CELL ACTIVATION AND SIGNAL INITIATION: RECEPTOR AND PHOSPHOLIPASE CONTROL OF INOSITOL PHOSPHATE, PAF AND EICOSANOID PRODUCTION

Organizers: Edward Dennis, Michael Berridge and Tony Hunter
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Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

Keynote Address: Triggering Events and Consequences (joint)

S 001 Protein Kinase C: Structure, Function and Mechanism of Regulation by Lipid Second Messengers. Robert M. Bell, Duke University Medical Center, Department of Biochemistry, Durham, N.C. 27710.

Protein kinase C is activated by sn-1,2-diacylglycerol (DAG) produced from phosphatidylinositol-4,5-bisphosphate and glycerolipids and is inhibited in vitro and in cells by sphingosine and lysosphingolipids, breakdown products of cellular sphingolipids. The goals of our studies are to understand the mechanism of protein kinase C regulation by phospholipids, Ca^{2+}, DAG (phorbol-esters) and the physiological significance of sphingosine inhibition/pharmacology. The structure of protein kinase C isoenzymes (80 kDa) revealed by cDNA cloning and sequencing and biochemical studies have provided knowledge of two functional domains, the catalytic fragment (51 kDa) and the lipid binding (32 kDa) fragment. Mixed micellar techniques were employed to investigate phospholipid and DAG cofactor specificity and stoichiometry and to deduce oligomeric state. The proposed model, a 4PS-Ca^{2+}-DAG-PKC complex, was tested by preparation of 17 phosphatidylserine derivatives. These analogues support and extend the model. Studies on sphingosine inhibition in vitro were studied by autophosphorylation and with histone substrates. Those in cells, platelets, neutrophils, HL60 cells, and A4B1, demonstrated protein kinase C inhibition occurred without blocking other regulatory pathways, thus suggesting specificity. The available data for sphingosine/lysosphingolipids functioning as second messengers and for a sphingolipid cycle will be presented. Emphasis will be on the relationship between sphingolipids and tumor formation. The role of protein kinase C-I transfection of 3T3-NIH cells in altering cell growth and enhancing tumorigenicity is consistent with protein kinase C involvement in critical events of carcinogenesis. The detailed model of regulation has lead to the discovering of several inhibitors, many of which are anti-tumor agents. Supported by CA46738 and GM38738.

S 002 MUTATIONS TEST A STRUCTURAL AND FUNCTIONAL MODEL OF THE α CHAIN OF G_S (α_S). H. R. Bourne, R. T. Miller, S. B. Masters, and K. A. Sullivan. Departments of Pharmacology and Medicine and the Cardiovascular Research Institute, San Francisco, CA 94143, USA.

We have proposed a structural model of the G protein α chain and predicted specific locations for its functional domains (Masters et al., *Protein Engineering* 1:47, 1986). Now we have tested the model using mutations in the α_S polypeptide chain of S49 mouse lymphoma cells. The *unc* and H21a mutations resulted from selective pressure that abolished interactions of G_S with receptors or adenylyl cyclase, respectively. cDNA sequences indicate that the *unc* mutation encodes proline in place of arginine at the sixth position from the carboxy terminus of α_S. This mutation causes the observed change in charge of the *unc* polypeptide and could kink a predicted carboxy-terminal α helix. Pertussis toxin-catalyzed ADP-ribosylation of the α chains of G_i, G_O, and transducin, at a site near the carboxy terminus, uncouples these G proteins from their receptors. In parallel, the *unc* mutation strongly suggests that the carboxy terminus of α_S, like that of other G protein α chains, interacts with receptors. The H21a mutation substitutes alanine for glycine at a hinge region in the presumptive GDP-binding domain (residue 208). We propose that normal movement at this presumed hinge is required for GTP-dependent changes in conformation. This proposal is supported by the observation that binding of GTP analogs (but not of GDP) prevents tryptic cleavage of wild type α_S, but not of H21a α_S, at a site six residues from the mutated amino acid. Thus the H21 mutation appears to prevent α_S from stimulating adenylyl cyclase by preventing it from distinguishing between GTP and GDP. A third test of the model utilizes expression of a chimeric α chain nucleotide sequence encoding the amino terminal 60% of α_i linked (in frame) to the carboxy terminal 40% of α_S. The α_i/α_S chimeric polypeptide, expressed *via* a retroviral vector in S49 *cyc*$^-$ cells (which lack α_S mRNA and polypeptide), couples β-adrenoceptors to stimulation of cAMP synthesis, a phenotype similar to that produced by normal α_S in wild type S49 cells. This phenotype suggests that the substantial differences in amino acid sequence between the amino terminal portions of α_i and α_S do not affect coupling either to the β-adrenoceptor or to adenylyl cyclase itself.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 003 INOSITOL(1,3,4,5)TETRAKISPHOSPHATE - THE OTHER SECOND MESSENGER FROM INOSITOL LIPIDS? Robin F. Irvine, Department of Biochemistry, AFRC Institute of Animal Physiology & Genetics Research, Babraham, Cambridge, CB2 4AT, U.K.

The concept is now generally accepted that inositol(1,4,5)trisphosphate is an intracellular second messenger whose function is to mobilize Ca^{2+} from intracellular stores. However, more recent investigations have revealed that Ins(1,4,5)P_3 is not alone metabolically, and furthermore it is now emerging that it is not alone functionally either. Some Ins(1,4,5)P_3 is rapidly phosphorylated to Ins(1,3,4,5)P_4 (refs. 1,2) and this novel inositol phosphate can synergize with Ins(1,4,5)P_3 in some tissues to regulate Ca^{2+} entry, and probably also Ca^{2+} mobilization (refs. 3,4). Our current understanding of how Ins(1,3,4,5)P_4 works is that it controls the Ca^{2+} content of the intracellular Ca^{2+} store which is mobilized by Ins(1,4,5)P_3, and thus the two inositol phosphates work as a duet to regulate intracellular Ca^{2+} levels.

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Phospholipase Mechanism

S 004 PHOSHOLIPASE MECHANISM, ACTIVATION AND INHIBITION, E.A. Dennis, F.F. Davidson, L.J. Reynolds, M.D. Lister, and R.A. Deems, Department of Chemistry, University of California, San Diego, La Jolla, CA 92093.

The biosynthesis of protaglandins, leukotrienes, lipoxins and related eicosanoids depends on the availability of free arachidonic acid. The simplest and most obvious candidate for the enzyme responsible for the release of free arachidonic acid would be a phospholipase A_2 (1), although other phospholipases are possible. Our laboratory has been studying the mechanism of action of phospholipase A_2 (2). Recently, we have turned our attention to inhibitors of phospholipase A_2, both because they provide mechanistic insight about the enzyme and because of their potential therapeutic importance. We and others studying inhibitors of phospholipase A_2 have relied almost exclusively on the extracellular phospholipase A_2 from cobra venom or mammalian pancreas because they are pure, and can be studied mechanistically. The cobra venom enzyme is activated by phosphorylcholine-containing lipids and the pancreatic enzyme by anionic lipids (3). Recently, we have extended our kinetic studies to a membrane-bound phospholipase A_2 isolated from a macrophage-like cell line (4). We have studied a number of irreversible inhibitors such as p-bromophenacylbromide and manoalide (5) and reversible inhibitors such as dyes, fatty acids (3) and non-hydrolyzable substrate analogues. Recent studies on new synthetic analogues of these inhibitors which help elucidate their mode of action and answer mechanistic questions will be presented.

The anti-inflammatory activity of corticosteroids has been explained by the induction of lipocortin synthesis and lipocortins have been claimed to be specific, non-competitive inhibitors of phospholipase A_2. However, we (6) have shown that lipocortin and calpactin inhibition of pancreatic phospholipase A_2 activity can be best explained by a "substrate depletion" phenomenon. Recent kinetic studies confirm this explanation for other substrate systems. Thus, studies of lipocortin inhibition of extracellular phospholipases cannot be used to support non-competitive inhibition as an explanation for the anti-inflammatory effect of corticosteroids.

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Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 005 CRYSTALLOGRAPHY OF PHOSPHOLIPASE A_2: STRUCTURE/FUNCTION RELATIONSHIPS. D. Scott, A. Achari, P. Barlow, J. C. Vidal, S. Brunie*, Z. Otwinowski and P. B. Sigler. Department of Biochemistry and Molecular Biology, The University of Chicago, 920 East 58th Street, Chicago, Illinois 60637; *Ecole Polytechnique, Palaiseau, France.

The transduction of signals from external receptors to the cell's internal metabolic control centers is mediated primarily by 'second messengers' (e.g., arachidonate, IP_3, diacyl glycerol) formed by the phospholipase-catalyzed breakdown of phospholipids in the cytosolic leaflet of the plasma membrane. To understand the chemistry of this process requires that we understand the structural basis for an enzyme's interaction with the polar face of a membrane or a membrane-like aggregate. The paradigm we have used to understand this process is extracellular phospholipase A_2, which releases headgroup components if, and only if, the phospholipid is condensed in lamellar aggregates. These enzymes (some of which are potent neurotoxins) produce exceptionally good crystals that yield highly resolved and functionally intriguing structures.

We have determined a series of high resolution crystal structures of phospholipase A_2 (PLA_2) and their physiologically important complexes aimed at answering the following questions: (1) What surface of the enzyme binds the phospholipid aggregate? (2) What features of the aggregated phospholipid are responsible for enhancing enzyme action? (3) What is the stereochemical basis for the essential role of the calcium ion in binding and catalysis? (4) What is the catalytic mechanism? Attempts to answer these questions will be based on crystallographic results as well as kinetic studies with constrained substrates. So far we have identified at least one region of dimeric PLA_2 that almost certainly interacts specifically with the lamellar phospholipid aggregate but is not involved in the binding or catalysis of monomeric phospholipids. We are currently contrasting the nearly isomorphous crystal structure of a calcium-containing PLA_2 with that of the same molecule that lacks this essential cofactor. Progress will be reported on attempts to prepare and analyze cocrystals containing PLA_2 and stable substrate analogues designed to emmulate aggregated phospholipid.

Phospholipase Activation and Inhibition

S 006 INTERRUPTION OF PLATELET DERIVED GROWTH FACTOR SIGNAL TRANSDUCTION IN NIH 3T3 CELLS EXPRESSING THE EJ-ras ONCOGENE, Robert R. Gorman, Christopher W. Benjamin, W. Gary Tarpley, Patricia L. Olinger, Vincent E. Groppi and Alice H. Lin, Cell Biology Department, The Upjohn Company, Kalamazoo, MI 49001.

Platelet derived growth factor (PDGF) elicits a diverse group of biochemical responses in NIH-3T3 cells. PDGF initiates phospholipase C (PLC) activation, inositol trisphosphate (IP_3) and diacylglycerol (DAG) formation. These two mediators stimulate Ca^{2+} mobilization and protein kinase C respectively, which in turn lead in proto-oncogene induction and mitogenesis. This set of responses is altered in NIH-3T3 cells expressing the EJ-ras oncogene. Exposure of EJ-ras transformed cells to PDGF results in essentially no PLC activation as measured by IP_3 formation. Measurement of PDGF-stimulated Ca^{2+} mobilization in single cells using digital imaging techniques and the fluorescent indicator fura-2 shows that stimulated PDGF Ca^{2+} mobilization is also attenuated in EJ-ras transformed cells. Microinjection of IP_3 stimulates Ca^{2+} mobilization in both control and EJ-ras transformed cells, and microinjection of recombinant ras proteins attenuates Ca^{2+} mobilization in control cells. In addition, Northern analysis shows much reduced levels of PDGF-stimulated c-fos mRNA induction in EJ-ras transformed cells. Despite this overall dampening of PDGF-stimulated responses, EJ-ras transformed cells still display a mitogenic response to PDGF. Our experiments suggest that mutated ras does not stimulate PLC as postulated by others, but actually blocks growth factor-stimulated PLC activity and Ca^{2+} mobilization. These data appear to dissociate PLC activation from PDGF-stimulated mitogenesis in EJ-ras transformed cells.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 007 PHOSPHOLIPASE D SPECIFIC FOR GLYCOSYL-PHOSPHATIDYLINOSITOLS, Martin G. Low Department of Physiology and Cellular Biophysics, College of Physicians and Surgeons of Columbia University, New York NY 10032.
Glycosyl-phosphatidylinositols (GPI) are utilised as membrane anchors for a functionally diverse group of cell surface proteins (1). Similar lipid molecules may also act as a source of insulin second messengers. It has been proposed that one function of the GPI protein anchor is to permit release of proteins from the cell surface as the result of specific degradation by endogenous phospholipases. Production of lipid molecules with biological signalling potential (e.g. 1,2-diacylglycerol and phosphatidic acid) might be important consequences of protein release by this mechanism. A soluble enzyme activity which may perform this function has been identified in several mammalian tissues (2,3). This anchor degrading enzyme can be distinguished from phosphoinositide-specific phospholipase C activity since it is relatively insensitive to inactivation by heating at 60°C and is inhibited by 1,10 phenanthroline. It can also be resolved from the phospholipase C activity by gel filtration chromatography on Sephacryl S-300. Several GPI-anchored proteins (e.g. alkaline phosphatase, 5'-nucleotidase, variant surface glycoprotein) are substrates for the enzyme but phosphatidylcholine and phosphatidylinositol are not hydrolysed. The lipid product of anchor degradation is phosphatidic acid indicating that the enzyme is a GPI-specific phospholipase D (GPI-PLD). The GPI-PLD activity is relatively high in plasma and serum of several mammalian species suggesting that this is its normal location. The GPI-PLD has relatively low activity against GPI-anchored proteins in membranes compared to that observed with the purified detergent-solubilized protein. The precise reason for this has not been identified but experiments with alkaline phosphatase reconstituted into phospholipid vesicles suggest that, in common with other phospholipases, the lipid environment has a major influence on activity. However, the mechanisms which might regulate the ability of the GPI-PLD to release proteins from the cell surface remain unknown and of great interest.

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S 008 REGULATION OF PHOSPHATIDYLINOSITOL 4,5-BISPHOSPHATE PHOSPHOLIPASE C. C.O. Rock and S. Jackowski. St. Jude Children's Research Hospital, Memphis, TN 38101.
A variety of hormones and polypeptide growth factors stimulate the formation of inositol 1,4,5-trisphosphate and diacylglycerol leading to an increase in cytosolic calcium concentration and protein phosphorylation. The regulated step in the cascade is the ligand-promoted activation of phospholipase C leading to the breakdown of phosphatidylinositol 4,5-bisphosphate (PtdIns-P_2). The relationship between PtdIns catabolism and cell growth was examined in a Chinese hamster ovary cell inositol auxotroph (CHO-K1-Ins). Metabolic labeling experiments indicated that polyphosphoinositides and inositol phosphates were the critical metabolites required for cell growth and function because (1) the cellular content of PtdIns species was conserved at the expense of the soluble pool; and (2) the level of PtdIns decreased whereas the concentrations of inositol polyphosphates, PtdIns-P and PtdIns-P_2 were preserved during inositol starvation. The addition of α-thrombin to growth-arrested (serum-starved) CHO-K1-Ins cells stimulated the incorporation of [^{3}H]thymidine into DNA to the same extent as that observed following the re-addition of serum. γ-Thrombin was also an effective mitogen whereas active site-inhibited α-thrombin did not stimulate significant DNA synthesis. Both α- and γ-thrombin, but not active site-inhibited α-thrombin, initiated PtdIns turnover *in vivo* and enhanced the activity of a membrane-associated PtdIns-P_2 phospholipase *in vitro*. The α- and γ-thrombin dose-response curves for mitogenesis, PtdIns turnover *in vivo*, and phospholipase C activation *in vitro* were comparable implicating a thrombin-regulated PtdIns-P_2 phospholipase C as the mediator of the mitogenic response of CHO cells to thrombin. PtdIns-P_2 phospholipase C activity was significantly stimulated by PtdIns-P, PtdIns-P_2 and phosphatidic acid (PtdOH) at low substrate concentrations. Kinetic data were consistent with cooperative regulation of phospholipase C by substrate and PtdOH. Effective activators of phospholipase C required the presence of both hydrophobic (fatty acid) and negatively charged (phosphate) moieties. Structural analogs of PtdOH (lipopolysaccharide and lipid X) possessed these structural requirements and enhanced PtdIns hydrolysis. PtdOH was also a CHO cell mitogen and potentiated the α-thrombin activation of phospholipase C *in vitro* indicating that the generation of PtdOH following hormone stimulation may provide a mechanism to amplify the response. These data indicate that the stimulation of kinases that increase the membrane concentration of PtdIns-P, PtdIns-P_2, or PtdOH would activate and/or enhance hormone-dependent PtdIns-P_2 degradation and initiate the mitogenic cascade. (Supported by ACS and ALSAC).

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

Inositol Phosphate Metabolism and Calcium Mobilization

S 009 PHOSPHATIDYLINOSITOL KINASES AND PI TURNOVER Lewis Cantley, Malcolm Whitman, David Kaplan*, Gerda Endemann, Leslie Serunian, Leona Ling, John Schulz, Steven Dunn, and Thomas Roberts*. Department of Physiology, Tufts University School of Medicine, Boston, MA. and *Department of Pathology and Laboratory of Neoplastic Disease Mechanisms, Boston, MA.

Resynthesis of polyphosphoinositides following hormone or growth factor stimulation of inositol-1,4,5-P_3 production is essential for a prolonged response in many tissues. We have purified phosphatidylinositol (PI) kinases and phosphatidylinositol-4-phosphate (PIP) kinases from several tissues in an attempt to understand regulation of these enzymes. Three distinct PI kinases with strikingly different enzymatic properties have been characterized and named type I, type II and type III. The type I enzyme has a low K_M for ATP (10 uM), is inhibited by detergent, and is only weakly inhibited by adenosine. This enzyme copurifies with an 85 KD peptide which is phosphorylated on tyrosine in platelet derived growth factor stimulated or polyoma virus transformed fibroblasts. We have not detected this enzyme in human red cells or bovine brain. The type II enzyme has a somewhat higher K_M for ATP (35 uM), is activated by detergent and is inhibited by adenosine (K_I = 15 uM). This enzyme has a native molecular weight of approximately 50 KD and is found in fibroblasts, brain, and red cells. The type III enzyme has a very high K_M for ATP (750 uM) and high K_I for adenosine (1.5 mM) and is activated by detergent. The apparent molecular weight of the native type III enzyme is 230 KD. We have detected this enzyme only in bovine brain. The type II and type III enzymes exclusively phosphorylate the 4' position on PI while the type I enzyme produces a novel PIP not previously described. We have also purified a PIP kinase to homogeneity from human red cells. This enzyme has little if any PI kinase activity. A 53 KD peptide copurifies with the PIP kinase activity and antibodies against this peptide precipitate the activity. Possible mechanisms of regulation of these enzymes and their roles in synthesis of polyphosphoinositides will be discussed.

S 010 REGULATION OF PHOSPHOINOSITIDASE C AND SYNTHESIS OF INOSITOL PENTAKISPHOSPHATE IN AVIAN AND MAMMALIAN CELLS, C. Peter Downes, Leonard Stephens, Philip T Hawkins, Christopher P Berrie, Jose Boyer* and T. Kendall Harden* Department of Cellular Pharmacology, Smith Kline & French Research Ltd, The Frythe, Welwyn, Hertfordshire. AL6 9AR, U.K. * and Department of Pharmacology, University of North Carolina, Chapel Hill, NC 27514, U.S.A.

Phosphoinositidases C (PICs) in a variety of tissues are apparently regulated by a guanine nucleotide dependent protein (G-protein)[1,2]. We have recently proposed[3] that the turkey erythrocyte membrane represents a valuable model system in which the molecular and kinetic details of PIC regulation may be studied. Hydrolysis of endogenous [^{3}H]-inositol labelled phosphoinositides to inositol phosphates is stimulated 20-40 fold by 1μM GTPγs in the presence of an ATP regenerating system. The enzyme appears to be highly specific for PtdIns(4,5)P_2, there being little direct hydrolysis of PtdIns4P and no detectable breakdown of PtdIns. Turkey erythrocytes also possess a population of purinergic P_{2Y} receptors that are coupled to PIC activation. Agonist- dependent hydrolysis of PtdIns(4,5)P_2 can therefore be studied using both intact cells and isolated membranes. The response to purinergic agonists in isolated membranes is entirely dependent upon guanine nucleotides and the agonists greatly potentiate the response to GTPγs alone. This large enhancement of the guanine nucleotide-dependent hydrolysis of PtdIns(4,5)P_2 by agonists is currently being used to determine the mechanism by which agonists and G-proteins combine in the activation of PIC.

In addition to at least two inositol trisphosphate isomers, certain mammalian and avian cells also contain substantial quantities of inositol (1,3,4,5,6) pentakinsphosphate ($InsP_5$)[4]. We have identified a novel inositol tetrakisphosphate isomer (L-myo inositol (1,4,5,6) tetrakisphosphate) which is converted to $InsP_5$ by a widely distributed kinase. The enzyme from rat brain has been partially purified and some of its properties will be described.

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Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 011 PHOSPHATIDYLINOSITOL-DERIVED MESSENGER MOLECULES, Philip W. Majerus, Division of Hematology-Oncology, Washington University School of Medicine, St. Louis, MO 63110.
The inositol phospholipids serve as precursors or storage forms of molecules that function in intracellular signaling. Arachidonate and related fatty acids yield icosanoids, diglyceride activates protein kinase C, and inositol phosphates function as intracellular effectors. The number of different inositol phosphates that are possible is 66. Thus, an enormous potential array of second messengers can be produced from these products of lipid breakdown. In cells, at least 20 different inositol phosphates have been identified to date. These compounds can be identified by a combination of electrophoresis, and ion exchange chromatography. Specific phosphatases and kinases that form and degrade these compounds have been discovered. These enzymes are also useful in characterizing inositol phosphates. Thus far, an Ins(1,4,5)P_3 3-kinase has been identified. Specific phosphatases include inositol monophosphatase, Ins(1,4,5)P_3 5-phosphomonoesterase that removes 5 phosphates from cyclic and non-cyclic tris- and tetraphosphates, Ins(1:2-cyclic)phosphate hydrolase, Ins polyphosphate 1-phosphate that cleaves Ins(1,4)P_2 to Ins(4)P and Ins(1,3,4)P_3 to Ins(3,4)P_2. Ins polyphosphate 4-phosphatase and 3-phosphatase enzymes also have been identified. The large number of specific phosphatase enzymes that metabolize inositol phosphates infers that these many compounds may play unique, albeit undefined, roles as second messengers.

S 012 INOSITOL PHOSPHATES AND Ca^{2+}-MOBILIZING RECEPTORS. J.W. Putney, Jr., A.R. Hughes, D.A. Horstman and H. Takemura, Calcium Regulation Section, Laboratory of Cellular and Molecular Pharmacology, National Institute of Environmental Health Sciences, PO Box 12233, Research Triangle Park, NC 27709, USA.
Activation of Ca^{2+}-mobilizing receptors is associated with breakdown of phosphatidylinositol 4,5-bisphosphate, and release of inositol 1,4,5-trisphosphate ((1,4,5)IP_3). (1,4,5)IP_3 is believed to signal the release of intracellular Ca^{2+}, and may also be responsible, at least in part, for agonist-activated Ca^{2+} entry. Recently, other inositol polyphosphates, such as inositol 1,3,4,5-tetrakisphosphate ((1,3,4,5)IP_4) and inositol cyclic 1:2,4,5-trisphosphate ((c1:2,4,5)IP_3), have been shown to be formed in activated cells, and roles for these compounds in cellular Ca^{2+} regulation have been suggested. Muscarinic receptor stimulation of rat parotid acinar cells results in the accumulation of both (1,4,5)IP_3 and (c1:2,4,5)IP_3. (c1:2,4,5)IP_3 is formed at only about 1 % of the rate of (1,4,5)IP_3, but due to its slow rate of metabolism, (c1:2,4,5)IP_3 accumulates to about 2/3 the level of (1,4,5)IP_3 at steady-state. The addition of atropine causes an apparent first-order decline in the levels of these inositol polyphosphates from which half-times for their rates of turnover can be estimated. Turnover of cellular (1,4,5)IP_3 has a half-time of 7.6 sec, while turnover of (c1:2,4,5)IP_3 has a half-time of almost 10 min. Under similar experimental conditions in which cytosolic [Ca^2] was monitored by fura-2 fluorescence, atropine caused cytosolic [Ca^{2+}] to return to basal level within 30 sec. Also, the agonist-sensitive Ca^{2+} pool was restored to its initial level within 5 min. Thus, (c1:2,4,5)IP_3 probably does not appear to directly regulate Ca^{2+} signalling in the parotid. In acinar cells which had been stimulated with methacholine for 40 min, cytosolic [Ca^{2+}] returned to basal levels somewhat more slowly than in those stimulated for only 5 min. This may indicate that Ca^{2+} mobilization in the parotid acinar cell is under coordinate control of more than one inositol polyphosphate. In AR42J pancreatoma cells, substance P gives rise to a 10-fold increase in (1,4,5)IP_3. However, neither (1,3,4,5)IP_4 nor any of its metabolites are formed. Homogenates of AR42J cells cannot convert (1,4,5)IP_3 to (1,3,4,5)IP_4, but can convert (1,3,4,5)IP_4 to all of its predicted metabolites. These cells give a substantial Ca^{2+} mobilization response when activated with substance P. These findings indicate that in the AR42J cells, Ca^{2+} mobilization may be activated by (1,4,5)IP_3 without any participation of (1,3,4,5)IP_4 or other products of the 3-kinase pathway.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

Protein Phosphorylation

S 013 TARGETS FOR SIGNAL-TRANSDUCING PROTEIN KINASES, Tony Hunter, Kathy Gould, Clare Isacke, Peter Van der Geer, Ellen Freed, and Rick Lindberg. The Salk Institute, P.O. Box 85800, San Diego, California 92138

Protein phosphorylation is a major mechanism whereby signals are transduced from external stimuli into cellular responses. Many growth factor receptors are ligand-stimulatable protein-tyrosine kinases (PTK), while other PTKs, such as $pp60^{c\text{-}src}$, located on the inner face of the plasma membrane have also been implicated in signal transduction. Protein kinase C (PKC), a DAG-regulated protein-serine kinase also binds to the inner face of the plasma membrane where it can respond to external stimuli that induce the turnover of PI. We have identified p81 as a substrate for both the EGF receptor and $pp60^{v\text{-}src}$. p81 is localized to the core of surface microvillar spikes. Together with Tony Bretscher (Cornell), we have obtained p81 cDNA clones, and their sequence is being used to elucidate p81 function, and to assign Tyr phosphorylation sites prior to mutagenesis. Among the PKC substrates we have identified is a 180K surface glycoprotein against which we have raised several monoclonal antibodies. p180 is present in coated pits, which suggests that it may be a receptor. We have immunoaffinity purified p180 and shown that PKC phosphorylates it at a single major Ser site which is also phosphorylated in TPA-treated cells. We have determined primary sequences of several p180 tryptic peptides and are now trying to obtain p180 cDNA clones. Among the family of cell adhesion receptors that recognize the sequence Arg.Asp.Gly in their ligand, are the vitronectin and fibronectin receptors. We have found that the vitronectin receptor β subunit is phosphorylated at a single Ser in TPA-treated cells. Purified vitronectin receptor is phosphorylated in vitro by PKC at the same Ser, and we are determing if this phosphorylation is of functional consequence.

The signal-transducing protein kinases are themselves substrates for other protein kinases. Thus the EGF receptor and $pp60^{c\text{-}src}$ are both phosphorylated by PKC. PDGF treatment of quiescent fibroblasts induces rapid phosphorylation of 5% of $pp60^{c\text{-}src}$ at an unknown Tyr and at 2 novel, but unassigned, Ser sites in its N-terminal 18K, which together cause a slightly retarded gel mobility, as well as an increase in phosphorylation of Ser 12 of all $pp60^{c\text{-}src}$ molecules, due to activation of PKC by PDGF-induced PI turnover. These changes correlate with a 3-fold increase in $pp60^{c\text{-}src}$ kinase activity. We are determining whether the N-terminal Tyr in $pp60^{c\text{-}src}$ is phosphorylated by the PDGF receptor itself, and whether these additional phosphorylations play a role in the PDGF mitogenic response.

We have begun to identify novel PTKs by screening cDNA libraries with oligonucleotide probes to consensus sequences, and with anti-phosphotyrosine antibodies. Several candidate PTK cDNA clones have been isolated. Such PTKs may also be involved in signal transduction.

S 014 THE STRUCTURE, LOCALIZATION AND POSSIBLE FUNCTION OF MULTIPLE SPECIES OF PROTEIN KINASE C, Ushio Kikkawa[1] and Yoshitaka Ono[2], [1]Department of Biochemistry, Kobe University School of Medicine, Kobe 650, [2]Biotechnology Laboratories, Central Research Division, Takeda Chemical Industries, Osaka 532, Japan

Protein kinase C has been shown to exist as a large family of multiple subspecies with subtle individual characteristics by complementary DNA analysis and biochemical studies. This heterogeneity comes from different genes as well as from alternative splicing of a single RNA transcript. The subspecies have closely related structures having several highly conserved regions among this protein kinase family. Biochemical studies have shown that their mode of activation and catalytic properties differ slightly from one another. By using a combination of biochemical and immunocytochemical techniques, their differential regional and cellular expression are shown especially in the nervous tissues. Each subspecies of this enzyme family may have a specialized function in transducing various physiological signals into different cell types.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

PAF and Ether Phospholipid Role

S 015 Observations on the Interaction of Platelet Activating Factor with its Receptor and Its Modulation. By Donald J. Hanahan, Junko Sugatani and Danielle Nunez, Department of Biochemistry, University of Texas Health Science Center, San Antonio, Texas 78284-7760.

This study has centered attention on the biochemical events associated with the interaction of platelet activating factor (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine; AGEPC) with its putative receptor. Initial interest was directed towards the possible involvement of proteolytic enzymes in the activating process. It was observed that chymotryptic protease inhibitors but not tryptic protease inhibitors could block the AGEPC induced alterations on platelets. Phenylmethylsulfonyl fluoride (PMS), diethylnitrophenyl phosphate (DNNP) and N-acetyl-l-tryptophan ethyl ester (NATrpEE) were particularly effective if added to platelets before addition of AGEPC. If these reagents were added to platelets and the cells washed with buffer prior to AGEPC, then no inhibition of platelet activation was noted. The binding of AGEPC to these cells and their cyclic AMP levels were not affected by these chymotryptic type inhibitors. Interestingly, tryptic protease type inhibitors, e.g. pamino benamidine and benzoyl arginine methyl ester were ineffective in blocking AGEPC induced activation of platelets. It appears that these protease inhibitors block a reaction developed after AGEPC has bound to the cell and that a chymotryptic type serine protease might be involved. The latter inhibitors, do not block binding of AGEPC to platelets. There was no effect if the inhibitors were introduced after the AGEPC. The data were supportive of an AGEPC-induced activation of a protease. Another important modulator of AGEPC action on human as well as rabbit platelets, namely zinc ions, has been investigated in some detail. In rabbit platelets, there is a preferential inhibition of serotonin secretion induced by AGEPC, whereas with the latter agonist only a low level of inhibition of aggregation is noted. Even more dramatic, zinc ions, in the 10 to 20 μ molar range, are potent inhibitors of AGEPC-induced aggregation and serotonin secretion in human platelets even in the presence of 1.33 mM Ca^{2+}. There is a concentration dependence (AGEPC vs. Zn^{+2}) to the interaction and other cations, such as La, Cu, Mg, Cd showed no inhibition at the 10 to 20 μM level. In the human platelet, zinc does not inhibit thrombin-, arachidonic acid- or ADP-induced aggregation or secretion. Of particular interest, addition of zinc to human platelets undergoing AGEPC-induced aggregation can cause a complete reversal of this latter phenomenon. The significance of these observations as they apply to agonist interaction and modulation will be discussed.

This investigation was supported by a grant, AQ887, from the Robert A. Welch Foundation.

S 016 METABOLISM AND REGULATION OF PLATELET ACTIVATING FACTOR (PAF), RELATED METABOLITES, AND PRECURSOR POOLS. Fred Snyder, Ten-ching Lee, Merle Blank, Boyd Malone, Tomio Kawasaki, and David Vallari. Med Hlth Sci Div, Oak Ridge Assoc Univ, Oak Ridge, TN 37831

PAF can be formed de novo in most mammalian tissues and by a remodeling pathway in certain cell types (e.g., neutrophils, macrophages, differentiated HL-60 cells) after they are stimulated by appropriate agents. Recent characterization of the properties of an acetyltransferase, a phosphohydrolase, and a cholinephosphotransferase in the de novo pathway have shown they are distinctly different from the corresponding enzymes that utilize 1-alkyl-2-lyso-sn-glycero-3-phosphocholine, phosphatidate, and diacylglycerols, respectively, as substrates. These experiments have also clearly demonstrated the specificities of the enzymes that catalyze the final reactions in both the remodeling and de novo pathways determine the type of PAF species produced in various cells. Enzymes responsible for catalyzing the rate-limiting steps in the de novo synthesis of PAF have been identified as cytidylyltransferase (activated by fatty acids) and acetyl-CoA:1-alkyl-2-lyso-sn-glycero-3-P acetyltransferase and indirect evidence obtained with 20:4-depleted and 20:4-supplemented HL-60 cells (differentiated) suggest an arachidonoyl specific phospholipase A_2 catalyzes the rate-limiting step for PAF production in the remodeling route. Factors that provide crucial signals for turning on and off the various enzymatic activities involved in PAF metabolism include divalent cations (Ca^{2+}, Mg^{2+}), pH, compartmentalization within the cell, and thiol compounds. Catabolic enzymes (acetylhydrolase, lyso-phospholipase D, Pte H_4-dependent alkyl cleavage monooxygenase) are also important in regulating PAF levels.

Alkylacylglycerophosphocholines (alkylacyl-GPC), a stored precursor form of PAF in the remodeling pathway, is produced when radiolabeled PAF, lyso-PAF, or alkylacetylglycerols are incubated with a variety of cell types. With PAF or lyso-PAF the alkylacyl-GPC formed mainly contains polyunsaturated acyl groups at the sn-2 position, whereas alkylacetylglycerol produces mostly saturated species. These results are consistent with the concept that the more saturated species of alkylacylglycerophosphocholines are derived via acyl-CoA:1-alkyl-2-lyso-sn-glycerol-3-P acyltransferase and the polyunsaturated species originate from the acylation of lyso-PAF via a well documented CoA-independent transacylase. There also appears to be a precursor reservoir of acetylated lipids that exists for the de novo pathway. 1-Alkyl-2-acetyl-3-acyl-sn-glycerols have been shown to be synthesized from alkylacetylglycerols by intact HL-60 cells and by a specific acyltransferase from these cells. The relationship of this neutral lipid PAF precursor to the remodeling pathway of PAF biosynthesis is presently being investigated. Support: US DOE (DE-AC05-76OR00033), NHLBI (27109-06, 35495-02); and ACS (BC-70R).

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

Summation: Receptor Functioning (joint)

S 017 REGULATION OF THE EPIDERMAL GROWTH FACTOR RECEPTOR, Gordon N. Gill, Department of Medicine, University of California, San Diego, La Jolla CA 92093.

Cellular responses depend on the concentration of both growth factors and their cognate receptors. Under physiological conditions, both are extensively regulated to give graded proliferative and other responses. Constitutive expression of ligand EGF or over-expression of EGF receptor results in transformation and enhanced tumor growth. Measured biological responses to EGF, including Ca^{2+} transport, gene induction, and cell proliferation, depend on activation of the intrinsic protein tyrosine kinase activity of the receptor. Mutational inactivation of intrinsic protein tyrosine kinase activity and microinjection of monoclonal anti-phosphotyrosine antibodies inhibit ligand-induced internalization and consequent receptor desensitization, indicating that receptor metabolism also depends on intrinsic protein tyrosine kinase activity. An unanswered question is whether this requirement for ligand-induced internalization reflects self-phosphorylation as an essential part of the allosteric change in the receptor or phosphorylation of cellular substrates. Phosphorylation of the EGF receptor via protein kinase C results in loss of high affinity binding and decreased EGF-stimulated tyrosine phosphorylation. Expression of the EGF receptor gene is increased by ligand EGF, tumor promoters, cAMP and retinoic acid, and is decreased by glucocorticoids. The complex multilevel control of EGF receptor expression allows appropriate biological responses under a variety of physiological conditions. In response to ligand binding, the protein tyrosine kinase is activated via allosteric changes necessary for transmembrane signaling and involves self-phosphorylation of a regulatory/inhibitory carboxyl terminus.

S 018 HUMAN LEUKOCYTE RECEPTORS FOR LEUKOTRIENES, Edward J. Goetzl, Jeffrey W. Sherman, Jeanne P. Harvey, and Catherine H. Koo, Howard Hughes Medical Institute and Departments of Medicine and Microbiology-Immunology, University of California Medical Center, San Francisco, California 94143-0724.

Human polymorphonuclear leukocytes (PMNLs) express receptors of three distinct specificities for the potent leukotriene (LT) mediators of hypersensitivity and inflammation. PMNL receptors for LTB_4 are localized in plasma membranes and exist in two affinity states with respective mean equilibrium dissociation constants (Kds) of 0.3 nM and 61 nM. A mean of 4400 high-affinity receptors per PMNL transduce chemotactic and surface adherence responses to nM LTB_4, whereas a mean of 270,000 low-affinity receptors per PMNL signal the degranulation and oxidative burst elicited by higher concentrations of LTB_4. Guanine nucleotides convert high-affinity receptors to the low-affinity state reversibly and LTB_4 enhances the binding of guanine nucleotides to specific N proteins in PMNL membranes, demonstrating the functional relevance of the interactions of N proteins with LTB_4 receptors.

In contrast to receptors for LTB_4, PMNL receptors for LTC_4 and LTD_4 are each a single population of approximately 30,000 per PMNL with a mean Kd of 30 nM. The subsets of receptors for LTC_4 and LTD_4 are distributed identically with approximately 1/3 on membranes and 2/3 on lysosomal granules of PMNL. The PMNL receptors for LTD_4 transduce increases in the cytosolic concentration of calcium and in adherence to surfaces, without altering other cellular activities. LTC_4 has no effects alone on PMNL functions, so that receptors for LTC_4 are dedicated solely to specific uptake of LTC_4 for intracellular oxidative and peptidolytic degradation.

Affinity cross-linking of LTB_4 and LTB_4 analogs to receptors on PMNL and HL-60 cell-derived myelocytes labels one membrane protein of 60-80 kD. Purification of the solubilized binding proteins of PMNL and HL-60 myelocyte receptors for LTB_4 by affinity-chromatography with columns bearing LTB_4 and mouse monoclonal antibodies to framework determinants of the receptors has yielded a predominant 60-80 kD protein for each type of leukocyte. The purified receptor proteins bind [^{3}H]LTB_4 and are recognized by rabbit anti-idiotypic antibodies to the combining site of LTB_4 receptors. The cellular properties of leukotriene receptors thus are explained by the structures of the binding proteins and their association with guanine nucleotide-binding proteins.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 019 THE FUNCTION OF GLYCOSYL-PHOSPHOINOSITIDES IN HORMONE ACTION,

Alan R. Saltiel, The Rockefeller University, New York, NY 10021.

It has recently been discovered that over thirty proteins of diverse origin and function are anchored to the plasma membrane by covalent attachment to glycosyl-phosphoinositides. In these novel structures, proteins are linked via an amide bond to ethanolamine, which is coupled to an oligosaccharide glycosidically linked to phosphatidylinositol(PI). We have found a structurally homologous form of this glycolipid that apparently is not attached to protein. This molecule undergoes a phosphodiesteratic hydrolysis catalyzed by a specific phospholipase C, giving rise to diacylglycerol and an inositol phosphate glycan. In liver, muscle and fat cells, this hydrolysis reaction is acutely stimulated by insulin. The resulting inositol-glycan modulates the activities of several insulin-sensitive enzymes involved in metabolic regulation, and mimics certain of the actions of insulin in intact cells Kinetic and mechanistic studies on the generation and action of the inositol glycan suggest a possible role as a second messenger for some of the acute actions of insulin. The glycosyl-PI precursor for this putative second messenger has been isolated and characterized. Glycosyl-PI synthesis can be detected in a liver microsomal fraction and its kinetic characterization is underway. The specific phospholipase C responsible for the insulin-sensitive hydrolysis of the glycosyl-PI has been purified from liver plasma membranes. New data suggest that certain glycosyl-PI anchored proteins may be released in response to insulin, perhaps due to the activation of one or more specific phospholipases. Elucidation of the functional role of glycosyl-PI in the generation of second messengers or the release of proteins from biological membranes may provide further insights into the pleiotropic nature of insulin action.

Late Addition

S 020 TRANSFER AND EXPRESSION IN VIVO OF HUMAN ADA cDNA IN MURINE HEMATOPOIETIC CELLS,

David A. Williams[1,2], Bing Lim[1,2], Jane F. Apperley[1] and Stuart H.Orkin[1,2], The Children's Hospital and Dana Farber Cancer Institute, Harvard Medical School[1] and Howard Hughes Medical Institute[2], Boston MA 02115.

Severe combined immunodeficiency disease secondary to adenosine deaminase (ADA) is an inherited fatal disease currently being studied as a model for somatic gene therapy. Our laboratory has developed simplified recombinant retrovirus vectors which transfer the human ADA cDNA into murine hematopoietic stem cells. Transplantation of these cells leads to expression of human ADA at levels near endogenous murine levels in primary CFU-S-derived spleen colonies (MCB 7, 1987).

Using prestimulation of cells with Wehi-3B conditioned media to improve efficiency of gene transfer into hematopoietic stem cells, we can demonstrate human ADA expression in the peripheral blood of 24/24 recipients who receive unselected transduced hematopoietic stem cells five weeks after transplantation. In addition, simplified vectors have been modified by the addition of the herpes thymidine kinase promoter driving the neomycin-resistance gene, and these vectors also lead to expression of human ADA in vivo in murine peripheral blood samples. Such results are promising with respect to the possible application of retrovirus-mediated gene transfer to somatic gene therapy for ADA deficiency.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

Phospholipases, PAF

S 100 THE PHOSPHATASES OF HUMAN T-LYMPHOCYTE MEMBRANES, Denis Alexander, Mark Hexham, Richard Marais*, Jozef Goris+, Wilfried Merlevede+ and Michael J. Crumpton. Imperial Cancer Research Fund, Lincoln's Inn Fields, London, *The Ludwig Institute of Cancer Research, London, +Afdeling Biochemie, Katholieke Universiteit te Leuven, Belgium.

The early events of human T lymphocyte antigen recognition and activation are mediated by the polypeptides of the Ti-CD3 antigen receptor complex. Activation of T cells results in stimulation of protein kinase C (pkC) and the subsequent phosphorylation of the CD3 γ polypeptide (Cantrell, D. et al. Nature 325, 540, 1987; Davies, A. et al. J. Biol. Chem. 262, 10918, 1987). In order to investigate the possible role(s) of protein phosphorylation/dephosphorylation in T cell signal transduction more fully, we have undertaken a study of human T cell phosphatases. It has been shown that dephosphorylation of the CD3 γ chain can occur in T cell microsomal fractions, pointing to the possible role of a membrane associated phosphatase. Immunoblotting of purified plasma membranes has revealed the presence of membrane-bound forms of the ATP, Mg-dependent phosphatase (AMD;type 1), polycation stimulated phosphatase (PCS;type 2A) and calcineurin (type 2B). In order to determine which of these phosphatases dephosphorylates CD3, peptide analogues of the γ chain were phosphorylated with pkC and used as phosphatase substrates. Initial characterisation of these phosphopeptides as substrates for purified phosphatases obtained from muscle and brain demonstrated their striking selectivity for PCS phosphatases in contrast to AMD phosphatase and calcineurin. Furthermore, phosphopeptide phosphatases from Jurkat-6 cell-line microsomes co-eluted through 5 purification steps with PCS phosphorylase phosphatases. The data suggest that membrane associated PCS phosphatases may be involved in dephosphorylating the CD3 γ polypeptide in T cells.

S 101 MOLECULAR CLONING AND SEQUENCE ANALYSIS OF RAT PHOSPHOINOSITIDE-SPECIFIC PHOSPHOLIPASE C. C.F.Bennett, J.M. Balcarek, and S.T. Crooke, Dept. of Molecular Pharmacology, Smith Kline and French Labs, Philadelphia, PA 19101.

We have previously reported the purification and characterization of a 62,000 molecular weight phosphoinositide-specific phospholipase C (PI-PLC I) from guinea pig uterus (C.F. Bennett and S.T. Crooke, (1987) J. Biol. Chem. 262, 13789). PI-PLC I preferentially hydrolyzed PIP_2 at nM concentrations of calcium and was found to be tightly associated with membrane fractions from several tissues, thus exhibiting properties expected of a PI-PLC coupled to agonist-stimulated phosphoinositide hydrolysis. Monospecific PI-PLC I antibodies were used to screen a λ gt11 cDNA expression library constructed from rat basophilic leukemia cell (RBL-1) mRNA. Twenty strongly immunoreactive plaques were identified and purified; several overlapping clones were selected for further study. Antibodies selected against the ß-galactosidase fusion proteins reacted specifically with PI-PLC I on immunoblots of total cytosolic proteins. The deduced amino acid sequence encoded by the 1.8 kb cDNA clone contains peptides with identical sequence to guinea pig uterus PI-PLC I tryptic peptides, including the amino-terminal sequence. The 1.8 kb clone hybridizes to a moderately abundant 2.1 kb mRNA species from RBL-1 cells. Tissue distribution analysis of PI-PLC I mRNA revealed that the 2.1 kb mRNA was most abundant in testis and intestine and least abundant in skeletal muscle and heart.

S 102 BRADYKININ-INDUCED ACTIVATION OF SENSORY NEURONES MAY BE MEDIATED BY PROTEIN KINASE C, Gillian M. Burgess, Humphrey P. Rang & Mary McNeill, Sandoz Institute for Medical Research, London WC1E 6BN, UK.

Bradykinin (Bk), which stimulates the peripheral terminals of nociceptors, depolarised cultured sensory neurones from rat dorsal root ganglia (DRGs). This depolarisation was associated with an inward current and an increase in membrane conductance. Bk increased the rate of entry of ^{45}Ca through voltage-sensitive Ca^{2+} channels, and levels of inositol(1,4,5)trisphosphate and of diacylglycerol (DAG) also rose. DAG activates protein kinase C (PKC), and as PKC regulates several membrane ion channels, the possibility that direct activation of PKC with phorbol esters could mimic some of the actions of Bk was tested. It was found that application of β-phorbol dibutyrate (PDBu) to DRGs did in fact lead to a depolarisation and inward current associated with an increase in membrane conductance. PDBu also increased ^{45}Ca entry into the cells via voltage-sensitive Ca^{2+} channels. The responses to maximal concentrations of Bk and PDBu were not additive, and the Bk-induced increase in ^{45}Ca uptake was blocked by the putative PKC inhibitor staurosporine (IC_{50} 20nM). A possible sequence of events might be as follows. The Bk-induced elevation of DAG activates PKC, which in turn opens a novel membrane conductance. This leads to depolarisation and the firing of action potentials, and eventually to increased Ca^{2+} entry through voltage-sensitive Ca^{2+} channels. These studies suggest that PKC may play a role in sensory transmission by nociceptive neurones.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 103 AGONIST-INDUCED DIACYLGLYCEROL (DG) FORMATION FROM PHOSPHATIDYLCHOLINE (PC) BY A PHOSPHOLIPASE-D (PL-D) PATHWAY AND THE EXTENT OF PROTEIN KINASE C (PKC) INVOLVEMENT, Myles C. Cabot, Clement J. Welsh and H-t. Cao*, W. Alton Jones Cell Science Center, Lake Placid, NY 12946, *Visiting Scientist, Shanghai Institute of Biochemistry, Shanghai, China.

We have recently shown that phorbol esters and vasopressin (VP) elicit PC degradation and collateral DG formation in cultured cells (Biochim. Biophys. Acta, in press). We now provide information on the enzymatic pathway of DG production, and show phorbol esters induce PC degradation by a PKC-directed mechanism, whereas the VP-induced reaction is, in part, PKC-independent. PC metabolism was followed in REF52 cells prelabeled with either glycerol, choline, or myristic acid. Treatment of cells with phorbol diesters or VP caused an increased release of [^{3}H]choline which was accompanied by increases in cellular phosphatidic acid (PA). The temporal pattern of agonist-induced PA and DG formation is characteristic for a precursor (PA) product (DG) relationship for a PC degradative sequence initiated by PL-D. Propranolol, a phosphatidic acid phosphatase inhibitor, blocked agonist-stimulated DG formation but did not decrease PA production. Treatment of cells with phorbol dibutyrate (PDBu), which decreases PKC activity in many cell types, reduced, upon rechallenge, the subsequent PDBu-induced PC hydrolysis by 85%; however, less inhibition of VP-induced PC hydrolysis occurred (~25%). VP + PDBu elicited an additive PC degradative response, indicative of bifricated mechanisms of action. Agonist-induced PC hydrolysis may provide a second source of DGs, downstream from the phosphoinositide event, important in temporal regulation of PKC activity.

