SIGNAL TRANSDUCTION BY G PROTEINS

Organizer: Lutz Birnbaumer and Ravi Iyengar

February 9-15, 1990

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Signal Transduction by G Proteins

Overview of Signal Transduction through G-Proteins

G 001 GTP-REGULATORY PROTEINS HAVE PROPERTIES OF CYTOSKELETAL ELEMENTS: A THEORY OF MECHANO-CHEMICAL SIGNALLING BY G-PROTEINS, Martin Rodbell, Sherry Coulter, Kazutaka Haraguchi, Fernando Ribeiro-Neto, Section on Signal Transduction, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709

G-proteins play a critical role in chemical signalling at the cell membrane. Purified G-proteins are heterotrimeric proteins consisting of α-subunits that bind and degrade GTP to GDP+ Pi, and a lipophilic complex of two proteins (β/γ) that form associations with the α-proteins. Treatment with non-hydrolyzable analogs of GTP (GTPγS or Gpp(NH)p) or with AlF_4^- induces release of the α-subunits. The current notion is that hormones and GTP also induce the same dissociative phenomenon; the released α-subunits and β/γ complexes exert independent actions on various effectors in the cell membrane.However, there is no evidence that hormones and GTP stimulate release of α-proteins from native membranes or cells. Nor is there proof that the heterotrimeric G-proteins exist in native membranes or how they are associated with the cell membrane. When native membranes or cells are extracted with octyl-β-glucoside(OG) a different picture emerges. The extracted α-proteins (α_s, α_i, α_o) sediment along with cytoskeletal proteins typical of the cell in question whereas the β/γ complexes remain in the OG-containing solution. The degree of sedimentation of α-proteins and of tubulin, as an example of a cytoskeletal protein, is affected by temperature and Mg^{2+}. These findings suggest that α-proteins exist as polymeric structures which dissociate into lower molecular weight structures depending on temperature and Mg^{2+}. When membranes are pretreated with GTPγS (but not GTP) and Mg^{2+} the polymeric α-proteins dissociate into monomers which are completely soluble in OG. Studies with the cross-linking agent p-phenylenedimaleimide (PDM) lend credence to the view that the α-proteins pre-exist as polymeric structures. When membranes or cells are treated with PDM, the immunoblottable material representing the monomeric form of α-proteins disappears with corresponding appearance of higher molecular weight material (minimally homodimers). The β/γ complexes, by contrast, are poorly cross-linked; any detectable cross-linked material does not associate with the immunoblottable material representing the α-proteins. Pretreatment of membranes with GTPγS and Mg^{2+} results in no cross-linking, consistent with dissociation of the polymeric structures into monomers. Based on these findings coupled with previous target analysis studies suggesting that receptors and G-proteins are oligomeric structures, it is proposed that receptors are linked to polymeric forms of α-proteins (β/γ complexes may confer linkage to the membrane's lipid matrix); hormones and GTP act in concert to induce movement of the entire transduction structure to various effectors in the membrane. Hydrolysis of GTP to GDP and Pi is essential since rate and direction of movement is the result of differential binding affinities of GTP and products to various regions of the polymeric structure. In this way hormone action on G-proteins may induce mechano-chemical signalling, the multiple effects of which are responsible for the vast changes in cellular structure and function in response to hormones and other extracellular signals.

Nucleoside Di- and Triphosphate Cycle of Proteins and Regulation of Protein Function

G 002 MUTATIONS IN α_s INHIBIT GTP HYDROLYSIS AND CONTRIBUTE TO TRANSFORMATION, Henry R. Bourne, Claudia A. Landis, Susan B. Masters, Ian Zachary, and Lucia Vallar, Departments of Pharmacology and Medicine, University of California, San Francisco, CA 94143.

Many oncogenic mutations promote tumor growth by inducing autonomous activity of proteins that normally transmit proliferative signals initiated by extracellular hormones and growth factors. By analogy, the role of cyclic AMP (cAMP) as the intracellular second messenger of several trophic hormones and its ability to stimulate growth of certain cells in culture predict that oncogenic mutations should be found in genes for proteins that control cAMP synthesis. We have identified mutations that cause autonomous cAMP synthesis in 18 of 42 Growth Hormone (GH) secreting human pituitary tumors. The mutations cause constitutive activation of α_s, the GTP binding subunit of the stimulatory regulator of adenylyl cyclase, G_s, by inhibiting its GTPase. α_s mutations in the tumors are found in either of two codons: Mutations in two tumors replaced Gln-227 by Arg (these mutations are termed Q227R). α_s codon 227 specifies a glutamine residue cognate to Gln-61 of $p21^{ras}$; replacement of this residue in $p21^{ras}$ by other amino acids inhibits GTP hydrolysis and causes oncogenic transformation. These mutations indicate close similarities between the mechanisms of GTP hydrolysis in α_s and $p21^{ras}$ and imply similar structures of GTP binding sites in the two proteins. Mutations in 16 additional tumors replaced Arg-201 in α_s by Cys or His residues (termed R201C or R201H). Arg-201 is the residue ADP-ribosylated by cholera toxin; this covalent modification inhibits GTPase activity of α_s. Expression in S49 *cyc*$^-$ cells and Swiss 3T3 cells of α_s carrying Q227R, R201C, or R201H mutations produces constitutively elevated adenylyl cyclase. The R201X mutations point to a region of α_s that is intimately involved in GTP hydrolysis and that probably acts in α_s as a built-in counterpart of the separate GTPase Activating Proteins (GAPs) required for GTP hydrolysis by $p21^{ras}$ and other small GTP binding proteins.

G 003 FUNCTIONAL ANALOGIES BETWEEN ACTIN, TUBULIN AND G-PROTEINS. Marie-France Carlier and Dominique Pantaloni, Laboratoire d'Enzymologie du C.N.R.S., 91198 Gif-sur-Yvette, France.

The regulatory function of G-proteins is exerted via two essential reactions: GTP hydrolysis accompanies the interaction with an effector, and regulates its catalytic activity ; exchange of GTP for bound GDP occurs upon interaction with a signal protein. These two processes, nucleotide hydrolysis and nucleotide exchange, are also important in the function of actin filaments and microtubules, i.e. they regulate the dynamic state of these polymers in the cell.

Actin and tubulin bind ATP and GTP respectively and hydrolyse it to ADP (GDP) upon polymerization in filaments and microtubules. Nucleotide hydrolysis is not tightly coupled to the polymerization process and develops on the polymer in two successive elementary reactions : cleavage of XTP and P_i release. The liberation of P_i is the critical event that regulates the dynamics of the polymer; P_i release is linked to the destabilization of actin-actin and tubulin-tubulin interactions in F-actin and microtubules. Therefore, as for G-proteins, nucleotide hydrolysis has a switch role and its rate modulates the extent of dynamic instability of microtubules which is a key feature in their motile functions. In the case of actin, P_i at physiological concentrations binds to F-actin filaments and reconstitute the stable F-ADP-P_i state of the polymer. The structural change of the actin filament accompanying P_i release has been investigated by cryoelectron microscopy, using AlF_4^- and $BeF_3^-H_2O$ as high affinity analogs of P_i.

The rate of nucleotide exchange on monomeric actin and tubulin affects the steady-state monomer concentration ; this reaction is involved in the dependence of the apparent critical concentration on the number concentration of polymers. However agents affecting the rate of nucleotide exchange on actin or tubulin are not known.

Other functional analogies include the ADP-ribosylation of G-actin and tubulin, and the possibility for tubulin to mimic the G-protein of adenylate cyclase. Although consensus sequences for G-proteins are not present in actin nor tubulin, overall homologies in domain structure and geometry of bound nucleotide can be found between all these proteins in relation with their function.

G 004 PERSISTENTLY ACTIVATED $G\alpha_o$ REGULATION OF PHOSHOLIPASE C MEDIATED FUNCTIONS IN XENOPUS OOCYTES. Ravi Iyengar, Spencer D. Kroll, Gilla Omri and Emmanuel M. Landau. Departments of Pharmacology and Psychiatry, Mount Sinai School of Medicine New York, NY and the Bronx V.A. Medical Center Bronx, NY.

The Xenopus oocyte contains a pertussis toxin sensitive G-protein regulated phospholipase C that can be stimulated by endogenous muscarinic receptors as well as a variety of exogenous receptors expressed in the oocytes. We have found that the bovine brain GTP binding protein G_o can couple muscarinic receptors to the phospholipase C pathway. The three G_is were ineffective. Injection of activated α_o evokes the IP_3 mediated Ca^{2+} dependent Cl^- current within 300-500 msec. After 8-16 hours, activated α_o induces germinal vesicle breakdown. The growth effects of activated α_o can be blocked by the injection of a specific peptide inhibitor of protein kinase C. These data indicate that the activated α_o can stimulate both the IP_3 as well as the diacylglycerol pathways. Furthermore, persistent activation of the G protein results in persistent activation of the entire pathway. This may have important implications in a number of cellular processes including growth regulation.

G 005 SIGNAL TRANSDUCING GTP BINDING PROTEINS: COMMON MOTIFS OF STRUCTURE AND FUNCTION. Yoshito Kaziro, Institute of Medical Science, University of Tokyo, Minatoku, Tokyo 108, Japan.

GTP binding proteins are the superfamily consisted of several families including translational factors, signal transducing heterotrimeric G proteins, and low molecular weight GTP binding proteins such as protooncogenic ras p21. In their primary structures, several regions are found to be highly conserved among all GTP binding proteins even from far distant organisms, Especially, the regions responsible for GTP hydrolysis (P site) and GTP binding (G, G', and G'' sites) are remarkably homologous.

The basic mechanism of the reactions catalyzed by GTP-binding proteins appears to be analogous to that proposed originally for translational elongation factors (Kaziro, 1978). The GTP bound form is an active conformation which transmits the signals to the amplifiers, and the hydrolysis of bound GTP to GDP is required to shift the conformation to an inactive form, i.e. to shut off the signal transduction. Many examples of this type of reaction mechanisms have been reported in a variety of systems either dependent on GTP or ATP.

The ratio of the GTP bound form of the protein to the one bound to GDP is regulated by two mechanisms. One involves the regulation of the release of GDP tightly bound to the protein to exchange with an external GTP, and the other involves the stimulation of hydrolysis of bound GTP to GDP and inorganic phosphate.

Structures and properties of various GTP binding proteins from mammalian and yeast cells will be discussed.

Heterotrimeric G-Proteins

G 006 A NOVEL FORM OF A G PROTEIN α SUBUNIT: CLONING, SITES OF EXPRESSION, INTERACTION WITH $\beta\gamma$ DIMERS AND POSSIBLE FUNCTIONAL ROLE. Juan Codina*, Walter Hsu#, Uwe Rudolph*, Philippe Bertrand*, Jack Sanford*, Larry G. Moss#, Aubrey E. Boyd, III,# and Lutz Birnbaumer*, Departments of Cell Biology* and Medicine#, Baylor College of Medicine, Houston, TX 77030. A homology screen of a hamster insulin secreting tumor cell (HIT cell) cDNA library made in lambda gt11 has revealed the presence of an unrecognized form of a G protein α subunit. The novel sequence was found initially in two phages, neither of which contained an insert with a complete open reading frame. The full open reading frame was reconstructed from the inserts found in two additional phages and encodes a polypeptide chain of 354 amino acids with a putative pertussis toxin ADP-ribosylation site (cysteine 351). Northern analysis of RNA's prepared from a variety of tissues and cells reveals a widespread distribution, although not as wide as the α subunit of G_s which represents the only G protein α chain found in essentially all somatic cells studied thus far.

G 007 STRUCTURE OF G ALPHA GENES. H. Itoh and Y. Kaziro, Institute of Medical Science, University of Tokyo, Minatoku, Tokyo 108, Japan.

We have cloned cDNAs encoding α-subunits of G_s, two G_i subtypes (G_{i2} and G_{i3}), and G_o from rat C6 glioma cells. Nucleotide sequence analysis revealed the structure of $G_{s\alpha}$, $G_{i2\alpha}$, $G_{i3\alpha}$, and $G_{o\alpha}$, consisting of 394, 355, 354, and 354 amino acid residues, respectively, with M_r of 45,663, 40,499, 40,522, and 40,068. Homologies between them are striking. The regions responsible for GTP binding and GTP hydrolysis are well conserved among G proteins, *ras* proteins, and translational factors. We have also obtained from a rat brain cDNA library two clones; one clone codes for $G_{i1\alpha}$ which is the most abundant G_i subtype in brain (41K) but absent in C6 cells, and unique G_α clone which apparently code for the pertussis toxin-insensitive G_α (tentatively designated as $G_{s\alpha}$). $G_{s\alpha}$ codes for a protein of 355 amino acids with an M_r of 40,879, and the Cys residue in the 4th position from the C-terminus is replaced by Ile.

Human genomic library was screened with above cDNAs and corresponding human genes were isolated. The human $G_{s\alpha}$ gene spans 20 kb and is composed of 13 exons and 12 introns. The $G_{s\alpha}$ gene generates four types of different $G_{s\alpha}$ mRNAs by alternative splicing. The human $G_{i2\alpha}$, $G_{i3\alpha}$, and $G_{o\alpha}$ genes are composed of 8 coding exons and 7 introns and possess a completely identical exon-intron organization. The human $G_{i1\alpha}$ gene was partially characterized. It appeared to be identical with the $G_{i2\alpha}$, $G_{i3\alpha}$, and $G_{o\alpha}$ genes in organization. The human $G_{s\alpha}$ gene is composed of 2 exons and only 1 intron within the coding region. A single copy of each G_α gene is present per human haploid genome. Structural feature of human chromosomal genes for various G proteins will be discussed.

By cross-hybridization using the cDNAs for the α-subunit of mammalian G proteins as probes, we isolated two G protein homologous genes from *Saccharomyces cerevisiae*. One gene, *GPA1*, encodes a protein of 472 amino acid residues with M_r of 54,075 and another one, *GPA2*, encodes a protein of 449 amino acid residues with M_r of 50,516. The homology of yeast G proteins with mammalian $G_{i\alpha}$ or $G_{o\alpha}$ is about 66%. *GPA1* is expressed only in haploid cells and its product is involved in mating factor-mediated signal transduction.

