Signal Transduction, Effectors and Targets in Tumor Cells: The Interface between Basic Research and Therapeutic Intervention

S10-01

C-RAF PROTEIN KINASE AS SIGNAL TRANSDUCER

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The c-Mil/Raf kinase is the normal cellular homologue of the viral oncogene v-mil/raf which codes for a serine/threonine-specific protein kinase and plays a role in signal transduction. Interaction of c-Mil/Raf protein with potential regulators and substrates is being analyzed by coimmunoprecipitation. The Raf Kinase is negatively regulated by PKA, binds to activated Ras, to 14-3-3 proteins, to a 90kD heat shock protein, a protein kinase of unknown origin, a 34kD protein which is phosphorylated in a cell-cycle dependent fashion. In certain cell-types c-Raf binds directly to the transcription factor Jun - which is also phosphorylated by the Raf kinase *in vitro*, suggesting a regulatory effect of c-Raf on nuclear transcription factors. Inhibition of the Raf-mediated signal pathway by dominant negative mutants transduced by retroviral vectors into tumor cells resulted in delayed tumor formation in nude mice. *In vitro* the tansformed phenotype was reverted with no change of cell proliferation.

S10-02

RHO GTPASES AND THEIR ACTIVATORS THE DBL ONCOGENES

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DBL-homology proteins act as guanine nucleotide exchange factors for members of the Rho family of small guanosine triphosphatases (GTPases). RhoGTPases regulate the organization of the actin cytoskeleton; Rho controls the assembly of actin stress fibres and focal adhesion complexes, Rac regulates actin filament accumulation at the plasma membrane to produce lamellipodia and membrane ruffles and Cdc42 stimulates the formation of filopodia. When microinjected into quiescent fibroblasts Rho, Rac and Cdc42 stimulate cell cycle progression through G_1 and subsequent DNA synthesis. Furthermore, microinjection of dominant negative forms of Rac and Cdc42 or of the Rho inhibitor C3 transferase, blocks serum-induced DNA synthesis. Unlike Ras, none of the Rho GTPases activate the mitogen-activated protein kinase cascade. Instead Rac and Cdc42, but not Rho, stimulate a distinct MAP kinase, the c-Jun kinase JNK/SAPK. Rho, Rac and Cdc42 control signal transduction pathways that are essential for cell growth.

S10-03

DEVELOPMENT OF PROTEIN-FARNESYLTRANSFERASE INHIBITORS AS POTENTIAL THERAPEUTIC AGENTS.

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Post translational addition of a farnesyl moiety to the Ras oncoprotein is essential for its efficient cell transforming activity. Therefore, inhibitors of the enzyme which catalyzes this modification, protein-farnesyltransferase (PFTase), may be effective agents in the treatment of human tumors. We have identified a mimetic of the Ras CAAX tetrapeptide, L-739,750, which is a potent and selective inhibitor of human PFTase *in vitro*. An ester prodrug of this compound, L-744,832, inhibited Ras processing in cells in culture and blocked the anchorage independent growth of human tumor epithelial cells and *ras*-transformed rodent fibroblasts. In animals, daily administration of L-744,832 inhibited the growth of H-*ras* dependent tumor xenografts in nude mice and caused tumor regression in MMTV-v-Ha-*ras* transgenic mice. No systemic toxicity was found upon necropsy of any L-744,832 treated mice. These results suggest that PFTase inhibitors may be safe and effective anti-tumor agents in some cancers.

S10-04

PROTEIN-TYROSINE KINASES AS THERAPEUTIC TARGETS.

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Protein-tyrosine kinases play a crucial role in the signal transduction pathways required for mitogenesis, transformation and cell differentiation. Uncontrolled signaling from growth factor receptor and intracellular tyrosine kinases has been implicated in various cancers as well as in non-malignant proliferative diseases such as atherosclerosis, restenosis and psoriasis. Protein-tyrosine kinases and their signaling pathways have therefore been identified as potential targets for drug design. Due to the involvement of tyrosine kinases in many signal transduction pathways, a major challenge is the development of highly selective inhibitors. Although the kinase domain is the most conserved domain of receptor and cytoplasmic tyrosine kinases and contains several residues that form a typical ATP-binding site, we have been able to develop novel selective inhibitors for a variety of protein-tyrosine targets. Examples of potent inhibitors with selectivity for the PDGF receptor, EGF receptor and Bcr-Abl tyrosine kinases will be shown. The emerging success in using tyrosine kinase blockers which inhibit growth of cancer cells *in vitro* and *in vivo*, will be discussed.

S10-05

Wortmannin inactivates PI 3-kinase via modification of Lys 802

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Wortmannin (Wt) inhibits phosphoinositide 3-kinase (PI 3-K) at nM concentrations[1] and has been used extensively to demonstrate the role of this enzyme in diverse signal transduction processes. At higher concentrations, the inhibitor was recently shown to block the ataxia telangiectasia gene (ATM)-related DNA-dependent kinase (DNA-PK_{cs}). We identified here the site of interaction of Wt on the catalytic subunit p110α. At pH 6 to 8 Wt reacted specifically with p110α. PI(4,5)P_2, ATP, adenine and FSBA competed effectively with Wt, suggesting that the Wt target site is close to the substrate-binding site. Proteolytic fragments of Wt-treated p110α were mapped using α-Wt- and α-p110α peptide antibodies, thus limiting the target site to a 10 kD fragment within the ATP-binding site. Site-directed mutagenesis of all candidate residues within this region showed that only the conservative Lys802 to Arg mutation abolished Wt-binding. Inhibition of PI 3-K occurs therefore by the formation of an enamine following the attack of Lys802 on the furan ring of Wt. The Lys802Arg mutant was unable to bind FSBA, and was catalytically inactive in lipid and protein kinase assays, indicating a crucial role for Lys802 in the phosphotransfer reaction. The above results, and a structural model derived thereof, provide the basis for the design of novel and specific inhibitors of an enzyme family including PI 3-Ks and ATM-related genes, that play a central role in many physiological processes. [1]Arcaro, A. and Wymann, M.P. 1993 *Biochem. J.* 296:297; Wymann, M.P. and Arcaro, A. 1994 *Biochem. J.* 298:517.

S10-06

ACTIVITY OF MAP KINASE IS NEGATIVELY REGULATED BY PHOSPHATASE 2A IN VENTRICULAR CARDIOMYOCYTES

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We studied the role of phosphatase 2A (PP2A) in regulating the activity of mitogen-activated protein kinase (MAPK) during stimulation of protein kinase C in cultured, spontaneously-beating, ventricular cardiomyocytes from neonatal rats. Phosphorylation of enzymes was investigated by immunoprecipitation and electrophoresis. MAPK activity was determined by phosphorylation of myelin basic protein and, phosphatase activity by dephosphorylation of previously labelled [^{32}P]-histone IIIS. Incubation of cardiomyocytes with 100 nM phorbol 12-myristate-13-acetate (PMA) induced a transient suppression of PP2A activity at 5 min, an effect that was reversed after 15 min of exposure to PMA. This inactivation was accompagnied *by a rapid and reversible* phosphorylation of PP2A. The transient inactivtion of PP2A was correlated with a transient increase in the phosphorylation level and activity of 42-kDa MAPK. The presence of the PP2A inhibitor okadaic acid (1μM) blocked the decrease in the phosphorylation level and activity of MAPK occurring after 5 min of exposure to PMA, while in unstimulated cardiomyocytes okadaic acid induced a sustained activation of MAPK. Our results indicate that in cardiomyocytes MAPK activity is negatively regulated by PP2A.

S10-07

CONSTITUTIVE ACTIVATION OF HGF/SFR (C-MET) IS A NECESSARY BUT NOT SUFFICIENT CONDITION FOR LIVER COLONIZATION BY B16-LS9 CELLS

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Serial selection of B16 cells *in vivo* for liver colonization resulted in a cell line, B16-LS9, which overexpresses a constitutive active form of the Hepatocyte Growth Factor/Scatter Factor receptor (HGF/SFr), the product of the c-met proto-oncogene. Overexpression of c-met in B16-LS9 cells results in a stronger response to HGF treatment in terms of motility and invasion, and a higher inducibility of proteolytic activities (uPA and gelatinase) as compared to its parental line B16-F1. To further investigate the role of c-met in liver metastasis, several F1 and LS9 clones with either high or low c-met expression, have been analyzed for their organ colonization ability after tail vein injection in syngeneic mice. Results show that elevation of c-met expression and activation better correlates with the colonization efficiency rather than the organotropism. However, further analysis of c-met in tumors developing at different sites suggests that overexpression of c-met could be required at later stages during the process of liver colonization.

S10-08

THE ISOLATION AND CHARACTERIZATION OF A NOVEL HEREGULIN FAMILY MEMBER FROM HUMAN BREAST CANCER CELLS

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We isolated recently from medium conditionned by the MDA-MB-231 breast cancer cell line a novel heregulin familly member that we denominated Mammamodulin (MM). We could show that this 52kDa protein acts as a potent mitogen on estrogen receptor(ER)-positive breast cancer cells. MM produces morphological changes on ER-positive cells (membrane ruffling) and stimulates transcription of the proto-oncogenes c-*myc* and c-*fos* but, as opposed to heregulin β1, it down-regulates the estrogen receptor at the mRNA and protein level without activating it. The heregulins are known ligands of the ErbB family of receptors. The ErbB-2 receptor is often overexpressed in breast cancer and is a marker for poor prognosis. Preliminary experiments indicate that our purified growth factor specifically binds to the ErbB-2 protein whereas the other heregulins are shown to bind specifically to the ErbB-3 and ErbB-4. The isolation of MM cDNA from a MDA-MB-231 cDNA library is currently under investigation. (supported by the SNF grants Nr 31-32510.91 (H.M.) and 3100-037633.93 (U.E.).)

S10-09

POINT MUTATIONS OF THE GABA(A) RECEPTOR: HOMOLOGY BETWEEN THE BINDING SITES FOR CHANNEL AGONISTS AND BENZODIAZEPINE LIGANDS.

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Clinically relevant benzodiazepines allosterically stimulate neurotransmitter evoked chloride currents at the gamma-aminobutyric acid type A (GABA(A)) receptor. Rat wild type or mutated α1-, β2-, and γ2S-subunits were coexpressed in Xenopus oocytes and investigated electrophysiologically. Mutation of one of four amino acid residues (two on α, one on β and one on γ) resulted in about a three-fold increase in potentiation by the benzodiazepine diazepam. The response to the imidazopyridine zolpidem was increased in three mutant channels containing the mutated α- and β- subunits but was nearly absent in channels containing the mutated γ-subunit. The amino acid residues influencing benzodiazepine effects are close to or identical to homologous residues implicated in GABA binding. Therefore, they may be part of the benzodiazepine binding site and may be located at subunit interfaces.

S10-10

Antibodies Against Membrane-Associated IL-1ß and Soluble IL-1 Receptor Stimulated IL-6 and IL-8 Expression in Human Dermal Fibroblasts

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IL-1β, the predominant form of IL-1 released by human monocytes, is related to various infectious, inflammatory, or hematological diseases. Pro-IL-1β (33 kD) is normally proteolytically cleaved by IL-1β converting enzyme (ICE) to generate the secreted mature form of IL-1β (17.5 kD). Fibroblasts express IL-1β mRNA upon stimulation with tumor necrosis factor-α (TNF-α) or lipopolysaccharides (LPS), but they do not process or secrete IL-1 β. Using human dermal fibroblasts (HDF), we could demonstrate, by immunocytochemistry, a membrane-bound form of IL-1β. This membrane-bound IL-1β on glutaraldehyde-fixed HDF was able to stimulate IL-6 production in viable T cells by a juxtacrine mechanism. If untreated, glutaraldehyde-fixed T cells were cocultured with viable HDF, an increase of the basal IL-6 production by HDF was demonstrated. Monoclonal antibodies against IL-1β or soluble IL-1 receptor stimulated HDF to increase their IL-6 and IL-8 mRNA expression. Therefore the membrane IL-1β has a functional role, not only as a ligand, which acts in a juxtacrine fashion but also as a cell surface receptor on HDF, which may be important in triggering cytokine production.