S 104 DEMONSTRATION OF RECEPTOR COUPLED PHOSPHOLIPASE C AND INOSITOL POLYPHOSPHATE PHOSPHATASE ACTIVITIES IN ISOLATED B LYMPHOCYTE MEMBRANES, John C. Cambier and Millie M. Chien, National Jewish Center for Immunology and Respiratory Medicine, Denver, CO 80206.

Recent studies have documented ligand-receptor mediated activation of phosphatidylinositol 4,5-bisphosphate ($PtdInsP_2$) specific phospholipase C (PLC) activities in isolated cell membranes in a number of tissues. Studies described here extend these findings by demonstrating that antibodies specific for B lymphocyte antigen receptor, i.e., membrane immunoglobulin (mIg), stimulate PLC activities which hydrolyze phosphatidylinositol (PtdIns) and $PtdInsP_2$ as well as an inositol bisphosphate ($InsP_2$) and inositol trisphosphate ($InsP_3$) phosphatase activity. Anti-Ig stimulation of ^{3}H-PtdIns labeled membranes from B lymphocytes led to release of inositol 1-phosphate (^{3}H-InsP), which was detectable within one minute and continued through the course of the ten-minute assay. Equivalent stimulation of ^{3}H-$PtdInsP_2$ labeled membranes led to rapid release (< 30s) of ^{3}H-$InsP_3$ which was immediately dephosphorylated to ^{3}H-$InsP_2$ and ^{3}H-InsP unless appropriate phosphatase inhibitors were present. Stimulation of membranes in the presence of exogenous ^{3}H-$InsP_3$ led to rapid dephosphorylation of this species as well as the ^{3}H-$InsP_2$ to yield ^{3}H-InsP. Further analysis revealed that the PLC activities were optimally activated only in the presence of ligand plus Ca^{++} (0.1 μM) and GTP (10 μM). The ligand induced phosphatase activity was independent of both Ca^{++} and GTP. These findings suggest that anti-immunoglobulin binding to B cells lead to signal transduction via activation of hydrolysis of both PtdIns and $PtdInsP_2$ and dephosphorylation of $InsP_3$ and $InsP_2$. Thus, ligation of receptors leads to both generation and catabolism of the Ca^{++} mobilizing second messenger $InsP_3$ as well as diacylglycerol generation from both PtdIns and $PtdInsP_2$.

S 105 PAF STIMULATES PHOSPHOINOSITIDE GENERATION IN RAT ANTERIOR PITUITARY CELLS, Lindsey Grandison and Phyllis Callahan, RWJ Med Sch, Piscataway, NJ 08854.

In a previous study PAF was observed to stimulate the secretion of prolactin and growth hormone from cultures of rat anterior pituitary cells. Stimulation of secretion was dose dependent, time related and reversed by PAF receptor antagonists. Release of other anterior pituitary hormones, luteinizing hormone and thyroid stimulating hormone, was not effected by PAF. In this study PAF was observed to induce the generation of phosphoinositides in cultures of rat anterior pituitary cells. Stimulation of phosphoinositide generation occurred over the dose range of 1 to 100 nM PAF during a 5 min incubation. HPLC fractionation of radiolabelled phosphoinositides showed that inositol 1,4,5 phosphate and its metabolites were formed. A time course indicated that stimulation persisted for approximately 15 mins and thereafter no further generation of phosphoinositides occurred. This short term response to PAF contrasts with the prolonged response produced by TRH or bombesin, two well characterized stimulators of phosphoinositide generation in the anterior pituitary. The short action of PAF was not due to metabolism of PAF by the pituitary cells, or by inactivation of pituitary cells by PAF since cells exposed to PAF still responded to TRH. Preliminary data suggest that PAF causes a rapid desensitization of pituitary cells to PAF but not other receptor ligands. In summary PAF can induce secretion of anterior pituitary hormones as a result of polyphosphatidylinositol hydrolysis and consequently PAF may act as a modulator of anterior pituitary function. This work was supported by NIH grant DK 38027.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 106 PURIFICATION OF PHOSPHATYDILINOSITOL KINASE FROM HUMAN ERYTHROCYTES. Andrea Graziani and Lewis Cantley. Departement of Physiology, Tufts University School of Medicine, Boston, MA.

The content of polyphosphoinositides during the stimulation of phosphatydilinositol turnover by a variety of cellular stimuli is very tightly controlled; it is therefore likely that the phosphatydilinositol (PI) and phosphatydilinositolphosphate (PIP) kinase activities play a crucial role in order to mantain or increase the pools of PIP and PIP_2 during a prolonged response.

Different PI kinases (referred as type I, II and III) with strikingly different enzymatic properties and leading to the phosphorylation of PI in different position of the inositol ring, have been characterized from various tissues. Human red blood cells (RBC) membranes appear to contain only type II PI kinase activity, which features a relatively high K_M for ATP, is strongly inhibited by adenosine, activated by non ionic detergent and phosphorylates PI in the D-4 position. Type II PI kinase activity has been solubilized from human erythrocytes ghosts with Triton X-100, chromatographed on an anion exchange column and then on a cation exchange column. This procedure afforded a 2500 fold purification of the PI kinase from the RBC ghosts; the specific activity is 200 nmoles/min/mg protein. The resulting activity exhibited a major silver stained band in SDS-PAGE electrophoresis with an apparent 57.000 dalton molecular weight. To test the identity of the 57.000 dalton band, sections of the gel have been assayed for PI kinase activity. After electrophoresis the gel has been sectioned, omogenized and incubated in presence of phospholipids to allow renaturation. PI kinase has been then assayed in presence of ATP and found to reside exclusively in the 56.000-58.000 dalton region of the gel.

S 107 SUBCELLULAR DISTRIBUTION OF PHOSPHOLIPASE C ACTIVITY IN PLANTS, H. Pfaffmann, E. Hartmann and D. J. Morre, Allgemeine Botanik, University Mainz, 6500 Mainz, FRG; and Department of Medicinal Chemistry and Pharmacognosy, Purdue University, West Lafayette, IN 47907, USA (D.J.M.)

The recent interest in phospholipases of plants has been stimulated by their general importance in signal transduction mechanisms and the increasing evidence for a regulative function of inositol phosphates in plant cells. We isolated and characterized a phosphatidylinositol specific phospholipase C in both lower (mosses) and higher plants (soy beans, bush beans). As in mammalian cells, the phospholipase occurred in both a soluble and a membrane-associated form. The membrane-associated activity seems to be preferentially located in the plasma membrane and showed a 10 times higher enzymatic activity compared with the endo-membrane fraction. The plasma membranes were prepared both by aqueous two-phase partitioning and by preparative free-flow electrophoresis. The modulation of enzyme activity and of polyphosphoinositides levels by physiological stimuli emphasizes the involvement in signal transduction processes in plant cells.

H. Pfaffmann, E. Hartmann, A. O. Brightman, and D. J. Morre 1987 Phosphatidylinositol specific phospholipase C of plant stems. Plant Physiol 85: 1151-1156

S 108 SPECTROPHOTOMETRIC COUPLED ENZYME ASSAY OF PHOSPHOLIPASE A_2 ACTIVITY SPECIFIC FOR PLASMALOGEN, Yutaka Hirashima, Marianne S. Jurkowitz and Lloyd A. Horrocks, The Ohio State University, Columbus, OH 43210

Phospholipases A_2 that very actively hydrolyze plasmalogens have been demonstrated in snake venoms and various mammalian tissues including rat brain.

We developed a spectrophotometric coupled enzyme assay specific for plasmalogen using Naja naja venom phospholipase A_2. This procedure is rapid, continuous, sensitive, and not expensive. In this method, the product of the phospholipase A_2 catalyzed reaction, lysoplasmalogen, is quantitated by the use of two auxiliary enzymes, rat liver lysoplasmalogenase and horse liver alcohol dehydrogenase. Rat liver lysoplasmalogenase was partially purified from solubilized microsomes using DEAE-cellulose chromatography. In these coupling reactions, choline lysoplasmalogen is hydrolyzed by lysoplasmalogenase to glycerophosphocholine and free aldehyde. The free aldehyde is quantitatively converted to the alcohol by alcohol dehydrogenase with the oxidation of NADH. The disappearance of NADH is measured spectrophotometrically at 340 nm.

The data obtained with this spectrophotometric method are in good agreement with the data obtained with two-dimensional TLC separations with phosphorus assays. Supported in part by NIH Research Grant NS-10165 and Training Grant NS-07091.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 109 ISOLATION OF TWO DIFFERENT FORMS OF PLC FROM RAT BRAIN, Homma,Y., Imaki,J., Nakanishi,O., and Takenawa,T., Tokyo Metropol.Inst.Gerontol., Tokyo 173, Japan.
Three peaks with activity of inositol phospholipid-specific phospholipase C (PLC) have been resolved from a crude extract of rat brain and two of them have been purified 2810-fold and 4010-fold. The resultant preparations each contained homogeneous enzyme with a molecular weight of 85,000 as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. One of these enzymes (PLC-II) was found to hydrolyze phosphatidylinositol-4,5-bisphosphate (PIP_2) at a rate of 15.3 μmol/min/mg of protein and also phosphatidylinositol-4-monophosphate (PIP) and phosphatidylinositol (PI) at slower rates. For hydrolysis of PI, this enzyme was activated by an acidic pH and a high concentration of Ca^{2+} (>2mM), and showed a Vmax value of 19.2 μmol/min/mg of protein. The other enzyme (PLC-III) catalyzed hydrolysis of PIP_2 preferentially at a Vmax rate of 12.9 μmol/min/mg of protein and catalyzed that of PIP slightly. This enzyme required a very low concentration of Ca^{2+} with an apparent Km for Ca^{2+} of 2×10^{-7} M. The rate of PIP_2 hydrolysis by PLC-III in the presence of octyl glucoside (1%) was three times greater than that in the absence, and exceeded that of PI hydrolysis under all conditions tested. Neither of these enzymes had any activity on phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine or phosphatidic acid. These two enzymes showed not only biochemical but also structural differences. Western blotting showed that antibodies directed against PLC-II and did not react with PLC-III. Furthermore, the two enzymes gave different peptide maps after digestion with α-chymotrypsin or Staphylococcus aureus V8 protease. These results suggest that these two forms of PLC belong to different families of PLC.

S 110 PURIFICATION AND CHARACTERIZATION OF INOSITOL POLYPHOSPHATE 1-PHOSPHOMONOESTERASE FROM BOVINE BRAIN. Roger C. Inhorn and Philip W. Majerus, Washington University Medical School, St. Louis, MO 63110

Inositol polyphosphate 1-phosphomonoesterase was purified to homogeneity from bovine brain. The pure protein has a specific activity of approximately 50 μmoles $Ins(1,4)P_2$ hydrolyzed/min/mg protein. The enzyme migrates at 45,000 daltons on sodium dodecylsulfate - polyacrylamide gel electrophoresis. The protein also has an apparent molecular weight of 45,000 daltons as determined by gel filtration chromatography, suggesting that inositol polyphosphate 1-phosphomonoesterase is a monomeric enzyme. As described previously, inositol polyphosphate 1-phosphomonoesterase hydrolyzes both $Ins(1,4)P_2$ and $Ins(1,3,4)P_3$, and the hydrolysis of $Ins(1,4)P_2$ is inhibited by both Ca^{++} and Li^+ (Inhorn and Majerus (1987) J. Biol. Chem. 262, 15946-15952). Ca^{++} also inhibits hydrolysis of $Ins(1,3,4)P_3$ with an ID_{50} identical to that determined for $Ins(1,4)P_2$. The mode of Li^+ inhibition was further investigated. Lithium inhibits $Ins(1,3,4)P_3$ hydrolysis uncompetitively, which is the same mechanism previously observed using $Ins(1,4)P_2$ as substrate. The K_i of lithium inhibition for $Ins(1,3,4)P_3$ hydrolysis, however, is lower than that for $Ins(1,4)P_2$.

S 111 EFFECTS OF ATP ON THE SPECIFIC [^{3}H]PDBu-BINDING TO PROTEIN KINASE C, Kazuo Irita, Pedro Cuatrecasas and G. Matthew Hebdon, Department of Chemotherapy,Glaxo Inc., Research Triangle Park, NC 27509.

The binding of protein kinase C(PKC) to the membrane is thought to be a prerequisite of the activation of PKC. Although Ca^{2+}, phosphatidylserine(PS) and PKC activators, diacylglycerol or tumor promoters, are thought to modulate the membrane-binding of PKC, the precise mechanism is still unclear. At physiological concentrations of free Ca^{2+}, ATP was found to modulate the specific [^{3}H]PDBu-binding to soluble PKC in a reconstituted system with PS. ATP had both a stimulatory and an inhibitory effect on the PDBu-binding depending on concentration. These effects of ATP were also modified by free Ca^{2+}, PS ,Mg, incubation time and pH. Nonhydrolyzable analogues of ATP were also effective, which suggested that the autophosphorylation of PKC was not involved. ATP had no effect on the PDBu-binding activity of PKC which was pre-bound to PS liposome by calcium. These data indicate the possibility that ATP regulates the binding of PKC to the membrane. The physiological implications of these findings will be discussed.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 112 PHORBOL ESTER RECEPTOR HETEROGENEITY IN GH4C1 CELLS, Susan Jaken and Susan Kiley, W. Alton Jones Cell Science Center, Lake Placid, NY 12946.

GH4C1 cells are a line of rat pituitary somatotrophs that respond to a variety of effectors including stimulators of inositol lipid metabolism. We have previously reported evidence for heterogeneity within the phorbol ester receptor population, and presumably protein kinase C (PKC), in these cells: 1) Binding studies with [3H]phorbol dibutyrate (PDBu) suggested an apparently homogeneous receptor population; however, competition for [3H]PDBu by mezerein revealed two populations. 2) Surprisingly, PDBu receptor down modulation did not decrease the PDBu effect on EGF binding. This indicates that the PDBu receptor population which is resistant to down modulation is functionally associated with this response. We now report biochemical and immunochemical evidence for heterogeneity. 3) Two PDBu receptor populations were separated by hydroxylapatite chromatography, one of which reacts with anti-brain Type 3 PKC monoclonal antibodies (mAbs). 4. We developed a method for separating Ca2+-sensitive from Ca2+-insensitive receptors. In cytosols from cells lysed in EGTA, Ca2+ increases receptor affinity without affecting total binding capacity. Lysis in the presence of excess Ca2+ causes a redistribution of PDBu receptors from cytosol to membranes. Cytosolic receptor number is decreased to 30% of that found with EGTA lysis. This subpopulation of receptors is resistant to the effect of Ca2+ on affinity. No evidence for Type 3 PKC or PKC fragments was found with the mAbs. Further experiments are directed towards isolating the molecular species associated with this Ca2+-independent PDBu binding activity, and determining whether there is type-specific regulation and/or activation of these receptor populations.

S 113 A PHYSIOLOGICAL REGULATOR OF DIACYLGLYCEROL KINASE. I. Jeng, N. Klemm, and C. Wu. MD Inst. of Psychiatry, St. Louis, MO 63139

Diacylglycerol (DG), one of the critical activators for protein kinase C, is catabolized very quickly to phosphatidate. The phosphorylation of DG is catalyzed by DG kinase. We are interested in how DG kinase is regulated. We attempted to purify DG kinase. Subcellular fractionation of rat brain homogenate yielded a greater than 100% recovery of DG kinase activity in the P_2 supernatant. We hypothesized that there was an inhibitor in the P_2 pellet. The P_2 pellet was extracted with 4 volumes of chloroform:methanol (2:1,v/v). After evaporation of the solvent, it was determined that the aqueous layer contained all the inhibitory activity while the organic layer was inactive. We characterized the putative inhibitor of DG kinase in the P_2 pellet. The inhibitor was apparently a macromolecule as its activity was retained in the tubing when dialyzed. Also, when it was fractionated on a P-60 column, the activity appeared in the void volume. We varied the concentrations of DG and ATP and found that they did not enhance or reduce the inhibitors effect, indicating that the inhibitor did not interfere with DG or ATP. We also investigated the possibility of the inhibitor being ATPase. The inhibitor was incubated with ATP and no degradation of ATP was found. Further characterization of this inhibitor suggested that it may play a significant role in control DG concentration during development.

S 114 MCF-7 MULTIDRUG-RESISTANT HUMAN BREAST CANCER CELLS, BUT NOT THE PARENT CELLS, SHOW INSENSITIVITY TO INHIBITORS OF GTP-BINDING PROTEINS. Marti Jett, R. L. Fine, Carole Jelsema, Kenneth C. Cowan, Carol Poston, and Bruce Chabner. Walter Reed Army Inst. Res., Wash. D.C. 20307, Clin. Pharmacol. Br., NCI, and Lab. Cell Biol., NIMH, Bethesda, MD 20896.

Tumor cells exposed to one drug of the natural product type (such as adriamycin) develops cross-resistance as a stable genetic trait to a host of other unrelated antineoplastic drugs (such as actinomycin D, colchicine, vinca alkaloids, etc), and this is known as multidrug-resistance (MDR). Because protein kinase C levels have been shown to be elevated in MDR cells, we examined the events of the cascade which eventually leads to activation of protein kinase C, viz, stimulation of signal transduction through hydrolysis of phosphoinositides. We found that oxytocin, vasopressin, bombesin, Con A, and other agonists produced a vigorous response of accumulation of inositol phosphates (insulin was synergistic) in the MCF-7 MDR cells while the parent cell line responded poorly to these same agonists. Using cholera toxin or pertussis toxin to probe the GTP-binding proteins, we observed classical inhibition of phosphoinositide hydrolysis with both toxins in the parent cell line however in the MDR cells, phosphoinositide hydrolysis was unaffected by either toxin. Antibody probes of the GTP-binding proteins showed quantitative and qualitative differences in levels of specific subunits cross-reacting with antibodies to the GTP-binding proteins.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 115 PURIFICATION AND CHARACTERIZATION OF PHOSPHOLIPASE A2 FROM HUMAN PLACENTA
L.Jordan-Tankou and F. Russo-Marie, North Carolina A & T State University, Greensboro, NC 27411 ; Institut Pasteur,Paris France 75015.
Purification of five isoforms of phospholipase A2 from human placenta has been done. The purification of these enzymes involves the use of Sepharose 6B Chromatography, DEAE-Cellulose Chromatography followed by Affinity Chromatography using an alkyl phospholipid. These proteins chromatographed as high molecular weight aggregates with an apparent molecular weight of 70 KDa. The native molecular weight of the dissociated aggregate has an apparent molecular weight of 15 KDa. The purified protein samples are specific for the sn-2-acyl position of phospholipids as determined from thin layer chromatography analyses. Three of the isoforms required calcium for activity, and the other two forms showed over fifty percent of the maximal activity without calcium present.

S 116 ALTERED SIGNAL TRANSDUCTION IN erbB-TRNSFORMED CELLS,
Miwako Kato, Sadaaki Kawai, and Tadaomi Takenawa, Tokyo Metropolitan Institute of Gerontology, Itabashi-ku, Tokyo 173, Inst. of Medical Science, University of Tokyo, Minato-ku, Tokyo, JAPAN
To clarify the signal transduction mechanism of the erbB gene(virus oncogene) products leading to cell growth and transformation, the alteration of signal transduction induced by enhanced inositol phospholipid metablism was studied in chick embryo fibroblast cells(CEF cells) transformed by gag-fused erbB gene-carrying virus(GEV cells). In GEV cells, the activities of lipid kinases such as phosphatidylinositol(PI), PIP, and diacylglycerol(DG) kinases were increased. Moreover, the 1,2-DG content and the activity of protein kinase C was also elevated in GEV cells. Immunoprecipitates of a GEV cell lysate with antisera that reacted with the erbB gene product had PI kinase activity, but the activity was very weak compared with the total cellular activity. These results suggest that the erbB gene product enhances inositol phospholipid metabolism and subsequent signal transduction, but that it is not involved directly in lipid kinases,although it is closely associated with lipid kinase.

S 117 ON THE PAF-INDUCED MODULATION OF GRANULOCYTE ACTIVATION. Leo Koenderman, Anton Tool, Carel M. Eckmann, Dirk Roos and Arthur J. Verhoeven. Central Lab. Netherlands Red Cross Blood Trans. Service, Amsterdam, The Netherlands.

After addition of platelet-activating factor (PAF) the respiratory burst of human granulocytes does not become activated, but shows an enhanced rate after subsequent activation with heterologous stimuli. Investigating this priming phenomenon, we found that a transient rise in the cytosolic free Ca^{2+} concentration ($|Ca^{2+}|_i$) is sufficient to irreversibly prime human granulocytes. Thus PAF induced a transient rise in $|Ca^{2+}|_i$ and primed the cells to an enhanced respiratory burst upon subsequent stimulation with either formyl-methionyl-leucyl-phenylalanine (fMLP) or phorbol-myristate acetate (PMA). Priming induced by the Ca^{2+} ionophore ionomycin was completely abolished when $|Ca^{2+}|_i$ changes were buffered during exposure of the cells to the ionophore. The priming induced by PAF was, however, only partially inhibited under $|Ca^{2+}|_i$-buffering conditions during priming, indicating multiple pathways in the priming by PAF. Priming of the respiratory burst was not observed when serum-treated zymosan particles (STZ) were used as a stimulus. Moreover, in contrast to fMLP, STZ did not cause the production of IP_3, IP_4 or 1,2-diacylglycerol upon stimulation of human granulocytes. These results indicate that with respect to the respiratory burst of human granulocytes not only multiple pathways of priming exist, but also multiple pathways of activation. Only the "classical pathway of signal-transduction" seems sensitive for priming by PAF.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 118 PURIFICATION AND CHARACTERIZATION OF PHOSPHOLIPASE A_2 FROM HUMAN PLATELETS. Ruth M. Kramer and R. Blake Pepinsky, Biogen Res. Corp., Cambridge, MA 02142. Phospholipase A_2 (PLA_2) plays a key role in many physiological processes and has been implicated in inflammation and tissue injury associated with various diseases. In our attempt to isolate an intracellular PLA_2 involved in generation of eicosanoids and PAF, we have studied two PLA_2 activities from human platelets: 1) A "physiological" PLA_2 was solubilized from platelet lysates in the presence of octylglucoside and EGTA at pH 7.4 and partially purified using chromatography on DEAE-cellulose, Sephacryl S-200 and Heparin-Sepharose. This enzyme readily degraded sonicated dispersions of phosphatidylcholine (PC) hydrolyzing 1-acyl- or 1-alkyl-linked, 2-arachidonoyl-PC equally well. It was stimulated greater than 4-fold by diacylglycerol and reached optimal activity with micromolar concentrations of Ca^{2+}. The PLA_2 activity exhibited an apparent molecular weight of 60,000 (by gel filtration) and was sensitive to detergents, chaotropic agents and pH less than 5. 2) An "acid-stable" PLA_2 was extracted from platelet lysates at pH 1 and purified to homogeneity using chromatography on Fast S Sepharose and G-50 Sephadex, followed by reverse-phase HPLC and SDS-PAGE. This enzyme readily hydrolyzed oleate labeled E.coli phospholipids, but did not act on PC liposomes. It required millimolar concentrations of Ca^{2+} for maximal activity, had a molecular weight of 13,000 and was detergent stable. The partially purified "physiological" PLA_2 was converted to the "acid-stable" form upon dialysis against acetate buffer pH 4.5, suggesting that other protein components or acid-labile cofactors may mediate the Ca^{2+} sensitivity and substrate specificity of the PLA_2 enzyme and thus may play a physiological role in the control and regulation of PLA_2 activity.

S 119 CLONING AND EXPRESSION OF A PHOSPHOLIPASE C TRANSCRIPT, Mark Stahl, Catherine R. Ferenz, Kerry S. Kelleher, Ronald W. Kriz, and John L. Knopf, Genetics Institute Inc., 87 Cambridge Park Drive, Cambridge, Mass 02140.

The binding of various agonists to their specific cell surface receptors rapidly induces the production of the two second messenger molecules derived from phosphidylinositol 4,5-bisphosphate: diacylglycerol, and inositol 1,4,5-triphosphate. The production of these second messenger molecules is mediated by activated phosphatidylinositol specific phospholipase C (PLC) enzymes. Here we report the cloning of a bovine brain cDNA encoding an enzyme PLC-148 with a molecular mass of 148,311 daltons characterized by calcium dependent and phosphatidylinositol specific phospholipase C activity when expressed in mammalian cells. Bovine brain mRNA contains a 7.5 kb transcript corresponding to the isolated cDNA; a related transcript of the same size is present in mRNA from some but not all human cell lines tested. Analysis of the bovine genome using Southern blots suggested that there are at least two genes which hybridize to the cloned PLC-148 cDNA. Sequence analysis has revealed several domains on the molecule which may serve as an initial focus for the generation of mutant enzymes, the analysis of which may provide important insight into the role of this enzyme in signal transduction.

S 120 REGULATION OF THE METABOLISM OF PLATELET ACTIVATING FACTOR (PAF) AND 1-ALKYL-2-ACETYL-SN-GLYCEROLS (G) BY RABBIT PLATELETS. T-c. Lee, B. Malone, M. L. Blank, V. Fitzgerald, and F. Snyder. Med Hlth Sci Div, Oak Ridge Assoc Univ, Oak Ridge, TN 37831

1-Alkyl-2-acetyl-sn-G is converted to PAF and at least 8 other metabolites by rabbit platelets and the amount of PAF produced is increased several fold when saponin-permeabilized platelets are supplemented with CDP-choline. Addition of dithiothreitol with CDP-choline to the permeabilized platelets further potentiated the formation of PAF. These results along with other evidence suggest the formation of PAF from alkylacetyl-G is controlled by the availability of CDP-choline and the activity of alkylacetyl-G:CDP-choline cholinephosphotransferase; this is consistent with our earlier findings that this cholinephosphotransferase is stimulated by dithiothreitol.

[^{3}H]PAF was converted to [^{3}H]alkyllyso[glycerophosphocholine] (GPC) and [^{3}H]alkylacyl-GPC in saponin-permeabilized platelets, albeit at a slower rate than untreated platelets. Inclusion of Ca^{2+} in the incubation media increased the production of alkyllyso-GPC under both conditions. These data suggest a Ca^{2+}-dependent phospholipase A_2 or acetylhydrolase (both membrane associated) may also contribute to the degradation of PAF in platelets. The major molecular species of alkylacyl-GPC generated after incubating [^{3}H]hexadecylacetyl-GPC with rabbit platelets was 16:0-20:4 (75%), while the primary molecular species of alkylacyl-GPC produced from both [^{3}H]alkylacetyl-G and [^{3}H]alkyl-G was 16:0-16:0 (67-71%). These data indicate different types of species of alkylacyl-GPC are formed by PAF inactivation (polyunsaturated species) and de novo synthesis (saturated species). Support: US DOE (DE-AC05-76OR00033); NHLB (HL-27109-07, HL-35495-02).

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 121 PURIFICATION AND PROPERTIES OF AN ARACHIDONOYL-HYDROLYZING PHOSPHOLIPASE A_2 FROM A MACROPHAGE CELL LINE, Christina C. Leslie, Jacqueline Y. Channon, Margaret M. Wall and Pearlanne T. Zelarney, National Jewish Center, Denver, CO 80206.
The first step in platelet-activating factor production and arachidonic acid release from stimulated inflammatory cells is thought to be the activation of a phospholipase A_2 (PLA_2). The mouse macrophage cell line, RAW 264.7 contained a calcium-dependent, cytosolic PLA_2, maximally active at pH 8.0, that hydrolyzed ^{3}H-arachidonic acid from 1-O-alkyl-2-(^{3}H)arachidonoyl-glycerophosphocholine (-GPC). To better characterize the properties of the PLA_2, it was purified to near homogeneity. After ammonium sulfate precipitation, the PLA_2 was chromatographed over sephadex G150, eluting with an apparent molecular weight of 70kDa. The enzyme was then eluted from DEAE cellulose (pH 8.0) at 0.18M NaCl. Further purification was achieved by the following sequence of chromatographic steps: phenyl sepharose, Q-sepharose, sephadex G150 and hydroxylapatite. PLA_2 purification >1000-fold has been achieved resulting in a specific activity of 2 umoles/min/mg protein. The hydroxylapatite purified PLA_2 showed one dominant band on non-reduced polyacrylamide gels with a molecular weight of 70 kDa. On reduced gels the molecular weight shifted lower to 60 kDa. In contrast to snake venom PLA_2 enzymes, the macrophage PLA_2 retained activity in the presence of reducing agents. The purified PLA_2 was inactive in the presence of EGTA but showed significant activity in the presence of nM calcium concentrations. The purified PLA_2 exhibited greatest activity against sn-2 arachidonoyl-containing phospholipid substrates. Supported by NIH grant HL 33755.

S 122 **PHORBOL MYRISTATE ACETATE (PMA) INDUCES ACETYLTRANSFERASE ACTIVATION AND PAF-ACETHER BIOSYNTHESIS IN HUMAN NEUTROPHILS AND MIMICKS THE OPSONIZED ZYMOSAN (OPZ) STIMULATION, S. Leyravaud, M-J. Bossant, F. Joly, G. Bessou, J. Benveniste and E. Ninio, INSERM U 200, Université Paris-Sud, 92140 Clamart, France.**
Challenge of neutrophils with OPZ (2 mg/ml) induced lyso paf-acether production (10 pmol/1x10^6 cells at 5 min) and acetyltransferase activation (1.6-fold the basal level at 10 min). These events led to the formation of 19 pmol of paf-acether (paf) per 1x10^6 cells after 15 min of stimulation. At this time, 21% of the total formed paf was already released. Similar results were obtained when cells were treated with PMA (10 ng/ml). Acetyltransferase basal level (73 pmol paf/min per 1x10^6 cells) was then increased by 2.3-fold when measured at 45 min. Lyso paf (26 pmol/1x10^6 cells at 30 min) and paf (44 pmol at 60 min of which 23% was released) were formed. Higher concentrations of PMA inhibited production of the phospholipids . The lyso paf synthesis, then the acetyltransferase activation induced by PMA preceeded the paf formation as already described using OPZ, although the response to PMA challenge was slower. Paf species from cells treated with each stimulus were analyzed by gas chromatography-electron capture detection. OPZ and PMA probably implicate the same pools of 1-alkyl-2-acyl-sn-glycero-3-phosphocholine for the paf biosynthesis since 80 to 90% of alkyl chains were composed of 16 carbons and 10 to 20% of 18 carbons. Thus, PMA mimicked the OPZ-induced biosynthesis of paf by stimulating both phospholipase A_2 and acetyltransferase steps.

S 123 PURIFICATION AND CHARACTERIZATION OF THE PHOSPHATIDYLINOSITOL-4-PHOSPHATE KINASE FROM RED BLOOD CELL MEMBRANES, Leona E. Ling, John T. Schulz, and Lewis C. Cantley, Department of Physiology, Tufts University School of Medicine, Boston, Massachusetts 02111.
The membrane bound form of phosphatidylinositol-4-phosphate (PIP) kinase was purified 4300 fold from human red blood cells. The purified PIP kinase preparation contained a single 53 Kd protein as analyzed by SDS-polyacrylamide gel electrophoresis. Since PIP kinase activity was renatured from the 43-55 Kd area of these SDS gels, this 53 Kd protein was identified as the PIP kinase. Gel filtration of the native, purified enzyme showed an apparent size of 150 Kd indicating an oligomeric structure. Antibodies prepared against the 53 Kd protein precipitated both the 53 Kd peptide and PIP kinase activity from red blood cell membranes. This enzyme could by extracted from membranes with high salt in the absence of detergent indicating that it is not an integral membrane protein. Under these conditions, the red cell PI kinase remains membrane-bound. The purified PIP kinase requires ATP (K_m=2) and divalent cations ($Mg^{2+} > Mn^{2+}$) for activity. Its activity is inhibited by PIP_2, its product, and activated by phosphatidylserine. The enzyme is specific for PIP and will phosphorylate phosphatidylinositol-4-phosphate at the 5' hydroxyl. However, the enzyme can also phosphorylate phosphatidylinositol-3-phosphate produced by the middleT/c-src associated PI kinase.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 124 KINETIC STUDIES OF A MEMBRANE-BOUND PHOSPHOLIPASE A_2 ISOLATED FROM THE P388D$_1$ MACROPHAGE-LIKE CELL LINE. M.D. Lister, Y. Watanabe, R.J. Ulevitch, R.A. Deems, and E.A. Dennis. Department of Chemistry, University of California, San Diego, La Jolla, CA 92093, and Department of Immunology, Research Institute of Scripps Clinic, La Jolla, CA 92037.

The synthesis of eicosanoids such as prostaglandins, thromboxanes and leukotrienes are dependent on the availability of arachidonic acid (AA). This fatty acid (FA) is normally found esterified at the *sn-2* position of glycerophosphatides and its release is thought to be the rate-limiting step. One possible candidate for the enzyme responsible for AA release is an alkaline pH-optimum, Ca^{2+} dependent, membrane bound phospholipase A_2 (PLA_2). We have now isolated such an enzyme from the macrophage. The enzyme was solubilized from the membrane fraction with octylglucoside and further purified by butanol extraction and HPLC. This yields a 2,500 fold purification of the membrane-bound phospholipase A_2 with a 25% recovery and a specific activity of about 800 nmol min^{-1} mg^{-1} toward 100 μM dipalmitoyl PC in mixed micelles. When this material was subjected to analysis on a Superose 12 sizing column, the molecular weight of the active fraction was approximately 18,000 daltons. A sixty fold purified preparation was found suitable for kinetic characterization based on similar behavior to the purified preparation. Kinetic studies toward palmitoyl, oleoyl and arachidonoyl containing PC vesicles have revealed two distinct activities. Below 10 μM, the data appeared to follow Michaelis-Menten behavior while at higher concentrations, the data could best be fit to a Hill equation with a Hill coefficient of about two. K_m's and V_{max}'s will be reported. Time course studies showed linearity to 15% hydrolysis for saturated acyl containing PC but only 5% for unsaturated. This decrease was due to inhibition of PLA_2 by released unsaturated fatty acid. AA, the most inhibitory, was shown to be a competitive inhibitor with a K_I of about 5 μM suggesting a regulatory role. Compounds of the AA cascade, $PGF_2\alpha$, 6-keto-$PGF_1\alpha$ and TxB_2 showed no inhibition.

S 125 Evidence for the Presence of the Phosphoinositide Cycle in the Lens, Marjorie F. Lou and S. Vivekanandan, Alcon Laboratories, Inc., Fort Worth, TX, 76134

In the lens, free inositol is present at high concentrations. The lens transports inositol from the extracellular source but can also synthesize inositol from glucose via inositol-1-phosphate. In the membrane, the inositol containing phospholipid (phosphoinositides) constitutes only 10% of the total phospholipid and was suggested to play some key role in the cellular differentiation. Recently, one of the phosphoinositides, PIP_2, was located in the epithelial cells but not in fiber cells. Prostaglandin, which uses one of the phosphoinositide metabolites, diacylglycerol, as a precursor in its biosynthesis was also found in the lens. These evidences, although scanty, do provide some clues to the possibility that lens may contain a phosphoinositide cycle similar to retina and cornea. Recently we discovered that rabbit lens epithelial cell layer in culture could incorporate ^{3}H-Inositol and ^{32}P into the membrane and the label accumulates predominantly in the lipids in the form of PI, some in the PIP and PIP_2. The incorporation of ^{3}H-Inositol increased as a function of time and also as a function of the concentration of ^{3}H-Inositol in the media. In a cell free system lens epithelial homogenate could incorporate (γ-^{32}P) ATP into the phosphoinositides with the highest incorporation in the PIP and PIP_2. The membrane bound phospholipase C was activated in the presence of Ca^{++} and could hydrolyze the phosphoinositides into free inositol and its respective inositol phosphates (IP, IP_2, IP_3). Phospholipase C was also stimulated by EGF, glucagon but not insulin.

S 126 RECEPTOR-MEDIATED HYDROLYSIS OF PHOSPHATIDYLCHOLINE BY PHOSPHOLIPASES C AND D IN 1321N1 CELLS. E.A. Martinson and J.H. Brown. UC San Diego, La Jolla, CA 92093.

Previous work from our laboratory has shown that stimulation of muscarinic receptors by carbachol increases $InsP_3$, mobilizes Ca^{2+} and activates protein kinase C in 1321N1 astrocytoma cells. We have recently found that activation of the muscarinic receptor in [^{3}H]choline-labelled 1321N1 cells also leads to an increase in the appearance of both [^{3}H]phosphorylcholine ([^{3}H]cholP) and [^{3}H]choline in the cell medium. Most of the release of [^{3}H]cholP occurs within the first three minutes after carbachol addition suggesting a rapid and transient activation of phospholipase C. The production of [^{3}H]choline also occurs in the first few minutes but continues for at least 30 min such that the [^{3}H]choline release response predominates with time. Since [^{3}H]CholP added to the cell medium is not converted to [^{3}H]choline, the [^{3}H]choline formed upon addition of carbachol may come from hydrolysis of phosphatidylcholine by phospholipase D. The Ca^{2+}-ionophore ionomycin enhances [^{3}H]cholP production in a manner similar to carbachol. On theother hand, phorbol ester treatment leads to the release of [^{3}H]choline, but does not increase [^{3}H]cholP production. We suggest that the increase in intracellular Ca^{2+} that occurs in response to carbachol may be the mechanism whereby phospholipase C is activated to hydrolyze phosphatidylcholine. Activation of protein kinase C may be a mechanism by which muscarinic receptor stimulation leads to hydrolysis of phosphatidylcholine by phospholipase D. Further experiments will address the relationship between the effects of muscarinic receptor stimulation on phosphoinositides, calcium and protein kinase C and the effect of this same receptor on the hydrolysis of phosphatidylcholine.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 127 PLATELET-ACTIVATING FACTOR AND LEUKOTRIENE B_4 SYNTHESIS BY HUMAN NEUTROPHILS: APPARENT PROTEIN KINASE C ACTIVATION OF PHOSPHOLIPASE A_2 ACTIVITY. Thomas M. McIntyre, Sandra L. Reinhold, Stephen M. Prescott and Guy A. Zimmerman, University of Utah, Salt Lake City, UT 84112.

Neutrophils synthesize Platelet-Activating Factor (PAF) and Leukotriene B_4 when stimulated by A23187. Phorbol myristate acetate (PMA) enhances this induction by A23187, but by itself does not stimulate autocoid synthesis. Long chain amines (e.g., sphingosine, stearylamine or palmitoylcarnitine) inhibit Protein Kinase C (PKC) activity in vitro and PMA-induced neutrophil responses. These amines also prevented the synthesis of PAF and LTB_4 by A23187-stimulated neutrophils. These amines did not permeabilize the cells as they still excluded trypan blue. PMA, but not inactive 4α-phorbol didecanoate (PDD) overcame this inhibition in a dose-dependent manner. Half-maximal recovery occurred at $3x10^{-8}$M PMA.

A23187 induces [^{3}H]arachidonic acid release from pre-labeled neutrophils. Sphingosine prevented [^{3}H]arachidonate release from the phospholipids of pre-labeled neutrophils. PMA, but not PDD, reversed this inhibition. Sphingosine did not prevent the synthesis of [^{3}H]LTB_4 from neutrophils presented with [^{3}H]arachidonate at the time of A23187 stimulation. Therefore, while PKC activation alone does not induce autocoid synthesis, it is required to couple the rise in internal Ca^{+2} to activation of a phospholipase A_2-like activity that mobilizes arachidonate and 1-alkyl-sn-glycero-3-phosphocholine from their phospholipid stores.

S 128 THE PAF-ACETHER ANTAGONISTIC ACTIVITY OF WEB 2086 MEASURED EX VIVO IN MAN

Adamus W.S., Heuer H., Meade C.J. and Brecht H.M. (presented by C.J. Meade)
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WEB 2086 is a novel platelet activating factor antagonist. As part of tolerability and efficacy studies in man, it was administered, in double blind placebo-controlled studies, by either the oral, intravenous or inhalative routes. Ex vivo platelet aggregation induced by paf-acether (normally 5 x 10^{-8} M) was used to monitor pharmacological activity. In an oral single rising dose study (doses 1.25, 5, 20, 100, 200, 400 mg), paf-acether induced aggregation 105 min after dosage was totally inhibited from dose 20 mg upwards, and 62% inhibited at 1.25 mg. In a study monitoring effects of single doses of 5, 30 or 90 mg over 24 hours, inhibition was dose dependent, maximal after 1-2 hours and still significant after 10 hours. Multiple oral dosage (3 x 100 mg/day or 3 x 40 mg/day, dose interval 8 h) caused almost complete inhibition throughout a 7 day study period. Intravenous administration (over 30 minutes) of 0.5, 2, 10, 20 or 50 mg resulted in significant inhibition (measured at the end of the infusion period) at all doses tested, and virtually total inhibition at 10, 20 or 50 mg. Inhalative administration of 0.05 mg, 0.25 mg, 0.5 mg or 1.0 mg WEB 2086 inhibited platelet aggregation (induced in this case by 1 x 10^{-8}M paf-acether) significantly at the upper two doses. In all studies, no clinically significant, drug-related changes of laboratory or vital parameters or adverse reactions were observed, and all subjects completed the study. To summarise, WEB 2086 is a potent and well tolerated paf antagonist in man, active by the oral, intravenous or inhalative route.

S 129 POSSIBLE INVOLVEMENT OF GTP-BINDING PROTEIN IN ARACHIDONIC ACID RELEASE BY PHOSPHOLIPASE A_2 IN HUMAN PLATELET, Shigeru Nakashima, Yukio Okano and Yoshinori Nozawa, Department of Biochemistry, Gifu University School of Medicine, Gifu 500, Japan.

The release of arachidonic acid by phospholipase A_2 was examined in [^{3}H]arachidonic acid-labeled human platelets. GTP or GTPγS was found to enhance thrombin-induced [^{3}H]arachidonic acid release in saponin-permeabilized platelets. Each nucleotide alone caused small but significant liberation of arachidonic acid in permeabilized but not in intact platelets. Inhibitors of phospholipase C/diacylglycerol lipase pathway of arachidonic acid release were not effective to reduce the [^{3}H]arachidonic acid release. The loss of [^{3}H]arachidonate radioactivity from phosphatidylcholine was almost complementary to the increment of released [^{3}H]arachidonic acid, suggesting hydrolysis of phosphatidylcholine by phospholipase A_2. GTP analogues stimulate phospholipase C via GTP-binding protein to produce [^{3}H]1,2-diacylglycerol (DG). Thrombin(0.2-2 U/ml)-induced arachidonic acid liberation was almost completely inhibited by pretreatment of pertussis toxin (10 ug/ml), whereas DG production was decreased by only 20-40% in the toxin-treated platelets. Although, GDPβS suppressed thrombin-induced arachidonic acid release and DG formation in a dose-dependent manner(1-1000 uM), the half maximal inhibition required less than 10 uM for arachidonic acid release but more than 100 uM for DG production. Moreover, the dose-response effects of NaF on arachidonate liberation and DG formation were different. These results indicate that arachidonic acid release by phospholipase A_2 might be linked to GTP-binding protein which is different from one coupled to phospholipase C.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 130 CHARACTERIZATION OF A HORMONALLY-REGULATED FORM OF PHOSPHOLIPASE A_2 IN RENAL MESANGIAL CELLS. R.A. Nemenoff, J.H. Gronich, and J.V. Bonventre, Massachusetts General Hospital, Harvard Medical School, Boston, Mass. 02114

Arginine vasopressin (AVP) stimulates arachidonic acid release and prostaglandin production in rat renal mesangial cells, presumably by activating phospholipase A_2 (PLA_2). This effect can be mimicked by phorbol myristate acetate (PMA) in conjunction with the calcium ionophore A23187. This suggests that both second messengers generated by activation of phospholipase C are involved in the AVP- induced stimulation of PLA_2. To directly examine hormonally-induced changes in PLA_2 enzyme activity, cell free extracts were prepared from control and hormone-stimulated cells. PLA_2 activity was assayed using exogenous radio-labeled phospholipids. A soluble from of PLA_2 was identified whose activity is stimulated 2-4 fold in extracts of cells treated with AVP or PMA when assayed at saturating calcium concentrations. Stimulated activity was recovered following fractionation by anion exchange chromatography or gel filtration. The stimulated enzyme migrated at 60,000 daltons and has an absolute requirement for calcium. Soluble extracts prepared form whole kidney showed a major peak of PLA_2 activity comigrating with the regulated enzyme from mesangial cells. The kidney enzyme has been purified several hundred-fold by sequential column chromatography. The ability of PMA to stably alter PLA_2 activity suggests phosphorylation of PLA_2 or a PLA_2 modulatory protein by protein kinase C.

S 131 DESIGN AND SYNTHESIS OF A GENE FOR BOVINE PROPHOSPHOLIPASE A_2, J. Noel[1], D. Dean[2], and M.-D. Tsai[1], Departments of Chemistry[1] and Biochemistry[2], The Ohio State University, Columbus, OH 43210.

We have constructed a gene coding for bovine prophospholipase A_2 by the "shotgun ligation" approach of Grundstrom et. al. [Nucl. Acids Res., 13, 3305-3316]. The gene was designed with a variety of unique, regularly spaced restriction sites which will facilitate future mutagenesis studies by replacement of short restriction fragments with mutagenized cassettes. Codons previously shown to be utilized to a large extent in prokaryotic systems were chosen whenever possible. The total gene spans 404 base pairs, and was constructed from two shorter fragments of 224 and 180 base pairs. The fragments were put together from a set of synthetic oligonucleotides by the "shotgun ligation" technique. The gene and gene fragments were identified in a preliminary restriction endonuclease mapping step prior to dideoxy sequencing. Various expression systems are currently being pursued. Prokaryotic systems that allow secretion of the recombinant product are being focused on. Once expressed, the enzyme will be engineered to probe the catalytic mechanism and substrate binding properties of phospholipase A_2.

S 132 ACTIVATION OF THE LEUKEMIC T CELL LINE JURKAT. COMPARISON OF THE TRANSMEMBRANE SIGNALLING MEDIATED VIA THE CD3-T CELL RECEPTOR COMPLEX, THE CD2 AND CD28 (Tp44) MOLECULES. Daniel Olive[1], Giuseppe Pantaleo[2], Marcello Bagnasco[3], Jacques Nunes[1], Alessandro Moretta[2] and Claude Mawas[1], [1]INSERM U.119, 13009 Marseille, France ; [2]Ludwig Institute for Cancer Research, Lausanne, Switzerland ; [3]Universita di Genova, Italy.

The activation of human T lymphocytes is initiated via the CD3-T cell receptor (TCR) complex but also by the CD2 and CD28 molecules. 1) The mAbs directed against the CD3-TCR complex and the T11.1 + T11.2 epitopes of the CD2 molecule induced an increase in inositol triphosphates (IP3), 1-2 diacylglycerol (1-2 DAG) and intracellular calcium (Ca^{2+}) concentration in the leukemic T cell line Jurkat. In addition, anti-CD3 mAbs induced the translocation of the protein kinase C (PKC). Altogether, these events suggest the linkage of the CD3-TCR complex and of the CD2 molecule to a phospholipase C (PLC). On the contrary, we couldn't detect any IP3 and 1-2 DAG increase after activation by the CD28 pathway, but an increase in Ca^{2+} which occurred only in the presence of extracellular Ca^{2+}. 2) We have investigated the action of cholera toxin on these pathways. Cholera toxin induced an important decrease in the expression of the CD3-TCR and CD28 molecules, while CD2 was unaffected. These events were not associated with the intracellular increase in cyclic AMP since forskolin didn't affect their surface expression. Cholera toxin inhibited profoundly the increase in Ca^{2+} mediated via the three pathways of activation. The half maximal inhibition were stikingly different. Altogether these data suggest that cholera toxin may act on different substrates, possibly G proteins distinct from $G_{s\alpha}$, that could regulate the Ca^{2+} increase induced by the CD3-TCR complex, the CD2 and CD28 molecules.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 133 ACTIVATION OF PHOSPHOLIPASE D BY CHEMOTACTIC PEPTIDE IN HL-60 GRANULOCYTES, Jin-Keon Pai, Marvin I. Siegel, Robert W. Egan and M. Motasim Billah, Schering-Plough Corporation, Bloomfield, NJ 07003.

Activation of phospholipase D (PLD) has been investigated in dimethylsulfoxide differentiated HL-60 granulocytes labeled in endogenous 1-O-alkyl-2-acyl-sn-glycero-3-phosphocholine (alkyl-PC) by incubation with [^{3}H]alkyl-lysoPC. Stimulation of these labeled cells with the chemotactic peptide, N-formyl-Met-Leu-Phe (fMLP), induces rapid generation of [^{3}H]phosphatidic acid (PA) and slower formation of [^{3}H]diglyceride, suggesting hydrolysis of alkyl-PC by PLD. A unique feature of PLD is its ability to transfer the phosphatidyl moiety of phospholipids to alcohols (transphosphatidylation). This characteristic has been exploited to identify PLD activity. For example, when ethanol is present during stimulation of the HL-60 cells, [^{3}H]phosphatidylethanol (PEt) is formed with a concomitant decrease in [^{3}H]PA. Cells incubated with [^{32}P]orthophosphate to label the terminal phosphate of ATP do not incorporate ^{32}P into PEt, consistent with the [^{3}H]PEt not being synthesized from [^{3}H]diglyceride. Furthermore, PEt synthesis closely parallels PA formation and both are inhibited by an fMLP receptor antagonist, suggesting that both PA and PEt are derived from agonist-stimulated PLD action. These observations demonstrate receptor-linked activation of PLD in HL-60 granulocytes.

