Instituto Juan March
de Estudios e Investigaciones

139 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Lipid Signalling: Cellular Events and their Biophysical Mechanisms

Organized by

E. A. Dennis, A. Alonso and I. Varela-Nieto

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J. Chun
S. Cockcroft
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Introduction

E. A. Dennis, A. Alonso and
I. Varela-Nieto

Lipid signaling cellular events and their biophysical mechanisms

The last decade has witnessed the discovery of a large number of lipid molecules that play specific roles in cell signaling. Ceramide, sphingosine phosphate, lysophosphatidic acid and others have joined the previously known phosphatidylinositol derivatives, diglyceride, and phosphatidic acid in their roles as metabolic regulators. Thus, phospholipids and sphingolipids, in addition to their structural involvement in membranes, are now viewed as important reservoirs of lipid second messengers.

Current research in this field includes studies in at least three areas, namely cell biology, biochemistry and biophysics, sometimes without the desirable degree of interaction between them. From the point of view of cell physiology, lipid signals have been found to mediate an amazing variety of processes, from cell activation to apoptosis, including ion channel regulation, intracellular membrane trafficking and membrane adhesion. Simultaneously, metabolic studies of these substances have led to significant advances in understanding the mechanism and regulation of biosynthetic and degradative enzymes, notably phospholipases A2, C and D, sphingomyelinases and CTP:phosphocholine cytydyltransferase. Finally, recent biophysical studies have led to important discoveries in the structure of some of the involved enzymes (e.g. PIP3 kinase, PI-phospholipase C), in the behaviour of lipid signals in bilayers, (e.g. ceramide segregation into domains and rafts), or in the regulation of important enzymes through membrane physical properties (e.g. PI-specific phospholipase C), that may provide the molecular foundation for an understanding of critical lipid-mediated cellular processes.

Moreover the genomics revolution has been impressive and innovative for the biological sciences and as difficult as it was, it has only had to deal with a finite number of genes, estimated to range from 30,000-50,000 for humans. The proteomics revolution is upon us, but the number of discrete proteins is enormous and certainly not finite. Proteins come in many forms; they are acylated, acetylated, phosphorylated, and ubiquinated and exist as preproteins and proproteins, and they can be altered in subtle manners by interaction with other proteins. The metabolomics revolution is next, but the number of distinct metabolites is astronomical. Even if one just thinks of the lipid metabolites, the number of unique structures is difficult to fathom. It is clear that the next decade will enlighten us with numerous novel new lipids and diverse new functions for them. Certainly the last decade moved the interest in lipids from just their traditional roles in energy storage and membrane structure to a central role in all of cell signaling.

Our workshop focused on LIPIDS, both their biophysical and cellular aspects. Our hope was to share the parallel evolution of biophysical approaches to understanding the physical parameters of lipids and the structural parameters of the proteins that make or interact with them. Then, we hoped to integrate this information with our evolving knowledge of the cellular and physiological actions of a variety of lipids as they interact with cellular proteins and with other lipid assemblies. During the course of the workshop, we considered a vast array of proteins that degrade lipids, that transfer lipids, and that synthesize lipids and

the cellular responses of activation, proliferation, differentiation, inflammation, and apoptosis. We heard the latest results from both the biophysical and the cellular directions and most importantly, the interplay of both. The meeting succeeded in bringing together lipidologists working in the often-separate sphingolipid and phospholipid fields and there was much new information to exchange. We all greatly benefited from excellent discussions that were fostered by the intimate atmosphere provided by the Juan March Foundation venue.

Edward Dennis, Isabel Varela and Alicia Alonso

Session 1: Phospholipases (I)
Chair: Isabel Varela-Nieto

Structure and regulation of phospholipase A_2 in lipid signaling

Edward A. Dennis, Yasuhito Shirai, Jesus Balsinde, and Maria Balboa

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Phospholipase A_2 (PLA_2) now constitutes its own superfamily (Dennis (1997) *TIBS 22*, 1-2) which is comprised of twelve Groups, several of which contain subgroups (Six *et al.* (2001)*BBA* ***1488***, 1-19). Murine P388D_1 macrophages exhibit a delayed prostaglandin biosynthetic response when exposed for prolonged periods of time (up to 20 h) to bacterial lipopolysaccharide (LPS) that is entirely mediated by cyclooxygenase-2 (COX-2). Both the constitutive Group IV cytosolic phospholipase A_2 ($cPLA_2$) and the inducible Group V secretory phospholipase A_2 ($sPLA_2$) are involved in the COX-2-dependent generation of prostaglandins. By using a selective $sPLA_2$ inhibitor and an antisense oligonucleotide specific for Group V $sPLA_2$ we have found that induction of COX-2 gene expression is strikingly dependent on Group V $sPLA_2$. This was further confirmed by experiments in which exogenous Group V $sPLA_2$ was added to the cells. Exogenous Group V $sPLA_2$ was able to induce significant arachidonate mobilization on its own and to induce expression of the COX-2 gene. Not only delayed prostaglandin production but also COX-2 gene induction are dependent on a catalytically active Group V $sPLA_2$. COX-2 expression was also found to be blunted by the Group IV $cPLA_2$ inhibitor methyl arachidonyl fluorophosphonate (MAFP), which we have previously found to block Group V $sPLA_2$ induction as well. Collectively, the results support and expand on our original model (Balsinde *et al.* (1996) *J. Biol. Chem. 271*, 6758-6765) whereby Group IV $cPLA_2$ activation regulates the expression of Group V $sPLA_2$, which in turn is responsible for delayed prostaglandin production by regulating COX-2 expression. We have now transfected P388D_1 macrophages to produce RFP or GFP fusion proteins for the Group IV $cPLA_2$ and the Group V $sPLA_2$ as well as the Group VI Ca^{2+}-independent phospholipase A_2 ($iPLA_2$). These fusion proteins as well as immunohistochemistry with anti-Group V $sPLA_2$ have been followed using confocal microscopy to determine the ultrastructural localization of all three enzymes in response to LPS and other agents as well as the role of PIP_2 in cell activation.

Biological effects of secretory phospholipase A_2 type IIA

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Arachidonic acid (AA) is a unique fatty acid in view of its ability to yield derivatives displaying a wide scope of biological activities following physiological stimulation by a variety of chemical signals. Until very recently, availability of both AA and lyso-phospholipids regulated by phospholipases A_2, in particular the cytosolic phospholipase A_2 isoform ($cPLA_2$), has been considered as the limiting step for both eicosanoid and platelet-activating factor production, but this might be an oversimplification, in part by the emergence of cyclooxygenase-2 (COX-2) as an enzyme regulated at the transcriptional level, and also by the description of biological actions for phospholipases A_2 of the low molecular weight family, the effects of which are independent of their catalytic activity and rather depend on the ability of these proteins to interact with structures expressed on the cell membranes. Among the low molecular weight phospholipases A_2, type IIA phospholipase A_2 ($sPLA_2$) is the most abundant element in human. $sPLA_2$ behaves as a ligand for a group of receptors pertaining to the C-type multilectin mannose receptor family and also interacts with heparan sulfate proteoglycans such as glypican and decorin, thus being able to internalize to specific compartments within the cell and producing biological responses. Moreover, $sPLA_2$ is an acute phase reactant, which is expressed in many tissues displaying inflammatory features, namely arterial walls in atherosclerosis, intestine in Crohn's disease, and synovial membrane in rheumatoid arthritis. This makes a relevant question addressing the biological effects of this enzyme on different cell types. $sPLA_2$ activates the p42-MAP module of the mitogen-activated protein (MAP) kinase cascade and c-Jun N-terminal kinase in human monocytes and astrocytoma cells. The activation of this kinase shows both an early and a late peak, different from the pattern of activation elicited by other agonist, which in many cases only produce the first peak. This is accompanied by activation of the oxidative metabolism of arachidonic acid, and the induction of COX-2 expression. $sPLA_2$ also elicits the production of the chemokine monocyte chemoattractant protein-1 (MCP-1) and shows a synergistic effect with TNF-α as regards both COX-2 induction and MCP-1 production. $sPLA_2$ up-regulates the expression of Fas ligand, but it does not influence Fas expression nor monocyte survival. Taken together, these data indicate that the effects of $sPLA_2$ on different cell types are exerted by engagement of a $sPLA_2$-binding structure. As regards human monocytes, this involves engagement of the M-type receptor for secretory phospholipases A_2, activation of the MAP kinase cascade, induction of a pro-inflammatory phenotype, and up-regulation of the surface display of Fas ligand.

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Biophysical mechanisms of phospholipase A2 activation and their use in liposome-based drug delivery

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Secretory phospholipase A2 is an ubiquitous small water-soluble enzyme found in e.g. venom and pancreatic fluid. Moreover, secreted lipases of this type are known to play a role in cellular signaling cascades and to be upregulated at diseased sites in the body, e.g. in cancerous and inflammed tissue. Phospholipase A2 is an interfacially active enzyme that catalyses the hydrolysis of glycerophospholipids in the *sn-2* position, leading to the formation of lysolipids and fatty acids. Since the enzyme is only weakly active at monomeric substrate but very active on organized types of substrate, e.g. micelles or bilayers, it is not surprising that the enzyme's activity and mode of action are controlled by the physical properties of the substrate [1].

Results obtained from a variety of experimental and theoretical studies of phospholipase A2 activity on lipid-bilayer substrates are presented which provide insight into the dependence of the enzyme activity of bilayer composition, lateral structure, and thermodynamic conditions. Particular attention is paid to the lag-burst kinetics and how the lag time and degree of hydrolysis can be varied by changing the physical properties of the substrate. The results discussed are obtained from fluorescence spectroscopy, real-time HPLC analysis, calorimetry, atomic force microscopy, and molecular modeling and computer simulation techniques.

Firstly, evidence is presented for the presence of the small-scale structures of lipid bilayers in the nano-meter range [2-4]. It is then shown how phospholipase A2 activity is sensitive to this small-scale structure which can be varied systematically by varying acyl-chain length of the phospholipids, the temperature, and the lipid composition [5-9]. Starting with the original observation, that phospholipase A2 activity is enhanced at polymer-covered `Stealth` liposomes [10], results of systematic studies of the effect of polymer coverage and length, liposome surface charge, liposome composition, and enhancer effects are described [11-13].

The insight into the biophysical mechanisms of phospholipase A2 activivation has recently let to the design of a novel principle for liposomal drug targeting, release and absorption by secretory phospholipase A2 [14]. The principle takes advantage of an elevated level of secretory phospholipase A2 at the diseased tissue. The phospholipase hydrolyses a lipid-based prodrug or proenhancer liposome leading to products that in a synergistic fashion promote liposome destabilization and drug release at the same time as the permeability of the target membrane is enhanced. The drug-delivery system can be made thermosensitive and offers a rationel way for developing smart liposome-based drug-delivery systems by incorporating into the the liposome carrier specific lipid-based proenhancers, prodestabilizers

or prodrugs, e.g. anticancer etherlipids that automatically become activated by phospholipase A2 at the diseased target sites, such as inflammed or cancerous tissue.

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Roles for lysophospholipid signaling: a view from receptor mutants in mice

Jerold Chun

The lysophospholipid molecules known as lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P) [1,2] are extracellular ligands for a family of similar G protein-coupled receptors (GPCRs), $LPA_{1/2/3}$ and $S1P_{1/2/3/4/5}$ [3,4]. LPA and S1P receptors are 7-transmembrane domain, integral membrane proteins that couple to several classes of G-proteins and activate ligand-dependent signaling pathways. These pathways in turn affect numerous biological phenomena that include changes in cell morphology, ionic conductances, cell proliferation and cell survival. The first lysophospholipid receptor, LPA_1, was identified from studies on developing brain [3,5]. To better understand the developmental and physiological roles of receptor-mediated lysophospholipid signaling, we are generating mice deficient for LPA and S1P receptors, in combination with a range of analytical approaches [4,6,7]. From these combined analyses have emerged information on the in vivo roles of these receptors, particularly during development and within the nervous system. New data on additional functions revealed by receptor combinations will also be presented.