G 008 ANTIBODIES AS TOOLS TO STUDY G-PROTEIN STRUCTURE AND FUNCTION, Allen M. Spiegel, Molecular Pathophysiology Branch, National Institutes of Health, Bethesda, MD 20892.

Antisera directed against synthetic decapeptides corresponding to the carboxy-termini of G-protein alpha subunits were raised and characterized. Such antisera block receptor-G-protein coupling, but can immunoprecipitate activated G-alpha-effector complexes. This offers a novel approach to defining the specificity of receptor-effector coupling by G-proteins in native membranes. Carboxy-terminal decapeptide antisera can also be used to immunoprecipitate metabolically labeled G-alpha subunits in *cos* cells and other cultured cell lines. Using this approach, we find that both Gs and Gi alpha subunits are primarily membrane-associated, but only the latter undergo myristylation. Mutagenesis of the myristylation site (gly-2 to ala) in Gi1 alpha abolishes myristylation and membrane attachment. The mutant protein, now localized to the cytosol, remains capable of interaction with beta/gamma subunits. Thus, myristylation is critical for membrane attachment of Gi, but not Gs, alpha subunits. In further studies to be discussed, we have probed the mechanisms for membrane attachment of Gs-alpha , as well as beta/gamma subunits.

Coupling Mechanisms Utilizing Heterotrimeric G-Proteins

G 009 DISTINCT FUNCTIONS OF MULTIPLE FORMS OF MUSCARINIC RECEPTORS, Tom I. Bonner, Laboratory of Cell Biology, National Institute of Mental Health, Bethesda, MD 20892

The genes and cDNAs of five different muscarinic acetylcholine receptors (m1-m5) have been cloned from both the human and rat genomes. Expression of the clones in Xenopus oocytes and mammalian cell lines have so far indicated only two classes of functional responses. The odd numbered receptors stimulate metabolism of phosphoinositides and independently cause release of arachidonic acid. They also produce increased levels of cAMP as a secondary effect of of calcium mobilization due to IP_3 production. The even numbered receptors preferentially cause decreased levels of cAMP but have been observed to stimulate phosphoinositide metabolism at high doses of agonist or when large numbers of receptors are expressed per cell. Parallel differences have been observed in electrophysiological responses. These functional specificities are reflected in the primary structure of the receptors in that the odd numbered receptors are more closely related to each other than to the even numbered ones and vice versa. This pattern of relatedness extends to the first and last 15-20 amino acids of the third cytoplasmic domain of the receptors, areas which have been shown through deletion mutations to be necessary for second messenger coupling in the β2-adrenergic receptor (1). The third cytoplasmic domain has also been shown through the use of chimeric receptors to be sufficient for specifying the characteristic electrophysiological response of the m1 and m2 receptors when expressed in Xenopus oocytes (2). Chimeras of the m2 and m3 receptors have therefore been examined to determine the role of these 20 amino acid domains in specifying the biochemical responses. Since there is little similarity in the corresponding sequences from other G protein-coupled receptors which would allow one to predict the second messenger, we have examined whether it is possible to obtain a second messenger response when the third cytoplasmic domain is exchanged between receptors for dissimilar ligands. The ability to do so in at least some cases indicates that it may be possible to identify the second messenger for a cloned receptor of unknown ligand by inserting its third cytoplasmic domain into a receptor for a known ligand.

1) C.D. Strader et al. J. Biol. Chem. 262, 16439-16443 (1987)
2) T. Kubo et al. FEBS Lett. 241, 119-125 (1988)

G 010 DISCRIMINATION OF G_i SUBTYPES BY THE D_2-DOPAMINE RECEPTOR IN A RECONSTITUTED SYSTEM, Susan E. Senogles and Marc G. Caron, Dept. Cell Biol., Duke Univ. Med. Ctr., Durham, NC 27710

The major consequence of dopaminergic action in the pituitary is the inhibition of prolactin release from the anterior lobe. The action of dopamine in this tissue and other target tissues is presumably mediated through several transmembrane signalling pathways. D_2 dopamine receptors have been shown to couple to inhibition of adenylyl cyclase and activation of K^+ channels. These effects of dopamine on various signalling systems are pertussis toxin sensitive, implicating the involvement of the G_i/G_o family of proteins. Given the multiplicity and ubiquity of the G-protein subtypes the question of whether the specificity of receptor-effector coupling resides at the receptor-G protein level, the G protein-effector level or both is an interesting one. We have shown previously that partial purification of the pituitary D_2 dopamine receptor by affinity chromatography results in co-purification of the receptor with a pertussis toxin substrate of M_r 40,000 (alpha subunit) (Senogles et al., J. Biol. Chem. 262: 4860-4867, 1987). To further evaluate this question the selectivity of D_2 dopamine receptor-G protein coupling was studied by reconstitution techniques utilizing purified D_2 dopamine receptors from bovine anterior pituitary and resolved G proteins from bovine brain, bovine pituitary and human erythrocyte. Titration of a fixed receptor concentration with varying G protein concentrations revealed two aspects of receptor-G protein coupling. First, G_{i2} appears to selectively couple with the D_2 receptor with ~10 fold higher affinity than any other tested G_i subtype. Secondly, the G proteins differed in the maximal receptor mediated agonist-stimulation of the intrinsic GTPase activity. G_{i2} appeared to be maximally stimulated by agonist-receptor complex with turnover numbers of ~2 min^{-1}. The other G_i subtypes, G_{i1}, and G_{i3} could be only partially activated resulting in maximal rates of GTPase of ~1 min^{-1}. Agonist-stimulated GTPase activity was not detected in preparations of G_o from bovine brain. The differences in maximal agonist-stimulated GTPase rates observed between the G_i subtypes could be explained by differences in agonist-promoted guanyl nucleotide exchange. Both GTPγS binding and GDP-release parameters were enhanced several fold for the G_{i2} subtype over the other G_i subtypes. These results suggest that even though several types of pertussis toxin substrate may exist in most tissues, a receptor may interact discretely with G proteins thereby dictating signal transduction mechanisms.

G 011 G PROTEIN-MEDIATED SIGNAL TRANSDUCTION BY CHEMOATTRACTANT RECEPTORS IN NEUTROPHILS,

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Neutrophils are activated by a variety of agents such as formyl peptides, leukotriene B_4, complement C5a, platelet-activating factor and neutrophil-activating protein, each compound acting on distinct membrane receptors. These receptors when activated by their specific ligands induce activation of phosphoinositide-hydrolyzing phospholipase C, increase in cytosolic Ca^{2+} concentration, production of superoxide, chemotaxis and secretion of granular enzymes. The action of these receptors, out of which that for formyl peptides serves as a model receptor, can be blocked by pertussis toxin treatment of the cells, suggesting an interaction of these receptors with pertussis toxin-sensitive G proteins in these membranes as the initial step of neutrophil activation. Out of the pertussis toxin-sensitive G proteins identified so far, neutrophil membranes contain apparently only G_{i2} (major form) and G_{i3} (minor form) at rather high concentrations. These two G_i proteins are also substrates for cholera toxin-catalyzed ADP-ribosylation, but only when studied in cell-free preparations. Although the above mentioned receptors interact with and activate the neutrophil G_i proteins, they do not induce inhibition of adenylyl cyclase.
The following data and hypotheses will be discussed in detail: Interaction of high and some low affinity agonist-binding receptors with G proteins; activation of G proteins by high and low affinity agonist-binding receptors; apparent activation of G proteins by empty, i.e. agonist-unoccupied receptors; coupling of apparently one agonist-liganded receptor to two distinct G_i proteins; evidence for differential coupling of different neutrophil receptors to a common pool of G_i proteins; extent and mechanisms of amplification of signal transduction in the intact membrane milieu at the receptor G protein level; evidence for participation of energy-rich phosphate transfer reactions in receptor-controlled G protein activation; finally, activation of neutrophil phospholipase(s) C by G proteins.

Supported by the Deutsche Forschungsgemeinschaft and the Fritz Thyssen-Stiftung.

G 012 COUPLING MECHANISMS UTILIZING HETEROTRIMERIC G PROTEINS,

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The muscarinic receptor of the frog oocyte is coupled to phospholipase C via a pertussis toxin(PTX)-sensitive G protein. The activation of phospholipase C leads to the formation of inositol tris-phosphate (IP_3) and the activation of a calcium-dependent chloride current. The chloride current was studied by voltage clamp technique and various component of G protein were directly injected into the oocyte. We find that the muscarinic response of the oocyte is blocked in a graded manner by injection of G protein $\beta\gamma$ subunits, indicating that the G protein mediating the muscarinic response is heterotrimeric, similar to G_s. Direct injection of holo-G proteins into the oocyte shows that G_o but not G_{i1},G_{i2} or G_{i3} can increase the response to acetylcholine, indicating that G_o can mediate the effects of muscarinic stimulation. Injection of activated α_o evokes the chloride current after a very short latency, indicating that the activated form of G_o may directly stimulate phospholipase C in the oocyte. The effect of α_o cannot be mimicked by any of the α_i's, denoting a specificity of action. The involvement of the phospholipase C- IP_3 pathway in mediating the effect of α_o can be demonstrated by the ability of pre-injected IP_3 to desensitize the response to α_o. In view of these results and the recent cloning of α_o from an oocyte library[1] we conclude that G_o could be the G protein mediating the muscarinic response in this cell. Since some receptors (e.g. the vasopressin receptor) may utilize both PTX-sensitive and -insensitive pathways to link to phosphlipase C, we suggest that the PTX-insensitive responses are coupled through G proteins with a structure closely resembling that of G_o.

1. Olate et al. FEBS Lett.**244**,188-192(1989).

G 013 STRUCTURE AND FUNCTION OF G PROTEIN SUBUNITS, Eva J. Neer, Carl Schmidt, Suzanne Garen-Fazio, Yung-Kang Chow, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, and Department of Neurobiology, Harvard Medical School, Boston, MA 02115

Heterotrimic GTP binding proteins, composed of a GTP binding α subunit and a $\beta\gamma$ subunit, transmit signals from many receptors for hormones and neurotransmitters to a variety of intracellular enzymes or ion channels. Receptors on the cell surface are very specifically activated by their ligands. However, interactions with G proteins appear to be less precise. Many receptors can interact with the same G protein and conversely, a single G protein is capable of interacting with several effectors and receptors. Additional complexity comes from the fact that dissociation of the GTP binding protein can give rise to two signalling molecules, the α and the $\beta\gamma$ subunits. A current challenge is to define the structural features and the cellular organization which form the basis of discrete hormonal responses in intact cells.

All cells contain several kinds of G protein α subunits. The α_s, α_{i-2} and α_{i-3} forms are ubiquitous, while other α subunits have a more restricted distribution. Apart from the G proteins of sensory receptors, the protein with the most skewed distribution is G_o whose α and $\beta\gamma$ subunits together make up 0.5 - 1% of brain particulate protein. In the mammalian central nervous system, α_o is localized in the neuropil. In other tissues, α_o is either absent or present at very much lower concentrations than it is in the nervous system.

The function of α_o is not known, although the protein has been implicated in regulation of phospholipase C and ion channels. Identification of α_o in an organism amenable to genetic analysis could provide a tool with which to define a cellular role. We have now isolated cDNA from Drosophila which encodes a protein that is 80% identical to mammalian α_o. In situ hybridization of mRNA shows that, like mammalian α_o, this protein is primarily found in the nervous system. Immunocytochemical studies using a polyclonal anti-Drosophila α_o antibody (raised against the protein expressed in E. coli) show that, like mammalian α_o, the insect protein is localized in the neuropil. The similarity of structure and localization suggests conservation of function of α_o between flies and mammals.

Pathways Mediated by Heterotrimeric G-Proteins

G 014 GUANINE NUCLEOTIDE BINDING PROTEINS IN DIABETES AND INSULIN-RESISTANT STATES. Miles D. Houslay, Suzanne Griffiths, Brian Lavan, Derek Strassheim & Mark Bushfield. Molecular Pharmacology Group, Institute of Biochemistry, University of Glasgow, Glasgow G12 8QQ, Scotland, UK

We have assessed the functioning and the expression of G-proteins in both the hepatocytes and the adipocytes of animal models which exhibit insulin resistance, such as streptozotocin-diabetic rats and obese Zucker rats [1,2]. This has involved using anti-peptide antibodies to identify G-proteins and synthetic oligonucleotide probes to identify the mRNA coding for these species. In hepatocytes, from diabetic animals, we observed a marked reduction in the expression of both G_i-2 and G_i-3 [1] and in the mRNA for these species. This was coupled with a loss in "G_i" function of the residual "G_i" activity when assessed by the ability of guanine nucleotides to inhibit adenylate cyclase activity. In contrast to this, using adipocytes from these animals adipocytes, we saw no reduction in the expression of the three forms of G_i despite the fact that "G_i" function was ablated when assessed by the ability of guanine nucleotides to inhibit adenylate cyclase activity [3]. However, the lesion in "G_i" appears not to affect the ability of inhibitory receptors to function: the action of the lesion being rather to remove the tonic GTP-mediated inhibitory action of "G_i". We suggest that the phosphorylation of α-G_i-2 may provide a possible molecular basis for the crippled "G_i" function under conditions where G_i forms are still expressed. This leads to a loss in GTP-, but not receptor-, mediated inhibitory "G_i" responses upon adenylate cyclase activity. One result of this, an enhanced action of stimulatory hormones can be observed upon adenylate cyclase activity. We have demonstrated [4] that G_i can be phosphorylated in intact hepatocytes. We now know that this is restricted to the α-subunit of G_i-2. This action is elicited by agents which stimulate the inositol phospholipid pathway and/or activate protein kinase C, and through the action of pure C-kinase.

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G 015 ROLE OF A G PROTEIN IN YEAST PHEROMONE RESPONSE, Janet Kurjan, Christine Dietzel, and Jeanne Hirsch, Department of Biololgical Sciences, Columbia University, New York, NY 10027.