S 134 INTERLEUKIN-1 STIMULATION OF CELLULAR INOSITOL PHOSPHATE TURNOVER, Subhash S. Rashatwar and Eugene Mochan, University of Medicine and Dentistry of New Jersey, Camden, N.J. 08103

The biochemical events accompanying IL-1 activation of cellular metabolism following receptors occupancy are poorly understood. In this study, we present evidence that one important early metabolic event may be the stimulation of inositol phosphate hydrolysis. Various human cell cultures (synovial cells (HSC), gingival fibroblasts, (HGF), osteosarcoma cells (KHOS)), were incubated with 2uCi/ml ^{3}H-myo-inositol for 48 hrs. and then exposed to human recombinant IL-1. Newly synthesized cellular inositol monophosphate (IP_1), bisphosphate (IP_2) and trisphosphate (IP_3) were characterized and quantitated by Dowex affinity chromatography. All three cell lines exposed to IL-1 (100 Units/ml) showed an increase in levels of IP_1, IP_2, and IP_3 when compared to untreated cells as follows: HSC-IP_1 (78%), IP_2 (111%), IP_3 (162%); HGF-IP_1 (301%), IP_2 (200%), IP_3 (56%); KHOS-IP_1 (305%), IP_2 (169%), IP_3 (92%). With HSC, a rapid increase in all three cellular phosphoinositides occurred within two minutes, which then gradually declined and returned to the basal level at 15 minutes. On the other hand, with HGF and KHOS cells IL-1 continuously elevated cellular phosphoinositides levels for at least 30 minutes. The IL-1 stimulation for all three phosphoinositides in these cell lines was dose dependent with similar EC_{50} (i.e., 50% maximum stimulation), values (20-50 U/ml). These EC_{50} values were similar to those reported for IL_1 activation of thymocyte proliferation, prostaglandin E_2 synthesis and neutral protease production. These results indicate that IL-1 is capable of stimulating inositol phosphate turnover in several different cell types at doses similar to those observed for other IL-1 induced biochemical events. Therefore, the IL-1 induced generation of IP_3 and its co-product diacylglycerol, which subsequently leads to mobilization of calcium and activation of protein kinase C, respectively, could be an early biochemical event that links IL-1 receptor occupancy to further activation of multiple metabolic events.

S 135 CHARACTERIZATION OF A CELL-FREE PHOSPHOLIPASE A_2 ACTIVITY IN RAT PERITONEAL EXSUDATES, Peter W. Schindler and Birgit Jordan, Hoechst AG, 6230 Frankfurt/M 80, FRG.

When rats are challenged by an intraperitoneal injection of caseinate this results in a massive infiltration of the peritoneum by polymorphonuclear leukocytes (PMN's). These cells have been shown to produce large quantities of inflammatory mediators such as eicosanoids and PAF. Since the precursers of these mediators (arachidonic acid and lyso-lipids) are derived from lipids predominantly by the action of phospholipase A_2 (PLA_2) we investigated the presence of this enzyme in casein-elicited rat peritoneal exsudates. Assays were performed using a 2-nitrobenzofuranylaminoacyl-labeled phosphatidyl choline in the presence of taurocholate. As expected, PLA_2 was shown to be present in intact PMN's. However, even more PLA_2 activity could be detected in the cell-free 150 x g supernatant of the peritoneal lavage. Initial experiments indicated that the cell-free enzyme was present in a high molecular weight form (> 100,000 dalton) as judged from the elution pattern from Superose TM 12 FPL-chromatography. In addition, about 50% of the total activity in fresh exsudates was routinely recovered in the pellet after centrifugation for 20 min at 40,000 x g. When this pellet was treated with 1 M KCl, FPLC analysis on Superose TM 12 showed that PLA_2 had quantitatively been converted into a low-molecular weight form with an apparent molecular weight of 12,000 to 13,000 daltons. Our data indicate that the cell-free supernatant of casein-elicited rat peritoneal exsudate provide an abundant source of inflammation-related PLA_2.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 136 CHROMOSOMAL MAPPING OF HUMAN PANCREATIC PLA_2 AND A HOMOLOGOUS PLA_2 EXON.
Seilhamer, J.J., Randall, T.L., Johnson, L.K. California Biotechnology Inc., 2450 Bayshore Parkway, Mountain View, CA, 94043 and Lusis, A., Sparkes, R.S., and Heinzman, C. Dept. of Medicine, UCLA, Los Angeles, CA 90024.

We described previously the cloning and DNA sequence of the human gene encoding pancreatic phospholipase A_2 (DNA 5, 519). When pancreatic PLA_2 cDNA was used to screen a human genomic library, two sets of clones were obtained. One set encoded the pancreatic enzyme, and a second set encoded the second exon of an apparently related PLA_2. A homologous sequence in both murine and porcine genomic DNA was detected by DNA blot hybridization, and the corresponding gene fragments were cloned and sequenced. Within the deduced amino acid sequences, the presence of known functional residues, along with the high degree of interspecies conservation strongly suggests the genes encode an important and active PLA_2 enzyme form. This enzyme lacks Cys_{11}, as do the "type II" viperid venom and other non-pancreatic mammalian PLA_2 enzymes. The sequence differs from those of the enzymes found in human pancreas or synovial fluid, as well as the recently published sequences of the rabbit ascites and rat platelet enzymes.

Hybridization of DNA probes containing sequences from these genes to genomic DNA blots of mouse/human fusions permitted chromosomal assignment for both. The pancreatic gene mapped to human chromosome 12, and the homologous gene mapped to chromosome 1.

S 137 PHOSPHOINOSITIDE KINASE ACTIVITIES IN CELL GROWTH AND TRANSFORMATION, L.A. Serunian, K.R. Auger, M. Whitman, and L.C. Cantley, Department of Physiology, Tufts University School of Medicine, Boston, MA. 02111.

Products of the PI turnover pathway have been implicated as potent mediators of cell growth and differentiation in several systems. Previous studies have shown that immunoprecipitates from polyoma virus middle T transformed cells and partially purified PDGF receptor preparations can catalyze two potential regulatory steps in the PI pathway: the phosphorylation of PI to PIP and the phosphorylation of PIP to PIP_2. The kinase activities associated with the middle T/$pp60^{c\text{-}src}$ complex depend on a transformation-competent middle T product, while those associated with the PDGF receptor depend on PDGF stimulation of cells. In both cases, a novel PI kinase (Type I) specifically associates with tyrosine kinases and is biochemically distinct from the predominant PI kinase (Type II) activity in fibroblasts. In addition, a middle T-associated PIP kinase activity has been investigated in middle T transformed cells and in nontransformed cells.

Both *in vivo* and *in vitro*, we have determined that the phosphatidylinositol phosphates generated by the Type I and Type II PI kinases are chemically separable using HPLC techniques. Moreover, we have found that the PIP_2 product of the middle T-associated PIP kinase can be distinguished from that produced by normal cell PIP kinase. Recent evidence suggests that the distinct products of the Type I PI kinase and the middle T-associated PIP kinase may not serve as substrates for a highly purified phosphoinositidase C. Thus, the existence of these chemically different forms of phosphoinositides suggests a novel pathway for PI turnover that may be active in stimulated and transformed cells.

S 138 SUBSTRATE SPECIFICITY OF A PURIFIED LYSOPHOPHOLIPASE FROM A MACROPHAGE-LIKE CELL LINE $P388D_1$: RELATIONSHIP TO THE CRITICAL MICELLE CONCENTRATION OF LYSOPHOSPHOLIPIDS R. E. Stafford and E. A. Dennis, Department Chemistry, University of California, San Diego, La Jolla, CA 92093.

Lysophospholipids are membrane-perturbing intermediates of phospholipid metabolism which act as detergents. Lysophospholipid production has been associated with inflammation, and lysophospholipids are degraded by lysophospholipases. The latter may therefore be important enzymes in inflammatory responses. A lysophospholipase was identified in the macrophage-like cell line $P388D_1$ which was more active than the other phospholipases. It was subsequently purified to homogeneity by chromatographic steps reported (see poster, Zhang and Dennis). Complex kinetics were observed toward highly purified lysophospholipid substrates. The critical micelle concentration (cmc) of these lysophospholipids was measured by surface tension and ^{31}P NMR. The cmc's of lyso PC's, lyso PE's and lyso PG's of various chain lengths will be reported. In all cases the enzyme kinetics are consistent with cooperative type kinetics. This may be indicative of a relationship to lipid aggregation or a direct binding of lysophospholipid to multiple binding sites on the enzyme.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 139 Ca^{2+}-INDEPENDENT BINDING OF ^{3}H-PHORBOL DIBUTYRATE TO PKC IS SUPPORTED BY PROTAMINE AND OTHER POLYCATIONS. N.T.Thompson, R.W.Bonser, H.F.Hodson and L.G.Garland, Wellcome Research Laboratories, Beckenham, Kent, U.K.

The activity of the Ca^{2+}- and phospholipid-dependent protein kinase, protein kinase-C (PKC), can be modulated by diacylglycerols and phorbol esters. The association of these agents with PKC is, in turn, generally understood to be dependent upon Ca^{2+} and phospholipids. Certain substrates e.g. protamine sulphate, are known to undergo cofactor-independent phosphorylation by PKC. We report here that, in the presence of such substrates, PKC bound 1,2-dihexanoylglycerol and phorbol dibutyrate in a Ca^{2+}-independent manner. Histone IIIs, which is phosphorylated by PKC only in the presence of Ca^{2+} and phospholipid, also supported Ca^{2+}-independent binding of 1,2-dihexanoylglycerol and phorbol dibutyrate to PKC, but to a lesser extent than protamine. Support for Ca^{2+}-independent binding was also exhibited by non-peptide polyamines (e.g. DEAE cellulose), indicating that recognition of the catalytic site is not a prerequisite for this effect. The affinity of PKC for phorbol dibutyrate and 1,2-dihexanoylglycerol was found to be unchanged by the presence of polyamines. It is proposed that, in the absence of Ca^{2+}, polyamines favour expression of the diacylglycerol/phorbol ester binding site by stabilising the active conformation of PKC.

S 140 PURIFICATION AND PROPERTIES OF PHOSPHATIDYLINOSITOL-SPECIFIC PHOSPHOLIPASE C FROM BACILLUS CEREUS. Hans Volwerk, Peter Wetherwax, Lori Evans, Andreas Kuppe and Hayes Griffith. Institute of Molecular Biology, University of Oregon, Eugene, OR 97403.

Phospholipase C specific for (poly)phosphoinositides (PI-PLC) is a key enzyme in transmembrane signal transduction involving calcium-mobilizing membrane receptors of eukaryotic cells. PI-PLC activity also is found in the culture medium of the bacterium Bacillus cereus together with two other phospholipase C-type activities; phosphatidylcholine-hydrolyzing phospholipase C (PC-PLC) and sphingomyelinase (SM-PLC). Polyacrylamide gel electrophoresis followed by enzyme assays of gel slices shows that three different proteins are responsible for these activities; the PI-PLC and SM-PLC activities running closely together with apparent molecular weights of approx. 35 kDa and the PC-PLC activity with an apparent molecular weight of 28 kDa. Using a combination of ion exchange and hydrophobic interaction chromatography we have purified to homogeneity the PI-PLC secreted by B. cereus. The final preparation contains a single 35 kDa polypeptide and has a specific enzyme activity that is considerably higher than previously reported in the literature. Preliminary data on the molecular and enzymological properties of the B. cereus PI-PLC are reported. (Supported by NIH Grant GM 25698)

S 141 VASOPRESSIN STIMULATES A SUSTAINED ELEVATION OF DIACYLGLYCEROL IN VASCULAR SMOOTH MUSCLE CELLS, Clement J. Welsh, Hui-ting Cao*, Holly Chabbott and Myles C. Cabot, W. Alton Jones Cell Science Center, Lake Placid, NY 12946 and *Visiting Scientist, Shanghai Institute of Biochemistry, Shanghai, China.

We have recently shown that vasopressin (VP) stimulates hydrolysis of phosphatidylcholine (PC) that results in the accumulation of a cellular second messenger, diacylglycerol (DG) (Biochim. Biophys. Acta, in press). Using primary cultures and an established cell line (A-10), we now show that VP stimulates hydrolysis of PC and the sustained elevation of DG in vascular smooth muscle cells. In cells prelabeled with [^{3}H]glycerol, treatment with VP for 10 min elevated [^{3}H]DG levels to 2.2 x control values, and [^{3}H]DG remained elevated at 20 min (2 x control levels). Treatment with phorbol esters gave similar results; thus, the activation of protein kinase C (PKC) may promote the sustained increase in DG. In an effort to examine the enzymatic sequence responsible for the DG production, cellular levels of phosphatidic acid (PA) were also quantitated. Time course experiments show that most of the [^{3}H]PA evolves prior to the appearance of maximal levels of [^{3}H]DG. These findings are consistent with a precursor/product sequence in which PA (precursor) is converted to DG (product). Such a mechanism may involve VP stimulating the hydrolysis of PC by a phospholipase D to give PA which is subsequently converted to DG. This enzymatic sequence could provide the DG necessary to stimulate the PKC activity that initiates the "second phase" agonist responses.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 142 EFFECT OF PHORBOL DIESTER ON IONOPHORE-INDUCED PAF PRODUCTION. Howard E. Wey, University of Cincinnati, Cincinnati, OH 45267.

Pretreatment of macrophages with phorbol diester (TPA) has been shown to enhance arachidonic acid metabolism in response to agonist stimulation. In these cells, a large percent of esterified arachidonate is found in 1-O-alkyl-2-arachidonoyl-phosphatidylcholine, and the action of phospholipase-A_2 yields the precursor of platelet activating factor (PAF). The purpose of this study was to determine if TPA pretreatment would alter calcium ionophore A23187-induced incorporation of [^{3}H]acetate into PAF. Cultured peritoneal macrophages from female CD-1 mice were preincubated in Krebs buffer and 25uCi [^{3}H]acetate with or without TPA (10ng/ml) for 10 minutes and subsequently incubated with or without 0.1uM A23187 for 0.5 to 10 minutes. Buffer and cells were extracted together, PAF resolved by normal phase HPLC, and radioactivity cochromatographing with authentic PAF determined. Neither the solvent control nor TPA alone resulted in significant acetate incorporation into PAF, and 0.1uM A23187 alone caused minimal incorporation (688dpm/10^6cells). Exposure to TPA and A23187 resulted in a greatly enhanced incorporation (11,861dpm/10^6cells). Extraction of PAF, treatment with phospholipase-C and acetylation revealed that A23187 alone (1uM) produced PAF comprised of 72% 1-O-acyl and 28% 1-O-alkyl, and A23187 (0.1uM) following TPA produced 81% 1-O-acyl and 19% 1-O-alkyl. Less than 2% of the radioactivity of "1-O-acyl" PAF was in the acyl moiety. These data suggest that the acetyltransferase does not possess specificity for 1-O-alkyl-2-lyso-PC and that availability of specific species of lyso-phospholipid may determine the kind of "PAF" produced. In addition the results indicate that kinase C may be involved in control of PAF synthesis.

S 143 EVIDENCE FOR A NEW CIRCULATING POOL OF ARACHIDONIC ACID COMPLEXED WITH GC (VITAMIN D-BINDING PROTEIN), M.H. Williams, G.M.P. Galbraith and R.M. Galbraith, Medical University of South Carolina, Charleston, SC 29425.

Arachidonic acid (AA) and eicosanoids have potent effects on a variety of cells. Hitherto, it has been believed that this unsaturated fatty acid (UFA) is largely derived from membrane phospholipids, eg by PLA_2. However, released AA binds to Gc, and this major serum protein shows substantial sequence homology with albumin. Gc purified from normal serum was therefore examined for associated UFA by chloroform:methanol extraction and gas chromatography. This showed large amounts of AA (20:4), and also 16:1, 18:1, 18:2. Gc demonstrated native properties (MW 56K; pI 4.8-5.1, and binding of the known Gc ligands - 25-(OH) D_3 and G-actin) after delipidation, and re-bound [^{3}H]-AA as demonstrated by gel filtration. Because of the poor solubility of AA, more detailed binding experiments were performed with the water-soluble fluorescent probe, toluidinylnaphthylene sulfonate (TNS). TNS bound native FA-bearing Gc, but in contrast to albumin, binding was negligible with delipidated Gc. This effectively prevented measurement of stoichiometry and affinity. However, although the interaction appeared extremely stable, FA were readily displaced from native Gc by both 25-(OH) D_3 and G-actin. Finally, tracer [^{3}H]-AA added to whole plasma or serum bound 70-80% to Gc, and 10-20% to albumin. Since Gc is a major circulating protein (300-500 $\mu g\ ml^{-1}$), and <10% is normally complexed with either Vitamin D_3 or G-actin, these results indicate a potentially important new extracellular pool of AA which can be readily mobilized.

S 144 PURIFICATION AND CHARACTERIZATION OF A LYSOPHOSHPOLIPASE FROM THE P388D_1 MACROPHAGE-LIKE CELL LINE. Y.Y. Zhang, R.E. Stafford, and E.A. Dennis, Department of Chemistry, University of California, San Diego, La Jolla, CA 92093.

Lysophospholipids are detergent-like intermediates of phospholipid metabolism that exhibit potent cytolytic and membrane-perturbing effects. Accumulation of lysophospholipids is therefore strictly regulated and at least five types of enzyme are directly involved in producing or removing them. Lysopholipases are likely to be important enzymes in a number of physiological and pathological processes. Two lysophospholipase activities (designated LPL-I and LPL-II) were identified in the macrophage-like cell line P388D_1. LPL-I was purified to homogeneity by DEAE Sephacel, Sephadex G-75, Blue Sepharose, and chromatofocusing chromatography. LPL-II was separated from the LPL-I in the Blue Sepharose step. The apparent molecular weights of LPL-I and LPL-II are 27,000 and 28,000 daltons, respectively, determined by SDS-PAGE. Their pI's were 4.4 and 6.1, respectively, as determined by isoelectric focusing. LPL-I exhibited a broad pH optimum between 7.5 - 9.0. The purified enzyme did not have phospholipase A_1, phospholipase A_2, acyltransferase or lysophospholipase-transacylase activity. No activity was detected toward triacylglycerol, diacylglycerol, p-nitrophenol acetate, p-nitrophenol palmitate, or cholesterol ester. The enzyme did, however, hydrolyze monoacylglycerol although at a rate twenty fold less than lysophospholipid. The LPL-I was inhibited by fatty acids but not by glycerol-3-phosphorylcholine, glycerol-3-phosphorylethanolamine, or glycerol-3-phosphorylserine. Triton X-100 decreased the enzymatic activity, although this apparent inhibition can be explained by a "surface dilution" effect.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

Protein Kinases

S 200 ASSOCIATION OF PROTEIN KINASE C WITH PHOSPHOLIPID MONOLAYERS. Mohammad D. Bazzi & Gary L. Nelsestuen. Department of Biochemistry, University of Minnesota, St. Paul, MN,55108.
The association of purified protein kinase C (PKC) with phospholipid (PL) monolayers showed properties indicating insertion of PKC into the hydrocarbon region of the PL monolayer and formation of an irreversible protein-PL complex. Injection of PKC into the subphase below PL monolayers caused an increase in the surface pressure due to PKC-monolayer association. PKC associated with phosphotidylserine (PS) monolayers in a Ca^{2+}-dependent manner when the PL monolayers were spread at an initial surface pressure of 26 dynes/cm or greater. Below 26 dynes/cm, PKC associated with the air-water interface in a Ca^{2+}-independent manner. In agreement with this property, PKC alone (absence of PL) caused a surface pressure change of about 26 dynes/cm. The Ca^{2+}-dependent surface pressure change showed a critical pressure (pressure where the protein could induce no further change) of 42 dynes/cm, which is close to the collapse pressure of PS. PL-bound PKC was not released by EGTA addition. Subphase depletion of PKC activity was more rapid than was the surface pressure change which suggested that the binding of PKC to monolayers consisted of two steps: a fast step involving Ca^{2+}-dependent PKC-PS binding without significant insertion, followed by a slower process that coincided with insertion and irreversibility. Histone associated with PS monolayers with a critical pressure of 50 dynes/cm. The activity of PKC was examined in a system containing PL-associated PKC, phorbol ester, Ca^{2+} and histone. PKC did not phosphorylate histone to a detectable level in this system. However, protamine sulfate was able to dissociate PKC from the monolayer and to form aggregates in the subphase that were rapidly phosphorylated. These results suggested that PKC was unable to phosphorylate histone (or other common in vitro substrates) on a planar membrane that was not aggregated. The irreversible PKC-PL association step might correlate with formation of the EDTA-resistant membrane-bound PKC population that has been observed in vivo. (Supported by grant GM-38819).

S 201 A SECOND STEREOCHEMICAL CENTRE IN THE DIACYLGLYCEROL STRUCTURE DEFINES INTERACTION AT THE "RECEPTOR" ON PROTEIN KINASE-C. R.W.Bonser, N.T.Thompson, H.F.Hodson and L.G.Garland, Wellcome Research Laboratories, Beckenham, Kent, U.K.
The interaction of several novel diacylglycerol (DAG) analogues with the DAG recognition site on protein kinase C (PKC) has been analysed using a [^{3}H]-phorbol dibutyrate (PDBu) binding assay and a mixed lipid/Triton X-100 PKC activation assay. Analysis of the 3-methyl analogues of 1,2-dihexanoyl-sn-glycerol (HHG) has revealed a preferred stereochemical configuration at the 3-position in this modified DAG structure. The two epimers differ by more than 50-fold in their ability to displace ^{3}H-PDBu and activate PKC. Our other chemical modifications have confirmed known structure/activity relationships and show also that cyclic groups (cyclopentyl, cyclohexyl, phenyl) can substitute for the alkyl moieties of the acylating groups. Thus, most, if not all, of the chemical functionalities in the DAG molecule are required for high affinity interaction with the "receptor" on PKC and stereochemical specificity is present at <u>two</u> sites within the molecule. At present it is not possible to separate the requirements for affinity from those needed for efficacy.

S 202 PHOSPHORYLATIONS OF Mr 34,000 AND 40,000 PROTEINS BY PROTEIN KINASE C IN MOUSE EPIDERMIS <u>in vivo</u>, Kazuhiro Chida, Ming Huang, and Toshio Kuroki, Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo 108, Japan.
Activation of protein kinase C and the resulting phosphorylations of proteins <u>in vivo</u> were examined in mouse epidermis, a target tissue of phorbol ester tumor promoters, such as 12-O-tetradecanoylphorbol-13-acetate (TPA). Treatment of mouse skin with TPA caused rapid translocation of protein kinase C from the cytosol to the membrane fraction of skin tissue, followed by its down regulation. Epidermal proteins were labeled locally with ^{32}P using the ring-shaped forceps technique that economizes on the amount of ^{32}Pi required. Treatment with TPA <u>in vivo</u> resulted in about 2-fold increases in the phosphorylations of epidermal proteins with Mrs of 34,000 and 40,000 and pIs of 4.7-5.1 and 5.2-6.2, termed p34 and p40, respectively. The phosphorylations of these proteins were also stimulated by an indolalkaloid tumor promoter, teleocidin B, which is also a strong activator of protein kinase C. Inhibitors of protein kinase C, such as chlorpromazine, quercetin, and staurosporine, inhibited these increases in phosphorylations of p34 and p40 on TPA treatment. Furthermore, p34 and p40 were phosphorylated by purified protein kinase C in a cell-free system. These results indicate that p34 and p40 are phosphorylated by protein kinase C in mouse epidermis <u>in vivo</u> and may be involved in tumor promotion.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 203 [3H] GLYCEROL-LABELLED DIACYLGLYCEROL IS RECOVERED IN BOTH CYTOSOLIC AND MEMBRANE FRACTIONS, AND IS ASSOCIATED WITH C-KINASE ACTIVITY FOLLOWING INSULIN TREATMENT OF BC3H-I MYOCYTES, Denise R. Cooper, Govindan Nair, Mary L. Standaert, Robert J. Pollet, Robert V. Farese, J.A. Haley V.A. Hospital and Depts. of Medicine and Biochemistry, USF College of Medicine, Tampa, FL 33612.

Increases in *in vitro* cytosolic and membrane-bound C-kinase activity have been observed in the action of insulin (J. Biol. Chem. 262:3633, 1987), but the mechanism of these changes are uncertain. We have recently shown that insulin increases diacylglycerol (DAG) in BC3H-1 myocytes, partly through increases in *de novo* phosphatidic acid (PA) synthesis and direct conversion of PA to DAG. We presently examined the subcellular localization of total and newly synthesized DAG. Myocytes were stimulated with insulin; within 1 min, [^{3}H] glycerol-labelled DAG increased several fold in both cytosol and membrane fractions. Surprisingly, cytosol contained 25-35% of total and newly synthesized [^{3}H] DAG and insulin-treatment did not influence this distribution. (Various homogenization methods did not change this subcellular distribution.) [^{3}H] labelled lipid was not present in cytosolic lipid droplets obtained by discontinuous sucrose gradient centrifugation. Cytosolic [^{3}H] DAG remained associated with fractions containing protein kinase C following anion-exchange chromatography and sucrose-density-gradient centrifugation. [^{3}H] DAG and [^{3}H] phospholipid counts were significantly greater in insulin-treated samples both before and after purification of C-kinase. Cytosolic as well as membrane-bound DAG mass (quantified by DAG kinase method) increased 50-100% following insulin treatment. Presumably, this cytosolic DAG could activate C-kinase, analagously to that currently accepted to occur through membrane-associated DAG. Thus, C-kinase may be activated in the absence of apparent "translocation" of the enzyme to the membrane and insulin-induced increases in C-kinase activity may be a reflection of cellular increases in DAG.

S 204 REGULATION OF PROTEIN KINASE C ACTIVITY IN OLIGODENDROCYTES, Glyn Dawson, T. Vartanian, R. Farrer and Sara Szuchet, U. of Chgo., Chicago, IL 60637.

In oligodendrocytes, protein kinase C (PK-C) is activated by adhesion to polylysine, and inhibited by both increasing cyclic AMP levels and by addition of lysoglycosphingolipids to the culture medium. A major in vivo substrate for PK-C in oligodendrocytes is myelin-basic protein (MBP); its phosphorylation is inhibited by both cyclic AMP and psychosine in a manner reversible by phorbol esters such as TPA. In contrast, another myelin-specific protein, 2',3' cyclic nucleotide phosphohydrolase (CNPase) is a substrate for both PK-C and cyclic AMP-dependent PK (PK-A), but phosphorylation is inhibited by psychosine. Elevation of cyclic AMP with forskolin specifically inhibited both phosphatidylinositol turnover and diacylglycerol (DAG) release, thus inhibiting PK-C indirectly. In contrast, lysoglycosphingolipids (which can be shown to occur in vivo by their reaction with fluorescamine) have been shown by Bell et al. to inhibit PK-C by direct competition with DAG for PK-C binding sites. In neuroblastoma x Chinese hamster brain hybrid cells (NCB-20) TPA-stimulated phosphorylation of the major PK-C substrate, an 80KDa protein, is also inhibited by both raising cyclic AMP levels (PK-A) and by addition of psychosine. Although the biological role of phosphorylation of MBP, CNPase and the 80KDa protein is not yet clearly understood, the role of PK-A in inhibiting DAG release appears to be widespread and of physiological significance. (Supported by USPHS Grants HD-06426 and HD-04583).

S 205 DIFFERENTIAL EFFECTS OF BRYOSTATINS AND PHORBOL ESTERS ON ARACHIDONIC ACID METABOLITE RELEASE AND EPIDERMAL GROWTH FACTOR BINDING IN C3H/10T1/2 CELLS. Marie L. Dell'Aquila, Cherry L. Herald, Yoshiaki Kamano, George R. Pettit, and Peter M. Blumberg, National Cancer Institute, Bethesda, MD 20892.

The bryostatins, a group of macrocyclic lactones isolated on the basis of antineoplastic activity, activate protein kinase C in vitro and block phorbol ester binding to this enzyme. In some cellular systems, bryostatins mimic phorbol ester action while in others they display only marginal agonistic action and, instead, inhibit phorbol ester-induced responses. At least in primary mouse epidermal cells, a transient duration of action of bryostatin 1 could rationalize these differences. To determine whether this model of transient activation could explain the dual actions of bryostatin 1 in other cell systems, we have examined the effects of bryostatin 1 on short term responses in C3H/10T1/2 mouse fibroblasts. Even at very short exposures (30 min), bryostatin 1 blocked phorbol ester-induced arachidonic acid metabolite release and induced only minimal release when assayed alone. In contrast, epidermal growth factor binding was markedly and rapidly decreased in bryostatin 1-treated C3H/10T1/2 cells, and this decrease showed only limited reversal 16 hours after initial exposure. Bryostatins 2, 3, 4, 10, and several of their derivatives caused variable arachidonic acid metabolite release (10-60% of phorbol ester control) and correspondingly variable inhibition of phorbol ester action. Our findings argue against transient activation of the protein kinase C pathway as the sole explanation of bryostatin 1 action. They indicate, moreover, differences in the structure-activity relations of the bryostatins for the phorbol ester-mimetic and phorbol ester-inhibitory actions.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 206 **OVEREXPRESSION OF αPKC LEADS TO A SUPERINDUCTION OF uPA SYNTHESIS IN LLC-PK1 CELLS**, Fabbro D.[1], Wartmann M.[1], Jans D.A.[2], Nagamine Y.[2], Eppenberger U.[1], Parker P.J.[3], Hemmings B.[2]
1:University Clinic Medical School,CH-4031 Basel;2:Friedrich Miescher Institute,CH-4002 Basel;3:Ludwig Institute for Cancer Research,London.

Phorbol esters induce the expresssion of the urokinase-type plasminogen activator (uPA) in LLC-PK1 cells. The role of PKC in uPA synthesis was studied by transfecting LLC-PK1 cells with bovine brain αPKC cDNA. Four out of 19 neomycin resistant cell clones overexpressed αPKC as determined by phorbol-ester binding, Western, Northern and Southern blot analysis. PKC was overxpressed from 3 to 20-fold and roughly correlated with elevated levels of αPKC-m-RNA. Transcription of uPA-mRNA was found to be enhanced 3 to 5-fold following PKC activation by TPA of stably transfected as compared to the untransfected LLC-PK1 cells. These data indicate that superinduction of the uPA gene may be a consequence of PKC overexpression due to an altered phosphorylation state of the putative transacting factors (APs).
This work was supported by the Swiss National Science Foundation grant 3.404-0.86

S 207 **MEMBRANE BILAYER BALANCE AND PLATELET SHAPE.** James E. Ferrell, Jr., K. T. Mitchell, and W. H. Huestis, Dept. of Chemistry, Stanford University, Stanford CA 94305.

Activated platelets adopt a characteristic spiculate morphology. A wide variety of anionic and zwitterionic amphipathic compounds were found to effect a similar shape change. Several cationic amphipaths reversed thrombin-, PAF-, and amphipath-induced spiculation and restored the discoid shape. Higher concentrations of cationic amphipaths caused the cells to assume spheroid and indented forms. One compound, dilauroylphosphatidylserine, caused both types of shape change in succession. For three amphipaths studied in greater detail, the observed morphological effects could not be accounted for by changes in protein phosphorylation and inositide metabolism. To account for these findings, we propose that platelet shape can be influenced by changes in the plasma membrane bilayer balance. Agents that accumulate in the membrane outer monolayer are accommodated by spiculation; those that accumulate in the inner monolayer are accommodated by sphering. Changes in bilayer balance of a similar magnitude are calculated to arise from thrombin-induced inositide hydrolysis. Thus bilayer balance changes may mediate physiologically relevant shape changes as well as amphipath-induced shape changes in platelets.

S 208 A PHORBOL ESTER AND PHOSPHOLIPID-ACTIVATED, CALCIUM-UNRESPONSIVE PROTEIN KINASE. CHARACTERIZATION, PARTIAL PURIFICATION AND SEPARATION FROM PROTEIN KINASE C.
Michael Gschwendt, Walter Kittstein, Friedemann Horn and Friedrich Marks, German Cancer Research Center, Institute of Biochemistry, 6900 Heidelberg, F.R.G.
The phosphorylation of a Mr 82,000 protein (p82) in the Triton X100-extract of the particulate fraction of mouse epidermis is dependent on the phorbol ester TPA or diacylglycerol and phospholipid and, contrary to protein kinase C (PKC)-catalyzed phosphorylation, cannot be activated by calcium plus phospholipid. In spite of their almost identical behaviour on polyacrylamide gel electrophoresis, p82 and PKC are different proteins according to peptide mapping. Thus, p82 phosphorylation is not an autophosphorylation of PKC. In contrast to PKC, the novel kinase system is exclusively located in the particulate fraction and it has a higher metabolic turnover rate than PKC. Moreover, it is significantly less suppressed by the PKC-inhibitor K252a than PKC. The p82-kinase can be partially purified and separated from PKC by chromatography on DEAE-cellulose, hydroxyapatite and phenyl sepharose. Separation of the p82-kinase from PKC is proved by the application of a PKC-specific antibody. Like PKC, the p82-kinase phosphorylates its substrate at serine and threonine residues. Similar to PKC, the p82-kinase system is down-modulated after TPA treatment of mouse skin, with a half life of around 5 hours. Down-modulation is also accomplished by the phorbol ester RPA, but not by the Ca^{2+}-ionophore A23187, and it is inhibited by the immunosuppressive agent cyclosporin A. In addition to down-modulation TPA treatment of the animals activates a phosphatase that dephosphorylates phosphorylated p82 in the extract of the particulate fraction.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 209 THE ROLE OF PROTEIN KINASE C IN THE CONTROL OF THROMBIN AND ALUMINIUM FLUORIDE MEDIATED GENERATION OF INOSITOL PHOSPHATES IN ENDOTHELIAL CELLS. Haraldur Halldórsson, Magnús K. Magnússon, Matthias Kjeld and Guðmundur Thorgeirsson. University of Iceland, Reykjavik, Iceland.

Endothelial cells previously stimulated to produce prostacyclin (PGI_2) by a variaty of stimuli become desensitized and do not respond to a repeat stimulation. Recent work in our laboratory has shown that the phosphoinositide messenger system is not activated during a repeat stimulation and that a short pretreatment with the phorbol ester TPA inhibits thrombin mediated generation of inositol phosphates (H.Halldórsson et al. Arteriosclerosis, in press). We have now further investigated the involvement of protein kinase C in the control of inositol phosphate generation and PGI_2 production. Long term (24 hours) treatment with TPA, wich in other cell types has been shown to down regulate protein kinase C, reduces both the inositol phosphate generation and the PGI_2 production in response to thrombin. Furthermore, long term TPA treatment abolishes the inbitory effect of short term TPA treatment on thrombin mediated actvation of inositol phosphate generation. This inhibition was also prevented by treatment with the protein kinase C inhibitor H7, further indicating that the effect of TPA in endothelial cells is mediated by protein kinase C. To localize the protein kinase C involvement we have used aluminium fluoride, a nonspecific actvator of G proteins, with and without TPA. Aluminium fluoride caused a great inositol phosphate generation and PGI_2 production. While TPA inhibited the generation of inositol phosphates, the PGI_2 production was increased. The results indicate that the inhibition of inositol phosphate generation accomplished by activation of protein kinase C has its effect distal to the receptor either at the level of a G protein or phospholipase C.

S 210 A REVERTANT OF V-FES TRANSFORMED MINK CELLS EXHIBITING ELEVATED LEVELS OF PHOSPHOTYROSINE-CONTAINING PROTEINS. Joel R. Haynes[1], and James R. Downing[1,2], Departments of Tumor Cell Biology,[1] and Pathology,[2] St Jude Children's Research Hospital, Memphis, TN 38105.

A revertant of a Gardner-Arnstein Feline sarcoma virus (GA-FeSV) transformed mink cell line (F3C17) was isolated by fluorescence activated cell sorting following staining with rhodamine 123. This strategy took advantage of differences in mitochondiral dye retention between transformed and nontransformed epithelial cells. The revertant line exhibited a decrease in growth rate and saturation density and a complete absence of anchorage independent growth. In addition, the revertant was refractory to retransformation by $p110^{gag\text{-}fes}$, and v-Ki-ras but not by $p85^{gag\text{-}fes}$ and v-mos. Evidence that the revertant contains an unaltered GA-FeSV provirus was obtained by virus rescue experiments and the demonstration of identical levels of the v-fes product and tyrosine kinase activities between the revertant and transformed cells. The observation of similar levels and patterns of phosphotyrosine-containing proteins between the two cell lines suggests that the revertant contains a lesion in a gene required for expression of the transformed phenotype and that the block in transformation occurs at a site downstream of substrate phosphorylation.

S 211 IMMUNOLOGICAL ANALYSIS OF PROTEIN KINASE C IN SWISS 3T3 CELLS: INHIBITION OF PHORBOL ESTER-INDUCED DOWN REGULATION BY CYCLOSPORINE A, Friedemann Horn, Katja Greif, and Friedrich Marks, German Cancer Research Center, Im Neuenheimer Feld 280, D-6900 Heidelberg, Federal Republic of Germany

Using synthetic peptides as antigens we raised polyclonal antibodies against protein kinase C (PKC) in rabbits. Antiserum JP1 was generated against the peptide DPNGLSDPYVKLK common to the regulatory domain of all known mammalian PKC isozymes. Antisera KP3 and KP8 were made against the sequences NVPIPEGDEEGNV which is specific for the alpha type PKC and FVNSEFLKPEVKS, the carboxy terminus of beta II type PKC, respectively. On immunoblots, all three antisera reacted with PKC purified from mouse brain as well as with a corresponding 81kD band in whole brain extracts. In Swiss 3T3 cell extracts JP1 and KP3 but not KP8 recognized a 81kD protein suggesting that alpha type but not beta II type PKC is present in these cells. Immunoreactive PKC was found to be located in the soluble fraction of homogenates prepared from quiescent 3T3 cells. Treatment of the cells with 10^{-7}M 12-O-tetradecanoylphorbol-13-acetate (TPA) caused a rapid translocation of the 81kD protein to the particulate fraction. Prolonged exposure to TPA resulted in a complete lost of immunoreactive PKC from the cells within 24 hours. The immunosuppressive agent cyclosporine A (CsA) which has been reported to antagonize many TPA-induced effects did not alter PKC in control cells nor did it influence the TPA-induced translocation of PKC. We could, however, observe a pronounced reduction of the decrease rate of PKC in TPA-treated cells by CsA in a concentration-dependent manner suggesting that the degradation of PKC is inhibited by CsA.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 212 IMMUNOCHEMICAL CHARACTERIZATION OF PROTEIN KINASE C ISOZYMES, K.-P Huang, F. L. Huang, H. Nakabayashi, and Y. Yoshida, NIH, Bethesda, MD 20892.

Protein kinase C (PKC) plays an important role in controlling several cellular processes. At least three cloned PKC cDNAs, named α, β, and τ, have been identified from several brain gene libraries and three PKC isozymes, designated type I, II, and III, have been purified from several animal brains. Monoclonal and polyclonal antibodies specific for each PKC isozyme were used for immunochemical analysis of these enzymes. Immunoblot analysis of PKC expressed in COS cells transfected with α, β, and τ cDNA revealed that they were recognized by antibodies for type III, II, and I PKC, respectively. The type I PKC was found only in brain, whereas type II and III PKC were detected in brain as well as peripheral tissues. Immunocytochemical analysis of rat cerebellum showed the presence of type I PKC in the cell body and dendrites of the Purkinje cells and the type III PKC mainly in the cell body of this cell type. The cerebellar granule cells contained both type II and III PKC without detectable type I PKC. Several neurons in rat cerebrum were found to contain all three types of PKC isozymes. Coexistence of multiple PKC isozymes in a cell type was also found in non-neuronal cells. The histamine-secreting RBL 2H3 cells contained both type II and III PKC, which underwent differential down-regulation in response to tumor-promoting phorbol ester. These results demonstrate that these three PKC isozymes have distinct tissue, cellular, and subcellular distributions and different responses to a stimulus. Based on these findings we predict that each PKC isozyme may participate in the regulation of specific cellular function.

S 213 The adenylate cyclase and the C kinase activities in rat thyroid epithelial cells infected by *ras* genes expressing virus.

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M.T.Berlingieri, A.Fusco and M.Grieco - Centro di Endocrinologia ed Oncologia Sperimentale del CNR - 2°Facoltà di Medicina e Chirurgia Napoli -Italy.

We report the results of our studies both on the cytosolic and membrane-bound Kinase activities in a Fisher rat thyroid differentiated epithelial cell line (PC-Cl3): both of them appear increased in the *ras*-virus infected cells, particularly the kinase fraction, either soluble in the cytoplasm, or membrane-bound, which is Ca^{++}-phospholipid dependent and dyacilglycerol activated. Previonsly we had demonstrated that in the same cells the *ras*-virus infection is linked to an increased membrane-bound adenylate cyclase activity, resulting in a higher intracellular (more than double) cAMP level.

Our results suggest the following chain of events subsequent to *ras*-virus infection and expression: the over-expressed p21 *ras* proteins stimulate the C kinase activity, which in turn affects the adenylate cyclase activity, phosphorilating a yet unidentified component of the enzymic sistem; the resulting increased intracellular cAMP level turns on tne competent kinase activities.

S 214 HUMAN T LYMPHOCYTE ACTIVATION BY TUMOR PROMOTERS: ROLE OF PROTEIN KINASE C, Noah Isakov and Amnon Altman , Ben-Gurion University of the Negev, Beer-Sheva, Israel, and The Research Institute of Scripps Clinic, San Diego, CA.

Protein kinase C (PKC) has a major role in a ligand-receptor-mediated signal transduction system in a variety of cell types including T lymphocytes. One of the early phenotypic changes associated with T cell activation is the expression of cell surface receptors for interleukin 2 (IL2). To test the role of PKC in regulation of IL2 receptor (IL2-R) expression and T cell activation in general, we used tumor promoters (TP) as modulators of PKC and compared their effects on intact human T cells and on the enzymatic activity of T-cell derived PKC in a cellfree system. We found that TPA induced IL2-R expression and proliferation associated with cytosol-to-membrane PKC translocation. A dose of TPA (1 to 4 ng/ml) that induced about 50% of the maximal activation of PKC in the enzymatic assay also induced half-maximal effects on cell proliferation, IL2-R expression, and PKC redistribution in intact T cells. Structure-function studies with different TP directly correlated tumor promotion activity with the ability to activate PKC and induce IL2-R. An inhibitor of PKC, chlorpromazine, was found to suppress TPA-mediated proliferation and IL2-R expression, and inhibited T cell-derived PKC by competing with the phospholipid. The results thus demonstrate a direct correlation between the effects of various chemicals on: a) subcellular redistribution of PKC in T cells; b) induction of T cell proliferation and IL2-R expression; and c) activation of T cell-derived PKC in vitro. These data provide further support for the role of PKC in transduction of activation signals in T cells and in regulation of IL2-R exoression.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 215 SPECIFIC AND NONSPECIFIC EFFECTS OF SPHINGOSINE ON EPINEPHRINE STIMULATION OF ADENYLATE CYCLASE, J.A. Johnson and R.B. Clark, The University of Texas Health Science Center, Graduate School of Biomedical Sciences, Houston, TX 77030. Desensitization of the β-adrenergic receptor (βAR) involves uncoupling, internalization-sequestration and down regulation. Recent data demonstrate that 4β-phorbol 12-myristate 13-acetate (PMA), an activator of protein kinase C (PKC), sensitizes adenylate cyclase (AC) by functionally inactivating the G_i coupling protein, and desensitizes by inhibiting βAR function. Since 50 μM D-sphingosine (SGO) has been reported to specifically inhibit PKC, we evaluated the effects of SGO on epinephrine (Epi) and PMA-induced alterations of cAMP accumulation in S49 WT and kin$^-$ (lacking cAMP-dependent protein kinase) cells. We found that: i)Pretreatment of cells with 50 μM SGO inhibited Epi-stimulated cAMP accumulation by 80-95%; ii) simultaneous additions of 20 μM SGO and 50 nM Epi led to a gradual augmentation of cAMP accumulation in S49 WT cells, consistent with a partial blockade of the desensitization of AC; iii) 20 μM SGO, added to S49 WT membranes, inhibited Epi-stimulated AC demonstrating nonspecific, detergent-like effects of SGO; and iv) 20 μM SGO prevented PMA (5 nM)-induced sensitization of Epi-stimulated cAMP production in S49 kin$^-$ cells, suggesting that SGO at this concentration specifically blocks PMA activation of PKC. Our results demonstrate that SGO at the proper concentration may be useful to study PKC-mediated events in cells but its nonspecific effects must be evaluated. Since signal transduction mechanisms are highly conserved, the nonspecific effects of SGO we have observed on AC may occur in other systems. These effects should be evaluated prior to interpretation of results. This work was supported by Grant AU-878 from the Robert A. Welch Foundation.

S 216 EFFECTS OF ANGIOTENSIN II AND OF PHORBOL ESTER ON PROTEIN KINASE C AND ON PROSTACYCLIN PRODUCTION IN AORTIC SMOOTH MUSCLE CELLS, Ursula Lang and Michel B. Vallotton, Division of Endocrinology, University Hospital, CH-1211 Geneva, Switzerland.

To assess the role of protein kinase C (PKC) as second messenger in cultured rat aortic smooth muscle cells, we studied the effects of angiotensin II (Ang II) and phorbol-12-myristate-13-acetate (PMA) on the subcellular distribution of PKC activity and on prostacyclin production. PKC activity was measured in both the cytosol and the solubilized membranous fraction after DEAE cellulose chromatography using a linear NaCl gradient. Ang II increased basal activity by about 50% and 100%, respectively. This Ang II-induced increase was dose-dependent in the range of 1-50 nM. It occurred rapidly reaching a plateau within 10 minutes, as did prostacyclin production. Treatment with PMA suppressed cytosolic PKC activity, while increasing basal membranous PKC activity by about 100% within 5 minutes. Measurements of PKC activity at various calcium concentrations showed that membranous PKC activity from untreated and Ang II-stimulated cells was highly calcium-dependent. In contrast, membranous PKC activity from PMA-stimulated cells was largely calcium-independent. PMA induced a dose-dependent (10 nM - 1 μM) prostacyclin production which occurred only 15 minutes after PMA addition. In the presence of 10 nM PMA, smooth muscle cells were about 10 times more sensitive to Ang II-stimulation, and maximal values of Ang II-induced prostacyclin production were increased by about 60%. Membranous PKC activity of cells treated with Ang II and PMA was increased by about 30% when compared to cells treated with Ang II alone, but cytosolic PKC activity was suppressed. Our observation suggest that Ang II-induced changes of the cellular PKC system may be more complex than those caused by PMA. They also indicate that an increase of the membranous but not of the cytosolic PKC activity represents an important intracellular signal during Ang II stimulation.

S 217 **PDGF ACTIVATES 3 KINASE SYSTEMS IN SWISS 3T3 CELLS: PDGF-SPECIFIC, EGF-LIKE AND KINASE-C-LIKE.** R. M. Levenson, H. B. Sadowski, and D. A. Young, Endo/Metab Unit, Dept of Med, University of Rochester School of Medicine, Rochester, NY 14642.

