal contraction and LES tone in intact muscle and in single cells isolated by enzymatic digestion. We found that spontaneously elevated LES tone, is associated with elevated $Ins(1,4,5)P_3$ formation and diacylglycerol (DAG) levels produced by spontaneous but submaximal activity of phosphatidyl-inositol specific phospholipase C, and that they decrease in response to the phospholipase C antagonist U-73122. We therefore tested submaximal doses of $Ins(1,4,5)P_3$ and DAG in permeabilized LES muscle cells and found that they act synergistically, their interaction depends on calcium release and is mediated through a PKC-dependent pathway. DAG induced contraction of LES muscle cells permeabilized by saponin was inhibited by preincubation with antiserum against the Ca^{++}-dependent PKCβ, but not by antisera raised against other PKC isozymes. In contrast esophageal contraction in response to cholinergic stimuli depends on influx of extracellular Ca^{++}, since it is abolished by Ca^{++} channel blockers and in the absence of extracellular Ca^{++}. Ca^{++} influx is required to activate multiple phosphatidylcholine specific phospholipases, namely phospholipase D, C, and the cytosolic phospholipase A2. DAG and arachidonic acid, produced by these phospholipases interact synergistically to activate a PKC-dependent pathway. DAG induced contraction of esophageal muscle cells permeabilized by saponin was inhibited by preincubation with antiserum against the Ca^{++}-independent PKCε, but not by antisera raised against other PKC isozymes. We conclude that different types of contractile activity in the esophagus and LES are mediated by different phospholipases, phospholipid substrates and PKC isozymes. Sustained LES tone is mediated by phosphatidylinositol specific phospholipase C and by the Ca^{++}-dependent PKCβ, while phasic contraction of esophageal muscle is mediated by phosphatidylcholine specific phospholipase C, phospholipase D and cytosolic phospholipase A2, and by the Ca^{++}-independent PKCε.

Q 401 SHORT CHAIN CERAMIDES REGULATE THE RESPIRATORY BURST IN HUMAN ADHERENT NEUTROPHILS. Laurence A. Boxer,Tadashi Nakamura, Akira Abe, Suzanne J. Suchard, and James A. Shayman. Departments of Internal Medicine and Pediatrics, University of Michigan, Ann Arbor, MI 48109-0676

Recent studies have demonstrated potentially important roles for sphingolipids such as sphingosine and ceramide on cell signalling. The effects of soluble short chain sphingolipids on adherent neutrophils were studied. H_2O_2 generation in formyl-Met-Leu-Phe (fMLP)-stimulated adherent human neutrophils was suppressed in a concentration-dependent manner by several free sphingoid amines and short chain ceramides. Sphingosine, dihyrosphingosine, phytosphingosine, *N*-acetylsphingosine and *N*-acetylphytosphingosine, but not *N*-acetyldihyrosphingosine inhibited formyl peptide stimulated oxidant production. The half maximal inhibitory concentrations of *N*-acetylsphingosine and *N*-acetylphytosphingosine were 0.51 μM and 0.38 μM respectively. Sphingosine, dihyrosphingosine and phytosphingosine were less potent inhibitors with half maximal inhibitory concentrations of 1.78 μM, 15.4 μM and 1.48 μM respectively. The 4β-phorbol 12β-myristate 13α-acetate-induced respiratory burst was inhibited by 5 μM of sphingosine but not 5 μM of *N*-acetylsphingosine. The effects of *N*-acetyl conjugated sphingols (C_2 ceramides) on phosphatidylcholine-specific phospholipase D (PLD) and phosphatidic acid phosphohydrolase were markedly different than the effects of the related sphingoid bases. Both C_2 ceramides and sphingoid bases partially inhibited the diradylglycerol formation via PLD pathway. Under the same conditions, however, *N*-acetyldihyrosphingosine and dihydrosphingosine failed to suppress H_2O_2 generation in fMLP-stimulated neutrophils. The effect of ceramide in inhibiting the respiratory burst is structurally specific since either a 4,5-*trans* double bond or 4-hydroxy group is required for the suppression of oxidant generation. These findings demonstrate that C2 ceramides inhibit H_2O_2 generation in fMLP-stimulated neutrophils via PKC- or sphingoid base-independent mechanisms. Ceramides are potential candidates for the cellular regulation of oxidant formation.

Q 402 SECOND MESSENGERS, CALCIUM AND THE REGULATION OF BILE FLOW IN NORMAL AND CHOLESTATIC RAT LIVER, Fyfe L Bygrave and Ari Karjalainen, Division of Biochemistry and Molecular Biology, Faculty of Science, Australian National University, Canberra, ACT 0200, Australia

Bile flow is an integral component of the enterohepatic circulation. How such flow is controlled is not fully understood. Recent work has provided indications that certain metabolic hormones that mobilise calcium might be involved but the mechanism(s) by which this occurs is not clear.

Glucagon and vasopressin have profound (synergistic) effects on bile flow when co-administered to the perfused rat liver[1]. The response is one that results in an enhanced flow of bile that peaks by approx. 40s and begins to decay immediately thereafter to less than basal rates by approx. 3 min following administration of the hormones. The effects are observed by concentrations of the hormones as low as 10^{-10} M, suggesting that the events might be of physiological significance. Acute pre-administration of cholestatic and choleretic bile salts for 10 min. attenuates and enhances, respectively, the initial hormone-induced transient response[2]. In addition, when cholestasis is induced in rats with ethynylestradiol, the response is also attenuated. By concomitantly monitoring the changes in perfusate (extracellular) calcium, we are able to gain insights also into the extent to which calcium mobilisation is involved in the regulation of bile flow.

We conclude that crosstalk between the second messenger systems generating cyclic AMP and inositol phosphates[3] plays a regulatory role in bile flow and that this mechanism is perturbed in pathological conditions involving cholestasis.

1 Hamada, Y, Karjalainen, A, Setchell, B, Millard, J & Bygrave, FL (1992) Biochem. J. 281, 387-392

2 Hamada, Y, Karjalainen, A. Setchell, B, Millard, J & Bygrave, FL (1992) Biochem. J. 283, 575-581

3 Bygrave FL & Benedetti, A (1993) Biochem. J. In press

Q 403 NEUTRAL SPHINGOMYELINASE ACTION STIMULATES SIGNAL TRANSDUCTION OF TUMOR NECROSIS FACTOR-α IN THE SYNTHESIS OF CHOLESTERYL ESTERS IN HUMAN FIBROBLASTS, Subroto Chatterjee, Department of Pediatrics, Johns Hopkins University, School of Medicine, Baltimore, Maryland 21287-3654 U.S.A.

We have investigated biochemical mechanisms of tumor necrosis factor (TNF)-α signalling in cultured human skin fibroblasts. We found that TNF-α signalling may involve activation of a cell membrane neutral sphingomyelinase (N-SMase). In that, within 2.5-5 min of treatment of cells with TNF-α there was a 2 fold increase in the activity of N-SMase compared to control. This reaction led to the hydrolysis of sphingomyelin as evidenced by a decrease in sphingomyelin mass and in the radioactivity associated with [^{14}C] choline labelled sphingomyelin. This was accompanied by a 4 fold increase in the formation of cholesteryl [^{14}C] oleate within 2.5 min of incubation with TNF-α. This reaction also stimulated the mobilization of cell surface associated [^{3}H] cholesterol and its utilization in the synthesis of [^{3}H] cholesteryl esters via acyl coenzyme-A cholesterol acyltransferase (ACAT). Gas chromatographic analysis revealed that the cellular level of cholesteryl esters increased about 2.5-3 fold following treatment with TNF-α compared to control. Cholesteryl ester synthesis was compromised upon incubation of cells with antibody against N-SMase and remained unaltered with TNF-ß and fibroblast growth factor. Furthermore, TNF-α mediated stimulation of cholesteryl ester synthesis was compromised by incubation of cells with an inhibitor of ACAT. These findings suggest a possible biological role of N-SMase in the signal transduction of TNF-α in the synthesis of cholesteryl esters in human fibroblasts.

Q 404 INHIBITION OF PHOSPHOLIPASES AND CELL PROLIFERATION BY DEMETHOXYVIRIDIN.