S10-11

LIGAND-INDEPENDENT ACTIVATION OF STEROID RECEPTORS IN YEAST

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Steroid receptors are ligand-regulated transcription factors. Surprisingly, in multicellular organisms, agents which stimulate intracellular phosphorylation pathways, such as EGF, dopamine, 8-Br-cAMP, okadaic acid, phorbol esters, etc., can activate steroid receptors in a ligand-independent manner. We have sought to recapitulate this phenomenon in the budding yeast *S. cerevisiae*. We find that treatment with the protein kinase C(PKC) activator 12-phorbol 13-myristate acetate(PMA) stimulates progesterone(PR) and estrogen receptor-mediated transcription in the absence of hormone in yeast. However, the target of PMA is not the yeast PKC1. The hormone binding domain(HBD) of PR is sufficient for responding to both PMA and progesterone. Thus, steroid-independent activation of steroid receptors can be studied in yeast. We are now screening for PMA response mutants to define the pathway.

S10-12

BRADYKININ-INDUCED GROWTH CONE COLLAPSE OF RAT PHEOCHROMOCYTOMA (PC12) CELLS

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Bradykinin (Bk) is a main mediator of inflammation and pain. It stimulates phospholipase C in target cells by activating specific G-protein coupled receptors. Here, we demonstrate that Bk causes transient growth cone collapse of NGF-differentiated rat pheochromocytoma (PC12) cells. The response is mediated by B2-type receptors and consisted of two consecutive events: (1) depolymerization of F-actin in the filopodia (EC_{50} = 1.5 pM) followed by (2) retraction of the growth cone. The collapse occurs in paralell with the Bk-induced Ca^{2+} release (EC_{50} = 2.5 nM). The significance of the Ca^{2+} signal is challenged, since retraction occurred in BAPTA/AM-loaded cells, in thapsigargin-treated cells or in Ca^{2+}-free medium, respectively. Moreover, the tyrosine kinase inhibitor genistein inhibited the reaction. Therefore, our data suggest that Bk initiates growth cone collapse by activating a tyrosine-kinase linked pathway.

S10-13

NORADRENALINE REDUCES, TROUGH α_2 ADRENERGIC RECEPTORS, SYNAPTIC TRANSMISSION BETWEEN THE ENTORHINAL CORTEX AND THE BASOLATERAL AMYGDALA

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Synaptic responses of 80 basolateral amygdala (BLA) neurones to either entorhinal cortex (EC), underlying white matter (WM) or direct BLA stimulations where studied in horizontal brain slices comprising the hippocampus, the EC and the BLA using intracellular recordings. Synaptic responses consist of a fast excitatory post-synaptic potential (fEPSP) followed by a strong fast inhibitory post-synaptic potential (fIPSP). fIPSPs time to peak increased from 20.5 ± 2.1 ms (sem, n= 7) to 22.2 ± 3.0 ms (n=14) and 32.9 ± 0.7 ms (n=18) with BLA, WM and EC simulations respectively. When the WM or the EC were stimulated, bath application of the glutamatergic antagonists CNQX (10 µM) and d-AP5 (100 µM) inhibited almost completely fIPSPs while fIPSPs generated in the BLA were resistant to this treatment. fIPSPs recorded after EC stimulation were reduced by application of 50 µM noradrenaline (NA) to 46.7 ± 4.6 % (n= 8) of the control response. NA reduction of the fIPSP was almost completly blocked by bath application of 10 µM of the α_2 andrenoreceptor antagonist yohimbine.These results suggest that significant components of the synaptic connections between the EC and the BLA are glutamatergic afferents to BLA interneurones and that NA, via α_2 adrenoreceptors, inhibits this connection.

Supported by the FNRS 31-040545.94

S10-14

GLUCOCORTICOIDS REGULATE THE EXPRESSION OF THE STRESSPROTEIN ALPHA B-CRISTALLIN

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αB-crystallin is a major component of the eye lens but is also found in many extralenticular tissues. In established fibroblasts it is synthesized in response to stress such as hyperthermia. Here we report that the treatment of NIH3T3 fibroblasts with the synthetic glucocorticoid (GC) hormone dexamethasone resulted in the accumulation of substantial amounts of αB-crystallin. αB-crystallin mRNA accumulated slowly and over a period of many days in response to prolonged hormone treatment. αB-crystallin promoter-reporter constructs were hormone responsive. A putative GRE within the analysed promoter region could bind the GC receptor as revealed from *in vitro* footprint analysis but is not involved in the hormone mediated gene activation. A deletion of 76 bp between -465 and -389 abolished GC-mediated gene induction. The absence of a GC receptor binding site within the GC-responsive region and the slow kinetics of gene activation suggest that the αB-crystallin gene responds in an indirect manner to hormone stimulation.

S10-15

PHORBOL MYRISTATE ACETATE INHIBITS THE BINDING OF COLONY-STIMULATING FACTOR-1 TO RAT OSTEOCLASTS.

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Colony stimulating factor-1 (CSF-1) is the growth factor for the cells of mononuclear phagocyte system and for osteoclasts (Oc). Phorbol myristate acetate (PMA), a protein kinase C (PKC) activating phorbol ester, downmodulates the CSF-1 receptor on macrophages. In this study, we tested whether PMA modulates the CSF-1 binding sites on mature osteoclasts.

Oc were isolated from femurs of 1 day old rats. This preparation is still contaminated with osteoblasts (OcM). Pure Oc(OcP) were picked up by micropipette. The OcM, OcP, OcP plus osteoblastic UMR106 cells were incubated ±PMA at 37^{0}C. The binding of CSF-1 was determined by incubation with [^{125}I]CSF-1 at 0^{0}C and quantified by autoradiography.

PMA decreased the binding of CSF-1 in a dose- and time-dependent manner. At the concentration of 10^{-7}M, it inhibited by 83% in OcM, by 59% in OcP and by 84% in OcP+UMR106 cells.

In conclusion, we have demonstrated that activation of PKC induces a down-regulation of CSF-1 binding sites on osteoclasts, the effect is enhanced when osteoblasts are present.

S10-16

Tumor Necrosis Factor α Modulates Mitogen-Activated Protein Kinase Activity of Epidermal Growth Factor-Stimulated MCF-7 Breast Cancer Cells.

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Tumor necrosis factor α (TNF-α) addition to epidermal growth factor (EGF)-stimulated MCF-7 breast cancer cells resulted in a dose-dependent inhibition of growth. Both EGF and TNF-α induced mitogen activated protein (MAP) kinase activity. However, MAP kinase activity was 50% inhibited within 3 minutes when TNF-α was added to EGF-stimulated cells. This observation indicates a regulation of MAP kinase activity in MCF-7 cells by inhibitory receptor pathways such as TNF-α. Since TNF-α did neither alter the autophosphorylation of the EGF receptor nor the tyrosine phosphorylation of EGF receptor substrates, such as PLC-γ-1 or ras-GAP, regulation of EGF-stimulated MAP kinase activity by TNF-α is likely to occur either downstream of the EGF receptor substrates or directly at the MAP kinase (supported by SNF grant Nr. 31-32510 (H.M.)).

S10-17

The role of protein kinase A and protein kinase C in epidermal growth factor (EGF) receptor signaling in human MCF-7 breast cancer cells.

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Activation of protein kinase C (PK-C) α, ß and γ by the specific agonist thymeleatoxin stimulated growth of MCF-7 cells considerably at concentrations between 10^{-5} and 10^{-6}M. The expression of the immediate early gene c-fos was activated by thymeleatoxin whereas the expression of c-myc was not affected. The specific PK-C blocker chelerythrine reversed the inhibitory effect of thymeleatoxin on cell growth and c-fos expression. In contrast, activation of protein kinase A (PK-A) by dBcAMP or the cyclase agonist forskolin did not stimulate growth of MCF-7 cells significantly but increased the expression of c-myc. The cAMP analogue RpcAMP served as a PK-A inhibitor and blocked the stimulation of PK-A induced c-myc expression. Taken together, these data indicate that PK-A and PK-C might have different roles in signaling pathways of MCF-7 human breast cancer cells. Although both kinases are activated by the same pathways, e.g. EGF receptors, PK-C seems to act as the stimulatory branch of the signaling pathway whereas PK-A might have a modulatory function on cell proliferation. (Supported by SNF grant Nr. 31-32510.91)

S10-18

VEGF IN HUMAN MAMMARY TUMORS

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Vascular endothelial growth factor (VEGF) was measured in extracts of human mammary tumors by a newly developed chemiluminescence immuno sorbent assay (CLISA). In 142 primary tumors, the median value was 0.24 ng VEGF/mg protein (0 to 12.27 ng/mg). In extracts of normal breast tissues adjacent to the tumors, VEGF levels were much lower than in the cancer tissues. VEGF concentrations of mammary tumors correlated significantly with prognostic factors: tumor size ($p<0.0001$, $r=0.334$), c-erbB2 ($p= 0.003$, $r= 0.250$), uPA ($p= 0.011$, $r= 0.214$), PAI-1 ($p= 0.028$, $r= 0.184$), cathepsin D ($p= 0.039$, $r= 0.173$) and progesterone receptor ($p=0.013$, $r= -0.208$). VEGF levels in tumors of axillary lymph node negative and positive patients were not significantly different. As shown by others, VEGF levels determined immunohistochemically correlate strongly with the grade of vascularization which in turn correlates with the incidence of metastasis and early death.Thus, the quantitation of VEGF may be of use as a prognostic tumor marker and as an indicator for future individual anti-VEGF treatment.

(Supported by Swiss Cancer League grant FOR 43.1991)

S10-19

IMIDAZOLINE RECEPTORS (IR) AND GFAP IN A CLONAL CELL LINE OF TYPE 1 ASTROCYTES.

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Astrocytic I_2-IR, activated by endogenous agmatine, appear to regulate the expression of GFAP. The aim of this study was to assess the modulatory role of I_2-IR on GFAP in type 1 astrocytes derived from rat diencephalon and transfected with a DNA construct containing the human GFAP promoter (cell line DI TNC_1; Radany et al., PNAS 89: 6467, 1992). In these cells, western blot analysis, using an anti-IR antibody, showed the presence of various IR proteins (29/30- and 45-kDa). In serum-free media, agmatine (100 µM, 4-24 h) did not alter the basal expression of GFAP immunoreactivity. Idazoxan, a mixed I_2-IR/α_2-adrenoceptor (α_2R) ligand, increased in a concentration (10-333 µM) and time (4-24 h) dependent manner the levels of GFAP (100 µM at 24 h: 65%). Alkylation of α_2R with EEDQ did not alter the basal levels of GFAP, but markedly reduced the stimulatory effect of idazoxan on GFAP (100 µM at 24 h: 19%). LSL 60101 (2-(2-benzofuranyl) imidazol), a selective I_2-IR ligand, did not alter the levels of GFAP in the range 1-100 µM (24 h) but at higher concentrations GFAP was decreased (333 µM at 24 h: 28%). Treatment with idazoxan and LSL 60101 (10 mg/kg for 7 days) was shown to increase GFAP and I_2-IR levels in rat brain. It is concluded that the clonal cell line DI TNC_1 is not a convenient model to study the I_2-IR selective modulation on GFAP.

S10-20

Characterization of the phosphorylation sites involved in the regulation of the α_{1B}-adrenergic receptor.