We propose (accompanying abstract) that in Swiss 3T3 cells the effects of EGF are independent of PK-C and that the overlap of 60 phosphorylations in response to EGF and PMA suggests either activation of a common kinase pathway or different kinase systems with similar substrate specificities. Both EGF and PDGF stimulate their receptor's tyrosine-kinase activity; however, PDGF also stimulates PI turnover and increases DAG. 10-min treatment with PDGF causes increases in $IP's_{1-3}$ (8,18,&2-fold), DAG (3-fold) and 80K phosphorylation (20-fold), as well as 92 protein phosphorylations (83 of 114 PMA-responsive, 2 "GF-responsive", and 7 "PDGF-specific"). In PK-C down-regulated cells PDGF induces 67 of these 92 phosphorylations (7 PDGF-specific, 2 GF-responsive and 58 of 60 seen with EGF in down-regulated cells). Thus, about 1/2 of the "PK-C dependent" phosphorylations (down-regulable PMA responses not seen with EGF) are also stimulated by PDGF in a down-regulable manner. These appear to be markers of PK-C activation. None of the growth-factor or PDGF-specific phosphorylations were stimulated by Bt_2-cAMP; however, a number of GF-induced tyrosine phosphorylations were detected by immunoblotting with anti-phosphotyrosine antibodies. One phosphotyrosine band seen with both PDGF and EGF corresponds (M.W.) with 1 of the 2 growth-factor-responsive phosphorylations and several bands seen only with PDGF correspond with PDGF-specific phosphorylations. We propose a model of signal transduction in which PDGF can activate a PK-C pathway, an EGF-like pathway and a PDGF-specific pathway. Supported by NIH grants: DK 16177 and 5T32-ES07026 and grants from the United Cancer Council and Wilmot Foundation.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 218 MYOSIN PHOSPHORYLATION BY MYOSIN LIGHT CHAIN KINASE AND PROTEIN KINASE C DURING SECRETION FROM INTACT AND PERMEABILIZED CELLS. R.I. Ludowyke, I. Peleg, R.S. Adelstein and M.A. Beaven. NHLBI, NIH, Bethesda, MD 20892.
Antigen stimulation of a rat basophil leukemic cell line (RBL-2H3) results in a stoichiometric increase in phosphorylation of the myosin heavy chains (200,000 Da) and light chains (20,000 Da). Two-dimensional tryptic peptide maps and two-dimensional gel electrophoresis of myosin light chains (MLCs) from unstimulated cells revealed that 0.3 to 0.4 moles of PO_4/mole MLC are incorporated at a serine site known to be phosphorylated by myosin light chain kinase (MLCK) in vitro. Antigen stimulation results in a transient increase in phosphate at the MLCK site and de novo phosphorylation of another serine site known to be phosphorylated by protein kinase C (PKC). Phosphorylation at the PKC site reaches a maximum of 0.8 to 0.9 moles of PO_4/mole MLC in 5-10 min. The time course of the hydrolysis of inositol phospholipids and the rise in cytosolic Ca^{2+} precedes that of kinase C phosphorylation which is concurrent with histamine secretion. Cells which have been permeabilized by streptolysin O still secrete histamine and hydrolyze inositol phospholipids when stimulated by antigen in potassium glutamate buffer containing 1 μM Ca^{2+}. The time courses and extent of both are similar to those in intact cells. MLCK and PKC phosphorylation of the light chains are also observed in permeabilized cells. GTPγS which stimulates phospholipid hydrolysis and histamine secretion only from permeabilized cells, also leads to phosphorylation of myosin light chains at the same sites as observed in the antigenic system.

S 219 ACTIVATION OF MEMBRANE-BOUND 1,2-DIACYLGLYCEROL KINASE IN 3T3 CELLS BY SERUM AND PMA, Anna Coco Maroney and Ian G. Macara, Dept. of Biochemistry, University of Rochester, Rochester, NY 14642.
1,2-Diacylglycerol (DAG) kinase (EC 2.7.1.107) is present in both the particulate and soluble fraction of Swiss 3T3 fibroblasts. It is not yet clear whether the cytosol and membrane form of the enzyme is the same protein. We now demonstrate that there is a significant increase in membrane bound DAG kinase activity after addition of 10% serum or 100 nM PMA to serum starved quiescent Swiss 3T3 fibroblasts. The inactive alpha form of PMA does not cause any change in DAG kinase activity. When protein kinase C is down regulated by PMA in serum starved cells, addition of 10% serum still results in a 56% increase in particulate activity above control levels, suggesting that the activation of DAG kinase is regulated by two different mechanisms. In vitro studies indicate that the soluble activity in rat brain binds to DAG-rich membranes produced by treatment of red cell ghosts with phospholipase C or calcium, suggesting that cytosolic DAG kinase may translocate to the membrane in vivo. Treatment of cells with serum, but not with PMA, leads to a rapid increase in cell DAG, and the increased membrane-associated DAG kinase activity is paralleled by a decrease in cytosolic activity, supporting the proposal that serum induces a translocation of cytosolic DAG kinase to the membrane.

S 220 **PLATELET TYROSINE-SPECIFIC PROTEIN PHOSPHORYLATION IS REGULATED BY THROMBIN.** James E. Ferrell, Jr. and G. Steven Martin, Dept. of Zoology, University of California, Berkeley CA 94720.

Platelets possess high levels of the c-src gene product $pp60^{c\text{-}src}$, a tyrosine-specific protein kinase whose physiological role is unknown. Here we present evidence that the platelet activator thrombin stimulates one or more protein-tyrosine kinases in intact human platelets. Thrombin treatment effected an increase in platelet phosphotyrosine content (to 132 - 176% of basal levels 3 minutes after thrombin treatment), and caused marked changes in the phosphotyrosine content of several individual protein bands. In dose/response studies, thrombin-stimulated tyrosine phosphorylation was correlated with secretion and secondary aggregation (rather than with shape change or primary aggregation) and thus may be involved in these processes.

Protein kinase C may play a role in activating a thrombin-stimulated protein-tyrosine kinase(s). Phorbol myristate acetate (PMA) caused an increase in platelet phosphotyrosine content and caused marked changes in the phosphotyrosine content of specific protein bands. One protein-tyrosine kinase that may be modulated by kinase C is $pp60^{c\text{-}src}$: both thrombin and PMA stimulate protein kinase C-mediated phosphorylation of $pp60^{c\text{-}src}$ at Ser 12.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 221 PMA HAS BOTH STIMULATORY AND INHIBITORY EFFECTS ON THE ANTI-Ig MEDIATED STIMULATION OF MURINE B CELLS, James J. Mond, Carl June, Fred D. Finkelman and Mark Brunswick, USUHS, Bethesda, MD 20814.

It has been reported that pretreatment of B cells for 30 min with PMA inhibits both the anti-Ig stimulated increases in $[Ca^{2+}]i$ and PIP_2 breakdown; however, overnight treatment with PMA enhances anti-Ig stimulated increases in both these events. While 24 hr culture of B cells with phorbol dibutyrate (PDBU) inhibits anti-Ig stimulated B cell proliferation, a 30 min coculture with PDBU followed by removal of PDBU significantly enhances anti-Ig but not LPS stimulated B cell proliferation. Under these conditions anti-Ig fails to stimulate additional increases in $[Ca^{2+}]i$ or in PIP_2 breakdown. To determine whether PIP_2 breakdown and increases in $[Ca^{2+}]i$ are essential early after culture with anti-Ig antibody for anti-Ig mediated B cell activation we cultured PDBU pretreated B cells for 60 min with anti-Ig to modulate sIg under conditions where PIP_2 breakdown was inhibited. Such sIgD modulated B cells proliferated normally in response to anti-IgD after removal of PDBU, even though PIP_2 breakdown and increases in $[Ca^{2+}]i$ were blocked by greater than 95%. These results suggest that activation of PKC may induce both stimulatory and inhibitory signals for anti-Ig stimulated B cells and that the large alterations in $[Ca^{2+}]$ and IP_3 that are induced early in B cell activation by anti-Ig may not be absolutely required for progression of B cells into DNA synthesis.

S 222 ROLE OF PROTEIN KINASE C (PKC) IN NK CELL ACTIVATION, Antonio D.G. Procopio, Angela Gismondi, Rossella Paolini, *Sergio Adamo, Mario Piccoli, Luigi Frati and *Angela Santoni, Dept. of Exp. Medicine University "La Sapienza", Rome, * Dept. of Biotechnology, University of L'Aquila, L'Aquila, Italy.

This work investigates the role of PKC in the early metabolic events involved in human NK cell activation.

Highly purified LGL were preincubated in the presence of TPA phorbol ester (10-1 ng/ml) or the diacylglycerol analog OADG (50-10 μg/ml), specific activators of PKC. These agents consistently elevated NK activity against K562 target cells. Sub-optimal doses of both TPA or OADG synergized with Ca^{2+} ionophores in the augmentation of cytotoxic activity. Pretreatment of LGL with H-7 (50-5 μM), a potent PKC inhibitor, greatly reduced basal and interferon-induced NK activity in a time and dose-dependent fashion. By contrast H-8 and HA-1004, potent inhibitors of cAMP and cGMP-dependent PK but almost uneffective on PKC, marginally reduced NK activity. Moreover almost complete abrogation of NK activity was observed when H-7 (10 μM) , but not HA-1004 (50 μM), was present in the NK assay. This functional evidence was supported by SDS-PAGE electrophoresis and autoradiographic detection of several endogenous substrates, phosphorylated within 10 min in TPA-treated LGL. A protein of 87 kDa, described by Albert et al. (Proc. Natl. Acad. Sci. USA, 1987, 84:7046) as a major specific substrate for PKC, has been identified together with other phosphorylated proteins with MW ranging from 80 to 25 kDa. H-7 but not HA-1004 almost completely inhibited the phosphorylation of these proteins, as analyzed by SDS-PAGE electrophoresis. The identity and the role of these PKC substrates in NK cells are under investigation.

These data suggest that selective activation of PKC plays an essential role in the activation mechanisms of NK cells.

S 223 MOLECULAR CLONING OF THE P47 PROTEIN KINASE C SUBSTRATE OF PLATELETS, M. Tyers, R.A. Rachubinski, R.G.L. Shorr*, M.L. McCaw, R.J. Haslam and C.B. Harley, McMaster University, Hamilton, Ontario, Canada L8N 3Z5 and *Smith Kline and French Laboratories, Philadelphia, PA 19101.

Stimulation of platelets causes immediate phosphorylation of an apparent 47 kDa protein (P47) by protein kinase C. Lambda gt11 cDNA libraries constructed from retinoic acid-differentiated HL-60 cells, in which P47 constitutes 0.07% of cellular protein, were screened with rabbit P47 antiserum. Immunoreactive clones hybridized to a 3 kb transcript regulated during HL-60 differentiation in a similar fashion to P47 protein. P47 clones were sequenced and confirmed by platelet P47 tryptic and CNBr peptide sequence. The initiator methionine, which could not be found from protein sequencing because the N-terminus of mature P47 is blocked, was located by the close match of flanking bases to the translation initiation consensus sequence. The putative translation start was confirmed by *in vitro* transcription and translation of P47 templates and expression of the predicted coding region of P47 in *E. coli*, both of which gave products that co-migrated with platelet P47. The 1050 bp open reading frame encodes a protein of Mr 40087 predicted to have a high α-helical content and a potential Ca^{2+}-binding EF hand at its C terminus. Southern analysis suggests that the P47 gene is conserved through vertebrate evolution. Searches against current protein and nucleic acid databases indicate the P47 sequence is unique. Supported by MRC of Canada.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 224 A REAPPRAISAL OF "RATIONALLY-DESIGNED" ACTIVATORS OF PROTEIN KINASE-C. R.W.Randall, R.W.Bonser, N.T.Thompson, H.F.Hodson and L.G.Garland, Wellcome Research Laboratories, Beckenham, Kent, U.K.

A recent analysis (1), using computer-modelling, of known activators of protein kinase-C (PKC), yielded compounds which were claimed to have affinity for the phorbol ester binding site and biological activity characteristic of phorbol esters. Two such compounds were 6-(N-decylamino)-4-hydroxymethylindole (DHI) and 3-(N-acetylamino)-5-(N-decyl-N-methylamino) benzyl alcohol (ADMB). We have estimated affinity and efficacy of these compounds for the phorbol ester receptor using partially purified rat brain PKC in a lipid/Triton X-100 mixed micelle system and measuring (i) displacement of bound ^{3}H-phorbol dibutyrate (PDBu), (ii) phosphorylation of histone IIIS. Under conditions in which PDBu, teleocidin and mezerein had affinity and stimulated phosphorylation, DHI and ADMB were inactive (concentrations up to 500 µM). Also, in HL60 granulocytes neither DHI nor ADMB displaced ^{3}H-PDBu. Nevertheless, DHI, but not ADMB, stimulated the production of O_2^-, a known PKC-dependent process. However, the rate of O_2^- production by DHI was different from that stimulated by PDBu. These results suggest that DHI and ADMB do not interact with the phorbol ester binding site on PKC; the mechanism by which DHI stimulates O_2^- production is unknown.

(1) Wender, P.A., et al. (1986) Proc. Natl. Acad. Sci. 83, 4214-4218.

S 225 SIGNAL TRANSDUCTION IN A PATIENT WITH SEVERE COMBINED IMMUNODEFICIENCY DISEASE, Ger T. Rijkers[1], Herman J. Neijens[2], Jacques J.M. van Dongen[3] and Ben J.M. Zegers[1], [1]University Hospital for Children and Youth "Het Wilhelmina Kinderziekenhuis", P.O. Box 18009, 3501 CA Utrecht, The Netherlands, [2]Sophia Children's Hospital and [3]Dept. of Cell Biology, Erasmus University, Rotterdam, The Netherlands

T cells from a male, 8 years old patient with ADA$^+$ severe combined immunodeficiency disease, display the CD3$^+$ CD8$^+$ phenotype, are oligoclonal in nature on Southern blot analysis with TCRβ probes, and do not proliferate in vitro upon stimulation with T cell mitogens. Proliferation is neither induced by a combination of phorbol esters (PMA) and Ca-ionophore (ionomycine). However, activation of T cells with PHA or Con A does lead to an increase of cytoplasmic free Ca^{++} (2-5 fold higher than in control CD8$^+$ T cells) as well as translocation of PKC from cytosol to the plasmamembrane. Patient T cells show an abnormal membrane phospholipid composition in that the PC/PS ratio is 5-fold higher than in normal T cells. The relevance of this finding for PKC activation (phosphorylation of cell surface proteins) and ultimate cell proliferation is currently under investigation.

S 226 MOLECULAR CLONING OF MOUSE PROTEIN KINASE C cDNA AND ITS EXPRESSION IN SWISS 3T3 FIBROBLASTS. Stefan Rose-John, Alexander Dietrich and Friedrich Marks, Dept. of Biochemistry, German Cancer Research Center, P 101949, 6900 Heidelberg, FRG.

Screening of a Mouse Swiss 3T3 cDNA library with synthetic oligonucleotides corresponding to human and rat Protein Kinase C (PKC) sequences led us to the identification and isolation of positive clones. Rescreening of 10^7 phages yielded more than 15 PKC cDNA clones. Sequence analysis revealed that all PKC inserts coded for alpha type PKC. The coding region shows very strong homology with the human alpha PKC at the amino acid level as well as at the nucleotide level. Our data suggest that Swiss 3T3 cells express only one type of PKC, the alpha type. Northern blot analysis showed that PKC expression in Swiss 3T3 fibroblasts is very low. The expression is not affected by TPA treatment. To our knowledge this is the first isolation of Mouse PKC cDNA and the first isolation of a PKC cDNA clone from tissue other than brain. There seems to be no apparent difference between alpha PKC from brain and from fibroblasts.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 227 CHARACTERIZATION OF A SPECIFIC FORM OF PROTEIN KINASE C (PKC) OVERPRODUCED BY A C3H10T½ CELL LINE. Susan A. Rotenberg, Robert S. Krauss and I. Bernard Weinstein, Columbia University, New York, NY 10032.

Recent DNA cloning studies indicate the existence of multiple forms of PKC which are differentially expressed and may differ in their biochemical properties. This laboratory has recently cloned a full length cDNA encoding the β_1 subspecies of PKC and inserted it into a retroviral expression vector, pMV7 (Cell 1988, in press). This vector was used to infect C3H10T½ murine fibroblasts to produce a series of clones that stably express very high levels of PKC-β_1. One of these lines, 10T½ PKC-4, which exhibits an 11-fold increase in PKC activity was used to characterize this form of PKC. The kinase activity produced by these cells is dependent on Ca^{2+} and phosphatidylserine, with a Ca^{2+} optimum of 1 millimolar. In the presence of TPA (100 ng/ml) or teleocidin (100 ng/ml), the calcium ion optimum is shifted to 10 micromolar. Chromatographic behavior of PKC-β_1 indicates that it elutes as a single peak from DEAE-Sephacel at about 40 millimolar NaCl and also as a single peak from phenyl Sepharose at about 150 millimolar NaCl. Further characterization of the enzyme is in progress. Thus, this cell system provides a unique opportunity to identify the specific biochemical and biological properties of a single form of PKC. (Supported by NIH CA 02656.)

S 228 **CONVERGENCE OF EGF AND PROTEIN KINASE-C-STIMULATED PROTEIN PHOSPHORYLATIONS.** Henry B. Sadowski, Richard M. Levenson and Donald A. Young, Endo/Metab Unit, Dept of Med, University of Rochester School of Medicine, Rochester, NY 14642.

To identify protein phosphorylation pathways shared by various growth factors as well as those unique to each we have performed experiments with Swiss 3T3 cells and "giant" 2-D gel electrophoresis of whole cell lysates. Numerous protein phosphorylations are stimulated by direct activators of PK-C; as the phorbol ester, PMA, induces over 60 phosphorylations (>3-fold) within 5 min, and the synthetic diacylglycerol, diC8, produces an identical response. Alpha-PMA, is inactive. EGF stimulates the phosphorylation of over 30 proteins by 5 min (28 also seen with PMA, + 2 "EGF-specific"). By 10 min, additional phosphorylations are detectable, as the number induced by PMA is 114 and by EGF 62 (60 also seen with PMA, + 2 EGF-specific). However, 10 min activation of PK-C by PMA leads to over 50 phosphorylations not seen in response to EGF. Down-regulation of assayable PK-C (96%) eliminates over 95% of the PMA-responsive phosphorylations; nevertheless, all of the EGF-stimulated phosphorylations remain intact. EGF has minimal effects if any, on: 80K phosphorylation (170% of control), diacylglycerol levels (174% of control), and inositol phosphate(s) formation ($IP'_{s_{1-3}}$; 145, 125 and 100% of control). These observations suggest that in Swiss 3T3 cells the phosphorylation responses to EGF are independent of PK-C. The large degree of overlap in protein phosphorylations stimulated by PMA and by EGF in a PK-C-independent manner, suggests either convergence to a common kinase pathway, or independent activation of two distinct protein kinases with similar substrate specificities. Supported by NIH grants: DK 16177 and 5T32-ES07026 and grants from the United Cancer Council and Wilmot Foundation.

S 229 SUBSTRATE SPECIFICITY IN THE ACTIVATION OF PROTEIN KINASE C BY ANIONIC SURFACTANTS, Diana D. Scala[1], Linda D. Rhein[1], Tanveer Abidi[2], Carol A. Faaland[2] and Jeffrey D. Laskin[2], [1]Colgate-Palmolive Research & Development Center, Piscataway, NJ 08854, [2]Comm. Medicine, UMDNJ-Robert W. Johnson Med. School, Piscataway, NJ 08854.

Two anionic surfactants, sodium lauryl sulfate (SLS) and linear alkylbenzene sulfonate (LAS), were found to strongly activate protein kinase C in the absence of calcium and phosphatidylserine in enzyme assays using partially purified rat brain protein kinase C. Activation with these compounds was dose-dependent in the uM concentration range. We have found that this occurred only when histone was used as the substrate in enzyme assays. When enzyme assays were performed using the endogenous proteins in the rat brain extract as the substrates, SLS and LAS failed to activate enzyme activity. Under these conditions, calcium and phosphatidylserine readily induced the phosphorylation of at least 8 distinct proteins ranging in molecular weight from 15,000 to 120,000. From these data it is apparent that, under appropriate conditions, protein kinase C can readily distinguish between different substrates. The specificity of protein kinase C for histone in the presence of SLS and LAS may be due to an interaction of the surfactants either directly with the enzyme protein or with the substrate.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 230 A PLATELET-ACTIVATING FACTOR-DEPENDENT PROTEIN KINASE MAY BE INVOLVED IN A NEW SIGNAL TRANSDUCTION CHAIN IN PLANTS. Günther F.E. Scherer, Georg Martiny-Baron, and Birgit Stoffel, University of Bonn, D-5300 Bonn, FRG.
Platelet-activating factor(=PAF) stimulates H^+ transport in plant microsomes (1). This prompted the search for the corresponding plant lipid which was identified by TLC and HPLC (2). It is chemically very similar and functionally equivalent to PAF in that it stimulates plant H^+ transport and platelet aggregation (3). PAF stimulates H^+ transport only in the presence of soluble plant protein. Upon partial purification by DEAE Sephacel chromatography this protein factor copurifies with a PAF-stimulated protein kinase (4). In the absence of calcium ions, a 53 kDa polypeptide and, in the presence of calcium ions, in addition to this a 35 kDA is also phosphorylated in a PAF-dependent manner in plant microsomes. In plants the major ion-translocating ATPases driving solute transport are the H^+-ATPases, one in the plasma membrane and homologous to the Na^+/K^+-ATPase, and one in the vacuolar membrane which is probably related to the endosomal/lysosomal H^+-ATPase. In marker enzyme studies with membranes separated on a sucrose gradient we found that it is likely that both types of H^+-ATPases are stimulated by the PAF and the PAF-dependent protein kinase (4). Recent evidence from many laboratories demonstrates the presence of the components of the PIP_2 cycle in plants, except for the diglyceride-stimulated protein kinase. We speculate that the phospholipid(PAF)-dependent protein kinase could be involved in signal transduction in plant membranes and may be linked in its function to the PIP_2 cycle in plant cells. (1) Biochem. Biophys. Res. Commun. 133, 1160 (1985). (2) Planta 172,127 (1987). (3) Hoppe Seyler's J. Physiol. Chem. (1988), in press. (4) Planta (1988), in press.

S 231 DIACYLGLYCEROLS MODULATE HUMAN POLYMORPHONUCLEAR NEUTROPHIL RESPONSIVENESS: EFFECTS ON INTRACELLULAR CALCIUM MOBILIZATION, GRANULE EXOCYTOSIS AND SUPEROXIDE ANION PRODUCTION, Robert J. Smith, Laurel M. Sam and James M. Justen, The Upjohn Company, Kalamazoo, MI 49001.
Activation of protein kinase C (PKC) by 1,2-diacylglycerol (DG) represents an integral phase of receptor-mediated signal transduction in polymorphonuclear neutrophils (PMNs). We report here that the synthetic DGs, sn-1,2-dihexanoylglycerol (diC_6), sn-1,2-dioctanoylglycerol (diC_8), and 1-oleoyl-2-acetylglycerol (OAG) stimulated the release of granule constituents from and superoxide anion (O_2^-) generation by PMNs. However, the DGs did not induce a rise in the cytosolic-free Ca^{2+} concentration ($[Ca^{2+}]_i$) as monitored by the fluorescence of PMNs loaded with the Ca^{2+}-sensitive dye, Fura-2. DiC_8 and OAG inhibited PMN degranulation elicited with the receptor-specific ligands, N-formyl-methionyl-leucyl-phenylalanine (FMLP), acetyl-sn-glyceryl-3-phosphorylcholine (AGEPC), 5(S),12(R)-dihydroxy-6,14-cis-8,10-trans-eicosatetraenoic acid (LTB_4) and the Ca^{2+} ionophore, A23187. DiC_8 also inhibited the rise in $[Ca^{2+}]_i$ elicited with FMLP, LTB_4, and AGEPC; and this effect as well as the DG-mediated suppression of degranulation could be reversed with the PKC inhibitor, H-7. In contrast, diC_8 and OAG enhanced FMLP, AGEPC and A23187-induced O_2^- production. Stimulation of the respiratory burst with PMA was unaffected by the DGs. These data indicate that in addition to possessing the intrinsic capacity to activate PMNs, DG may, via its capacity to activate PKC, function in a bioregulatory mode to influence PMN activation in a stimulus-specific manner by affecting certain components of receptor-coupled and receptor-independent signal transduction systems in a response-specific manner.

S 232 KINASE C-MEDIATED PROTEIN PHOSPHORYLATION AND ACTIVATION OF A PHOSPHOLIPASE A PATHWAY IN MOUSE MACROPHAGES
Roger Sundler, Jonny Wijkander and Adalsteinn Emilsson

Resident mouse peritoneal macrophages respond to activators of protein kinase C (pkC) as well as to activators of phosphoinositide phospholipase C with a distinct pattern of protein phosphorylation and rapid enhancement of a phospholipase A (plA) pathway mobilizing arachidonic acid. Down-regulation of pkC by prolonged pretreatment with phorbol ester abolishes most or all of the stimulus-induced phosphorylation response and inhibits by 70-100% the mobilization of arachidonic acid, depending on stimulus. Further experiments suggest that activation of pkC may cause an increase in the calcium-sensitiviy of the plA pathway but the virtual lack of phosphorylation of lipocortin I and II argues against a role for these proteins in the process.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 233 REGULATION OF GENE EXPRESSION BY PKC-ACTIVATORS IN MOUSE SKIN KERATINOCYTES, Staffan Bohm, Carl Pereswetoff-Morath, Anna Berghard and Rune Toftgård, Center for BioTechnology, Karolinska Institute, Huddinge University Hospital, F82, S-141 86 Huddinge, Sweden. The potent tumor promotor and protein kinase C activator 12-O-tetradecanoylphorbol-13-acetate (TPA) cause alterations of both epidermal differentiation and proliferation patterns in vivo as well as in cultured keratinocytes. To characterize early changes in gene expression a cDNA library representative for mRNAs expressed in mouse epidermis in vivo after TPA treatment was constructed and used for differential screening. Two cDNA clones corresponding to mRNAs increased at early time points after TPA treatment were characterized in more detail. Both mRNAs (size 0.6 kb and 5.6 kb) were increased already 1.5h after treatment and the mRNA levels reached their maximum between 4 and 8h. Several other treatments (12-O-retinoylphorbol-13-acetate, mezerein, the calcium ionophore A23187, sn-1,2-dioctanoylglycerol and dexamethasone) could to various extents regulate these mRNAs. Partial cDNA sequencing revealed that the cDNA clone recognizing the 5.6 kb mRNA contains a sequence belonging to the VL30 retroviral-like gene family with an LTR highly homologous to previously described VL30 LTRs. The cDNA clone recognizing the 0.6 kb mRNA encodes metallothionein II (MT-II). Using synthetic oligonucleotides as probes it was shown that also the MT-I mRNA level is increased after TPA treatment. Metallothionein protein was increased 6h after TPA treatment and was localized mainly to the cytoplasm in all living cell layers of mouse epidermis including hair follicle keratinocytes. These findings show that MT-I and MT-II mRNAs are coordinately induced by TPA in mouse keratinocytes and that essentially all keratinocytes are responsive. Most likely activation of protein kinase C is a key event in the regulation of the mRNA levels for both MTs and the VL30 sequence.

S 234 PROTEIN KINASE C REDISTRIBUTION IN INTACT CELLS IS STIMULATED BY Ca^{2+} MOBILIZATION, I. Trilivas and J.H. Brown, UC San Diego, La Jolla, CA 92093. It is widely accepted that Ca^{2+} is an important factor regulating the association of protein kinase C (PKC) with the plasma membrane. This step appears to be necessary for the activation of the enzyme by diacylglycerol (DAG). Since most studies demonstrating this role for Ca^{2+} have been carried out in vitro, we chose to examine how hormonally-stimulated increases in $[Ca^{2+}{}_i]$ affect PKC redistribution in vivo. We assessed changes in the association of PKC with the membrane by measuring the binding of ^{3}H-PDBu to intact 1321N1 cells. Carbachol, histamine, bradykinin and ionomycin, all known to elevate cytosolic free $[Ca^{2+}]$ in 1321N1 cells, increased ^{3}H-PDBu binding in proportion to which they increase $[Ca^{2+}{}_i]$. This increase in phorbol binding was determined by Scatchard analysis to represent an increase in B_{max} with no change in K_D for PDBu. Such an increase in B_{max} likely results from redistribution of PKC to a membrane compartment because ^{3}H-PDB does not bind to cytosolic PKC under our assay conditions. To demonstrate that the redistribution of PKC resulted from an increase in $[Ca^{2+}{}_i]$, cells were depleted of releasable Ca^{2+} by prior agonist treatment. Under these conditions, carbachol failed to increase $[Ca^{2+}]$ and 3H-PDBu binding. Phosphoinositide (PI) turnover was not inhibited by agonist pretreatment, suggesting that the loss of the ^{3}H-PDBu response is not due to a loss in DAG production. Whereas carbachol induces a rapid and transient increase in both $[Ca^{2+}{}_i]$ and ^{3}H-PDBu binding, it stimulates a slower and more sustained increase in DAG formation. Thus, it appears that it is the Ca^{2+} mobilization aspect of the PI metabolic pathway, and not DAG, which induces the redistribution of PKC in intact cells. Supported by GM 36927.

S 235 DOWN-REGULATION OF PROTEIN KINASE C ELIMINATES RESPONSES TO ACTIVATING STIMULI BUT NOT TO INTERLEUKIN 2 IN A MOUSE INDUCER T CELL CLONE, Viia E. Valge, Barry M. Datlof, Anthony J. Sinskey, and Anjana Rao, Division of Tumor Virology, Dana-Farber Cancer Institute and Department of Pathology, Harvard Medical School, Boston, MA 02115 and Department of Applied Biological Sciences, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139.

T lymphocyte proliferation is mediated by two classes of growth-promoting agents. Activating stimuli (lectins, antigen, antireceptor antibodies) induce synthesis of autocrine growth factors such as interleukin 2 (IL2), which directly stimulate proliferation of T cells bearing the appropriate receptors. Both the primary activation pathway and the IL2-mediated proliferation of T lymphocytes have been reported to involve protein kinase C (PKC), a Ca^{++} and phospholipid-dependent kinase with serine/threonine specificity. Using a mouse helper T cell clone, we have directly tested the role of protein kinase C in proliferation mediated by activating agents or IL2.

1. Chronic treatment of clone Ar-5 with 12-O-tetradecanoyl phorbol 13-acetate (TPA) strikingly decreased their level of protein kinase C, and caused a complete loss of responsiveness to activating agents (antigen, ConA). In contrast, the response of TPA-treated cells to IL2 was increased.
2. Gene expression induced by activating agent (ConA) was inhibited in the TPA-treated cells. Gene expression induced by IL2 was not affected by TPA treatment of cells.
3. Gene expression induced by activating agent (ConA) is independent of the interaction of IL2 with its receptor.

We conclude that while activation of T cells requires intact protein kinase C, their IL2 dependent proliferation is mediated by an alternative pathway not requiring intact protein kinase C. TPA-treated and control cells expressed similar numbers of cell-surface antigen receptors, and showed similar levels of calcium mobillization to Con A. We suggest that TPA-treated cells lack a distal proetin kinase C-mediated step of the activation process, which is required for induction of gene expression and proliferation in response to activating agents.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 236 1-O-ALKYL-2-O-METHYLGLYCEROL MAY INHIBIT TRANSMEMBRANE SIGNALLING VIA PROTEIN KINASE C, W.J.van Blitterswijk*, R.L. van der Bend*, IJ.M. Kramer**, A.J. Verhoeven**, H. Hilkmann* and J. de Widt*. *Divison of Cellular Biochemistry, The Netherlands Cancer Institute (Antoni van Leeuwenhoek-Huis) and **Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, 1066 CX Amsterdam, The Netherlands.

1-O-alkyl-2-O-methylglycerol (AMG) was detected as an intracellular metabolite of the anti-tumor drug 1-O-alkyl-2-O-methylglycero-3-phosphocholine (AMG-PC, an "alkyllysophospholipid"). Synthetic AMG (with a hexadecyl chain at the sn-1 position) inhibited the activation of highly purified human protein kinase C by diacylglycerol in the presence of phosphatidylserine. Furthermore, AMG also inhibited the receptor-specific binding of ^{3}H-phorbol-12,13-dibutyrate (PDBu) to human neutrophils and HL-60 promyeloid leukemia cells in a dose- and time-dependent fashion. AMG-PC was not or much less effective in these assays. AMG was found to inhibit the respiratory burst in human neutrophils as induced by sub-optimal concentrations (<100 nM) of PDBu. The inhibition is competitive in that it can be readily reversed by increasing the dose of PDBu. AMG also inhibited the respiratory burst response to suboptimal doses of other stimulants, such as serum-treated zymosan, 1-oleoyl-2-acetylglycerol and the chemoattractant formyl-methionyl-leucyl-phenylalanine (FMLP). The inhibitor had no effect on cell viability and did not affect the characteristics of the calcium response as induced by FMLP.

The activity of diacylglycerolkinase, partially purified from calf brain, was not significantly affected by AMG.

S 237 **POSSIBLE IMPLICATION OF ARACHIDONIC ACID METABOLISM AND PROTEIN KINASE C IN THE ACTIVATION OF ADRENOCORTICAL CELL STEROIDOGENESIS BY ANGIOTENSIN II.**
Isabelle Vilgrain, Jean-Marc PELOSIN and Edmond M. Chambaz, INSERM U 244, DRF/BRCE, Centre d'Etudes Nucléaires, 38041 Grenoble Cédex, France.

Development of a procedure based on the use of a high performance hydroxyapatite column chromatography allowed the resolution of an apparently homogeneous rat brain protein kinase C (PKC) preparation into four isozymic forms. A single form of PKC, with a chromatographic behavior similar to that of the brain type III isoform, was detected in bovine adrenal cortex. (i) Active phorbol esters (e.g. TPA) and combination of TPA and the calcium ionophore A-23187 (resulting in cellular protein kinase C activation) induced a rapid release of arachidonic acid from prelabeled BAC cell phospholipids. (ii) This effect is dose-dependent and paralleled the increase in BAC cell steroidogenic activity. (iii) A II elicited a similar arachidonate release, in a dose-response fashion (10^{-9} to 10^{-7} M) superimposed to that observed for cortisol production whereas this is not the case with ACTH. (iv) Exogenous arachidonate (1 to 100 µM) induced a dose-dependent increase in BAC cell cortisol production. (v) Specific inhibitors of the arachidonate-initiated lipoxigenase pathway blocked the BAC cell steroidogenic response to arachidonate, as well as to A II. Alltogether, these data suggest that arachidonic acid metabolism, (especially through the lipoxigenase pathway) may be involved in the mechanism of A II-activated BAC cell steroidogenesis, via a protein kinase C-mediated process. This hypothesis would point to phospholipase A_2-lipomodulin or diacylglycerol metabolism as possible targets regulated by protein kinase C in A II-activated cells.

S 238 ACTIVATION OF THE Ca PUMP ATPase OF INTACT RED BLOOD CELLS BY A23187, Frank F. Vincenzi, Ling Wu and Thomas R. Hinds, University of Washington, Seattle, WA 98195.

The well known Ca pump ATPase of plasma membranes has usually been studied in isolated membranes where it is expressed as a Ca^{2+} and Mg^{2+} dependent ATPase. In experiments presented here, ATPase activity was monitored by following the loss of ATP in intact human red blood cells (RBCs) (in the presence of 1mM iodoacetic acid [IAA] to prevent ATP resynthesis) exposed to the divalent ionophore, A23187 in the presence of Ca^{2+}. Under these conditions, within 2 minutes of the addition of ionophore, the loss of ATP from the cells obeyed pseudo first order decay with a rate constant of approximately 0.2 min^{-1} (half life approximately 3.5 min). In the absence of Ca^{2+} in the medium, A23187 induced no ATP loss. The rate constant for ATP loss differed in a characteristic manner, depending on the individual blood donor. The rate constants determined in intact cells from various donors correlated with the specific activities of the ATPase as determined in RBC lysates and/or isolated membranes from the same donors, respectively. The rate constant also differed in 'young' and 'old' RBCs (as selected by density). The rate constant for A23187-induced ATP loss was approximately 20% greater in young than in old RBCs. The results demonstrate that the activity of the plasma membrane Ca pump ATPase is activated by the influx of Ca in intact cells, that within two minutes of such activation, the enzyme appears to not be saturated with the substrate, ATP and that the rate of ATP loss can be taken as a measure of the Ca pump ATPase activity of intact cells. Funded by the National Dairy Board administered in cooperation with the National Dairy Council.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 239 REGULATION OF SMOOTH MUSCLE PROTEIN KINASE C, Michael P. Walsh and Gwyneth de Vries, University of Calgary, Alberta, Canada T2N 4N1.
Protein kinase C was purified from chicken gizzard smooth muscle by Ca^{2+}-dependent hydrophobic-interaction chromatography, ion-exchange chromatography and hydrophobic chromatography. Fast-performance liquid chromatography (gel filtration) revealed a Stokes radius of 33.7 Å, corresponding to a globular protein of M_r 61,500. Denaturing polyacrylamide gel electrophoresis indicated M_r = 80,000. Enzymatic activity was routinely assayed by the mixed-micelle assay of Bell et al [Methods Enzymol. 124, 353 (1986)] using histone III-S as the substrate but at a Triton X-100 concentration of 0.03% (w/v) which was found to be optimal. The isolated enzyme required Ca^{2+}, phospholipid and diacylglycerol (DG) for activity, with half-maximal activation at 4.62 ± 0.31 x 10^{-7}M Ca^{2+} (n=3). No activation by Ca^{2+} was observed in the absence of DG. The enzyme required free Mg^{2+} in addition to the $MgATP^{2-}$ substrate. The K_m for ATP was 19.9 ± 4.6 μM (n=4). Activity was highly sensitive to ionic strength with half-maximal inhibition at 70 mM NaCl. Phosphorylation of the physiological substrates, platelet P47 and gizzard vinculin, was more strongly dependent on Ca^{2+} and lipids than was histone phosphorylation. Partial digestion with trypsin yielded a constitutively active fragment, i.e. activity was no longer dependent on Ca^{2+} and lipids, as has been shown with other kinase Cs. A heat-stable protein inhibitor of kinase C was also identified in smooth muscle. Further studies will focus on the role of kinase C in the regulation of smooth muscle contraction, and structural and functional characterization of the inhibitor. (Supported by the Medical Research Council of Canada).

S 240 BRYOSTATIN 1 PHOSPHORYLATION PATTERN IN HL-60 CELLS CAN BE MIMICKED BY HIGH CONCENTRATIONS OF PHORBOL ESTER, Barbour S. Warren, Yoshiaki Kamano, George R. Pettit, and Peter M. Blumberg, Laboratory of Cellular Carcinogenesis and Tumor Promotion, National Cancer Institute, Bethesda, MD 20892 and The Cancer Research Institute and Department of Chemistry, Arizona State University, Tempe, Arizona 85287.
The bryostatins are a group of macrocyclic lactones isolated from the marine bryozoan *Bugula neritina*. Bryostatin 1, like the phorbol esters, activates protein kinase C; however, it partially or completely inhibits the phorbol ester induced differentiation of the human promyelocytic leukemic cell line HL-60. Treatment of HL-60 cells with phorbol 12,13-dibutyrate (PDBU) at typical concentrations (10^{-8} - 10^{-7} M) increased the phosphorylation of 7 proteins. Treatment with bryostatin 1 enhanced the phosphorylation of these same proteins. In addition, bryostatin 1 caused the appearance of 2 phosphorylated protein spots with molecular weights of 70 kDa and pIs of 6.3 - 6.4. These latter phosphorylations were evident after a 30 min exposure to bryostatin 1 at 6 nM. Concentrations of PDBU of 600 nM or greater, approximately 100-fold that necessary to induce differentiation, also induced appearance of these phosphoprotein spots. The 70 kDa phosphoproteins were located in the cytoskeletal fraction of the cells; their phosphorylation was almost totally on serine residues. We conclude that phorbol esters at high concentrations may mimic certain actions of bryostatin 1.

S 241 REGULATION OF ARACHIDONIC ACID RELEASE BY PROTEIN KINASE C IN MDCK CELLS, Barbara Weiss, Sandra Slivka, and Paul Insel, University of California, La Jolla, CA 92093
Protein kinase C (PKC) has been implicated as a mediator of several classes of membrane receptors. Using a cloned Madin-Darby kidney cell line (MDCK-D1), we have tested whether or not PKC regulates arachidonic acid and arachidonic acid metabolite (AA) release stimulated by the phorbol ester TPA (an exogenous activator of PKC), the calcium ionophore A23187, and the hormones epinephrine (acting at α_1-adrenergic receptors), and bradykinin. AA release studies were conducted using cells that had been labeled with ^{3}[H]-arachidonic acid. Cells were pretreated in the presence or absence of sphingosine, an inhibitor of PKC, prior to stimulation of AA release. Released counts stimulated by both TPA and bradykinin were predominantly prostaglandin E_2 (PGE_2). TPA, A23187, and bradykinin stimulated formation of lysophosphatidylcholine in MDCK-D1 cells prelabeled with 3[H]-choline, suggesting that part of the arachidonic acid was released by the action of phospholipase A_2 (PLA_2). TPA and A23187 both stimulated a several fold increase in ^{3}[H]-AA release that could be inhibited 70-80% by sphingosine. α_1-mediated ^{3}[H]-AA release was also inhibited by sphingosine (40-50%). In contrast, bradykinin stimulated ^{3}[H]-AA release several fold but this stimulation was only slightly inhibited (15-25%) by sphingosine. Thus, AA production in MDCK-D1 cells can be stimulated by hormonal and non-hormonal agents. Some of the arachidonic acid appears to be generated by activation of PLA_2. TPA-, A23187-, and α_1-mediated release are inhibited by sphingosine, whereas bradykinin-mediated release is relatively insensitive. This suggests that 1) PKC is an important cellular mediator of AA release in MDCK-D1 cells and 2) Hormonally-stimulated release of AA can result from more than one pathway in these cells.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 242 TYPE I PHOSPHATIDYLINOSITOL KINASE MAKES A NOVEL INOSITOL PHOSPHOLIPID, PHOSPHATIDYLINOSITOL 3 PHOSPHATE, Malcolm Whitman*, C.Peter Downes**, Marilyn Keeler, Tracy Keller, and Lewis Cantley; Department of Physiology ,Tufts University School of Medicine Boston MA 02111* Department of Biochemistry and Molecular Biology, Harvard University 7 Divinity Ave, Cambridge MA 02138 ;** Smith Kline and French Research Ltd.The Frythe, Welwyn Hertfordshire England AL6 9AR

A PtdIns kinase activity has been found to associate with several oncogene products , as well as with platelet derived growth factor receptor. We have previously identified two biochemically distinct PtdIns kinases in fibroblasts, and have found that only one of these, designated Type I, specifically associates with activated tyrosine kinases . An 85 kD protein which becomes phosphorylated on tyrosine in response to PDGF stimulation or oncogenic transformation of fibroblasts copurifies with the Type I PtdIns kinase .We have now found that Type I PI kinase specifically phosphorylates the D-3 position to generate a novel phospholipid, PtdIns-3-P. In contrast, the major PtdIns kinase of fibroblasts, designated Type II, specifically phosphorylates the D-4 position to produce PtdIns-4-P, previously considered to be the only form of PtdInsP . We have also identified a minor component of the total PtdInsP of intact fibroblasts which, when deacylated, comigrates with the deacylation product of PtdIns-3-P produced in vitro. We propose that Type I PtdIns kinase is responsible for the generation of PtdIns-3-P in intact cells, and that this novel phosphoinositide, which is presumably not in the pathway for generation of $Ins(1,4,5)P_3$, may play a role in the transduction of mitogenic/oncogenic signals.

S 243 ISOLATION OF THE PROTEIN KINASE C GENE OF DICTYOSTELIUM, J.G. Williams, H.M. Mahbubani, J.A. Kirk, M.M. Lloyd and P. Skehel, Imperial Cancer Research Fund, Clare Hall Laboratories, South Mimms, Potters Bar, Herts, EN6 3LD U.K.

The rise in extracellular cAMP levels which occurs during cellular aggregation induces the expression of a large number of Dictyostelium genes. Extracellular cAMP pulses, which induce premature expression of these genes, elevate intracellular cGMP, cAMP and Ca levels. There is pharmacological evidence to suggest an involvement of the calcium activated enzyme protein kinase C (PKC) in gene induction. In order to investigate the PKC transduction pathway we have cloned a Dictyostelium PKC gene. It was isolated from a genomic clone bank using a long synthetic oligonucleotide containing sequences derived from a cysteine-rich region which is highly conserved in the PKC genes of higher eukaryotes. The sequence of the isolated gene, in this region, is highly homologous to vertebrate and Drosophila PKC sequences. Multiple copies of a single gene were isolated but genomic hybridization at reduced stringency suggests the existence of at least one related gene and Northern transfer analysis indicates there to be multiple PKC transcripts.

S 244 PROTEIN KINASE C ISOENZYME FUNCTION STUDIES, R.M. Marais and P.J.J. Parker; Ludwig Institute for Cancer Research, (Middlesex Hospital/ University College Branch), Courtauld Building, 91 Riding House Street, London, W1P 8BT, U.K.

The existence of multiple isoenzymatic forms of protein kinase C has raised interesting questions as to the biochemical functions of different isoenzymes in individual cells. Using synthetic peptides corresponding to variable regions of each of the four isoenzymatic forms of protein kinase C; α, β_1, β_2 and γ we have raised monospecific polyclonal antisera with high titres which recognise individual isoforms in a protein-immunoblot system. These antisera have been used to identify individual purified isoenzymes separated on chromatographic columns. Initial biochemical analysis of the separated proteins indicates that there may be a difference in calcium dependence between the isoforms. Further results will be presented.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

Phospholipase Activators, Inhibitors, Lipocortins

S 300 **PLA$_2$-RELEASED LIPID MEDIATORS AND cAMP IN fMLP-ACTIVATED ALVEOLAR MACROPHAGES,** Gilbert BEREZIAT, Joëlle MASLIAH, Maria BACHELET, Odile COLARD and Boris B. VARGAFTIG, CNRS UA 524 CHU Saint Antoine and INSERM U 285 Institut Pasteur PARIS

The stimulation of cultured guinea-pig alveolar macrophages by the chemotactic peptide formyl-Methionyl-Leucyl-PhenylAlanine (fMLP) or by the platelet activating factor (PAFacether) induces an increase in the release of arachidonic acid (AA) or of its cyclooxygenase and lipoxygenase byproducts.

From studies using macrophages which have been labeled with [1-^{14}C]AA for a variable lengh of time, we demonstrated that AA was released (probably by a multistep process) from fast-turnover pools of AA molecular species of phosphatidylinositol (PI), alkylacylglycerophosphocholine (AlkAcPC) and diacylglycerophosphocholine (DAPC). In both fMLP- and PAFacether-stimulated macrophages, the NaCL 1M-extracted phospholipase A$_2$ (PLA$_2$) exhibited a higher activity than in control cells. Using as substrate [1-^{14}C]AA-labeled membranes cleared of PLA$_2$ by NaCl-extraction, we showed that the increase of PLA$_2$ activity in macrophages lead to an increase in the breakdown of AlkAcPC and of DAPC but not in the breakdown of PI. AlkénylAcylglycerophosphoethanolamine (PPE) in which AA transfer occured upon cell stimulation was not a substrate for NaCl 1M-extracted PLA$_2$. Then, the significance of this transfer to PPE remains to be elucidated.

Pretreatment of macrophages by Prostaglandin E$_2$ (PGE$_2$) decreased the AA release elicited by PAFacether and fMLP as well. Furthermore, PAFacether lowered the cellular content in cAMP of PGE$_2$-treated macrophages with a time-course which paralleled the release of AA.

From these results, we suggest that an initial stimulation of alveolar macrophages by a bacterial signal (fMLP) initiates the sequential activation of phospholipase C leading to PI breakdown and of PLA$_2$ leading to the release of PAFacether and icosanoïdes. These mediators might in turn modulate the respons by increasing (PGE$_2$) or decreasing (PAFacether) the macrophage content in cAMP.

S 301 THE ROLE OF MEMBRANE PHOSPHOLIPIDS IN THE TRANSDUCTION OF PHYSICAL FORCES INTO BIOCHEMICAL SIGNALS IN BONE CELLS, I. Binderman, U. Zor*, A.M. Kaye*, A. Harell and D. Somjen, Hard Tissues Unit, Ichilov Hospital, Tel Aviv 64239, and *Department of Hormone Research, The Weizmann Institute of Science, Rehovot 76100, Israel.

Physical forces applied to cultured bone cells induce the production of cAMP via PGE$_2$ formation. In this paper we show that antiphospholipid antibodies, which bind to membrane phospholipids, abolish cAMP formation induced by physical force. This inhibition is not seen in the presence of arachidonic acid. Increased cAMP production, due to physical stimulation, is also blocked by the antibiotic gentamicin, which is a known inhibitor of phospholipase A$_2$ activity due to binding to its substrates, the membrane phospholipids. These observations suggest that physical forces exert their effect via the following chain of events: 1) activation of phospholipase A$_2$, 2) release of arachidonic acid, 3) increased PGE synthesis, 4) stimulation of adenylate cyclase activity, 5) augmented cAMP production. Indeed, addition of exogenous phospholipase A$_2$ (but not phospholipase C) causes a similar increase in the formation of cAMP in bone cells, a process which is also inhibited by both antiphospholipid antibodies. These data strongly suggest that the synthesis of PGE$_2$ due to physical forces is the result of activation of phospholipase A$_2$, which releases arachidonic acid which activates prostaglandin synthetase.

S 302 EGF STIMULATION OF PROSTACYCLIN PRODUCTION IN A10 RAT AORTIC SMOOTH MUSCLE CELLS. Jonathan Blay and Morley D Hollenberg, Dept of Pharmacology and Therapeutics, Faculty of Medicine, University of Calgary, Alberta T2N 4N1, CANADA.

The A10 cell line is a clonal smooth muscle cell line derived from rat embryonic thoracic aorta. A10 cells have functional receptors for epidermal growth factor (EGF), which modulates their DNA synthesis and proliferation. We have investigated the ability of EGF to influence prostacyclin production by A10 cells. We measured the accumulation of 6-keto-PGF$_{1\alpha}$ (a stable product of prostacyclin) in the culture medium of butyrate-primed A10 cells by radioimmunoassay. EGF by itself had no effect on prostacyclin production, but when added together with vasopressin it markedly increased the production of 6-keto-PGF$_{1\alpha}$ over that seen with vasopressin alone. Levels of the prostaglandin in the medium rose to half-maximal at 1 h and reached a maximum after 3 h.