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Demethoxyviridin (DMV) has been reported to inhibit fMet-Leu-Phe stimulation of phospholipase C and, more potently, phospholipase D activity in the human neutrophil (Bonser *et al*, Br. J. Pharmacol 103: 1237, 1991). These phospholipases are believed to play a major role in the stimulation of mitogenic signal transduction by growth factors and oncogenes. We have therefore investigated the effects of DMV on the growth and activation of the phospholipases C, D and A_2 in bombesin-stimulated Swiss 3T3 fibroblasts. In a cell growth assay DMV inhibited bombesin plus insulin-stimulated proliferation of Swiss 3T3 cells. Significant growth delay was observed at concentrations as low as 10 nM, with an IC_{50} of 1μM. Membrane lysis, as detected by a [^{51}Cr]-Chromate release assay, was only significant at high concentrations of DMV (100μM). Inhibition of bombesin-stimulated PLC and PLA_2 activity was dose dependent with IC_{50}s of 200nM and 2nM respectively. Inhibition of PLD was also dose dependent however, only 55% of the response was inhibited with an IC_{50} of 50nM. DMV inhibited the tyrosine phosphorylation of a range of proteins in bombesin-stimulated Swiss 3T3 cells over the concentration range 10nM to 10μM in a dose dependent manner. Therefore, in Swiss 3T3 cells the antiproliferative effects of DMV are seen at concentrations that inhibit PLC, PLA_2 and PLD . DMV also acts as a tyrosine kinase inhibitor and it may be that this inhibition is responsible for the differential effect of the compound upon phospholipase activation.

Q 405 TIME COURSE OF LTB_4 AND PGE_2 PRODUCTION IN THE CHRONIC MOUSE EAR INFLAMMATION MODEL, Inger M. Darling, Lynda B. Davern and Kenneth M. Tramposch, Drug Discovery, Bristol-Myers Squibb Pharmaceutical Research Institute, Buffalo, NY 14213

The chronic skin inflammation model developed in mice has been used to screen prospective anti-inflammatory compounds. The model consists of multiple dose applications of 12-O-tetradecanoylphorbol-13-acetate (TPA) to mouse ears which induces an inflammatory reaction consisting of increased ear weight, inflammatory cell infiltration and epidermal hyperplasia. The mechanism by which TPA causes inflammation may be in part due to the release of eicosanoid mediators as a result of increased PLA_2 activity. The present study was designed to characterize the chronic inflammation model more fully by determining the time course of mediator production in comparison to myeloperoxidase (MPO, an indicator of neutrophil infiltration) in the mouse ears. Mouse ears were treated with TPA 5 times over an 11 day study period. On various days throughout the 11 day period, the animals were sacrificed and punch biopsies were taken from each ear. Ear homogenates were assayed for either MPO (spectrophotometric assay) or PGE_2 and LTB_4 (RIA of the appropriate fractions eluted from HPLC analysis). Consistent with previous studies (Stanley et al., 1991), the MPO concentrations peak after three days and level off after seven days. However, PGE_2 concentrations increased greater than 5 fold over baseline 6 hours after each TPA application and returned to baseline values after 30 hours throughout the 11 day study period. LTB_4 showed a significant increase from baseline on the last two days of the 11 day period. The effect of various potential inhibitors were examined in the model. These included: hydrocortisone-17-valerate (HCV), lonapalene, indomethacin and tacaryl. Animals were topically dosed with the drugs twice daily from the eighth until the eleventh day. PGE_2 was inhibited greater than 95% by indomethacin with lesser inhibitions by the other compounds. LTB_4 was inhibited greater than 70% by lonapalene, HCV and indomethacin. Overall, this data provides further evidence to suggest that chronic applications of TPA to mouse ears results in an increase in PLA_2 activity.

Q 406 NERVE GROWTH FACTOR (NGF) ACTIVATES THE SPHINGOMYELIN CYCLE: IDENTIFICATION OF A SIGNAL TRANSDUCTION PATHWAY FOR THE LOW AFFINITY NGF RECEPTOR, Rick T. Dobrowsky*, Alex Castellino†, Moses V. Chao† and Yusuf A. Hannun*, Departments of Medicine and Cell Biology*, Duke University Medical Center, Durham, NC 27710 and Department of Cell Biology and Anatomy †, Cornell University Medical College, New York, NY 10021

In T9 glioma cells, ceramide mimics the effects of NGF on cell growth and differentiation. We therefore investigated whether NGF activates the sphingomyelin cycle in T9 glioma cells. NGF binds to both high and low affinity membrane receptors. The high affinity NGF receptor, $p140^{trk}$, is a receptor linked tyrosine kinase whose expression is sufficient for NGF responsiveness. However, the role of the low affinity NGF receptor, $p75^{NGFR}$, in neurotrophin signaling is a matter of controversy; due in part to a lack of evidence coupling $p75^{NGFR}$ to cellular signal transduction pathways. We report that NGF treatment of rat T9 glioblastoma cells activates the sphingomyelin cycle specifically by interaction with $p75^{NGFR}$. This conclusion was supported by 1) the lack of $p140^{trk}$ expression in rat T9 glioma cells, 2) the ability of brain-derived neurotrophic factor, which also binds to $p75^{NGFR}$, to stimulate sphingomyelin hydrolysis in NIH-3T3 cells expressing $p75^{NGFR}$, and 3) NGF-did not induce sphingomyelin hydrolysis in mutated PC12 cells which express $p140^{trk}$ but lack an intact $p75^{NGFR}$ binding domain. Further, expression of EGF/NGF receptor chimeras in T9 cells imparted to epidermal growth factor (EGF) the ability to activate the sphingomyelin cycle. Activation of the sphingomyelin cycle by EGF in T9 cells expressing EGF/NGF receptor chimera correlated with EGF-induced growth inhibition and morphologic differentiation. These effects were similar to that induced by treatment of wild type T9 cells with either NGF or ceramide. Collectively, these data demonstrate that $p75^{NGFR}$ is capable of signaling and offers new insight into the molecular mechanism by which this receptor may mediate neurotrophin biology.

Q 407 HUMAN GROUP II PHOSPHOLIPASE A_2 TRANSGENIC MICE AS A DISEASE AND DRUG TESTING MODEL, Niles Fox, James Schrementi, John D. Sharp, Donald L. White, David W. Snyder, Lawrence W. Hartley, Donald G. Carlson and Jesse L. Bobbitt*, Departments of Pulmonary Research and *Biotechnology Research, Lilly Research Laboratories, Lilly Corporate Center, Indianapolis, IN 46285

The secreted group II phospholipase A_2 (sPLA2) hydrolyzes the sn-2 position of glycerophospholipids. Products of this hydrolytic activity include arachidonic acid and lysophospholipids that are further metabolized to a host of pro-inflammatory mediators. Blood and tissue levels of sPLA2 are significantly elevated in a number of clinical syndromes suggesting sPLA2 may be an important etiopathic factor in disease and a potential therapeutic target. To develop an animal model of sPLA2-mediated pathology and an in vivo sPLA2 inhibitor screen, we have created transgenic mice containing a mouse metallothionein promoter-human sPLA2 fusion gene. Northern blot and immunohistochemical analyses reveal a wide, yet cell-type-specific, transgene expression pattern. Blood levels of PLA2 enzyme activity are elevated 2-10 fold in the transgenic mice and are inducible (>4-50 fold) by $ZnSO_4$ treatment. Pathological changes are observed in the male reproductive tract and cause male infertility at an early age. These animals provide an attractive model for exploring the role of sPLA2 in disease and for the development of sPLA2 inhibitors.