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The α_{1B}-adrenergic receptor (α_{1B}-AR) is a member of the G protein-coupled receptor superfamily. Agonist- as well as phorbol ester-induced desensitization of the α_{1B}-AR is mediated by receptor phosphorylation on serine residues via the activation of distinct protein kinases. We previously showed that the carboxyl portion of the α_{1B}-AR plays a crucial role in receptor regulation as demonstrated by the fact that truncation of the last 149 amino acids abolished both agonist and phorbol ester-induced receptor phosphorylation and desensitization. To identify the serine residues involved in phosphorylation of the α_{1B}-AR, we constructed three receptor mutants: two truncated receptors (T469 and T412), lacking the last 46 and 103 amino acids, respectively, and a deletion mutant (Δ392-413) lacking a region of 21 amino acids containing seven serines. Both the T412 and T469 mutants were still able to undergo agonist- as well as phorbol ester-induced phosphorylation suggesting that the main phosphorylation sites are located before residue 412 of the α_{1B}-AR. On the other hand, the deletion mutant Δ392-413 was not phosphoryated either by the agonist or by phorbol esters. This provides evidence that the major phosphorylation sites of the α_{1B}-AR are represented by some of the seven serines located in the stretch of sequence including amino acids 392-412. Our future goal is to discriminate the serines phosphorylated upon stimulation of the α_{1B}-AR with the agonist *versus* those phosphorylated following phorbol ester-induced activation of protein kinase C.

S10-21

MUTATION OF THE HIGHLY CONSERVED DRY MOTIF CONSTITUTIVELY ACTIVATES THE α_{1B}-ADRENERGIC RECEPTOR.

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The α_{1B}-adrenergic receptor (AR) is a member of the large superfamily of the seven transmembrane domain (TMD) G protein-coupled receptors (GPCR). So far, very little is known about the transition of GPCRs from their "inactive" to "active" conformation. We have previously discovered that point mutations in the C-terminal portion of the third intracellular loop (iL) resulted in ligand-independent (constitutive) activation of different ARs. This initially suggested that the C-terminal portion of the third iL plays an important role in receptor activation. However, more recently spontaneously occuring activating mutations have been found in different regions of various GPCRs indicating that the transition of GPCRs from their "inactive" to "active" conformation relies on a series of intramolecular interactions among different receptor domains. In the attempt to assess the intramolecular mechanisms involved in receptor activation, we have simulated the "activation" process of the α_{1B}-AR by molecular modelling. Following the predictions of molecular dynamics simulation, we have mutated the highly conserved DRY motif in the cytosolic end of the third TMD of the α_{1B}-AR. Mutations of this region resulted in either loss of G protein coupling or in constitutive activation of the receptor. These results suggest that the DRY region is an additional domain crucially involved in the activation of the a_{1B}-AR as well as of other GPCRs.

S10-22

IN VITRO INTERACTIONS OF GLUCOCORTICOID RECEPTORS

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The glucocorticoid receptor (GR) is a hormone dependent transcription factor. We recently generated a dominant negative GR by changing the reading frame of a trinucleotide repeat in the N-terminal region (1). We hypothesize that the altered stretch induces a conformational change that affects the interaction of the receptor with other components of the transcriptional machinery. To test this, we expressed GST-GR fusion proteins in an eukaryotic system. We are performing in vitro transcription experiments with the purified receptors and pull down experiments with the receptors bound to a glutathione-agarose column to identify differentially interacting partners. We confirmed that a fusion of GST with the carboxy-truncated GR (missing the HBD) maintains its ability to transactivate in a transient reporter assay. However, we noticed that fusion of GST with full-length GR produces an inactive chimera that surprisingly acts itself as a dominant negative GR.

(1) Lanz et al.(1995), Nucl. Acids Res. 23, 138-145

S10-23

β_3-ADRENOCEPTOR STIMULATION INHIBITS *OBESE* GENE EXPRESSION IN MOUSE BROWN ADIPOCYTES DIFFERENTIATED IN CULTURE.

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The *ob* gene mRNA is highly expressed in mouse brown adipocytes differentiated in culture. The classical β_3-adrenergic agonist BRL 37344 decreased its level by 40 ± 9 and 88 ± 2 % at concentrations of 1 and 10 nM, respectively in 4 hours. (-)-Isoproterenol had the same effect but at concentrations about 2 orders of magnitude higher than those of BRL 37344. Adenosine deaminase, 2 UI/ml, which stimulated lipolysis more than the β-adrenergic agonists did not affect *ob* gene mRNA level. This data indicates that the down regulation of *ob* gene mRNA by β-adrenergic agonists is not secondary to a decrease in the cell lipid content.

S10-24

AN INHIBITORY FEEDBACK LOOP REGULATES SV40 LARGE T-ANTIGEN FUSION PROTEIN NUCLEAR TRANSPORT

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Nuclear protein import is central to eukaryotic cell function. It is dependent on ATP, temperature, cytosolic factors, and specific targeting sequences (nuclear localisation signals - NLSs). We studied nuclear import kinetics *in vitro* using digitonin permeabilised HTC rat hepatoma cells and a fluorescently labelled fusion protein carrying amino acids 111-135 of the SV40 large T-antigen (T-ag) including the NLS. Nuclear accumulation reached steady state after c. 80 min at 37°C; surprisingly, the maximal nuclear concentration was found to be directly proportional to the concentration of both the cytosolic extract and the cytoplasmic T-ag protein. If cells were allowed to accumulate T-ag protein for 1h prior to the addition of fresh transport medium containing different concentrations of T-ag protein and incubated for a further 1h, the maximal nuclear concentration did not change unless the concentration of T-ag protein in the second mix exceeded that in the first, in which case the nuclear concentration increased. Nuclear import of T-ag thus appeared (i) to be strictly uni-directional in the import direction over 2h at 37°C and (ii) to be regulated by an inhibitory feedback loop, the cytosolic concentration of protein directly determining the end-point of nuclear accumulation.

S10-25

***Src* KINASES IN GPI DOMAINS OF THE LYMPHOCYTE PLASMA MEMBRANE ARE IN A HIGHER STATE OF ACTIVATION**

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Plasma membrane domains enriched in glycosylphosphatidylinositol (GPI)-linked or transmembrane glycoproteins have been isolated by subcellular fractionation. The GPI domains contain a TX-100-resistant core made of glycosphingolipids, GPI-linked surface glycoproteins and *src*-family kinases. The lck and fyn kinases are associated with the plasma membrane, within GPI domains as well as without. The kinases in both domains are active in phosphorylating endogenous and exogenous substrates, but the specific activity of the kinases in GPI domains is considerably higher than that of kinases associated elsewhere with the plasma membrane. The *src* kinase activity of GPI domains is minimally perturbed by TX-100, while the same kinases bound to non-GPI domains undergo a marked increase in activity in the presence of TX-100. This suggests that *src* kinases bound to non-GPI plasma membrane domains are downregulated in their native membrane environment. GPI domains in lymphocyte plasma membranes could represent privileged sites where *src* kinases are maintained in an optimal state of activation to initiate transmembrane signalling. This property of the *src* kinases in GPI domains may underlie the known capacity of GPI-linked surface molecules to trigger cellular activation upon cross-linking.

S10-26

C-FOS IS UPREGULATED IN DISEASED HUMAN SKELETAL MUSCLES AND IN NORMAL MUSCLES AFTER ENDURANCE EXERCISE

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We have found the mRNA coding for the proto-oncogene c-fos to be increased up to 20 fold in biopsies of five untrained subjects after a 30 minute run at their anaerobic threshold. The concomitant activation of stress activated protein kinase suggests a transient increase in AP-1 activity. *In situ* hybridization indicated that c-fos was mainly increased in type I fibers, which are primarily recruited in this exercise. We hypothesize that this AP-1 increase is a prerequisite for the well known structural adaptations to endurance training.
In biopsies from patients with various muscle diseases c-fos mRNA was also upregulated when compared to normal, unstressed muscle. In all nine samples of diseased muscles studied so far, the ratio between an unspliced c-fos transcript and c-fos mRNA was at least 10 fold higher than in normal muscles after exercise. This could reflect a more continuous c-fos upregulation in diseased muscles. Preliminary *in situ* hybridizations on a muscle sample from a Duchenne patient indicate that c-fos is expressed by cells in areas of degeneration/regeneration, but also by some but not all fibers of normal size.

S10-27

WORTMANNIN SELECTIVELY INHIBITS RECEPTOR-LINKED INCREASES IN CYTOSKELETAL ACTIN IN HUMAN NEUTROPHILS

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The signals involved in chemoattractant-induced increases in F-actin and cytoskeletal actin in neutrophils are as yet unknown. Chemotactic stimuli activate the enzyme PI-3-kinase which is inhibited by nM concentrations of wortmannin. We have now found, that preincubation of neutrophils with 200 nM wortmannin inhibits the increase in cytoskeletal actin induced by chemotactic peptide in 1 min by 67 ± 11% (mean ± SD, n=3), and that by a 30 min incubation by 72 ± 28%. In contrast, the increase in cytoskeletal actin induced by 10 nM phorbol-myristate-acetate was not at all inhibited (101 ± 12% of the maximal response after preincubation with 200 nM wortmannin). These results suggest, that activation of PI-3-kinase, and the ensuing increase in phosphatidylinositol-3,4,5-tris-phosphate may be involved in receptor-linked actin reorganization in neutrophils.

S10-28

VARIATION OF CAVEOLIN EXPRESSION BETWEEN METASTATIC AND NON-METASTATIC HUMAN COLON CARCINOMA CELL LINES

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Caveolae or plasmalemmal vesicules are non-clathrin coated membrane invaginations particularly abundant in endothelial cells, smooth muscle cells and fibroblasts, that are enriched in a protein called VIP-21/caveolin. Caveolae also contain many proteins involved in signal transduction, suggesting a role for caveolin in such events. In addition, caveolin levels are reduced in oncogenically transformed NIH 3T3 cell lines, indicating that the presence of caveolin may modulate the ability of cells to proliferate in culture. Given that caveolin might be important for cellular signal transduction in general and growth control in particular, we analysed its expression in human colon carcinoma cell lines. We found significant differences at both the mRNA and protein level between various cell lines. In particular, metastatic cell lines were found to express higher levels of caveolin, suggesting that elevation of caveolin expression could be important for cell metastasis.

S10-29

EARLY GENE EXPRESSION INDUCED BY A GLUCOSE-cAMP STIMULUS REPONSIBLE FOR INSULIN SECRETION IN THE *B*-PANCREATIC INS CELLS.

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Physiological control of pancreatic *B*-cell activity involves two elements: blood glucose levels and neuropeptides which raise cAMP, such as GIP and GLP. The question arises as to how cAMP and glucose affect induction of immediate early genes, which are candidates to control long term *B*-cells responses. We investigated the induction of *nur-77*, *jun-B* and *zif-268* in clonal rat Ins-1 cells by Northern blotting. Nur-77 expression was activated by glucose in a physiological range (6-15 mM) within 60 min, provided the presence of 0.5 mM cpt-cAMP, which at low glucose (3 mM) caused less than 50% of the maximal glucose induced rise in *nur-77* mRNA levels. No glucose stimulation was found in the absence of cpt-cAMP. Preliminary data show a similar behavior for *jun-B* and *zif-268*. Demonstration of a synergistic activation of immediate early genes by glucose and cAMP opens the way to investigate their physiological role for *B*-cell function.