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Phosphatidic acid and the regulation of the Ras/Raf-1/Erk pathway

Bradley Andresen, Mark Rizzo and Guillermo Romero

Phosphatidic acid (PA) is one of the main products of the hydrolysis of phosphatidylcholine (PC) by phospholipase D (PLD). Recent work from our laboratory has shown that the activation and coupling of the Ras/Raf/Erk cascade requires the binding of PA to a specific site of Raf-1. We have examined the role of PLD activation and PA production in the regulation of the Erk1/2 cascade by receptor tyrosine kinases and G-protein linked receptors. Our data show that both insulin and angiotensin II (AngII) induce the production of PA by a mechanism that involves primarily the activation of PLD2. AngII-dependent activation of PLD2 was found to be downstream of the activation of Rho proteins in primary glomerular vascular smooth muscle cell cultures (PGSMC). In contrast, PLD2 activation was dependent on the activation of ARF proteins in insulin- and PDGF-stimulated HIRcB fibroblasts. ARF also mediated the activation of PLD2 in a vascular smooth muscle cell line (A10) by AngII. Imaging analysis demonstrated that stimulation of the cells with either insulin or AngII induced the accumulation of phosphorylated MEK (p-MEK) on endosome-like structures. In HIRcB cells, Raf-1 and phosphorylated Erk1/2 (p-Erk) co-localized with p-MEK on the surface of endosomes. In PGSMC, p-Erk was translocated to the nucleus by the action of EGF, whereas AngII induced the translocation of p-Erk to actin filaments. Mutational analysis of the putative PA-binding region of Raf-1 demonstrated that intact PA binding is essential for the coupling of Raf-1 activation to the phosphorylation of MEK. Likewise, overexpression of the PA-binding region inhibited the activation of MEK and Erk1/2 phosphorylation in HIRcB cells. A model for the role of PA in the coupling of the Ras/Raf/MEK/Erk signaling cascade is presented.

Session 2: Phospholipases (II)
Chair: Edward A. Dennis

The multiple roles of Pleckstrin homology domains in phospholipase C function

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Pleckstrin homology (PH) domains are small, 120 amino acid, structural modules that are found in a variety of signal transduction proteins including the four known forms of mammalian phospholipase C (PLC). The mammalian inositol-specific PLCs catalyze the hydrolysis of a minor component in lipid membranes, phosphatidylinositol 4,5 bisphosphate ($PI(4,5)P_2$) to produce two second messengers which result in the activation of protein kinase C and a rise in intracellular calcium. Our laboratory has focused on the role of the PH domains in the function of PLCβ and PLCδ. These types of PLCs are similar in their sequence organization expect that PLCβ contains a long (400 aa) C-terminal extension needed for activation by Gα subunits. The cellular regulation of PLCβ and PLCδ differ dramatically. PLCβ enzymes are regulated by $G\alpha_q$ and $G\beta_\gamma$ subunits while PLCδ is regulated by changes in intracellular calcium, RhoGAP and $G\alpha_h$ proteins.

PLCs are modular proteins and we have been particularly interested in the role that the PH domain plays in regulation the function of these proteins. Insight into the function of the PH domain of PLCδ came before PH domains were identified. It had been established that PLCδ will only bind strongly to membranes when its substrate, $PI(4,5)P_2$, is present. $PI(4,5)P_2$ -specific binding served to regulate protein function by anchoring the protein to membranes as long as substrate was present and the level of product in the aqueous phase was low. When a region encompassing the PH domain was cleaved, PLCδ remained active but lost its ability to bind to $PI(4,5)P_2$-containing membranes. This observation led to the suggestion that PH domains are $PI(4,5)P_2$-binding motifs. The crystal structure of PHδ bound to product verified that PHδ makes several specific contacts with to $PI(4,5)P_2$ give this high affinity binding. Alternately, studies using PLCβ showed that it bound strongly and non-specifically to lipid membranes regardless of the presence of $PI(4,5)P_2$. By expressing the isolated PH domains of both these proteins we established that both PH domains served as membrane-binding motifs with two very different specificities. Sequence comparison of the two proteins show that PHβ is missing the necessary residues for specific $PI(4,5)P_2$ binding. Computer models of PHβ based on PHδ offer some rationale for their membrane binding behaviors.

Studies of the β-adrenergic receptor kinase indicated that it PH domain served as a docking site for Gβγ subunits. Since PLCβ enzymes are also regulated by these proteins, we measured the interaction energies between PHβ, PHδ and Gβγ subunits. We find that these proteins all bound with high affinity, although the affinity between Gβγ and PHδ was ~4 fold weaker than PHβ. Thus, PHβ has the ability to bind both membrane and Gβγ subunits. We then constructed chimeric PLCs which contained the PH domain of PLCδ swapped into the catalytic domain of PLCβ (PHδPLCβ), and one which contained the PH domain of PLCβ swapped into the catalytic domain of PLCδ (PHβ PLCδ). The PHβ PLCδ chimer bound non-

specifically to lipid membranes and displayed a high affinity for Gβγ subunits, which is consistent with the behavior of the isolated PH domain. Interestingly, the PHδPLCβ chimera was activated by Gβγ subunits thus turning PLCδ into a G protein-regulated enzyme, and showing that the PH domain confers activation to the catalytic core. This idea was confirmed using the PHδPLCβ where we found this chimera to have $PI(4,5)P_2$ -specific binding and reduced Gβγ affinity. While this chimera was not acitivated by Gβγ subunits, as expected, it was no longer activated by Gα subunits, even though the docking and activation by these subunits take place in a region distinct from the PH domain. We conclude that the PH domain of PLCβ is closely coupled to the catalytic core enabling it play a key role in enzyme regulation as well as G protein activation.

Regulation of phospholipase D

J. H. Exton

Phospholipase D (PLD) is an enzyme of widespread distribution. There are two mammalian isoforms (PLD1 and PLD2) which occur as splice variants. They both contain PX and PH domains and two HKD motifs, which designate the PLD superfamily. They have different intracellular locations and differ significantly in their regulation. PLD1 is regulated in vitro by conventional PKC isozymes and members of the Rho and Arf families of small GTPases. On the other hand, PLD2 has higher intrinsic catalytic activity, but shows little or no response to PKC, Rho or Arf in vitro.

Mutational studies have shown that both HKD motifs of PLD1 are required for catalytic activity and molecular modeling based on the crystal structures of plant and bacterial PLDs demonstrates that the two motifs associate to form a catalytic center. In addition, a highly conserved sequence at the extreme C-terminus is absolutely required for activity, suggesting that in this sequence participates in catalysis. In unstimulated cells, PLD1 is basally phosphorylated on Ser/Thr residues in the N-terminal half and is also palmitoylated on two adjacent Cys residues. Neither of these modifications is required for catalytic activity, although they influence membrane association of the enzyme. PLD2 is also palmitoylated, but its basal phosphorylation is less than that of PLD1.

The α- and β-isozymes of PKC activate PLD1 in vitro, but surprisingly, ATP is not required, indicating that the activation occurs by a non-phosphorylating mechanism. Deletional mutagenesis of PKCα indicates that both the regulatory and catalytic domains of this enzyme are required for PLD1 activation in vivo, and that deletion of the last 23 residues of the kinase causes a loss of activation. The interaction sites for PKCα on PLD1 are localized to both the N- and C-termini of the phospholipase, as determined by activity measurements and/or co-immunoprecipitation. Express of mutant forms of PLD1 in which the N-terminal PKCα interaction site is deleted indicate that PKC plays an important role in the stimulation of PLD by many agonists in vivo.

The interaction site for RhoA on PLD1 is localized to a specific sequence in the C-terminus as demonstrated by binding and activity measurements. Forms of PLD1 in which this sequence is mutated also indicate a role for RhoA in the activation of PLD1 mediated by certain agonists that act through the heterotrimeric G protein G_{13}. Critical residues in the effector domain of RhoA are required for PLD1 activation and residues in the switch II region of the G protein are responsible for the greater efficacy of RhoA in activating the enzyme compared with Cdc42Hs.

Despite its inability to be stimulated by PKCα in vitro, PLD2, like PLD1, can be activated by phorbol ester treatment or expression of PKCα in intact cells. PLD2 is further phosphorylated on Ser/Thr in response to PMA treatment of cells or by incubation with PKCα in vitro. However, this phosphorylation inhibits rather than increases its activity. In addition, inhibitors of the kinase activity of PKCα abolish the PMA-stimulated phosphorylation of PLD2, but only partly inhibit the ability of PMA to stimulate the enzyme in vivo. These findings indicate that phosphorylation of PLD2 is not a major mechanism by which phorbol esters and PKCα activate this enzyme.

Session 3: PI-signaling (I)
Chair: Karel W. A. Wirtz

Investigation into the roles and regulation of phospholipase D

Michael J. O. Wakelam

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Phospholipase D (PLD) catalyses the hydrolysis of phosphatidylcholine (PtdCho) to generate phosphatidic acid (PtdOH) as a lipid messenger molecule. Two mammalian PLD genes have been cloned each of which exists as two splice variants, however no obvious difference exists between the spliced forms, though PLD1b and PLD2a appear to be the predominant forms. There is distinct regulation of PLD1 and PLD2 in cells and this is presumably related to different apparent physiological functions of the two enzymes. The control of distinct functions by the different PLD isoforms is surprising since HPLC-MS analysis has shown that the lipid product PtdOH from mammalian cells is essentially monounsaturated in both cases; analysis of lipids from Dictyostelium and S. Ceravisiae also identifies primarily monounsaturated PtdOH species as being generated by PLD activation. This points to there being be a family of PtdOH binding proteins that mediate the cellular effects of PLD activation, further the distinct functions of the PLD isoforms must be dependent upon their differing intracellular localisation in addition to their differing regulation.

We have examined PLD1 regulation in the RBL-2H3 mast cell line. Antigen stimulation of these cells induces secretion of serotonin and hexosaminidase. Inhibition by butan-1-ol but not butan-2-ol demonstrates the PLD dependence of this process. Confocal microscopy demonstrates that PLD1 localises to the secretory vesicles in these cells and following stimulation translocates to the plasma membrane where it colocalises with and is apparently activated by Rac1, Arf6 and protein kinase Cα. The PLD PH domain is necessary for membrane interaction whilst localisation of PLD1 to endosomal and/or secretory vesicles is dependent upon an intact PX domain, Studies with CTLA-4 in T-cells and CHO cells points to an important role for PLD1 in receptor recycling. PLD2 can also be activated by Arf6, however this is indirect and is dependent upon phosphatidylinositol 4-phosphate 5-kinase I α (PIPkinIα). PIPkinIα interacts with PLD2 and the generation of PtdIns(4,5)P_2 activates the phospholipase. Studies with alcohols and with catalytically inactive PLD mutants show that the adherence and spreading of RBL-2H3 cells is at least partly regulated by both PLD1 and PLD2. In the absence of mouse knock-outs we have been utilising Dictyostelium, which have provided additional support for key roles for PLD activation in membrane trafficking and cell movement.

Identification of the molecular targets for PLD activation points to the need to identify PtdOH binding domains. The characterisation and structural basis of a PtdOH binding domain will be described.