Q 408 PHOSPHOLIPASE A_2, CYCLOOXYGENASE-1 AND -2 EXPRESSION INCREASE IN HUMAN SKIN FOLLOWING UVB INJURY, A.T. Gresham*, S.Y. Buckman*, J. Masferrer†, and A.P. Pentland*, *Division of Dermatology, Department of Medicine, Washington University, †Monsanto Corp., St. Louis, MO 63110. Ultraviolet light B (UVB) induced inflammation may contribute to the capacity of light to act as a tumor promoter, and is characterized by dramatic increases in prostaglandin E_2 (PGE_2). Prostaglandin levels are typically increased 5-10 fold 6-9 hr. post-UV, and are maintained for approximately 24 hr. following acute UVB exposure. In order to understand how increased PGE_2 synthesis occur, we have developed specific polyclonal antibodies against $cPLA_2$, COX-1 and COX-2 to study the temporal expression and localization of these enzymes following UVB injury. Keratinocyte cytosolic proteins were metabolically labeled then immunoprecipitated at intervals following exposure to 30 mJ/cm^2 UVB to assess *de novo* protein synthesis. Maximal $cPLA_2$ and COX-2 synthesis occurred 3-6 hr. after irradiation and returned to baseline 24 hr. post-UV. However, PGE_2 levels were unaffected when keratinocytes were pre-treated with 2 μM dexamethasone prior to incubation with exogenous arachidonic acid, suggesting that COX-2 synthesis at early time points does not contribute significantly to prostaglandin formation. By comparison, COX-1 synthesis was increased 2-4 fold 24 hr. after UV injury, and prostaglandin data suggest that COX-1 activity may be important for the maintenance of PGE_2 levels 24 hours post-UV. Immunostaining of whole human skin explants demonstrated that $cPLA_2$, COX-1 and COX-2 are localized to the epidermis *in vivo*. Studies with lightly pigmented skin demonstrated enhanced staining density which correlated with the changes in synthesis observed by immunoprecipitation. Further studies have indicated a reduced induction of COX-1 and COX-2, and no induction of $cPLA_2$ with exposure to UVB in darkly pigmented, naturally photoprotected skin, implying that the synthesis of these enzymes are correlated to inflammation. Our results collectively suggest that increased $cPLA_2$ is important at early time points following UV injury, while induction of COX-1 synthesis is responsible for increased PGE_2 formation 24 hr. after acute UVB exposure.

Q 409 CHRONIC VALPROATE TREATMENT ALTERS CELL MORPHOLOGY AND PKC LEVELS IN RAT C6 GLIOMA CELLS AND IN HUMAN SK-N-SH NEUROBLASTOMA CELLS, David B. Hawver*, Guang Chen, Husseini K. Manji, and William Z. Potter, Section on Clinical Pharmacology., Experimental Therapeutics Branch, NIMH, and *NIGMS, PRAT Fellow, NIH, Bethesda, MD 20892

Valproic acid (VPA, or valproate) is a simple branched-chain fatty acid (2-propyl pentanoic acid) that has anticonvulsant effects in several types of seizure disorders and also shows antimanic properties in some bipolar patients. Despite decades of research, the precise molecular mechanisms responsible for valproate's clinical efficacy are still unknown. Given the recent explosion of knowledge on the importance of Protein Kinase C (PKC) in the regulation of cell excitability and responsiveness in the brain, we performed the present experiments to see if PKC might be involved in the mechanism of action of VPA. Previous work (C. M. Regan, 1985), had shown that chronic VPA treatment (1 mM for 1-2 wks) could induce morphological changes in rat C6 glioma cells *in vitro* such that they became elongated and aligned into swirling patterns. We observed such morphological changes in rat C6 glioma cultures treated for 3 days with .5-1 mM VPA, and somewhat similar changes in human SK-N-SH neuroblastoma cells after 7 days. Treatment of C6 and SK-N-SH cultures with phorbol ester (PMA), okadaic acid, or forskolin altered cell morphology, but did not produce the same pattern of elongation and alignment seen in VPA-treated cultures. Staurosporin, however, an inhibitor of PKC as well as other protein kinases, did produce a "VPA-like" pattern in C6 cultures (but not in SK-N-SH cultures). In addition, PMA, although failing to produce similar changes alone, dramatically potentiated the ability of VPA to induce elongation and alignment in C6 cultures. Western immunoblotting revealed that PKC-α levels in C6 cytosol were reduced 30-60% after 6 days of exposure to .5 mM VPA, 10 nM PMA, or 5 nM Staurosporin compared to control cultures. In contrast, treatment of SK-N-SH cells with VPA for 14 days produced substantial increases in levels of both PKC-α and PKC-β in the cytosol. We are presently investigating the effects of PMA and Staurosporin on PKC levels in SK-N-SH cells. We are also examining the possibility that VPA may affect the PKC pathway indirectly through effects on endogenous lipid PKC-modulators, since VPA has been shown to alter levels of phospholipids and sphingolipids (R. Davis et al, 1993; Bolanos and Medina, 1993).

Q 410 APOPROTEIN A-1 IS A NOVEL SUBSTRATE OF PKC, Oksana Holian, Raj Kumar and Bashar Attar, Hektoen Institute for Medical Research, Cook County Hospital, and University of Illinois, Chicago, IL 60612.

Apoprotein A-1 is the predominant protein constituent of high density lipoproteins (HDL) and is believed to participate in the efflux of cholesterol from peripheral tissues to liver and steroidogenic tissues. Human apoprotein A-1 does not contain the preferred amino acid sequence recognized by PKC. Nonetheless, lipid-free apoprotein A-1 is phosphorylated by purified PKC. This phosphorylation is independent of the known PKC activators phosphatidyl serine, diacylglycerol and calcium. Furthermore, the optimal pH of apoprotein A-1 phosphorylation by PKC is pH 5.

Apoprotein A-1 associated with the HDL particle acts differently than lipid free apoprotein A-1. HDL-bound apoprotein A-1 is not phosphorylated by PKC suggesting inhibition by the hydrophobic components of HDL. This was substantiated by the finding that lipid-free apoprotein A-1 phosphorylation was inhibited by both native and reconstituted HDL particles. In contrast, phosphorylation of lysine rich histones was stimulated by HDL in the presence of calcium. Phosphorylation of apoprotein A-1 was also suppressed by both anionic (oleic acid) and cationic (sphingosine and gangliosides) hydrophobic compounds. Among the individual lipid components of HDL only phosphatidyl choline was inhibitory toward apoprotein A-1.

Our findings lead us to conclude that apoprotein A-1 phosphorylation is independent of calcium, phosphatidyl serine and diacylglycerol, and that the other hydrophobic compounds inhibit apoprotein A-1 phosphorylation by PKC through their interaction with the substrate and not with the enzyme. It is hypothesized that a specific hydrophobic environment modulates a serine/threonine containing sequence of apoprotein A-1 in an inhibitory manner.

Q 411 *Abstract Withdrawn*

Q 412 ***DIFFERENTIAL REGULATION OF APOPTOSIS BY CERAMIDE AND DIGLYCERIDE IN HUMAN MYELOID LEUKEMIA CELL LINES.*** W. David Jarvis, Richard N. Kolesnick[1], Frank A. Fornari, David A. Gewirtz, and Steven Grant. *Medical College of Virginia, Richmond, VA, 23298 and [1]Sloan-Kettering Institute, New York, NY. 10021.*

The relative effects of ceramide and diglyceride on the regulation of programmed cell death, or *apoptosis*, were investigated in the human myeloid leukemic cell lines HL-60 and U937. Experimental increases in intracellular ceramide levels by treatment with exogenous sphingomyelinase (SM'ase; 0.001-100 mU/ml) for 1-24 hr were clearly cytotoxic, resulting in time- and concentration-dependent internucleosomal degradation of genomic DNA, as demonstrated by the formation and release of truncated double-stranded DNA fragments and a corresponding loss of integrity of bulk DNA; this effect did not extend to nascent DNA, however. Comparable apoptotic responses were elicited by exposure to synthetic ceramide (0.001-100 μM) for 1-12 hr. All of these responses entailed the manifestation of distinct apoptotic morphology and loss of clonogenicity. Parallel treatment with exogenous PL'ase C (0.001-10 mU/ml) or synthetic diglyceride (0.001-100 μM) failed to promote apoptosis. In fact, PL'ase C action was cytoprotective, as SM'ase-induced apoptosis was greatly reduced, or abolished, by PL'ase C co-treatment. Comparable interactions between synthetic diglyceride and ceramide were also observed. In addition, the apoptotic capacity of SM'ase was also attenuated by co-exposure to pharmacological activators of protein kinase C (PKC), including tumor-promoters such as phorbol dibutyrate (0.01-100 nM) and mezerein (0.01-100 nM), as well as the non-tumor-promoting bryostatin 1 (BRY; 0.001-100 nM). Moreover, 24-hr pretreatment with BRY completely down-regulated PKC activity, and consequently eliminated the cytoprotective influence of PL'ase C. Taken together, these findings indicate that apoptosis in myeloid leukemia cells is subject to reciprocal modulation by the lipid second messengers ceramide and diglyceride, and strongly support opposing effector roles for ceramide-activated protein kinase and PKC in the regulation of programmed cell death.