S10-30

EXPRESSION OF MEMBRANE-TYPE MATRIX METALLOPROTEAE-1 (MT-MMP1) BY HUMAN SYNOVIAL FIBROBLASTS IS UPREGULATED BY PROINFLAMMATORY CYTOKINES

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The molecular and biochemical basis of articular destruction in acute and chronic arthritis are not understood. MMPs play an important role in the destruction of the extracellular matrix. MT-MMP regulate localised proteolytic degradation of extracellular matrix to sites of cellular contact by activating progelatinase-A. We therefore investigated if synovial fibroblast (SF) cells express MT-MMP1, and whether cytokines can modulate its expression. Northern blot analysis showed constitutive basal expression of MT-MMP1 mRNA in SF cell lines derived from osteoarthritis synovial membrane. No basal MT-MMP1 expression was detected in the human gingival fibroblasts, and hepatocytes indicating that MT-MMP1 expression is cell-type and/or tissue specific. Expression was increased in a dose and time dependent fashion when SF cell lines were stimulated with the cytokines ILβ, IL6, TNFα, TGFβ, and PMA. Maximal expression was detected 3 to 8 hrs post stimulation. Maximal IL1β stimulation was observed at 10ng/ml, TNFα at 1ng/ml, TGFβ at 10μl/ml, IL6 at 10ng/ml and PMA at 50ng/ml. Western blot analysis of plasma membrane extracts of SF cells detected a specific polypeptide of 63 kDa, characteristic of MT-MMP1. Indirect immunofluorescence assay localised MT-MMP1 expression to the plasma membrane of SF cells. Gelatin zymogramme analysis revealed that SF cells secrete progelatinase-A which is subcequently activated by MT-MMP1. In control experiments no MT-MMP was detected in hepatocytes.

Our results showed that SF cells express MT-MMP, and that expression is upregulated by cytokines which are found in the inflammatory arthritis. This novel family of MT-MMPs may play a key role in regulating cell mediated matrix degradation by the synovium during the pathogenesis of arthritic diseases.

S10-31

MOLECULAR CHAPERONE HSP90 INTERACTION PARTNERS

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The molecular chaperone Hsp90 is one of the most abundant proteins in eukaryotic cells. The disruption of the two hsp90 genes in yeast is lethal, indicating an essential role of this protein for viability. Hsp90 appears to exist in a variety of different complexes and is likely to be involved in a multitude of cellular processes. To find proteins interacting with Hsp90 we have performed Ni^{2+}-affinity chromatography with protein extracts from yeast with disrupted hsp90 genes. Proteins could be found that copurify with 6xhis-hsp90 but do not appear in the control extract. We are currently generating anti-idiotypic antibodies to Hsp90 to test the copurifying proteins for direct interaction with HSP90. In addition we cloned a putative cyclophilin-40(Cyp40) which is thought to be part of various Hsp90 complexes. The knock-out of the cyp40 has no effect on viability and is not synthetical lethal with hsp90 mutations. Cyp40 is also dispensable for steroid and pheromone signaling which depend on Hsp90. Further biochemical and cell biological characterization of Cyp40 is in progress.

S10-32

INOSITOL IS INVOLVED IN THE CONTROL OF MATING AND SPORULATION OF *SCHIZOSACCHAROMYCES POMBE*

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The fission yeast *S.pombe* is auxotrophic for inositol. We show that mating and sporulation in this yeast is induced by high amounts of inositol. To elucidate mechanisms involved in inositol induced mating we selected mutants that do not respond properly to the inositol signal and cloned (by transformation and complementation) the corresponding genes. Three genes have been analyzed so far: *plc1* which codes for a putative phospholipase C (δ-form), *itr1* encoding a putative inositol transporter (12 membrane spanning protein) and *ksg1* which codes for a putative protein kinase that is responsible for growth, mating and possibly sporulation. We speculate that the gene products of *plc1* and *ksg1* are part of a signal transduction cascade.

S10-33

Characterization of Two Genes, *car1* and *car2*, in *Schizosaccharomyces pombe*, Responsible for Amiloride Sensitivity

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Amiloride inhibits growth in *S. pombe*. Its toxic effect is relieved by low concentrations of thiamine and the pyrimidine moiety of the thiamine molecule. By selecting amiloride resistant mutants and isolating the corresponding genes, we identified two putative proteins that are responsible for amiloride sensitivity. The *car1* product is a 12 transmembrane protein and is involved in the utilization of thiamine and its biosynthetic precursor pyrimidine. Its expression is repressed by thiamine and is under the genetic control of the genes *thi1*, *tnr1*, *tnr2*, and *tnr3*. The *car2* gene encodes a protein with an ATP/GTP-binding site and does not reveal any striking homology to an other protein. The *car1* and *car2* gene products seem to interact with each other and could form an ABC-like transport system. Thiamine inhibits mating and *car2* mutants are sterile. It is possible that mating regulation by thiamine occurs via the postulated ABC transporter.

S10-34

CYTOSKELETON REORGANIZATION INDUCES PLASMINOGEN ACTIVATOR INHIBITOR-1 IN RAT LIVER EPITHELIAL CELLS

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The rat liver epithelial cell line, BRL, can be induced to migrate and acquire a fibroblastoid morphology by treatment with EGF or TGF-α. This coincides with the induction of the PAI-1 gene and increased PAI-1 secretion in the culture medium. Transformation of this cell line with activated Ha-ras (3.1 cells) leads to constitutive migration and PAI-1 expression. Since previous reports have shown that in different cell systems cytoskeletal reorganization can induce the production of proteases such as urokinase type plasminogen activator (uPA), stromelysin or gelatinase, we intended to determine the role of the cytoskeleton in migration related gene expression. PAI-1 is induced in BRL and 3.1 cells after actin filament disassembly by cytochalasin D or after microtubule reorganization by colchicine or nocodazole. Microtubule stabilization by taxol apparently reduces the expression of PAI-1. Cytochalasin D shows the most pronounced effects and was thus analyzed more extensively. The induction of uPA, as previously shown in other cell lines, can also be confirmed for BRL cells. At the mRNA level the induction of PAI-1 by EGF is more rapid as by cytochalasin D. Similarly as for uPA, PAI-1 induction is preceded by an increase in c-Jun transcript levels.

S10-35

IL-15 AND IL-7 ARE GROWTH FACTORS FOR CUTANEUOS T CELL LYMPHOMA CELLS

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CTCL (cutaneous T cell lymphomas) consist of malignant T cells which remain during the longest time of the disease restricted to the skin . The growth factors IL-15 and IL-7 are produced in the skin and their receptors have some subunits in common with the IL-2 receptor. Using the IL-2 dependent CTCL cell line SeAx we found that IL-7 as well as IL-15 can replace IL-2 as a growth factor. IL-7 and IL-15 can synergize and are each a better growth factor for SeAx cells than IL-2. No synergism was observed with IL-2. In the absence of IL-7, IL-15 or IL-2 SeAx cells died within 8 days. Both IL-7 and IL-15 did not protect SeAx cells from apoptosis induced by the Ca2+ mobilizers thapsigargin and aluminium fluoride. The dependence on skin produced interleukins may explain the preference of malignant T cells for the skin in cutaneous T cell lymphomas. This assumption is corroborated by immuno-histochemical findings showing that the basal cell layer is the place where CTCL cells are found first in early stages is also the place of highest IL-15 production in the skin.

S10-36

A NOVEL TRANSMEMBRANE PROTEIN WHICH IS LOST IN TUMOR CELLS

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We have prepared a subtracted cDNA library starting from normal and from SV40 transformed fibroblasts. The objective of this approach is to identify novel proteins which are down-regulated in tumor cells and which may be responsible for the poor adhesion properties of these cells. Many of the cDNA clones derived from this library code for already known proteins of the extracellular matrix like fibronectin and collagen VI. Among the unknown proteins is a polypeptide of 1100 amino acids that is down-regulated in most of the tumor cell lines analyzed. The first 580 amino acids of the novel polypeptide are made up of 13 ANK-repeats, which have been identified in ankyrins and which may be involved in protein-protein interactions. The C-terminal portion consists of 7 putative transmembrane domains. This arrangement of domains is reminiscent of several ion channels. It is therefore possible that our novel protein represents an adhesion-dependent ion channel.

S10-37

CHARACTERIZATION OF ELEMENTS WITHIN THE C2 REGION THAT MEDIATE LIPID-INDEPENDENT PROTEIN KINASE Cγ-PROTEIN INTERACTIONS

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Protein Kinase C (PKC) is a family of serine-threonine specific protein kinases implicated in the control of cell growth and cell differentiation. In vitro, PKC activity is regulated through interactions with lipids. In vivo, isoform-specific function may additionally be determined by interactions with specific proteins present in distinct cellular compartments. Using the regulatory domain of PKCγ expressed as a fusion protein with glutathione-S-transferase (GST) in a ligand blot assay, binding proteins of molecular mass 14-18 and 28-33 kD present in several rat tissues and prominent in the detergent-insoluble fraction have been identified. Lipid-independent binding to these proteins was also seen using a fusion protein containing only a segment of the C2 region refered to as the CaLB (Calcium-dependent Lipid Binding) motif (amino acids 187-234). By deletion analysis we have determined, within the CaLB motif, elements critical for these interactions. Strong binding was seen for GST fusion proteins containing PKCγ peptides 187-200 and 217-234, but not for peptide 200-217. The latter CaLB element is a polybasic stretch containing multiple lysines and an arginine residue. Thus, electrostatic forces may be critical for interactions between the CaLB motif of PKCγ and the binding proteins detected.

S10-38

EXPRESSION AND CHARACTERIZATION OF INDIVIDUAL REGULATORY DOMAIN ELEMENTS OF PROTEIN KINASE C δ

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Protein kinase C δ (PKC δ) is a member of the non-calcium-dependent group of PKC isoforms (nPKCs). Activation *in vivo* is thought to result from specific interactions between the lipid activator diacylglycerol (DAG) and various elements of the regulatory domain in the presence of lipid cofactors. The activation by DAG can be mimicked by phorbol esters like phorbol-12,13-dibutyrate (PDBu). To identify regions critical for the control of PKC δ activity, different PKC δ regulatory domain elements were expressed as GST fusion proteins, affinity purified and characterized by measuring [^{3}H]-PDBu binding. The fusion proteins contained either individual or combinations of the following regulatory elements: first variable region, cysteine-rich regions (Cys1, Cys2), the hinge region. Surprisingly, all fusion proteins lacking the Cys2 motif were inactive as PDBu binding proteins, in both the vesicle and the mixed micellar assays. For all Cys2-containing GST-PKC δ fusion proteins, PDBu binding affinities in the range of 15-30 nM were measured in the mixed micellar assay. Thus, PDBu binding affinities of GST PKC δ fusion proteins containing the Cys2 element are similar to those previously determined for the fusion proteins GST-Cys1 and GST-Cys2 of the calcium-dependent PKC γ (Quest and Bell (1994) J. Biol. Chem. **269**, 20000). Our results suggest that Cys2 is the region critical for DAG-dependent activation of PKC δ. A model for PKC δ activation based on these results will be discussed.

S10-39

PKA BYPASSES cAMP RECEPTORS DEFICIENCIES IN *DICTYOSTELIUM DISCOIDEUM*.

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Chemotaxis and multicellular differentiation in *Dictyostelium discoideum* requires extracellular cAMP signalling which is sensed by four subtypes of surface receptors (cARs). PKA was shown to play a key role during differentiation. Overexpression of PKA in cAR disruption mutants rescued almost wild type phenotype. Thus PKA is a downsteam effector in extracellular cAMP signalling.

S10-40

C-TERMINAL PHOSPHORYLATIONS OF PKC-ALPHA

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Protein kinase C represents a protein family that participates in cellular signalling. Interactions of the PKC molecule with the membrane lead to an opening of the catalytic site and to the enzymatic activation. Besides activation by physical interactions with the membrane transphosphorylation and autophosphorylation of the molecule have been suggested to be needed for the permissive activation of PKC-alpha and -beta. Here we report that phosphorylation of Ser residues near the C-terminus of PKC-alpha which are different from the reported autophosphorylation sites are also involved in the regulation of the PKC-alpha activation. The kinase mutated at these serines shows a faster electrophoretic migration indicating less phosphorylation than the wild type. The Ser mutants also exhibit a substantial reduction of the kinase activity when compared to the wild type. Stable cellular transfectants overexpressing these mutant proteins have been produced. It is discussed how the mutations affect cellular localisation, translocation and down regulation of the PKC-alpha protein.