We thought that vasopressin's ability to support an EGF stimulation of prostacyclin production might be due to its capacity to stimulate polyphosphoinositide breakdown, leading to the activation of protein kinase C and the release of intracellular calcium. We therefore tested whether 12-O-tetradecanoylphorbol 13-acetate (TPA), a direct activator of protein kinase C, or A23187, a calcium ionophore, could substitute for vasopressin in this context. TPA and A23187 each increased 6-keto-PGF$_{1\alpha}$ production by A10 cells, but only A23187 supported a further effect of EGF. The ability of EGF to stimulate prostacyclin production in A10 smooth muscle cells may therefore be dependent upon a concurrent increase in intracellular calcium levels.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 303 A PHOSPHOLIPASE A_2 ACTIVATING PROTEIN (PLAP) ISOLATED FROM RHEUMATOID SYNOVIAL FLUID STIMULATES HUMAN NEUTROPHIL RELEASE OF LYSOSOMAL ENZYMES AND SUPEROXIDE, AND ERYTHROCYTE HEMOLYSIS, John S. Bomalaski, Daniel Baker, Lynne Brophy, Norman V. Resurreccion and Mike A. Clark, V.A. Medical Center, Med. College Penn., University of Pennsylvania, Philadelphia, PA 19104 and Washington University, St. Louis, MO 63110.

We have recently isolated a human phospholipase A_2 activating protein termed PLAP which shares antigenic and biochemical similarity with melittin, a well characterized bee venom phospholipase stimulatory peptide. This is the first description of a human phospholipase A_2 stimulatory protein. In order to explore the potential mechanisms of action of PLAP that extend beyond its effects on eicosanoid synthesis, we examined its effects on the release of human neutrophil lysosomal enzymes and superoxide, and on red blood cell hemolysis. Results were compared with melittin, which has been reported to induce enzyme release and hemolysis. PLAP induced neutrophils to release B-glucuronidase and metalloproteinase enzyme activities as well as produce superoxide ion in a dose and time dependent manner. Both PLAP and melittin induced these responses, but melittin was more potent. Eicosanoid synthesis inhibitors (indomethacin, esculetin, BW-755C) did not abrogate these responses. Concentrations of PLAP that induced these responses did not induce cellular toxicity as determined by light microscopy, LDH release, trypan blue dye exclusion or hemolysis as did melittin. In contrast, prolonged incubation with higher concentrations of PLAP induced neutrophil and erythrocyte death that was similar to that observed with melittin. These findings suggest that the mechanisms of action of PLAP extend beyond activation of the eicosanoid synthesis pathway.

S 304 **STUDIES ON THE CELLULAR DISTRIBUTION OF LIPOCORTINS 1, 2 AND 5,** J. Browning, A. Ribolini, L. Sinclair, D. Pratt, and B. Pepinsky, Biogen Research Corp., Cambridge, MA 02142.

The lipocortin family of calcium/phospholipid binding proteins is composed of at least six different members. These proteins share a four-fold repeated 70 amino acid domain that mediates phospholipid binding. The N-terminal amino acid sequence of lipocortins 1 and 2 differ, yet both contain sites for phosphorylation and appear to regulate functional aspects of the phospholipid binding domain. Lipocortin-5 lacks such a regulatory N-terminus. To evaluate whether these proteins may have specifically tailored functions, their distribution in various tissues and cultured cell lines was examined by western blotting. The tissue distributions of lipocortins 1, 2, and 5 are substantially different and dramatic variations were found in cultured cell lines. In the case of peripheral blood lymphocytes, expression of lipocortins 1 and 2 are independently regulated. These data support a model where the four-fold repeat domain of the lipocortins shares a common function; however, each protein may be tailored to individual functions via separate transcriptional/translational control and a specific N-terminus that targets each molecule to a specific task.

S 305 EFFECTS OF EPIDERMAL GROWTH FACTOR AND 12-O-TETRADECANOYL PHORBOL-13-ACETATE ON THE SUBCELLULAR DISTRIBUTION AND PHOSPHORYLATION OF LIPOCORTIN I IN T51B CELLS. Roberto Campos-Gonzalez, Martha Kanemitsu, Sharon Yokoyama, and Alton L. Boynton. Cancer Research Center of Hawaii, University of Hawaii, Honolulu, HI.

T51B rat liver epithelial cells can be stimulated to proliferate upon the addition of EGF or TPA. In these cells most immunoreactive lipocortin was located in the cytosol ($lipocortin^{cyt}$) of cells extracted in Ca^{2+}-free buffers. These cells however, had another pool of immunoreactive lipocortin in the particulate fraction that was Triton X-100 extractable ($lipocortin^{mem}$). Stimulation of cells with EGF resulted in a rapid translocation of $lipocortin^{cyt}$ to the plasma membrane. This translocation was extracellular Ca^{2+}-dependent. In vitro phosphorylation assays demonstrate that $lipocortin^{cyt}$ is phosphorylated by a Ca^{2+} and phosphatidylserine-dependent kinase (protein kinase C). The Ca^{2+} and phosphatidylserine-dependent phosphorylation of $lipocortin^{mem}$ in vitro, occurred only in TPA-treated cells. TPA also induced the phosphorylation of $lipocortin^{mem}$ in ^{32}P-phosphate labelled cells. These results suggest a possible role of $phospholipocortin^{mem}$ in the proliferation of T51B cells. Supported by NCI grant CA 39745.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 306 ARACHIDONATE RELEASE FROM PLATELET PHOSPHOLIPIDS UPON THROMBIN STIMULATION : POSSIBLE CONTROL BY TRANSFER REACTION. O Colard, M. Breton, H. Masrar and G. Bereziat. C.H.U. Saint-Antoine, Paris, FRANCE.

The increased rate of prostanoïd synthesis when platelets are stimulated is dependent on an enhanced amount of arachidonic acid (AA) available as substrate. AA can be released from membrane phospholipids through phospholipases, it can also be transferred between phospholipids by transacylases and it can be reacylated into membrane lipids by the acyl CoA acyltransferase. The available pool for prostanoïd synthesis is thus the result of these various enzymatic pathways. Upon stimulation of AA prelabeled platelets, AA appeared to be released from phosphatidylinositol (PI) and phosphatidylcholine (PC). However platelet phospholipase A_2 which has been thought to play the major role in the release of AA is independent on the fatty acids and is primarily active on diacyl phosphatidylethanolamine (PE). Studying changes in phospholipid mass following stimulation, it was recently shown that diacyl-PE contributed also to AA release.

In this paper, AA prelabeled rat platelets were stimulated with thrombin. We compared the specific activity of the released AA to the specific activities of the different classes of phospholipids in order to determine the time dependent arachidonate release from each platelet phospholipid. The various classes (alkyl, alkenyl and diacyl) of phospholipids were separated as their benzoilated diglyceride derivatives and analyzed by HPLC. The molecular species were quantitated by U.V absorption and counted on line using a scintillation counter. The incorporation rate of labeled AA in phospholipids was dependent on the length and unsaturation of the other fatty chain, on the polar head group and on the acyl, alkyl or alkenyl bond in the 1-position of the glycerol backbone. Depending on these parameters, large differences in the specific activities were observed following a short time arachidonate pulse-chase. Arachidonate release was measured by gaz liquid chromatography and counted for radioactivity, platelets being stimulated in the presence of BW 755 C to prevent arachidonate transformation. The results obtained could be consistent with a stepwise release, labeled arachidonate being first transferred from high specific-activity phospholipids to other phospholipids before its release.

S 307 LOSS OF INHIBITORY ACTION OF DEXAMETHASONE ON INOSITOL PHOSPHOLIPID (PI) BREAKDOWN AND HISTAMINE SECRETION IN PERMEABILIZED RBL-2H3 CELLS. Dolores Collado-Escobar, Jose Cunha-Melo, Hydar Ali, James J. Mond and Michael A. Beaven. NHLBI, Bethesda, MD 20892 and Dept. of Med. USUHS, Bethesda, MD 20814

Long term treatment of mast cells or a cultured analog, RBL-2H3 cells, with glucocorticoids inhibits IgE-dependent PI breakdown, Ca^{++} signal and release of histamine and arachidonic acid. Glucocorticoid receptors have been characterized in mast cells and it has been postulated that activation of these receptors induces synthesis of protein(s) that inhibit stimulatory components such as phospholipase C, phospholipase A_2 or coupling G-proteins. Another possibility is that the PI-stimulatory cascade is suppressed by induction of adenylate cyclase as appears to occur in some other types of cells such as platelets, neutrophils and lymphocytes. We have measured phospholipase C activity towards all inositol phospholipids from extracts of RBL-2H3 cells that were treated for 24 h with concentrations of dexamethasone ranging from 10^{-10} to 10^{-5}M and found that there was no reduction of phospholipase C activity. Indeed neither PI breakdown nor secretion of histamine was impaired when dexamethasone treated cells were permeabilized before stimulation with antigen or GTPγS-a stimulant of G-proteins. As the same cells, when left intact, showed markedly depressed responses to antigen, it seemed probable that dexamethasone acted through induction of soluble inhibitory factor(s). Possible candidates include cyclic AMP and protein kinase C both of which presumably would diffuse from permeabilized RBL-2H3 cells.

S 308 **IDENTIFICATION AND CHARACTERIZATION OF SIX LIPOCORTIN-LIKE PROTEINS IN HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS.** Christine Comera, Bernard Rothhut and Françoise Russo-Marie. Unité Associée Institut Pasteur/INSERM N°285, 25 rue du Dr Roux, 75015 Paris, France.

Normal mononuclear cells were obtained by isolation from human peripheral blood. The lipocortin-like proteins were purified by calcium precipitation-EGTA solubilization and separation on a monoQ column HR 5/5 (FPLC Pharmacia). In the NaCl gradient, 4 pure lipocortin-like proteins were eluted, named 35K, 36K, 32K and 73K, according to their apparent Mr and their order of elution. These proteins were different from lipocortin I and II which were found in the flow through. The 32K, 35K and 36K are recognized selectively by polyclonal antibodies directed to themself suggesting that they are different one another. These results were confirmed by the different two dimensional tryptic maps obtained with each protein. The 36K and 73K cross react with the anti 36K antibodies and the anti 68K calelectrin antibodies. In additon, the two dimensional tryptic maps of the 36K and 73K were very similar, demonstrating that the 36K is contained inside the 73K. The 32K is different from endonexin I as evidenced by its two dimensional electrophoretic migration, and is similar to chromobindin 7, endonexin II, placental anti coagulant protein and the inhibitor of blood coagulation. The 35K and 36K cannot be related to other described annexins. All these proteins inhibit phospholipase A2 in a dose dependent manner, they are specific of negatively charged phospholipids, and the inhibitions are reversed by high doses of substrates. The biological significance of the presence of these proteins in normal peripheral cells is not yet understood.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 309 ANTI-INFLAMMATORY EFFECT OF MICE LIPOCORTIN-LIKE PROTEINS IN A POLYACRYLAMIDE GEL-INDUCED INFLAMMATION. Mourad Errasfa and Françoise Russo-Marie. Unité Associée, Institut Pasteur/INSERM N°285, 25 rue du Dr Roux, 75015 Paris, France.
Lipocortins (LCs) are glucocorticoid-inducible proteins which inhibit PLA_2. A 36kDa lipocortin-like protein was purified from mice thymus by calcium precipitation-EGTA solubilization and separation on a MonoQ HR 5/5 (FPLC-Pharmacia). The anti-PLA_2 effect of this protein was assessed in vitro on labeled E.Coli membranes and on cellular PLA_2 of isolated cells. The anti-inflammatory effect of glucocorticoids is thought to be due in part to their induction of an anti-PLA_2 activity in the cells which could be mediated by the LCs. We have thus investigated the anti-inflammatory effect of the 36kDa anti-PLA_2 protein in a model of acute inflammation. The inflammatory reaction was induced by the injection of a suspension of a polyacrylamide gel in the dorsal subcutaneous area of mice. Before the injection of the gel suspension, the animals received intravenously the 36kDa protein (1-20 µg/animal) or its control solution. In addition, dexamethasone (0.2 and 2 mg/kg), aspirin (50 and 150 mg/kg) and indomethacin (0.5 and 5 mg/kg) were used according to the same protocol. After a defined period of time, mice were killed, and the cells (PMNs > 95% of total cells) which have migrated to the subcutaneous area of mice counted and icosanoids assayed. In this investigation, we report the anti-inflammatory effect (inhibition of the cell migration and icosanoid production) of the anti-PLA_2 protein (36kDa) which is compared to the effect of the other anti-inflammatory drugs.

S 310 INHIBITION OF PLA_2 ACTIVITY AND SUPEROXIDE ANION GENERATION OF ACTIVATED ALVEOLAR GUINEA-PIG MACROPHAGES BY LIPOCORTIN-LIKE PROTEINS. Mourad Errasfa, Maria Bachelet, Isabelle Maridonneau-Parini, B. Boris Vargaftig and Françoise Russo-Marie. Unité Associée Institut Pasteur/INSERM N° 285, 25 rue du Dr Roux, 75015 Paris, France.
Lipocortin-like proteins were purified from mice lung (36kDa "P", 40kDa) and thymus (36kDa "T") by calcium precipitation-EGTA solubilization and separation on a MonoQ column HR 5/5 (FPLC-Pharmacia). The anti-PLA_2 effect of these proteins was assessed "in vitro" using labeled E. Coli membranes as a substrate. The effect of these anti-PLA_2 proteins on the activation of guinea-pig alveolar macrophages by PAF-acether and the calcium ionophore A23187 was investigated. The activation of macrophages was assessed in vitro by the generation of superoxide anion and the release of ^{3}H-arachidonic acid from labeled cells. The anti-PLA_2 proteins (0.5-10 µg/$1x10^6$-$3x10^6$ cells) purified from mice lung and thymus were able to inhibit in a dose-dependent manner the release of ^{3}H-arachidonic acid and the production of superoxide anion from macrophages activated either by PAF-acether (5nM) or A23187 (1µM). The inhibition observed was decreased when PAF-acether and A23187 were used at high doses. The protein kinase C activator PMA had a synergistic effect with the calcium ionophore A23187 on the activation of macrophages. The pretreatment of macrophages with PMA (1µg/ml, which did not activate macrophages) resulted in a loss of the protein activity in a dose dependent manner. These results demonstrate that exogenous lipocortins modulate two parameters of activated macrophages involved in the inflammatory reaction.

S 311 IL-1-INDUCED ARACHIDONIC ACID MOBILIZATION IN HUMAN NEUTROPHILS, Gillian M.P. Galbraith and M.H. Williams, Medical University of South Carolina, Charleston, SC 29425.
Interleukin 1 (IL-1) exerts a number of effects on neutrophils including chemotactic response. Since activation of these cells is frequently accompanied by eicosanoid generation, we examined the effect of IL-1 on arachidonic acid (AA) mobilization in normal human neutrophils. Cells were prelabeled with [^{3}H]-AA and exposed to recombinant human IL-1 β (rIL-1). The cells responded by release of [^{3}H] products into the medium in a time and IL-1 concentration dependent manner. This response was also calcium dependent, in that it was reduced in the presence of 10mM EDTA or 200 µM TMB-8. Furthermore, IL-1-induced release of radioactivity was markedly enhanced in the presence of calcium ionophore A23187 used at concentrations insufficient alone to mobilize AA. Two-dimensional thin layer chromatography of neutrophil phospholipids indicated a relative loss of AA from phosphatidylinositol in cells stimulated with rIL-1. Although IL-1 receptors have not to date been demonstrated in normal neutrophils, these data suggest that interaction of IL-1 with such cells results in phospholipase activation and mobilization of AA.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 312 Modulation of Natural Killer Cell Phospholipase Activities by Lipid Peroxidation
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Eicosanoid production is virtually undetectable in RNH-16 natural killer cells (NK), a large granular lymphocyte leukemia cell line; yet these cells have an active phosphoinositide cycle *in situ*. To assess the potential role of lipolytic enzymes in NK cell function, phospholipase A_2 (PLA_2) and C (PLC) activities were examined in sonicates of RNH-16 cells using as phospholipid substrates: ^{14}C-oleate labelled autoclaved *E. coli* (PLA_2), 2-[1-^{14}C]-acyl phosphatidylethanolamine (PLA_2 and PLC), ^{14}C-inositol labelled phosphatidylinositol (PI-PLC) and N-methyl^{14}C sphingomyelin (SMase-PLC). No PLA_2 activity was detectable at neutral-to-alkaline pH with or without added Ca^{+2}, while substantial neutral-active, Ca^{+2}-dependent PI-PLC was noted (17.4 nmols/ min/mg). Both PLA_2 and PLC activity was noted at pH's 4.0-5.0 that was ion dependent and most likely of lysosomal or granule origin. PLC-mediated hydrolysis of air-oxidized phosphatidylethanolamine (PE) by NK sonicates at pH 5.0 was increased 800% vs the unperoxidized control, while PLA_2 activity at pH 5.0 was inhibited 90% by this treatment. Hydrolysis of both control and peroxidized PE by acid-active phospholipases was inhibited by the antioxidant sodium metabisulfite. It is hypothesized that peroxidative modulation of phospholipase activities may contribute to membrane perturbations during degranulation associated with killer cell cytolylic function.

S 313 PHOSPHOLIPID ANALOGUES AS MECHANISTIC PROBES OF PHOSPHOLIPASE A_2 CATALYSIS. Michael H. Gelb, Wei Yuan, and Karen Fearon. Departments of Chemistry and Biochemistry, University of Washington, Seattle, Washington 98195.
A number of phospholipid analogues have been prepared as inhibitors of snake venom phospholipase A_2 in an effort to understand the mechanism of enzymatic catalysis. Compounds were synthesized in which the ester at the 2-position of the glycerol backbone was replaced by a difluoromethylene ketone unit. These compounds were designed to be tight-binding inhibitors since the hydrated fluoro ketone is a structural mimic of the putative tetrahedral intermediate that forms in phospholipase A_2 mediated hydrolysis. Inhibitors were tested in a mixed-micelle system. It was shown that only those fluoro ketones that were hydrated in the micelle were good phospholipase A_2 inhibitors. The most potent compounds were found to bind some 1000-3000 fold tighter to the enzyme than dipalmitoyl phosphatidylcholine substrate. The snake venom enzyme is known to be activated by choline-containing lipids. It was shown that the hydrated fluoro ketone inhibitors bind only to the activated form of the enzyme. Additional phospholipase A_2 inhibitors were designed to probe the role of the calcium ion in catalysis. The results of these studies will also be discussed.

S 314 MOLECULAR PHARMACOLOGY OF MANOALIDE: LOCALIZATION OF THE MANOALIDE BINDING SITE ON PHOSPHOLIPASE A_2. Keith B. Glaser and Robert S. Jacobs, Dept. of Biological Science and Marine Science Institute, University of California, Santa Barbara, CA 93106.
The marine natural product manoalide (MLD), a potent inhibitor of phospholipases, completely inactivates bee venom phospholipase A_2 (PLA_2) by an irreversible mechanism. It has been proposed (Glaser and Jacobs, BIOCHEM. PHARMACOL., 36:2079, 1987) that the reaction of MLD with PLA_2 may involve the selective reactivity of MLD to a peptide sequence, possibly a Lys-X-X-Lys peptide. Upon MLD modification of PLA_2 the only change in amino acid content was an apparent loss of Lys, corresponding to approximately three of the eleven Lys residues present. Selective chemical modification of Lys residues with (^{14}C)maleic anhydride demonstrated all eleven Lys residues on PLA_2 were accessible to this reagent (11.6 mol maleyl group/mol PLA_2). Pretreatment of PLA_2 with MLD (<0.7% residual activity) resulted in a molar ratio of 8.7, also consistent with the loss of three Lys residues. These data suggest that MLD has a selective binding site on bee venom PLA_2 and that the mechanism of action of MLD does not involve non-selective amino (Lys) group modifying reactions. The cyanogen bromide (CNBr) peptide corresponding to residues 81-128, as determined by gas-phase sequence analysis, showed the most intense absorbance at 434 nm, corresponding to the MLD-Lys adduct chromophore, and sequencing failure was observed at Lys-88. The peptide corresponding to residues 42-80 had a minor absorbance at 434 nm and demonstrated a sequencing failure at Lys-47. These data suggest that Lys-47 and Lys-88 may correspond to two of the three MLD modified Lys residues. Further experiments with radiolabelled MLD are in progress to provide direct evidence of the MLD binding site.(Supported by NOAA, Sea Grant, NA 80AA-120 and State Resources R/MP-21)

S 315 FLUORIDE INHIBITS PHOSPHOINOSITIDE TURNOVER IN RAT CORTEX, P.P.Godfrey S.P.Watson and D.G.Grahame-Smith, Oxford University, Oxford, UK.

There is good evidence that G_s and G_i, the G-proteins coupled, positively and negatively respectively, to adenylate cyclase can be activated by fluoride when complexed with Al^{3+} to form AlF_4^-. It has recently been found that phospholipase C can also be activated by fluoride, and these results have provided evidence for the existence of a G-protein coupled to inositol phospholipid metabolism. In this study we have investigated the actions of fluoride on inositol phosphate production in rat cerebral cortex in order to determine possible roles of G-proteins in these responses.

NaF (10mM), in the presence or absence of Al^{3+}, caused a very small, though significant, increase in inositol phosphate (IP) levels over basal (23 ± 5%) during a 30 min incubation in the presence of 10mM LiCl. In addition NaF dose-dependently inhibited carbachol (1mM) stimulated IP formation. A maximal inhibition was seen at a fluoride concentration of 20mM and the IC_{50} was 1.2mM. 20mM NaF inhibited the carbachol response by over 90%. NaF also blocked the responses to noradrenaline (300uM), 5-HT (100uM) and K^+ (20mM). The relative ratios of IP_1, IP_2, IP_3 and IP_4 levels was not altered by NaF. The data suggests that NaF may activate a G-protein negatively coupled to phospholipase C in rat cortex.

S 316 GLUCOCORTICOIDS INHIBIT PROSTANOID SYNTHESIS NOT ONLY AT THE LEVEL OF PHOSPHOLIPASE A2, BUT ALSO AT THE LEVEL OF CYCLOOXYGENASE/PGE ISOMERASE

Margarete Goppelt-Struebe, Dirk Wolter and Klaus Resch, Medical School, D-3000 Hannover 61, West Germany

Prostanoid synthesis was induced in bone marrow-derived macrophages by various stimuli of phospholipase A2, such as phorbolester (TPA), ionophore or melittin When the cells were preinucbated with dexamethasone over night, prostaglandin synthesis was nearly completely inhibited. Phospholipase A2 activity was measured using labelled phospholipids as substrates. The enzyme activity was only moderately inhibited in dexamethasone treated macrophages compared to control cells. Neither the distribution of radio labelled arachidonic acid among the different phospholipid species nor the release of arachidonic acid from prelabelled cells were significantly impaired by pretreatment of the macrophages with dexamethasone. Thus inhibition of phospholipase A2 could not be considered to be the most prominent effect of dexamethasone on arachidonic acid metabolism.

Prostaglandin synthesis was also inhibited by dexamethasone, when it was induced by incubation of the macrophages with excess exogenous arachidonic acid. Therefore the enzyme activity of the cyclooxygenase/PGE isomerase was measured It was markedly inhibited in dexamethasone-treated cells compared to control cells. Thus glucocorticoids inhibit no only phospholipase A2, but interfere more specifically with arachidonic acid metabolism.

S 317 ALTERATED REGULATION OF PHOSPHOLIPASE A_2 (PLA_2) DURING DIFFERENTIATION OF THE HISTIOCYTIC LYMPHOMA CELL-LINE U937

R. Hass, L. Köhler, M. Goppelt-Strübe and K. Resch
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By treatment with 5 x 10^{-9}M 12-O-tetradecanoylphorbol-13-acetate (TPA) the human histiocytic lymphoma cell-line U937 can be induced to differentiate morphologically and functionally into macrophage-like cells. These changes are associated to differences in the lipid pattern after differentiation. Thus, an increased amount of cholesterol, unsaturated fatty acids and glycolipids as well as changes in the phospholipids were found in TPA-treated U937 as detected by thin-layer-chromatography. Of the enzymes involved in the deacylation-reacylation cycle of phospholipids an 2-fold increased activity of PLA_2 was observed in TPA-differentiated U937 cells, whereas the corresponding enzyme lysophospholipid-acyltransferase (LAT) was unaffected. PLA_2 was measured by incubating membranes of the cells with ^{14}C-arachidonoyl-phophatiditylcholine and by measuring the release of ^{14}C-arachidonic acid labeled cells. Interestingly, the increased PLA_2-activity in TPA-treated cells could be inhibited to 20% by preincubation of the cells with 10^{-6}M dexamethasone, whereas the PLA_2-activity in undifferentiated U937 was unaffected. These data suggest that PLA_2 is induced after this differentiation process and that the induction of PLA_2-activity is accompanied by an alterated regulation of PLA_2-inhibitory proteins which are induced by glucocorticoids.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 318 STIMULATION OF MONO- AND DIACYLGLYCEROL LIPASES BY BRADYKININ IN NEURONAL CELL CULTURES OBTAINED FROM MOUSE SPINAL CORD, Akhlaq A. Farooqui, Cheryl J. Flynn, Douglas Anderson*, Eugene Means* and Lloyd A. Horrocks, Ohio State University, Columbus, OH 43210, *VA Hospital, Cincinnati, OH 45220.

Mono- and diacylglycerol lipases hydrolyze the primary esters of mono- and diacylglycerols. These enzymes have attracted considerable attention in recent years because diacylglycerols regulate activities of many enzymes including protein kinase C which may be involved in cell division, differentiation, and signal transduction. Neuronal cultures of fetal mouse spinal cord (FMSC) and mouse neuroblastoma (N1E-115) display measurable activities of mono- and diacylglycerol lipases. Treatment of FMSC and N1E-115 cultures with bradykinin (10 nM) for 1-4 min resulted in a marked increase in activities of mono- and diacylglycerol lipases. Increase in activities of lipases was dose and time dependent. Direct incubation of bradykinin (up to 1 μM) with purified preparations of diacylglycerol lipase had no effect on enzyme activity. The stimulation of mono- and diacylglycerol lipases by bradykinin suggests that differences in basal and stimulated enzymic activities may be a useful model for studying differential regulatory mechanisms. Immunocytochemical studies of FMSC with a polyclonal antibody to purified bovine diacylglycerol lipase show intense staining of neuronal plasma membranes. Thus this enzyme is localized near the diacylglycerols released from polyphosphoinositides and is stimulated after the stimulation of phospholipase C activity. The lipases may be involved in the production of additional messengers synthesized from the released arachidonic acid.

Supported in part by NIH Research Grant NS-10165 and Training Grant NS-07091.

S 319 EVIDENCE THAT AMINOGLYCOSIDE INHIBITION OF LYSOSOMAL PHOSPHOLIPASE A OCCURS BY SUBSTRATE DEPLETION. Karl Y. Hostetler and Elisabeth J. Jellison. University of California, San Diego and the VA Medical Center, San Diego, CA. 92161

Aminoglycoside antibiotics are widely used in clinical medicine to treat infections with Gram-negative organisms. A major toxic side effect is damage to kidney proximal tubule cells where aminoglycosides accumulate and cause lysosomal phospholipid accumulation and other effects. To investigate their effects on lysosomal phospholipase A, we isolated and purified this enzyme from rat kidney. Highly purified phospholipase A (PLA) was used to assess the effects of gentamicin (GM) and tobramycin (TM) on the hydrolysis of sonicated liposomes of di[1-^{14}C]oleoylphosphatidylcholine (DOPC) alone or [^{14}C]DOPC plus unlabeled phosphatidylinositol (PI). Kidney lysosomal phospholipase A did not degrade PI in liposomes and catalyzed only the hydrolysis of [^{14}C]DOPC. GM and TM were weak inhibitors of PLA action with [^{14}C]DOPC as substrate (IC_{50} > 10 mM). However, when unlabeled PI was present in the liposome at 40 mole percent, GM and TM caused 50 percent inhibition at 0.03 or 0.01 mM, respectively. We have shown previously that GM binds to PI-containing liposomes but not to DOPC liposomes. When fixed concentrations of GM and TM were used and the substrate concentration increased, data were obtained which are consistent with GM and TM binding to PI at the liposome surface, interfering with PLA hydrolysis of [^{14}C]DOPC. This type of effect has been noted earlier by E.A. Dennis and coworkers in lipocortin inhibition of phospholipase A_2 which appears to be due to binding of lipocortin to the substrate resulting in a substrate depletion type of inhibition.

S 320 DIFFERENTIAL EFFECT OF DEXAMETHASONE ON PROSTANOID SYNTHESIS IN UNDIFFERENTIATED AND PHORBOLESTER-DIFFERENTIATED U937 CELLS

L. Köhler, R. Hass, M. Goppelt-Strübe, V. Kaever and K. Resch, Medical School, Hannover, FRG

The human histiocytic cell line U937 can be induced to differentiate into macrophage-like cells within 3 days by $5x10^{-9}$ M 12-O-tetradecanoylphorbol-13-acetate(TPA). Undifferentiated U937 cells secreted small amounts of prostanoids when challanged with arachidonic acid. In contrast TPA-differentiated U937 cells had a 20-fold increased capacity to produce prostanoids. Dexamethasone reduced prostanoid secretion in TPA-differentiated cells but not in undifferentiated U937 cells in the range of 10^{-6} M to 10^{-8} M. In TPA-differentiated cells the production of PGE_2, $PGF_{2\alpha}$ and TxB_2 were reduced by approximately 50% at 10^{-6} M dexamethasone, whilst prostaglandin release by undifferentiated cells was unaffected. In order to get further insight into the molecular mechanism of the observed differences we examined the enzymes involved in regulating the availability of the precursor fatty acid arachidonic acid for prostanoid production. The enzyme activities were measured in membranes of cells pretreated with 10^{-6} M dexamethasone overnight. Phospholipase A_2 activity was inhibited in TPA-differentiated cells in contrast to the undifferentiated cells. Lysolecithine-acyltranferase activity remained unchanged both in undifferentiated and differentiated cells. During differentiation to macrophage-like cells U937 thus became sensitive towards dexamethasone with respect to prostaglandin synthesis. As the inhibition of the prostaglandin synthesis appears to be caused by an inhibition of phospholipase A_2 activity the data suggest that differentiated cells aquire the capacity to synthesize phospholipase inhibiting proteins in response to dexamethasone, such as lipocortin.

S 321 POSSIBLE INVOLVEMENT OF Na^+/H^+-EXCHANGE IN A23187-STIMULATED ARACHIDONIC ACID (AA) AND HISTAMINE (Hi) RELEASE FROM RAT PERITONEAL MAST CELLS (RPMC).

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RPMC, preincubated with 14C-AA, released 14C-AA and Hi in the presence of the calcium ionophore A23187 in a calcium containing buffer. Suppression of Na^+/H^+-exchange by removal of extracellular Na decreased A23187-(2.0 µM)stimulated 14C-AA- and Hi-release from RPMC by 53% and 52% resp. In the presence of 1.0 mM amiloride 14C-AA- and Hi-release were inhibited by 49% and 72%, resp., in A23187-(0.5 µM)stimulated cells. A23187-(0.5 µM)stimulated 14C-AA- and Hi-release were decreased by 20% and 31% resp. by the protein kinase C-(PKC)inhibitor H-7 (100 µM), and by 23% and 20% resp. by the PKC-inhibitor sphingosine (1.0 µM). The results suggest that an activation of Na^+/H^+-exchange is involved in the pathway leading to Hi-release and phospholipase A_2-mediated AA-release in mast cells by A23187. A part of the A23187-stimulated release seems to be mediated via PKC-activation. The results further indicate that there is a PKC-independent mechanism for the stimlation of the Na^+/H^+-exchanger in RPMC that may involve calcium.

S 322 PHOSPHOLIPID METABOLISM IN DIPLOID AND POLYPLOID SMOOTH MUSCLE CELLS AND IN TRANSFORMED FIBROBLASTS

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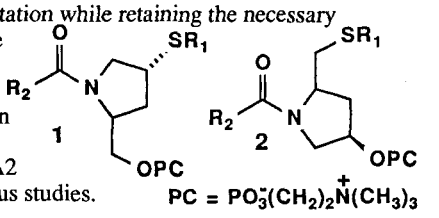
The actions of a variety of hormones and growth factors are mediated through the turnover of phosphatidylinositides. Transformation of 3T3 fibroblasts with the ras or sis oncogenes increases turnover of these regulatory phospholipids. We have found that induction of the cyclic AMP dependent protein kinase can reverse the transformation of 3T3 cells by the ras or sis oncogenes. Upon reverse transformation there is an immediate inhibition of inositol phospholipid turnover. Activation of protein kinase C synergistically enhances this effect. Such changes may be significant for cell growth in other proliferative disorders. Smooth muscle cell (SMC) growth is important in the pathogenesis of atherosclerosis and hypertension. Abnormal SMC proliferation is often accompanied by a transition from a diploid to a polyploid state. The cellular homologue of the sis oncogene, PDGF, may function in such proliferation. We have developed diploid and tetraploid cell lines from rat aortic smooth muscle which differ in their morphology and growth. The behavior of the phosphatidylinositol pathway in these cell lines should be valuable for understanding the role of cell ploidy in pathological smooth muscle cell proliferation.

S 323 ***DESIGN AND SYNTHESIS OF CONFORMATIONALLY RESTRICTED PHOSPHOLIPIDS AS PHOSPHOLIPASE A2 INHIBITORS.*** RONALD L. MAGOLDA and WILLIAM GALBRAITH; Central Research and Development Department and Medical Products Department, E. I. du Pont de Nemours & Co., Experimental Station, Wilmington, DE 19898.

Phospholipase A2 (PLA2) is an esterase that releases arachidonic acid, a putative mediator of inflammation, from membrane phospholipids. As part of our ongoing program to define the interaction between the substrate phosphatidylcholine and the active site of PLA2, we have prepared the conformationally restricted phopholipids **1** and **2**. Both **1** and **2** constrain the two lipid side chains into an anti (**1**) verses a syn (**2**) orientation while retaining the necessary phosphatidylcholine components. The synthetic route from 4-hydroxy proline and the inhibition of porcine pancreatic PLA2 for **1** and **2** will be described. Based upon the significantly different PLA2 inhibition profiles of **1** verses **2** along with the aid of computer molecular modeling, we will present a model for PLA2 substrate binding consistent with these and our previous studies.



Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 324 ARACHIDONIC ACID METABOLISM IN HUMAN NEUTROPHILS STIMULATED WITH A23187 AND FMLP. Vhundi F. Mahadevappa and William S. Powell*, Department of Nutritional Sciences, University of Guelph, Guelph, Ont., Canada N1G 2W1 and *Royal Victoria Hospital, McGill University, Montreal, P.Q., Canada H3A 1A1.

In this study, we have investigated the effect of FMLP on the metabolism of endogenous and exogenous arachidonic acid (AA) in human neutrophils. Furthermore, we also studied the effect of exogenous eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on AA metabolism that resulted in response to stimulation by A23187 and FMLP. Our results on endogenous AA release in the presence of BW755C (a dual inhibitor of cyclooxygenase and lipoxygenase) and its subsequent metabolism to eicosanoids (in the absence of BW755C) in neutrophils stimulated with A23187 and FMLP indicate that FMLP is relatively a very weak stimulus. In other words, we found very little stimulation of endogenous phospholipases in the presence of FMLP as monitored by endogenous AA release and eicosanoids formed in response to FMLP. However, exogenous AA was rapidly converted to leukotrienes and their isomers in the presence of FMLP although there were some differences between A23187 and FMLP. Furthermore, a significant conversion of exogenous AA to eicosanoids through 5-lipoxygenase occurred in response to FMLP even in the presence of exogenous EPA and DHA. In conclusion, our results support that FMLP stimulates the metabolism of AA although it does not cause a significant release of AA from endogenous sources through phospholipases. (Supported by the Natural Sciences and Engineering Research Council of Canada)

S 325 Phospholipase A_2-induced Mouse Paw Edema as a Model to Evaluate Anti-inflammatory Agents. L.A. Marshall, W. Calhoun, J. Yu, J. Chang, and R. P.Carlson. Wyeth-Ayerst Research, Princeton, NJ 0843

Phospholipase A_2 (PLA_2) is a key component of the inflammatory process due to its role in the generation of eicosanoids and platelet activating factor (PAF). Manipulation of PLA_2 activity offers a novel therapeutic approach for the development of anti-inflammatory agents. To define the effects of PLA_2 inhibitors in animals, there is a need for a suitable in vivo model. Injection of snake venom PLA_2 (Naja mocambique mocambique; $SVPLA_2$) into the mouse hind foot pad initiated a significant rise in paw edema within 10^2 min. (0.095 ml; 10 ug PLA_2/50 µl) compared to the phosphate buffer control (0.03 ml). This edema was dependent on enzyme concentration and was specific for PLA_2 since it was negated by enzyme pretreatment with p-bromophenacylbromide. Moreover, injection of other proteins does not result in edema, e.g. bovine serum albumin. When PLA_2 from different sources were compared, human synovial PLA_2 was more inflammatory than $SVPLA_2$ or cell-free platelet PLA_2. Oral administration of ibuprofen (50 mg/kg), indomethacin (10 mg/kg), cyproheptadine (4 mg/kg), BW 755c (50 mg/kg), or Rev 5901 (100 mg/kg) inhibited PLA_2-induced edema suggesting that inflammation induced by PLA_2 is a multi-mediator event.

S 326 p68: A NOVEL MEMBER OF THE LIPOCORTIN FAMILY, Stephen E. Moss, Mark R. Crompton and Michael J. Crumpton, Imperial Cancer Research Fund, Lincoln's Inn Fields, London, WC2A 3PX, U.K.

Interest in the lipocortin family of proteins, which includes protein II, and the tyrosine kinase substrates p35 (lipocortin I) and p36 (lipocortin II), has led to the rapid analysis of their primary structures. Many observers have reported another protein (p68) with similar calcium and phospholipid-binding properties which appears as a polypeptide doublet on SDS-PAGE. We now report the primary cDNA and derived amino-acid structures of murine and human p68, and show that it too belongs to the same gene family as protein II, p35 and p36. Murine and human p68 share 95% cDNA and amino acid sequence homology, and approximately 50% homology with the other proteins in this family. The protein sequence which may be divided into two halves (with 50% homology to each other), each contained four serially-repeating units, which consisted of a variable N-terminal sequence and a highly conserved core consensus sequence which fitted that described for protein II, p35 and p36. The results show that p68 is a new member of the calcium-regulated lipocortin family of proteins.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 327 GLUTAMATE RECEPTORS AND PI METABOLISM: STIMULATION VIA QUISQUALATE RECEPTORS IS INHIBITED BY NMDA RECEPTOR ACTIVATION. Elizabeth Palmer, Daniel T. Monaghan and Carl W. Cotman, Univ. of California, Irvine, CA 92717. Excitatory amino acid receptors have been shown to be coupled to phosphatidylinositol-4,5-diphosphate (PI) metabolism in rat brain. To elucidate the role of the different classes of glutamate receptors (quisqualic acid [QA], N-methyl-D-aspartate [NMDA], and kainic acid [KA]) in PI metabolism, the effect of various glutamate agonists and antagonists on PI turnover was studied in hippocampal slices from neonatal rats. Based on the following observations, we now suggest that glutamate has two opposite and simultaneous actions on PI metabolism. Glutamate acting at a QA receptor enhances PI turnover while glutamate acting at the NMDA receptor inhibits PI metabolism. QA (10 μM) stimulates PI turnover and NMDA (100 μM) inhibits this stimulation. The modulation of QA stimulation by NMDA is dependent on extracellular calcium and may be reversed by NMDA antagonists (3-((±)2-carboxypiperazin-4-yl)propyl-1-phosphonic acid [CPP] and D-2-amino-5-phosphopentanoate [D-AP5]). NMDA antagonists enhance glutamate stimulation of PI turnover, consistant with the observation that glutamate activates both QA and NMDA receptors. Since most hippocampal glutamate using pathways appear to use both QA and NMDA receptors these results suggest that PI metabolism may be differentially regulated by physiological conditions (e.g. changes in frequency of stimulation) which would either prefer QA receptor activation (stimulating PI turnover) and/or NMDA receptor activation (inhibiting PI turnover).

S 328 MOLECULAR CLONING, SEQUENCING, AND EXPRESSION OF MOUSE LIPOCORTIN I IN SWISS 3T3 FIBROBLASTS. Christine Philipps, Stefan Rose-John, Gabriele Rincke, Gerhard Fürstenberger, Friedrich Marks, Dept. of Biochemistry, German Cancer Research Center, P101949, 6900 Heidelberg, FRG.
Using synthetic oligonucleotides designed after the published human lipocortin I sequence, we isolated two positive clones (3.0 and 2.5 kb) from a λgt10 cDNA library. Sequencing of the 3.0 kb cDNA insert revealed strong homology with human lipocortin I. When confluent layers of Swiss 3T3 fibroblasts were stimulated with 0.1 μM glucocorticoid together with 10% serum, expression of lipocortin I was strongly stimulated. In parallel, DNA synthesis was induced with a peak at 24 hrs after glucocorticoid treatment; without serum we showed neither induction of DNA synthesis nor expression of lipocortin I. From our data we conclude that fetal calf serum contains an unidentified factor which acts synergistically with glucocorticoids on cell proliferation and lipocortin I expression.

S 329 EFFECT OF CHLORPROMAZINE ON PULMONARY Mg^{2+}-DEPENDENT PHOSPHATIDATE PHOSPHOHYDROLASE. Fred Possmayer and Paul A. Walton, U. of Western Ontario, London, Canada N6A 5A5. Chlorpromazine and other amphilphilic cationic drugs redirect the biosynthesis of phospholipids from triacylglycerol and phosphatidylcholine towards phosphatidylinositol. It has been suggested that, in addition to inhibiting phosphatidate phosphohydrolase, chlorpromazine can replace the Mg^{2+} requirement of this enzyme from rat liver (Sturton, R.G. and Brindley, D.N.; Biochim. Biophys. Acta 619:494-505, 1980). Rat lung contains both Mg^{2+}-dependent and Mg^{2+}-independent phosphatidate phosphohydrolase activities. Addition of 1.0 mM chlorpromazine led to a 6-fold stimulation of the Mg^{2+}-independent activity but a 30% decrease in the Mg^{2+}-dependent activity of rat lung microsomes. Incubating the microsomes at 55°C for 15 min abolished the Mg^{2+}-dependent activity but had only a slight inhibitory effect on the Mg^{2+}-independent activity. Chlorpromazine was equally effective in stimulating phosphatidate phosphohydrolase in the heat-treated microsomes, suggesting a selective stimulation of the Mg^{2+}-independent activity. Other experiments revealed that the drug abolished the Mg^{2+}-dependent phosphohydrolase activity in rat lung cytosol and diminished the Mg^{2+}-independent activity by half. Heat treatment abolished the Mg^{2+}-dependent activity. The remaining Mg^{2+}-independent phosphohydrolase activity in heat-treated cytosol was not affected by chlorpromazine. These results demonstrate that chlorpromazine inhibits Mg^{2+}-dependent phosphatidate phosphohydrolase activity in rat lung microsomes and cytosol and selectively stimulates the Mg^{2+}-independent activity in the microsomes. The results are consistent with the view that rat lung microsomes and cytosol contain a phosphatidate phosphohydrolase activity which exhibits an absolute dependency on Mg^{2+}.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 330 PHOSPHOLIPASE A_2 INHIBITION AND MODIFICATION BY MANOALIDE ANALOGUES.
L.J. Reynolds, B.P. Morgan, G.A. Hite, E.D. Mihelich and E.A. Dennis. Dept. of Chemistry, University of California at San Diego, La Jolla, CA 92093 and Lilly Research Laboaratories, Indianapolis, IN 46285.
Manoalide, an anti-inflammatory agent isolated from the sponge *Luffariella variabilis*, has been shown to inactivate cobra venom phospholipase A_2 (PLA_2) along with the modification of about four lysine residues (Lombardo and Dennis, (1985), *J. Biol. Chem. 260,* 7234-7240). We have synthesized several manoalide analogs and studied their ability to inactivate PLA_2 (Deems et al. (1987) *Biochim. Biophys. Acta. 917* 258-268). One of these compounds, (E,E)-2-[3-(2,5-dihydro-2-hydroxy-5-oxo-3-furanyl)propylidene]-6,10-dimethyl-5,9-undecadienal, which has been named "manoalogue", shows a partial, irreversible inactivation of cobra venom phospholipase A_2 concomitant with a modification of about three lysine residues. The concentration of manoalogue required for half-maximal inhibition is very close to that of manoalide. Manoalogue contains the terminal butenolide ring of manoalide as well as the α, β-unsaturated aldehyde which is present latently in manoalide in the hemiacetal ring. Either reduction of the aldehyde to an alcohol or methylation of the hydroxyl group on the butenolide ring abolished the molecule's ability to irreversibly inhibit phospholipase A_2 and to modify lysine residues. These results demonstrate that the presence of the α, β-unsaturated aldehyde and the opening of the lactone on the butenolide ring are necessary for inactivation to occur. N-terminal sequence analysis of the manoalogue-inhibited protein suggests that Lys-6 is one of the modified residues. A possible mechanism of inactivation of cobra venom phospholipase A_2 by manoalide and manoalogue consistent with these findings is proposed.

S 331 MOLECULAR MODELING IN THE DESIGN OF PHOSPHOLIPASE A2 INHIBITORS,
W. C. Ripka, W.J. Sipio, and W. G. Galbraith, Medical Products Department, E. I. du Pont de Nemours & Co., Wilmington, DE 19898.
The X-ray structures of pancreatic bovine and porcine phospholipase A2 (PLA2) have been used along with interactive computer graphics to design conformationally rigid, novel compounds (1-meta-hydroxybenzyl-2-substituted acenaphthenes) directed at the active sites of these enzymes. *In vitro* testing confirmed the designed compounds are potent inhibitors (10^{-7} M) of porcine PLA2 and exhibit both *stereoselectivity* and structure-activity relationships that are consistent with the proposed mode of binding. These compounds take advantage of a hydrophobic 'slot' positioned between residues Leu-2 and Tyr-69 while positioning hydrogen-bonding functionality directed at the - N of His-48. Experimental evidence shows a clear regioselective preference for this H-bond acceptor with a value for the interaction of 1.5 kcal mol-1. A second part of our strategy called for a tethered amine to displace the essential calcium of PLA2 providing a 'bisubstrate analog'.

S 332 A 32 kDa PROTEIN LIPOCORTIN ISOLATED FROM HUMAN BLOOD MONONUCLEAR CELLS IS A CALCIUM AND PHOSPHOLIPID BINDING INHIBITOR OF PHOSPHOLIPASES A_2,
B.Rothhut, C.Comera, B.Prieur, M.Errasfa, L.Jordan, F.Russo-Marie, Institut Pasteur/U285, Paris, France, F.Lederer Hôpital Necker, Paris, France, and A.J.Aarsman, G.Mynbeek, H.Van Den Bosch, Biochemistry Laboratory, Padualaan 8, 3584 CH Utrecht, The Netherlands.
Lipocortins are a family of antiinflammatory proteins acting by inhibiting the activity of phospholipase A_2 and thus preventing the formation of inflammatory mediators. Among this group of calcium, phospholipid and actin binding proteins, we isolated a 32-35 kDa species based on the capacity to inhibit phospholipases A_2 from pig pancreas and Naja Naja. The protein was inhibiting the enzymes in vitro in a dose dependent manner due to the binding on negatively charged phospholipids. The protein was also inhibiting arachidonic acid release from intact cells suggesting that the protein interacted with the membrane phospholipids leading to an inhibition of the enzyme. These results were reinforced by the inhibitory effect on two purified intracellular phospholipases A_2 from rat platelets and rat liver mitochondria. Finally the protein was recognized by a monoclonal antibody raised against a lipocortin from rat renal cells and therefore we propose to name this protein lipocortin V. The sequence of this protein is the same as an inhibitor of blood coagulation (IBC) which needs calcium and phospholipid binding to be active. These results raise the question of the role and specificity of action for these proteins. Nevertheless it is possible that activated cells exhibit a different phospholipid pattern on their surface (as in platelets), increasing the lipocortin interaction with the membrane. This could be a control mecanism of the cellular response.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 333 INFLAMMATORY MEDIATORS STIMULATE THE PHOSPHOINOSITIDE PATHWAY IN PRIMARY HUMAN KERATINOCYTES AND A431 CELLS. L.A. Wheeler, R.K. Gary, D.D. Goodrum, N.N. Horowitz and G. Sachs. Discovery Research, Allergan, Inc./Herbert Labs, Irvine, CA.