A small GTPase and tyrosine kinase co-regulated molecular switch in the phosphoinositide 3-kinase regulatory subunit

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Upon stimulation by many extracellular stimuli, PI3K type IA, a heterodimer of a catalytic subunit, p110, and a regulatory subunit, p85, catalyzes the synthesis of phosphatidylinositol-3,4,5-P3, a second messenger that regulates cell growth, proliferation, apoptosis and intermediary metabolism. Interestingly, the cellular levels of phosphatidylinositol-3,4,5-P3 are elevated in almost all cancers through the aquisition of mutations in tyrosine kinases, small GTPases, and the phosphatidylinositol-3 phosphatase, PTEN. Evidence obtained by experiments in this study, showed that p85 contains a GTPase-responsive domain (GRD), and an inhibitory domain which together form a molecular switch that regulates PI3K. H-Ras and Rac1 activate PI3K by targeting the GRD domain. The stimulatory effect of these molecules, however, is blocked by the p85 inhibitory domain, which can be neutralized by tyrosine phosphorylation, mediated by Src, or other tyrosine kinases. The complimentary effects of tyrosine kinases and small GTPases on the p85 molecular switch result in synergy between these two classes of molecules toward PI3K activation.

In conclusion, data in this report suggest a unifying model of PI3K activation by tyrosine kinases and small GTPases, according to which these two classes of molecules act in concert to trigger a molecular switch that regulates PI3K. Given the importance of phosphatidylinositol-3,4,5-P3 and its target Akt, in human cancer, the molecular switch described here, defines an important target for future chemotherapeutic drugs.

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Expression of a catalytically inactive form of diacylglycerol kinase alpha induces sustained signalling through RasGRP

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Regulating the generation and clearance of lipid second messengers such as diacylglycerol (DAG) is critical for the correct propagation of intracellular signalling pathways. In human Jurkat T cells stably transfected with the human muscarinic receptor (J-HM1-2.2 cells), both carbachol and T cell receptor (TCR) triggering were able to cause rapid and transient membrane translocation of the recently discovered Ras guanyl nucleotide release protein (RasGRP) and activation of mitogen-activated protein kinase (MAPK). However, when the same cells expressed green fluorescent protein (GFP)-tagged kinase-dead diacylglycerol kinase alpha (GFP-DGKalpha-kd) (J-HM1-2.2-GFP-DGKalpha-kd cells), similar agonist stimulation resulted in prolonged signalling through RasGRP and MAPK. These results suggested that the key factor controlling the activation through RasGRP-MAPK signalling was that of the level of agonist-stimulated DAG generation. This was confirmed when biochemical analyses were performed in both carbachol-stimulated J-HM1-2.2 and J-HM1-2.2-GFP-DGKalpha-kd cells. In the latter cell line, carbachol-stimulation led to a greater and more sustained accumulation of DAG. Taken together, these results suggest that modulation of the intracellular level of agonist-stimulated DAG generation in T lymphocytes, such as that presented here by the expression of GFP-DGKalpha-kd, is able to dramatically alter Ras activation and its downstream signalling, a process of utmost importance for sound immunological function.

Activation of *Drosophila* TRP channels: the role of lipid second messengers in regulating calcium influx

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The *Drosophila* light sensitive channels TRP and TRPL are prototypical members of a novel and widespread family of ion channels mediating calcium influx in eukaryotic cells. Although an essential role for phospholipase C activity in their activation is accepted, downstream events leading to opening of these channels is poorly understood. We have previously shown that a complete knockout of the only $InsP_3R$ gene in *Drosophila* has no discernable effect on phototransduction suggesting that $InsP_3$ induced Ca^{2+} release plays no role in activation of TRP and TRPL. Recent evidence suggests that lipid second messengers derived from PIP_2 might be involved in activation of TRP and TRPL. Polyunsaturated fatty acids, potential metabolites of diacylglycerol, have been shown to activate the light sensitive channels but their *in-vivo* significance as messengers of excitation remains to be established. *rdgA* mutants that lack diacylglycerol kinase (DGK) activity show constitutive activity of the light sensitive channels, excessive Ca^{2+} influx and severe retinal degeneration. Mutants lacking TRP, the major component of the light sensitive conductance rescues retinal degeneration in *rdgA* and *rdgA;;trp* double mutants, which can respond to light, show a specific defects in response termination. These results have suggested a model in which DGK controls the levels of a lipid second messenger that activates TRP and TRPL. However the identity of this messenger remains to be established and the biochemical basis of the *rdgA* phenotype remains unclear. Specifically, the relative importance of DAG accumulation versus phosphatidic acid (PA) deficiency in the *rdgA* phenotype is unknown. To address this issue, we have used transgenic techniques to (i) Supplement PA levels in *rdgA* photoreceptors by overexpressing phospholipase D and (ii) Deplete PIP_2 levels without producing DAG. The effect of these two manoeuvres on TRP channel activation and retinal degeneration are being studied. The results of these experiments will be presented and should contribute to our understanding of how lipid second messengers regulate calcium influx in eukaryotic cells.

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Session 4: PI signaling (II)
Chair: Michael J. O. Wakelam

The 3D-structure of phosphatidylinositol transfer protein α: from closed to open

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Phosphatidylinositol transfer protein α (PITPα) is a ubiquitous and highly conserved protein in multicellular eukaryotes (>98% sequence identity among mammalian species) that catalyzes the exchange of phospholipids between membranes *in vitro* and participates in cellular phospholipid metabolism, signal transduction and vesicular trafficking *in vivo*. The protein has a dual specificity with a distinct preference for phosphatidylinositol (PI) over phosphatidylcholine (PC). It has a lipid-binding cavity for accommodating a single PI or PC molecule. Recently we have succeeded in elucidating the 3D-structure of a phospholipid-free mouse-PITPα at 2.0 Å resolution. In contrast to the closed structure of the lipid carrying form, the apo-PI-TPα has an open conformation with a channel running through the protein. The phospholipid-binding cavity is part of this channel that due to conformational changes displays distinct recognition sites for the phosphorylinositol and phosphorylcholine head groups. The interaction with the PI polar head group is stabilized by extensive hydrogen bonding. The open relaxed conformation is stabilized at the proposed membrane-association site by hydrophobic interactions with a crystallographically related molecule, creating an intimate dimer. The relaxed conformation is consistent with a membrane-bound state and suggests a mechanism by which PI-TPα may extract a phospholipid molecule from a minimum-energy structure, ie. the membrane. In addition, the open structure is very suggestive as to how the protein may present PI to kinases and phospholipases after it being extracted from the membrane.

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Structure of apo-phosphatidylinositol transfer protein α provides insight into membrane association.

PITP proteins as lipid sensors: transfer function regulated by EGF

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Phosphatidylinositol transfer proteins (PITPs) bind one molecule of either phosphatidylinositol (PtdIns) or phosphatidylcholine (PtdCho) and can mediate their transfer between membrane compartments *in vitro*. The best-characterised mammalian PITPs are PITPα and PITPβ, two highly homologous proteins encoded by distinct genes . Genetic studies indicate that PITPβ is an essential housekeeping gene as ablation of the gene is embryonically lethal whilst ablation of PITPα leads to the birth of live mice which only survive for a couple of weeks (3) (1). Biochemical studies involving reconstitution of cytosol-depleted cell preparations have demonstrated the requirement of PITPα and β in signal transduction and in membrane traffic. From such analysis, a requirement for PITP has been identified in phospholipase C (PLC)-mediated phosphatidylinositol(4,5)bisphosphate (PtdIns(4,5)P_2) hydrolysis, in the synthesis of 3-phosphorylated lipids by phosphoinositide 3-kinases, in regulated exocytosis and in the biogenesis of vesicles at the Golgi. Studies aimed at elucidating the mechanism of action of PITP in each of these seemingly disparate functions have yielded a singular theme; the activity of PITP stems from its ability to transfer PtdIns from its site of synthesis to sites of cellular activity and to stimulate the local synthesis of phosphorylated forms of PtdIns including PtdIns(4)P, PtdIns(4,5)P_2, PtdIns(3)P and PtdIns(3,4,5)P_3 by delivering PtdIns to the lipid kinases involved in phosphoinositide synthesis (4). Using FLIM (Fluorescence Lifetime Imaging Microscopy) to measure FRET (Fluorescence Resonance Energy Transfer) between GFP-PITP proteins and fluorescently labelled phospholipids, we report that PITPα and PITPβ can dynamically interact with PtdIns or PtdCho at the plasma membrane only when stimulated with epidermal growth factor (EGF). In addition, PITPβ is localised at the Golgi, and upon EGF addition, the lipid environment is altered resulting in enhanced FRET (5). Our observations demonstrate that the transfer function of PITPα and PITPβ is a regulated process involving dynamic behaviour *in vivo*. Previous studies have shown that both PITPα and PITPβ can reconstitute PLC signalling (2) and the observation that both PITPα and PITPβ are found at the plasma membrane following stimulation emphasises that PITP proteins have overlapping functions in PLC signalling.

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Structure and specificity of the inositol polyphosphate 5-phosphatases

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The inositol polyphosphate 5-phosphatases are a diverse class of enzymes with key roles in synaptic vesicle recycling, signaling downstream of PI 3-kinases, Ca^{2+} signaling, cytoskeletal regulation, and other key cell processes. Their critical role is highlighted by pathological mutation of the 5-phosphatase domain of OCRL in Lowe's syndrome. Using a structural genomics-based approach we have cloned, characterized, and determined the structure of a novel member of the inositol polyphosphate 5-phosphatase family. This phosphatase has broad substrate specificity and is active against membrane-incorporated $PI(4,5)P_2$ and $PI(3,4,5)P_3$ and the soluble phosphoinositides $I(1,4,5)P_3$, $I(1,4,5,6)P_4$, and $I(1,3,4,5)P_4$. The 2.0 Å structure of the enzyme reveals a bilobed fold of approximate 2-fold symmetry, resembling that of DNase I, exonuclease III, and endonuclease HAP1. The structural conservation of catalytic His residues and divalent cation ligands supports a common enzymatic mechanism for these enzymes. The structure of the tungstate complex of the enzyme has been refined, revealing potential determinants for the 3- and 4-phosphate binding subsites. The putative determinants for 3-phosphoinositide specificity of the SH2 domain-containing inositol phosphatase SHIP and its relatives have been identified and analyzed by site-directed mutagenesis. The determinants for long-chain versus soluble phosphoinositide specificity have been mapped to a basic face of the enzyme surrounding the active site and have been analyzed by mutagenesis and *in vitro* and *in vivo* assays.

Phosphoinositide recognition by PX domains

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PX domains are 120-residue phosphoinositide-binding modules present in many proteins that have a role in vesicle trafficking, signal transduction and lipid modification. The module consists of al β-sheet subdomain followed by a helical subdomain. Most members of the PX domain family specifically recognise PtdIns(3)P, however, specificities for PtdIns(3,4)P_2, PtdIns(4,5)P_2 and PtdIns(4)P have also been observed. The PX domain from the cytosolic $p40^{phox}$ subunit of NADPH oxidase shows a dramatic PtdIns(3)P-dependent accumulation around the phagosome immediately upon closure of the phagocytic cup. The X-ray crystal structure of the PX domain from the $p40^{phox}$ subunit of NADPH oxidase bound to PtdIns(3)P shows the basis of PtdIns(3)P recognition. A network of hydrogen bonds involving the 3-phosphate and the 1-phosphate as well as the 4- and 5-hydroxyls enable the domain to bind PtdIns(3)P and exclude polyphosphorylated phosphoinositides. Unlike FYVE domains, the PX domain derives its membrane affinity mostly from headgroup interactions. The residue forming the most important interactions with 3-phosphate of the bound lipid is Arg 58 which is located at the junction of the β-sheet and helical halves of the domain. The small subset of PX domains that lack a basic residue analogous to Arg 58 include the PX domains from type II PI 3-kinase (CPK) and the yeast bud emergence protein Bem1 which have been shown to bind PtdIns(4,5)P_2 and PtdIns(4)P, respectively. A chronic granulomatous disease (CGD)-associated mutation in the $p47^{phox}$ PX domain that abrogates PtdIns(3,4)P_2 binding maps to a conserved Arg that does not directly interact with the phosphoinositide but instead stabilizes a critical lipid-binding loop. For $p47^{phox}$, an interaction between the poly-proline helix in the PX domain and the C-terminal SH3 domain has been proposed to affect lipid binding by the PX domain. In contrast, for $p40^{phox}$, we find that the SH3 domain present in the full-length $p40^{phox}$ protein does not affect soluble PtdIns(3)P binding to the $p40^{phox}$ PX domain.