S10-41

RAC CONTROLS ACTIN-GELSOLIN DISSOCIATION IN HUMAN NEUTROPHILS

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Chemoattractants like fMLP stimulate dissociation of gelsolin from the barbed ends of actin filaments in neutrophils, an event that has been proposed to trigger actin polymerization. We have studied the regulation of actin-gelsolin dissociation in a cell-free system. GTPγS (but not ATPγS) indirectly induced a significant dissociation of actin-gelsolin complexes in human neutrophil cytosol, which was not mediated by endogenous phospholipids. The GTPγS-induced actin-gelsolin-dissociation could be blocked by rhoGDI and mimicked by GTP-loaded activated rac (V12rac1). GDP-V12rac1, inactive V12A135rac1 and activated GTP-rhoA did not dissociate cytosolic complexes, and purified complexes from gelsolin and actin were not affected by activated rho or rac proteins. Taken together, the above results suggest that activation of rac triggers indirectly the dissociation of actin from gelsolin. This novel role for rac might provide a lipid-independent signal for the liberation of barbed ends and actin polymerization in human neutrophils.

S10-42

RAB3A AND MSS4 REGULATE INSULIN EXOCYTOSIS

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Rab3A is a small GTPase that regulate neurotransmitter release. We identified Rab3A by Western blotting in β-cells of rat pancreatic islets and in insulin-secreting cell lines. Immunoelectron microscopy revealed that Rab3A is associated with insulin-containing secretory granules. To study the role of this small GTPase in insulin secretion, HIT-T15 cells were transiently transfected with wild type Rab3A or with mutants defective in either GTP hydrolysis or in guanine nucleotide binding. Exocytosis from the cells expressing the Rab3A constructs was monitored by cotransfecting the hamster cell line with human proinsulin and by measuring human C-peptide release. Using this approach, we found that overexpression of Rab3A mutants, but not of Rab3A wild type, inhibits stimulated insulin release. This was observed both in intact and in permeabilized cells. Overexpression of Mss4, a regulatory protein that promotes GDP/GTP exchange in a subset of Rabs, including Rab3A, enhanced stimulated insulin secretion. These results indicate that Rab3A is involved in the regulation of insulin secretion and suggest that Mss4 may be one of the rate-limiting factors in the control of exocytosis.

S10-43

ANISOMYCIN MEDIATED INDUCTION OF THE IL-1R RELATED T1 MOUSE GENE.

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The *T1* gene give rise to two transcripts encoding a 62 kDa membrane-bound (T1-M) and a 37 kDa secreted (T1-S) protein. The T1-M shows high similarity to the IL-1R type I.

A serum responsive enhancer, which is essential for *T1* expression in fibroblasts, has been identified at position -3.6 kb. The transient serum response of *T1* has been shown to require ongoing protein synthesis, defining *T1* as a delayed early serum responsive gene. Furthermore we have shown that the serum response is blocked by the sustained expression of the *ras* oncogene.

Here we report that the *T1* gene is transcriptionally activated by the protein synthesis inhibitor anisomycin, even at concentrations which block protein synthesis completely. Anisomycin in contrast to serum can induce the *T1* gene also in *ras*-transformed NIH3T3 cells. These observations suggest that two different signaling pathways are used for the transcriptional activation of the *T1* gene by serum and anisomycin. Anisomycin has previously been shown to stimulate mainly the pathway leading to JNK activation. However, UV treatment, which activates this pathway efficiently does not trigger *T1* gene expression. Thus, we assume that an other yet unidentified signaling pathway is involved in the anisomycin mediated induction of the *T1* gene.

S10-44

PHOSPHATIDYLINOSITOL 3-KINASE IS INVOLVED IN THE COLONY-STIMULATING FACTOR-1-INDUCED SPREADING OF MACROPHAGES (MΦ) AND OSTEOCLASTS (Oc).

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Colony-stimulating factor-1 (CSF-1) is the growth factor for cells of the mononuclear phagocyte system and for Oc. Phosphatidylinositol 3-kinase (PI 3-K), a component of many signal transduction pathways, binds to the receptor activated by the ligand. In this study, it was investigated whether PI 3-K mediates the signal induced by CSF-1 leading to spreading and to increased endocytosis and pinocytosis.

The MΦ cell line BAC1.2F5, and Oc isolated from rat femurs were treated ± 5 nM CSF-1 (Chiron Corp., Emeryville, CA). Cell spreading, endocytosis, pinocytosis and PI 3-K activity were determined.

CSF-1 induced spreading of MΦ and Oc, and endocytosis and pinocytosis by MΦ. Wortmannin (donated by Dr. T. Payne, Sandoz, Basel), an inhibitor of PI 3-K, inhibited these processes by 50% at a concentration of around 10 nM. PI 3-K in MΦ was stimulated by CFS-1 within 1 min. Wortmannin blocked this effect.

The data suggest that PI 3-K is involved in the signal transduction leading to a change in MΦ and Oc behaviour after exposure to CSF-1.

S10-45

RAS-LIKE GTPases AS TARGETS OF Ca^{2+}-CALMODULIN

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Recently a new subfamily of Ras-related small GTP-binding proteins was discovered: Rad (Ras associated with diabetes); Gem, an immediate-early gene expressed in mitogen stimulated T-cells and Kir (kinase inducible ras-like). Kir appears to be highly related to Gem and is a close relative of the Rad gene product.

The C-terminus of the Kir/Gem and Rad proteins contains an extension of approximately 30 amino acids not present in other members of the Ras family. This sequence is highly hydrophobic with basic amino acids resembling a basic amphiphilic structure. We discovered that the C-terminal extension of the Kir/Gem and Rad proteins exhibits all the hallmarks typical for calmodulin (CaM)-binding peptides. In order to investigate the possibility that these Ras-like proteins indeed could be CaM-binding proteins, we synthesized the putative CaM-binding peptide of Kir/Gem and demonstrate a high affinity interaction using fluorescence spectrometry. Titration of Dansyl-CaM with the Kir/Gem peptide gave an affinity constant of 1 nM. Furthermore gel shift analysis showed that the complex formation between CaM and the Kir/Gem peptide is strictly calcium-dependent. Full-length Kir/Gem and Rad proteins bind ^{32}P-labeled GST-CaM in the overlay assay.

S10-46

THE LAR INTERACTING PROTEIN TRIO CONTAINS A RAC-SPECIFIC AND A RHO-SPECIFIC GUANINE EXCHANGE FACTOR DOMAIN

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Using the interaction trap assay to identify candidate proteins that bind the cytoplasmic domain of the LAR transmembrane protein tyrosine phosphatase, we isolated a cDNA encoding a 2861 amino acid protein with three putative enzymatic functions. TRIO contains two functional guanine-exchange factor (GEF) domains and a protein kinase domain. One TRIO GEF domain has rac-specific and the other rho-specific activity, indicating a role for TRIO in conjunction with LAR in coordinating cell-matrix interactions and cytoskeletal re-arrangements involved in cell movement.

S10-47

THE C. ELEGANS PHOSPHATIDYLINOSITOL 3-KINASE (VPS34).

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Phosphatidylinositol (3,4,5)P_3 is considered to be a novel 2nd messenger. A family of enzymes catalysing the phosphorylation of phosphoinositides at the D-3 position has been identified and cloned. Catalytic subunits of the p110α and β -subtypes have been found in tight association with regulatory p85 subunits and the p110γ is activated by βγ-subunits of G-proteins. While the p110α-γ accept PtdIns, PtdIns 4-*P* and PtdIns(4,5)P_2 as substrates, homologs of the yeast Vps34 gene product utilise solely PtdIns to produce PtdIns 3-*P* . Vps34p has been found to be indispensable for the sorting of various proteins to the yeast vacuole and is associated with the Vps15p serine/threonine kinase.

Although many PtdIns 3-kinases have been identified from various species, very little is known about their downstream targets and their role in developmental processes. Since the yeast system does not allow the study of multicellular organisation and the mammalian excludes genetics, we searched for PtdIns 3-kinase genes in *C. elegans* using nested PCR with degenerated primers from the conserved catalytic domain sequences. One PCR product gave the expected length and homology and was further used to screen a C. elegans cDNA library and lead to the isolation of a full length cDNA clone (P51Y) of 2900 bp encoding an ORF of 901 amino acids. The putative translation product of P51Y is homologous to the Vps34 family with high degrees of identity within the C-terminal conserved regions and significant similarities throughout the entire protein up to the N-terminus. The cDNA hybridized to cosmid F53D11, which localizes the gene to chromosome I, to a region where 3 lethal mutants have been mapped. Rescuing of these 3 mutant candidates, cellular localization studies of the gene product and lipid kinase assays are currently carried out in our labs.

S10-48

IDENTIFICATION OF YEAST RHO1 GTPASE AS A REGULATORY SUBUNIT OF 1,3-β-GLUCAN SYNTHASE

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Members of the Rho-family of Ras homologous small GTPases are involved in morphogenesis regulation in both yeast and mammalian cells. In the yeast Saccharomyces cerevisiae, temperature-sensitive mutants in the essential RHO1 gene displayed reduced and thermolabile *in vitro* synthesis of 1,3-β-glucan, a major structural component of the cell wall. By contrast, glucan synthesis was not affected in Cdc42 mutants, another Rho-family GTPase. Impaired glucan synthase in Rho1 mutants was restored by the addition of recombinant, purified Rho1 protein, but not Cdc42, in a GTP-dependent manner. Glucan synthase from mutants expressing constitutively active Rho1 (RHO1-Q68H) did not require exogenous GTP for activity. Rho1 copurified with 1,3-β-glucan synthase and was found to associate with the Fks1 subunit of this complex *in vivo*. In addition, both Rho1 and Fks1 were found to reside predominantly at sites of cell wall remodeling. Therefore, it appears that Rho1 is a regulatory subunit of 1,3-β-glucan synthase and is required for its activity.

S10-49

Reduction of $[Ca^{2+}]_i$ in skeletal muscle cells by prednisolone

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The glucocorticoid α-methylprednisolone (PDN) is the only drug to exert a beneficial effect in the chronic skeletal muscle degeneration exhibited by Duchenne muscular dystrophy (DMD) patients. Increased calcium (Ca^{2+}) influx has been incriminated as a potential pathological mechanism in *DMD*. We have therefore studied the influence of PDN on Ca^{2+} handling in the C2 skeletal muscle cell line. Treatment of myotubes with PDN led to a 2- to 4-fold decrease in cellular $^{45}Ca^{2+}$ uptake while no effect was seen on $^{45}Ca^{2+}$ efflux. This decrease was independent of the extracellular $[Ca^{2+}]$ applied to cells and took at least 24 h in order to become established. Cellular calcium ($[Ca^{2+}]_i$) accumulation was decreased as well in PDN-treated myotubes when exposed in a long term to $^{45}Ca^{2+}$-containing medium. PDN treatment led to a concomitant 58% decrease in basal $[Ca^{2+}]_i$ concentration. Antioxidant lazaroids induced a similar decrease in Ca^{2+} influx. Our results suggest that part of the beneficial effect of PDN in DMD could be due to a reduction of Ca^{2+}influx and of the size of Ca^{2+} pools in dystrophic muscle fibres.

Supported by the Swiss NSF (32-45004.95) and Swiss Fondation for Research on Muscular Diseases

S10-50

LOCAL EVENTS AND DIFFUSION IN CARDIAC CA SIGNALING

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Our goal is to model and understand Ca signaling in cardiac muscle cells. Computer models of Ca diffusion in the subsarcolemmal space and in the cytosol were developed. On the molecular level, we simulate the interactions of a single L-type Ca channel and SR Ca release channels in the junctional local environment. On the cellular level, the model is composed of a simplified sarcomere and includes a restricted subsarcolemmal and nonrestricted cytosolic space as well as the SR and a myofibrillar space with Ca buffers. Since the Ca transient originates in the junctional space between sarcolemma and SR, and the curvature of this cleft does not significantly affect the diffusion, the geometric boundary conditions for this space correspond to two infinite planes. Analytical as well as numerical solutions of the diffusion equation were derived. The results indicate that in the cleft the situation is fundamentally different from an unrestricted hemisphere. In this narrow space, an asymptotic steady-state Ca distribution is not established during opening of a single L-type Ca channel. In contrast, the Ca concentration can continue to increase as long as the channel remains open. This time-dependence of [Ca] may be important for the local [Ca] and for the generation of elementary events in cardiac Ca signaling and excitation-contraction coupling. Supported by SNF.