Mating between cells of opposite mating type (a and α) in the yeast *Saccharomyces cerevisiae* involves the peptide pheromones a-factor and α-factor, which are secreted by a and α cells, respectively. Each of the pheromones binds to a receptor present on the opposite mating type and elicits responses that are essential for mating. Results obtained in the past few years have indicated that this pathway is mediated by a G protein homologous to the G proteins involved in well-characterized vertebrate signal transduction pathways.

The *SCG1* (also called *GPA1*), *STE4*, and *STE18* genes encode homologs to the α, β, and γ subunits of mammalian G proteins. The phenotypes resulting from disruption of these genes indicate the Scg1 plays a negative role in the pheromone response pathway, and that Ste4 and Ste18 play positive roles. These and other genetic results are consistent with a model in which $\beta\gamma$ acts downstream of α to activate a currently unidentified effector. In the absence of pheromone, α binds to $\beta\gamma$ to keep the pathway inactive, consistent with its negative role. After activation of the pheromone receptors by exposure to pheromone and the resulting guanine nucleotide exchange, $\beta\gamma$ dissociates from α and activates the effector, consistent with its positive role.

Site-directed mutations have been constructed in *SCG1* that alter regions predicted to play a role in guanine nucleotide-binding, based on analogy to EF-Tu and ras, or that alter the carboxy terminus. The phenotypes of the resulting mutants indicate that *SCG1* mutations can result in either of two opposite phenotypes; recessive mutations that lead to constitutive activation of the pheromone response pathway (similar to the null phenotype), or dominant or partially dominant mutations that result in defects in pheromone response and mating. The phenotypes associated with carboxy terminal mutations are consistent with the proposed role of the carboxy terminus in receptor interactions. These and other mutants should be useful in investigating interactions between components of the system.

G 016 RECEPTOR-MEDIATED CALCIUM ENTRY, Timothy J Rink, Smith Kline & French Research Limited, The Frythe, Welwyn, Herts, AL6 9AR, U.K.

There is recent evidence from measurements of cytosolic Ca, and electrophysiological measurement in cells, membrane patches and vesicles incorporated into bilayers, for a diversity of receptor-mediated calcium entry processes[1] (RMCE), defined as, any influx consequent on receptor occupation and not dependent on depolarisation that generates a biologically significant increase in cell Ca. We can sub-divide RMCE into: (1) that through 'ROCC's' receptor-operated calcium channels, where the ligand acts directly on a component of the channel complex. (2) 'SMOC's', second messenger-operated channels, where a chemical mediator generated by receptor occupancy activated the Ca entry, (3) intermediate between these may be Ca entry activated via G-proteins. We might also regard circumstances where chemical modulation, e.g. phosphorylation, activates voltage-gated Ca channels without an actual change in membrane potential as another class of receptor-mediated Ca entry. Yet another complexity is the possibility that internal Ca stores may be refilled by entry of Ca from the external medium through a "special" pathway across both the plasma membrane and organelle membrane, by-passing the cytosol. Work on smooth muscle cells, parotid gland cells, and human platelets, neutrophils and endothelium will be described, which has explored RMCE using a variety of techniques in cells loaded with fluorescent Ca-indicator dyes, including stopped-flow fluorimetry, single cell micro-spectrophotometry, digital imaging, and the combination of patch clamping with single cell fluorescence.

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G 017 MOLECULAR BASIS FOR STIMULATORY AND INHIBITORY CONTROL OF SECRETION, Werner Schlegel, Fondation pour Recherches Médicales, Dept. of Medicine, University of Geneva, 64 ave. de la Roseraie, CH-1211 Geneva / Switzerland.

Endocrine secretion is controlled by a variety of G-protein linked receptors, which are coupled to ion channels, phospholipases, adenylate cyclase, and possibly other effector systems. Control may be exerted at several of the many steps leading from gene expression to exocytosis of mature secretory granules. Ligand - receptor interaction initiate a cascade of intracellular events leading to well defined alterations in intracellular 'messengers'; among them, cytosolic free Ca^{2+} stands out as an important element in the control of secretion. A combination of microfluorimetry with fast image processing and patch clamp recording techniques was used to analyze sub-second kinetics and intracellular localization of Ca^{2+} transients, and corresponding ion channel activities; these techniques permit the analysis during the application of extracellular receptor ligands or of intracellular 'messengers'. Ca^{2+} activated K^+ channels serve to monitor indirectly alterations of free Ca^{2+} at the cell periphery. In pituitary cells, stimulatory secretagogues (TRH, VIP, GnRH) act via G-protein coupled phospholipases, adenylate cyclase, and Ca^{2+} channels; inhibitory receptors (e.g. somatostatin) in addition activate G-protein linked K^+ channels. TRH triggers in a first phase Ca^{2+} mobilization from internal stores as a result of $Ins(1,4,5)P_3$ induced Ca^{2+} release, and Ca^{2+} induced Ca^{2+} release. Reinforcement by an $Ins(1,4,5)P_3$ independent mechanism which likely inhibits Ca^{2+} re-uptake will contribute to a massive and generalized rise in cytosolic free Ca^{2+}. Later on, and based mainly on a sustained DG production and protein kinase C activation, TRH modulates Ca^{2+} channel activity resulting in enhanced frequency and amplitude of Ca^{2+} dependent action potentials. In intact cells, VIP based on the stimulation of adenylate cyclase, appears to be able to produce the same repertoire of Ca^{2+} channel modulation as TRH. Somatostatin acts in parallel on adenylate cyclase, K^+ -, and Ca^{2+} channels to block electrical activity and counteract its stimulation. Furthermore, inhibitory control of exocytosis apparently mediated by G-proteins may be exerted at steps distal to the inhibition of Ca^{2+} transients. Pituitary tumor cells exposed to EGF, estradiol, and insulin leading to the apparition of prolactin storage granules (normal phenotype) show significantly reduced levels of immunoreactive G_s. The complex regulation of cytosolic free Ca^{2+} in endocrine cells demonstrates that G-protein linked effectors are part of an intertwined biochemical network with many apparently redundant parallel pathways. To describe the precise physiological function of defined G-proteins within this network in intact cells represents a major challenge for future research.

Pathways Involving Small Molecular Weight G-Binding Protein, SMGs

G 018 LOW MOLECULAR WEIGHT GTP BINDING PROTEINS OF THE NEUTROPHIL; rap AND rac, Gary M. Bokoch, Department of Immunology, Research Institute of Scripps Clinic, La Jolla, CA 92037.

A large number of 20-28,000 MW GTP binding proteins have recently been identified. These proteins specifically bind GTP with high affinity and exhibit intrinsic GTP hydrolysis activities. The biochemical properties of the low molecular weight GTP binding proteins (LMWG) indicate that they are likely to be regulated by mechanisms distinct from those of the "classical" oligomeric G proteins. The existence of factors which can influence both guanine nucleotide binding and/or hydrolysis activities of the LMWG is suggested, and several regulatory factors of this nature have been recently identified. The normal cellular functions of the LMWG have not been well defined, with the exception of their role in protein trafficking in the yeast system.

Human neutrophils contain a number of distinct LMWG. One of these proteins is a novel botulinum toxin substrate. This substrate is ADPribosylated by botulinum toxin C_3 ADPribosyltransferase in the presence of an additional protein cofactor,[3] and can be distinguished from the rho protein immunologically. The rac protein has been cloned from an HL-60 cell library and can be shown to serve as a botulinum toxin C_3 substrate. Rac exhibits ~53% amino acid sequence homology with rho, and contains an asparagine residue at position 41, which has been identified as the site for ADPribosylation of rho by botulinum C_3 toxin. The rap1 protein was also purified from human neutrophils. This LMWG serves as a substrate for cAMP-dependent protein kinase both in vivo and in vitro. Phosphorylation of rap1 does not change its guanine nucleotide binding or hydrolysis activities, nor its ability to interact with neutrophil rap-GAP. Phosphorylated rap1 does get released from the neutrophil plasma membrane, and this translocation may serve to modify rap function. Several lines of evidence suggest that in the neutrophil, rap1 may be able to interact with, and potentially regulate, superoxide formation by the NADPH oxidase system.

G 019 GTP-ACTIVATED COMMUNICATION BETWEEN CALCIUM SIGNALLING POOLS.
Donald L. Gill, Julienne M. Mullaney, and Tarun K. Ghosh. Department of Biological Chemistry, University of Maryland School of Medicine, Baltimore, MD 21201.

A sensitive and specific GTP-activated mechanism has been observed to mediate translocation of Ca^{2+} within cells[1]. Recent evidence reveals the existence of two discrete Ca^{2+}-signalling organelles distinguished by sensitivity or insensitivity to inositol 1,4,5-trisphosphate ($InsP_3$)[2]. In permeabilized N1E-115 neuroblastoma cells or DDT_1MF-2 smooth muscle cells, the GTP-activated Ca^{2+} translocating process induces the movement of Ca^{2+} between these organelles. This Ca^{2+} movement is believed to be mediated by GTP-induced communication via junctional processes between endoplasmic reticulum and an as yet unidentified organelle[3]. The nucleotide specificity, sensitivity, and requirement for terminal phosphate hydrolysis are all features similar to those now established for the role of monomeric G proteins in mediating trafficking events in cells[4]. Since the effect of GTP on Ca^{2+} translocation is reversible it is considered unlikely that the initial event is fusion between membranes. Investigation of the proteins involved in GTP-induced communication and Ca^{2+} regulation were undertaken to determine the possible mechanism of GTP-activated Ca^{2+} transfer and the significance of the two participating organelles in Ca^{2+} signalling. Using ER vesicles isolated from DDT_1MF-2 smooth muscle cells, the "reconstituted-blot" technique for labeling PAGE-separated SDS-solubilized proteins on nitrocellulose blots with [^{35}S]GTPγS, revealed eight discrete GTP-binding proteins between 21 and 29 kDa. The same proteins were identified within both rough and smooth ER-enriched membrane vesicle preparations all showing GTP-induced Ca^{2+} translocation. Labeling was highly specific and reflects a class of low M_r GTP-binding proteins believed to mediate trafficking between membranes. Western analyses revealed cross-reactivity with a MAb to the mammalian ypt1 protein (23 kDa). Preliminary studies reveal that this MAb specifically inhibits the GTP-activated Ca^{2+} translocation process without altering Ca^{2+} pumping, $InsP_3$-activated Ca^{2+} release, or other fluxes of Ca^{2+}; experiments are currently assessing whether this effect is due to interaction with the ypt protein. Western analysis also reveals a 110 kDa protein cross-reacting with MAbs to the slow cardiac Ca^{2+} pump, and a 170 kDa protein cross-reacting with an anti-calsequestrin MAb and corresponding with a high M_r form of skeletal muscle calsequestrin. Present studies are hoped to ascertain the link between GTP-activated Ca^{2+} translocation and those GTP-binding proteins thought to be involved in communication between membranes. (Supported by NIH grant NS19304 and NSF grant DCB-8510225).

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G 020 **REGULATION OF VESICULAR TRANSPORT BY A GTP-BINDING PROTEIN**, Peter Novick, Alisa K. Kabcenell, Nancy C. Walworth, Robert Bowser and Heike Muller, Department of Cell Biology, Yale University School of Medicine, New Haven, CT 06510

The 23.5 kD Sec4 protein is required for vesicular transport between the Golgi apparatus and the plasma membrane in *Saccharomyces cerevisiae*. In order to analyze its biochemical properties, we have purified the soluble pool of the wild-type protein from an overproducing yeast strain. At 30°C, Sec4p bound GTPγS with a rate of 0.18 min-1 in a reaction requiring micromolar concentration of free magnesium ions. The protein had high affinity for guanine nucleotides with Kd's for GTPγS and GTP of 3.7nM and 3.5nM respectively, and that for GDP of 77nM. The dissociation of [^{3}H]GDP from Sec4p occurred with a rate of 0.21 min-1 suggesting that the association of GTPγS was the result of exchange for prebound GDP. The release of GTP from Sec4p was slow and correlated with a low inherent GTPase activity of 0.0012 min-1. By analogy with other classes of GTP binding proteins, both the nucleotide exchange and hydrolysis activities of Sec4p may be modulated in vivo to facilitate its role in the regulation of intercompartmental membrane traffic. The rate of hydrolysis of GTP by Sec4 can be stimulated by a yeast membrane fraction and this stimulation can be inhibited by micromolar amounts of Sec4 prebound to GTPγS. In vivo, at normal levels of expression, Sec4 is associated predominantly with the cytoplasmic surface of secretory vesicles and the plasma membrane (1) by a mechanism requiring the two C-terminal cysteine residues (2). This attachment process has been reconstituted in vitro by addition of pure, soluble Sec4 to purified secretory vesicles. *SEC4* displays strong genetic interactions with several of the late acting *SEC* genes including *SEC2* and *SEC15* (3). The Sec15 protein is 105 kD and is peripherally associated with the inner surface of the plasma membrane. Overproduction of Sec15 inhibits cell growth and leads to the formation of a patch of Sec15 protein and a cluster of secretory vesicles (4). The ability of Sec15 to form a protein patch upon overproduction is dependent on the function of Sec2 and Sec4, but not on the function of the other late acting *SEC* gene products. Sec15 may normally act in conjunction with Sec2 to dock secretory vesicles bearing Sec4 onto the plasma membrane, thereby maintaining the specificity of the exocytotic fusion reaction.

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RAS Proteins: Structure and Function

G 022 **RAS FROM THE YEAST PERSPECTIVE:** Jeffrey Field, John Colicelli, Anne Vojtek, Roymarie Ballester, Ken Ferguson, Nick Chester, Tamar Michaeli, Jeffrey Gerst, Michael Riggs, Linda Rodgers and Michael Wigler. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724.

The RAS genes are highly conserved among eucaryotic organisms. We are studying RAS1 and RAS2, the two homologs found in S. cerevisiae. We previously demonstrated that RAS proteins are positive regulators of adenylyl cyclase. We reconstituted RAS regulation of adenylyl cyclase with purified proteins. RAS was purified from an E. coli expression system, and adenylyl cyclase was purified from a yeast expression system. The purified adenylyl cyclase consisted of a catalytic subunit of an apparent molecular weight of 200 kDa (the CYR1 gene product) plus a second subunit of an apparent molecular weight of 70 kDa.