The PI3K/PKB/Forkhead pathway controls cell proliferation/transformation

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Cell cycle progression is a process tightly controlled by internal and external signals. Environmental cues, such as those provided by growth factors, activate early signals that promote cell cycle entry. Cells past the restriction point become growth factor-independent, and cell cycle progression is then controlled endogenously. The phosphatidyl-inositol 3-kinase (PI3K)/PKB pathway must be activated in G1 to inactivate forkhead transcription factors (FKH-TF) and allow cell cycle entry. We show that subsequent attenuation of the PI3K/PKB pathway is required to allow transcriptional activation of FKH-TF in G2. FKH-TF activity in G2 controls mammalian cell cycle termination, as interference with FKH transcriptional activation by disrupting PI3K/PKB downregulation, or by expressing a transcriptionally inactive FKH mutant, induces cell accumulation in G2/M, defective cytokinesis, and delayed M-to-G1 transition. We demonstrate that FKH-TF regulate expression of mitotic genes such as *cyclin B* and *polo-like kinase* (*plk*). Our results support the pivotal role of forkhead in the control of mammalian cell cycle completion, and suggest that efficient execution of the mitotic program depends on downregulation of PI3K/PKB and consequent induction of FKH transcriptional activity.

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Session 5: Signaling by sphingolipids
Chair: John H. Exton

Membrane properties of sphingomyelins

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Sphingomyelins are important components of the external leaflet of cellular plasma membranes. Naturally occurring sphingomyelins consist of long-chain sphingosine (1,3-dihydroxy-2-amino-4-octadecene) or sphinganine (1,3-dihydroxy-2-amino-octadecane) bases, and long and highly saturated amide-linked acyl-chains. The gel-to-fluid (or order-to-disorder) transition temperatures of natural sphingomyelins are fairly high (30-45 °C) compared to other naturally occurring (glycero)phospholipids [1]. These features give natural sphingomyelins and glycosphingolipids the potential to introduce lateral heterogeneity in the membrane plane [1,2]. Studies with various cell membranes have suggested that sphingomyelins and glycosphingolipids may cluster with cholesterol to form raft-like domains (reviewed in [3,4]).

All naturally occurring sphingomyelins have the D-*erythro*-(2S,3R) configuration of the sphingosine base. However, the synthetic acyl-chain defined sphingomyelins that have been used in most studies on the properties of sphingomyelins in model membranes have been racemic, i.e. they contained both D-*erythro* and L-*threo* isomers. This is due to the fact that hydrolysis of sphingomyelin to yield lyso-sphingomyelin (the commonly used synthetic precursor in sphingomyelin synthesis) can give rise to an epimerization at C-3. Most of the commercially available synthetic sphingomyelins have therefore also been racemic. Racemic saturated sphingomyelins differ in their membrane packing properties compared to enantiomerically pure counterparts. There are also differences in the susceptibility to degradation by sphingomyelinases between pure and racemic sphingomyelins.

Sphingomyelin is highly enriched in the plasma membrane compartment, as is cholesterol [5]. In cultured cells (i.e. human skin fibroblast and baby hamster kidney cells) about 90-95 % of the sphingomyelins contain sphingosine as the long-chain base, whereas the remainder have sphinganine as the base. The latter sphingomyelins are also called dihydrosphingomyelins, and display an even higher melting temperature in membranes as compared to acyl-chain matched sphingomyelins. Dihydrosphingomyelins may therefore be raft constituents.

Sphingomyelins and cholesterol co-localize in cell membranes, and it has been shown that cholesterol prefers to interact with sphingomyelin over other glycerophospholipids [6]. The hydroxyl group at C-3 in sphingomyelin has been shown not to affect the interaction with cholesterol in model membrane studies. The amide group at C-2 in sphingomyelin, on the other hand, has been shown to be essential for the strong interaction with cholesterol, since replacement of this group (with a carbonyl ester linkage) increases the availability of cholesterol in mixed vesicles for oxidation by cholesterol oxidase [7]. The amide-linked acyl-chain, its length, degree of unsaturation and the position of the possible double bond, also markedly influence the interaction between sphingomyelin and cholesterol [8].

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Physical aspects of the interaction of sphingomyelinase with lipid bilayers

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In recent years, sphingomyelinases have attracted the attention of cell biologists because of their implication in the sphingomyelin-related signalling pathway. We have studied sphingomyelinase activity as a function of the physical state of its substrate. For this purpose, large unilamellar vesicles consisting of pure egg sphingomyelin (gel-to-liquid crystalline transition temperature ca. 39°C) have been treated with bacterial sphingomyelinase in the temperature range 10-70°C. The vesicles have also been examined by differential scanning calorimetry and infrared spectroscopy, among other techniques. Enzyme activity is much higher when the substrate is in the fluid than when it is in the gel state. This may be related not only to an increased mobility of the molecules but also to a change in conformation of the sphingomyelin amide group that is detected by IR spectroscopy at the transition temperature.

Addition of phosphatidylcholine or phosphatidylethanolamine decreases the gel-fluid transition temperature of sphingomyelin bilayers, thus increasing enzyme activity at 37°C. Cholesterol however does not allow any sphingomyelinase activity at 37°C unless the sterol mole fraction in the bilayer is of at least 0.33. This is the mole fraction at wich cholesterol abolishes the gel-fluid transition of sphingomyelin. Moreover, IR spectra show unequivocally that cholesterol modifies the conformation of the sphingomyelin amide group, which explains the strong interaction between both lipids

Signalling networks controlling apoptosis during development: role of ceramide and ceramide-1-phosphate

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Programmed cell death is a critical process for normal development and tissue homeostasis. While the basic program of apoptosis execution remains conserved, distinct regulatory signals have been described depending on the cell type and developmental stage. Particularly interesting are the opposite actions displayed by nerve growth factor (NGF) that acts either as a survival factor or as a death-inducing factor. A coherent understanding of the regulation of programmed cell death during development requires a coordinated study of some of the multiple signals acting on the cells.

Inner ear ontogenesis is an attractive model system to study which signals are implicated in the modulation of cell death and survival. The inner ear is a complex sensory organ responsible for sound detection in vertebrates. In the last few years the signaling networks responsible for the induction, growth and differentiation of the inner ear have started to be unraveled (world wide webs: URL: http://www.biologists.com/, URL: http:/www.ihr.mrc.ac). Members of the insulin family of growth factors (IGFs), NGF, retinoic acid and their different receptors are among the molecules involved in this process.

We are interested in the molecular mechanisms by which these signals initiate and pattern the vertebrate inner ear. IGF-1 is a member of a family of structurally related genes that have pleiotropic actions on embryonic cells. In vitro culturing and knock out mice analysis have determined that IGF-1 is critical for the proper development and maturation of the inner ear. IGF-1 stimulates the generation of lipidic second messengers, activates the Raf/mitogen-activated protein kinases cascade and increases AP-1 and PCNA levels leading to cell growth and survival. On the contrary, NGF, after binding to p75 low affinity receptors, activates Jun N-terminal kinase and increases ceramide levels, in a process that regulates apoptotic cell death. In this context, we have explored the interactions between the pathways activated by IGF-1 to prevent apoptosis and those activated by NGF to induce cell death. We propose that the dynamic balance between levels of ceramide metabolites and the consequent regulation of Akt phosphorylation are important factors that determine whether a cell survives or dies.

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Ceramide is required for DISC formation upon Fas stimulation

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The fundamental unit that forms at the cytoplasmic surface of ligated Fas, and is required for killing, is termed the death-inducing signaling complex or DISC. The DISC is comprised of the adaptor protein FADD and caspase 8, and its formation results in caspase 8 autoactivation, initiating the death process. However, recent data suggest that another event, the oligomerization of pre-formed Fas trimers, must also occur for death to ensue. The molecular ordering of the DISC formation relative to Fas oligomerization has not been defined. Prior studies from our and other groups showed that acid sphingomyelinase activation and ceramide generation were downstream of FADD and caspase 8, and hence by inference downstream of the DISC. In fact, by use of splenocytes from lpr-cg mice, which manifest a defect in DISC formation, and acid sphingomyelinase defective cells, it has been possible to show that ceramide generation and the formation of ceramide-enriched microdomains are obligate for DISC formation. Apparently, a small amount of caspase activity, initiated by pre-formed trimers, induces acid sphingomyelinase activation and ceramide generation, which provides the driving force for both Fas oligomerization and DISC formation.

TNF receptor-induced activation pathways of sphingomyelinases

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TNF is a functionally pleiotropic cytokine that regulates cellular differentiation, activation, proliferation and apoptosis. TNF action is strikingly overlapping with that of ceramide, a neutral lipid, that seems to be involved in the regulation of diverse signaling pathways. Within seconds after interaction with the p55 TNF receptor (TNF-R55), TNF stimulates the activation of at least two types of sphingomyelinases (SMase). The acid isoform (ASMase), produces ceramide within the endo- lysosomal compartments. A neutral isoform, NSMase, generates ceramide through sphingomyelin hydrolysis at the plasmamembrane. The activation of ASMase is mediated by two TNF-R55 adapter proteins, TRADD and FADD. In TNF-induced apoptosis, these two proteins are recruited via homologous death domain interactions to the death domain of TNF-R55, which results in binding and activation of caspase 8. However, caspase 8 is not involved in ASMase activation, indicating that the apoptotic caspase 8 pathways segregates from the ASMase activation pathway at the level of FADD. Notwithstanding, ceramide produced by ASMase has been shown to activate cathepsin D, that plays an active role in cytokine-induced apoptosis. Notably, ASMase deficient mice are especially susceptible to intracellular *L. monocytogenes* infection, suggesting that TNF exerts its antibacterial effects, at least in part, through ASMase.

The activation of NSMase emanates from TNF-R55 at a distinct motif of nine aminoacid residues at position 310-318, termed NSD. A WD-repeat protein, FAN specifically binds to the NSD and functionally couples TNF-R55 to NSMase. Structural analysis revealed that FAN neither contains a phospholipid binding site nor does it seem to exert enzymatic activity itself. Instead, FAN interacts with RACK1, a protein kinase C activating, WD-repeat protein. While some light could be shed on the NSMase activation pathway, the picture of the signaling events downstream of NSMase has become blurred. For example, MAP kinase activation previously assigned to NSMase signaling, has been shown to be triggered by the death domain of TNF-R55. Clearly, cDNA cloning of the plasmamembrane-associated NSMase is urgently required to provide pertinent genetic evidence for a possible function of this as yet orphan enzyme in TNF signaling.