S10-51

DUAL FUNCTION OF CALCIUM IN LAMININ-MEDIATED GROWTH CONE GUIDANCE: II. LATE SUSTAINED CA2+-SIGNAL.

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Laminin (LN)-coated beads provide unique guidance instructions to advancing chick DRG growth cones. Upon transient interactions, growth cones display a series of stereotype changes in their behavior and morphology, some lasting considerably beyond the time of actual contact (Kuhn et al., 1995). Among the most striking growth cone response are early turning and late, enhanced outgrowth after transient LN-contact. A pharmacological approach demonstrated that an early influx of extracellular Ca2+ is essential for growth cone turning. The two potential Ca2+ targets, calmodulin (CaM) and CaM-dependent kinase II (CaMK-II) regulated two independent growth cone responses. CaM was necessary for early turning while CaM-II was involved only in late, enhanced outgrowth. Fluorescent Ca2+ imaging revealed a small, LN-induced rise in intracellular Ca2+ only in surgically isolated filopodia but not in attached filopodia indicating that the parent growth cone played a key role in regulating Ca2+ signals received by individual filopodia. In addition, LN stimulated a second rise in intracellular Ca2+ with a distinct lag phase. This late Ca2+ signal was of small magnitude, sustained, restricted to growth cone bodies and strongly associated with late, enhanced outgrowth. Interestingly, inhibition of CaMK-II abolished this late,sustained Ca2+-signal. Taken together, the spatial, temporal and functional restriction of these two distinct Ca2+ signals suggest a dual function of Ca2+ in LN-mediated growth cone guidance. Furthermore, a sequential signaling cascade composed of CaM and CaMK-II links the early Ca2+ influx restricted to filopodia with the late, sustained rise in intracellular Ca2+ restricted to growth cone bodies.

S10-52

FAST CALCIUM WAVES IN PC12 NEURITES

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Bradykinin (Bk) and caffeine (Cf) evoked intracellular Ca^{2+} release in outgrowing neurites of nerve growth factor (NGF)-treated rat pheochromocytoma cells (PC12). Changes in $[Ca^{2+}]_i$ were measured using a 1:1 mixture of the Ca^{2+}-sensitive dyes, Fura-red and Fluo-3, respectively, in combination with confocal microscopy. The advanced imaging method allowed observation of very short neuritic Ca^{2+} transients evoked by the two agonists. The Bk-evoked Ca^{2+} signal showed a characteristic onset at the neck of the neurite. It propagated as a bidirectional wave towards the growth cone and the cell body. In contrast, the Cf-induced Ca^{2+} release showed simultaneous onset along the entire neurite. The phospholipase C inhibitor, U73122, inhibited the Bk-induced but not the Cf-induced Ca^{2+} release. Amplitude and wave speed, but not duration of the IP3-induced $[Ca^{2+}]i$ signal in PC12 neurites were amplified by additional Ca^{2+} release from the Cf-sensitive Ca^{2+} pool.

S10-53

MITOCHONDRIAL Ca^{2+} IN INSULIN SECRETING CELLS

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Glucose-induced insulin secretion depends on the sustained generation of ATP by the mitochondria. We tested whether the glucose-evoked rise in cytosolic Ca^{2+} ($[Ca^{2+}]c$) is associated with changes in mitochondrial Ca^{2+} ($[Ca^{2+}]m$), thought to link ATP supply to ATP demand. To test this, we used the Kreb's cycle intermediate succinate (Suc) in insulin secreting cells. We established an INS-1 cell line stably expressing the Ca^{2+}-sensitive photoprotein aequorin targeted to the mitochondria. Intact or staphylococcus α-toxin permeabilized cells were studied. Glucose or the cell permeant methyl-Suc caused parallel increases in $[Ca^{2+}]c$ and $[Ca^{2+}]m$. Blockade of L-type Ca^{2+} channels abolished these $[Ca^{2+}]c$ and $[Ca^{2+}]m$ rises. This suggests that $[Ca^{2+}]m$ changes by mitochondrial substrates depends on permissive $[Ca^{2+}]c$ levels. Indeed, in permeabilized cells, $[Ca^{2+}]m$ was increased by Suc only when the cells were superfused with stimulatory (2μM) but not basal (0.1μM) Ca^{2+}. In conclusion, the sustained production of metabolic coupling factors during nutrient-induced insulin secretion requires a rise in $[Ca^{2+}]m$ which is dependent on permissive $[Ca^{2+}]c$ levels.

S10-54

DIFFERENTIAL ROLES OF T- AND L-TYPE CALCIUM CHANNELS IN THE ACTIVATION OF THE ADRENAL GLOMERULOSA CELL

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The T-type (low threshold) voltage-dependent calcium channels play an important role in the potassium-induced stimulation of aldosterone production in adrenal glomerulosa cells.

Using the patch clamp method in the perforated patch configuration, combined with cytosolic calcium ($[Ca^{2+}]_c$) measurement by microfluorimetry (fluo-3), we studied, in voltage clamped bovine glomerulosa cells, the relationship between cytosolic free calcium concentration and membrane potential.

Under these conditions, we observed a sustained $[Ca^{2+}]_c$ increase at potentials above -60 mV. However, a discrepancy was observed between the depolarization-evoked $[Ca^{2+}]_c$ response and the predicted steady-state Ca^{2+} current through T channels (calculated from their activation and inactivation curves). This finding suggests an important contribution of L channels (high threshold) in the calcium response. Inhibition of L channels by specific blockers, such as nifedipine (200nM) or/and calciseptin (1μM), that did not affect T-type currents, resulted in an almost complete abolition of the calcium response to cell depolarization. On the other hand, aldosterone secretion was not significantly decreased by these inhibitors and therefore seems to depend only upon the activity of T-type channels.

In conclusion, it appears that in adrenal glomerulosa cells, 1) the cytosolic calcium response to a depolarisation is almost exclusively due to L-type channels (even at potentials as negative as -60 mV) and 2) this calcium response may be dissociated from the potassium-induced activation of aldosterone secretion. These results suggest moreover that the intracellular destination of calcium entering through L- or T-type channels may be different.

S10-55

CALRETININ AND CALRETININ-22K TRANSLOCATE INTO THE NUCLEUS UNDER SPECIFIC CONDITIONS IN WIDR CELLS

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The calcium-binding protein calretinin (CR) is expressed in several colon adenocarcinoma cell lines (e.g. WiDr, HT-29, SW620). In these cell lines an alternatively spliced transcript leading to a truncated form of calretinin, called calretinin-22k (CR-22k), has been detected. As the splice junction exon/intron 7 where the splicing occurs is in phase 2, a frame shift leads to the expression of a novel C-terminus consisting of 14 amino acids. A polyclonal antiserum was raised against a fusion protein containing these C-terminal amino acids of CR-22k. The antiserum was tested on fixed WiDr cells and detects this protein in the cytoplasm, as it is also known for the full-length protein.

Application of 1,25-dihydroxyvitamin-D3 (10^{-8}M) leads to a transient accumulation of CR and especially of CR-22k in the nucleus of WiDr cells as seen by immunohistochemical staining. The maximum is reached approximately 48h after the addition of 1,25-dihydroxyvitamin-D3. The accumulation of these 2 proteins was confirmed by Western blot experiments using isolated fractions of nuclear proteins. Similar results were obtained when WiDr cells were grown in the presence of sodium butyrate and *hexamethylene bisacetamide*, agents known to block proliferation and induce differentiation in these cells. Since mainly CR-22k is found in the nucleus after these treatments, a different physiological role for this alternatively spliced form of calretinin is suggested.

S10-56

Several steps of arachidonic acid metabolism are involved in smooth muscle reactivity of human bronchus.

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The influence of the phospholipase A_2-arachidonic acid (PLA_2-AA) pathway in cell activation was investigated in smooth muscle cells from human airways. The radiolabeled inositol phosphates (IP) formed following application of 10 µM histamine during 5 s were measured by HPLC. Various steps in the PLA_2-AA pathway were found to influence the activity of phospholipase C (PLC): (1) two PLA_2 inhibitors, quinacrine and 4bromophenacyl bromide, blocked histamine-induced generation of 1,4,5-IP_3, suggesting a tight coupling between PLC and PLA_2 in a membrane-delimited domain; (2) indomethacin, a cyclooxygenase inhibitor, modulated the IP production in a time-dependent manner, implying that prostaglandins (PGs) were also involved; (3) exogenous AA elicited the generation of IP_3, an effect abolished by indomethacin; this shows that PGs formed endogenously activated PLC. These observations suggest that several intermediates in AA metabolism can modulate airway reactivity by a direct effect in smooth muscle cells.

S10-57

Ca^{2+} release in human bronchial smooth muscle cells: different mechanisms revealed by caffeine and thapsigargin.

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The aim of the present study was to further characterize the mechanism of histamine-induced Ca^{2+} signal and to disclose the possible existence of several Ca^{2+} pools in these cells. The single cell fluorescence video imaging method was used to measure Ca^{2+}. Histamine, 10 µM, induces a single Ca^{2+} peak whose kinetic properties fit to those of 1,4,5-IP_3. The Ca^{2+} peak was suppressed following preincubation with thapsigargin, 5 µM. Caffeine, 10 mM, significantly enlarged the duration of histamine-induced Ca^{2+} peak with no effect on its amplitude. Following preincubation with both drugs, the amplitude and the duration of the Ca^{2+} response to histamine were enhanced, the drugs being added simultaneously or sequentially, thapsigargin or caffeine first place. Moreover, in the presence of caffeine, thapsigargin potentiated the effect of histamine. These observations suggest the occurrence of two Ca^{2+} pools: (i) a IP_3-activated, thapsigargin-sensitive pool, exhibiting fast kinetics, and (ii) a thapsigargin-insensitive pool, source of CICR, with slower kinetics, whose threshold is lowered by caffeine.

S10-58

Regulation of cytosolic calcium in dystrophin-lacking myotubes under conditions of stress

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The regulation of free cytosolic calcium concentration ($[Ca^{2+}]_c$) was studied in cultured myotubes isolated from the hind limbs of wild-type (C57BL10) and dystrophin-deficient mutant *mdx* mice. Resting $[Ca^{2+}]_c$ was similar in *mdx* and normal myotubes (35 ± 9 nM and 38 ± 11 nM, respectively). Lowering the osmolarity of the superfusion solution from 300 mOsm to 100 mOsm resulted in an about two times higher rise in $[Ca^{2+}]_c$ for *mdx* (243 ± 65 nM) than for C57BL10 (135 ± 37 nM) myotubes, which was entirely dependent on extracellular Ca^{2+}. Incubation of the cultures for 3-7 days with the protease inhibitor calpeptin (50 µM), α-methylprednisolone (10 µM) or creatine (20 mM) abolished or significantly reduced the rise in $[Ca^{2+}]_c$ in *mdx* myotubes upon exposure to a hyposmotic shock. The results suggest an increased permeability of *mdx* myotubes to Ca^{2+} under specific stress conditions. The ameliorating effect of PDN on $[Ca^{2+}]_c$ could explain, at least partly, the beneficial effect of this drug on DMD patients.