We cloned the 70 kDa subunit using an antibody raised against the purified protein. The predicted sequence of this protein was not homologous to any in the current data bases. Genetic and biochemical studies suggested that the 70 kDa protein is multifunctional, playing an important role in regulation of adenylyl cyclase and also in a second, as yet undetermined pathway.

We have also mapped functional regions of adenylyl cyclase through deletion analysis of the cloned gene. The catalytic domain mapped to the C-terminal 417 amino acids as previously reported (amino acids 1609 to 2026). Deletions from the NH_2-terminal up to amino acid 605 had no effect on RAS responsiveness, but deletions beyond that point resulted in loss of RAS responsiveness.

We also developed a genetic interference assay that appears to reflect the RAS binding region of adenylyl cyclase. In this assay we expressed non-catalytic forms of adenylyl cyclase in yeast and scored for reversion of the heat sensitivity phenotype, of $RAS2^{val19}$ containing yeast. Presumably, the hyperactive $RAS2^{val19}$ is sequestered from the wild type adenylyl cyclase by the non-catalytic adenylyl cyclase. The interfering region mapped within the RAS responsive region to a region known as the leucine rich repeat domain (amino acids 733 to 1301). This region is homologous to leucine rich repeat domains found in a number of other proteins where it is thought to be a protein binding site.

G 023 THE RELATIONSHIP BETWEEN RAS AND GAP FUNCTIONS, J.B. Gibbs, U.S. Vogel, M.D. Schaber, M.B. O'Hara, S. Moores, K. Matsumoto +, C. Molloy*, S. Aaronson*, E.M. Scolnick, R.A.F. Dixon, and M.S. Marshall, Merck, Sharp and Dohme Research Laboratories, West Point, PA 19486, + DNAX, Palo Alto, CA 94304, and *National Cancer Institute, Bethesda, MD 20892.

The GTPase activating protein (GAP) may function to regulate Ras or to confer downstream signals. There are many similarities between Ras/GAP binding and Ras/yeast adenylyl cyclase interaction, although this alone does not constitute proof of GAP as a protein downstream of Ras. To explore the function of GAP, we have focused on several biological approaches. In the yeast S. cerevisiae, GAP clearly functions to turn-off Ras activity. Expression of mammalian GAP in yeast complements ira gene disruptions. IRA acts as an upstream negative regulator of Ras function. Full ira suppression can be conferred by the C-terminal Ras binding domain of GAP which lacks the N-terminal Src homology 2 region. To explore the function of GAP in mammalian cells, bovine GAP was overexpressed 50-fold in normal NIH3T3 cells and in NIH3T3 cells transformed by overexpression of normal ras or [Val-12]ras. The biology of these 3 cell lines was apparently not affected by GAP overexpression suggesting that GAP levels are not rate-limiting and that other factors may regulate or activate GAP activity. The cellular response to growth factors may be one activating step. In quiescent NIH3T3 cells, PDGF stimulates tyrosine phosphorylation of GAP within 10 min, and this phosphorylation can be detected for several hours post-challenge. Furthermore, PDGF stimulation induces the association of GAP protein, which is normally cytosolic, with the particulate fraction and may coordinate Ras and GAP functions. In Xenopus oocytes, a cytosol-localized Ras mutant antagonizes [Val-12]Ras function, possibly by blocking a cytosolic factor such as GAP from interacting with membrane-bound Ras. Mutagenesis of GAP and the identification of other proteins associated with GAP and Ras should help to clarify the function of GAP.

G 024 MOLECULAR SWITCH FOR SIGNAL TRANSDUCTION BY *RAS* PROTEIN: A THREE-DIMENSIONAL VIEW, Sung-Hou Kim, Department of Chemistry and Chemical Biodynamics Division, Lawrence Berkeley Laboratory, University of California, Berkeley, CA 94720. *Ras* proteins play a crucial role of molecular switch in the early steps of the signal transduction pathway associated with cell growth and differentiation; the protein in its GTP complexed form is active in signal transduction, whereas it is inactive in its GDP complexed form. The comparison of the crystal structure of a *ras* protein complexed with a non-hydrolyzable GTP analog with that of the protein complexed with GDP reveals that the "on" and "off" states of the switch are distinguished by extensive conformational differences induced by the γ-phosphate. Both regions are highly exposed and form a contiguous strip on the molecular surface most likely to be the recognition sites for the effector and receptor molecule(s). The conformational differences also provide a structural basis for understanding the changes in biological and biochemical properties of the proteins due to oncogenic mutations, autophosphorylation, GTP hydrolysis and for understanding interaction with other proteins.

G 025 IN VITRO RECONSTITUTION OF CDC25-REGULATED S.CEREVISIAE ADENYLYL CYCLASE AND ITS KINETIC PROPERTIES

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We describe reconstitution of efficient guanyl nucleotide dependent adenylyl cyclase, by combining cell membranes or lysates from yeast strains defective in their COLC25 gene with cell membranes or lysates of yeast cells harbouring normal CDC25. The rate of adenylyl cyclase (CDC35) activation is first order and proportional to the number of CDC25 gene copies. These experiments provide direct biochemical evidence for the regulation of RAS dependent cyclase by CDC25. These experiemnts also provide an experimental tool to analyze in-vitro the mechanism of action of the CDC25 protein once it is purified. The reconstitution experiments and the kinetic approach also suggest a possible tool to study CDC25 homologues from higher eucaryotes.
In this study we also show that adenylyl cyclase activity in ras1ras2bcyl is found mostly in the souble fracton (100,000xg supernatant). Over expression of CDC25 in the ras1ras2bcy1 astrain relocalizes enzyme activity to the membrane fraction (100,000xg supennatant) suggesting a CDC25 to cyclase (CDC35) cross talk in the absence of RAS proteins.

G 026 REGULATION OF YEAST RAS, Kazuma Tanaka[1], Akio Toh-e[1], Masato Nakafuku[2], Yoshito Kaziro[2], Rosamaria Ruggieri[3] and Kunihiro Matsumoto[3], [1]Department of Fermentation Technology, Hiroshima University, Japan, [2]Institute of Medical Science, University of Tokyo, Japan, and [3]DNAX Research Institute, Palo Alto, CA 94304.

In Saccharomyces cerevisiae, the RAS proteins play a key role in the transduction of the signal for growth in response to nutrients. The signal is transduced by virtue of the RAS protein ability to bind and hydrolyze GTP. In the GTP-bound state, RAS activates adenylate cyclase. The CDC25 gene is proposed to be a positive regulator which exchanges the GDP bound to RAS proteins for GTP. On the other hand, IRA1 and IRA2 genes negatively regulate RAS activity. Quantitative analysis of guanine nucleotides bound to RAS proteins clearly indicates that IRA proteins function to decrease the population of RAS-GTP. Yeast IRA proteins have homology to mammalian ras GTPase activating protein (GAP). Overexpression of bovine GAP suppressed the phenotypes of ira disruption mutants, suggesting that yeast IRA proteins may be the counterpart of mammalian GAP.

Effectors of Heterotrimeric G-Proteins

G 027 REGULATION OF PHOSPHOLIPASES FOR PHOSPHOINOSITIDES AND PHOSPHATIDYLCHOLINE BY G-PROTEINS AND OTHER MECHANISMS, John H. Exton, Stephen B. Bocckino and Stephen J. Taylor, Howard Hughes Medical Institute and Department of Molecular Physiology and Biophysics, and Department of Pharmacology, Vanderbilt University School of Medicine, Nashville, TN 37232. It is now clear that many calcium-mobilizing agonists stimulate the hydrolysis of both phosphatidylinositol 4,5-bisphosphate (PIP_2) and phosphatidylcholine (PC) in their target cells. While there is abundant evidence that stimulation of PIP_2 breakdown by these agonists is transduced by G-proteins that couple the relevant receptors to a phosphoinositide-specific phospholipase, the specific G-proteins and phospholipase have not been unequivocally unidentified. Data on the purification and characterization of PIP_2 phospholipases from liver plasma membranes will be presented, with emphasis on that form responding to GTPγS stimulation. Efforts to define the G-protein specifically involved in regulating the phospholipase and in coupling to the vasopressin receptor will be described.

Studies utilizing hepatocytes indicate that PC hydrolysis is a major source of 1,2-diacylglycerol (DAG) in hepatocytes stimulated with Ca^{2+}-mobilizing agonists, especially at later times, and is therefore a major factor in the regulation of protein kinase C. Data obtained by HPLC analysis of DAG molecular species and using hepatocytes in which PC is labeled selectively using [^{3}H]alkyl-lyso-glycerophosphocholine will be presented. These demonstrate that PC hydrolysis is a major response of liver cells to certain agonists.

The mechanisms involved in agonist regulation of PC hydrolysis are unclear at present, but it is evident that both phospholipase C and D activities are involved. Control of these enzymes by a G-protein(s) in liver plasma membranes has been shown. Evidence will be presented showing that phorbol esters and ionophores stimulate PC hydrolysis to DAG and phosphatidate (PA) in hepatocytes, apparently via mechanisms involving protein kinase C and Ca^{2+}, and both phospholipases C and D. Data on the subcellular distribution and properties of PC phospholipases C and D in liver and other enzymes involved in the metabolism of DAG and PA will be presented.

G 028 REGULATION OF PLATELET MEMBRANE PHOSPHOLIPASES AND ADENYLYL CYCLASE BY GTP-BINDING PROTEINS, Richard J. Haslam, Kimberley A. Williams, Wanda Davis, Judy Sherwood and John Van der Meulen, Departments of Pathology and Pediatrics, McMaster University, Hamilton, Ontario, Canada L8N 3Z5

Agonist and GTP-dependent activation of phospholipase C (PLC) and inhibition of adenylyl cyclase (AC) were measured at the same time in rabbit platelet membranes containing [^{3}H]phosphoinositides. Thrombin or platelet-activating factor (PAF) simultaneously stimulated PLC and inhibited AC, whereas epinephrine only inhibited AC, suggesting that G_i does not mediate the stimulation of PLC. Activation of PLC required higher thrombin or PAF concentrations than did inhibition of AC by the same agonist. Rabbit platelets possess three pertussis toxin substrates. Under conditions in which inhibition of AC by epinephrine was blocked by pertussis toxin or N-ethylmaleimide, the actions of thrombin or PAF on PLC and AC were much less affected, implicating GTP-binding proteins that are not pertussis toxin substrates in the effects of the latter agonists on both enzyme activities. Addition of G-protein $\beta\gamma$ subunits inhibited AC but not PLC, suggesting a role for a non-heterotrimeric GTP-binding protein in the activation of PLC. Differential effects were also exerted by protein kinase C. Treatment of platelets with phorbol ester blocked both the activation of membrane PLC by thrombin or GTP[S] and the inhibition of AC by epinephrine, but did not block inhibition of AC by thrombin or GTP[S]. Measurement of phospholipase D (PLD) activity in membranes from rabbit platelets labelled with [^{3}H]glycerol showed that pretreatment of platelets with phorbol ester greatly potentiated activation of this enzyme by GTP[S] or GTP. However, addition of thrombin to isolated platelet membranes did not cause a GTP-dependent activation of PLD, suggesting that activation of PLD by thrombin in intact platelets may be a secondary effect of the stimulation of PLC and thus of protein kinase C. The results demonstrate the presence of independent pathways for the agonist-dependent regulation of platelet phospholipases and AC that involve distinct GTP-binding proteins. Supported by MRC Grant MT 5626.

G 029 GTP-BINDING PROTEINS AND ADENYLYL CYCLASE IN OLFACTORY SIGNAL TRANSDUCTION, Randall R. Reed, David T. Jones and Heather Bakalyar, Department of Molecular Biology and Genetics, Howard Hughes Medical Laboratories, Johns Hopkins School of Medicine, Baltimore, MD.

The vertebrate olfactory system is exquisitely adapted for the detection and recognition of small odorant molecules. The presence of odorant stimulated adenylyl cyclase and cyclic-nucleotide gated channels in olfactory cilia implicates an olfactory second messenger cascade analogous to that operating in the visual system. Previous biochemical studies suggest a role for a stimulatory GTP-binding protein and adenylyl cyclase in olfaction. In an effort to identify the G-protein which couples to olfactory receptors, we have isolated six G-protein a subunit cDNAs from rat olfactory neuroepithelium. Five of the cDNA clones encode $G_{\alpha s}$, $G_{\alpha o}$ and three distinct forms of $G_{\alpha i}$. None of these five G_α species are uniquely expressed in olfactory tissue.

The sixth G_α cDNA is a novel species that is highly homologous to $G_{\alpha s}$ (88% amino acid identity) and is, therefore likely to stimulate adenylyl cyclase. This $G_{\alpha s}$-like mRNA is expressed exclusively in olfactory tissue. Moreover, utilizing a neuron depletion technique, its expression appears to be confined to olfactory sensory neurons. With monospecific antisera to this novel G-protein (G_{olf}) we have localized G_{olf} protein to the cilia of the receptor neurons.

Recently, we have shown that the majority of the adenylyl cyclase activity in the olfactory epithelium resides within the receptor neurons. In an effort to characterize the adenylyl cyclase enzyme expressed in different tissues at the molecular level, we have collaborated with Dr. Alfred Gilman's laboratory to isolate cDNA clones which apparently encode three distinct forms of this enzyme. Expression of one of these forms is confined to olfactory neurons. The expression of these forms of adenylyl cyclase in a mammalian expression system has revealed distinct biochemical properties for each of the membrane proteins. In addition, we are examining the structure and biochemical properties of adenylyl cyclase cloned from a variety of other eukaryotic organisms.

The molecular cloning approaches described here support the notion that novel components of a second messenger cascade, G_{olf} and olfactory-specific adenylyl cyclase, couple to as yet unidentified receptors and mediate olfaction. The extension of these studies to other signal transduction systems will likely shed some light on the function of these molecular components.