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Session 6: Membranes: Spatial location and microdomains
Chair: Alicia Alonso

Ceramide – a unique lipid with unique properties

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Ceramide has been established as a second messenger in cell death, apoptosis. In addition, this lipid is a major constituent of the permeability barrier of skin. The chemical structure of ceramide is rather simple. However, the invoked physical properties are unique and striking. Accordingly, the small and weakly hydrated headgroup contains both hydrogen bond donors and acceptors, which together with the saturated alkyl chains results in strong intermolecular hydrogen bonding and high melting temperatures. Strong intermolecular hydrogen bonding also readily accounts for the negative spontaneous curvature of ceramide containing membranes. Partial phase diagram for C16:0 ceramide and dimyristoylphosphocholine (DMPC) revealed already small amounts of ceramide (< mole fraction 0.10) to significantly elevate the chain melting temperature of the mixed bilayer, together with the emergence of lamellar gel-fluid coexistence region, pertaining up to 45 °C. Coexisting ceramide enriched membrane microdomains were subsequently demonstrated in both large unilamellar vesicles as well as so-called giant liposomes. The precursor for ceramide in cells is sphingomyelin (SM), which is a ubiquitous component of cellular membranes. While sphingomyelin has high chain melting transition temperatures (above 37°C), the strongly hydrated phosphocholine headgroup can be assumed to represent a steric hindrance preventing intermolecular hydrogen bonding. In keeping with this sphingomyelin is not segregated into microdomains in mixed membranes with phosphatidylcholine (PC). Yet, the presence of sphingomyelin appears to enhance the lateral segregation of ceramide in tertiary alloys with PC, suggesting the cosegregation and enrichment of these two sphingolipids.

In cells SM is converted to ceramide by sphingomyelinase (SMase), which catalyzes the hydrolytic cleavage of the phosphocholine headgroup. Studies using giant liposomes consisting of SM and PC and subjecting these to the action of SMase applied by microinjection have demonstrated that the ceramide formed becomes rapidly segregated into microdomains. Furthermore, these microdomains form vesicles. Intriguingly, the latter process is vectorial and takes place on the surface opposite to the action of the applied SMase. More specifically, SMase applied onto the outer surface causes the formation of endocytotic vesicles whereas the enzyme injected into the giant liposome causes shedding of vesicles from the outer surface. The above studies using microinjection lack control of the exact amount of enzyme in contact with the substrate. This uncertainty was alleviated using SMase coupled covalently to polyacrylamide microbeads. These microbeads were held using micropipette and brought into contact with the giant liposome surface. After contact was established and allowing for the progression of catalysis, the formation of microdomains became visible, similarly to the studies using microinjected enzyme. Yet, the microdomains appeared distal to the contact site, thus revealing diffusion of the product from the site of enzymatic catalysis. Subsequently, the microdomains formed endocytotic vesicles inside the giant liposome. This technique represents a novel, topical approach to study enzyme reactions, allowing for the first time precise spatial and temporal control of catalysis.

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Dynamics of lipid raft domains and cross-talk to the insulin signalling cascade

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GPI proteins are expressed in eucaryotes from yeast to man and anchored to the outer leaflet of the plasma membrane by a covalently attached glycosylphosphatidylinositol (GPI) lipid moiety. Despite the lack of a transmembrane domain, GPI proteins have been implicated in signal transduction across the plasma membrane *via* either their aggregation or lipolytic cleavage (1). The finding that GPI proteins can associate with specialized lipid raft domains, so-called detergent-insoluble glycolipid-enriched rafts, DIGs, rather than with distinct transmembrane binding/linker proteins favors lipid-lipid interactions as the major coupling mechanism for signal transduction mediated by GPI proteins.Current evidence suggests that the basic structural element of DIGs is a lateral assembly of (glyco)sphingolipids and cholesterol which adopts a liquid-ordered organization distinct from that of adjacent liquid-disordered regions in the membrane lipid bilayer (2). Several types of DIGs seem to exist in the same cell. In addition to caveolae (3), lcDIGs of low cholesterol/caveolin content exhibiting high buoyant density (according to sucrose density gradient centrifugation) can be discriminated from typical hcDIGs with high cholesterol/caveolin content characterized by low buoyant density. In rat adipocytes, the major fraction of the GPI proteins, such as Gce1 and Nuc, as well as of dually acylated proteins, such as the non-receptor tyrosine kinase (NRTK), $pp59^{Lyn}$, are located at hcDIGs. Thereby $pp59^{Lyn}$ interacts *via* its caveolin-binding domain with the caveolar structural protein, caveolin, which seems to downregulate its activity (4).

Previous studies have elucidated the molecular mechanism of the efficient positive cross-talk of phosphoinositolglycans (PIG), PIG-peptides (PIG-P), the sulfonylurea drug, glimepiride, or the synthetic caveolin-binding domain peptide derived from $pp59^{Lyn}$ to the insulin signaling cascade in insulin target cells, such as cultured or primary adipocytes and myocytes (5). This involves the redistribution of $pp59^{Lyn}$ from hcDIGs to lcDIGs and its concomitant dissociation from caveolin resulting in tyrosine phosphorylation of the insulin receptor substrate proteins (IRS) by activated $pp59^{Lyn}$. In isolated rat adipocytes the primary target of PIG(-P) is localized in hcDIGs. Radiolabeled PIG-P, Tyr-Cys-Asn-NH-(CH_2)2-O-PO(OH)O-6Manα1(Manα1-2)-2Manα1-6Manα1-4GluN1-6Ino-1,2-(cyclic)-phosphate and the radiolabeled and lipolytically cleaved GPI protein, lcGce1, from yeast used for preparation of the radiolabeled PIG-P bind in saturable fashion to hcDIGs but not lcDIGs, microsomes and total plasma membranes from isolated rat adipocytes and are displaced by excess of chemically synthesized unlabeled PIG-P. Specific binding of both YCN-PIG and lcGce1 is completely blocked by pretreatment of the adipocytes with trypsin and subsequent washing with NaCl or N-ethylmaleimide prior to isolation of the hcDIGs but considerably increased in hcDIGs from adipocytes pretreated with (G)PI-specific phospholipases C. Thus cross-talk of PIG(-P) to the insulin signaling cascade *via* the DIGs-caveolin-$pp59^{Lyn}$-IRS pathway apparently requires a proteinaceous hcDIGs-associated receptor, which may act to concentrate GPI proteins as the authentic ligands at hcDIGs *vs.* lcDIGs. Since GPI protein acyl groups are restricted to the exoplasmic leaflet of the lipid bilayer, while the $pp59^{Lyn}$ myristoyl and palmitoyl groups reside within the cytoplasmic leaflet, the lipid moieties of the

exoplasmic and cytoplasmic leaflets of the bilayer seem to communicate *via* a molecular mechanism which is presently not understood. It is possible that lipid-lipid interactions between the exoplasmic and cytoplasmic leaflets of the DIGs membrane serve to alter the relationship between the proteins to which they are bound, ultimately affecting their function. This assumption is compatible with the fact that there is no known protein-protein interaction between GPI proteins and caveolin or $pp59^{Lyn}$. However, it seems more likely that the acyl group-dependent association of caveolin-1 with cholesterol links the cytoplasmic and exoplasmic leaflets of the lipid bilayer, since cholesterol is present in both leaflets and can potentially form dimers that span the membrane, acting as a bridge. Thus, caveolin-1 in complex with cholesterol may function as the postulated linker mediating transmembrane signaling *via* GPI proteins to components at the inner leaflet of the plasma membrane.

The following model for a role of the PIG(-P) receptor in regulation of redistribution of GPI proteins is suggested. In unstimulated cells, GPI proteins partition between lcDIGs, hcDIGs and non-raft domains of the adipocyte plasma membrane in a random receptor-independent fashion. This equilibrium is dramatically shifted to hcDIGs upon specific interaction of the PIG-P structure within the GPI anchor with the corresponding binding sites of the PIG(-P) receptor. In case of distinct binding sites of the PIG(-P) receptor for the PIG and the peptidylethanolamidyl moieties, interaction with the PIG portion of one GPI protein and the carboxy-terminal tripeptidylethanolamidyl portion of another GPI protein would result in crosslinking of the GPI proteins and lead to the observed concentration of GPI proteins in hcDIGs. Interestingly, for certain GPI proteins their crosslinking by bi- or multivalent antibodies has been reported to significantly increase efficiency of recruitment to DIGs which presumably are identical with hcDIGs. On the other hand the saturated fatty acyl chains of the GPI anchor seem to be necessary and sufficient for accumulation in lcDIGs *vs.* non-raft domains. Thus, high affinity binding and crosslinking to the PIG(-P) receptor may favor lateral movement of GPI proteins from lcDIGs to hcDIGs. According to this model, the PIG(-P) receptor and the PIG(-P) constituent of the GPI anchor are required for retention of GPI proteins in hcDIGs *vs.* lcDIGs. In consequence, incubation of intact rat adipocytes with excess PIG(-P) acting as agonists/antagonists of the PIG(-P) receptor will displace GPI proteins from the receptors and abrogate their crosslinking ultimately leading to redistribution of the GPI proteins from hcDIGs to lcDIGs. In conclusion, the apparent dynamics between hcDIGs, lcDIGs and caveolae may represent a putative target for modulation of the redistribution of lipid-modified signalling proteins between these plasma membrane microdomains and thereby for regulation of their activity state by small drug molecules. This is exemplified by the peripheral insulin-independent blood glucose-lowering activity of glimepiride which bypasses the insulin resistance at the level of the insulin receptor and IRS proteins as prevalent in type II diabetic patients by positive cross-talk from DIGs *via* $pp59^{Lyn}$ to IRS (6).

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Sphingosine-1-phosphate, an important lipid mediator

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Sphingosine-1-phosphate (S1P) is a potent lipid mediator produced from ATP and sphingosine in a reaction catalyzed by sphingosine kinase, an enzyme activated by growth factors, cytokines, and many other stimuli. S1P is the natural ligand of five G-protein coupled receptors, known as S1PRs, that are coupled to different G proteins and mediate a variety of important biological effects. It has also been suggested to have second messenger actions, regulating several signal transduction pathways leading to calcium mobilization, cell growth and suppression of apoptosis. These effects of S1P have remained controversial as intracellular targets of S1P have not been identified and intracellular S1P can activate its receptors by an 'inside-out action". Enforced expression of sphingosine kinase to specifically increase intracellular S1P levels has proved to be a useful tool to dissect intra and extra cellular functions of S1P. Little is still known of the mechanism of regulation of sphingosine kinase although one clue has emerged recently from our studies of its intracellular distribution. Sphingosine kinase is a cytosolic protein, while the hydrophobic substrate sphingosine is membrane-associated. Indeed, we recently found that several growth factors induce translocation of sphingosine kinase to the plasma membrane. Importantly, this translocation leads to activation of S1PRs, indicating that intracellularly generated S1P can act in an autocrine and/or paracrine manner. In agreement with the observation that sphingosine kinase expression expedites the G_1/S transition, we found that it also translocates to nuclear membranes as cells enter S phase. Thus, it is expected that anticancer therapeutics targeting sphingosine kinase will be useful clinically. Indeed, inhibition of sphingosine kinase has been shown to suppress gastric tumor growth and conversely, over-expression of sphingosine kinase increases tumorigenicity. Moreover, S1P has also been shown to regulate angiogenesis, or new blood vessel formation, which is critical for tumor progression. Hence, sphingosine kinase may not only protect tumors from apoptosis, it may also increase their vascularization, further enhancing growth.

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Spatial and temporal control of lipid second messenger signaling

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Activation of phosphatidyl-inositol-3'-OH-kinase (PI3K) and the resulting production of phosphatidy-linositol-3,4,5-trisphosphate (PIP3) are ubiquitous signaling steps that link various cell surface receptors to multiple intracellular targets. In fat and muscle cells, the insulin triggered insertion of glucose transporter GLUT4 into the plasma membrane relies on the same PI3K pathway that also regulates metabolic enzymes, proliferation and differentiation[1,2]. Here we developed an evanescent-wave microscopy method to simultaneously measure GLUT4 insertion and PIP3 production in individual 3T3L1 adipocytes in order to test the hypothesis that specificity of insulin signaling is achieved by a filtering mechanism based on timing and amplitude of PIP3 signals. While insulin stimulation and direct PI3K activation could both trigger maximal PIP3 signals and GLUT4 insertion, transporters were neither inserted for small amplitude persistent PIP3 signals nor for large amplitude short PIP3 signals. The resulting threshold rejection explains the selective advantage of insulin over other hormones for inducing GLUT4 insertion. Our study suggests that the same PI3K pathway controls different cell functions by relying on effector systems that are tuned to particular receptor encoded time-courses and amplitudes of PIP3 signals.