Supported by the Swiss Foundation for Research on Muscular Diseases

S10-59

INCREASE IN CAPACITATIVE Ca^{2+} ENTRY BY BAPTA

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Capacitative Ca^{2+} entry is induced by depletion of the intracellular stores. This can be provoked by either inositol 1,4,5-trisphosphate-induced Ca^{2+} release following receptor activation, by blockade of the ER-Ca^{2+}-ATPase with thapsigargin or by pretreatment with an extracellular Ca^{2+} chelating agent. The mechanism leading to the opening of the corresponding channels, however, is not clear. Additionally, receptor-mediated Ca^{2+} entry is small and difficult to quantify.

We demonstrate here that stimulated $^{45}Ca^{2+}$ influx in vascular smooth muscle cells (A7r5 cell line) induced either by receptor activation with [Arg^8]vasopressin or by thapsigargin was increased more than threefold if cells were preloaded with the intracellular calcium chelator BAPTA. This is probably due to an attenuation of negative feedback of Ca^{2+} on its own entry accompanied by increased Ca^{2+} storage capacity of BAPTA-loaded cells leading to diminished cellular Ca^{2+} release. We propose that BAPTA preloading could be a useful approach to investigate receptor-mediated Ca^{2+} influx.

Supported by the SNSF (grant Nr. 31-36514.92).

S10-60

MECHANISM OF CYCLOSPORIN A POTENTIATION OF $[Ca^{2+}]_c$ SIGNALLING IN SMOOTH MUSCLE

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Drug induced local vasoconstriction appears to be responsible for the hypertensive side effect of the immunosuppressant cyclosporin A (CsA). In vascular smooth muscle cells, the calcium response (measured by fura-2 fluorescence or $^{45}Ca^{2+}$ flux experiments) and inositol phosphate (Ins-P) formation induced by different vasoconstrictors was potentiated by CsA. The effect of CsA took several hours to become completely established. Actinomycin D and cycloheximide completely inhibited the CsA effect suggesting a possible effect of CsA on gene expression.

Cyclosporin H, a CsA analog devoid of immunosuppressive activity was active in both, the potentiation of Ins-P metabolism and the Ca^{2+} response, while the potent immunosuppressant FK520 did not show potentiation. There appears to be no correlation between the immunosuppressive effect and the side effect. It is likely that the potentiation of Ins-P formation -via a change in gene expression- leading to an increase in $[Ca^{2+}]_c$ is responsible for the hypertensive side effect.

Supported by the Swiss NSF (grant Nr. 31-36514.92) and Sandoz Pharma.

S10-61

BACTERIAL EXPRESSION OF MUTANTS OF μCANP

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Two mutants of the large catalytic subunit of the human low-Ca^{2+}-requiring form of calpain (μCANP) were expressed in *E.coli*: one mutant (L-μCANPΔ3) lacking the domain III, whose function is still unknown, the other one (L-μCANPΔ4) lacking the calmodulin-like domain IV. Expression of both constructs and of the wild-type L-μCANP was always followed by formation of inclusion bodies. The expressed products were solubilized from inclusion bodies with the use of the nonionic detergent Zwittergent 3-14.
Activity was measured fluorimetrically in the absence of the small subunit (S-CANP). L-μCANPΔ4 showed the same features as L-μCANP: a low activity, the need for very high [Ca^{2+}] (30 mM), and a slow rate of autolysis. L-μCANPΔ3 showed high activity also in the absence of Ca^{2+}, accompanied by rapid autolysis. All the activities measured could be succesfully inhibited by calpain inhibitors.

S10-62

ROLE OF cAMP AND cGMP IN ENDOTHELIN-STIMULATED VENTRICULAR CARDIOMYOCYTES

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We investigated cellular responses to endothelin-1 (ET-1) in spontaneously beating ventricular cardiomyocytes from neonatal rats. ET-1 increased ANP release in a concentration-dependent manner. Calcium fluorometry using the calcium-sensitive fluorescent probe fura-2/AM, revealed that cultured cardiomyocytes behaved like a syncytium, such that each contraction of the whole cell population was accompagnied by a single Ca++ transient. Incubation with 100 nM ET-1 caused an increase of approximately 350% in cAMP formation which enhanced contraction frequency and ANP secretion by 50% and 40%, respectively.The calcium channel antagonist nifedipine abolished cell contractions and strongly reduced ET-1-induced ANP secretion. Cardiomyocytes bear ANP receptors, the activation of which leads to the formation of cGMP.
Incubation of cells with 1 μM of the cGMP analogue 8-bromo cGMP (8-Br-cGMP) suppressed ET-1- or forskolin-induced ANP secretion and inhibited cell contraction frequency. Our results indicate that ET-1 stimulates ANP secretion via an increase in cAMP formation activating a cAMP-dependent, nifedipine sensitive calcium channel. They further suggest that ANP regulates its own secretion by cGMP-mediated inhibition of cell contraction and Ca++ influx.

S10-63

$G_{\alpha 16}$ ANTISENSE EXPRESSION REVEALS DUAL SIGNALING CAPACITY OF THE P_{2U}-PURINOCEPTOR IN HEL CELLS

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In order to assess the role of G_{16}, a trimeric G-protein exclusively expressed in hematopoietic cells, $G_{\alpha 16}$ antisense mRNA was stably expressed in human erythroleukemia (HEL) cells. Western blot analysis showed that in transfected cell lines the expression of endogenous $G_{\alpha 16}$ was suppressed while the expression of $G_{\alpha q/11}$, $G_{\alpha i2}$, and $G_{\alpha i3}$ remained unaffected. Stimulation of parental HEL cells with ATP, UTP, or prostaglandin E1 (PGE1) transiently increased intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$). ATP and UTP showed cross-desensitization, and no additivity was observed when maximum effective nucleotide concentrations (100 μM) were applied, indicating that both nucleotides stimulated the same receptor, most likely the P_{2U} purinoceptor. In addition, pretreatment of cells with ATP or UTP enhanced the effect of PGE1 on $[Ca^{2+}]_i$. In the $G_{\alpha 16}$-suppressed cell lines, PGE1 still mobilized Ca^{2+} as it did in parental HEL cells. However, ATP or UTP failed to increase $[Ca^{2+}]_i$ in $G_{\alpha 16}$-suppressed cells although pretreatment of cells with ATP or UTP still enhanced PGE1-induced Ca^{2+}-mobilization as in parental cells. Thus, the data demonstrate i) that $G_{\alpha 16}$ antisense expression selectively blocks $G_{\alpha 16}$ expression, and ii) that in HEL cells, the P_{2U} purinoceptor functionally couples to G_{16} (increasing $[Ca^{2+}]_i$), and to at least one other G-protein (resulting in a potentiation of the effect of PGE1 on $[Ca^{2+}]_i$).

S10-64

CYTOSOLIC Ca^{2+} OSCILLATIONS IN RAT HEPATOCYTES ARE MODULATED BY INTRACELLULAR pH

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Single isolated rat hepatocytes were used to investigate the influence of intracellular pH (pH_i) on cytosolic Ca^{2+} oscillations following α_1-agonist stimulation. Simultaneous measurements of Ca^{2+} concentration and pH_i were performed at 37°C by video-fluorescence microscopy using Fura-2 and BCECF, respectively. While pH_i did not change in a measurable manner following phenylephrine stimulation and during the subsequent train of Ca^{2+} oscillations, manipulations of pH_i induced dramatic alterations in the frequency of oscillations. Increasing the resting pH_i using ammonium chloride (5-20mM) reduced the frequency of oscillations. The same effect was observed after alkalinization of the cell using trimethylammonium (6-10mM) or triethylammonium (10mM). A ΔpH_i >+0.3 was sufficient to reversibly inhibit oscillations. Rapid washout of ammonium in the continuous presence of agonist produced a transient cellular acidification (ΔpH_i<-0.5) that was accompanied by a frequency increase of the oscillations, often leading to an over-stimulated state of oscillations. Frequency increase was also observed after moderate acidification ($\Delta pH_i \approx -0.2$) induced by acetate and butyrate (10-20mM). This study indicates that intracellular pH exerts a strong influence on the frequency of Ca^{2+} oscillations through a mechanism that remains to be elucidated.

S10-65

DEFECTIVE PROTEINTRANSPORT IS THE PROBABLE CAUSE OF THE PERIPHERAL NEUROPATHY IN THE *TREMBLER* MOUSE

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The autosomal dominant *trembler* mutation (*Tr*) manifests as a Schwann cell defect characterized by severe hypomyelination and continuing Schwann cell proliferation throughout life. Affected animals move clumsily and develop tremor and transient seizures at a young age. It has been shown that *Tr* animals carry a point mutation in a putative transmembrane domain of the peripheral myelin protein 22 (PMP22), which leads to a amino acid substitution from a glycine to an aspartic acid. PMP22 is a hydrophobic glycoprotein of 160 amino acids with four putative transmembrane domains which is most abundantly expressed in compact peripheral myelin.
We have generated mammalian expression vectors for wildtype PMP22 and *Tr* and have transiently expressed the proteins in COSN cells. In contrast to PMP22 isolated from sciatic nerve, protein produced by the transfectants stains only intracellularly and is not complex glycosylated, indicating localization in the endoplasmic reticulum (ER). We were able to overwrite a putative ER-retrieval signal -RKRE at the carboxyterminus of the PMP22 protein by additon of an eight amino acid-tag originating from the vesicular stomatitis virus (VSV) G-protein. Extensive plasmamembrane staining and colocalization with lysosomal marker proteins was observed in cells transfected with the PMP22VSV expression construct using an antibody directed against the epitope tag. However, TrVSV protein remained localized to the ER of transfectants and colocalized completely with ER-marker proteins but not with the plasmamembrane or lysosomes.
These findings suggest that the Tr protein is incorrectly processed and remains in the ER, thereby causing a peripheral neuropathy.

S10-66

THE ROLE OF KUPFFER CELLS IN THE REGIO-SPECIFIC TOXICITY OF CHEMICALS IN LIVER.

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After exposure to xenobiotics, frequently a regiospecific toxicity within liver lobules can be observed in which nonparenchymal cells (NPC) might be involved. This was investigated in the recently established periportal (PP)- and pericentral (PC)-equivalent rat hepatocyte cultures. Kupffer cells were isolated by a pronase-collagenase liver perfusion, a discontinuous gradient centrifugation followed by centrifugal elutriation. Their viability was more than 90% and approx. 65% of the cells expressed a peroxidase activity. The Kupffer cells were cocultured with hepatocytes in ratios of 1:10 up to 1:2. This resulted in a reduced albumin secretion of the hepatocytes (25-50%), a reduced cytochrome-c reductase activity (50%), preferentially in pc-cocultures, without an increase in cytotoxicity (LDH-release). These changes were further downregulated by the addition of 500ng/ml endotoxin (LPS). This indicates that the coculture system responds in an in vivo equivalent manner and will allow the role of Kupffer cells in liver toxicity to be investigated.

S10-67

THE ROD AND THE CARDIAC SODIUM-CALCIUM EXCHANGERS: EXPRESSION AND COMPARISON THEIR PROPERTIES

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The sodium-calcium exchangers remove Ca^{2+} from the cytosol by coupling the transport of this ion to the outside of the cell with the entry of the Na^{+}. Two families of exchangers have been described: the cardiac exchanger and the retinal rod exchanger. The two proteins show a very low degree of homology at the primary structure level, but are thought to have an almost identical membrane topology: 11 transmembrane domains (TMs) and a large cytosolic loop between TM5 and TM6. The two proteins were expressed with the help of the vaccinia virus system. A mutated version of the cardiac exchanger lacking most of the cytosolic loop was shown to be partially active. A chimeric construct was prepared in which the cytosolic loop of the cardiac exchanger was replaced with that of the rod counterpart. Since the cytosolic loop is apparently important for its regulation, the insertion of the cytosolic loop of the rod exchanger is likely to influence the regulation of the protein.