Q 413 IMMEDIATE ACTIVATION BY SPHINGOSINE OF CALCIUM-MEDIATED MECHANISMS IN WASHED HUMAN PLATELETS, Jan-Henrik Mäkelä and Boris Isomaa, Department of Biology, Åbo Akademi University, Biocity, Artillerigatan 6, FIN-20520 TURKU, Finland.

The amphiphilic sphingolipid-metabolite sphingosine (SFI) was previously applied as an inhibitor of protein kinase C (PKC) in signal transduction studies in isolated human platelets. In further studies of the effects of amphiphilic compounds, including SFI, on signal transduction in isolated human platelets, we have encountered several immediate effects mediated by the application of SFI to platelets:

1. A rapid and transient elevation of $[Ca^{2+}]_i$, which occurs in the presence of extracellular EGTA, indicating SFI-mediated release of Ca^{2+} from intraplatelet stores. In the presence of extracellular Ca^{2+} the elevation of $[Ca^{2+}]_i$ is biphasic, indicating Ca^{2+}-influx.

2. Phosphorylation of myosin light chain (MLC). MLC can be phosphorylated by the Ca^{2+}/calmodulin-regulated myosin light chain kinase. This makes possible the assembly of functional actomyosin-complexes as well as subsequent contractile activity needed for shape change, secretion and clot retraction.

3. Degradation of cytoskeletal substrates of the Ca^{2+}/calmodulin-regulated protease calpain. This effect is not dependent on extracellular Ca^{2+}.

4. Release of pre-loaded [^{14}C]-5-HT. This effect reaches maximum (about 90-100% of total) only after 5 min of treatment with 40 µM SFI, and can not be blocked by a pre-treatment of the platelets with the cyclo-oxygenase-inhibitor aspirin, or the intracellular Ca^{2+}-chelator BAPTA. The release of a cytoplasmic marker, lactate dehydrogenase (LDH), from platelets treated with 40 µM SFI does not exceed 10% of the total LDH content in platelets even after an incubation of the platelets for 1 h, indicating that membrane damage is not the cause of [^{14}C]-5-HT-release.

Q 414 DIETARY FISH OIL REDUCES ADENYLATE CYCLASE ACTIVITY IN NORMAL AND ISCHEMIC DOG HEART. Joseph C Miller, Kathryn H Muntz*, and Claude R Benedict. Univ Texas Health Sci Ctr, Houston, TX 77225, and *Univ Texas Southwestern Med Ctr, Dallas, TX 75235.

Myocardial ischemia decreases activity of the ß-adrenergic receptor-adenylate cyclase complex, increases catecholamine release, and increases ßAR density. To test the effects of omega-3 polyunsaturated fatty acids on this signal transduction pathway, dogs were fed standard chow supplemented with menhaden oil to provide eicosapentenoic (20:5) plus docosahexenoic (22:6) acids totalling 100 mg/kg/d for 70 weeks. cAMP formation was measured (nmol/min/g, mean±SEM, n=5/10) in homogenates *in vitro* under basal conditions and with NaF, isoproterenol plus GTP, and forskolin stimulation. The w3-PUFA-supplemented diet reduced ACase activity 27-38% in non-ischemic heart compared to 9-21% reductions produced by 60 min of circumflex coronary artery occlusion. Ischemia did not lower ACase activity further in hearts from the w3-PUFA-supplemented dogs. Membrane ßAR density measured by ICYP ligand binding (control, non-isch: 75±9 fmol/mg) and ßAR-ACase coupling were unchanged. These data suggest that w3-PUFAs reduce the response of normal and ischemic myocardium to ßAR stimulation. Therefore, alterations in the myocardial plasma membrane may minimize the physiological impact of increased catecholamine levels observed during ischemia by decreasing ACase activity, or other signal transduction pathways.

		Basal	NaF	Iso/GTP	Forskolin
Control	Non-Isch	5.0±0.4	14.4±0.8	10.4±0.9	39.5±2.4
	Ischemic	4.6±0.6	12.2±1.4	9.5±1.5	31.4±3.9
Fish Oil	Non-Isch	3.1±0.4	10.5±1.0	7.0±0.8	28.3±2.6
	Ischemic	3.5±0.2	10.9±1.0	8.5±1.0	26.7±3.3

Q 415 ROLE OF LIPID MESSENGERS IN INTRACELLULAR SIGNALLING OF TNF-α. G. Müller, J. Rennecke, P. Scheurich, K. Pfizenmaier. Institute of Cell Biology and Immunology, University of Stuttgart, Germany.

Two distinct receptor subtypes for TNF-α, TR60 and TR80 have been identified which are independently active in signal transduction. One known signalling pathway of TNF-α is the activation of a plasma membrane located sphingomyelinase which releases ceramide as intracellular messenger. Ceramide has been assigned as the signalling lipid of the TR60 receptor. The signal transducing mechanisms of the TR80 receptor however, have not yet been identified. Therefore, the role of lipid messengers specific for the TR60 and the TR80 receptor subtypes for TNF-α were investigated. The signalling response to agonistic receptor specific antibodies revealed a time dependent decrease of sphingomyelin induced by TR60, while the TR80 antibodies increase the sphingomyelin fraction possibly replacing the degraded sphingomyelin and indicating an early divergence of TR60 and TR80 mediated signalling pathways. The metabolic route of the lipid messenger ceramide was followed by incubating cells with [^{14}C]-ceramide revealing a slow deacylation with appearance of the [^{14}C]-palmitate in the phosphatidylcholine pool suggesting that ceramide might be a long-persisting intracellular messenger. The involvement of a phospholipase A_1 in ceramide degradation is discussed. TNF-α reduces the binding capacity of cells for [^{14}C]-ceramide. This suggests that binding sites are either downregulated or occupied by TNF induced endogenous ceramide. Downstream to the production of lipid messengers is a rapid stimulation of several serine/threonine kinases. By employing specific antibodies against novel protein kinase C subtypes we investigated whether there is a differential activation of protein kinase C isozymes by the two distinct TNF-receptors. The novel PKC zeta binds [^{14}C]-ceramide. Data will be presented concerning the role of ceramide as selective activator of PKC zeta.

Q 416 EVIDENCE FOR THE INVOLVEMENT OF INTRA-CELLULAR PLATELET-ACTIVATING FACTOR BINDING SITES IN THE REGULATION OF PHOSPHOLIPASE A_2 IN fMET-LEU-PHE-CHALLENGED HUMAN NEUTROPHILS

Santosh Nigam, Hong Zhang, Stefan Müller, Hans Weitzel

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We have recently shown that the inhibition of protein kinase C by staurosporine and calphostin C stimulated PLA_2-, 5-lipoxygenase- and acetyl-CoA:lyso-PAF acetyltransferase activities dose-dependently, as measured by [^{14} C]AA-release, production of LTB_4 metabolites and PAF formation in FMLP stimulated human neutrophils. Moreover, a sustained elevation of cytosolic Ca^{++} level was observed within 5 minutes, although the initial mobilization of intracellular Ca^{++} was not affected by PKC inhibitors, suggesting the dire need of Ca^{++} for the activation of above Ca^{++}-dependent enzymes. Pretreatment of cells with specific PAF antagonists WEB 2086 and BN 50739 caused an inhibition of staurosporine- or calphostin C-induced potentiation of AA-release and PAF formation as well as elevation of intracellular Ca^{++} and degranulation in FMLP-stimulated cells. Thus, we conclude that (a) PKC isozymes and other protein kinases, which can be inhibited by staurosporine and calphostin C, are apparently not involved in the activation of calcium dependent enzymes, (b) prolonged activation of PLA_2 by FMLP in presence of staurosporine or calphostin C is partially attributed to the autostimulatory role of endogenously synthesized PAF, which acts via specific high affinity cytosolic PAF binding sites, and (c) a staurosporine and calphostin C-sensitive cytosolic PKC isozyme other than ß- and n-PKC might be involved in the regulation of PLA_2 in human neutrophils.