G 030 T CELL ANTIGEN RECEPTOR SIGNAL TRANSDUCTION MUTANTS, Arthur Weiss, Jim Fraser, Dev Desai and Mark Goldsmith. Departments of Medicine and of Microbiology and Immunology, Howard Hughes Medical Institute, University of California, San Francisco, Ca. 94143. The T cell antigen receptor (TCR) is a seven chain molecular complex involved in antigen recognition and signal transduction. The TCR activates two signal transduction pathways: the inositol phospholipid pathway and a tyrosine kinase pathway. In an effort to understand how the structure of the TCR relates to these signal transduction pathways, we have isolated a family of somatic cell mutants (J.CaM1-3) from the Jurkat T cell leukemic line which define three distinct complementation groups and are all defective in the TCR-stimulated inositol phospholipid pathway. One (J.CaM2) still is capable of activating the tyrosine kinase pathway. Whereas, all three mutants have grossly normal TCR proteins, J.CaM1 and J.CaM3 fail to associate with two recently identified ligand-induced TCR associated proteins, suggesting a proximal defect in these cells. Moreover, the human muscarinic receptor subtype 1, which activates the inositol phospholipid pathway, functions normally in all three mutants. These studies suggest that the mutations in all three of these cells are in elements of the inositol phospholipid pathway that are TCR specific. Finally, in addition to identifying components of the TCR signal transduction pathway, these mutants may prove to be useful in understanding to how the signal transduction events initiated by the TCR relate to cellular responses.

Sensory Signal Transduction

G 031 INTERACTION OF TRANSDUCIN WITH THE cGMP-PHOSPHODIESTERASE IN RETINAL RODS. Marc Chabre, Philippe Deterre* and T. Minh Vuong. Institut de Pharmacologie CNRS, Sophia Antipolis, F-O6560, France, and *Labo. de Biophysique Moléculaire et cellulaire, CENG, BP 85, 38041 Grenoble, France. After the catalysis by photoexcited rhodopsin of GDP/GTP exchange on transducin and release of the TαGTP subunits, the second stage of the phototransduction cascade is the activation by TαGTP of the cGMP-PDE. TαGTP interacts with a small inhibitory subunit (I) of the PDE, which maintained the basal PDE activity to a low level in the dark, and displaces it from the cGMP catalytic site. *In vitro*, the stoichiometric TαGTP-I complex can be detached from the activated PDE by low ionic strength extraction, but it is not excluded that *in situ* the complex remains loosely bound to the catalytic subunits PDEαβ. The native PDE complex is regulated by two copies of I, and is thus activated in two steps by two TαGTP. The two inhibitors may have different affinities for the PDE complex, but the two PDE catalytic sites that they uncover do not seem to differ much in their V_{max} nor in their K_m for cGMP. The activity of the intermediate state PDEαβ-I is half of that of fully activated PDEαβ. This precludes that inhibitor exchange between different states of activation: PDEαβ-I_2 + PDEαβ- ⇔ 2 PDEαβ-I contributes to the fast blocking of the light-induced PDE activity. The activation is terminated when the hydrolysis of GTP governs the return to the TαGDP conformation that has a low affinity for I and a higher one for Tβγ. I then returns to inhibit the PDE, and Tα binds back to Tβγ to regenerate native holotransducin. Recent microcalorimetric measurements demonstrate that in native rod membranes the hydrolysis of GTP in Tα is completed within a second of a flash activation. The interaction with the PDE inhibitor may be an accelerating factor for the GTP hydrolysis.

G 032 STRUCTURE-FUNCTION RELATIONSHIPS OF RETINAL ROD GTP-BINDING PROTEIN, G_t: SITE AND MECHANISM OF INTERACTION WITH PHOTOISOMERIZED RHODOPSIN. H. E. Hamm, Dept. of Physiol. and Biophys., Univ. of Ill. Coll. of Med., Chicago, IL 60612.

Rhodopsin photoisomerization (R*) leads to a conformational change on its cytoplasmic surface, allowing a high-affinity binding to and consequent activation of G_t. Interaction of G_t with R* catalyzes a conformational change in G_t resulting in the release of GDP from the nucleotide binding pocket of the α subunit (α_t) and a high-affinity interaction with R*. Subsequent uptake of GTP into the GTP binding pocket on α_t leads to the dissociation of the R^*G_t complex and release of activated α_t-GTP. To begin to understand these events which underlie the speed and amplification of visual excitation, the mode of interaction between the two proteins is being studied.

Recent studies have identified a region α_t-311-328 near the C-terminal of α_t, by monoclonal antibody blockade and epitope mapping studies[1], that is a part of the R* binding site on α_t. The role of this region in rhodopsin binding has been studied using synthetic peptides corresponding to this region as molecular decoys to map the R^*G_t interaction site in more detail[2]. A synthetic peptide, α_t-311-328, competitively blocks the light-activated R^*G_t interaction. A modified peptide, acetyl-311-329-NH_2, blocks with a higher potency, and at higher concentration causes stabilization of metarhodopsin II, mimicking the effect of G-protein binding. A peptide corresponding to the carboxyl terminal, α_t-340-350, similarly blocks and also mimics R^*G_t interaction. The binding of the two peptides is cooperative, suggesting that these two regions of α_t bind close together on rhodopsin's surface. The conformation of the bound peptides is being determined spectroscopically to give insight into the conformation of the receptor binding domain of G_t.

Studies in other signal transducing systems indicate that similar regions of homologous G proteins are important in interaction with their cognate receptors. Monoclonal antibodies that block R^*G_t interaction also block Rs-Gs and Ri-Gi interaction[3]. Synthetic peptides corresponding to the homologous sequences of Gs also block Rs-Gs interaction.

The dynamics of G_t activation subsequent to interaction with R* are being studied using a variety of biophysical approaches. Structural details of the GDP-bound R^*G_t complex suggest that at least one other region of G_t is important in R*G interaction.

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[2]Hamm et al., Sci. 241:832, 1988); [3]Hamm et al., J. Biol. Chem. 264:11475, 1989

G 033 THE cGMP-GATED CHANNEL IN PHOTORECEPTORS: PRIMARY STRUCTURE AND PHYSIOLOGICAL FUNCTION. U.B. Kaupp, Institut für Biologische Informationsverarbeitung, KFA Jülich, D-5170 Jülich, FRG

Photoreceptors respond to stimulation by light with a brief transient change in the membrane voltage. A decrease in the Na^+ permeability of the plasma membrane generates the electrical signal in vertebrate photoreceptors due to the closing of a *light-regulated* ion channel in the plasma membrane. This ion channel has been also designated *cGMP-gated channel* because cGMP, the internal messenger substance of visual transduction activates it directly without involving other enzymatic reactions, i.e. phosphorylation by a cGMP-dependent protein kinase (cGPK).

The cGMP-gated channel does not discriminate very well between alkali ions and even allows the passage of Ca^{2+} and Mg^{2+}. The single-channel conductance in physiological saline is ~ 25 pS, but decreases significantly in the presence of divalent cations. One interpretation of this blocking effect implicates that Ca^{2+} or Mg^{2+} bind to a high affinity site inside the channel, thereby inpeding the flow of other, more conductive ions.

The isolated channel protein consists of a single polypeptide of M_r 63,000 which is functional when reconstituted into phospholipid vesicles or artificial planar bilayers.

The complete amino-acid sequence of the cGMP-gated channel has been deduced by cloning DNA complementary to bovine mRNA coding for the 63 K polypeptide. Similarity matrix analysis of the cGMP-gated channel detects no extensive amino-acid sequence similarity to other ionic channels, membrane transporters and G-protein-coupled receptors. A hydrophobicity plot reveals 6 hydrophobic domains which may represent transmembrane segments. By analogy with other channels of known sequence, we favour the view that the cGMP-gated channel has either six (like the potassium channel) or four transmembrane segments (like subunits of neurotransmitter-gated ionic channels). A segment of approximately 80 amino acids at the C-terminal end exhibits significant sequence similarity with the proposed binding sites for cGMP in cGPK. The channel is cooperatively activated by at least three probably more cGMP molecules (Hill coefficient n > 3). cGPK which is cooperatively stimulated by two cGMP molecules per monomer contains two tandem homologous cGMP-binding regions, whereas the cGMP-gated channel contains only one such region. This finding suggests that the cGMP-gated channel is a homooligomeric complex, each constituent polypeptide having a single cGMP-binding site.

cGMP-stimulated channel activity has been expressed in *Xenopus* oocytes by injection of mRNA derived from cloned cDNA. The properties of the expressed channel are similar to those observed *in situ*. These experiments suggest that the 63 K polypeptide alone is sufficient to form the functional channel.

G 034 SIGNAL-PROCESSING OLFACTORY-SPECIFIC ENZYMES: FROM G_s TO GLUCURONYL TRANSFERASE, Doron Lancet, Department of Membrane Research, The Weizmann Institute of Science, Rehovot, 76100 Israel, and the Laboratory of Developmental Pharmacology, National Institutes of Health, Bethesda, Maryland 20892.

Olfactory signal generation has been shown to involve a G_s-protein, adenylate cyclase (AC) and cation channels directly gated by cAMP. The olfactory G_s-protein has recently been cloned (1,2) and shown to be a new type (G_{olf} or G_{s2}) uniquely expressed in olfactory neurous. AC_{olf} is also an olfactory-unique species, corresponding to a 180kDa polypeptide, different from AC in other tissues (3). The molar concentration of this enzyme is 100 fold higher in olfactory cilia compared to brain and myocardium. The molecular nature of olfactory cAMP channels remains to be elucidated, but it is possible that they resemble the visual photoreceptor cGMP-gated channels.

The molecular mechanisms of olfactory signal termination remain elusive. We recently discovered and subjected to molecular cloning two highly enriched and olfactory-specific forms of biotransformation enzymes. These are olfactory cytochrome P-450 (P-450olf1) and olfactory UDP-glucuronyl transferase ($UDPGT_{olf}$) (4,5). They are localized in secretory cells of olfactory neuroepithelium. We propose a model, whereby these enzymes catalyze rapid conversion of lipophilic, membrane penetrable odorants, that may partition into and linger in cells throughout the tissue, to secretable, hydrophilic glucuronates. An array of such broadly specific biotransformation enzymes could thus allow efficient termination of diverse chemosensory signals.

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G 035 SENSORY TRANSDUCTION IN THE Drosophila EYE, Randall D. Shortridge, Jaeseung Yoon, Brian T. Bloomquist, Stephan Schneuwly, and William L. Pak, Department of Biological Sciences, Purdue University, W. Lafayette, IN 47906. To study phototransduction, we use a combination of various techniques to analyze Drosophila mutants expressly isolated for the purpose. The basic strategy is to isolate mutations that affect phototransduction, identify the genes in which the mutations have occurred, clone the genes, and analyze them in detail. The first gene to yield to this approach was the ninaE gene encoding opsin in the majority class of photoreceptors, R1-6 (O'Tousa et al., 1985; Zuker et al., 1985). More recently, another gene, norpA, was isolated using the same basic approach (Bloomquist et al., 1988). Strong alleles of norpA have long been known to abolish the photoreceptor potential (Pak et al., 1970; Hotta and Benzer, 1970), rendering the fly homozygous for the mutant gene blind. Molecular analysis of the gene revealed that it encodes a photoreceptor-specific phospholipase C (PLC), providing critical evidence that PLC is an important component of phototransduction cascade in Drosophila. In addition to cloning genes that are identified by mutations, we have utilized low-stringency hybridization techniques to isolate other members of the gene families to which the norpA protein and other known components of phototransduction cascades belong. Among the genes isolated by this approach are one that encodes another member of the PLC family and one encoding a G protein α subunit exhibiting strong sequence similarity to mammalian $G_o\alpha$ (Yoon et al., 1989).

Regulation of Ionic Channels

G 036 G-PROTEIN ACTIVATION OF EPITHELIAL Na^+ CHANNELS IS MEDIATED VIA PHOSPHOLIPID METABOLITES, Dennis A. Ausiello, Charles R. Patenaude, and Horacio F. Cantiello. Renal Unit, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts, 02114.

The ability of G proteins to regulate epithelial, amiloride-sensitive Na^+ channels on the apical membrane of the epithelia cell line, A6, has recently been demonstrated in our laboratory using the patch-clamp technique. Na^+ channels were studied in excised inside-out patches. Single-channel activity, expressed as the % of total open time, was reduced by 85% after 1 min perfusion with pertussis toxin (PTX) and NAD (46±9 (7) vs 7±2 (5), $p<0.001$) and declined to zero in ~ 3 min. The single-channel conductance remained constant at 9.35±2.35 (7) vs 8.84±2.19 (5) pS. In contrast, GTP (10^{-4} M) increased spontaneous channel activity from 6% to 50% of open time. GDPβS decreased spontaneous channel activity by 84% (44±14 (3) vs 7±4 (5), $p<0.025$), also without effect on the ionic conductance. The purified, GTPγS-activated human α_i-3 subunit (20 pM), reversed the effect of PTX or GDPβS: 32±13 (3) % open time as compared to the control in the presence of PTX or GDPβS, 0%, $p<0.05$. This is in contrast to the lack of effect of the purified, activated α_i-2 subunit. ATP, cAMP, and phorbol esters had no direct effect on the activation process induced by G-proteins.

G-protein activation of phospholipase (PL) as a means of modulating this Na^+ channel was determined. In patches where spontaneous Na^+ channel activity was eliminated by PTX, the PL agonist, mellitin (1mg/ml), increased % open time of Na^+ channel activity in 5/6 patches from 0 to 37±16 (5), $p<0.01$. In contrast, mepacrine (10 μM), a PL antagonist, decreased either spontaneous or mellitin-stimulated Na^+ channel activity from 45±15 to 8±4 (4) % open time, $p<0.025$. Neither GTPγS nor α_i-3 were capable of stimulating the Na^+ channel in the presence of mepacrine. The addition of arachidonic acid (AA) in the presence of mepacrine and/or PTX increased Na^+ channel activity from 0 to 26±7 (6) % open time. The effect of AA was reversed by the 5-lipoxygenase inhibitor, NDGA, whose inhibition in turn could be overcome by the leukotriene precursor, 5-S-HPETE (100 nM). Na^+ channel stimulation was also accomplished by the direct addition of the leukotriene, LTD_4, in the presence of PTX or NDGA, thus suggesting that the apical Na^+ channel is regulated by G protein modulation of phospholipase(s) via the 5-lipoxygenase pathway and its metabolites.