S10-68

OSTEOBLASTS MEDIATE THE BISPHOSPHONATE (BP) INHIBITION ON BONE RESORPTION THROUGH SYNTHESIS OF AN INHIBITOR OF OSTEOCLASTIC (OC) RESORPTION

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Present knowledge indicates that osteoblasts (OBs) play a central role in osteoclastic bone resorption through an osteoclast-stimulating activity (OSA), produced in response to osteotropic factors. Previously, we have shown that the inhibiting action of BP on bone resorption in part is mediated by OBs. The aim of the present study was to resolve whether the BP generated inhibition on resorption was caused by BP acting on the OB-derived OSA, or due to OBs synthesizing an OC-inhibitor, in response to BP. As target cell we used the osteoblastic cell line CRP 10/30 which produces OSA, constitutively. The results obtained show that CM of cells treated with BP reduce the number of resorption pits by 52±5%, as compared to CM of control cells. With ultrafiltration, CM of BP-treated cells could be separated into 2 fractions, one containing stimulatory the other an inhibitory activity on OC resorption. With gel chromatography, the MW of the inhibitory activity was found to be between 3 and 10kDa. The inhibitor is proteinase and heat labile. Because the reduction in resorption pits was paralleled by a decrease in TRAP+ multinuclear cells, and since the area resorbed/lacunae was not changed, suggests that the inhibitor affects OC formation, rather than the OC resorption activity.

In conclusion, beyond their role of mediating OC resorption promoters, OB apparently are also involved in inhibiting bone resorption through synthesis of an OC resorption inhibitor.

S10-69

ASSAY OF TYROSINE HYDROXYLASE ACTIVITY IN RAT AND PIG ADRENAL GLAND USING THIN LAYER CHROMATOGRAPHY (TLC)

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Tyrosine hydroxylase (TH) [EC 1.14.16.2] activity is currently employed as a marker of the adrenomedullar catecholamine-synthesizing capacity and thus, as an indicator of a chronic stress exposure in various animal species. We have developed a TLC procedure for its assay in adrenal gland of rats and large animals which considerably improves some drawbacks of the currently employed methods, and facilitates a routine use. Preparation of tissue samples was adapted for rats and pigs. The activity of the enzyme is estimated, as in the majority of published procedures, as the rate of the TH-catalyzed tyrosine hydroxylation to 3',4'-dihydroxyphenalalanine (DOPA) using tritium labeled tyrosine, in the presence of cofactors and a DOPA decarboxylase inhibitor. DOPA production is measured after 5 min incubation. The subsequent separation of the radioactive product (DOPA) from the substrate (tyrosine) is accomplished by TLC on silicagel plates, in n-butanol-acetic acid-water solvent system (4:1:1). Radioactivity in the scraped zones, in which DOPA has been detected by means of an internal standard, is measured by β-counting. Advantage of this procedure is its simplicity, reliability, and convenience for routine assays.

Supported by the ETH grant and by the SNSF (grant No. 32-36244.92).

S10-70

POTASSIUM INDUCES MULTIPLE STEROIDOGENIC ENZYMES IN CULTURED RAT ZONA GLOMERULOSA CELLS

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The aldosterone production in primary cultures of rat zona glomerulosa cells depends critically on a high extracellular potassium concentration. The induction of the aldosterone synthesis is associated with the appearance of aldosterone synthase. This cytochrome P-450 catalyzes the conversion of DOC to aldosterone. At a potassium concentration of 6.4 mmol/l, aldosterone synthase mRNA and aldosterone production were not detectable. Upon increasing the potassium concentration to 18 mmol/l, the mRNA and aldosterone synthesis became measurable within 1 day and reached a plateau within 4 days. Additionally, marked increases of the mRNA levels of 11β-hydroxylase, side chain cleavage enzyme and 21-hydroxylase were measured. According to these results, the gene expression of the aldosterone synthase in cultured rat zona glomerulosa cells depends crucially on a high extracellular potassium concentration. The expression of the other three steroidogenic enzymes is also stimulated by high extracellular potassium.

S10-71

STUDY OF THE SECOND MESSENGER MEDIATING THE NEURONAL ACTION OF OXYTOCIN.

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The hormone oxytocin acts on peripheral target cells by binding to oxytocin receptors and by activating a phospholipase C. In the rat brain, oxytocin can generate a sustained nonspecific cationic current in preganglionic vagal motoneurons. The second messenger involved in this effect is not yet known. To address this question, we have used whole-cell recordings in brainstem slices. When loaded with GTPγS, a nonhydrolysable analogue of GTP, almost all vagal neurons developed a persisting inward current in voltage-clamp, and initiated spontaneous membrane potential oscillations in current-clamp. These effects were accompanied by the disappearance of the response to oxytocin, whereas the sensitivity to the selective glutamate receptor agonists, AMPA and NMDA, was not altered. This GTPγS-induced occlusion of the oxytocin effect suggests that the peptide acts via a G protein. In vagal neurons loaded with a calcium chelator, BAPTA, the oxytocin-evoked current was still present and had an amplitude comparable to that measured in control neurons. By contrast, I_{AHP}, a calcium-dependent outward aftercurrent, was suppressed in all cells. Thus, the oxytocin-specific current is not elicited by an increase in the intracellular calcium concentration. We are presently investigating whether the peptide response is due to the activation of a protein kinase C or an adenylyl cyclase.

S10-72

SPONTANEOUS ACTIVITY AND FIRING PATTERNS OF CAT PRIMARY AUDITORY CORTEX NEURONS IN VIVO

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We performed intracellular and extracellular recordings from single neurons in the primary auditory cortex (AI) of halothane anesthetized cats. The large majority of AI neurons (N=153, 86%) were firing spontaneously. Average firing frequency was 4.8 ± 0.6 Hz (Mean ± SD), ranging from 0 to 41 Hz. Mean firing frequencies did not significantly differ inbetween cortical layers. Three basic patterns of spontaneous firing were observed: (1) Quiet cells (N=24, 14%) which did not fire spontaneous action potentials when unstimulated. (2) Bursty cells (N=111, 62%) displaying bursts of spikes together with single action potentials. These neurons were further classified as predominantly bursty (N=54, 30%) and predominantly non-bursty (N=57, 32%) based on the relative amount of burst firing. (3) Some cells (N=8, 4%) fired single action potentials exclusively. When recorded intracellularly, all three groups of neurons showed many highly varible membrane oscillations, probably reflecting subthreshold excitatory postsynaptic potentials (EPSPs) and inhibitory postsynaptic potentials (IPSPs) from the cortical network.

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S10-73

THALAMIC STIMULATION EVOKES DIFFERENT RESPONSE PATTERNS IN CAT AI NEURONS IN VIVO

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We performed intracellular and extracellular recordings from single neurons in the primary auditory cortex (AI) of halothane anesthetized cats. These neurons reponded to stimulation of the ipsilateral medial geniculate body (MGB) with a complex synaptic pattern of excitation and inhibition. In the majority of AI neurons (N=116, 80%), the response to a single pulse MGB stimulation consisted of a period of excitation followed by an inhibition and occasionally by a second excitation. In 17 neurons (10%) the MGB stimulation initially evoked a long duration inhibition which in some cells was followed by rebound activity. Response patterns were strongly dependent on stimulus intensity. Onset latencies within groups of MGB stimulation evoked EPSPs and IPSPs in AI showed a bimodal distribution. In 39 neurons (22%) up to six groups of repetitive discharges followed the initial MGB stimulation induced response pattern for a maximum of 1.5 s. Besides the orthodromic response, MGB stimulation also triggered an antidromic spike in 22 (13%) AI neurons.

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S10-74

TWO-PHOTON SPECTROSCOPY OF FLUORESCENT CA DYES

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Many fluorescent indicators require excitation with UV-light (e.g. Indo-1, Fura-2). Problems with chromatical aberrations of the objectives put serious constraints on their use in confocal microscopy. Recently, it has been shown that these dyes can be excited by simultaneous absorption of two red photons. However, the absorption and emission spectra prevailing under these conditions do not necessarily correspond to those with UV-illumination. We modified a laser-scanning confocal microscope to allow spectroscopy from diffraction limited sub-femtoliter volumes. A Ti:sapphire laser generated the required short pulses for two-photon excitation. The aperture of a fiber-optic spectrometer collected the light in the primary image plane. Emission spectra at various Ca concentrations were recorded *in-vitro* for Indo-1 and Fluo-3 (1 mM; excitation 705 nm; pulse-width ≈ 100 fs. Compared to published spectra, the Ca bound form of both indicators exhibited a reduced emission, consistent with a smaller absorption cross-section. This leads to a reduced dynamic range (F_{max}/F_{min}) for Fluo-3 and a significant shift of the isosbestic point towards shorter wavelengths for Indo-1. We changed the duration of the pulses by varying the degree of negative group velocity dispersion in the laser. At constant average power (26 mW) an inverse linear relationship was found between the emission and duration of the pulses (from 80 fs to ≈ 175 fs). These results suggest that pulse-width and emission spectra need to be considered when implementing a two-photon confocal microscope. Supported by SNF.

S10-75

PROTECTIVE ROLE OF CALRETININ IN DIFFERENTIATED P19 CELLS EXPOSED TO EXCITATORY AMINO ACIDS

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In some neurological diseases, injury to neurons is related to overstimulation of receptors for excitatory amino acids (EAA). This overstimulation may disturb the Ca^{2+} homeostasis leading to an abnormal prolonged increase of its intracellular concentration. Various reports claim a protective role of calcium binding proteins (CaBP) in these pathologies. In our study we tested the capacity of the CaBP calretinin (CR) to buffer Ca^{2+} and to protect nerve cells. For this, we transfected P19 mouse embryonal carcinoma cells, which have the capability of differentiating into neuron-like structures expressing functional glutamate receptors, with CR cDNA. The presence of CR in cloned transfected cells was verified by immunohistochemical staining and Western blot experiments. After EAA exposure, $[Ca^{2+}]_i$ was measured using the ratiometric dye fura-2. After an initial rise of Ca^{2+} in transfected and control cells from 60 to 400 - 500 nM, $[Ca^{2+}]_i$ decreased in transfected cells to 150 - 200 nM within 5 min, while $[Ca^{2+}]_i$ remained at the initial high level in control cells. The survival rate of a CR transfected P19 cell clone was compared with that of control P19 cells using the lactate dehydrogenase assay. The results indicate a protective effect of CR within the first hour following EAA stimulation. However, this protection is transient and fails to rescue P19 cells after prolonged stress. Thus, calretinin may help to delay the onset of cell death after EAA stimulation.

S10-76

THE CYTOSOLIC CALCIUM-BINDING PROTEINS CALRETININ AND CALRETININ-22K ARE DETECTED IN THE SERUM OF CANCER PATIENTS

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Calretinin (CR), a member of the EF-hand family of calcium-binding proteins, as well as calretinin-22k (CR-22k), an alternatively spliced form of calretinin (M_r 22 kDa) are expressed in several colon adenocarcinoma cell lines as evidenced by several methods (Immunohistochemistry, Western blots, Northern blots). An antibody for the unique C-terminal amino acids of CR-22k localises this protein in the cytoplasm, as it is also known for the full-length protein.
Until now there was no indication for an extracellular localisation of CR or CR-22k. We found evidence for the presence of both proteins in the serum of some cancer patients by a sandwich ELISA technique using two polyclonal antisera against calretinin or CR-22k. In some cases the concentration of calretinin was as high as 0.15 µg per ml of serum. The identity of the protein specifically bound to the titer plates was confirmed by Western blot experiments and was shown to migrate on SDS-gels at the same position as human recombinant CR. The highest blood serum concentrations of CR and CR-22k were detected in blood collected from colon carcinoma or breast carcinoma patients. For the moment it is unknown if the proteins are specifically secreted or present in the serum as a result of necrosis of calretinin-producing tumor cells. Immunohistochemical studies on tumor tissue sections from these patients will help to address these questions.