Psoriasis is an inflammatory benign hyperplasia of the epidermis. Many inflammatory mediators have been detected in lesioned skin: Histamine (H), Leukotrienes (B_4, C_4, D_4), 12-HETE, Platelet activating factor (PAF), Prostaglandin (PGE_2) and Bradykinin (BK). We tested whether these mediators cause Ca^{2+} mobilization and phosphoinositide (PI) turnover in keratinocytes. Neonatal human foreskins were incubated in trysin-EDTA overnight, after which epidermis was separated. Single cell suspensions of human keratinocytes (HK) (10^6 cells/ml) were incubated with 1μM fura-2-AM for 15 min at 37°C to measure free cytosolic Ca^{2+} ($[Ca^{2+}]_i$). For PI turnover experiments KC were incubated with 10μCi/ml ^{3}H-myo-inositol for 3 hrs at 37°C. H (10^{-5}-10^{-4}M, LTB_4 (10^{-9}-10^{-6}M), 12-HETE (1μM), PAF (1μM) and BK (10^{-9}-10^{-6}M) but not PGE_2, LTD_4 and LTC_4 stimulated Ca^{2+} mobilization. BK and LTB_4 induced rapid transient rises in $[Ca^{2+}]_i$ from a baseline of 122 ± 14nM that decayed to baseline in 2-3 min and were independent of medium Ca^{2+}. BK also resulted in a rapid transient stimulation of inositol triphosphate (150%) and inositol tetraphosphate (160%) that peaked at 15-30 sec and had largely subsided at 60 sec. Similar results were observed in A431 cells. Pretreatment of HK with 1μM manoalide (a novel anti-inflammatory compound) for 5 min completely inhibited changes in $[Ca^{2+}]_i$. These results suggest that inflammatory mediators may stimulate the growth of HKC by their effects on PI turnover and Ca^{2+} mobilization. The data provide a molecular link between inflammation and epidermal hyperplasia. Compounds that block this response may be useful drugs in psoriasis.

S 334 TWO GROWTH FACTOR-INITIATED PATHWAYS OF PHOSPHOLIPASE C ACTIVATION, Terukatsu Sasaki and Hiroko Sasaki, Sapporo Medical College, Sapporo 060, Japan. The mode of phospholipase C activation by platelet-derived growth factor (PDGF) has been studied in comparison with that by vasopressin and bombesin in a rat fibroblast line, WFB. On stimulation of WFB cells with PDGF, there was a lag period of about 10 s before an increase in $[Ca^{2+}]i$. No measurable lag period was observed in the $[Ca^{2+}]i$ response induced by vasopressin or bombesin. Pretreatment of WFB cells with phorbol ester(PMA) profoundly inhibited the vasopressin-, and bombesin-induced inositol phosphate formation, but enhanced to some extent the PDGF-induced inositol phosphate formation. In membranes prepared from WFB cells, GTP markedly augmented inositol polyphosphate formation induced by vasopressin and bombesin. The effects of guanine nucleotides and their analogs on the polyphosphoinositide hydrolysis stimulated with growth factors were studied in WFB cells made permeable to nucleotides by treatment with either saponin or pseudomonal cytotoxin. GDPβS markedly reduced the bombesin- and vasopressin-stimulated production of inositol phosphates. However, the PDGF-stimulated production of inositol phosphates was not affected by the addition of GDPβS. $GTP\gamma S$ enhanced largely the vasopressin- and bombesin-stimulated hydrolysis of inositol lipids. In the presence of 100 μM $GTP\gamma S$, the PDGF-stimulated hydrolysis of inositol lipids was reduced to about half of the control level. These effects were found specific to $GTP\gamma S$: no other nucleoside triphosphates was effective. These results indicate that a G-protein couples the vasopressin- and bombesin-receptors to the activation of phospholipase C. These results also suggest that coupling of the PDGF-receptor to phospholipase C is not mediated through a G-protein.

S 335 ACCUMULATION OF DIACYLGLYCEROL PRECEDES ASBESTOS-INDUCED CELLULAR PROLIFERATION, Ann Sesko, Myles C. Cabot*, and Brooke T.Mossman, Univ. of Vermont, Burlington, VT 05405 and W. Alton Jones Cell Science Center, Lake Placid, NY 12946*. Asbestos, a putative tumor promoter in the development of bronchogenic carcinoma, induces abnormal differentiation and proliferation of epithelial cells of the respiratory tract. Certain proliferative effects of asbestos on cultured cells appear to be mediated, at least in part, by protein kinase C (J. Marsh and B.T. Mossman, Cancer Res.,in press). This study was performed to determine whether the stimulation of cellular growth by asbestos is directed initially through perturbations in lipid metabolism that result in the formation of diacylglycerols (DG), endogenous activators of protein kinase C. Hamster tracheal epithelial (HTE) cells (progenitor cells of bronchogenic carcinoma) were labelled with [3H]-glycerol and subsequently stimulated with either crocidolite asbestos, 12-O-tetradecanoylphorbol-13-acetate (TPA), or phospholipase C (B.cereus) for various periods of time. Results revealed that a significant increase in radioactivity occurred in the DG fraction of cells exposed for 2 hr to concentrations of asbestos causing proliferative alterations. Increased label also was noted in mono- (MG) and triacylglycerol (TG) fractions upon exposure to asbestos, suggesting a generalized perturbation in lipid metabolism. As expected, HTE cells exposed to TPA did not demonstrate an increase in DG, but did express an accumulation of TG. We conclude that asbestos stimulates phospholipases leading to the formation of DG whereas TPA mimics DG and activates protein kinase C directly. Thus, both fibrous (asbestos) and soluble (TPA) tumor promoters may activate protein kinase C albeit by differing mechanisms. Supported by NCI #R01CA33501 and #T32CA0928607.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 336 ACTIVATION OF PHOSPHOLIPASE A2 BY PLATELET ACTIVATING FACTOR IN VITRO
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Dept. of Medical Chemistry, University of Helsinki, SF-00170 Helsinki, Finland

The interactions of synthetic platelet activating factor (PAF) with phosphatidylcholine (PC) and phosphatidylglycerol (PG) were studied using monolayer and fluorescent techniques. In addition, the effects of PAF on phospholipase A2 (PLA2) reaction were investigated using a fluorometric assay. Monolayer experiments revealed that PAF could form stable monolayers at an air-water interface. The isotherms on PAF were continuous and expanded, with a lift-off point at 150 A^2/molecule. A collapse pressure of 34.8 mN m^{-1} was observed. PAF could penetrate into PC and PG monolayers up to the critical surface pressure of 30 mN m^{-1}. When increasing amounts of PAF was added to 1-palmitoyl-2-pyrenehexanoyl-sn-glycero-3-phosphocholine (PPHPC) liposomes a decrease in pyrene excimer/monomer emission ratio (Ie/Im) was evident. When PAF was added to PPHPG liposomes a maximum in pyrene Ie/Im was seen at 2:1 molar ratio of PG to PAF. At the same PG/PAF molar ratio the hydrolysis of PG by pancreatic PLA2 was enhanced 4.5-fold at a low $CaCl_2$ concentration. When 4 mM $CaCl_2$ was included PAF did not have any effect on the hydrolysis of PPHPG by PLA2. Regardless of $CaCl_2$ concentration PAF inhibited the hydrolysis of PC. The reason (s) for the activation of PLA2 is(are) at present unknown. Yet, the studies on the interaction of PAF with PG suggest that PAF may induce changes in the physicochemical state of PG which result in an enchanced PLA2 activity towards this lipid. PAF activated the hydrolysis of pyrene PS and -PI by PLA2 1.7 and 5.7-fold, respectively.

S 337 **EFFECT OF CYCLOOXYGENASE INHIBITORS ON ANION ANTIPORT.**
Tor Inge Tønnessen, Kirsten Sandvig and Sjur Olsnes. Dept. of Biochemistry at the Norwegian Radium Hospital, Oslo, Norway.
Vero cells regulate their pH_i by at least three antiports viz. Na^+/H^+ antiport and Na^+-dependent anion antiport (probably as $NaCO_3^-/Cl^-$ exchange) to normalize pH_i after acidification of the cytosol, and Na^+-independent Cl^-/HCO_3^- exchange to regulate pH_i back to normal after alkalinization of the cytosol. The latter antiport is strongly regulated by the internal pH. It is in a high activity state at alkaline pH_i, but turns to a low activity state when the pH_i < 7.0. The aspirin-like drugs (cyclooxygenase inhibitors) inhibit anion transport probably by binding extracellularly to the anion antiporters. In addition to this, the drugs gain access to the cytosol and interact with the regulation of the activity of the antiporters. Thus, the Na^+-independent antiport which in the absence of drugs is in a state of low activity when the internal pH is below neutrality, is kept in a state of higher activity in the presence of drugs. The Na^+-linked antiport which is more active at acidic pH_i, is inhibited by the presence of drugs intracellularly. The net effect of the drugs on the antiports results in the lowering of pH_i.

S 338 **THE PRESENCE OF LIPOCORTIN IN PLATELETS.** L. Touqui, B. Rothhut, A.M. Shaw, A., B.B. Vargaftig and F. Russo-Marie. Unité Associée Pasteur/INSERM n° 285; Institut Pasteur, 28, rue Dr. Roux, 75015 Paris.// The activation of platelets leads to phosphorylation of various proteins among whose a 40 K protein, substrate of protein kinase C (PKC). The phosphorylation of the 40 K protein is associated with numerous platelet responses, such as phospholipase A_2 (PLA_2) activation. The existence in various mammalian cells of PLA_2 inhibitory proteins (lipocortins, LC) has been reported. Western blot experiments using anti-LC antibodies showed that both rabbit and human platelets contain 2 proteins related to LC, LC I (38 Kd) and LC V (32 Kd) exhibiting anti-PLA_2 activities. Incubation of platelets with increasing concentrations of PMA (a specific activator of PKC) leads to a dose-dependent loss in the anti-PLA_2 activity of LC I concommitantly to its phosphorylation. However, no phosphorylation has been observed with LC V. Treatment of platelet LC I by alkaline phosphatase restaured its ability to inhibit PLA_2. The PMA-dependent loss of LC I activity was parallel to the release of arachidonate by the combination of PMA and the calcium ionophore A23187. In addition, recent studies reported a protein similar to LC V in placenta. This protein exhibited an anti-coagulant activity which has been attributed to its ability to bind phospholipids (PL). In conclusion, we suggest that LC I and LC V found in platelets may have a potential role in the control of platelet activation and probably in the coagulation process. The mechanisms of interaction between LC, PLA_2 and PL in platelets is yet under investigation.

S 339 USE OF SHORT CHAIN CYCLOPENTANO-PHOSPHATIDYLCHOLINES TO PROBE THE MODE OF ACTIVATION OF PHOSPHOLIPASE A_2 FROM BOVINE PANCREAS AND BEE VENOM, M.-D. Tsai, G. Lin[1], J. Noel[1], W. Loffredo[1], and H. Sable[2], [1] Department of Chemistry, The Ohio State University, Columbus, OH 43210, [2] Department of Biochemistry, Case Western Reserve University, Cleveland, OH 44106.

A great mystery in the mechanism of phospholipase A_2 (PLA2) and many other lipolytic enzymes is the "interfacial activation" induced by micellar but not monomeric substrates. Equally mysterious is the lack of interfacial activation in bee venom PLA2, as opposed to PLA2s from pancreas and other sources. We have probed these problems using short-chain cyclopentano-analogues of diacylphosphatidylcholine (Cp-DC_nPC, all-trans isomer). In the reaction catalyzed by bovine pancreatic PLA2, Cp-DC_8PC behaved differently from DC_8PC in that its monomers and micelles showed comparable activities (but lower than the activity of DC_8PC). This result supports the substrate conformation model [Wells, M. A. (1974) Biochemistry 13, 2248], but raises a question as to whether Cp-DC_8PC mimics monomers or micelles of DC_8PC. Conformational analysis by ^1H NMR revealed that monomeric Cp-DC_8PC was conformationally restricted near the carbonyl region, a property characteristic of micelles. The kinetic pattern of Cp-DC_8PC was similar to that of DC_8PC in the hydrolysis catalyzed by PLA2 from bee venom. This suggests that, unlike PLA2 from other sources, bee venom PLA2 is not sensitive to the conformation of monomeric and micellar substrates.

S 340 SYNTHESIS AND STEREOSPECIFICITY OF PHOSPHOROTHIOATE ANALOGUES OF PLATELET ACTIVATING FACTOR, M.-D. Tsai[1], T. Rosario-Jansen[1], R.-T. Jiang[1], and D. J. Hanahan[2], [1]Department of Chemistry, The Ohio State University, Columbus, OH 43210, [2]Department of Biochemistry, The University of Texas Health Center, San Antonio, TX 78284.

R_p and S_p isomers of 1-O-hexadecyl-2-acetyl-3-thiophosphocholine (AGEPsC) have been synthesized. The activity of these isomers in platelet aggregation and serotonin secretion was compared with that of 1-O-hexadecyl-2-acetyl-3-phosphocholine (AGEPC). The results show that (S_p)-AGEPsC have the same activity as AGEPC within experimental errors in both assays. The R_p isomer, however, is only 0.6-2 % as active as AGEPC in platelet aggregation and serotonin release. The results suggest that the phosphate group of AGEPC is likely to be involved in the interactions with its receptor, at least in the events leading to platelet aggregation and secretion.

S 341 SITES IN HUMAN LIPOCORTIN I THAT ARE PHOSPHORYLATED BY PROTEIN TYROSINE KINASES AND PROTEIN KINASES A AND C

Lyuba Varticovski*, Suresh B. Chahwala*, Malcolm Whitman*, Lewis Cantley*, E. Pingchang Chow+, Lesley K. Sinclair+ and R. Blake Pepinsky+; *Department of Physiology, Tufts University School of Medicine, 136 Harrison Ave, Boston MA 02111; +Biogen Research Corp., 14 Cambridge Center, Cambridge, MA 02142

Lipocortins are members of a large family of calcium and phospholypid-binding proteins. Lipocortin I is a 39 kDa membrane associated protein that in A431 cells is phosphorylated on tyrosine in response to epidermal growth factor (EGF). We have used purified recombinant human lipocortin I as an in vitro substrate for several protein kinases and identified phosphorylated residues by a combination of peptide mapping and sequence analysis. Lipocortin I was phosphorylated near the amino terminus at tyr-21 by recombinant $pp60^{c-src}$, polyoma middle t/$pp60^{c-src}$ complex and by EGF receptor/kinase in A431 cells. The primary site of phosphorylation by protein kinase C was also near the amino terminus at ser-27. The major site of phosphorylation by the catalytic subunit of cAMP dependent protein kinase (with micromolar Km) was on the carboxy terminal at thr-216. These sites will be compared to the phosphorylation sites previously located in the structurally related protein, lipocortin II.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 342 INHIBITION OF PHOSPHOLIPASE A_2 ACTIVITY IN HUMAN MONOCYTES BY GAMMA INTERFERON BLOCKS ENDOGENOUS PROSTAGLANDIN E_2 DEPENDENT COLLAGENASE PRODUCTION, Larry M. Wahl, Marta L. Corcoran and David S. Finbloom, NIDR, NIH, Bethesda, MD 20892 and WRAIR, Wash., DC 20307.

Previous studies from our laboratory have demonstrated that exposure of human monocytes (Mϕ) to concanavalin A (Con A) results in the production of the enzyme, collagenase, through a prostaglandin E_2 (PGE_2) dependent pathway. Since recombinant gamma interferon (rINF-γ) has been shown to modulate Mϕ prostaglandin synthesis, we examined the effect of rINF-γ on the activation sequence leading to collagenase production. The addition of rIFN-γ (10-1000 U/ml) to Con A stimulated Mϕ resulted in a dose dependent inhibition of PGE_2 and collagenase synthesis. When PGE_2 was added to rIFN-γ treated cultures collagenase production was restored. Moreover, the addition of phospholipase A_2 (PLA_2) reversed the inhibition of both PGE_2 and collagenase synthesis caused by rIFN-γ. These data suggested that rIFN-γ inhibited the release of arachidonic acid (AA) from phospholipids which would result in a decrease in the release of all AA metabolites. This was confirmed by HPLC analysis of Mϕ labeled with ^3H AA in which the release of both cyclooxygenase and lipoxygenase products were decreased by rIFN-γ. Measurement of Mϕ PLA_2 revealed that rIFN-γ significantly reduced the cellular levels of this enzyme. The findings presented here demonstrate that rIFN-γ inhibits PLA_2 in activated Mϕ and as a result blocks PGE_2 dependent collagenase synthesis.

S 343 INFLAMMATORY MEDIATORS STIMULATE THE PHOSPHOINOSITIDE PATHWAY IN PRIMARY HUMAN KERATINOCYTES AND A431 CELLS. L.A. Wheeler, R.K. Gary, D.D. Goodrum, N.N. Horowitz and G. Sachs. Discovery Research, Allergan, Inc./Herbert Labs, Irvine, CA.

Psoriasis is an inflammatory benign hyperplasia of the epidermis. Many inflammatory mediators have been detected in lesioned skin: Histamine (H), Leukotrienes (B_4, C_4, D_4), 12-HETE, Platelet activating factor (PAF), Prostaglandin (PGE_2) and Bradykinin (BK). We tested whether these mediators cause Ca^{2+} mobilization and phosphoinositide (PI) turnover in keratinocytes. Neonatal human foreskins were incubated in trysin-EDTA overnight, after which epidermis was separated. Single cell suspensions of human keratinocytes (HK) (10^6 cells/ml) were incubated with 1μM fura-2-AM for 15 min at 37°C to measure free cytosolic Ca^{2+} ($[Ca^{2+}]_i$). For PI turnover experiments KC were incubated with 10μCi/ml ^3H-myo-inositol for 3 hrs at 37°C. H (10^{-5}-10^{-4}M, LTB_4 (10^{-9}-10^{-6}M), 12-HETE (1μM), PAF (1μM) and BK (10^{-9}-10^{-6}M) but not PGE_2, LTD_4 and LTC_4 stimulated Ca^{2+} mobilization. BK and LTB_4 induced rapid transient rises in $[Ca^{2+}]_i$ from a baseline of 122 ± 14nM that decayed to baseline in 2-3 min and were independent of medium Ca^{2+}. BK also resulted in a rapid transient stimulation of inositol triphosphate (150%) and inositol tetraphosphate (160%) that peaked at 15-30 sec and had largely subsided at 60 sec. Similar results were observed in A431 cells. Pretreatment of HK with 1μM manoalide (a novel anti-inflammatory compound) for 5 min completely inhibited changes in $[Ca^{2+}]_i$. These results suggest that inflammatory mediators may stimulate the growth of HKC by their effects on PI turnover and Ca^{2+} mobilization. The data provide a molecular link between inflammation and epidermal hyperplasia. Compounds that block this response may be useful drugs in psoriasis.

S 344 INHIBITION OF CELL MEMBRANE PHOSPHOLIPASE A_2 AND CELLULAR SECRETION, Saul Yedgar, Nurit Reisfeld, Phyllis Dan and Arie Dagan, Hebrew University Medical School, Jerusalem, Israel.

To study the role of cell membrane phospholipase A_2 (PLA_2) in cellular secretion, a cell impermeable inhibitor of PLA_2 was designed and prepared by linking N-derivatized phosphatidylserine to dextran-80 (Dex-PS) via a spacer. The derivatised PS (a PLA_2 inhibitor) incorporates into the cell plasma membrane but its internalization is prevented by the macromolecular carrier. PLA_2 activity was measured in intact cells by the hydrolysis of a fluorescent analogue of phosphatidylcholine, C_6-NBD-PC, that contains NBD-caproic acid (C_6-NBD) at the 2 position. When given to cells this substrate is hydrolyzed to produce only one fluorescent product, C_6-NBD, which is not further metabolized. Thus, the fluorescent fatty acid produced is a direct measure of PLA_2 activity. In cultured hepatocytes and rat basophilic leukemic (RBL) cells, C_6-NBD-PC incorporates predominantly into the plasma membrane and hydrolyzed by PLA_2 in that locus. Application of the cell-impermeable inhibitor blocks the cell membrane PLA_2 activity concomitantly with inhibition of the secretion of lipoproteins or lysosomal enzymes from liver cells and of histamine from RBL cells. Dex-PS inhibits platelet aggregation and thromboxane production as well. These findings provide direct evidence as to the linkage between cell membrane PLA_2 and cellular secretion.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 345 INVOLVEMENT OF (Na+K) ATPase ON LYMPHOCYTE ACTIVATION, Vera Lucia G. de Moraes*, B. Olej, Lucia de La Rocque and Vivian M. Rumjanek, Dept. of Biochemistry, ICB, Federal University of Rio de Janeiro* and Basic Research Centre, National Cancer Institute, Rio de Janeiro.

There is strong evidence that the plasma membrane is the primary site where early events leading to cell activation occur. These events have been related to an increase of ion fluxes through the membrane, mediated by transporter proteins such as (Na+K) ATPase. The participation of this enzyme in different cellular processes has been studied through its specific inhibitor, the cardiac glycoside ouabain. This work compares the effect of the drug on lymphocyte proliferation with that on natural killer (NK) cytotoxic activity. NK activity was measured against K562 cells previously incubated with ^{51}Cr. Cell proliferation was assayed by $[^{3}H]$ Thymidine incorporation in whole blood or peripheral blood lymphocytes (PBL) cultures after PHA stimulation. The results showed that NK activity was not sensitive to ouabain, even after stimulation by the phorbol-ester TPA or Interferon. Nevertheless, lymphocyte proliferation, whether tested in whole blood or in a PBL population, was totally inhibited by very low ouabain concentrations when compared to those observed to be inhibitory to human heart (Na+K) ATPase. This effect was observed throughout the incubation time, except when the drug was added at the last 6h concomitantly with the $[^{3}H]$ Thymidine pulse. Proliferation inhibition by ouabain was partially reverted by K+ addition. The results presented here suggested that (Na+K) ATPase plays a very important role in cell proliferation, but other cellular processes involving activation, such as natural killer activity, seem to occur without such a close dependence.

S 346 PLATELET ACTIVATING FACTOR (PAF) IS AN ESSENTIAL AUTOCOID FOR EARLY EMBRYONIC DEVELOPMENT. Chris O'Neill, Human Reproduction Unit, Royal North Shore Hospital of Sydney, St. Leonards, NSW, 2065, Australia.

The mammalian embryo produces and secretes 1-0-alkyl-2-acetyl-sn-glyceryl-3-phosphocholine (PAF) soon after fertilization which persists at least until the time of embryo implantation. Its production is de novo from glucose and can be followed by feeding embryos ^{14}C glucose. Culture in vitro results in reduced PAF production and reduced embryo viability. Supplementation of embryo culture medium with PAF causes a 90% increase in glucose metabolism, enhanced cell division rates and implantation rates by mouse embryos. By comparison specific PAF receptor antagonists inhibit embryo metabolism and implantation in vitro and in vivo. Hence the production and response of embryos to PAF is essential for normal embryogenisis.

Receptors, G-Proteins

S 400 INHIBITION OF T CELL ANTIGEN RECEPTOR (TcR)-MEDIATED SIGNALLING BY PHORBOL ESTERS, Robert T. Abraham, Steffan N. Ho and David J. McKean, Mayo Clinic/Foundation, Rochester, MN 55905.

Multivalent interaction of cell surface TcRs with specific ligands (antigens, mitogens anti-TcR antibodies) triggers the phospholipase C-mediated hydrolysis of phosphoinositides, with the concomitant generation of at least 2 second messengers, 1,2-diacylglycerol (DG) and inositol-1,4,5-trisphosphate (IP_3). DG-mediated protein kinase C (pkC) activation and IP_3-induced intracellular Ca^{2+} mobilization represent synergistic intracellular events for activation of resting T cells. In this study, we have utilized a murine T lymphoma cell line, LBRM-33, to examine the regulation of signal output from the TcR. Heterologous activation of pkC in LBRM-33 cells by phorbol ester exposure rapidly uncoupled TcR perturbation from phospholipase C activation, IP_3 release and intracellular Ca^{2+} mobilization. Densitization of the TcR by phorbol esters was reflected in the mixed agonist/antagonist properties of these agents on stimulus-induced IL2 production. Inhibition of the TcR-dependent increase in $[Ca^{2+}]_i$ by phorbol ester was attributable to the selective suppression of IP_3-mediated intracellular Ca^{2+} mobilization. Phorbol ester treatment had no direct effects on LBRM-33 cell phospholipase C activities, measured with exogenous phosphoinositides as substrates. Although phorbol ester induced partial downregulation of TcR expression, TcR desensitization by phorbol esters was not due solely to TcR sequestration. These results indicate that pkC activation impairs TcR-mediated signalling by altering both TcR-phospholipase C coupling and downregulating TcR expression.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 401 CYTOPLASMIC AND MEMBRANE-ASSOCIATED ONCOGENES PRODUCE COMMON ALTERATIONS IN PHOSPHOINOSITIDE SIGNALLING PATHWAYS
E. Santos and T. Alonso, NIAID, NIH, Bethesda,MD 20892

The role of different oncogene products in signal transduction was assessed by studying both phosphoinositide (PI) metabolism and PI-mediated cellular responsiveness to agonists in cells transformed by nuclear, cytoplasmic and membrane-associated oncogenes. The same specific alterations were observed in the PI cycle of fibroblasts transformed by either membrane-associated oncogenes such as ras, src, met or trk or the cytoplasmic oncogenes mos and raf; the nuclear oncogenes fos and myc did not produce these changes. The alterations included: (a) stimulation of phospholipase A2 activity as indicated by elevated levels of glycerophosphoinositol (GPI) and non-esterified arachidonic acid (AA) and (b) specific uncoupling between surface receptor-mediated stimulation by PDGF, bombesin or serum and activation of intracellular phospholipase C. These findings suggest the existence of common biochemical pathways for transformation by cytoplasmic and membrane-associated oncogenes and are not consistent with the notion that p21 ras proteins are direct or unique regulatory elements of phospholipase C or phospholipase A_2 in PI signal transduction pathways.

S 402 PARTICIPATION OF MEMBRANE-ASSOCIATED GTP-BINDING PROTEIN IN COLLAGEN-INDUCED PLATELET ACTIVATION. Lilly Y.W. Bourguignon and Gary Walker, Department of Anatomy and Cell Biology, University of Miami Medical School, Miami, Fl. 33101.
Recent data from our laboratory indicate that collagen stimulates a rapid loss of PIP_2 and generation of IP_2 and IP_3 in human platelets. The production of IP_2 and IP_3 occurs as early as 5-10 sec following the addition of collagen to the platelets and is immediately followed by the accumulation of free intracellular Ca^{2+}. These results indicate that the binding of collagen stimulates the inositol phospholipid-specific phospholipase C during platelet activation. Furthermore, the fact that (1) GTP or GTP-γ-S augments and (2) pertussis toxin inhibits collagen-induced IP_3 formation suggests that a GTP-binding protein is directly involved in the regulation of phospholipase C-mediated phosphoinositide turnover. Additional analysis using azido-$GT^{32}P$ photoaffinity binding assays, $AD^{32}P$-ribosylation by pertussis toxin, two-dimensional gel electrophoresis and peptide mapping, indicates that platelets contain a membrane-associated 41 kDa protein which has a number of structural and functional similarities to the regulatory α subunit of the GTP-binding protein isolated from bovine brain. Most importantly, the addition of antibody against the GTP-binding protein, both precipitates the platelet 41 kDa protein and blocks the collagen-induced IP_3 formation. These data provide strong evidence that the 41 kDa platelet is a GTP-binding protein and is involved in collagen-induced signal transduction during platelet activation.

S 403 PERTUSSIS TOXIN-SENSITIVE INHIBITION OF ADENYLATE CYCLASE ACTIVITY AND Ca^{2+} INFLUX BY ADENOSINE A1 RECEPTORS IN GH_3 CELLS, Christine L. Boyajian, Kevin K. Caldwell, Werner Schlegel* and Dermot M.F. Cooper, University of Colorado Health Sciences Center, Denver, CO 80262 and *University of Geneva, Geneva, Switzerland.
Receptors that are inhibitorily coupled to adenylate cyclase decrease Ca^{2+} influx in a number of cell types. We thus addressed the possibility that cyclase inhibitory receptors generally serve to modulate Ca^{2+} influx. In the presence of >0.5μM GTP, phenylisopropyladenosine (PIA) inhibited vasoactive intestinal polypeptide (0.5μM) stimulated adenylate cyclase activity in GH_3 plasma membranes via an A1 adenosine receptor. Somatostatin (SST), whose effects on GH_3 cell cyclase activity are GTP-dependent, inhibited the enzyme to a similar extent as PIA; co-administration of maximally effective concentrations of these agents did not show significant additive effects. Pretreatment of GH_3 cells with pertussis toxin (PT) attenuated the inhibition of cyclase activity by both PIA and SST, suggesting the involvement of a PT-sensitive GTP regulatory protein(s). Analysis of PT substrates in GH_3 plasma membranes by SDS-PAGE revealed two proteins of M_r=41,000-Da and 40,000-Da. In cell suspensions of GH_3 cells loaded with quin2, both PIA and SST inhibited thyroid releasing hormone-induced elevations in intracellular Ca^{2+}, via a PT-sensitive action. Maximal effects of PIA did not preclude a further effect of SST, suggesting that at least in part, distinct mechanisms serve to lower Ca^{2+} influx. Although it is not yet clear which of the GH_3 cell PT substrates may be selectively associated with Ca^{2+} currents and cyclase inhibition, we conclude that two distinct signalling pathways may be subject to regulation by the occupation of a single inhibitory receptor.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 404 PHOSPHORYLATION OF YEAST RAS1 PROTEIN AND ITS POSSIBLE ROLE IN THE REGULATION OF PI TURNOVER, Alexander R. Cobitz and Fuyuhiko Tamanoi, The University of Chicago, Chicago, IL. 60637. Two homologues of ras-oncogenes, termed RAS1 and RAS2, have been identified in Saccharomyces cerevisiae. Whereas the RAS2 protein is involved in the regulation of adenylate cyclase, the RAS1 protein has been proposed to play a major role in the regulation of PI turnover. As a first step toward investigating the PI turnover in yeast, we have concentrated on characterizing the RAS1 protein. Here we report that the protein is phosphorylated. Our conclusion is based on the following observations. (1) The immuno-purified RAS1 protein appears as multiple species; in addition to the species having the predicted molecular weight (36K), multiple higher molecular weight species have been detected. These species can be converted to the 36K species by alkaline phosphatase treatment. (2) Only the high molecular weight species can be labeled with radioactive phosphates. Acid hydrolysis of the phosphate-labeled proteins has demonstrated that the phosphates are exclusively on serine residues. Subcellular fractionation of the cells suggests that the phosphorylated RAS1 proteins are membrane localized. These results demonstrate that the RAS1 protein is phosphorylated and further raise the possibility that the PI turnover in yeast is regulated by the phosphorylation of one of the components. In marked contrast to the RAS1 protein, no phosphorylation of the RAS2 protein is detected.

S 405 Rapid biochemical changes occur during GM-CSF induction of differentiation in a myeloid leukemia cell line. Seth Corey and Philip M. Rosoff, Dept of Physiology, Tufts University School of Medicine, Boston,Ma 02111 (P.M.R.) and Dana-Farber Cancer Institute, Boston, Ma 02115 (S.C.)

Granulocyte-macrophage colony stimulating factor (GM-CSF), a 22 kdD glycoprotein produced by activated mononuclear and stromal cells, has pleiotropic effects on myeloid cells ranging from stimulating proliferation and enhancing mature cell function to inducing differentiation in progenitor cells. A single class of high-affinity binding sites has been described on GM-CSF-senstive cells. The biochemical mechanisms underlying the multiple effects of this cytokine are poorly understood. We have examined the early biochemical events occuring in the human myeloid leukemic cell line U937, after treatment with physiological concentrations of recombinant human GM-CSF. This system offers the advantage of being able to study a physiological hormone as opposed to chemical inducers of differentiation such as dimethylsulfoxide. This cell line can be induced to differentiate into functionally mature cells as assayed by their ability to reduce nitroblue tetrazolium after 96 hours. This response requires only a short initial exposure to GM-CSF as removal of the cytokine after 1 hour, or addition of an inhibitory anti-GM-CSF antibody, had no effect on induction of differentiation. GM-CSF did not produce any rapid changes in intracellular free (Ca^{2+}) or pH, but did lead to significant decrease in diacylglycerol within 15 minutes. No changes in phosphatidylinositol-4-phosphate or phosphatidylinositol-4,5-bisphosphate were observed. These data suggest that irreversible commitment to differentiation in these cells occurs rapidly and is associated with concomitant changes in phospholipid metabolism. This work was supported by NIH training grant 2T32CA09172-12 (S.C.). P.M.R. is a Pew Scholar in the Biomedical Sciences.

S 406 PURIFICATION OF A PERTUSSIS TOXIN-INSENSITIVE GTP-BINDING PROTEIN FROM RAT LIVER, T.J. Fitzgerald, C.J. Lynch and J.H. Exton, Howard Hughes Med. Inst. and Dept. of Mol. Physiol. Biophys., Vanderbilt Univ. Sch. Med., Nashville, TN 37232 U.S.A. Receptors for Ca^{2+}-mobilizing hormones are coupled to a polyphosphoinositide phospholipase C in rat liver plasma membranes through a pertussis and cholera toxin-insensitive GTP-binding protein. A protein with these characteristics was solubilized from these membranes using 1% Na cholate and purified to apparent homogeneity. The [^{35}S]GTPγS-binding activity in the membrane extract was precipitated with polyethyleneglycol (6-10%) and the redissolved pellet applied to DEAE Sephacel and eluted with a linear NaCl gradient (0-250 mM). The active fractions eluting about 100 nM NaCl were then gel filtered on Sephacryl S300 and the fractions showing binding activity were subjected to chromatography on hydroxylapatite using a linear 0-150 mM KH_2PO_4 gradient. This yielded several peaks of GTPγS binding activity. SDS-PAGE of the first peak revealed several 39-43 kDa proteins, but none that could be ADP-ribosylated by pertussis toxin, whereas the second peak contained a 41 kDa protein which was a substrate for the toxin and is probably the α-subunit of G_i. When the first peak was purified further on octyl sepharose using a gradient with decreasing NaCl (200-50 mM) and increasing cholate (0.25-1.2%), substantial purification of the GTPγS binding activity was achieved. A final step on Q-Sepharose with a 0-400 nM NaCl gradient yielded one major peak of GTPγS binding which contained 41 kDa and 35 kDa proteins on SDS-PAGE, corresponding to the α and β subunits of the GTP-binding protein. Reconstitution studies with purified PIP_2 phospholipase C and vasopressin receptor from liver are currently being undertaken to establish that the purified protein is the GTP-binding protein that transduces the signal between these activities.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 407 A COMPARISON OF CYCLOSPORIN-BINDING BY CYCLOPHILIN AND CALMODULIN AND THE IDENTIFICATION OF A NOVEL 45 KD BINDING PHOSPHOPROTEIN IN JURKAT CELL, Brian M.J. Foxwell and Bernhard Ryffel, Preclinical Research, Sandoz Ltd., CH-4002 Basle, Switzerland.

Cyclosporin A (CSA) inhibits the synthesis of lymphokine mRNA during the process of T-lymphocyte activation. Although the detailed molecular mechanism is unknown, two proteins have been identified as putative intracellular CSA-receptor proteins. One of these, calmodulin, is an important Ca++ binding-protein and enzyme cofactor and the other, cyclophilin, is a novel protein which is reported to have protein kinase activity. In this study the CSA-binding capacity of both these proteins has been assessed using CSA-coated ELISA plates and CSA-affinity-gel matrices. CSA-binding was shown by cyclophilin where as no CSA-calmodulin binding could be detected under identical conditions. However, it was not possible to demonstrate any cyclophilin associated protein kinase activity. In Jurkat cells, in addition to the 17 KD protein-CP, a $_{32}$previously unidentified CSA-binding 45 KD phosphoprotein was precipitated from ^{32}P-labelled Jurkat cells. Our results would support cyclophilin as the major, intracellular receptor protein for CSA. The relationship between binding of CSA to cyclophilin and/or the 45 KD phosphoprotein and the immunosuppressive effects of CSA are still unknown.

S 408 DOWN-REGULATION OF EGF BINDING BY ACTIVATION OF THE src ONCOGENE PRODUCT pp60$^{v\text{-}src}$, George M. Gray and Ian G. Macara, Environmental Health Sciences Center, Division of Toxicology, University of Rochester, Rochester, NY.

The relationship between oncogenic transformation and signal transduction systems is not well understood although it is known that many oncogene products are involved in the transmission of signals from outside of the cell. In this study NIH 3T3 cells infected with a virus encoding a temperature sensitive mutant of the v-src oncogene product were used to assess the effect of pp60$^{v\text{-}src}$ on the binding of ^{125}I-EGF (Epidermal Growth Factor) to its receptor. Changing cells from the restrictive temperature (40°C) to the permissive (transforming) temperature (35°C) resulted in a rapid loss of binding of ^{125}I-EGF to its receptor. Binding dropped to just 35 percent of control in 15 minutes and slowly declined to 15 percent of control over 24 hours. Two distinct processes contribute to the down-modulation by pp60$^{v\text{-}src}$. The first is rapid and transient, while the second requires protein synthesis and persists long after inactivation of pp60$^{v\text{-}src}$. The decrease in binding is not a consequence of the expression of autocrine factors. Surprisingly, both mechanisms are protein kinase C independent. Both operate by decreasing the affinity of the EGF receptor for its ligand. Supported by NIH grant CA38888.

S 409 RAS PROTEINS MEDIATE INSULINE-INDUCED PHOSPHORYLATION OF RIBOSOMAL PROTEIN S(6). Tohru Kamata[1] and Hsiang-fu Kung[2]; [1]Program Resources Inc.,NCI-FCRF ;[2]Lab.of Biochemical Physiology, NCI-FCRF., Frederick, MD 21701.

Oncogenic ras protein and insulin,like progesterone, are known to induce the maturation of Xenopus oocytes. By utilizing this system, the biochemical pathway influenced by ras proteins has been studied. Microinjection of purified oncogenic T24 ras protein into Xenopus oocytes stimulates phosphorylation of ribosomal protein S(6) on serine residues, whereas normal ras protein is without effect. In addition, insulin also indúces S(6) phosphorylation and the induction is suppressed by injection of anti-ras antibody which blocks GTP binding activity of ras proteins. These result suggest that ras proteins mediate the insulin action on ribosomal protein S(6) phosphorylation which is possibly required for protein synthesis leading to oocyte maturation.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 410 PURIFICATION AND CHARACTERIZATION OF A G-PROTEIN FROM BOVINE LUNG, Yasunori Kanaho, Stanley T. Crooke and Jeffrey M. Stadel, SK&F Laboratories, Philadelphia, PA 19101
A predominant G-protein that is a pertussis toxin (PT) substrate was partially purified from bovine lung and compared to purified PT substrates from brain, G_i and G_o. The partially purified G-protein from lung (G_L), like G_i and G_o, appears to be a heterotrimer with α and $\beta\gamma$ subunits. On 10% SDS-polyacrylamide gels, $G_{L\beta}$ (35/36Kda) comigrated with β of G_i and G_o whereas $G_{L\alpha}$ migrated between $G_{i\alpha}$ and $G_{o\alpha}$. $G_{L\alpha}$, [^{32}P]ADP-ribosylated by PT, also migrated between the corresponding $G_{i\alpha}$ and $G_{o\alpha}$ and comigrated with the [^{32}P]-ADP-ribosylated G-protein in neutrophil membranes. The isoelectric focusing pattern of the [^{32}P]ADP-ribosylated $G_{L\alpha}$ was different from that of $G_{i\alpha}$ and $G_{o\alpha}$, but similar to that of the corresponding G-protein in neutrophil membranes. On immunoblots, $G_{o\alpha}$-antiserum raised against the purified $G_{o\alpha}$ from bovine brain reacted with $G_{o\alpha}$ and strongly crossreacted with $G_{i\alpha}$, but did not react with $G_{L\alpha}$ nor G-protein in neutrophil membranes. In contrast, G_{i-2}-antiserum raised against a synthetic decapeptide predicted by G_{i-2} cDNAs from human, rat and mouse libraries reacted with $G_{L\alpha}$ and G-protein in neutrophil membranes but did not with $G_{i\alpha}$ and $G_{o\alpha}$. Kinetics of [^{35}S]GTPγS binding to G_L was quite different from that to brain G_i or G_o; half maximal binding times were approximately 5, 15 and 30 min for G_o, G_i and G_L, respectively. The rate of hydrolysis of bound [γ-^{32}P]GTP by G_L ($t_{1/2} \simeq 1$ min) was faster than that by brain G_i or G_o ($t_{1/2} \simeq 4$ min). Thus the predominant PT substrate purified from lung was structurally and functionally different from the previously identified PT substrates in brain, G_i and G_o, and structurally similar to or the same as the PT substrate of neutrophils.

S 411 LIGAND INDEPENDENT SELF-TYROSINE PHOSPHORYLATION OF EGF-RECEPTOR AND THE erbB-2/neu PROTO-ONCOGENE PRODUCT IS INDUCED BY HYPEROSMOTIC SHOCK; C. Richter King, Ivan Borrello, Laura Porter, Paolo Comoglio, and Joseph Schlessinger.

The epidermal growth factor receptor and the protein product of the erbB-2 proto-oncogene become phosphorylated on tyrosine, when intact cells are subjected to high concentrations of ionic and non-ionic solutes. This self-phosphorylation occurs in the absence of EGF or other growth factors. High concentrations of solutes do not activate phosphoryla-tion of either isolated EGF-receptor or EGF-receptor solubilized by non-ionic detergents. In contrast to EGF stimulation, hyperosmotic shock does not result in the rapid internalization of EGF-receptor suggesting a different fate for receptor. No evidence for receptor dimerization was found in response to hyperosmotic shock. Since receptor dimers have been implicated in the EGF induced activation of EGF-receptor, hyperosmotic shock may activate EGF-receptor by a different mechanism.

S 412 RECEPTOR-MEDIATED PHOSPHOLIPASE C ACTIVATION, VIA A PERTUSSIS TOXIN INSENSITIVE G-PROTEIN, IS NECESSARY BUT NOT SUFFICIENT TO TRIGGER cGMP ACCUMULATION IN THE GUINEA PIG MYOMETRIUM. Denis LEIBER, Sylvie MARC, Sylvain BOURGOIN and Simone HARBON, Endocrinologie et Régulations Cellulaires, CNRS UA 1131, Université Paris Sud, 91405 Orsay Cedex, France.

In the guinea pig myometrium, carbachol (CB) enhanced phospholipase C (PLC) activation with an increased generation of inositol triphosphate (IP_3) and diacylglycerol (EC_{50} = 10 μM) and induced two concomitant Ca^{2+}-dependent responses viz. contraction and cGMP accumulation (an arachidonic acid-mediated event). Activation of muscarinic receptors also trigged an inhibition of cAMP accumulation due to prostacyclin (EC_{50} = 10 nM). Two distinct guanine nucleotide regulatory proteins (G-proteins) seem to be coupled to adenylate cyclase inhibition and PLC activation. NaF + $AlCl_3$ mimicked the Gi-mediated CB inhibitory effect on cAMP. Similarly fluoroaluminates reproduced an increase in the generation of IP_3 and induced contraction as well as cGMP accumulation. CB-mediated inhibition of adenylate cyclase was abolished by pertussis toxin (PT) ; by contrast the generation of IP_3 and similarly the concomitant cGMP and contractile responses were insensitive to PT. The data indicated that the increased phosphoinositide metabolism caused by CB may be relevant to the two Ca^{2+}-dependent responses (contraction and liberation of arachidonic acid involved in the generation of cGMP). However oxytocin which stimulated the accumulation of IP_3 and contraction (both events being insensitive to PT) could not evoke any increase in cGMP. This further suggested that CB could activate additional specific phospholipase (s) independently of the PLC-diacylglycerol pathway.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 413 ACTIVATION OF BASAL AND RECEPTOR-LINKED PHOSPHOINOSITIDE HYDROLYSIS IN RAT BRAIN BY GUANINE NUCLEOTIDES. Peter P. Li, Andrew S. Chiu, Jerry J. Warsh, University of Toronto, Canada.

Studies in non-neuronal tissues indicate that a G-protein is involved in receptor-coupled phosphoinositide (PI) hydrolysis. However, as yet there is little direct information available to support the occurence of such a mechanism in CNS. In the present experiments, we examined the effect of guanine nucleotides on PI hydrolysis in rat cerebral cortical slices or membrane preparations prelabelled with [^{3}H]inositol. PI hydrolysis was measured by the accumulation of inositol phosphates with either AG1-X8 column chromatography or HPLC. GTP-γ-S stimulated PI hydrolysis in cortical slices in a dose dependent manner with a EC_{50} value of 0.8 mM. Other guanine nucleotides (1 mM) were less effective but also activated PI hydrolysis, whereas the adenine nucleotides were ineffective. No measurable effect on PI hydrolysis was found with forskolin (10 μM) or dibutyryl cAMP (1-5 mM), indicating that the adenylate cyclase system is not involved in the effect of guanine nucleotides. NaF also stimulated PI hydrolysis in cortical slices, further suggesting a role of G-protein in regulating PI hydrolysis. The coupling between receptor activation and PI hydrolysis was studied in membranes treated with GTP-γ-S and/or carbachol. GTP-γ-S (0.1 mM) caused a 5-fold stimulation of PI hydrolysis. Although carbachol (0.1 mM) had no effect by itself, its combination with GTP-γ-S led to a 8-fold stimulation of PI hydrolysis. Thus the functional coupling of muscarinic receptor to PI hydrolysis is demonstrable in this cell-free system. In summary, the above data strongly suggest the involvement of G-protein(s) in regulating both basal and receptor-linked hydrolysis in CNS. (P.P. Li is an OMHF Fellow).

S 414 A TYROSINE-KINASE DEFECTIVE MUTANT ALLOWS TO SEPARATE BETWEEN HIGH AFFINITIES, EFFICIENT ENDOCYTOSIS AND THE MITOGENIC RESPONSE. Etta Livneh, Nachum Reiss, Eva Berent, Axel Ullrich[+] and Joseph Schlessinger. Dept. of Chemical Immunology, The Weizmann Institute of Science, Rehovot 76100, Israel, and [+]Genentech Incorporation, South San Francisco, California, U.S.A

Cultured NIH-3T3 cells devoid of endogenous EGF-receptors were transfected with cDNA constructs encoding normal human EGF-receptor and with a construct encoding an insertional mutant of the EGF-receptor containing four additional amino acids in the kinase-domain after residue 708. Unlike the "wild type" receptor expressed in these cells which exhibits EGF-stimulatable protein tyrosine-kinase activity, the mutated receptor lacks protein tyrosine-kinase activity both *in vitro* and *in vivo*. Despite this deficiency the mutant receptor is properly processed, it binds EGF and it exhibits both high and low affinity binding sites. Moreover, it undergoes efficient EGF-mediated endocytosis. However, EGF fails to stimulate DNA synthesis and is unable to stimulate the phosphorylation of S6 ribosomal protein in cells expressing this receptor mutant. Hence, it is proposed that the protein tyrosine-kinase activity of EGF-receptor is essential for the initiation of S6 phosphorylation and for DNA synthesis induced by EGF. However, EGF-receptor processing the expression of high and low affinity surface receptors and receptor-endocytosis appears to require neither kinase-activity nor receptor autophosphorylation. Interestingly, phorbol ester (TPA) fails to abolish the high affinity state of this kinase-mutated receptor. Moreover, TPA is also unable to stimulate the phosphorylation on serine or treonine residues of this receptor mutant. We are currently examining the possible mechanisms that underlay the failure of this mutant receptor to be phosphorylated by protein kinase C.

S 415 SITE-DIRECTED MUTAGENESIS AND PEPTIDE SYNTHESIS DEFINE ACTIVE SITES OF HUMAN GRANULOCYTE-MACROPHAGE COLONY STIMULATING FACTOR (GM-CSF) A.F. Lopez*, L.K. Eglinton*, M.F. Shannon*, S. Milton*, I. Clark-Lewis§, L.B. To∆, J.R. Gamble*, J. Schrader§ and M.A. Vadas*, Divisions of *Human Immunology and ∆Haematology, and the §Biomedical Research Centre, The University of British Colombia, Vancouver, Canada. Institute of Medical and Veterinary Science, Box 14, Rundle Mall Post Office, Adelaide, S.A. 5000.

Granulocyte-macrophage colony stimulating factor (GM-CSF), a 127 amino acid glycoprotein with two disulphide bonds, is responsible for the stimulation of proliferation and differentiation of haemopoietic progenitor cells and for the activation of mature cells. The chemical synthesis of GM-CSF peptides of different lengths has previously revealed a region between amino acids (aa) 14 and 25 which is critical for function. We have now used site-directed mutagenesis of the GM-CSF cDNA to examine this region in more detail. Mutant GM-CSF protein, produced firstly by using an ***in vitro*** transcription/ translation system and secondly by transfection of COS cells, was analysed for activity in several biological assays.

Results have shown that deletion of aa 14-24 abolishes GM-CSF activity, indicating a substantial correlation with the data obtained by peptide synthesis. In addition we have found that deletion of aa 14-18 does not abolish activity, implicating the area between aa 19-25 as critical for GM-CSF function. Deatiled experiments are currently being conducted to determine the importance of two hydrophilic residues (aa 20 and 21) within this region by deletion and substitution mutagenesis. In addition the roles of the C-terminal end and the four cysteine residues of the GM-CSF protein will be discussed.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 416 MEMBRANE SIGNAL TRANSDUCTION AND PROTEIN PHOSPHORYLATION DEFECTS IN CYCLIC HEMATOPOIESIS, C.D. Lothrop Jr., J.B. Jones, M.G. Low and R.C. Carroll. University of Tennessee, Knoxville, TN 37901 and Oklahoma Medical Research Foundation, Oklahoma City, OK 73104.