Cell adhesion and signaling through glycosphingolipid microdomain during development and cancer

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Glycosphingolipids (GSLs) show strong trends of interaction among themselves: (i) GSL-to-GSL *cis* interaction in membrane, which is stronger than that of glycerophospholipids or sphingomyelin, due to higher ability of GSL to donate as well as accept hydrogen bonds. This provides a basis for formation of GSL clusters. (ii) GSL-to-GSL *trans* interaction through complementary carbohydrate structure, often catalyzed by bivalent cation (Ca^{2+}). This provides a basis for GSL-dependent cell-to-cell adhesion through GSL clusters (1). Such GSL cluster, *i.e.*, GSL-enriched microdomain (GEM), preferentially includes proteolipids and lipophilic signal transducers (Src family kinases, small G-proteins, focal adhesion kinase), and is sometimes associated with a complex of ganglioside/ integrin/ tetraspanin, or a complex of ganglioside/ growth factor receptor (2,3). Examples of GSL-dependent cell adhesion coupled with signaling, during ontogenesis and oncogenesis, are described below.

1. Cell adhesion mediated by GEM during the compaction process. Compaction, the first strong cell adhesion event in embryogenesis, occurs in the morula stage and induces dramatic phenotypic changes. It is mediated by multiple molecular systems: (i) Le^x-to-Le^x interaction (4); (ii) globo-series interaction (*e.g.*, Gb5-to-nLc_4; Gb4-to-Gb5); (iii) E-cadherin to E-cadherin interaction. Here, I will present an example of globo-series interaction in GEM that activates signaling leading to strong enhancement of CREB (5).

2. Tumor cell adhesion through tumor-associated GSL antigen, leading to enhanced tumor cell invasiveness. (i) Adhesion of B16 melanoma cells to Gg3- or LacCer-coated plate, through GM3-Gg3 or GM3-LacCer interaction in GEM, causes activation of cSrc, RhoA, and RasH, leading to activation of haptotactic and phagokinetic motility. Since Gg3 and LacCer are present in microvascular endothelial cells of lung, this provides a model of B16 metastasis to lung (6,7). (ii) Sialyl-Gb5, enriched in breast cancer cell line MCF7, causes activation of cSrc and FAK when MCF7 cells are treated with anti-sialyl-Gb5 monoclonal antibody RM1, leading to increased invasiveness as assayed by penetration through collagen gel and other criteria (Steelant W, Kawakami Y, Handa K, Bruyneel EA, Mareel M, Hakomori S, unpubl. data).

In order to assess basic mechanisms of GSL function in membrane, reconstitution of membrane showing GSL-dependent adhesion and associated cSrc activation was performed using model membranes having GM3, cSrc, sphingomyelin, and phospholipid, with or without addition of cholesterol (8).

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POSTERS

Forkhead transcription factors contribute to execution of the mitotic program in mammals

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Cell cycle progression is a process tightly controlled by internal and external signals. Environmental cues, such as those provided by growth factors, activate early signals that promote cell cycle entry. Cells past the restriction point become growth factor-independent, and cell cycle progression is then controlled endogenously. The phosphatidylinositol 3-kinase (PI3K)/PKB pathway must be activated in G1 to inactivate forkhead transcription factors (FKH-TF) and allow cell cycle entry. Our data illustrates that PI3K activation coordinately controls cell growth and cell cycle entry. We also show that after cell cycle entry subsequent attenuation of the PI3K/PKB pathway is required to allow transcriptional activation of FKH-TF in G2. FKH-TF activity in G2 controls mammalian cell cycle termination, as interference with FKH transcriptional activation by disrupting PI3K/PKB downregulation, or by expressing a transcriptionally inactive FKH mutant, induces cell accumulation in G2/M, defective cytokinesis, and delayed M-to-G1 transition. We demonstrate that FKH-TF regulate expression of mitotic genes such as cyclin B and polo-like kinase (plk). Our results support the pivotal role of forkhead in the control of mammalian cell cycle completion, and suggest that efficient execution of the mitotic program depends on downregulation of PI3K/PKB and consequent induction of FKH transcriptional activity. Finally, these results point out at FKH-TF as a potential target for the treatment of human cancer.

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The crystal structure of the PX domain from p40(phox) bound to phosphatidylinositol 3-phosphate

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More than 50 human proteins with a wide range of functions have a 120 residue phosphoinositide binding module known as the PX domain. The 1.7 A X-ray crystal structure of the PX domain from the p40(phox) subunit of NADPH oxidase bound to PtdIns(3)P shows that the PX domain embraces the 3-phosphate on one side of a water-filled, positively charged pocket and reveals how 3-phosphoinositide specificity is achieved. A chronic granulomatous disease (CGD)-associated mutation in the p47(phox) PX domain that abrogates PtdIns(3)P binding maps to a conserved Arg that does not directly interact with the phosphoinositide but instead appears to stabilize a critical lipid binding loop. The SH3 domain present in the full-length protein does not affect soluble PtdIns(3)P binding to the p40(phox) PX domain.

H_2O_2 induces caspase 3-independent degradation of Akt/PKB through the generation of reactive oxygen species and ceramide

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Oxidative stress is a major contributor to apoptosis of neural cells and a potential cause of neurodegeneration and senescence. However, hydrogen peroxide induces the activation of Akt/PKB, a kinase involved in promotion of cell survival. We analyzed early and late effects of H_2O_2 in PC12 cells. H_2O_2 produced early activation of Akt/PKB but also DNA damage and long-term production of reactive oxygen species (ROS) and activation of a glutathione-sensitive sphyngomyelinase. The rise in ceramide correlated with enhanced dephosphorylation of Akt/PKB. Following Akt/PKB dephosphorylation, we observed the proteolysis of Akt/PKB, p110PI3 kinase and ERK1/2. Specific inhibitors of caspase groups I and III but not calpain inhibitor II prevented proteolysis of Akt/PKB when compared to poly (ADP-ribose) polymerase. Surprisingly, the Akt/PKB double point mutant Akt(D108A/D119A), that is resistant to *in vitro* caspase 3-cleavage, was also degraded in H_2O_2-treated cells, suggesting the additional involvement of other caspases. A membrane-targeted myr-Akt1 was more resistant to H_2O_2-induced dephosphorylation and proteolysis and attenuated apoptosis induced by H_2O_2. Our results suggest that cells activate the Akt/PKB survival pathway to tolerate low oxidative injury and that strong oxidative insults generate intracelular ROS and ceramide that in term lead to down-regulation of this kinase by dephosphorylation and caspase-dependent proteolysis.

Ceramide signaling in cannabinoid action

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Cannabinoids, the active components of *Cannabis sativa* (marijuana) and their endogenous counterparts, exert their effects by binding to specific Gi/o-protein-coupled receptors that modulate adenylyl cyclase, ion channels and extracellular signal-regulated kinase (1). Recent research from our laboratory has shown that the CB1 cannabinoid receptor is also coupled to the generation of the lipid second messenger ceramide via two different pathways: sphingomyelin hydrolysis and ceramide synthesis *de novo* (2). Ceramide in turn mediates different cannabinoid actions such as stimulation of metabolism in primary astrocytes and induction of apoptosis in glioma cells, depending on the origin and characteristics of the generated ceramide pool. Of importance, cannabinoid-induced apoptosis of glioma cells in vitro correlates with their ability to induce tumor regression of malignant gliomas *in vivo* (3). These findings provide a new conceptual view on how seven-transmembrane cannabinoid receptors signal, and raise exciting therapeutic and physiological questions.

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Serum deprivation increases ceramide levels and induces apoptosis in embryonic hippocampal HN9.10e cells

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Sphingolipid metabolites have been involved in the regulation of proliferation, differentiation and apoptosis. While cellular mechanisms of these processes have been extensively analysed in postmitotic neurones, little is known about proliferating neuronal precursors. We have taken as a model of neuroblasts the embryonic hippocampal cell line HN9.10e. In undifferentiated cells, apoptosis was induced by serum deprivation and by treatment with N-acetylsphingosine (C2-Cer). The intracellular levels of ceramide peaked at one hour following serum deprivation. We observed translocation of Bax from cytosol to mitochondria after one hour of serum withdrawal followed, two hours later, by cytochrome c release from mitochondria. These events occurred without mitochondrial membrane potential loss nor mitochondrial calcium raise. As calcium is implicated in several cell death pathways, we analysed intracellular calcium levels after longer periods of serum deprivation. After six hours, an increase of cytosolic Ca^{++} was detected in HN9.10e cells loaded with the Ca^{++} indicator Fluo3-AM. We have also measured caspase-3 activity and we have found caspase-3 activation after 24 hours but not after 4 hours of serum deprivation or C2-Cer treatment. Cells serum-deprived for four hours and then grown in complete medium for twenty hours fully recovered viability. Summarising, in HN9.10e cells, apoptosis involves increases in ceramide levels, translocation of Bax, release of cytochrome c, and maintenance of mitochondrial functionality followed by calcium deregulation. The lack of caspase-3 activation, in addition to the maintenance of mitochondrial function could allow the reversibility of death commitment and provide neuroblasts a longer temporal window to decide their fate.

Mechanisms of phospholipase A2 regulated TNF- or IL-1β-induced activation of NF-κB

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Inflammation is the common symptom of chronic diseases, such as rheumatoid arthritis, cardiovascular disease, asthma, inflammatory Bowel diseases and psoriasis. The inflammatory reaction is regulated by lipid mediators, eicosanoids and lysophospholipids, derived from membrane phospholipids by the action of phospholipase A2 (PLA2). The molecular mechanisms of proinflammatory lipid action are not understood in detail. Elucidation of such mechanisms will identify novel targets for mechanism-based drug development.

Psoriasis is an inflammatory skin disorder, possibly of autoimmune origin. We have shown overexpression of specific PLA2 isoenzymes in human psoriatic skin [1,2]. Cytokines induce expression of proinflammatory and immunologic important genes by activation of nuclear transcription factor, NF-κB. We have found that IL-1β or TNF induced activation of NF-κB in keratinocytes is inhibited by selective inhibitors against either group IIa secretory or group IV cytosolic PLA2 [3]. Further investigation of the mechanisms of cytokine induced PLA2-regulated activation of NF-κB has revealed that the two PLA2 enzymes act in sequential manner, where the secretory enzyme regulates phosphorylation and activation of the intracellular cytosolic enzyme [4]. The sPLA2-generated signaling molecules include leukotriene B4 (LTB4) interacting with its G-protein-coupled cell surface receptor to induce intracellular kinase cascades involving phosphatidylinositol 3-kinase (PI 3-K), atypical protein kinase C (PKC) and p38 MAPK regulating phosphorylation of cPLA2 [5]. Understanding the molecular mechanisms of proinflammatory lipid mediator action may lead to identification of new targets for development of next generation anti-inflammatory drugs.