G 037 REGULATION OF IONIC CHANNELS BY G PROTEINS, A.M. Brown and L. Birnbaumer, Department of Molecular Physiology and Biophysics, Baylor College of Medicine, Houston, Texas 77030. Ionic channels are subject to a number of acute regulatory processes. A process that has only recently been elucidated is membrane-delimited or direct; it uses signal transducing, guanine nucleotide binding (G) proteins as activators or modulators. Direct gating of ionic channels by G proteins is widespread and must be distinguished from another form of regulation by G proteins which is indirect and uses cytoplasmic second messengers. The simplest way to make this distinction is to reconstitute G protein effects in cell-free membrane systems containing the ionic channel of interest and possibly the receptor-G protein apparatus, too. Using recombinant α subunits expressed in bacteria, we established that G_s had at least two effectors, adenylyl cyclase (AC) and Ca^{2+} channels, some G_is probably have at least two effectors, and α_is are not specific for the K^+[ACh] channel. Direct G protein gating may be obligatory, i.e. necessary and sufficient for channel opening, or modulatory, i.e. neither necessary nor sufficient for channel opening. In heart, the G protein G_k is obligatory for the atrial muscarinic K^+ channel (K^+[ACh]) which slows heart rate and the G proteins G_s and G_o are modulatory for the hyperpolarization-activated cardiac pacemaker current, I_f. An interesting question is the role of the dual direct and indirect modulatory G protein pathways for cardiac Ca^{2+} channels. Theory predicts and experiments confirm that the direct pathway is much faster. In fact, it operates as the direct G_k-K^+[ACh] channel pathway. The direct pathways can explain second-to-second neural regulation of heart rate.

G 038 MOLECULAR PROPERTIES OF VOLTAGE SENSITIVE Na^+ AND Ca^{++} CHANNELS. Catterall, William A., Department of Pharmacology, University of Washington, Seattle, WA 98195.

Electrical signals in the form of transmembrane ionic currents are important regulators of cellular function. The voltage-gated ion channels include the Na+ channels, Ca^{++} channels, and K^+ channels. Voltage-sensitive Na^+ and Ca^{++} channels mediate inward movements of Na^+ and Ca^{++} that are responsible for the depolarizing phase of the action potential in excitable cells, and Ca^{++} moving into the cell through Ca^{++} channels acts as a signal for multiple intracellular events. Activation of K^+ channels terminates the action potential and hyperpolarizes the cell. Na^+ channels isolated in functional form from mammalian brain consist of a complex of an α subunit (260 kDa) in association with a $\beta 1$ subunit (36 kDa) and disulfide-bonded to a $\beta 2$ subunit (33 kDa). The α subunit is common to Na^+ channels from all tissues that have been studied, and mRNA encoding it is sufficient to direct the synthesis of functional sodium channels in *Xenopus* oocytes, and mammalian cells. Ca^{++} channels isolated from skeletal muscle transverse tubules consist of an α_1 subunit (175 kDa), which is similar is primary structure to the α subunit of the Na^+ channel, can form a functional voltage-gated channel, and contains the receptor sites for calcium channel modulators. It is associated with a disulfide-linked glycoprotein complex of α (143 kDa) and δ (27 kDa), β (54 kDa) and γ (30 kDa).

The homologous primary structures of the α subunit of the Na^+ channel and the α_1 subunit of the Ca^{++} channel contain 4 repeated homologous domains containing multiple transmembrane segments. Site-directed, antipeptide antibodies have been used to probe the transmembrane organization of these proteins, and identify separate sites on the Na^+ channel at which cAMP-dependent phosphorylation, binding of α-scorpion toxins, and the binding of specific antibodies modulate rapid Na^+ channel inactivation. Highly conserved S4 α-helical segments in each domain of all members of the ligand-gated channel family are both hydrophobic and positively charged and are postulated to traverse the membrane and form the voltage-sensing elements of the channels according to a "Sliding Helix" model of voltage-dependent gating. These studies begin the development of a functional and topological map for the principal subunit of the voltage-sensitive ion channels.

G 039 GTP-BINDING PROTEIN-MEDIATED INHIBITION OF NEURONAL CALCIUM CHANNELS AND PEPTIDE SECRETION. George G. Holz IV, Department of Physiology, Tufts University School of Medicine, 136 Harrison Avenue, Boston, MA 02111.

Presynaptic inhibition of excitation-secretion coupling in the spinal dorsal horn results from activation of α_2-adrenergic and $GABA_b$ receptors located on terminal arborizations of peripheral sensory neurons. In a subset of these neurons, receptor occupancy results in a suppression of depolarization-induced calcium influx through ω-conotoxin-sensitive calcium channels, thereby blocking calcium-dependent exocytosis of substance P.[1] Patch clamp analysis (whole-cell configuration) and release experiments (radioimmunoassay for substance P) of cultured peripheral sensory neurons demonstrated that both types of presynaptic inhibitory response are blocked either by intracellular application of GDP-*B*-S or by prior exposure to *Bordetella pertussis* toxin (PTX).[2,3] The action of PTX is associated with ADP-ribosylation of substrates migrating as a doublet of M_r 39-41 kDa on SDS-PAGE (1-D gel electrophoresis). Isoelectric focusing combined with SDS-PAGE (2-D gel electrophoresis) demonstrates that this doublet is resolvable as four distinct spots with pI of ca. 5-6. A role for G_i and/or G_o proteins as mediators of presynaptic inhibition is confirmed by immunoblot analysis using affinity-purified antisera directed against synthetic peptides corresponding to amino acid sequences predicted from cDNAs for PTX-sensitive G protein α-subunits (AS/7, $T_{1\alpha}$ 341-350 and GO/1, $G_{o\alpha}$ 345-354). In Lubrol-solubilized homogenates these antisera specifically immunoprecipitate α-subunits radiolabeled with either ^{32}P-ADP-ribose or ^{35}S-GTP-γ-S, thereby allowing an assessment of the molecular nature of G protein-effector coupling. Subcellular fractionation demonstrates that these ribosylatable, immunoreactive G proteins are enriched in synaptosomes (ca. 62% of total), whereas sucrose density gradient centrifugation of lysed synaptosomal homogenates indicates that G_i and/or G_o are included not only in the plasma membrane fraction (15% of total), but also in the vesicular fractions (35% of total). This subcellular distribution of PTX-sensitive G protein α-subunits is most compatible with an important presynaptic inhibitory role for G_i and G_o as regulators of peptide secretion not only at the level of calcium channel modulation, but also at steps subsequent to the entry of calcium ions across the plasma membrane.

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G 040 PARTICIPATION OF PERTUSSIS TOXIN-SENSITIVE G-PROTEINS IN THE INHIBITION AND STIMULATION OF VOLTAGE-DEPENDENT Ca^{2+} CURRENTS BY HORMONES AND NEUROTRANSMITTERS, Walter Rosenthal, Jürgen Hescheler, Stefan Offermanns, Anke Schmidt, Klaus-Dieter Hinsch, Karsten Spicher and Günter Schultz, Institut für Pharmakologie, Freie Universität Berlin, D-1000 Berlin.

We examined the possible involvement of pertussis toxin-sensitive G-proteins in the modulation of voltage-dependent Ca^{2+} currents by receptor agonists. Applying the whole-cell clamp technique, we observed an inhibitory Ca^{2+} current modulation in neuroblastoma x glioma hybrid (NG) cells, a pituitary cell line (GH_3) and an insulin-producing cell line (RINm5F). Inhibitory receptor agonists included the synthetic opioid, D-Ala^2, D-Leu^5-enkephalin (DADLE; NG cells), somatostatin (NG and GH_3 cells) and adrenaline (NG and RINm5F cells). Inhibition of Ca^{2+} currents in NG and GH_3 cells was abolished by treatment of cells with the main exotoxin of *Bordetella pertussis* (pertussis toxin). The inhibition of NG cell Ca^{2+} currents by intracellular application of the GTP analog, guanosine 5'-O-(3-thiotriphosphate) was promoted by extracellular application of DADLE; intracellular application of the GDP analog, guanosine 5'-O-(2-thiodiphosphate) rendered NG cells insensitive to DADLE. By the use of highly specific antisera raised against synthetic peptides, the G-protein, G_o, and G-proteins of the G_i family were identified in membranes of NG, GH_3 and RINm5F cells. Inhibitory agonists stimulated incorporation of the GTP-analogue, [α-^{32}P]GTP azidoanilide, into 39 and 40 kDa proteins comigrating with the α-subunits of the G-proteins, G_o and G_{i2}, respectively, in membranes from all three cell types. In NG cells, the rank orders of potency of receptor agonists with regard to Ca^{2+} current inhibition and photolabeling of the 39 kDa protein were identical (DADLE > somatostatin > adrenaline » bradykinin). In contrast, the rank order of potency of receptor agonists for photolabeling of the 40 kDa protein (DADLE > somatostatin = adrenaline = bradykinin) did not match with that for Ca^{2+} current inhibition. The inhibition of Ca^{2+} currents in pertussis toxin-treated NG cells by DADLE was restored by intracellular infusion of purified G-proteins (G_i-type G-proteins and G_o) among which the α-subunit of G_o exhibited the greatest potency. The data suggest that G_o functionally couples receptors for inhibitory agonists and voltage-dependent Ca^{2+} channels in neuronal and certain endocrine cells. In the adrenocortical cell line, Y1, angiotensin II stimulated voltage-dependent Ca^{2+} currents. This stimulatory effect was not observed in pertussis toxin-treated cells. Membranes of Y1 cells were found to be devoid of G_o but possessed G_i-type G-proteins, i.e. G_{i2} and another G_i subtype, possibly G_{i3}. The data suggest that a G_i-type G-protein is involved in the pertussis toxin-sensitive stimulation of voltage-dependent Ca^{2+} currents. This may also apply to GH_3 cells in which LHRH stimulates Ca^{2+} currents. Like the inhibition of Ca^{2+} currents by somatostatin, the stimulation of Ca^{2+} currents by LHRH was abolished by treatment of cells with pertussis toxin. In membranes from GH_3 cells, not only the inhibitory agonist, somatostatin, but also the stimulatory agonists, LHRH and TRH, stimulated a high affinity-GTPase; the effects on GTPase activity of LHRH and TRH were reduced and that of somatostatin was abolished in membranes prepared from cells treated with pertussis toxin. Thus, receptor agonists may stimulate and inhibit voltage-dependent Ca^{2+} channels within one cell type by distinct pertussis toxin-sensitive G-proteins. So far, intracellular signal molecules involved in the pertussis toxin-sensitive modulations of voltage-dependent Ca^{2+}-channels have not been identified. It is therefore conceivable that pertussis toxin-sensitive G-proteins may control the activity of voltage-dependent Ca^{2+} channels by a membrane-confined mechanism.

Structure-Function Analysis of G-Protein Coupled Receptors

G 041 ANALYSIS OF LIGAND BINDING TO G-PROTEIN COUPLED RECEPTORS THROUGH THE USE OF ENGINEERED RECEPTOR PROTEINS, Richard A.F. Dixon and Catherine D. Strader, Departments of Molecular Biology and Biochemistry, Merck, Sharp and Dohme Research Laboratories, West Point, PA 19486 and Rahway, NJ 07065.

The cloning of many small molecule and peptide hormone receptors which couple to G-proteins has revealed that these receptors have common structural features including the presence of seven putative transmembrane segments. A combination of genetic and biochemical studies have demonstrated that the ligand binding site of the beta-adrenergic receptor (BAR) for small molecule agonists and antagonists is contained within these transmembrane domains. Site directed mutagenesis studies of the BAR coupled with structure-activity studies of novel ligands have allowed the identification of several residues within the BAR which are important for ligand recognition and binding. In addition, hybrid receptor proteins have been utilized to identify regions of the receptor important for subtype determination. These studies have led to the construction of a model for the ligand binding site of the BAR.

G 042 THE LUTROPIN/CHORIOGONADOTRIN RECEPTOR (LH/CG-R). Deborah L. Segaloff, The Population Council, New York, NY 20021.

The LH/CG-R, present in gonadal tissues, recognizes both the pituitary hormone LH and the homologous placental hormone hCG. LH and hCG are large (28 and 38 kDa) glycoproteins related to two other pituitary hormones (TSH and FSH), all of which are composed of an identical alpha subunit and a distinct beta subunit. Upon binding either hCG or LH, the LH/CG-R activates adenylyl cyclase, which ultimately leads to an increase in steroid biosynthesis in the target cells.

From biochemical studies on the LH/CG-R, we had previously determined that this receptor is a single polypeptide with a molecular weight (on SDS gels) of 93,000 and that it is glycosylated. Using amino acid sequences determined from purified rat luteal LH/CG-R, we have recently cloned the cDNA for this receptor. The results of these studies suggest that the LH/CG-R is composed of a large (341 amino acids) N-terminal extracellular domain (which is presumably involved in ligand binding) attached to a region that is homologous to other receptors known to couple to G proteins and thought to transverse the plasma membrane seven times. The intracellular loops and the C-terminal cytoplasmic tail contain numerous potential phosphorylation sites.

Expression of the LH/CG-R cDNA in 293 cells results in expression of a cell surface receptor that specifically binds hCG or oLH with high affinity and activates adenylyl cyclase when occupied. Furthermore, the expressed receptor shows other properties characteristic of the native LH/CG-R, such as hCG and phorbol ester induced desensitization of hCG-stimulated adenylyl cyclase activity, receptor-mediated internalization of hCG, and hCG-induced down-regulation of receptor numbers. These studies indicate that the cDNA isolated encodes for a fully functional LH/CG-R and allows for future studies on the elucidation of the structure/function relationships of this receptor.

G 043 MOLECULAR MECHANISMS OF RECEPTOR DESENSITIZATION AND INTERNALIZATION IN YEAST. K.J. Blumer, W.E. Courchesne, H.G. Dohlman, D. Kaim and J. Thorner, Division of Biochemistry and Molecular Biology, University of California, Berkeley, CA 94720.