Cyclic hematopoiesis (CH) is a genetic disease of the hematopoietic stem cell in man and dogs, characterized by cyclic increases and decreases in circulating blood cells. The hematopoietic cycles result from periodic production of blood cells by the pluripotent stem cell pool. Collagen, thrombin and platelet activating factor (PAF)-evoked platelet activation are significantly decreased in CH dogs. Protein kinase C phosphorylation of a 40kd substrate and myosin light chain kinase phosphorylation of 20kd myosin light chain as measured by ^{32}P-orthophosphate incorporation were normal after thrombin stimulation of CH platelets. Phosphorylation of the 40kd substrate but not the 20kd substrate was markedly decreased in CH platelets after collagen stimulation. Quantitation of phosphotidic acid (PA) and 40kd phosphorylation in ^{32}P-orthophosphate labeled CH platelets demonstrated that the 40kd phosphorylation defect was associated with decreased PA formation. Abnormalities in cAMP, cGMP or Ca^{2+} metabolism were not observed in CH platelets. These results suggest the platelet activation and protein phosphorylation abnormalities in CH platelets may be due to a regulatory defect in the G-protein mediated phospholipase C/protein kinase C signal transduction cascade.

S 417 RECOMBINANT PDGF DOES NOT ACTIVATE PHOSPHATIDYLINOSITOL TURNOVER BUT STIMULATES DIACYLGLYCEROL KINASE IN SWISS 3T3 CELLS, Ian G. Macara, Department of Biophysics, Environmental Health Sciences Center, Univ. of Rochester Medical Ctr., Rochester, NY 14642.

Platelet-derived growth factor (PDGF) has been widely reported to stimulate phosphatidylinositol (PI) turnover, leading to the production of IP_3, which triggers Ca^{2+} release, and of diacylglycerol, which activates protein kinase C. Most of these studies, however, have relied on partially purified PDGF, which may contain contaminants that can potently stimulate the PI cycle. We now demonstrate, using pure recombinant PDGF, that this growth factor does not stimulate diacylglycerol or inositol phosphate production, nor can we detect increased turnover of PIP or PIP_2 by non-equilibrium labeling with ^{32}P-phosphate. We conclude that earlier evidence for the activation of the PI cycle by PDGF is artefactual. rPDGF does, however, increase phosphate uptake into 3T3 cells, increases ATP turnover, and dramatically stimulates the phosphorylation of diacylglycerol to phosphatidic acid. Supported by NIH grant CA38888.

S 418 IDENTIFICATION OF PLUMBAGIN AS A G PROTEIN INHIBITOR IN AN ANTIINFLAMMATORY CHINESE HERB, D.K. Miller, T. Zhen, S. Sadowski, M. Chang, and S.-S. Pong, Merck Sharp & Dohme Research Laboratories, Rahway, NJ 07065 and Beijing Medical University, China

A survey of extracts of Chinese herbs with reputed antiinflammatory and analgesic activity was made with a recently developed CXBG mastocytoma assay measuring inhibition of leukotriene biosynthesis. Methylene chloride extracts of herbs were incubated at 15 μg/ml with 2 x 10^6 CXBG cells/ml medium 199, the cells were stimulated with 5 μg/ml A23187, and aliquots of the cell suspension were tested for the presence of LTC_4 by ELISA. The extract of Plumbago zeylanicum L was found to have potent inhibitory activity of LTC_4 production with an IC_{50} of 0.6 μg/ml. Following silica gel column and preparative thin layer chromatography, the active ingredient plumbagin (2-methyl, 5-hydroxy, 1,4 naphthaquinone) was isolated with an LTC_4 inhibition of 0.15 μg/ml (0.8 μM) for LTC_4 production in the CXBG cell assay. When both the original extract and plumbagin were tested for their inhibition of fMet-Leu-Phe (FMLP) activated rat peritoneal neutrophil responses, they were not only potent inhibitors of LTB_4 production, but they were also sensitive inhibitors of all other tested responses of neutrophil activation, namely degranulation (myeloperoxidase release), adhesion, and thromboxane production (see below). Neutrophils activated by platelet activating factor were similarly inhibited by plumbagin. Because a common inhibition of all the neutrophil responses suggested an inhibition at an early postreceptor event, plumbagin was tested as an inhibitor of neutrophil FMLP and LTB_4 receptor activated GTPase activity where it was found to be active with an IC_{50} of 4 μg/ml.

	LTB_4	MPO	Adhesion	TXB_2
% Inhibition by 15 μg/ml extract	100	101	98	87
IC_{50} of plumbagin (μg/ml)	0.07	0.7	0.6	0.5

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 419 AGE-RELATED DEFECTS IN T LYMPHOCYTE ACTIVATION. Richard A. Miller, Boston University School of Medicine, Boston, MA 02118
Although T lymphocytes from old mice do not proliferate strongly when exposed to mitogenic lectins, they do respond strongly to intracellular signals (e.g. PMA plus ionomycin), suggesting age-specific defects in one or more of the early biochemical events that mediate the activation process. We find that increases in intracellular calcium ion concentration, in responses to Concanavalin A or to anti-T cell receptor (T3) antibody, are diminished in T cells from old mice, as is the expression of activation-sensitive oncogenes (c-myc, c-fos) and tissue specific genes (IL-2, IL-2 receptor). Con A-induced generation of inositol phosphates (IP_1, IP_2, and IP_3), however, proceeds normally in old mice, in both short-term (1 - 5 minute) and longer term (30 - 60 minute) experiments. Surprisingly, the age-related defect in Ca^{2+} concentrations is seen even in cells treated with ionomycin. This finding, together with the sparing of lectin-induced IP production, suggests that the activation defect in T cells from old mice may reside not so much in alterations of receptor-mediated signal transduction per se as in changes in the transport processes that maintain calcium compartmentalization.

S 420 EGF ACTIVATES PHOSPHOINOSITIDE TURNOVER AND PROTEIN KINASE C IN NONTRANSFORMED BALB/MK KERATINOCYTES, Jorge Moscat, Christopher J. Molloy, Timothy P. Fleming and Stuart A. Aaronson, National Cancer Institute, Bethesda, MD 20892.
BALB/MK mouse epidermal keratinocytes require EGF for growth and terminally differentiate in the presence of calcium concentrations >1.0 mM. Using a defined medium culture system, we investigated the role of EGF on phosphoinositide metabolism in these cells. The results show that EGF rapidly activated PL-C mediated PI metabolism resulting in the generation of the second messengers inositol 1,4,5-trisphosphate and diacylglycerol. This was accompanied by protein kinase C activation as evidenced by the phosphorylation of the acidic 80-kd endogenous protein substrate specific for this kinase. In contrast, insulin, which acts in concert with EGF to cause BALB/MK cell proliferation, had no effect on phosphoinositide metabolism nor led to any additional stimulation when added in combination with EGF. Taken together, our results show that phosphoinositide metabolism and subsequent protein kinase C activation are involved in the normal mitogenic response of epithelial cells to EGF. Preliminary studies on the effects of calcium on these signalling pathways will also be described.

S 421 COUPLING OF ANTAGONISTIC SIGNALLING PATHWAYS IN REGULATION OF NEUTROPHIL FUNCTION. Heinz Mueller and Larry A. Sklar. Res. Inst. of Scripps Clin. La Jolla, CA 92037.
The activation of neutrophils during inflammatory events and host defense is fine-tuned in vivo by a balance between stimulatory and inhibitory agents. Modulation of neutrophil activation by catecholamines may reflect this fine-tuning by coupling inhibitory (beta-adrenergic) and stimulatory (N-formyl peptide) receptor pathways. Modulation of cell function by Isoproterenol (ISO) is mimicked by agents that elevate intracellular cAMP concentration. Preincubation of cells with 50 uM forskolin or 1 mM dibutyryl cAMP inhibited peptide stimulated superoxide (O_2^-) generation similar to the inhibition by 1uM ISO. Preincubation of cells with the protein kinase antagonist H-9 blocked these inhibitors up to 70%. Thus, H-9 prevents cAMP-mediated inhibition of O_2^- generation. On the other hand, formyl peptide receptor binding is not affected by ISO. ISO did not inhibit O_2^- generation activated by 1uM ionophor A23187, the protein kinase C activators phorbol ester (100ng/ml) and oleoylacetylglycerol (50 uM) and the G-protein activator NaF (40 mM). Thus, intracellular calcium , protein kinase C and the G-protein appear not to be the target of the inhibitory pathway. These observations raise the possibility of the involvement of a cAMP-dependent kinase in inhibition of the signal transduction at the formyl peptide receptor or in blocking the interaction of this receptor with its G-protein.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 422 TGFβ MODULATES CALCIUM INFLUX AND PHOSPHATIDYLINOSITOL TURNOVER IN RAT-1 FIBROBLASTS. Leslie L. Muldoon, Karin D. Rodland, and Bruce E. Magun. Oregon Health Sciences University, Portland, OR, 97201.

TGFβ stimulates a variety of cellular processes, ranging from growth arrest and differentiation to transformation and mitogenesis, depending on the system studied. We have examined second messenger levels to determine if altered signal transduction mechanisms may mediate the transforming effects of TGFβ and modulate the TGFβ effects on EGF-stimulated processes. Addition of TGFβ (0.1-10 ng/ml) to serum-deprived, confluent Rat-1 cells increased the rate of ^{45}Ca influx 2-3 fold, measured over a 5 min interval. The effect was maximal after 4 h of exposure to TGFβ. Treatment with EGF also increased the rate of ^{45}Ca influx while addition of EGF to TGFβ-pretreated cells produced an additive increase in ^{45}Ca influx. ^{45}Ca influx stimulated by either growth factor was inhibited by cobalt or EGTA but not by more specific Ca^{2+}-channel blockers such as nifedipine or verapamil, suggesting that TGFβ and EGF do not act via L-type Ca^{2+} channels. Incubation of Rat-1 cells with 10 ng/ml TGFβ also produced an elevation of cellular inositol trisphosphate (IP_3) levels, which reached a maximum at 4 h and were subsequently maintained for at least 8 h. TGFβ pretreatment and EGF stimulation appeared to act synergistically in elevating IP_3 levels. The increases in IP_3 stimulated by TGFβ and EGF were sensitive to either cobalt or EGTA, but not to the specific Ca^{2+}-channel blockers. Measurement of intracellular free Ca^{2+} with fura-2 indicated that EGF stimulated a marked rise in free Ca^{2+} in TGFβ-pretreated cells, which was dependent on the influx of extracellular Ca^{2+}. We conclude that TGFβ increases the rate of Ca^{2+} influx, and that Ca^{2+} influx is responsible for the increased production of IP_3. Influx of Ca^{2+} from the extracellular medium is required for the changes in IP_3 and intracellular Ca^{2+} in response to both EGF and TGFβ and may be important in modulating some of the effects of EGF in Rat-1 cells. (Supported by NIH grant CA39360).

S 423 REGULATION OF RECEPTOR-MEDIATED Ca^{2+} INFLUX ACROSS THE PLASMA MEMBRANE IN A HUMAN LEUKEMIC T-CELL LINE: EVIDENCE FOR ITS DEPENDENCE ON AN INITIAL Ca^{2+} MOBILIZATION FROM INTRACELLULAR STORES. Janet Ng, Bertil Fredholm, Mikael Jondal and Tommy Andersson. Dept. of Immunology, Karolinska Institute, S104 01, Stockholm, Sweden.

Stimulation of a human leukemic T-cell line, Jurkat by both lectin (PHA) and anti-T3 antibody (OKT3) leads to a rise in the cytosolic free Ca^{2+} concentration. This Ca^{2+} transient results from both intracellular mobilization and influx of Ca^{2+} through specific membrane channels. In this study, we have examined how receptor-mediated influx of Ca^{2+} is regulated in Jurkat cells which have been reported to lack "voltage-gated Ca^{2+} channels". We used the Ca^{2+}-chelating properties of quin2 and BAPTA to study its effects on agonist-induced $^{45}Ca^{2+}$ influx. Increasing concentrations of both chelators caused a pronounced inhibition of OKT3- and PHA-induced $^{45}Ca^{2+}$ influx. At a concentration of 15 uM quin2/AM or 30 uM BAPTA/AM, agonist induced $^{45}Ca^{2+}$ influx was almost totally abolished. At these concentrations of quin2/AM and BAPTA/AM, both agonists could still cause a rise in cytosolic free Ca^{2+} above 200 nM. Using a concentration of La^{3+} (200 uM) that totally abolished the agonist-induced $^{45}Ca^{2+}$ influx, both agonists were able to raise the cytosolic free Ca^{2+} concentration above 200 nM by mobilizing Ca^{2+} from intracellular stores alone. Also, neither OKT3- nor PHA-induced $InsP_3$ formation was affected under conditions when $^{45}Ca^{2+}$ influx was abolished. The data suggest that both OKT3 and PHA, via increased $InsP_3$ formation and intracellular mobilization of Ca^{2+} raised the cytosolic free Ca^{2+} concentration to above 200 nM and this Ca^{2+} is the rate-limiting step for the initiation of receptor-mediated Ca^{2+} entry.

S 424 PHORBOL ESTER DOWN-REGULATES β-ADRENERGIC RECEPTORS BUT RESULTS IN ENHANCED cAMP PRODUCTION IN NEONATAL RAT VENTRICULAR MYOCYTES: POSSIBLE ROLE FOR PROTEIN KINASE C IN THE REGULATION OF cAMP. Charlotte Reupcke, Norman Honbo and Joel S. Karliner. Department of Medicine, VAMC and CVRI, University of California, San Francisco, CA.

Tumor-promoting phorbol esters stimulate protein kinase C (PK C) activity and can regulate β-adrenergic receptors (AR) and βAR-mediated signals in different cell types, but the results have been inconsistent. We investigated the effects of short-term (10 min) 12-O-tetradecanoyl-phorbol-13-acetate (TPA, 100 nM) exposure on cultured neonatal rat ventricular myocytes. TPA produced a 35% decrease in βAR density (^{3}H-CGP 12177 binding) from 51.8 ± 12.2 (mean ± SEM) to 33.6 ± 5.5 fmol/mg protein (n=11, p<.02). To our surprise isoproterenol-stimulated (1μM) cAMP content increase by 42% from 27.6 ± 2.2 to 39.3 ± 3.6 pmol/100μl (n=23, p<.0001). The enhanced response to isoproterenol was maintained over a concentration range of 1 nM to 10μM. Competition curves showed no change in agonist affinity but antagonist Kd was decreased from 3.74 ±1.17 to 2.03 ± 0.58 nM in TPA-pretreated cells (n=11, p<.05). Both down-regulation of βAR and TPA-enhanced cAMP accumulation were suppressed by preincubation with the PK C inhibitor H7. Stimulation by forskolin and GppNHp (1nM to 10μM) also resulted in an increase of cAMP content after preincubation with TPA. We conclude that TPA enhances βAR responsiveness despite loss of β-receptors. These observations implicate PK C in the regulation of cAMP generation in intact myocardial cells. The enhanced responses both to forskolin and to GppNHp support the possibility that one site of modulation by PK C may be beyond the βAR at the level of the guanine nucleotide regulatory proteins.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 425 NORMAL HUMAN B CELL ACTIVATION AND GROWTH, Rhiannon Sanders, Håkan Hedman and Erik Lundgren, University of Umeå, S-901-87 Umeå, Sweden.

We have studied the in vitro activation and long term growth of human B cells along with the expression of the nuclear antigen Ki-67. Normal B lymphocytes isolated from peripheral blood and depleted of T cells, could be induced to enter the cell cycle either by a mixture of B cell growth factor (BCGF), a sepharose-coupled anti-IgM antibody (anti-IgM) and interleukin 2 (IL-2) or by the combination of a phorbol ester (phorbol dibutyrate, PBt_2) and a calcium ionophore (ionomycin). A substantial fraction of the cells activated by either of these protocols progressed through the S phase of the cell cycle (as measured by cytofluorography) and expressed the nuclear antigen Ki-67. On average 75% of the cells treated with BCGF, anti-IgM and IL-2 expressed Ki-67, most prominently over the nucleolus. The combination of PBt_2 and ionomycin was a more potent inducer of DNA synthesis and was significantly more effective in inducing Ki-67 expression (88.6% Ki-67$^+$ were obtained on average). A parallel measure of IL-2 receptor expression (using an anti-Tac antibody) revealed that presentation of IL-2 receptor increased in cell populations whether treated with BCGF, anti-IgM and IL-2 (67% IL-2R$^+$) or with PBt_2 and ionomycin (90% IL-2R$^+$), but the increase was greater in the latter cells. Although the activated B cells showed a DNA staining profile characteristic of cycling cells no real increase in cell number was observed in keeping with previous observations of other workers. However, it was found that after pretreatment with PBt_2 and ionomycin for 4 hours the cells could be propagated for extended periods of time (60 days) using BCGF and IL-2. B cells grown in this way were shown to be uncontaminated by T cells (less than 1% CD4, CD8 or CD3 positive cells), to express the CD20 antigen (86%), to be capable of being induced to secrete antibodies and to show no detectable expression of EBNA.

S 426 ENDOCYTOSIS FROM COATED PITS IS INHIBITED BY ACIDIFICATION OF THE CYTOSOL. EVIDENCE FOR TWO DIFFERENT PATHWAYS OF RECEPTOR-MEDIATED ENDOCYTOSIS. Kirsten Sandvig[1], Sjur Olsnes[1], Ole W. Petersen[2], and Bo van Deurs[2]. 1. Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, Oslo 3, Norway. 2. The Panum Institute, University of Copenhagen, DK-2200 Copenhagen, Denmark.

Binding and endocytosis of the ligands transferrin, epidermal growth factor and ricin were measured in a number of different cell lines under conditions where the cytosolic pH was lowered. Low pH in the cytosol strongly inhibited endocytosis of transferrin and EGF, whereas entry of ricin was much less affected. Also the endocytosis of lucifer yellow was only slightly reduced. The effect of acidification of the cytosol on endocytosis was rapidly reversible. Acidification of the cytosol increased the number of transferrin receptors on the cell surface in a Ca^{2+} dependent manner. Data from electron microscopy studies indicated that the formation of coated vesicles from coated pits is inhibited by low cytosolic pH, and the results are consistent with the existence of a pathway of endocytosis which involves formation of vesicles from uncoated areas of the membrane.

S 427 CYTOPLASMIC AND MEMBRANE-ASSOCIATED ONCOGENES PRODUCE COMMON ALTERATIONS IN PHOSPHOINOSITIDE SIGNALLING PATHWAYS
E. Santos and T. Alonso, NIAID, NIH, Bethesda,MD 20892

The role of different oncogene products in signal transduction was assessed by studying both phosphoinositide (PI) metabolism and PI-mediated cellular responsiveness to agonists in cells transformed by nuclear, cytoplasmic and membrane-associated oncogenes. The same specific alterations were observed in the PI cycle of fibroblasts transformed by either membrane-associated oncogenes such as ras, src, met or trk or the cytoplasmic oncogenes mos and raf; the nuclear oncogenes fos and myc did not produce these changes. The alterations included: (a) stimulation of phospholipase A2 activity as indicated by elevated levels of glycerophosphoinositol (GPI) and non-esterified arachidonic acid (AA) and (b) specific uncoupling between surface receptor-mediated stimulation by PDGF, bombesin or serum and activation of intracellular phospholipase C. These findings suggest the existence of common biochemical pathways for transformation by cytoplasmic and membrane-associated oncogenes and are not consistent with the notion that p21 ras proteins are direct or unique regulatory elements of phospholipase C or phospholipase A_2 in PI signal transduction pathways.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 428 PI METABOLISM ASSOCIATED WITH LTB_4 INDUCED $[Ca^{2+}]_i$ MOBILIZATION IN U937 CELLS, H.M. Sarau, J.J. Foley, P.D. Henriksen, J.D. Winkler and S.T. Crooke, SKF Labs, Phila, PA 19101

LTB_4 receptors are expressed upon differentiation of U937 cells with DMSO and $[Ca^{2+}]_i$ mobilization occurs in response to LTB_4. The PI metabolites produced by LTB_4 in cells loaded with ^3H inositol were analyzed by HPLC. EC_{50}s for IP_3 production and $[Ca^{2+}]_i$ mobilization were similar (2-4 nM). IAP sensitive G proteins were involved as both LTB_4 induced $[Ca^{2+}]_i$ mobilization and IP_3 production were blocked by IAP treatment. $I(1,4,5)P_3$ increased (2-3 fold above basal levels) for 5-10 sec then decreased rapidly to basal levels by 30 sec. $I(1,3,4)P_3$ and IP_4 were maximal at 20 sec then decreased slowly and remained above basal levels for > 60 sec. The initial $[Ca^{2+}]_i$ mobilization response followed $I(1,4,5)P_3$ response but decreased more slowly in a manner equal to $I(1,3,4)P_3$ levels. Homologous desensitization was observed in LTB_4 pretreated cells, in that no significant $[Ca^{2+}]_i$ mobilization or $I(1,4,5)P_3$ production occurred with a second LTB_4 stimulation. LTD_4, a closely related agonist that interacts with different receptors, induced an unchanged increase in $[Ca^{2+}]_i$ mobilization and $I(1,4,5)P_3$ production following an LTB_4 stimulation. Results indicate that LTB_4 receptors are coupled through a G protein to phospholipase C and that a transient increase in $I(1,4,5)P_3$ is necessary for $[Ca^{2+}]_i$ mobilization.

S 429 Ligand induced stimulation of EGF-receptor mutants with altered transmembrane regions. Azriel Schmidt, Ofra Kashles, Daniel Szapary, Axel Ullrich and Joseph Schlessinger. Rorer Biotechnology Inc., Rockville, MD 20850.

The EGF-receptor is a transmembrane glycoprotein composed of a large extracellular ligand binding domain connected to the cytoplasmic kinase domain be a single transmembrane (TM) domain composed of 23 hydrophobic amino acids. To explore the mechanisms governing the activation of cytoplasmic kinase domain by ligand binding to the extracellular domain several mutant receptors with altered transmembrane domain were generated utilizing in vitro site directed mutangenesis. The TM region of two mutant receptors was either extended (i626-3) or shortened (d626-3) by three hydrophobic amino acid residues. In the other two mutants hydrophobic amino acids were substituted by charged residues, i.e., Val627 was replaced by glutamic acid (v627E and Leu642 was substituted by an arginine residue (L642R). We find that the mutant receptors are expressed on the cell surface as polypeptides of 170'000 MW exhibiting both high and low affinity binding sites for ^{125}I-EGF and phorbol ester (PMA) abolished the high affinity binding sites for EGF. EGF was able to stimulate the kinase activity of wild type and mutant receptors in a similar manner both in vitro and in living cells. Moreover, the mutant receptors were also able to undergo EGF-induced receptor dimerization as revealed by cross-linking experiments. It was concluded that the transmembrane region has a passive role in signal transduction and that receptor activation is probably mediated by an intermoleculer rather than intramolecular process.

S 430 CHARACTERIZATION OF THE BINDING PROPERTIES OF DETERGENT-SOLUBILIZED VASOACTIVE INTESTINAL PEPTIDE (VIP) RECEPTORS, G. Velicelebi, S. Patthi, and S. Simerson, SIBIA (Salk Institute Biotechnology/Industrial, Assoc., Inc.) 505 Coast Blvd S., La Jolla, CA 92037.

Previously, we demonstrated that nondenaturing detergents can be used effectively to solubilize rat lung VIP receptors that had been covalently labeled with ^{125}I-VIP prior to solubilization (Patthi, S., Akong, M., and Velicelebi, G. (1987) J. Biol. Chem. 262:15740). We demonstrate here the specific binding of ^{125}I-VIP to detergent-solubilized VIP receptors subsequent to their extraction from the membrane. Furthermore, we describe the binding properties of detergent-solubilized receptors, determined through equilibrium and kinetic studies. Scatchard analyses of competitive binding data indicated the presence of two classes of binding sites in the detergent-solubilized receptors similar to that seen in the membrane-bound receptors (Provow, S. and Velicelebi, G. (1987) Endocrinology 120:2442). The values for the dissociation constant (K_d) were 200pM and 8 nM while the values for the binding capacity (B_{max}) were 200 fmoles/mg and 2300 fmoles/mg protein for Class I and II sites, respectively. Thus, based on a molecular size of 55,000 daltons determined in our earlier study (Patthi, et al, 1987), it is estimated that 8,000-fold purification is required to purify the solubilized receptor to homogeneity. The ability to extract the VIP receptor from the membrane in the native state is an important first step towards purification of the protein.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 431 Involvement of G-protein in the regulation of vascular smooth muscle response to histamine. B. Vollrath, R. Sherry, V. Hughes and D.A. Cook. Dept. of Pharmacology, University of Alberta, Edmonton, Alberta, Canada, T6G 2H7.

Aluminium is a required cofactor for fluoride activation of G proteins. We have confirmed the Al^{3+} requirement for fluoride activation of G-protein in vascular smooth muscle of canine aorta and demonstrated that the contractile response to histamine, which is mediated by the formation of inositol phosphates, was significantly potentiated by ALF_4^-. The synergism of AlF_4^- with Hi suggests an interaction between the H_1 histamine receptor, a G protein and phospholipase C. The effect of AlF_4^- on aortic smooth muscle contraction was potentiated by 2,3-bisphosphoglyceric acid (10 μM), an inhibitor of phosphomonoesterases, indicating that AlF_4^- stimulated $Ins(1,4,5)P_3$ formation in vascular smooth muscle cells.

Further studies with ^3H $Ins(1,4,5)P_3$ in cell free preparations have demonstrated that $Ins(1,4,5)P_3$ is phosphorylated to inositol tetrakisphosphate [$Ins(1,3,4,5)P_4$] by an ATP dependent kinase, H.P.L.C. analysis revealed that $Ins(1,3,4,5)P_4$ is hydrolysed first to an inositol trisphosphate isomer $Ins(1,3,4)P_3$ by a 1-phosphatase and then to inositol bisphosphate $Ins(1,3)P_2$. Pretreatment of broken cell preparation with 2,3 bisphosphoglyceric acid inhibited dephosphorylation of $Ins(1,4,5)P_3$ and $Ins(1,3,4)P_3$ to the corresponding inositol bisphosphate. These data are consistent with the idea that a guamine nucleotide regulatory protein is involved in receptor-mediated stimulation of vascular smooth muscle contraction.

S 432 BIDIRECTIONAL ACTIONS OF FLUORIDE ON PHOSPHOLIPASE C IN GUINEA-PIG ILEUM S P Watson, A Stanley, T Sasaguri & P P Godfrey*. Dept. of Pharmacology & *Dept. of Clinical Pharmacology, OXFORD, U.K.

Fluoride, when complexed with Al to form ALF_4^-, activates a number of GTP-binding proteins including G_s, G_i and G_p. In the present study fluoride has been used to investigate the possible involvement of a G-protein in the activation of phospholipase C and in the onset of contraction in guinea-pig ileum.

Sodium fluoride (10mM)-induced a 102 ± 13.1% (n = 16) increase above basal in the levels of [^3H]inositol phosphates in guinea-pig ileum longitudinal smooth muscle during a 30 min incubation in the presence of Li (10 mM). Under the same conditions, carbachol (1mM) raised the levels of the inositol phosphates by 918 ± 76 % (n = 4). In the presence of fluoride (10 mM), however, the response to carbachol (1 mM) was significantly reduced ($p < 0.01$) to 460 ± 43.6 % (n = 4). The formation of inositol phosphates by fluoride was associated with a slow contractile response which peaked after approx. 5 min and then declined back to basal; the peak level of contraction was 35 ± 2 % (n = 5) of the response to a maximally effective concentration of carbachol. Contraction was blocked in the presence of nifedipine (1 uM) or Cd^{2+} (0.3 mM).

These data provide evidence that a G-protein is involved both in the activation of phospholipase C and in the onset of contraction in the guinea-pig ileum suggesting that these two events may be linked. The mechanism of the fluoride-induced contraction involves the entrance of extracellular Ca^{2+} through voltage operated channels; the role, if any, of IP3 or DG in stimulating the onset of the depolarisation which leads to the opening of these channels is not known. In addition, the present study has observed a novel inhibitory action of fluoride on carbachol-induced formation of inositol phosphates. The mechanism of this effect is unknown but could be related to the activation of a novel G-protein which inhibits phospholipase C.

S 433 A FAMILY OF $p21^{ras}$-LIKE GDP BINDING PROTEINS IN SWISS 3T3 PLASMA MEMBRANES, Alan Wolfman and Ian G. Macara, Environmental Health Sciences Center, Department of Biophysics, University of Rochester Medical Center, Rochester, NY 14620.

A large family of GDP-binding proteins has been discovered in 3T3 cell plasma membranes with properties similar to those of $p21^{ras}$. The partially purified plasma membranes bind about 50 fmols α-^{32}P-GDP/μg protein, with an apparent $K_{1/2}$ of 40 nM. In the presence of Mg^{2+}, exchange of α-^{32}P-GDP with prebound ligand is very slow, but, as with $p21^{ras}$, exchange is dramatically accelerated by excess EDTA. GDP binding is inhibited by GTP-γ-S, but not by ATP, ADP, c-GMP, or GMP. In the presence of Mg^{2+}, two classes of binding sites are distinguishable, with a rapid (2.3×10^{-3} sec^{-1}) and a slow (1.5×10^{-4} sec^{-1}) GDP off-rate. The k_{on} for the slowly exchanging class is about 3.9×10^3 M^{-1} s^{-1}. High resolution anion exchange chromatography separates at least ten distinct species of GDP binding proteins in CHAPS-solubilized membranes. Gel filtration analysis resolves multiple peaks of 68,000 to 20,000 daltons. One of the GDP-binding species has been tentatively identified as $p21^{c\text{-}Ha\text{-}ras}$. Surprisingly, the inactivating anti-$p21^{ras}$ monoclonal antibody, Y13-259, depletes solubilized membranes of at least 5 GDP-binding species. The results suggest that a family of ras-related proteins is present in 3T3 cell membranes and that the Y13-259 antibody detects a subclass of these proteins in addition to $p21^{ras}$.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 434 GENERATION AND CHARACTERIZATION OF T CELL HYBRIDOMAS DEFECTIVE IN SIGNAL TRANSDUCTION THROUGH THE T CELL RECEPTOR, Edward T.H. Yey, Arthritis Unit, Massachusetts General Hospital, Boston, MA 02114.

Activation of T lymphocytes is a process only partially understood that involves triggering of the T cell receptor, generation of secondary signals, and activation of specific gene programs. However, the precise pathways of signal transduction are not well characterized. In order to gain more insight into the process of normal T cell activation, we have generated a panel of T cell hybridomas that are defective in signalling through the T cell receptor. One class of mutants are deficient in the expression of certain surface activating molecules, such as Thy-1 or Ly-6. Another class of mutants are phenotypically indistinguishable from the wild type, but can not be activated through the T cell receptor. However, these mutants do secrete IL-2 in response to PMA and calcium ionophore. Thus, the defects are located proximal to the protein kinase C step. These mutants will be further characterized.

S 435 G-PROTEIN-MEDIATED MECHANISM OF REGULATION OF NICOTINIC ACETYLCHOLINE (ACH) RECEPTOR IN CULTURED MYOTUBES, B.M. Zani,C. Ciccarelli F. Eusebi,S. Adamo & M. Molinaro, Inst. Histol. Gen. Embryol. Univ."La Sapienza" Rome, Italy.

Many cellular responses are mediated by G-protein regulated activation of phospholipase C (PLC), which in turn catalyzes the hydrolysis of phosphatidilinositol bisphosphate to inositol trisphosphate (IP_3) and diacylglicerol. We here report that Ach induces IP_3 release in intact cells as well as in crude membrane preparation from ^{3}H-inositol prelabelled myotubes. The effect of Ach in intact cells is not abolished in Ca^{2+}-free medium, is not affected by atropine while is blocked by curare or α-bungarotoxin. The data suggest the presence of active nicotinic Ach receptor coupled with PLC in cultured myotubes. A G-protein appears to be coupled to nicotinic Ach receptor as shown by both biochemical and electrophysiological data as follows: 1) pertussis toxin administered to cultured myotubes drastically lowers the effect of Ach on IP_3 release; 2) GTPγS (but not GDPβS) increase the production of IP_3 in crude membrane preparation; 3) GTPγS (but not GDPβS) is sinergic with Ach in the production of IP_3 in crude membrane preparation; 4) the effect of GTPγS on the IP_3 release is blocked in crude membrane preparation from cells pretreated with pertussis toxin; 5) GTPγS (but not GDPβS or cAMP), intracellularly perfused, affects the Ach-induced single channel similarly to Ach, when the transmitter is applied to extrapatch membrane under cell-attached patch-clamp recording condition.

S 436 EVIDENCE FOR A HORMONE-MEDIATED PHOSPHOINOSITIDE TURNOVER ON ISOLATED CELL MEMBRANES OF *DAUCUS CAROTA* L., A PLANT

Bernd A. Zbell, Cornelia Walter-Back and Hubert Bucher, Botanical Institute of the University, Im Neuenheimer Feld 360, D-6900 Heidelberg, Federal Republic of Germany.

Using [γ-^{32}P]ATP as label for the polyphosphoinositides a rapid decrease of radioactivity of the phospholipid fraction extracted from membranes of carrot suspension cells could be detected in the presence of auxin. The phytohormone effect was caused by a simultaneous release of inositol polyphosphates and not by an inhibition of the lipid phosphorylation reactions. These findings point to an auxin-mediated control of a phospholipase C-like reaction being similar to the hormone-stimulated phosphoinositide response in animals. Exogenously applied (1,4,5)IP_3 was found to release Ca^{2+} from preloaded membrane vesicles which originate from endomembranes of carrot cells. It is hypothezised that *in vivo* the release of Ca^{2+} from internal stores leads to a transient increase of the cytoplasmic Ca^{2+} level which could initiate auxin-mediated cellular processes like exocytosis or mitosis. The detection of the auxin-stimulated phosphoinositide response as well as an (1,4,5)IP_3-mediated Ca^{2+} release on isolated carrot cell membranes do offer new experimental approaches for the identification of the putative auxin receptor and its signal transduction pathway.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

Inositol Phosphates

S 500 ARACHIDONIC ACID CONTENT OF PHOSPHOINOSITIDES IN RAT LIVER NODULES
Monica B Andersson and Lennart C Eriksson, Dept of Pathology, Huddinge Hospital, S-141 86 HUDDINGE, SWEDEN.

One system available for the sequent step-by-step analysis of carcinogenesis, is the resistant hepatocyte model, where hepatocarcinogenesis in the rat is initiated and promoted with chemicals. In this model, the behaviour of the persistent hepatocyte nodules as the progression to cancer occurs, is studied. The nodules have a higher rate of cell division than normal liver, and have shown several morphological and biochemical differences from normal liver. In this model we have investigated the fatty acid composition of phospholipids and phosphoinositides, with special interest focused on arachidonic acid, in comparison with normal liver and regenerating liver, ie after partial hepatectomy.
Our results show a slight, overall decrease of arachidonic acid content in phospholipids from the nodules, compared to normal liver. When studying the phosphoinositides, we found a decrease of arachidonic acid in the polyphosphorylated forms of PI, in normal liver from 36,1 % of total fatty acid in PI to 11,1 % in PIP and 8,0 % in PIP_2. In liver nodules the values are 27 % in PI, 10,7 % in PIP and 5,9 % in PIP_2.

This work was supported by the Swedish Cancer Society.

S 501 PHOSPHOINOSITIDE KINASE ACTIVITIES IN CELL GROWTH AND TRANSFORMATION, L.A. Serunian, K.R. Auger, M. Whitman, and L.C. Cantley, Department of Physiology, Tufts University School of Medicine, Boston, MA. 02111.

Products of the PI turnover pathway have been implicated as potent mediators of cell growth and differentiation in several systems. Previous studies have shown that immunoprecipitates from polyoma virus middle T transformed cells and partially purified PDGF receptor preparations can catalyze two potential regulatory steps in the PI pathway: the phosphorylation of PI to PIP and the phosphorylation of PIP to PIP_2. The kinase activities associated with the middle T/$pp60^{c\text{-}src}$ complex depend on a transformation-competent middle T product, while those associated with the PDGF receptor depend on PDGF stimulation of cells. In both cases, a novel PI kinase (Type I) specifically associates with tyrosine kinases and is biochemically distinct from the predominant PI kinase (Type II) activity in fibroblasts. In addition, a middle T-associated PIP kinase activity has been investigated in middle T transformed cells and in nontransformed cells.

Both in vivo and in vitro, we have determined that the phosphatidylinositol phosphates generated by the Type I and Type II PI kinases are chemically separable using HPLC techniques. Moreover, we have found that the PIP_2 product of the middle T-associated PIP kinase can be distinguished from that produced by normal cell PIP kinase. Recent evidence suggests that the distinct products of the Type I PI kinase and the middle T-associated PIP kinase may not serve as substrates for a highly purified phosphoinositidase C. Thus, the existence of these chemically different forms of phosphoinositides suggests a novel pathway for PI turnover that may be active in stimulated and transformed cells.

S 502 A MONOCLONAL ANTIBODY (PL/IM 430) THAT INHIBITS Ca^{2+} SEQUESTRATION IN PERMEABILISED PLATELETS WITHOUT AFFECTING RELEASE OF Ca^{2+} BY IP(3).
Kalwant S. Authi, N. Hack and N. Crawford, Royal College of Surgeons, London, WC2A 3PN, UK.

We describe studies using PL/IM 430, a mAb which immuno recognises a 100 kD polypeptide of platelet intracellular membranes identical to the Ca^{2+} Mg^{2+} ATPase which translocates Ca^{2+} into the endoplasmic reticulum. Studies are carried out using saponin permeabilised platelets and measurements of ^{45}Ca uptake into the endoplasmic reticulum (E.R.) stores using mitochondrial inhibitors. Uptake of ^{45}Ca into the E.R. of saponin-permeabilised (25 µg/ml) platelets is inhibited by 50% by 20 µg/ml PL/IM 430. This inhibition is specific and not shown by other mAb's to platelet components or control mouse IgG (L11/135). Inhibition of ^{45}Ca uptake is linked to degree of permeabilisation of the cells as measured by loss of cytoplasmic enzyme LDH and is also related to specific binding to intracellular sites of ^{125}I-labelled PL/IM 430. IP_3 is potent in releasing Ca^{2+} from permeabilised platelets that have been allowed to sequester ^{45}Ca to steady state levels. In the presence of PL/IM 430 where a lower steady state level is achieved, IP_3 still releases Ca^{2+} with the same kinetics. The EC_{50} for Ca^{2+} release by IP_3 (alone 0.5 µM) was not appreciably different in the presence of PL/1M 430 (EC_{50} = 1.4 µM) when compared to the control mouse IgG L11/135 (EC_{50} = 1.8 µM). These studies indicate that the channel used for Ca^{2+} sequestration is distinct from that used by IP_3 in causing Ca^{2+} release.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 503 INHIBITORY ACTION OF GUANOSINE 5'-O-(2-THIODIPHOSPHATE) ON THROMBIN INDUCED ACTIVATION IN INTACT AND PERMEABILISED PLATELETS. Kalwant S. Authi, G. H. R. Rao and N. Crawford, Royal College of Surgeons, London, WC2A 3PN, U.K.
In saponin permeabilised platelets the stimulatory guanine nucleotide analog GTPγS (60 μM) is an effective stimulus for "Gp" in activating PLC activity as measured by formation of ^{32}P phosphatidic acid (P.A.) and causes aggregation and [^{14}C] 5HT secretion. The inhibitory analogue GDPβS totally inhibits GTPγS stimulated PLC activity, aggregation and 5HT secretion. The ability of GDPβS to inhibit thrombin induced phosphoinositide turnover and functional responses was also studied. GDPβS (1 mM) inhibits thrombin (0.1 U/ml) induced ^{32}P.A formation and aggregation and secretion in saponised platelets supporting a role for Gp in linking receptor occupancy with PLC activation and functional responses. At higher doses of thrombin (> 0.5 U/ml) inhibition of all 3 parameters is progressively reduced. Interestingly GDPβS is also inhibitory to similar extents towards thrombin induced aggregation and secretion in intact platelets even though GDPβS does not penetrate the cell and PLC activity is unaffected indicating that the analogue has additional inhibitory actions on the surface. To probe this further Fura II labelled cells were used. GDPβS inhibits thrombin induced Ca^{2+} mobilisation in a dose dependent manner. Its major effect was in blocking Ca^{2+} entry. Studies were also carried out on clot formation and retraction in platelet rich plasma. GDPβS did not affect clot formation by thrombin but effectively inhibited platelet dependent clot retraction providing further evidence of extracellular inhibitory sites of action.

S 504 PLATELET ACTIVATING FACTOR SIGNALS RAPID TURNOVER OF PHOSPHATIDYLINOSITOL IN P388D1 MACROPHAGES, Paul Bankey, Ann Carlson, Ravinder Singh, and Frank Cerra, University of Minnesota, Minneapolis, 55455.
Recent investigation has linked turnover of membrane phosphatidylinositol (PI) and mobilization of intracellular calcium with cell activation. Specific phospholipases present in the plasma membrane have been described that hydrolyze phosphatidylinositol producing inositol triphosphate, a second messenger for calcium mobilization. We were interested to determine if this signal transduction pathway had a role in macrophage activation.

Turnover of PI was measured in P388D1 macrophages stimulated with platelet activating factor (500nM) and lipopolysaccharide (10 ug/ml). Cells were labelled with H3-inositol and triggered in lithium (10mM) containing media for 5 minutes. Cells were then harvested, extracted with chloroform/methanol, and the aqueous phases fractionated using ion-exchange chromatography. Labelled IP-3 was used as a standard for chromatography. Paired t-test used to determine p-value.

TREATMENT	TOTAL INOSITOL PHOSPHATES (n=3)	IP-3 (% total IP's) (n=2)
Control	77080+/-8535 (CPM+/-SD)	3.0+/-1.3%
LPS	75327+/-12269	4.2+/-0.2%
PAF	97630+/-8422 p=.041 vs.control	7.0+/-0.1% p=.048 vs.control

These results suggest that macrophage activation by PAF is signalled by PI turnover and production of IP-3 while activation by LPS is not signalled by rapid turnover of phosphatidylinositol.

S 505 THE EFFECT OF INOSITOL 1,4,5 TRISPHOSPHATE AND TPA ON INTRACELLULAR CALCIUM AND THE INITIATION OF DNA SYNTHESIS IN RAT LIVER CELLS, **Alton L. Boynton, Timothy D. Hill and Jean Zwiller, Cancer Research Center of Hawaii, University of Hawaii, Honolulu, HI 96813.**
Successful G1-->S transition of most if not all non-neoplastic cells requires an optimal level of extracellular calcium (0.2-0.5 mM). Mitogen stimulation of confluent T51B rat liver cells results in the initiation of DNA synthesis if the extracellular Ca^{2++} level is > 0.2 mM. When the extracellular Ca^{2++} is between 0.05-0.1 mM the mitogen-induced initiation of DNA synthesis is blocked unless Ca^{2++} is added back to the extracellular medium by 8-10 hours. The tumor promoter and protein kinase C activator TPA is able to overcome this Ca^{2++} deprivation and promote G1-->S transition, however, if the extracellular Ca^{2++} is further reduced to < 0.05 mM, TPA is unable to promoter G1--> S transition. Under these low $Ca2^{++}$ (< 0.05 mM) conditions the combination of TPA + A23187 or TPA + Ins(1,4,5)P_3 is able to promote the initiation of DNA synthesis. The effect of both TPA and Ins(1,4,5)P_3 on intracellular Ca^{2++} using digital video imaging of single cells will be presented. Supported by NIH grants CA39745 and CA42942.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 506 **SPECIFIC BINDING OF [^{3}H]INOSITOL(1345)TETRAKISPHOSPHATE TO HL-60 CELL MEMBRANES**
Peter G. Bradford[1] and Robin F. Irvine[2], [1]Hahnemann University, Philadelphia, PA 19102, USA and [2]AFRC Institute of Animal Physiology, Babraham, Cambridge CB2 4AT, UK.

Inositol(1345)tetrakisphosphate [Ins(1345)P_4] is a recently discovered inositol phosphate with potential physiological importance. Like Ins(145)P_3, the second messenger molecule regulating calcium ion release from the endoplasmic reticulum, Ins(1345)P_4 is formed rapidly in a wide variety of cell types in response to calcium-mobilizing hormones and growth factors. Furthermore, Ins(1345)P_4 has been shown to have specific biological activity in sea urchin eggs and mouse lacrimal glands which suggests that in these cells and perhaps in others, Ins(1345)P_4 regulates calcium influx into an Ins(145)P_3-sensitive store. The present study identifies specific binding sites for radiolabeled Ins(1345)P_4 in HL-60 cell membrane preparations. The binding is reversible and saturable exhibiting nanomolar affinity (K_d=90nM) and a density of 750 fmol/mg protein. The apparent affinity of the binding sites for Ins(1345)P_4 is over 200-fold greater than for Ins(145)P_3. Competition studies utilizing a wide variety of inositol polyphosphates indicate that the presence and correct grouping of the phosphates are critical for high affinity recognition. The rank order potency of the inositol polyphosphates in inhibiting [^{3}H]Ins(1345)P_4 binding correlates well with the biological activity of these inositol polyphosphates in sea urchin egg. The data are consistent with these binding sites being biologically relevant and further experiments are being conducted to confirm this.

S 507 THE EFFECTS OF CHRONIC LITHIUM TREATMENT ON AGONIST-STIMULATED INOSITOL PHOSPHOLIPID HYDROLYSIS IN RAT BRAIN, Tamara L. Casebolt and Richard S. Jope, University of Alabama, Birmingham, Alabama 35294.

Lithium is used for treatment of manic-depressive illness, and the therapeutic effects of this drug may be mediated by the inositol phospholipid (PI) second messenger system. We examined the effects of chronic lithium treatment on PI hydrolysis in rat brain slices. [^{3}H]inositol phosphate production was measured in slices from cortex (CX), hippocampus (HC), and striatum (SM) prepared from rats fed diets containing LiCl (1.696g/kg diet) for 1 month and from untreated controls. Chronic lithium treatment significantly reduced the PI response to norepinephrine in each region. The effects of serotonin were reduced in the HC and SM but not the CX. The carbachol-induced response was reduced only in the SM after chronic lithium treatment. These changes were not due to reduced PI substrates or to increased metabolism of inositol phosphates. The NaF-induced response, which is not receptor-mediated, was not altered. These results demonstrate that after chronic lithium treatment, receptor and region-specific changes in PI hydrolysis occur, primarily affecting catecholaminergic-mediated responses. We propose that selective reductions in responses may be due to alterations of receptors or receptor-coupling mechanisms.

S 508 INOSITOL PHOSPHATES AND INOSITOL PHOSPHOLIPIDS FROM THE LEAF-MOVING ORGAN OF A PLANT, Gary G. Coté, Richard C. Crain, M.J. Morse, Anthony DePass, and Ruth L. Satter, The University of Connecticut, Storrs, CT 06268

All components of the phosphatidylinositol (PI) cycle were detected in extracts of pulvini (leaf-moving organs) of the legume, Samanea saman. The soluble inositol phosphates were analyzed by anion-exchange HPLC using a gradient of ammonium formate. The mono-, bis-, and tris-phosphates of inositol were all detected in aqueous extracts of the pulvini. Another metabolite of inositol, tentatively identified as a uronic acid, was also resolved and partially characterized. The inositol phospholipids were analyzed by anion-exchange HPLC using a gradient of ammonium acetate in chloroform/methanol/water. PI, its mono- and bis-phosphate derivatives, and lyso PI were detected in the organic extracts of pulvini. Two species of PI were resolved, presumably differing in attached fatty acids. We are currently investigating temporal variations in the levels of the cycle components and in the sensitivity of PI turnover to illumination.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 509 THE EFFECT OF EGF AND THROMBIN ON PHOSPHATIDYLINOSITOL METABOLISM AND CELL DIVISION IN T51B CELLS, Nicholas M. Dean, Timothy D. Hill, and Alton L. Boynton, Cancer Research Center of Hawaii, University of Hawaii, Honolulu, Hawaii.
The mitogenic effect of EGF on proliferatively quiescent T51B rat liver epithelial cells has previously been shown to be entirely dependent upon extracellular Ca^{2+}, and treatment of cells with EGF results in elevated intracellular Ca^{2+} in an EGF dose dependent manner. The mechanism by which EGF elevates intracellular Ca^{2+} is unclear, however pretreatment of cells with La^{3+} (50 μM) completely blocked the rise. This is in contrast to Ca^{2+} mobilization by thrombin which was completely unaffected by La^{3+} treatment. Recent evidence suggests an involvement of inositol(1,3,4,5)P_4 in modulating membrane Ca^{2+} channels. We have investigated the effects of EGF and thrombin on inositol phosphate production in T51B cells and compared the efficacy of these agents in inducing mitogenesis to enable any involvement of phosphatidylinositol metabolism in cell division to be demonstrated.