The study was supported by Deutsche Forschungsgemeinschaft (Ni 242/9-1).

Q 417 CERAMIDE IS AN INDUCER OF CELLULAR SENESCENCE, Lina M. Obeid and Mark Venable, Department of Medicine, Duke University Medical Center, Durham, NC 27710

Cellular senescence is defined as the inability of cells to undergo DNA synthesis and proliferate. The underlying molecular mechanisms involved in cellular senescence are beginning to be investigated. Indications are that signal transduction and cell regulatory pathways are altered during senescence and could induce the senescent phenotype. Recently the sphingolipid ceramide has emerged as a critical molecule in signal transduction and regulation of cell growth and proliferation. Here we show that ceramide is significantly elevated in senescent human diploid fibroblasts as compared to young cells and that there is a direct correlation between ceramide levels and the population doublings of human diploid fibroblasts. Ceramide is able to induce senescence in young human diploid fibroblasts as assayed by its ability to inhibit their growth and prevent young human diploid fibroblasts from incorporating [^{3}H]thymidine in response to serum stimulation. These studies demonstrate that ceramide may play a critical role in organism aging and may impart the senescent phenotype.

Q 418 INDUCTION BY LPS AND CYTOKINES OF ENDOTHELIAL CELL GLYCOSPHINGOLIPIDS AND THEIR RELATIONSHIP TO HUMAN DISEASE, Tom G. Obrig, Chandra B. Louise, Susan A. Kaye and Clifford A. Lingwood, Department of Microbiology and Immunology, University of Rochester Medical Center, Rochester, NY, 14642 and Hospital; for Sick Children, Toronto M5G 1X8, Canada.

Endothelial cells are capable of synthesizing neutral glycosphingolipids. However, the functional role of these compounds in endothelial cell physiology remains to be determined. In one case, globotriaosylceramide (Gb_3) has been identified as the high-affinity receptor for the bacterial Shiga toxins. These toxins have been implicated as causal agents in *E. coli* O157:H7-related hemolytic uremic syndrome, with their putative target being renal microvascular endothelial cells. Recently, we demonstrated that bacterial lipopolysaccharide or the cytokines TNF-alpha or IL-1 beta sensitized human umbilical vein endothelial cells (HUVEC) to Shiga toxin. The present study was conducted to determine if the sensitization of HUVEC to the toxin could be due to induction of Gb_3 and other glycosphingolipids by LPS, TNF, or IL-1.

The results indicate that LPS, TNF and IL-1 individually induced HUVEC glycosphingolipids of the Gb_3 pathway. This induction was both dose- and time-dependent. During the 24 h induction period, their was a 2 to 10-fold increase in glucosylceramide, lactosylceramide, Gb_3, and globotetraosylceramide content of HUVEC as determined by HPLC analysis. All three inducing agents appeared to favor the accumulation of Gb_3 vs. the other glycosphingolipids. Removal of LPS, TNF or IL-1 from the induced HUVEC cultures resulted in a partial decay of the glycolipids within the following 48 h period. Signal transduction mechanisms involved in the induction process and the role of these glycosphingolipids in endothelial cell physiology are presently under investigation.

Q 419 CERAMIDE INDUCED-PROTEIN PHOSPHORYLATION IN HUMAN LEUKEMIA HL-60 CELLS, [1]Toshiro Okazaki, [2]Hirofumi Sawai, [2]Masaro Tashima, [2]Hirofumi Sawada, [2]Minoru Okuma and [1]Naochika Domae. [1]Department of Medicine, Osaka Dental University, 1-5-17 Otemae, Cyuo-ku, Osaka, 540, Japan. [2]First Division of Internal Medicine, Faculty of Medicine, Kyoto University, 54 Syogoin Kawaramachi, Sakyo-ku, Kyoto, 606, Japan.

It was discovered that sphingomyelin decreased two hours after treatment with 1,25 dihydroxyvitamin D3 and returned to the control level at four hours and that ceramide, sphingomyelin breakdown product by sphingomyelinase increased reciprocally in HL-60 cells (Okazaki, T. et al. J. Biol. Chem., 264: 19076-19080, 1989). Ceramide could induce HL-60 cells to monocytic differentiation without any inducers (Okazaki, T. et al. J. Biol. Chem.,265: 15823-15831, 1990). We tried to investigate the downstream of ceramide-related signal transduction and found out that ceramide-induced HL-60 cell differentiation was enhanced by protein tyrosine phosphatase (PTPase) inhibitor, vanadate but not by tyrosine kinase (TKase) inhibitor, herbimycin A. Then a phosphorylation/dephosphorylation by ceramide was investigated by using anti-phosphotyrosine antibody. The data showed that at least three proteins were phosphorylated 5-60 min. after treatment with ceramide. These results suggest that a protein tyrosine phosphorylation /dephosphorylation by a balance of TKase/PTPase activity modulated by ceramide plays an important role in cell differentiation.

Q 420 INHIBITION OF TUMOUR CELL MITOGENESIS BY DEMETHOXYVIRIDIN; A PUTATIVE INHIBITOR OF PHOSPHOLIPASE D. JA Plumb, M Cross[2], V Brunton, G McAulay[1], DJ Kerr[2] and MJO Wakelam[2], CRC Dept of Medical Oncology and [1]Dept of Chemistry, University of Glasgow, Glasgow G12 8QQ, and [2]Dept of Clinical Oncology, University of Birmingham, Birmingham, B15 9TH, UK

Most oncogene products are components of intracellular signal transduction pathways and transfection of cells by a number of oncogenes has been shown to subvert the regulation of pathways controlling second messenger concentration. If the key sites of regulation of these pathways can be identified they might serve as targets for novel anticancer agents. Following mitogen stimulation protein kinase C is activated by diacylglycerol (DAG) derived from phospholipase C mediated breakdown of phosphatidylinositol 4,5-bisphosphate. This pathway is rapidly desensitised and subsequent activation is thought to be from the breakdown of phosphatidylcholine catalysed by phospholipase D (PLD) to phosphatidic acid (PA) and subsequently to DAG by the action of phosphatidate phosphohydrolase. Since PA may be an important mediator of the mitogenic signal we have identified PLD as a novel target for anti-cancer drug development.

The respiratory burst induced by fmet-leu-phe in neutrophils is inhibited by the fungal metabolite demethoxyviridin (DMV) through inhibition of agonist stimulated PLD activity. We have studied the effects of DMV on growth factor stimulated mitogenesis in human tumour cell lines.

DMV was cytotoxic to a number of cell lines. Growth of the human small-cell lung cancer cell line H69 is stimulated by bombesin and stimulation resulted in 10 fold increase in sensitivity to DMV (ID_{50} 0.2 and 2uM). Similarly, sensitivity of the human breast cell line MCF7 was increased 3 fold (ID_{50} 10 and 33uM) when growth was stimulated by insulin and oestradiol. EGF stimulated mitogenesis in the squamous cell carcinoma line SiHa was inhibited by a much lower concentration of DMV (0.01uM) than required for cytotoxicity (ID_{50} 3uM). Similar results were obtained with the glioma cell line T98G for which EGF, FGF and PDGF are mitogenic. We have also shown that DMV does not induce expression of p53 suggesting that cytotoxicity is not due to DNA damage.

These results indicate that DMV is a potent inhibitor of growth factor stimulated mitogenesis in tumour cell lines. Although the mechanism of activity is not known the effect of DMV on components of cellular signal transduction pathways is under investigation.