The α-factor pheromone of Saccharomyces cerevisiae is a 13-residue peptide that exerts its effects on its target cells (MATa haploids), which include arrest of the cell cycle at the G1 phase, by binding to a plasma membrane receptor encoded by the STE2 gene. STE2 protein has 7 transmembrane helices and a Ser/Thr-rich cytosolic tail that is hyperphosphorylated rapidly after ligand binding. The receptor is thought to interact with a set of proteins encoded by the GPA1, STE4, and STE18 genes, which resemble in sequence the α, β, and γ subunits, respectively, of mammalian G proteins.

MATa cells arrested by α-factor eventually resume growth, even when pheromone is still present. The rate of this recovery or adaptation can be influenced in several ways. Truncations of the STE2 C-terminal tail retard recovery; overexpression of GPA1 product promotes recovery. Cells expressing a complete receptor tail deletion (ste2Δ296) are unable to recover. The recovery defect manifest by the ste2Δ296 mutant receptors is almost completely suppressed by coexpression in the same cells of wild-type STE2 receptors. The recovery defect of ste2Δ296 can be partially suppressed by production in the same cells of an independent tail mini-protein targeted to the plasma membrane (because it contains an N-terminal consensus sequence for myristoylation, derived from $pp60^{v\text{-}src}$), but not by a version of the tail mini-protein lacking the myristoylation site. In contrast, overproduction of GPA1 product does not promote recovery of the ste2Δ296 mutant cells. These results suggest that in some way the receptor tail in the membrane and the GPA1 product interact to regulate the rate of receptor desensitization. Unlike normal STE2 receptors, the ste2Δ296 mutant receptors do not display ligand-induced endocytosis upon α-factor binding. Deletion analysis indicates that the signals within the C-terminal tail for internalization are redundant. Site-directed mutagenesis is being used to pinpoint the residues essential for endocytosis. Heterologous probes are being used to determine if yeast cells possess homologs of arrestin and kinases that are specific for the agonist-occupied or activated forms of mammalian receptors.

In vitro assays have been developed that demonstrate GTP-dependent coupling between the STE2 protein and the GPA1 product. Treatment of partially purified membranes with GTPγS converts receptor from a high affinity state (K_d= 7 nM) to a low affinity state (K_d= 80 nM), as judged by the rate of ligand dissociation, equilibrium binding, or by antagonist competition. Use of gpa1, ste4, and ste18 mutations indicates that all three subunits of the G protein are required for functional coupling in vivo. Supported by NIH grant GM21841 to J.T.

Heterotrimeric G-Proteins, their Receptors and Effectors; Small Molecular Weight G-Proteins

G 100 CARBOXYL-METHYLATION OF THE BRAIN G-PROTEIN GAMMA SUBUNIT. Peter S. Backlund, Jr.*, William F. Simonds**, and Allen M. Spiegel**. *Natl. Inst. of Mental Health, and **Natl. Inst. of Diabetes and Digestive and Kidney Diseases, Natl. Inst. of Health, Bethesda, MD 20892.
The enzymatic methylation of the G-protein gamma subunit was investigated in brain membranes. Rabbit brain membranes were incubated with [^{3}H-methyl]-AdoMet, and the G-protein βγ-complex was isolated using an antisera directed against the β-subunit to assay for the βγ-complex throughout the purification. The isolated βγ-complex was carboxyl-methylated specifically on the γ-subunit. The methylated βγ-complex was demonstrated to bind to the α-subunit of transducin, in a fluoride dependent manner. Posttranslational modification of the ras protein has previously been shown to involve removal of three amino acids at the C-terminal and carboxyl-methylation of the resulting C-terminal cysteine. The processing of the C-terminal of ras appears to be necessary for the association of ras with the membrane. The structural similarity of the C-terminal of ras and the G-protein γ-subunit suggests that similar modifications may occur with the G-protein γ-subunit. The carboxyl-methylation of the G-protein γ-subunit reported here may represent one of the posttranslational modifications of the C-terminal of the G-protein γ-subunit in brain, and may be involved with anchoring the G-proteins to the membrane.

G 101 A NOVEL ADENYLYL CYCLASE MAY MEDIATE ODORANT DETECTION Heather A. Bakalyar and Randall R. Reed, Dept. of Molecular Biology and Genetics, Johns Hopkins School of Medicine, Baltimore, MD 21205

Previous biochemical studies have demonstrated that certain odorants stimulate a GTP dependent increase of cAMP levels in olfactory neuronal cilia. Our laboratory has identified a novel $G_{s\alpha}$ subunit termed "$G_{olf\alpha}$" expressed exclusively in olfactory sensory neurons (Science vol 244, pp790-795). Recently, in a collaborative effort we have cloned a bovine brain adenylyl cyclase (type I, Science vol 244, pp1558-1564). Low stringency hybridization of a rat olfactory cDNA library with the type I clone revealed the existence of a distinct form of cyclase, adenylyl cyclase type III. The two cyclases share considerable amino acid similarity and each has a primary protein sequence which predicts 12 membrane spanning domains. Expression of the type III protein in a human kidney cell line results in increased cyclase activity in response to both forskolin and AlF_4^-. Northern analysis indicates that type III mRNA transcripts are confined to the primary sensory neurons of the olfactory epithelium. Thus, like G_{olf}, this olfactory specific adenylyl cyclase appears to be a component in a signal transductory cascade specialized for odorant detection.

G 102 EFFECT OF GLUCOCORTICOID ON GUANINE NUCLEOTIDE BINDING PROTEIN EXPRESSION IN THE RAT KIDNEY Peter Brecher, Fred Mamuya, and Kelvin Lam, Department of Biochemistry, Boston University School of Medicine, Boston, MA 02118
To determine if adrenal steroids have a role in regulating G protein expression in the kidney, northern blot analysis were performed on total renal RNA using specific cDNA probes for the alpha subunits of 4 different G proteins. Adrenalectomy (Adx) markedly decreased steady-state mRNA levels for Gia1 and Gia3, but had essentially no effect on Gsa or Gia2 mRNA levels. Administration of dexamethasone by Alzet minipumps to Adx rats prevented the Adx-induced changes in G protein mRNA levels whereas aldosterone had no effect. Rats made hypertensive by deoxycorticosterone/salt treatment had changes in G-protein mRNA levels similar to those observed during adrenalectomy. Pertussis toxin catalyzed ADP-ribosylation in renal membrane fractions were performed to determine if changes in protein levels concommitant with those of mRNA could be documented. No detectable change in pertussis-induced ADP-ribosylation of Gi was found following either adrenalectomy or DOC/salt induced hypertension. The data suggest that glucocorticoids influence the expression of at least 2 genes in the G-protein family, possibly at the level of transcription. It also appears likely that at least a portion of renal Gia1 is insensitive to pertussis toxin catalyzed ADP-ribosylation.

G 103 STRUCTURE-FUNCTION STUDIES OF HUMAN β_2-ADRENERGIC RECEPTOR EXPRESSED IN *ESCHERICHIA COLI*. Richard M. Breyer, A. Donny Strosberg, and Jean-Gérard Guillet. Laboratoire de Biologie Moléculaire des Récepteurs, Institut Pasteur 75725 Paris France. Human β_2-adrenergic receptor (β_2AR) is an integral membrane protein which binds catecholamines. We are interested in understanding the structure of the β_2AR in the membrane and the molecular basis of its ligand binding properties. Our laboratory has developed a number of vectors to express functional adrenergic receptors in *E. coli*. These constructs have fused the β_2AR gene to *lac*Z, *mal*E and *lam*B genes which encode cytoplasmic, periplasmic and outer membrane proteins respectively. For structure-function studies of the receptor it would be advantageous to express the receptor protein without fusion to a heterologous protein. We therefore have cloned the native human β_2AR gene under transcriptional control of the bacteriophage T7 promoter. *E. coli* expressing the β_2AR protein bind the radiolabeled ligand ^{125}I cyanopindolol (ICYP) with high affinity (K_D = 3 pM). ICYP binding can be competitively displaced by a number of β_2AR agonists and antagonists with typical order of affinity. Expression of functional β_2AR in *E. coli* colonies can be detected by binding of radiolabeled ligand. This system will provide a method for rapidly screening receptor variants for ligand binding activity.

G 104 INDIVIDUAL MEMBERS OF THE G_i AND G_o SUBFAMILIES HAVE DISTINCT GUANINE NUCLEOTIDE BINDING PROPERTIES. Donna J. Carty, Elena Padrell, and Ravi Iyengar. Department of Pharmacology, Mount Sinai School of Medicine, NY, NY 10029. Individual members of G-protein subfamilies were resolved on FPLC, and sequence-specific antisera were used to characterize the proteins and to ensure the purity of the preparations. Thus three forms of G_i and two forms of G_o were isolated. All three forms of G_i were capable of activating the atrial K^+ channels and both forms of G_o were capable of specifically increasing muscarinic stimulation of the IP_3-mediated Cl^- current in *Xenopus* oocytes. However, the individual G-proteins showed distinct guanine nucleotide binding properties. Both members of the G_o subfamily bound GTPγS faster than the three G_i proteins. When no added Mg^{2+} was present , G_{o1} bound GTPγS twofold faster than G_{o2}. G_{o1} reached steady state binding within two minutes and G_{o2} in three minutes. At 3 mM Mg^{2+}, both G_{o1} and G_{o2} bound GTPγS at similar fast rates, reaching steady state within 1.5 minutes. At 3 mM Mg^{2+}, G_{i2} bound GTPγS 5 to 7 fold faster than G_{i1} and G_{i3}, and G_{i1} bound GTPγS twofold faster than G_{i3}. G_{i1}, G_{i2}, and G_{i3}, however, did not reach steady state binding until 65, 20, and 80 minutes respectively. Rates of GDP release also differed among members of the G-protein sub-families. Differences in the rates of GDP release generally were similar to the rates of GTPγS binding. These data indicate that rates of GTP binding and GDP release are intrinsic properties of the individual G_i or G_o proteins. Since members of the sub-families have similar capabilities to interact with receptors and effectors, the relative proportions of G-protein subfamily members in a cell will be a crucial factor in determining the kinetics and extent of signal transduction through G-protein-coupled receptors.

G 105 **Production of Go Alpha Subunit in Insect Cells using Baculovirus Vector.** M. Dennis, J. Labrecque, M. Caron and K. Torossian. Biotechnology Research Institute 6100 Royalmount Ave. Montreal, Quebec H4P 2R2.
The heterotrimeric G protein Go has been implicated in signal transduction between numerous receptors and effectors in mammalian cells. In order to examine the structure and function of this protein, we have established a high-level expression system for th α subunit (αo) using a baculovirus vector and insect host cells. The coding segment of rat αo cDNA was inserted into the transfer vector IpDc126, flanked by promoter and 3' sequences of the viral polyhedrin gene. Following co-transfection into SF9 insect cells of plasmid and wild type viral DNA, recombinant viruses harboring αo inserts were isolated by successive plaque purifications using hybridization and visual screening. Cultures of SF9 cells infected in suspension with αo recombinant (5moi) expressed a ca. 40kd protein reactive with polyclonal anti-αo antisera. Immunoreactive material was present in both membrane and soluble fractions following cell lysis. Supernatants showed high levels (> 0.15nmol/mg protein) of GTPγ S^{35} binding over wild type controls. Work is in progress to purify the soluble, active material for further characterization.

G 106 OVERPRODUCTION OF THE YEAST STE12 PROTEIN LEADS TO CONSTITUTIVE PHEROMONE RESPONSE, Joseph W. Dolan and Stanley Fields, Department of Microbiology, State University of New York at Stony Brook, Stony Brook, New York 11794. Haploid **a** and α cells of the yeast Saccharomyces cerevisiae respond to the pheromones α- and **a**-factor, respectively, by triggering the dissociation of a receptor-coupled G protein. This response is dependent on the activity of several genes, including STE12, whose product binds to the pheromone response element located within the regulatory DNA sequences of inducible genes. We have assayed the effects of overproducing the STE12 protein in both STE^+ cells as well as cells carrying mutations in several other STE genes. We find that overproduction leads to the increased transcription of pheromone-inducible genes and to the cell cycle arrest and morphological changes associated with pheromone treatment, similar to the effect of previously characterized mutations in subunits of the pheromone-responsive G protein. In addition, this overproduction is able to suppress the mating defect caused by mutations in several other components of the pheromone response pathway. These results suggest that the observed responses of yeast cells to pheromone is due to transcriptional induction mediated by the STE12 protein.

G 107 ISOLATION AND CHARACTERIZATION OF THE PORCINE G PROTEIN $\alpha_{i\text{-}3}$ SUBUNIT GENE PROMOTER, Louis Ercolani, Brian W. Soper, Dennis A. Ausiello, and Eliezer J. Holtzman, Renal Unit, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114
We have shown that the G protein $\alpha_{i\text{-}3}$ subunit is developmentally expressed in renal porcine LLC-PK_1 cells and modulates amiloride-sensitive Na^+ conductance. Our group has recently demonstrated that the activated $\alpha_{i\text{-}3}$ subunit can directly modulate apically located Na^+ channels in the epithelial cell line A6. To understand $\alpha_{i\text{-}3}$ subunit expression, DNA segments required for its transcriptional regulation were isolated from an EMBL-3 porcine genomic library. The 5′ flanking porcine DNA segment displayed a 71%, and 81% sequence identity to the human $\alpha_{i\text{-}3}$ gene and the rat olfactory $\alpha_{i\text{-}3}$ mRNA, respectively. No typical TATA-like sequences were found. However, several "CAAT" boxes and "GC" boxes were identified as potential cis acting control elements in the 5′ flanking area of the porcine gene. To determine the requisite regulatory sequences, 5′-flanking DNA segments of 0.3 kb and 4 kb were ligated in both sense and antisense orientations to a plasmid that contained a firefly luciferase gene but lacked a promoter. These plasmids were transiently transfected into LLC-PK_1 cells. Only transfectants with the 0.3 kb and the 4 kb of putative sense 5′-flanking sequence displayed an increase in luciferase activity as compared to cells transfected with the promoterless plasmid. Transcription of the sense 0.3 kb 5′-flanking chimeric gene was found to be correctly initiated from its $\alpha_{i\text{-}2}$ promoter. The minimal promoter was found within 300 bases upstream of the ATG translation start site. These findings are the first to demonstrate the minimal DNA segments of the $\alpha_{i\text{-}3}$ subunit gene necessary for its basal transcription in LLC-PK_1 cells.