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Ceramide mediates insulin resistance by tumor necrosis factor alpha in brown adipocytes

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Tumor necrosis factor (TNF)alpha caused insulin resistance on glucose uptake in fetal brown adipocytes. We have explored the hypothesis that some effects of TNF could be mediated by the generation of ceramide, since TNF treatment induced the production of ceramide in these primary cells. A short-chain ceramide analogue, C2-ceramide, completely precluded insulin-stimulated glucose uptake and insulin-induced GLUT4 translocation to plasma membrane, either determined by Western blot or by immunofluorescent localization of GLUT4. These effects were not produced in the presence of a biologically inactive ceramide analogue, C2-dihydroceramide. Analysis of the phosphatidylinositol (PI) 3-kinase signaling pathway indicated that C2-ceramide was precluding insulin stimulation of Akt kinase activity, but neither PI3-kinase nor PKCzeta activities. C2-ceramide completely abolished insulin-stimulated Akt/PKB phosphorylation on both regulatory residues Thr308 and Ser473 as TNF did, as well as inhibited insulin-induced mobility shift in Akt1 and Akt2 separated in PAGE. Moreover, C2-ceramide seems to be activating a phosphatase involved in dephosphorylating Akt since 1) a PP2A activity was increased in C2-ceramide and TNF-alpha-treated cells, 2) treatment with okadaic acid concomitantly with C2-ceramide completely restored Akt phosphorylation by insulin and 3) transient transfection of a constitutively active form of Akt did not restore Akt activity. Our results indicate that ceramide produced by TNF induced insulin resistance in brown adipocytes by maintaining Akt in an inactive dephosphorylated state.

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Dynamics of DGKz translocation in T cells

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The recognition of specific membrane lipids by discrete protein modules is an essential mechanism for signal transduction. The diacylglycerol kinases (DGK) regulate diacylglycerol (DAG)-based signals by phosphorylating this key lipid intermediate to phosphatidic acid (PA). In mammals there are nine different DGK isoforms, grouped in five subtypes according to the presence of distinct domains with regulatory properties. Type IV DGKs, comprising the z and i isoforms, contain a domain homologous to MARCKS as well as four Ankyrin repeats and a PDZ-binding sequence. Here we have investigated the spatial and temporal regulation of diacylglycerol kinase z (DGKz) in living Jurkat cells expressing a muscarinic type I receptor. Using real time videomicroscopy we demonstrate the rapid translocation of a EGFP-DGKz chimera from the cytosol to the plasma membrane following receptor stimulation. The generation of a panel of truncations, deletions and point mutations of the enzyme allowed us to examine the requirements of each of its defined structural motifs both for activity and for receptor-regulated translocation. The data show that DGKz has strict requirements for intact zinc fingers and catalytic domain for full enzymatic activity. PKC-driven MARCKS domain phosphorylation and intact zinc fingers are in turn essential for plasma membrane translocation. DGKz does not translocate to the membrane following stimulation of the endogenous T cell receptor, and the specificity in terms of receptor response is provided by the regulatory motifs present at the carboxy-terminal domain of the protein. This is the first report to show in vivo DGKz translocation in response to agonist stimulation and establishes the role of the different domains in enzymatic activity and in selectivity of the response to receptors.

Membrane restructuring via ceramide results in enhanced solute efflux

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The capacity of ceramides to modify the permeability barrier of cell membranes has been explored. Membrane efflux induced either by in situ generated ceramides (through enzymatic cleavage of sphingomyelin), or by addition of ceramides to pre-formed membranes has been studied. Large unilamellar vesicles composed of different phospholipids and cholesterol, and containing entrapped fluorescent molecules,have been used as a system to assay ceramide-dependent efflux. Small proportions of ceramide (10 mole % of total lipid) that may exist under physiological condiditions of ceramide-dependent signalling have been used in most experiments. When long-chain (egg derived) ceramides are used, both externally added or enzymatically produced ceramides induce release of vesicle contents. However, the same proportion of ceramides generated by sphingomyelinase induce faster and more extensive efflux than when added in organic solution to the pre-formed vesicles. Under our conditions 10 mole % of N-acetylsphingosine (C2-ceramide) did not induce any efflux. On the order hand, sphingomyelinase treatment of bilayers containing 50 mole % sphingomyelin gave rise to release of fluorescein-derivatised dextrans of M=20 kDa, i.e. larger than cytochrome c. These results have been discussed in the light of our own previous data (Ruiz-Argüello et al., J. Biol. Chem. 271, 26616-26621, 1996) and of the observations by Siskind and Colombini (J. Biol. Chem. 275, 38640-38644, 2000). Our spectroscopic observations appear to be in good agreement with the electrophysiological studies of the latter authors. Furthermore, some experiments in this paper have been designed to explore the mechanism of ceramide-induced efflux. Two properties of ceramide, namely its capacity to induce negative monolayer curvature and its tendency to segregate into ceramide-rich domains, appear to be important in the membrane restructuring process.

The redistribution approach is utilizing intracellular protein translocation to discover novel drugs

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BioImage has developed a new drug discovery approach, Redistribution, for discovering drugs that specifically modulate the function of individual intracellular signalling proteins without affecting their catalytic activity. Redistribution allows identification of drug candidates against signalling targets, which previously have proven difficult to modulate with high selectivity. Translocations occur in most signalling pathways and are often causatively linked to the functional output of said pathway. At BioImage we have selected a library of signalling proteins that show patho-physiological functions and where such function involves an intracellular translocation event. The specific translocation of the selected molecule labelled with a proprietary Green Fluorescent Protein (GFP) is quantified in living cells in high throughput. Screening campaigns of diverse chemical libraries against several translocation targets has revealed that selective molecules can be identified. As an example we show how PKB-alpha/AKT1 can be inhibited without directly targeting its catalytic activity. PKBalpha translocates from the cytoplasm to the membrane upon cell stimulation. PKBalpha activity is frequently elevated in primary carcinomas of the prostate, breast and ovary. Upon screening our in-house library we found 17 compounds belonging to the same chemical class that can inhibit PKBalpha translocation. These compounds do not show toxicity when tested in toxicity assays. The biological specificity of the compounds is currently being validated in functional assays. Our newly identified lead compounds prove the Redistribution method to be a potential strategy in finding novel and selective drugs acting at difficult targets.

Activation and regulation of diacylglycerol kinase-theta by noradrenaline in intact rat small arteries

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Noradrenaline (NA) stimulates phosphoinositide (PIP2) hydrolysis [1] and activates diacylglycerol kinases (DGK) in vascular smooth muscle [2,3]. DGKs convert DG to phosphatidic acid (PA) resulting in: i) termination of protein kinase C (PKC) activation, ii) replenishment of cellular PIP2 and iii) formation of the second messenger PA. DGKs therefore play a crucial role in maintaining normal cell function. We have identified the NA-induced DGK activity as DGKtheta, present in both smooth muscle and endothelial cells of rat mesenteric small arteries (RMSA). Subcellular fractionation and immunoblot analysis revealed that DGK-theta was present in nuclear, plasma membrane (both Triton-X 100 soluble and insoluble components) and cytosolic fractions. Following sodium carbonate pH 11 extraction and discontinuous sucrose gradient fractionation DGK-theta was detected in a low-density fraction which also contained cholesterol, sphingomyelin, caveolin and src tyrosine kinase, markers of lipid rafts/caveolae. NA (15microM) induced a rapid translocation of DGK-theta to the plasma membrane (2.5 fold increase at 30s) followed by activation (2.5 fold at 60s), both effects returning to near basal levels at 5min. Membrane translocation and DGK-theta activation were differently regulated: NA-induced DGK-theta activation, but not translocation, was inhibited by wortmannin (0.1microM) and LY294002 (10microM) demonstrating dependence on phosphoinositide 3-kinase (PI3K) activity. In addition, following NA stimulation DGK activity co-immunoprecipitated with Akt/PKB suggesting that a signalling complex was formed. Inhibition of PI3K activity with LY294002 reduced the contractile response to NA and also reduced by approximately 30% the increase in PA. We conclude that in RMSA, PI3K and its downstream effector Akt/PKB is responsible for regulating NA-induced DGK-theta activation and that rapid phosphorylation of DG by DGK-theta, following NA stimulation would lead to an early attenuation of DG levels and presumably PKC activation. The rapid agonist-induced activation of DGK-theta suggests that this pathway may have a physiological role in vascular smooth muscle function.

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CD161 (hNKRP-1A) interacts with and positively regulates acid sphingomyelinase activity of human natural killer cells

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Natural killer (NK) cell plays a major role in innate immunity by killing a variety of virally infected and transformed target cells to which they have not been previously sensitised and have an inadequate expression of MHC class I molecules (Moretta et al., 2002). NKRP-1A , a C-type lectin-like NK receptor, is apparently involved in the regulation of NK and NKT cells function, but its mechanisms of action and signalling pathways are poorly understood. In an attempt to delineate the molecular details of the intracellular signals triggered by the human NKRP-1A (CD161) receptor, a two-hybrid screening assay using a spleen cDNA library was carried out and the enzyme acid sphingomyelinase (acid SMase) identified as a partner for the hNKRP-1A receptor.

The sphingomyelinases represent the catabolic pathway for ceramide generation, an emerging second messenger with key roles in induction of proliferation and differentiation, induction of apoptosis as well as cell-cycle arrest (Kolesnick and Fuks, 1995) (Adam et al., 2002). In the context of the immune system, ceramide has been recognized as a common intracellular second messenger for various cytokines (TNF-a, IL-1), IFNg or CD28 and CD95 (Fas/APO-1) receptor triggering.

In the present work, we found a new interaction between hNRP1-A and acid sphingomyelinase. The genetic evidence was confirmed by immunoprecipitacion followed by western blot analysis or acid sphingomyelinase enzymatic activity measurements using stable transfected YT or 293T with the full-length coding hNKRP-1A receptor. Acid sphingomyelinase activity was stimulated after CD161 cross-linking showing a fast time course peaking at 3 minutes. In contrast, neither CD161 cross-linking nor secondary anti-mouse IgG1 activated the neutral isoform of the human sphingomyelinase. Interesting, cross-linking of CD161 stimulated human NK primary cultures. Taken together, our data strongly suggest a new direct, physiologically relevant interaction between CD161 and acid sphingomyelinase.

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Essential contribution of CD3γ to T-cell receptor mediated phosphorylation, adhesion and apoptosis in human mature T lymphocytes

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CD3 proteins may have redundant as well as specific contributions to the intracellular propagation and final effector responses of TCR-mediated signals at different checkpoints during T-cell differentiation. We report here on the participation of CD3γ in the activation and effector function of human mature T lymphocytes at the antigen recognition checkpoint. Following TCR/CD3 engagement of human CD3γ-deficient T cell lines, and despite their lower TCR/CD3 surface levels compared to normal controls, mature T-cell responses such as the regulation of expression of several cell surface molecules, were either normal or only slightly delayed. In contrast, an early extinction of protein tyrosine phosphorylation was observed. Other physiological responses like the specific adhesion and concomitant cell polarization on ICAM-1-coated dishes were selectively defective, and activation-induced cell death was increased. Our data indicate that CD3γ contributes essential specialized signaling functions to certain mature T cell responses. Failure to generate appropriate interactions may abort cytoskeleton reorganization and initiate an apoptotic response.

Triton X-100 resistant bilayers: effect of lipid composition and relevance to the raft phenomenon

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The molecular basis for the existence of the so-called "detergent-resistant membranes" has been explored. With that aim, vesicles composed of phosphatidylcholine, sphingolipid and cholesterol were treated with the nonionic detergent Triton X-100 either at 4 °C or at 37°C, and tested for solubilization using turbidity and centrifugation methods. Bilayer fluidity was systematically measured as fluorescence anisotropy of a diphenylhexatriene derivative of phosphatidylcholine. Putative sphingomyelin-cholesterol interactions were explored using infrared spectroscopy. The combined experimental evidence clearly indicates that these lipid mixtures are solubilized more easily at 4°C than at 37°C, that an increased membrane fluidity does not correlate with an easier solubilization, and that sphingomyelin-cholesterol interactions are essential for insolubility. Sphingolipids by themselves do not hinder detergent solubilization, and some of them, e. g. gangliosides, actually increase bilayer solubility in the presence of detergents. At least with some lipid compositions, there is a range of detergent concentrations at which partial solubilization occurs concomitantly with major changes in bilayer architecture (lysis and reassembly). Moreover, a nonsolubilized residue of composition phosphatidylcholine:sphingomyelin:cholesterol of ca. 1:1:1: (mole ratio) is recovered by centrigugation after detergent treatment of vesicles with very different original lipid compositions. These observations do not preclude the presence of liquid-ordered domains in the cell membrane, but support the idea that the "detergent-resistant membranes" obtained after detergent treatments may well be the result of bilayer partial solubilization and reassembly, instead of corresponding precisely to structures pre-existing in the cell membrane.