S 510 PHOSPHOINOSITIDE METABOLISM IN THE INITIAL STAGE OF DOCA-SALT HYPERTENSION, Hoda Eid and Jacques de Champlain, Université de Montréal, Montréal Canada H3C 3J7.
The phosphoinositide (PI) pathway activity was investigated in right (RA) and left atria (LA), in right (RV) and left ventricle (LV) and in the mesenteric artery (MA) during the development of one model of hypertension (HT) in the rat (DOCA and sodium). During the first week of DOCA and sodium treatment, no abnormalities were observed in the 3H-inositol (3HI) incorporation in any tissues under basal condition or following NE activation. However following 2 weeks of treatment, at a time when blood pressure started to rise, and when cardiac hypertrophy started to develop, the basal and NE stimulated accumulation of IP was found to be increased in RA, LA, RV and MA in the HT rats, while no changes were found in the LV. The incorporation of 3HI was also found increased in the membrane fraction of RA, LA, RV, MA as well as in the LV in both basal and NE activated tissues. The increase in PI pathway activity seems to be dissociated from the number and affinity of α1 receptors since these were found to be normal in the atria and ventricles of HT rats 4 weeks after the beginning of hypertension when IP formation under NE activation was found to be markedly increased in the heart and artery of HT rats. These studies therefore suggest a NE mediated hyperactivation of PI pathway in the cardiovascular tissues of rats treated with DOCA and sodium. Although it is not possible to determine the exact mechanism underlying the PI pathway hyperactivity, it is likely that this alteration could contribute to the development of an α1 adrenoceptor type of hypersensitivity which could facilitate the development of HT and cardiac hypertrophy in this model. (Supported by MRC).

S 511 PARTIAL PURIFICATION OF A PHOSPHATIDYLINOSITOL KINASE ACTIVITY FROM THE YEAST *Saccharomyces cerevisiae*. CATHERINE A. FLANAGAN and JEREMY THORNER, University of California, Berkeley, CA 94720. Little is known about the role of phosphatidylinositol (PI) and its turnover in the yeast *Saccharomyces cerevisiae*. Inositol-requiring auxotrophs (*ino*1 mutants) die rapidly when depleted of exogenous inositol (Culbertson and Henry, *Genetics* 80; 23-40, 1975) and insertional mutations in the gene encoding phosphatidylinositol synthase (*pis*1) are lethal (Nikawa et al. *J. Biol. Chem.* 262: 4876-4881, 1987), indicating that formation of PI is necessary for viability. The critical function of PI may be that it is an indispensable component of cellular membranes. Alternatively, it may be that PI is required for the production of phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol-4,5-*bis*-phosphate (PIP_2) which may participate in an essential signal transduction pathway. It has been reported that readdition of glucose to glucose-starved yeast cells induces PI turnover (Kaibuchi et al. PNAS 83: 8172-8176, 1986), suggesting that PI or its derivatives may have some role in growth control. To delineate the functions of PI and the PI-derived signalling molecules it would be informative to construct a mutation in phosphatidylinositol kinase (PI kinase). Such a mutant would be capable of synthesizing PI itself, but unable to generate PIP (or PIP_2). Toward this end, we have undertaken the purification of PI kinase from yeast cells with the ultimate goal of cloning the structural gene for this enzyme. First, a sensitive and reliable assay for measuring the incorporation of phosphate from γ-^{32}P-ATP into PI was developed. The product comigrates with authentic PIP upon thin layer chromatography in several different solvent systems. In agreement with previous preliminary observations (Talwalkar and Lester, *B.B.A.* 360: 306-311, 1974; McKenzie and Carman, *J. Bacteriol.* 156: 421-423, 1983) two PI kinase activities (one particulate and one soluble) can be separated by differential centrifugation (at 100,000 x g). The interrelationship between these two forms is unclear, and currently under investigation. The soluble activity has been partially purified using both anionic and cationic exchange chromatography. This preparation (~1000 pmol PIP formed/min/mg) has a pH optimum of 7.5, a temperature optimum of 34°C, and an apparent Km for ATP of ~150 μM. As estimated by gel filtration, the activity has an apparent MW >250,000 Kd, suggesting that the enzyme is quite large, or is associated with other proteins.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 512 CELL CYCLE DEPENDENCE OF INOSITOL PHOSPHATE LEVELS IN NEUROBLASTOMA CELLS. Laurie F. Fleischman and Lewis Cantley, Tufts University School of Medicine, Dept. of Physiology, Boston, Ma. 02111.
A role for phosphatidylinositol turnover in growth regulation has been proposed on the basis of the ability of certain growth factors to stimulate this response when added to quiescent (G_o/G_1 arrested) cells. It remains unclear whether PI turnover is a transient response to growth factors in G_0 arrested cells or is continuously activated through the cell cycle or shows cell cycle fluctuations. Exponentially growing neuroblastoma (NB-2a) cells have a relatively fast (11-12 hrs) cell cycle and can be synchronized by a mitotic shakeoff technique that avoids the use of drugs, inhibitors or starvation protocols. In order to investigate the timing of inositol lipid turnover in relation to the cell cycle, inositol phosphates and lipids were measured in NB-2a cells that were pre-labelled with ^{3}H-inositol and synchronized by mitotic shakeoff. Distinct early and late phases of inositol phosphate production were observed. The early peak occurs between the 2nd and 4th hour after mitosis near the G_1/S transition. A later peak occurs around the peak of S phase (DNA synthesis), at 7 ± 8 hours after mitosis. These results suggest that second messengers generated by PI turnover may play a role in signalling progression through the cell cycle.

S 513 INOSITOL PHOSPHATE METABOLISM AND CA^{2+} SIGNALLING IN HEPATOCELLULAR CARCINOMA-DERIVED CELL LINES EXPRESSING DIFFERENT LEVELS OF EGF RECEPTOR. A.L. Gilligan*, M. Prentki**, M.C. Glennon**, and B.B. Knowles*. *The Wistar Institute of Anatomy and Biology, Philadelphia PA 19104 and **The Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia PA 19104. A subline (NPLC/PRF/5) of the human hepatocellular carcinoma derived cell line, PLC/PRF/5, expresses approximately 20 times as many EGF receptors as the parental line. EGF-induced rises in cytosolic Ca^{2+} and in inositol phosphate production were measured to determine whether altered EGF receptor levels lead to changes in signal transduction. Cytosolic Ca^{2+} was measured with the Ca^{2+} indicator, fura 2. Inositol phosphates were isolated from cells metabolically labelled with tritiated myo-inositol and the various isomers separated by anion exchange HPLC. Upon addition of EGF, both cell types exhibit a rapid rise in Ca^{2+} which is independent of extracellular Ca^{2+}. After an initial decrease from peak levels, cytosolic Ca^{2+} remains elevated for at least 10 min. That later component of the Ca^{2+} response is abolished by EGTA pretreatment. NPLC/PRF/5 cells require less EGF to reach maximum cytosolic Ca^{2+} levels. At EGF levels that induce maximal cytosolic Ca^{2+} increases in both cell types (25 ng/ml), inositol 1,4,5-trisphosphate production is stimulated to about the same extent in both cell types 10 to 15 sec after EGF addition. This isomer declines to basal levels in PLC/PRF/5 cells within 2 min but remains elevated in NPLC/PRF/5 cells. Inositol 1,3,4,5-tetrakisphosphate and inositol 1,3,4-trisphosphate are present at low levels 10 to 15 sec after EGF addition but have accumulated to high levels in both cell types within 2 min of EGF addition. At later time points, the levels of these isomers have decreased in PLC/PRF/5 cells but remain elevated in NPLC/PRF/5 cells. EGF binding to either cell type activates inositol phosphate metabolism and generates a complex Ca^{2+} signal. EGF receptor overexpression appears to increase the sensitivity of cells to EGF stimulation and to prolong the inositol phosphate response.

S 514 STEADY STATE LEVELS OF INOSITOL PHOSPHATES AND DIACYLGLYCEROL IN CELLS EXPRESSING POLYOMA VIRUS MIDDLE T PROTEIN, Frank R. Gorga and Thomas L. Benjamin, Dept. of Pathology, Harvard Medical School, Boston, MA 02115. Alterations in phosphatidylinositol (PI) metabolism have been implicated in the action of several oncogenes. Work in other labs as well as our own has shown that PI kinase is associated with the middle T protein (mT) of polyoma virus (Kaplan et al(1987) Cell 50: 1021; Courtneidge & Heber(1987) Cell 50:1031) and that this interaction is dependent upon phosphorylation of mT by c-src (Talmage et al, ms in preparation). In order to directly investigate the role of mT in the regulation of PI metabolism we have measured the steady state levels of inositol phosphates (IPx) and diacylglycerol (DAG) in F-111 rat fibroblasts transformed by polyoma virus (PyF cells) and in rat fibroblasts which express mT protein under control of the regulatable MMTV promoter (MT-1 cells). The levels of all three IPx's were found to be 2 to 3-fold higher in cells expressing the mT protein as compared to control cells. In contrast, no change in DAG levels were found in mT expressing cell lines compared to controls. Analysis of the inositol containing lipids following HClO3 extraction of cells do not show consistent changes due to expression of mT. These results provide direct evidence for mT affecting the activity of enzymes in the "PI cycle", including PI kinase. Since ras transformed cells show increased DAG and no change in IPx, the transformed phenotype can be associated with increased levels of either IPx or DAG but not necessarily both.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 515 Ins(1,3,4,5)P_4 REVERSES ELEVATED Ca^{2+} LEVELS STIMULATED BY Ins(2,4,5)P_3 IN PERMEABILIZED NEOPLASTIC RAT LIVER EPITHELIAL CELLS, Timothy D. Hill and Alton L. Boynton, *Cancer Research Center of Hawaii, University of Hawaii, Honolulu, Hawaii.*
The influence of Ins(1,3,4,5)P_4 on the action of inositol trisphosphates was investigated in electropermeabilized neoplastic rat liver epithelial (261B) cells. Addition of 1 μM Ins(1,4,5)P_3 to 261B cells induced a typical biphasic response (measured with fura-2) composed of a rapid Ca^{2+} mobilization (300-400 nM), and a slower reduction of the $[Ca^{2+}]$ to resting state through activation of Ca^{2+}-ATPases of the ER. 1 μM Ins(2,4,5)P_3 induced a 100-200 nM rise in $[Ca^{2+}]$ which was unaccompanied by sequestration producing a prolonged elevation of free Ca^{2+}. 2 μM Ins(1,3,4,5)P_4 added 1 min prior to 1 μM Ins(2,4,5)P_3 turned the monophasic Ca^{2+} response into the biphasic response produced by Ins(1,4,5)P_3. Ins(1,3,4,5)P_4 had no effect on the $[Ca^{2+}]$ if added after Ins(2,4,5)P_3; or if added with Ins(1,4,5)P_3 (up to 10 μM IP_4 tested). These results indicate that Ins(1,3,4,5)P_4 is capable of altering the $[Ca^{2+}]$ through a stimulation of the Ca^{2+} sequestration mechanism. A continued elevated $[Ca^{2+}]$ induced by the non-metabolized Ins(2,4,5)P_3 is thought to be caused by constant interaction at the receptor mediating Ca^{2+} release. Ins(1,3,4,5)P_4 might compete for the binding site with Ins(2,4,5)P_3, which permits a closure of Ca^{2+} channels and effective movement of Ca^{2+} by Ca^{2+}-ATPases into storage sites. This suggests that the Ins(1,4,5)P_3-induced rise in $[Ca^{2+}]$ is made transient through its metabolism to Ins(1,3,4,5)P_4. Ins(1,3,4,5)P_4 could, as well, mediate its effects by direct interaction with Ca^{2+} release channels or Ca^{2+}-ATPases apart from any influence on binding receptors.
Supported by NIH grant no. CA42942.

S 516 *β-adrenorecptor-mediated $[Ca^{2+}]_i$ mobilization in dispersed rat parotid acinar cells.* V.J. Horn*, B.J. Baum and I.S. Ambudkar, CIPCB, NIDR, NIH, Bethesda, Maryland 20892 U.S.A.

β-adrenoreceptor stimulation of dispersed rat parotid acinar cells by the agonist, isoproterenol, induced a rapid (∿8 sec), 1.6 fold increase (peak) in free cytosolic Ca^{2+} ($[Ca^{2+}]_i$), measured by Quin 2. The $[Ca^{2+}]_i$ response was transient, independent of external Ca^{2+} ($[Ca^{2+}]_e$) and inhibited by the β-adrenergic antagonists, propranolol ($\beta_1+\beta_2$) and ICI-118-551 (β_2). *The muscarinic-cholinergic agonist, carbachol, induced a* rapid (∿6 sec) 3.5 fold elevation (peak) in $[Ca^{2+}]_i$, which was also independent of $[Ca^{2+}]_e$. The $[Ca^{2+}]_i$ increase obtained by successive additions of isoproterenol and carbachol was equal to that elicited by the addition of carbachol alone. The $[Ca^{2+}]_i$ response induced by carbachol, when added after isoproterenol was ∿50% lower in the absence of $[Ca^{2+}]_e$ as compared to that in normal $[Ca^{2+}]_e$. This indicated that isoproterenol and carbachol mobilize the same intracellular Ca^{2+} pool. IP_3 production was increased ∿3.5 fold by carbachol and ∿1.5 fold by isoproterenol. 8 Bromo-cAMP mimicked both the $[Ca^{2+}]_i$ and IP_3 responses mediated by isoproterenol, while propranolol inhibited the IP_3 response significantly. These data are suggestive of an interaction between the β-adrenergic and the muscarinic-cholinergic receptor associated signalling mechanism.

S 517 **THE MODULATION OF PHOSPHATIDYLINOSITOL KINASE ACTIVITY BY HYDROPHILIC AND AMPHIPHILIC CATIONS,** **Eystein S. Husebye and Torgeir Flatmark, Department of Biochemistry, University of Bergen, Arstadvn. 19, N-5009 Bergen, NORWAY.**

The effects of hydrophilic and amphiphilic cations on the activity of phosphatidylinositol (PI) kinase (EC 2.7.1.67) of chromaffin granule membranes were investigated. Free calcium inhibited PI kinase activity in a calmodulin-independent manner. Half maximal inhibition was found at 4 and 280 nM free calcium at 1 and 5 mM Mg^{2+}, respectively. The inhibition was reversible, and no evidence for the involvement of calcium-mediated protein phosphorylation was found. Other cations had more unspecific effects on PI kinase activity. A pure stimulatory effect was observed for Mg^{2+}, as well as for spermidine and the amphiphilic polypeptide mastoparan. Trifluoperazine and structurally related cationic amphiphiles, as well as neomycin and poly-L-lysine, also stimulated the enzyme activity. However, at higher concentrations of these compounds, a progressive inhibition was observed. We conclude that calcium has a specific inhibitory effect on PI kinase activity, indicating that free calcium in the physiological concentration range may provide a negative feedback control on the synthesis of polyphosphoinositides during cell stimulation. Other cations stimulate the enzyme activity rather unspecifically by binding to a membrane component different from that mediating inhibition by calcium. These two sites are probably located on the PI kinase itself. The inhibitory effect of the cationic amphiphiles represents a perturbation of the membrane structure, but not a solubilization of enzyme or phosphoinositide from the membrane. The inhibitory effect of hydrophilic cations is due to chelatation of ATP.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 518 α_1-ADRENERGIC SIGNAL TRANSDUCTION AND PHOSPHATIDYLINOSITOL METABOLISM IN MYOCARDIAL CELL HYPOXIA, Toshifumi Kagiya, Joel S. Karliner, Norman Honbo and Krishna Rocha-Singh. VA Medical Center and Cardiovascular Research Institute, University of California, San Francisco, CA 94121.

Altered adrenergic receptor regulation during myocardial ischemia may be of critical importance in determining myocardial cell viability after an ischemic insult. We investigated the effects of hypoxia on myocardial α_1-adrenergic receptors and phosphatidylinositol(PI) metabolism in single-cell cultures from neonatal rat myocardium. The cultured cells were incubated in normoxic(95% O_2/5% CO_2) or hypoxic (95% N_2/5% CO_2) conditions at 37 ℃ for 2 hr. Medium pO_2 averaged 47 mmHg during hypoxia. α_1-Adrenergic receptors were identified using ^{125}I-HEAT. To assess PI metabolism the cultures were preincubated with [^{3}H]myoinositol for 24 hr and the accumulation of inositol monophosphate (IP1), inositol bisphosphate (IP2) and inositol trisphosphate (IP3) was measured in the presence of 20mM $LiCl_2$. IP1, IP2 and IP3 were separated using anion exchange chromatography. Norepinephrine (NE) stimulation increased the accumulation of IP1, IP2 and IP3, and was completely antagonized by terazosin, an α_1-selective antagonist. Pertussis toxin (1 μg/ml, 24 hr) had no effect on NE-stimulated accumulation of all three IPs. Hypoxia for 2 hr increased the number of α_1-receptors (22.4 ±3.7 to 33.0±5.6 fmol/ mg protein; mean± SEM, n=6, p<.01), whereas Kd was unchanged. Basal levels of IPs were increased in the hypoxic cells compared with the normoxic cells (IP3 59.2±1.2 to 91.6±7.1 fmol/mg protein; n=3, p<.05). The content of all three IPs was not significantly different after NE stimulation (1 μM) between the normoxic and the hypoxic cells. These results indicate that prolonged hypoxia augments basal PI metabolism. Despite a 47% increase in α_1-receptor density, NE-stimulated IP accumulation is not enhanced, suggesting that hypoxia results in uncoupling of α_1-receptor mediated signal transduction.

S 519 BRADYKININ AND THROMBIN EFFECTS ON POLYPHOSPHOINOSITIDE HYDROLYSIS AND PROSTACYCLIN PRODUCTION IN ENDOTHELIAL CELLS. K. Bartha, L.A.A. van Rooijen and R. Müller-Peddinghaus, Department of Inflammation Research and Neurobiology Department, Troponwerke GmbH & Co. KG, Cologne, F.R.G.

Bovine aortic endothelial cells (BAEC) possess receptors for bradykinin (BK) and thrombin (T). Both compounds are known to stimulate inositol phosphate (IP_n) production in several cell types. In the present study we followed the production of ^{3}H-IP_n (IP_1, IP_2, IP_3) from cultured BAEC after isotopic labelling with [^{3}H]-myo-inositol and separation on Dowex anion-exchange chromatography. BK caused a rapid 3-fold increase in IP_3 and IP_2, within 30 sec, returning to baseline in 3 min. Stimulation by BK of IP_1 was much slower and lasted for at least 10 min. T caused only minimal stimulation of IP_n. BK also induced the production of prostacyclin measured as 6-keto-prostaglandin $F_{1\alpha}$ in BAEC. Prostacyclin synthesis followed the IP_3 and IP_2 formation, reaching a maximum at 5 min. The BK stimulation was dose dependent and similar for the IP_n and prostacyclin responses. T on the other hand induced a minimal rise in prostacyclin and IP_n production. In contrast, in human umbilical vein endothelial cell (HUVEC) culture, T induced a rise in IP_3 and IP_2 and stimulated prostacyclin production, thus supporting the notion that BAEC and HUVEC respond differently to T. In other experiments using the more sensitive HPLC analysis of the IP_n, an increase in IP_3 and IP_2 after T stimulation of BAEC could be detected, in an antithrombin III sensitive manner. Following stimulation with T as well as with BK, the effects were observed on the 1,4-IP_2 and 1,4,5-IP_3 isomers, while accumulation of 1,3,4-IP_3 was not apparent. Studies are underway to investigate the involvement of possibly differential IP_n responses in endothelial cell activation by T and BK.

S 520 INOSITOL LIPIDS IN INTACT VASCULAR SMOOTH MUSCLE, Edward F.LaBelle,The Graduate Hospital,Philadelphia,PA. 19146

The thoracic aorta of the rat was divided into rings of 4mm in length and each ring incubated with $^{32}P_i$. The rings were then placed in a mortar that had been prechilled with liquid N_2 and homogenized with a chilled pestle together with frozen 2.4M HCl. After the mixture had been thawed it was extracted with $HCCl_3$/MeOH and the lipids separated by TLC. The most rapidly labeled lipids were PIP_2 and PIP which became uniformly labeled within 2-3 hrs. PI and PC became labeled more slowly after a brief lag. Labeled lipids were detected that appeared to migrate in the same position on the TLC plate as did the PI glycans detected recently in liver. When rings were pre-equilibrated with $^{32}P_i$ and treated with vasopressin, the vasopressin stimulated the breakdown of PIP and PIP_2 within 30s. When rings were pre-equilibrated for 6 hrs with [^{3}H] inositol norepinephrine was shown to greatly increase the concentration of IP and IP_2 within the rings. Studies currently in progress will determine the sensitivity of inositol lipid metabolism in the rings to neomycin. Mechanical measurements on the aortal rings should help to prove that hormones such as vasopressin stimulate the contraction of vascular smooth muscle via the enhanced breakdown of PIP_2. (Supported by Grant HL 37413 from NIH)

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 521 A VERY RAPID DECREASE OF PHOSPHATIDYLINOSITOL TURNOVER OCCURS ON INDUCTION OF NEUROBLASTOMA CELL DIFFERENTIATION. M. Lanciotti and M. Ponzoni. Ped. Oncol. Research Lab., G. Gaslini Institute, Genova (Italy).

The cellular levels of certain phosphatidylinositol (PI) metabolites play key roles in the control of proliferation, transformation and differentiation. We investigated the role of PI turnover in differentiation using LAN-1 cells, a human neuroblastoma cell line which can be induced to differentiate along the neuronal pathway by retinoic acid (RA).
The occurrence of inositol phospholipid hydrolysis upon treatment with RA was investigated by monitoring the generation of the two putative second messengers, inositol (1,4,5) trisphosphate (IP_3) and (1,2) diacylglycerol (DG). LAN-1 cells, prelabelled for 24 hours with myo(1,2-^{3}H)-inositol, were subsequently treated with RA in the presence of an excess amount of cold inositol and extracted at different times for analysis of water soluble radioactivity. A very rapid decrease in the amount of labelled inositol 1-monophosphate inositol 1,4 bisphosphate and inositol 1,4,5 trisphosphate were observed in RA-treated cells. Using (^{3}H)-glycerol we found a rapid decrease of DG within 1-2 minutes of induction of LAN-1 cell differentiation. These findings indicate the occurrence of decreased inositol phospholipid turnover in RA treated LAN-1 cells and suggest that phosphoinositide-derived metabolites may constitute a "second messenger" in the RA response.

S 522 AUXIN INDUCES RAPID CHANGES IN PHOSPHATIDYLINOSITOL METABOLITES L.Lehle and C.Ettlinger, Universität Regensburg, 84 Regensburg, FRG

The plant growth hormone auxin is involved in regulating such diverse processes as cell elongation, cell division and differentiation. Evidence for auxin receptors at the plasmamembrane has been reported, but the signal-transduction pathway is not known, for this nor for other plant hormones. We have found that auxin can generate transient changes in Ins(1,4,5)P_3 and $InsP_2$ within minutes in Catharanthus roseus cells arrested in G_1. These changes are accompanied by a redistribution within the polyphosphoinositide fraction. The biologically inactive auxin analogue 3,5-D did not trigger hydrolysis of phosphoinositides. When Li^+ was added, a second trisphosphate isomer with the chromatographic behaviour of Ins(1,3,4)P_3 was observed. This may indicate that the alternative pathway for metabolizing Ins(1,4,5)P_3 via formation of inositol1,3,4,5-tetrakisphosphate found recently in animal cells also exists in plants. As the physiological response to auxin addition is to relieve the arrest in G_1, it is suggested that these effects are an element in the signal transduction of this plant hormone

S 523 ISOLATION OF 1-MONOMETHYLPHOSPHOINOSITOL 4,5-BISPHOSPHATE (A PRODUCT OF METHANOLYSIS OF INOSITOL 1,2-(CYCLIC)-4,5-TRISPHOSPHATE) FROM SWISS MOUSE 3T3 CELLS, Daniel L. Lips, Teresa E. Bross, and Philip W. Majerus.

We have noted two previously undescribed inositol polyphosphates in neutral methanol extracts from Swiss mouse 3T3 cells that were grown in [^{3}H]inositol and stimulated with PDGF. They have been identified as 1-monomethylphosphorylinositol 4,5-bisphosphate and 1-monomethylphosphorylinositol 4-phosphate by comparison to a synthesized standard using HPLC chromatography, paper electrophoresis, and enzymatic dephosphorylation with inositol polyphosphate 5-phosphomonoesterase and intestinal alkaline phosphatase. We propose that these compounds are formed by methanolysis of inositol 1:2(cyclic)4,5-trisphosphate and inositol 1:2(cyclic)4-bisphosphate present in the cells. Inositol cyclic phosphates did not react with neutral methanol in the absence of cells which are required for the methanolysis reaction. These findings suggest a possible role for inositol cyclic phosphates as reactive compounds that are added to as yet unidentified cellular acceptors.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 524 HIGHLY COOPERATIVE GATING OF Ca^{2+}-EFFLUX BY (1,4,5)-INOSITOLTRISPHOSPHATE: A MOLECULAR MECHANISM FOR RAPID INTRACELLULAR Ca^{2+}-TRANSIENTS

T.Meyer+, D.Holowka*, L.Stryer+; +Stanford University,Stanford, CA 94305; *Cornell University, Ithaca, NY 14853.

We investigated Ca^{2+}-efflux rates from the endoplasmic reticulum in saponin-permeabilized tumor mast cells (RBL) using a fluorescence assay in which Fura-2 served as a Ca^{2+} indicator. The initial Ca^{2+}-efflux rates after additions of IP_3 were determined. The rate of Ca^{2+}-efflux increases with the third power of the IP_3 concentration, indicating that channel opening requires the binding of three IP_3 molecules. Complete depletion of the Ca^{2+} stores was achieved within a few seconds after addition of 20 nM IP_3. These experiments were performed under conditions where no Ca^{2+} uptake into the ER and no degradation of IP_3 could be observed (11 °C,no ATP, no Mg^{2+}). Kinetic arguments derived from these flux studies and a high transport rate of Ca^{2+} per ^3H-IP_3 bound (determined in binding studies) emphasize the activation of an IP_3-receptor/channel complex. Based on these results we present a model that can explain the observation that many receptor stimulated cells do not increase their Ca^{2+}-levels gradually but fire rapid and periodic Ca^{2+}-transients (N.M.Woods et al. Nature, 319:600-602). This model postulates a positive feedback mechanism involving known enzymatic activities: receptor and Ca^{2+}-stimulated phospholipase C, cooperative IP_3-gated Ca^{2+}-release, ATP-driven pumping of Ca^{2+} out of the cell and into the endoplasmic reticulum, a steady Ca^{2+}-leakage over the plasma membrane and a IP_3-phosphatase activity. Two major predictions of this model are: 1. The intracellular stored Ca^{2+} is released by a switch mechanism. When the receptor activation of phospholipase C reaches a certain threshold, all stored Ca^{2+} is released on a subsecond time scale. 2. The frequency of Ca^{2+}-spikes depends on the degree of activation of phospholipase C.

S 525 LIGHT-MODULATED PHOSPHATIDYLINOSITOL TURNOVER IN THE LEGUME SAMANEA SAMAN, M.J.Morse R.C.Crain, G.G.Cote and R.L.Satter, University of Connecticut,Storrs, CT 06268

The circadian rhythmic leaf movements of Samanea saman are driven by an endogenous clock but are modulated by light. Light signals may utilize accelerated phosphatidylinositol turnover as a means of transducing the signal within plant cells. We have evidence that a brief (30 second) pulse of white light stimulates phosphatidylinositol turnover as measured by decreases in polyphosphoinositides and increases in inositol phosphates. We are now using specific wavelengths of light to assess the relative contribution of phytochrome and cryptochrome in light absorption and signal reception. We also have very preliminary evidence of light-stimulated changes in the level of diacylglycerol.

S 526 ARGININE VASOPRESSIN STIMULATES THE FORMATION OF PHOSPHATIDIC ACID AND INOSITOL PHOSPHATES IN RAT LEYDIG CELLS.

Jane R. Nielsen, Harald S. Hansen and Benny Jensen, Biochemical Laboratory, Royal Danish School of Pharmacy, Universitetsparken 2, 2100 Copenhagen Ø, Denmark.

We have previously demonstrated that arginine vasopressin (AVP) via V_1-receptors stimulates phosphatidic acid-turnover in rat Leydig cells (1). These studies have now been extended.

Leydig cells were preincubated with [^{14}C]-arachidonic acid. In the presence of 10^{-6}M AVP for 2 minutes we observed a significant rise in labelled phosphatidic acid and a significant fall in the label of phosphatidylinositol (PI) and phosphatidylinositol 4-phosphate + phosphatidylinositol 4,5-bisphosphate (PIP + PIP_2).

Futher, Leydig cells have been preincubated with myo-[2-^{3}H]-inositol for 22 hours and with 10 mM LiCl for 10 minutes. Exposure to AVP (10^{-6}M) induced a rapid rise in labelled inositol trisphosphate, inositol bisphosphate and inositol phosphate. These results together with our previous results (1) provide evidence for a V_1-receptor mediated phospholipase C stimulation in rat Leydig cells.

(1) Jane R. Nielsen et al. (1987) FEBS Letters 218, 93-96.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 527 TURNOVER OF INOSITOL PHOSPHATES DURING NEOPLASTIC DEVELOPMENT
Helena E Nilsson and Lennart C Eriksson, Dept of Pathology, Huddinge Hospital, S - 141 86 Huddinge, SWEDEN.

The formation and breakdown of different phosphorylated inositol compounds - inositol phosphates and their corresponding phospholipids - have during the last ten years been shown to be an important step in the transducing of different signals from the cell surface to its interior. In many cases, these signals are closely related to growth control, either by proliferation stimulation or inhibition.
In some cell populations with altered growth properties, eg tumor cell lines, it has been shown that the inositol system is somewhat changed, both in regard to total amounts and turnover pattern after agonist stimulation.
We have studied these two parameters in normal rat hepatocytes and in liver nodules. These nodules are regarded as preneoplastic lesions which, besides having a larger than normal growth fraction, also show an altered response to growth factors. Preliminary results indicate some interesting differences in the answer to agonist stimulation between the two cell types, eg the nodular hepatocytes seem to be uncapable of synthesizing inositol tetrakisphosphate (IP_4). In response to the hormone vasopressin, the normal cells show a six-fold increase of (IP_4), which we have not been able to find in nodular cells. The formation of inositol trisphosphate (IP_3) is quicker than normal in the nodular cells, but extend to the same level.

This work was supported by grants from the Swedish Cancer Society.

S 528 A ROLE FOR INOSITOL TRISPHOSPHATE IN LIGHT-REGULATED GRAVITROPISM, Donna O. Perdue, A. Carl Leopold, Cornell University and Boyce Thompson Institute, Ithaca NY 14853.

Certain corn varieties require exposure of root caps to red light in order to exhibit positive gravitropic behavior. In the hybrid variety "Merit", red light switches the transductive mode from diageotropic to orthogravitropic; we find that Ca++ fluxes have an essential role in this light-regulated switching. It is possible to mimic the effect of red light on etiolated roots by treatments known to trigger Ca++ influx into the cytosol of corn root cells (A23187, cold or heat shock) or by treatments known to cause turnover of inositol phospholipids (serotonin, deoxycholate, 2,4-D). We hypothesized that the phototransduction system for red light may be the inositol trisphosphate (IP^3) second-messenger system. Labelled root tips were exposed to light, physical, or chemical treatments and analyzed for inositol phosphates. All of the following treatments caused an elevation of IP^3 levels: red light, heat shock, deoxycholate, 2,4-D. Our red light data agree with the report of Morse et al (PNAS 84: 7075-7078) demonstrating red-light-stimulated increases in IP^3 levels accompanied by inositolphospholipid turnover in green plant tissues. The other treatments described here mimic red light effects on etiolated "Merit" roots at two levels: at a physiological level, detected by their effects on root gravitropism, and at a biochemical levle, as seen by their stimulation of IP^3 release. These results suggest that the IP^3 second-messenger system may function in the transduction of many diverse stimuli in plants. Such results further suggest that the treatments capable of substituting for red light achieve their physiological effects by their action as agonists of the same second-messenger as that used in phototransduction.

S 529 HIGH AND LOW AFFINITY INOSITOL TETRAKISPHOSPHATE BINDING SITES IN BOVINE ADRENAL CORTICAL MICROSOMES, Peter Enyedi and Gordon H. Williams, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115.

Accumulating evidence suggests that inositol-1,3,4,5-tetrakisphosphate ($InsP_4$) can have intracellular messenger function. $InsP_4$ formation in response to Ca-mobilizing hormones has been detected in several cell types including adrenal cortical cells and we have searched for $InsP_4$ binding sites in this tissue. ^{32}P-labelled $InsP_4$, with high specific radioactivity was used as the tracer, prepared from ^{32}P-$InsP_3$ by phosphorylating it with a specific kinase. A crude microsomal fraction, prepared from bovine adrenal cortex was used in the binding experiments. The incubations were performed at °C in a medium composed of KCl, 100 mM; NaCl, 20 mM; EDTA, 1mM; bovine serum albumin, 0.1%; buffered with sodium phosphate, 25 mM (pH 7.2). The bound and unbound radioactivity were separated by filtration through GF/B (Whatman) filters. Binding of ^{32}P-$InsP_4$ to the microsomes was rapid, with equilibrium reached by 15 min. Subsequent addition of excess amount of unlabelled $InsP_4$ resulted in a time dependent decrease in the bound radioactivity with kinetics indicating that $InsP_4$ binds to two kinetically distinct biding sites having 12 sec and 2.5 min half lifes respectively. Scatchard analysis of the displacemet curve (under equlibrium condition) also reveals the existence of high ($3.68\pm1.02\times10^{-9}$ M) and low ($9.02\pm1.71\times10^{-8}$ M) affinity binding. Competition studies with other inositol phosphate analogs ($InsP_5$, $Ins(1,3,4)P_3$, and $Ins(1,4,5)P_3$) indicate that the binding is specific, with $InsP_5$ showing the highest crossreactivity being about 40 times less effective than $InsP_4$. Thus the characteristics of the binding sites are compatible with their possible receptor function.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 530 PURIFICATION AND CHARACTERIZATION OF A PHOSPHATIDYLINOSITOL 4-KINASE FROM BOVINE UTERI, Forbes D. Porter, Yue-Sheng Li and Thomas F. Deuel, Jewish Hospital at Washington University Medical Center, St. Louis, MO 63110.
The involvement of products of the inositol lipids in signal transduction by growth factors such as platelet derived growth factor (PDGF) has been recently established. In order to study the role of inositol lipid metabolism in cell activation by growth factors, we have purified and characterized a phosphatidylinositol 4-kinase (PtdIns 4-kinase) from bovine uteri. This enzyme has been purified over 10,000 fold to near homogeneity by ion exchange and hydroxylapatite chromatography, and by chromatofocusing. The purified enzyme has a molecular mass of ~55 kDa on SDS/PAGE, appears to be monomeric, and has a specific activity of ~2.7 μmol/mg/min. Kinetic analyses of the enzymatic activity demonstrated apparent Km's for ATP and phosphatidylinositol of 18 μM and 22 μM respectively, and a sigmoidal dependence of enzymatic activity on $[Mg^{2+}]$. Ca^{2+} inhibited the enzyme at non-physiological concentrations with 50% inhibition observed at a free calcium concentration of ~300 μM. The purified PtdIns 4-kinase utilized both ATP and 2'-deoxyATP and to a lesser extent 2',3'-dideoxyATP as a phosphoryl donor, and specifically phosphorylated phosphatidylinositol on the fourth position. No phosphatidylinositol-4-phosphate 5-kinase activity was observed in the purified enzyme preparations. The purified enzyme is being used to study possible regulation by divalent cations, protein kinases, phosphoinositides, and phosphoinositide metabolites.

S 531 REGULATION OF POLYPHOSPHOINOSITIDE METABOLISM IN ISOLATED PLANT MEMBRANES. Anna Stina Sandelius and Marianne Sommarin, Departments of Plant Physiology, Universities of Göteborg and Lund, SWEDEN.

Signals causing fluxes of Ca^{2+} in animal cells utilize turnover of a plasma membrane localized phospholipid, phosphatidylinositoldiphosphate (PIP_2) to transfer the signal information from the cell exterior to the interior. Ca^{2+} is known to affect a number of physiological processes also in plants and we are investigating the possible role of polyphosphoinositide metabolism in connecting signal perception and mobilization of Ca^{2+} in plant cells. We have established the presence of phosphatidylinositol (PI) and phosphatidylinositolmonophosphate (PIP) kinases in plasma membranes isolated from shoots and roots of wheat seedlings (Sandelius and Sommarin 1986 FEBS Lett. 201, 282) and shown that there are close resemblances between the characteristics of PI and PIP kinase activities of plant plasma membranes with corresponding activities reported for various animal membranes, in eg. ATP dependency, ion requirement and pH optima (Sommarin and Sandelius 1988 BBA, In Press). Furthermore, the isolated plant plasma membranes possess Ca^{2+}-stimulated phospholipase C activities, hydrolysing PIP and PIP_2 at similar rates, but PI only marginally (Melin, Sommarin, Sandelius and Jergil 1987 FEBS Lett. 223, 87). The phospholipase C activities of the isolated membranes depended on whether the plants had been grown in light or in darkness, while the substrate specificity remained unaffected by the growth conditions.

S 532 HIGH EXTRACELLULAR CA^{2+} AND OTHER DIVALENT CATIONS STIMULATE $InsP_3$ ACCUMULATION AND INHIBIT PARATHYROID HORMONE RELEASE IN BOVINE PARATHYROID CELLS, Dolores Shoback and Linda Membreno, Veterans Administration Medical Center, University of California, San Francisco, CA 94121.
Changes in the extracellular (EC) $[Ca^{2+}]$ regulate the secretion of parathyroid hormone (PTH). The addition of Ca^{2+} or other divalent cations to bovine parathyroid cells rapidly increases intracellular free Ca^{2+} ($[Ca^{2+}]i$) and suppresses PTH release. To determine whether polyphosphoinositide turnover might mediate the responses of parathyroid cells to these cations, we quantified inositol polyphosphates (InsPs) by anion-exchange chromatography after labelling parathyroid cells with ^3H-myoinositol. Raising EC $[Ca^{2+}]$ from 0.5 to 2.0 or 3.0 mM produced 95 and 134% increments in $InsP_3$ respectively within 5-15 seconds. $InsP_2$ and $InsP_1$ increased 2-4 fold thereafter. Within 10 seconds of adding 2 mM $BaCl_2$, 2 mM $MnCl_2$, or 4 mM $SrCl_2$ to parathyroid cells at 0.5 mM EC Ca^{2+}, $InsP_3$ rose 56, 152, and 160% respectively. High EC Ca^{2+} as well as Ba^{2+}, Mn^{2+}, and Sr^{2+} inhibited PTH release ~50-75% compared to maximal, low Ca^{2+}-stimulated PTH release (at 0.5 mM Ca^{2+}). Since Ca^{2+} and divalent cations increase $[Ca^{2+}]i$ and depolarize parathyroid cells, we tested the effects of the Ca^{2+}-ionophore ionomycin and high EC K^+ on InsPs. While ionomycin inhibited PTH release in a dose-dependent manner, neither ionomycin (500 nM) nor depolarizing concentrations of EC K^+ (40 mM) reproduced high EC Ca^{2+}- or divalent cation-induced increments in InsPs. We conclude that parathyroid cells express membrane sensors for Ca^{2+} and other divalent cations linked to intracellular Ca^{2+} mobilization, $InsP_3$ formation, and inhibition of PTH release.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 533 MANGANESE ACUTELY ATTENUATES SECRETAGOGUE-MEDIATED INOSITOL-1-PHOSPHATE ACCUMULATION IN AR42J CELLS. Steven A. Siwik and Murray Korc, University of Arizona College of Medicine, Tucson, Arizona 85724.

The exocrine pancreas has been a useful model for studying stimulus-secretion coupling and receptor-mediated phosphatidylinositol (PI) hydrolysis. Cholecystokinin octapeptide (CCK_8) and the nonapeptide bombesin bind to distinct cell surface receptors in the pancreas and stimulate PI turnover, $^{45}Ca^{2+}$ efflux, and digestive enzyme secretion. The divalent cation manganese (Mn) also enhances pancreatic enzyme secretion and $^{45}Ca^{2+}$ efflux, but is not believed to activate membrane PI metabolism. In the present study we examined the effects of CCK_8, bombesin, and Mn on PI metabolism in the AR42J cell line, which consists of a homogeneous population of relatively well differentiated rat pancreatic cancer cells. CCK_8 and bombesin induced an accumulation of tritiated inositol-1-phosphate [^{3}H]I-1-P in a dose-dependent manner. Maximal stimulation for both agonists occurred at a concentration of 30 nM, resulting in a 19- and 16-fold increase in [^{3}H]I-1-P over control values for CCK_8 and bombesin, respectively. In contrast, Mn (1uM - 10 mM) did not alter [^{3}H]I-1-P levels when incubated with cells for up to 1 hour at 37°C. When added to the incubation medium 5 min prior to the addition of polypeptide secretagogues, however, Mn attenuated CCK_8- and bombesin-mediated [^{3}H]I-1-P accumulation by 25 percent ($p<0.005$) and 40 percent ($p<0.05$), respectively. These findings suggest that the AR42J cell line will provide a useful model for further characterizing the effects of Mn on receptor-mediated PI turnover.

S 534 PTH INDUCES CREATINE KINASE ACTIVITY AND DNA SYNTHESIS VIA THE PHOSPHOINOSITOL PATHWAY. D. Somjen, U. Zor*, A.M. Kaye*, A. Harell and I. Binderman, Hard Tissues Unit, Ichilov Hospital, Tel Aviv 64239 and *Department of Hormone Research, The Weizmann Institute of Science, Rehovot 76100, Israel.

PTH stimulates the activity of adenylate cyclase and creatine kinase BB (CKBB) and increases DNA synthesis in osteoblast-like cells in culture. The mechanism by which PTH induces CKBB activity and DNA synthesis is not known, since it is not mediated by cAMP production. Confluent osteoblast-like cell cultures, derived from rat embryo calvaria, were treated with PTH which increased cAMP formation (within 15'), CKBB activity and DNA synthesis (within 24 hours). The following results lead us to conclude that PTH induction of CKBB and DNA synthesis is via activation of membranal phospholipid metabolism as was suggested for the action of growth factors: 1) Blocking of phospholipid activity by gentamicin (100 µg/ml) or antiphospholipid antibodies (0.1%), abolishes the stimulation of CKBB and DNA synthesis but not cAMP production by PTH. 2) Treatment of cell cultures with phospholipase C (but not A or D) increased CKBB and DNA synthesis but not cAMP production; this reaction was blocked by antiphospholipid antibodies. 3) PTH and phospholipase C had an additive effect on stimulation of DNA synthesis (but not on CKBB). 4) Oleyldiacylglycerol, together with Ca^{2+} ionophore, induces CKBB and DNA synthesis in bone cells. This effect was not blocked by antiphospholipid antibodies; PTH had no additional effect. 5) Phorbol ester also induced CKBB and DNA synthesis. These data strongly suggest that the induction by PTH of CKBB and DNA synthesis is via activation of membranal phospholipase C leading to activation of protein kinase(s) which via specific protein phosphorylation presumably modulate protein and DNA synthesis.

S 535 COMPARISON OF INOSITOL PHOSPHATE ACTION IN AN INTACT CELL. Bradley J. Stith, and William H. Proctor*, University of Colorado at Denver and*Univ. of Colorado Health Sci. Center, Denver, CO 80202.

Stock concentrations (100-1000 uM) of inositol trisphosphate (IP3), inositol tetrakisphosphate (IP4), and cyclic-inositol trisphosphate (cIP3; gift of P. Majerus) were pressure-injected (Picospritzer II, General Valve) into Xenopus oocytes while the membrane potential was continuously recorded. All three inositol phosphate compounds produced an early (D_1) and late (D_2) membrane depolarization. The dose-response relationship of each inositol was determined by the intracellular concentration (EC50) which produced a half-maximal late depolarizing response (D_2). The mean EC50 ± s.e.m. and the number of cells (n) tested for each compound were as follows: IP3, 88 ± 16.5 nM (n=10); cIP3, 86 ± 29 nM (n=7); and IP4, 3440 ± 55 nM (n=7). Microinjection of calcium chloride mimicked the early and late depolarizations whereas coinjection of EGTA with each inositol phosphate derivative blocked these responses. Injection of inositol-1-phosphate alone produced no detectable membrane change. Therefore, these results suggest that IP3, cIP3, and IP4 can each trigger an increase in intracellular calcium that results in an increase in chloride efflux through calcium-sensitive chloride channels. Although IP4 is capable of producing this effect, it must be present at a 40-fold higher concentration than IP3 or cIP3.

Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production

S 536 AN INOSITOL TETRAKISPHOSPHATE CONTAINING PHOSPHOLIPID IN ACTIVATED NEUTROPHILS Alexis E. Traynor-Kaplan, Anna L. Harris, Barbara L Thompson*, Palmer Taylor*, Larry A. Sklar Dept. of Immunol. Scripps Clinic and Res. Fdn., La Jolla, CA 92037, *Dept. of Pharmacol., Univ. of Cal., San Diego, La Jolla, CA 92093.

Although sought as a source for $InsP_4$, $PtdInsP_3$ has not been identified in any specific cell type. Using two methods for deacylation of phospholipids we have identified an inositol phosphate coeluting with Ins 1,3,4,5 P_4 in the hydrolyzates of deacylated phospholipids from activated but not from unstimulated neutrophils. Phospholipids from neutrophils labelled with ^{32}P and stimulated for 45 sec. with formyl peptide were separated by TLC. HPLC of the deacylated eluates from TLC scrapings of an area beneath $PtdInsP_2$ has been found to contain $GroPIns(1,4)P_2$, $GroPIns(1,4,5)P_3$ and an unknown peak. The unknown peak was collected, the glycerol was removed and the product resubjected to HPLC. 98% of the counts in the unknown peak now coeluted with Ins 1,3,4,5 P_4. We have gone on to measure $InsP_4$ containing lipid directly in phospholipid extracts using monomethylamine instead of Na methoxide as a means of deacylation. After the subsequent hydrolysis of the glycerol, a peak eluting with Ins 1,3,4,5 P_4 was again observed. Therefore we have identified an $InsP_4$ containing phospholipid using two separate methods of degradation.

S 537 INOSITOL PHOSPHATE GENERATION DURING HUMAN NATURAL KILLER CELL ACTIVATION. K. P. Windebank, R. T. Abraham, T. J. Barna and P. J. Leibson, Mayo Clinic, Rochester, MN 55905.

Natural killer (NK) cells can recognize and kill a wide variety of malignant and virally-infected cells. In order to determine the signalling events that initiate this lethal process, we evaluated the generation of intracellular second messengers during NK cell-mediated cytotoxicity. Cloned human NK cells ($CD16^+/CD3^-$) were pre-labelled with ^3H-myo-inositol and then mixed with selected tumor targets. Within seconds of recognizing NK-sensitive targets, natural killer cells exhibited marked increases in phosphoinositide hydrolysis, as measured by accumulation of inositol phosphates. Conversely, targets which were resistant to lysis by NK cells did not stimulate inositol phospholipid turnover. Although elevations in cAMP are known to modify NK cell activation, treatment of NK cells with forskolin/IBMX did not significantly alter their ability to generate inositol phosphates. This suggests that cAMP exerts its effects on biochemical events distal to activation of phospholipase C.

NK cells also mediate antibody-dependent cellular cytotoxicity via a surface receptor (CD16) for immunoglobulin constant regions (Fc receptors). Activation of the human NK cell lines via CD16 (using anti-CD16 monoclonal antibody) also resulted in intracellular inositol phosphate generation. Taken together, these data indicate that polyinositide-derived second messengers play a central role in the signalling mechanism that couples target cell recognition by NK cells to subsequent NK cell-mediated lysis.

S 538 STIMULATION BY INOSITOL TRISPHOSPHATE, TETRAKISPHOSPHATE AND CALMODULIN OF A PROTEIN PHOSPHATASE, **Jean Zwiller and Alton L. Boynton, Cancer Research Center of Hawaii, University of Hawaii, 1236, Lauhala Street, Honolulu, HI 96813.**

Several inositol trisphosphate isomers (inositol (1,4,5) trisphosphate being the more potent) and inositol tetrakisphosphate activate a rat brain phosphoprotein phosphatase, using phosphohistone as well as phosphorylase kinase as substrate. Inositol mono- and bisphosphate have no effect. This protein phosphatase is also activated by calmodulin in the absence of calcium. The protein phosphatase is not calcineurin since it exhibited calcium-independency; was separable from calcineurin on ion-exchange chromatography; and was inhibited by inhibitor-2, suggesting that it may correspond to type-1. It is suggested that the catalytic subunit of the protein phosphatase is stimulated on distinct sites by calmodulin and by inositol (1,4,5) trisphosphate. This implies that the activation of the calcium messenger system results in a high rate of dephosphorylation induced by these two second messengers. Supported by NIH grant CA39745.