Q 421 PROTECTION OF ENDOTOXIC SHOCK IN MICE BY PHARMACOLOGIC INHIBITION OF THE GENERATION OF PHOSPHATIDIC ACID Glenn C. Rice, Paul A. Brown, Richard J. Nelson, James A. Bianco, Jack W. Singer and Stuart Bursten, Cell Therapeutics, Inc. Seattle, WA 98177

The mammalian cell membrane associated enzyme, lysophosphatidate acyl-CoA:acyltransferase (LPAAT), catalyzes the transfer of acyl CoA from lysophosphatidic acid (lyso-PA) to phosphatidic acid (PA). Certain PA species function as lipid intermediates in cell activation and may function directly as intracellular signaling molecules. PA can be subsequently dephosphorylated to 1,2-sn- diacylglycerol (DAG) via phosphatidate phosphohydrolase (PAPh). Treatment of various cell types including murine P388 monocytic leukemia cells with bacterial lipopolysaccharide (LPS) rapidly stimulates large increases in PA and PA-derived DAG. Pentoxifylline (PTX), a 1-(5-oxohexyl) substituted 3,7-dimethylxanthine, inhibits LPS stimulated formation of PA in P388 cells at high concentrations (IC_{50} = 500 μM). CT-1501R, a 1-(5-R-hydroxyhexyl) methylxanthine, is a unique metabolite of PTX in humans, and is over 800-fold more active as an inhibitor of PA formation than PTX (IC_{50}=0.6 μM). Since large amounts of lyso-PA accumulate following addition of CT-1501R following stimulation, the inhibitory mechanism is most likely inhibition of LPAAT. CT-1501R does not inhibit LPS induced activation of phosphoinositol (PI)-directed phospholipase C (PLC) and generation of PI-derived DAG. CT-1501R but not PTX significantly protects Balb/c mice from endotoxin lethality when administered even 4 hours following a lethal administration of LPS. Under these circumstances, control mice (n=10 experiments/109 mice) had a survival of 3%, mice treated with PTX had a survival of 0% (n=2 experiments/20 mice), whereas mice treated with CT-1501R had a survival of 37% (n=4 experiments/35 mice: $P < 10^{-3}$ vs. control & PTX treated mice). This protective effect is independent of either agent's effect on suppression of plasma tumor necrosis factor-α. These data suggest that inhibitors of PA formation may have significant clinical potential in the treatment of sepsis and septic shock.

Q 422 NEUTRAL AND ACIDIC SPHINGOMYELINASE CONTRIBUTE TO DIFFERENT TNF-SIGNALING PATHWAYS

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Sphingomyelinases (SMase) have recently been implicated in a number of TNF responses including stimulation of cell growth and differentiation, as well as triggering cytotoxicity and apoptosis. The metabolic cleavage product of SMase, ceramide is recognized as a novel multifunctional lipid second messenger, capable to induce various signaling systems. The pleiotropic activities of ceramide may be due to specific SMase species involved. Our present study aimes to identify the selective role of membrane-bound neutral and endo-lysosomal acidic SMase in TNF signaling. Exogenous ceramide is capable of activating the mitogen-activated kinase (MAP-kinase)-cascade, phospholipase A_2, and the nuclear transcription factor NF-κB in U937 and Jurkat cells. The *in-vivo* contribution of TNF-responsive SMase species to these events was assayed using different lysosomotropic agents: chloroquine, monensin and NH_4Cl prevented TNF-mediated activation of acidic SMase and NF-κB. In contrast, TNF-induced PC-PLC, neutral SMase, MAP-kinase and PLA_2-activation remained unaffected. Furthermore, prevention of TNF-induced 1,2-diacylglycerol-production by the xanthogenate D609 also prevented acidic SMase but had no effect on either neutral SMase, MAP-kinase or PLA_2 activities. In concert, these results indicate that 1,2-DAG-regulated acidic SMase, associated with the endo-lysosomal compartment represents an important relais for TNF-induced NF-κB activation, while DAG-independent neutral SMase may gain entry to the MAP-kinase cascade.

Q 423 INCREASED ACTIVITY OF PHOSPHATIDATE PHOSPHOHYDROLASE IN HUMAN COLORECTAL TUMOURS. P.H.Scott[1], A.Martin[2], D.N.Brindley[2] and J.A.Plumb[11] CRC Dept.of Medical Oncology, University of Glasgow, G61 1BD and [2]Dept.of Biochemistry and Lipid and Lipoprotein Research Group, 328 HMRC, Edmonton, Canada T6G 2S2

Point mutations in members of *ras* oncogene family and elevation of *src* kinase activity are commonly observed in colon cancers and it has been suggested that these changes may play a role in malignant transformation. However, whilst cells transformed with these oncogenes show increased levels of the second messenger diacylglycerol (DAG), an endogenous activator of protein kinase C (PKC), decreased levels of DAG and PKC activity have been observed in colon tumours. Since a major source of DAG in mitogen stimulated cells is thought to be derived from the breakdown of phosphatidylcholine (PC) by the sequential action of phospholipase D and phosphatidate phosphohydrolase (PAPH), decreased levels of DAG might be explained by decreased activity of one of these enzymes. We have, therefore, determined the activity of PAPH in 21 surgically resected human colorectal cancers and adjacent macroscopically normal tissue. There are two forms of PAPH: PAPH-1, found in the cytosol, is predominantly involved in glycerolipid syntheis, whilst PAPH-2, located in the plasma membrane, is involved in signal transduction. Activities of these enymes are measured by differential sensitivity to N-ethylmaleimide. The levels of PAPH-2 activity (2.28 nmolsDAG/min/mg protein) were consistently higher than those of PAPH-1 (0.39 nmolsDAG/min/mg protein) in the normal mucosa and this reflects the relative activities of the two forms observed in mouse intestinal mucosa. Increased specific activities of both forms were seen in all 21 human colorectal cancers when compared to that in adjacent normal tissue. The increase was greater for PAPH-1 (3.7 fold) than for PAPH-2 (2 fold) which may be significant since we have recently shown that PAPH-2 plays a key role in regulating the balance between phosphatidic acid (PA) and DAG, both of which are second messengers. Subsequently, we are examining signal transduction within human colon cancer by measuring mass levels of important lipids and activity of several enzymes involved in the metabolism of PA and DAG. It is hoped that a detailed study of signaling in colon carcinomas will provide information with respect both to the cause of uncontrolled growth and to possible sites for therapeutic intervention.

Q 424 CERAMIDE IS NEITHER SUFFICIENT NOR NECESSARY FOR NF-kB ACTIVATION, James A. Shayman, Jonathan C. Betts, Adam B. Agranoff, and Gary J. Nabel, Department of Internal Medicine and Howard Hughes Medical Institute, University of Michigan, Ann Arbor, MI 48109-0676

The participation of cell ceramide in TNF-α-stimulated NF-kB activation in Jurkat T cells and HL-60 cells was studied. TNF-α readily stimulated NF-κB activity as assayed by electrophoretic mobility shift and use of a HIV-CAT reporter construct in both cell lines. TNF-α did not change cell ceramide levels. The exogenous addition of N-acetylsphingosine to Jurkat cells and HL-60 cells had no effect on NF-κB activity. When Jurkat T cells were exposed to the glucosylceramide synthase inhibitor, 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), endogenous ceramide levels increased 4 fold. The increase in ceramide, however, neither resulted in NF-κB activation nor potentiated TNF-α or phorbol ester-stimulated activity. We conclude that TNF-α-induced NF-κB activation occurs in Jurkat and HL60 cell lines which do not demonstrate an increase in TNF-α-induced ceramide. Increasing ceramide levels by the addition of short chain ceramides or the use of a glucosylceramide synthase inhibitor neither activates NF-κB nor potentiates TNF-α-induced NF-κB activation.

Q 425 ROLE OF PLATELET ACTIVATING FACTOR (PAF)-ANTAGONISTS TO PREVENT LOCAL AND SYSTEMIC COMPLICATIONS FOLLOWING THROMBOLITIC TREATMENTS, L. Silvestro[1], G. Montrucchio[2], G. Alloatti[3], G. Camussi[4], [1]Res Pharma Pharm. Res. s.r.l., Via Belfiore 57, 10125 Torino Italy [2]Lab. Immunopatologia, Medical School, Univ. di Torino [3]C Lab. di Fisiologia, Dip. di Biologia Animale, Università di Torino, [4]Cattedra di Nefrologia Sperimentale Dip. Biochimica Biofisica, I Fac. di Medicina, Napoli Italy.

In the last years thrombolitic treatments with streptokinase (SK), recombinant-TPA (rTPA) and urokinase (UK) proven to be effective therapies in myocardial infarction (MI) and other thrombotic pathologies. Meanwhile complications, both local (reocclusion) and systemic (thrombocitopenia hypotension), have been observed. Different drugs, i.e. heparin, were included in many protocols of treatment mainly to prevent reocclusion with conflicting results. Recently we clinically observed (1) that thrombocitopenia and hypotension during SK treatment of patients with MI is related to PAF release and "in vitro" we confirm that plasmin induce PAF synthesis from endothelium. The observed complications of thrombolitic therapy may, at least in part, depend on PAF production. In the present study, using animal models for thrombosis, we evaluate the role of PAF-antagonists, WEB 2170 and SDZ 63-675, to improve the therapeutic results and minimize the side effects of SK or rTPA .