CtBP3/BARS, a lysophosphatidic-acid acyl transferase inducing membrane fission in the Golgi complex, interacts with microtubules

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CtBP3/BARS is a component of the machinery responsible for the fission of Golgi membranes (Nature, 402: 429, 1999). When added to isolated Golgi membranes together with palmitoyl-CoA, this protein induces the formation of fission intermediates (zones of extreme constriction in Golgi tubules) and fragmentation of Golgi membranes. CtBP3/BARS is also able to transfer an acyl moiety from acyl coenzyme A to lysophosphatidic acid increasing the levels of phosphatidic acid in the membranes. The lysophosphatidic acid acyl transferase activity is thus proposed as an essential molecular process mediating membrane fission.

CtBP3/BARS is a member of the CtBP family of proteins, which includes regulators of gene transcription. In contrast to CtBP2, which is specifically localized in the nucleus, CtBP3/BARS, similarly to CtBP1, is mainly localized in the cytoplasm, and colocalizes with the Golgi complex. We are mainly interested in understanding the mechanisms of membrane fission. Thus, to identify other components of the fission machinery, we have searched for CtBP3/BARS-interacting proteins by co-immunoprecipitation and pull-down approaches. We find that CtBP3/BARS is able to co-precipitate with glyceraldehyde-3-phosphate dehydrogenase (GAPDH), tubulin, the microtubule-associated protein 2 (MAP2), and alpha-internexin. The interaction with microtubules is also confirmed by colocalization of CtBP3/BARS and microtubules in CtBP3/BARS-transfected cells. Our finding suggests that CtBP3/BARS and microtubules can cooperate in the formation of transport intermediates.

Ceramide 1-phosphate increases intracellular free calcium concentrations in thyroid FRTL-5 cells. Evidence for an effect mediated by intracellular sphingosine 1-phosphate

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Sphingolipid derivatives cause diverse effects towards the regulation of intracellular free calcium concentrations ($[Ca^{2+}]_i$) in a multitude of nonexcitable cells. In the present investigation, the effect of C-2 ceramide 1-phosphate (C1P), on $[Ca^{2+}]_i$ was investigated in thyroid FRTL-5 cells. C1P evoked a concentration-dependent increase in $[Ca^{2+}]_i$, both in a calcium-containing and a calcium-free buffer. A substantial part of the C1P-evoked increase in $[Ca^{2+}]_i$ was due to calcium entry. The effect of C1P was attenuated by overnight pretreatment of the cells with pertussis toxin. Similar results were obtained with C-8 ceramid 1-phosphate, although the magnitude of the responses was smaller than with C-2 C1P. C1P did not invoke the production of inositol phosphates, and the effect of C1P on $[Ca^{2+}]_i$ was inhibited neither by Xestospongin C, 2-aminoethoxydiphenylborate (2-APB) nor neomycin. C1P mobilized calcium from an inositol-1,4,5-triphosphate-sensitive calcium store, as C1P did not increase $[Ca^{2+}]_i$ in cells pretreated with thapsigargin. However, the effect of C1P on $[Ca^{2+}]_i$ was potently attenuated by dihydrosphingosine and dimethylsphingosine, two inhibitors of sphingosine kinase, and stimulating the cells with C1P evoked an increase in the production of intracellular sphingosine 1-phosphate (S1P). In cells treated with the phosphatase inhibitor calyculin A, the C1P-evoked increase in $[Ca^{2+}]_i$ was enhanced. C1P did not modulate DNA synthesis or the forskolin-evoked production of cAMP. The results show that C1P potently mobilizes sequestered calcium, possibly by an increase in intracellular S1P. The results indicate that C1P may be an important sphingolipid participating in cellular signalling.

Extended lipid anchorage and lipid binding of cytochrome c

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Binding of cytochrome c (cyt c) to fatty acids and acidic phospholipid membranes produces pronounced and essentially identical changes in the spectral properties of cyt c, revealing conformational changes in the protein. The exact mechanism of the interaction of fatty acids and acidic phospholipids with cyt c is unknown. Binding of cyt c to liposomes with high contents (mole fraction $X > 0.7$) of acidic phospholipids caused spectral changes identical to those due to binding of oleic acid. Fluorescence spectroscopy of a cyt c analog containing a Zn^{2+} substituted heme moiety and brominated lipid derivatives (9,10)-dibromostearate and 1-palmitoyl-2-(9,10)-dibromo-sn-glycero-3-phospho-rac-glycerol demonstrated a direct contact between the fluorescent [Zn^{2+}-heme] group and the brominated acyl chain. These data constitute direct evidence for interaction between an acyl chain of a membrane phospholipid and the inside of the protein containing the heme moiety and provide direct evidence for the so-called extended-lipid anchorage of cyt c to phospholipid membranes. In this mechanism, one of the phospholipid acyl chains protrudes out of the membrane and intercalates into a hydrophobic channel in cyt c while the other chain remains in the bilayer.

Insulin-induced UCP-1 expression is mediated by IRS-1/PI 3-kinase/Akt signaling pathway in brown adipocytes

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To investigate the role of IRS-1 and its signaling in insulin-induced thermogenic differentiation of brown adipocytes, we reconstituted fetal IRS-$1^{-/-}$ brown adipocytes with IRS-1^{wt}. Insulin failed to induce IRS-1-associated PI3-kinase activity, Akt phosphorylation and activation of α-, β- and γ-Akt isoforms in IRS-$1^{-/-}$ brown adipocytes. Reconstitution of IRS-$1^{-/-}$ cells with IRS-1^{wt} restored IRS-1/PI3-kinase/Akt signaling. Treatment of wild-type cells with insulin for 24 h up-regulated UCP-1 expression and transactivated the UCP-1 promoter, this effect being abolished in the absence of IRS-1 or in the presence of an Akt inhibitor, and further recovered after IRS-1^{wt} reconstitution. Insulin stimulated AP-1 and C/EBPα DNA binding activities in wild-type brown adipocytes, but not in IRS-$1^{-/-}$ cells, these effects being restored after IRS-1^{wt} reconstitution. Retrovirus-mediated reexpression of C/EBPα and PPARγ in IRS-$1^{-/-}$ brown adipocytes up-regulated UCP-1 and transactivated the UCP-1 promoter. Both C/EBPα and PPARγ reconstituted basal AP-1 activity and FAS mRNA expression, but only C/EBPα restored insulin sensitivity in the absence of IRS-1. Finally, reconstitution of IRS-$1^{-/-}$ brown adipocytes with the IRS-1 mutants IRS-1^{Y895F}, which lacks IRS-1/Grb-2 binding, but not IRS-1/p85-PI 3-kinase binding, or IRS-$1^{Y608/Y628/Y658}$ which only binds p85-PI 3-kinase, induced UCP-1 and transactivated the UCP-1 promoter.

These data provide evidences for an essential role of IRS-1 through PI 3-kinase/Akt signaling in initiating and/or maintaining insulin-induced thermogenic differentiation of brown adipocytes during development.

Developmental programmed cell death is balanced by nerve growth factor and insulin-like growth factor-I through the control of ceramide levels

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Nerve growth factor induces cell death in organotypic cultures of otic vesicle explants with a restricted pattern that reproduces the *in vivo* occurring apoptosis reported during inner ear development. In this work, we show that binding of nerve growth factor to its low affinity p75 neurotrophin receptor is essential to achieve the apoptotic response. Blocking antibodies of binding to p75 receptor neutralized nerve growth factor-induced cell death, as measured by immunoassays detecting the presence of cytosolic oligonucleosomes and by TUNEL assay to visualize DNA fragmentation. Nerve growth factor also induced a number of cell death-related intracellular events including ceramide generation, caspase activation and poly-(ADP ribose) polymerase cleavage. Again, p75 receptor blockade completely abolished all of them. Concerning the intracellular pathway, C2-ceramide increase depended on initiator caspases while its actions depended on both initiator and effector caspases as shown using site-specific caspase inhibitors. Conversely, insulin-like growth factor-I, which promotes cell growth and survival in the inner ear, abolished apoptosis induced by nerve growth factor. Insulin-like growth factor cytoprotective actions were accomplished, at least in part, by decreasing endogenous ceramide levels and activating Akt. Taken together, these results strongly suggest that regulation of nerve growth factor-induced apoptosis in the otocysts occurs via p75 receptor binding and it is strictly controlled by the interaction with survival signalling pathways.

Functional interaction of caveolin-1 with bruton's tyrosine kinase and bmx

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Bruton's tyrosine kinase (Btk), a member of the Tec family of protein tyrosine kinases, has been shown to be crucial for B cell development, differentiation, and signaling. Mutations in the Btk gene lead to X-linked agammaglobulinemia (XLA) in humans and X-linked immunodeficiency (*xid*) in mice. Using a co-transfection approach, we present evidence here that Btk interacts physically with caveolin-1 (cav-1), a 22-kDa integral membrane protein, which is the principal structural and regulatory component of caveolae membranes. In addition, we found that native Bmx, another member of the Tec family kinases, is associated with endogenous caveolin-1 in primary human umbilical vein endothelial cells (HUVEC). Secondly, in transient transfection assays, expression of caveolin-1 leads to a substantial reduction in the *in vivo* tyrosine phosphorylation of both Btk and its constitutively active form, E41K. Furthermore, a caveolin-1 scaffolding peptide (amino acids 82-101) functionally suppressed the auto-kinase activity of purified recombinant Btk protein. Thirdly, we demonstrate that mouse splenic B-lymphocytes express substantial amounts of caveolin-1. Interestingly, caveolin-1 was found to be constitutively phosphorylated on tyrosine 14 in these cells. The expression of caveolin-1 in B-lymphocytes and its interaction with Btk may have implications not only for B cell activation and signaling, but also for antigen presentation.

Cannabinoids protect astrocytes from ceramide-induced apoptosis

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Cannabinoids are known to protect neurons from toxicity as induced by different insults. However, the possibility that cannabinoids protect astrocytes from death has not been studied to date. Here, we tested whether cannabinoids are cytoprotective in a model in which primary astrocytes are exposed to C2-ceramide, a cell-permeable analogue of the pro-apoptotic lipid ceramide, to trigger apoptosis. Data show that (i) D9-tetrahydrocannabinol and other cannabinoids rescued astrocytes from ceramide-induced apoptosis, and (ii) this effect was CB1 receptor-mediated since SR141716 abolished the cannabinoid effect. Next we wondered on the mechanism involved in this anti-apoptotic effect of cannabinoids. We have recently shown that in transfected cells the CB1 receptor is coupled to the activation of PI3K/PKB, a pathway which is widely involved in cell survival. Data show that the anti-apoptotic action of cannabinoids in primary astrocytes relied on PI3K activation, via PKB and RSK stimulation. In conclusion, our data indicates that, while in glioma cells cannabinoids induce apoptosis via sustained ceramide generation and ERK activation (Nature Med. 6, 313-319, 2000), in primary astrocytes cannabinoids prevent ceramide-induced apoptosis by activating the PI3K pathway.

LIST OF INVITED SPEAKERS

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