With both PAF-antagonist we observed a clear reduction side effects if administered before the thrombolitic therapy. On these basis we suggest that PAF-antagonists can be promising pharmacological agents to improve thrombolitic treatments in patients.

1) G. Montrucchio, S. Bergerone, F. Bussolino, G. Alloatti, L. Silvestro et Al. 1993, Circulation In press.

Q 426 ALTERATIONS IN THE POTENCY OF HUMAN RECOMBINANT NONPANCREATIC SECRETORY PHOSPHOLIPASE A2 (hPLA2) ON GUINEA PIG LUNG PLEURAL STRIPS, D.W. Snyder, C.D. Sommers, L.W. Hartley, D.G. Carlson and J.L. Bobbitt, Pulmonary Research, Lilly Research Labs., Indianapolis , IN 46285.

We have previously demonstrated that hPLA2 acts catalytically to induce contractions of guinea pig lung pleural strips which were mediated by products of arachidonic acid (J. Pharmacol. Exp. Ther. 266:1147-1155, 1993). The potency of hPLA2 was less than that obtained with snake venom PLA2. The present study was designed to alter the potency of hPLA2 in this tissue bath assay. Pretreating the tissues with inactivated hPLA2 using para-bromophenacyl bromide (pBPB) shifted the hPLA2 concentration-response curves to the left. In contrast, PLA2 from snake venom inhibited with pBPB failed to alter contractile responses induced by hPLA2 or snake venom PLA2. Protamine (0.1-100 μM) shifted hPLA2 curves to the left in a concentration-related manner. The optimal concentration of protaime to induce a leftward shift was 10 μM. This concentration of protamine failed to alter concentration-response curves induced by snake venom PLA2, histamine or arachidonic acid. In the presence of protamine, hPLA2 responses were inhibited by the combination of indomethacin (3 μM), BW A4C (3 μM) and SQ 29548 (0.1 μM). The effects of protamine and pBPB-treated enzyme appeared additive and resulted in >3-fold leftward shift on hPLA2 curves. Heparin (0.1-10 μg/ml) inhibited the hPLA2-induced contractile responses in a concentration-related manner. Snake venom PLA2 responses were not altered by heparin. Interestingly, in an isolated enzyme assay, neither protamine nor heparin altered the catalytic activity of hPLA2. The results suggest that protamine and heparin are interfering with the tissue substrate/enzyme interface which selectively alters the contractile actions of hPLA2. We propose that protamine and pBPB-treated hPLA2 selectively enhance the contractile responses of hPLA2 by inhibiting non-specific binding sites of hPLA2 on the pleural strips so that more of the active protein is available to release arachidonic acid. Likewise, heparin acts by interfering with the tissue substrate to limit the liberation of arachidonic acid.

Q 427 EICOSAPENTAENOIC ACID SUPPRESSED THE PROLIFERATION OF VASCULAR SMOOTH MUSCLE CELLS THROUGH THE MODULATION OF SIGNAL TRANSDUCTION BY PDGF. Tamura Y, Terano T, Shiina T, and Yoshida S. The 2nd Department of Internal Medicine, Chiba University School of Medicine, 1-8-1 Inohana, Chuo-Ku Chiba, 260 Japan

Introduction: Eicosapentaenoic acid is one of polyunsaturated fatty acids(PUFA) and is known to exert its anti-atherogenic action through the modulation of various cell functions including vascular tissues. In general PUFA have been reported to inhibit the proliferation of vascular smooth muscle cells(VSMC) but the difference of potency between PUFAs and the mechanisms which are involved have not been yet fully understood. In this study,we concentrated on the effect of EPA and DHA. In order to mimic the conditions which pertain in physiological extracellular fluid, TG-emulsified forms of PUFAs were prepared and used for preparing PUFA rich cells. Using these cells, DNA synthesis, PDGF binding on its receptor and proto-oncogene expression were examined to clarify the mechanisms of PUFA to suppress VSMC proliferation. Materials and methods: Rat VSMC in culture were obtained from the thoracic aorta of Wistar rats by explant method. VSMC were incubated with EPA-TG(40-160 μ M) for 24 hr. And then VSMC were stimulated by PDGF. DNA synthesis was measured by ^{3}H-thymidine uptake, PDGF binding was measured using ^{125}I-PDGF, and c-fos and c-myc expression was measured by northern blot analysis. Results: EPA pretreatment increased the content of EPA. By the pretreatment of EPA-TG(40-80 μ M), DNA synthesis by PDGF was dose dependently suppressed, but protein synthesis was not influenced. EPA pretreatment suppressed the binding of PDGF on the receptor of VSMC in dose dependent manner. Scatchard analysis for PDGF receptor revealed that number of maximal bindind site(MBX) was reduced by EPA treatment in dose dependent manner, but did not affect the Kd value. EPA suppressed the expression of c-fos mRNA. Summary: EPA would suppress the proliferation of VSMC through the inhibition of the growth factors such as PDGF on its receptor. This might be derived from the change of membrane fluidity through the increase in EPA. This effect of EPA might partly explain the anti-atherosclerotic effect of EPA.

Q 428 INHIBITOR OF PHOSPHOLIPASE A_2 BLOCKS EICOSANOID AND PLATELET-ACTIVATING FACTOR BIOSYNTHESIS AND HAS ANTI-INFLAMMATORY ACTIVITY, K.M. Tramposch, F.H. Chilton, P.L. Stanley, R.C. Franson, M.B. Havens, L.B. Davern, I.M. Darling, and R.J. Bonney, Dermatology Drug Discovery, Bristol-Myers Squibb Pharmaceutical Research Institute, Buffalo, NY 14213

Activation of Phospholipase A_2 (PLA_2) is a key step in the production of lipid mediators of inflammation. Inhibition of this enzyme could result in the suppression of three important classes of inflammatory lipids: prostaglandins, leukotrienes and Platelet-Activating Factor (PAF), and offers an attractive approach toward developing novel agents for treating inflammatory diseases. BMS-181162 [4(3'-carboxyphenyl)-3,7-dimethyl-9(2",6",6"-trimethyl-cyclohexenyl),2Z,4Z,6E,8E-nonatetraenoic acid] is a novel inhibitor of phospholipase A_2 and the effect of this compound on leukotriene, prostaglandin, and PAF biosynthesis in isolated cells and *in vivo* was determined. BMS-181162 reversibly inhibited the 14kDa PLA_2 purified from human synovial fluid with an IC_{50} = 8 μM. In A23187-stimulated human polymorphonuclear leukocytes (PMNs), BMS-181162 blocked arachidonic acid release with an IC_{50} = 5.6 μM. LTB_4 and PAF biosynthesis in these cells was also inhibited. In a phorbol ester-induced chronic mouse skin inflammation model, topically applied BMS-181162 markedly lowered the tissue levels of LTB_4 and PGE_2 and dose dependently inhibited leukocyte infiltration (ED_{50} = 180 μg/ear). BMS-181162 is a specific inhibitor of PLA_2 and may prove to be a useful tool in the delineation of the role of PLA_2 in the inflammatory process.

Q 429 PLATELET-ACTIVATING FACTOR (PAF) INDUCES CYCLOOXYGENASE (CO) ACTIVITY IN HUMAN TRACHEAL EPITHELIAL (HTE) CELLS, Ronald W. Walenga, Rohit Dwivedi, Departments of Pediatrics and Physiology Biophysics, Case Western Reserve University School of Medicine, Cleveland OH 44106

Platelet-activating factor (PAF) is a potent phospholipid mediator of inflammation which can evoke many of the pathophysiological responses seen in chronic airway inflammatory diseases. PAF stimulates PGE_2 production by *in vitro* cultures of HTE cells, in a concentration dependent manner (EC_{50}=15nM), which is inhibited by PAF-receptor antagonists (JC Carl, RW Walenga 1993 Am Rev Respir Dis 147:A449). We have found that at concentrations as low as 1nM, PAF leads to elevated levels of CO activity in these cells (measured by immunoassay of PGE_2 production from 30μM arachidonic acid). That increase is time dependent, requiring 6-8 hours for maximum induction (up to 300% of basal), and dependent on protein synthesis, since it is inhibited by cycloheximide. RNA synthesis inhibitors such as actinomycin D only partially inhibited the increase in CO activity, suggesting that translational regulation may play a role in the increased CO activity. BN52021, a PAF receptor antagonist, abolished the increase in CO in response to PAF, while the stable PAF agonist, pyrolidino-PAF, elevated CO levels at 0.1 nM. In both untreated and PAF-stimulated cells, antisera with specificity toward PGHS-2 reacted with two bands between 70-75kD in Western blots of microsomal protein, while antisera specific for PGHS-1 showed little reactivity. These results suggest that normal human airway epithelial cells can respond to inflammatory stimuli, not only with the immediate release of an anti-inflammatory agent, PGE_2, but also by increasing their capacity for subsequent synthesis of that homeostatic eicosanoid.

Q 430 INFLUENCE OF SYNTHETIC PHOSPHOLIPID ANALOGUES ON PHOSPHATIDYLCHOLINE AND PHOSPHATIDYLETHANOLAMINE BIOSYNTHESIS

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Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are quantitatively the most important phospholipids in mammalian tissues, especially in MDCK cells [1]. Both lipids are synthesized from diacylglycerol which reacts with CDPcholine or CDPethanolamine to yield PC and PE, respectively. Since PC and PE are essential components of cellular membranes, a balanced synthesis of membrane phospholipids seems to be important for optimal growth of the cell and regulation of the different biosynthetic pathways of PC and PE is necessary [for review see 2]. For example, the biosynthesis of PC is maximally activated under choline-deficient conditions whereas the formation of PE seems to be inhibited [3]. Previously, we have shown that the alkylphosphocholine hexadecylphosphocholine (HePC) inhibits PC-biosynthesis in MDCK cells [4] and antagonizes phorbol-ester induced PC-biosynthesis in the same cell line [1]. In the present study, we investigated the influence of three different phospholipid analogues (Hexadecylphosphocholine (HePC), octadecylphosphocholine (OcPC) and hexadecylphosphoethanolamine (HePE)) on choline incorporation into PC and ethanolamine incorporation into PE. Our results show that only the analogues with a choline headgroup inhibit PC-biosynthesis whereas HePE has no influence. On the other hand, HePE inhibits incorporation of ethanolamine into PE. In contrast, HePC and OcPC slightly stimulate the biosynthesis of PE from ethanolamine. These results suggest that the biosynthetic pathways of PC and PE are tightly coupled.

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Q 431 INHIBITION OF CoA-INDEPENDENT TRANSACYLASE (CoA-IT) INTERRUPTS ARACHIDONATE (AA) MOVEMENT INTO ETHER PHOSPHOLIPIDS (PL) AND INHIBITS LIPID MEDIATOR PRODUCTION. James D. Winkler, Alfred N. Fonteh†, Chui-Mei Sung, Lisa Huang, Lisa A. Marshall and Floyd H. Chilton †. Dept. Pharmacol., SmithKline Beecham Pharm., 709 Swedeland Rd., King of Prussia, PA, 19406, USA and † Dept. Pulmonary Med., Bowman Gray School of Medicine, Medical Center Blvd., Winston-Salem, NC, 27103, USA.

Evidence has accumulated that inflammatory cells remodel AA from 1-acyl PL into 1-ether PL, transferring AA into major PL pools from which it is released upon cell activation. The enzyme CoA-IT has been hypothesized to mediate this process. To test this hypothesis, we used a tool compound, SK&F 98625 (diethyl 7-(3,4,5-triphenyl-2-oxo-2,3-dihydro-imidazol-1-yl)heptane-phosphonate). This compound inhibited microsomal CoA-IT activity (IC_{50} 9 μM), but not the activities of acetyltransferase or either soluble or cytosolic PLA_2s. To test the effect of CoA-IT inhibition on AA movement, human neutrophils (PMN) were pulsed with [^{3}H]AA for 5 min, washed and the movement of [^{3}H]AA followed over two hrs without stimulation. During this time, there was a remodeling of [^{3}H]AA into 1-ether PL. SK&F 98625 produced a concentration-dependent blockade in the movement of [^{3}H]AA from 1-acyl PL into 1-alkyl-PC and 1-alkenyl-PE. Because the rapid movement of AA in 1-ether PL has been proposed to be important for lipid mediator production, the effects of CoA-IT inhibition were examined. Treatment of PMN with SK&F 98625 for 5 min followed by stimulation with 2 μM A23187 was found to block the release of AA (IC_{50} 10 μM). There has been increasing evidence over the last 3 years, in studies utilizing broken cell preparations, that CoA-IT, and not PLA_2 , catalyzes the formation of lyso PAF and hence regulates PAF production. SK&F 98625 inhibited PAF production in A23187-stimulated PMN (IC_{50} 12 μM). This work is the first to utilize a specific inhibitor to explore the role of CoA-IT in inflammatory cells and inflammation. We conclude that CoA-IT is required for the movement of AA into 1-ether PL, the production of PAF and the release of AA. Thus inhibition of CoA-IT represents a novel therapeutic mechanism for reducing lipid mediators of inflammation.

Late Abstracts

ACTIVATION OF PI3K THROUGH THE PDGF RECEPTOR; EFFECTS OF RECEPTOR MUTANTS AND WORTMANNIN, Brian C. Duckworth, Andrius Kazlauskas, and Lewis C. Cantley, Division of Signal Transduction, Department of Medicine, Beth Israel Hospital, Boston, MA 02115

Upon binding of PDGF to the PDGF receptor, phosphatidylinositol 3-kinase (PI3K) is recruited to the receptor and there is a coordinate synthesis of $PI3,4P_2$ and PIP_3, two lipids not found in resting fibroblasts. Wortmannin is a fungal toxin which has recently been shown to inhibit partially purified PI3K at low nanomolar concentrations. It has also be given at similar concentrations to PC12 cells or neutrophils *in vivo* and shown to inhibit synthesis of PIP_3.

We have looked at signaling through the PDGF receptor via PI3K using receptor mutants and wortmannin. HepG2 epithelial cells expresssing mutant receptors which do not bind PI3K do not synthesize $PI3,4P_2$ or PIP_3 in response to PDGF, although these cells have high background levels of PIP_3. Nanomolar concentrations of wortmannin added to A31 fibroblasts completely abolish PDGF stimulated synthesis of $PI3,4P_2$ and PIP_3, but only partially inhibit PDGF receptor associated, pTyr associated, or total cellular PI3K activity. At varying wortmannin concentrations there is a good correlation between the remaining level of PDGF receptor-associated PI3K activity and the remaining level of PDGF-stimulated thymidine incorporation.

Role of regulatory phospholipase A2's in inflamed human skin

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The phospholipases A2 (PLA2) comprise a group of enzymes releasing unsaturated fatty acids from the *sn*2-position of membrane phospholipids. Once released, the fatty acids are acted upon by various enzymes and turned into biologically very important signalling molecules, the eicosanoids, which play an important role in inflammation. The level of PLA2 enzyme is elevated both locally and in circulation during infections, inflammatory diseases, tissue damage and brain dysfunction, and correlate with the severity, magnitude and duration of this disorders. Uncontrolled or excessive PLA2 activity may promote chronic inflammation, allergic reactions, tissue injury and pathophysiological complications. PLA2 play an important proinflammatory role in psoriasis, and we have investigated the presence and expression patterns of two regulatory (cytosolic and non-pancreatic) PLA2s in normal and psoriatic skin. We have detected expression of both non-pancreatic and cytosolic PLA2 in normal human skin, both transcripts by RNA hybridization and mature protein by immunhistologic techniques. In involved psoriatic skin, the non-pancreatic PLA2 is overexpressed in cells resident in skin (fibroblasts and keratinocytes), and different levels of expression is detected. From the localization and overexpression of npPLA2 in vascular dermis, the enzyme may play an important role in T cell activation. Differences in expression patterns are believed to result from the presence of various up- and down-regulators of npPLA2 gene expression, known to be overproduced in psoriatic skin. Induction studies of epidermal and connective tissue cells are being performed.