G 108 ISOLATION OF THE GRP RECEPTOR FROM SWISS 3T3 FIBROBLASTS, Richard I. Feldman, James M. Wu, Elaina Mann and James C. Jenson, Triton Biosciences Inc., 1501 Harbor Bay Pkw., Alameda CA 94501. Previous observations have indicated that the GRP receptor in Swiss 3T3 cells is G-protein coupled. We have characterized the binding of ^{125}I-GRP to Swiss 3T3 cell membranes and have confirmed the role of G-proteins in ligand binding to the receptor. GMPPNP, GTP, and GDP, but not adenyl nucleotides were able to convert the receptor from a high affinity form to a low affinity form. Ligand dissociation kinetics indicated that the two forms differ in affinity by about 10 fold. The GMPPNP promoted conversion of the receptor to the low affinity form does not require Mg^{2+}.

The GRP receptor was solubilized from Swiss 3T3 membranes by the detergent CHAPS in a form that bound GRP with about the same affinity as the receptor in membranes. ^{125}I-GRP binding to the soluble receptor was competed by a series of GRP-related peptides with potencies similar to those observed with the membrane bound form of the receptor. The ligand affinity of the receptor in the solubilized extracts was unaffected by guanyl nucleotides indicating that it has lost the ability to couple to G proteins. In addition, the receptor was purified to homogeneity via a combination of lectin and ligand affinity chromatography. No significant change in the receptor's ligand binding characteristics occurred as a result of purification. The purified receptor exhibited a single band on SDS PAGE with a molecular weight of about 75-100 kD, in agreement with studies crosslinking ^{125}I-GRP to the receptor in both whole cells and isolated membranes. Isolation of the GRP receptor in an active form is an important step towards identifying accessory proteins involved in its signal transduction pathway by reconstitution of purified components.

G 109 INTERACTION OF PURIFIED SUBUNITS OF G-PROTEINS MEASURED BY FLUORESCENCE ENERGY TRANSFER. Marga Fröhlich, Helmuth Heithier, Martin Baumann, Christian Dees and Mirko Hekman, Department of Physiological Chemistry, University of Würzburg Medical School, D-8700 Würzburg, Fed. Rep. Germany.
GTP-binding proteins consist of three different subunits (α, β, γ) and are assumed to exist as heterotrimers in the inactive state. The aim of our experiments was to measure quantitatively dissociation of G-protein subunits after activation with GTPγS using fluorescence energy transfer. For this purpose, the purified subunits α and $\beta\gamma$ of bovine brain G_O were covalently modified with fluoresceinisothiocyanate and tetramethylrhodaminemaleimid, respectively. The activity of the subunits was retained following modification as proven by functional tests. Energy transfer could be measured between the labelled components in detergent solution and in lipid vesicles. The measurements allowed the calculation of binding constants for two distinct populations having high and low affinity. The high affinity component was abolished by the addition of GTPγS through the dissociation of the α-subunit from the $\beta\gamma$-complex. Further experiments are being carried out in a stable reconstituted system with fluorescently labelled β_1-receptor.

G 110 A MUTATION OF THE β_2-ADRENERGIC RECEPTOR IMPAIRS AGONIST ACTIVATION OF ADENYLYL CYCLASE WITHOUT AFFECTING HIGH AFFINITY AGONIST BINDING, W.P. Hausdorff, M. Hnatowich, B.F. O'Dowd, M.G. Caron, and R.J. Lefkowitz, Depts. of Medicine, Cell Biology, and Biochem, HHMI, Duke Univ. Med. Ctr., Durham, NC 27710.
Activation of G proteins by hormones and neurotransmitters appears to require the formation of high affinity agonist-receptor-G protein ternary complexes. In the case of the β_2-adrenergic receptor (β_2AR), multiple regions of the molecule have been implicated in coupling to the stimulatory G protein G_s, but it is unclear whether distinct regions may play different mechanistic roles in G_s activation. We have expressed in a mammalian cell line a mutant β_2AR comprising a seven amino acid deletion in the carboxyl terminal region of its third cytoplasmic loop (D267-273), a region proposed to be critically involved in coupling to G_s. When tested with β-adrenergic agonists, the maximal adenylyl cyclase response mediated by this mutant receptor was less than half of that seen with the wild-type receptor. Nevertheless, D267-273 exhibited high affinity agonist binding and underwent agonist-induced sequestration in an identical fashion as the wild type receptor. These findings indicate that the formation of high affinity agonist-receptor-G_s complexes is not sufficient to fully activate G_s. Instead, an additional stimulatory signal appears to be required from the receptor. Our data thereby suggest that the molecular determinants of the β_2AR involved in formation of the ternary complex are not identical to those that transmit the agonist-induced stimulatory signal to G_s.

G 111 ISOLATION AND CHARACTERIZATION OF THE PORCINE G PROTEIN α_{i-2} SUBUNIT GENE PROMOTER, Eliezer J. Holtzman, Brian W. Soper, Dennis A. Ausiello, and Louis Ercolani, Renal Unit, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114
We have shown that the G protein α_{i-2} subunit is developmentally expressed in renal porcine LLC-PK$_1$ cells and modulates adenylyl cyclase. To understand α_{i-2} subunit expression, DNA segments required for its transcriptional regulation were isolated from an EMBL-3 porcine genomic library. The 5' flanking porcine DNA segment displayed a 78%, 85%, 54.4% sequence identity to: the human α_{i-2} gene, the rat olfactory α_{i-2} mRNA, and the human c-Ha-ras proto-oncogene 5'-flanking area sequences, respectively. No typical TATA-like sequences were found. However, two "CAAT" boxes, four "GC" boxes, two TGTGG sequences, and a 38 bp seqment sharing 76 % similarity to the c-Ha-ras proto-oncogene promoter sequence were identified as potential cis acting control elements in the 5' flanking area of the porcine gene. S_1 nuclease analysis of LLC-PK$_1$ mRNA revealed major and a minor transcriptional start sites 131 and 171 bp upstream of the translation initiation site. To determine the requisite regulatory sequences, 5'-flanking DNA segments of 0.6 kb and 11 kb were ligated in both sense and antisense orientations to a plasmid that contained a firefly luciferase gene but lacked a promoter. These plasmids were transiently transfected into LLC-PK$_1$ cells. Only transfectants with the 0.6 kb and the 11 kb of putative sense 5'-flanking sequence displayed a 17 and 34-fold increase in luciferase activity as compared to cells transfected with the promoterless plasmid. Transcription of the sense 0.6kb 5'-flanking chimeric gene was found to be correctly initiated from its α_{i-2} promoter. Negative regulatory cis-elements were found by deletions 3' to the major transcriptional start site, whereas 5' deletions revealed two other areas containing enhancers. The minimal promoter was found between - 130 bp and - 60 bp from the major transcription start site. The apparent motifs required for basal transcription were a "GC" box at - 94 bp and a "TGTGG" sequence at - 114 bp. These findings demonstrate the requisite α_{i-2} subunit gene regulatory elements for its basal transcription in LLC-PK$_1$ cells.

G 112 DETECTION OF POINT MUTATIONS IN THE Gs α CHAIN OF HUMAN NEOPLASIA, John Lyons, Frank McCormick, Claudia Landis, Henry R. Bourne, Griffith Harsh, Lucia Vallar, and Anna Spada; Departments of Human Genetics, Molecular Biology, Cetus Corporation, Emeryville, CA 94608; Departments of Pharmacology, Neurosurgery, University of California Medical Center, San Francisco, CA 94143; Departments of Pharmacology, Endocrinology, University of Milan, Italy.

Activation of one of two different amino acids in the α-chain of the Gs protein in pituitary tumors has been shown to be possibly one of the molecular genetic alterations giving rise to these tumors. The point mutations occur at position 201, which corresponds to the site of ADP-Ribosylation of the α-chain by cholera toxin, and position 207, which is biochemically equivalent to the 61st codon in the *ras* genes. Mutations at both positions destroy the GTPase activity of the Gs α-chain. We have now applied PCR and oligonucleotide hybridization technology to the detections of such activating point mutations in human pituitary neoplasia, as well as a number of other human tumor tissues. Confirmation of positive samples has been achieved by direct sequencing.

G 113 MOLECULAR CLONING AND ANALYSIS OF *GPA1*, A G PROTEIN α SUBUNIT GENE FROM *Arabidopsis thaliana*

Hong Ma, Martin F. Yanofsky and Elliot M. Meyerowitz
Division of Biology, California Institute of Technology, Pasadena, CA 91125

Although heterotrimeric G proteins function in a variety of signalling processes of eukaryotic organisms, from yeasts to humans, they have not been identified previously from any plant species. There are reasons to believe, however, that G proteins also exist in plants, because G proteins are known to be present in many eukaryotic species, and because there is evidence suggesting G protein activity in signalling processes in plants. In order to identify and study G proteins in plants, we chose as our experimental organism *Arabidopsis thaliana*, a small flowering plant that has become a model system for plant genetics and molecular biology. We have taken a molecular approach of cloning genes encoding G protein subunits, using the polymerase chain reaction (PCR) technique. Based on the amino acid sequence similarity of several animal and yeast G protein α subunits, we chose two highly conserved regions to derive sequences for degenerate oligonucleotides. Using a specific PCR generated fragment as a probe, we have isolated both genomic and cDNA clones representing a single genomic locus. Sequence analysis indicates this gene is indeed structurally similar to known G protein α subunit genes; therefore, we have named this gene *GPA1*, for G protein α subunit. This is the first Gα gene identified in any plant. We are in the process of characterizing the structure and function of *GPA1*, and the results will be presented.

G 114 CLONING AND EXPRESSION OF GTP-BINDING PROTEINS IN MAMMALIAN CELLS. L. MERCKEN (1), V. MORAS (1), P. SOUBIGOU (2), J.F. MAYAUX (1) and B. TOCQUE (2). Labor. of Molecular Biology (1) and Labor. of Molecular Oncology (2). Rhône-Poulenc Santé. 13, Quai J. Guesde. F - 94400 Vitry-sur-Seine.

A cDNA clone containing the complete sequence of Chinese Hamster a_s was obtained by screening a cDNA library by oligonucleotides that recognize conserved regions in the α-subunits of GTP-binding proteins. Directed mutagenesis was performed on this α_s-subunit in order to introduce mutations corresponding to the mutations observed in the activated *ras* oncogene. Mutated G_s-proteins were expressed under the control of the SV40 promoter in Chinese Hamster lung fibroblasts (CCL39 cells). Measurements of adenylate cyclase activity allow the identification of mutations that enhance the activity of the α-subunits of GTP-binding proteins.

A strategy defined on the PCR methodology was used to clone cDNA sequences corresponding to the α-subunits of the G-proteins expressed in B-lymphocytes. Sequences corresponding to α_s, α_{12}, α_{13} and of an unidentified α-subunit (named α_1) were obtained. The sequence of α_1 presents less than 50 % homology with the other α-subunits. Cloning and sequencing of this cDNA in under progress.

G 115 G PROTEINS OF DROSOPHILA MELANOGASTER: AN IMMUNOLOGICAL AND MOLECULAR CHARACTERIZATION, Franklin Quan, Nina Thambi, William J. Wolfgang and Michael A. Forte, VIABR, L474, Oregon Health Sciences University, Portland, OR 97201

The sophisticated genetics and ease of manipulation of Drosophila make it an attractive system in which to study the role of G proteins in complex biological processes. Using vertebrate cDNAs as probes, we have isolated cDNAs coding for the α subunits of Drosophila Gs, Gi, and Go. The Drosophila proteins are approximately 70% identical to their vertebrate equivalents at the amino acid level. cDNAs coding for multiple forms of Gsα and Goα have been identified. Two forms of Drosophila Gsα differ by the inclusion or deletion of 3 amino acids near the carboxy terminus. This heterogeneity occurs in a region distinct from that of the vertebrate proteins and is the result of alternate splicing involving an unusual splice site at the 3'-end of intron 7 of the Gsα gene. Two classes of cDNA for Goα differ in their 5'-untranslated regions and code for proteins which differ in 7 amino acid residues at the amino terminus. Both forms of Goα appear to be pertussis toxin substrates, but the Drosophila Giα protein is not. Northern and in situ hybridization analysis demonstrate that Gsα is expressed predominately in the CNS. Both neuronal and non-neuronal Goα transcripts have been identified. Giα transcripts appear to be predominately non-neuronal. These results have been confirmed and extended by immunocytochemical localizations using affinity purified antibodies generated to synthetic oligopeptides unique to each subunit.

G 116 ASSESSMENT OF G-PROTEIN AND ADENYLYL CYCLASE INVOLVEMENT IN IL1-SIGNAL TRANSDUCTION K. P. Ray, N. Thompson, P. Rollins, S. Grenfell, S. Witham, N. Kennard and R. Solari, Cellular Science Department, Glaxo Group Research, Greenford, Middlesex, UB6 0HE, UK.

To assess the possible involvement of G-protein(s) and adenylyl cyclase in the signal transduction pathway for IL1, we have examined the effects of bacterial toxins on responses to IL1 in EL4 cells and investigated the ability of IL1 to affect cyclic AMP formation and G-protein function in membrane preparations from these cells. EL4 murine T cells express high numbers of IL1 receptors (2-3 x 10^3/cell) and respond to IL1 by producing IL2 which can be measured in a bioassay. IL1α (6pM) - induced IL2 production was partially inhibited by cholera toxin (65% inhibition at 200ng/ml) and completely blocked by pertussis toxin (2ng/ml) treatment. Part of the inhibitory effect of cholera toxin may be secondary to an increase in cyclic AMP levels since forskolin (10μM) and 8-bromocyclic AMP (100μM) also inhibited IL1-induced IL2 production. IL1α alone did not alter cellular cyclic AMP levels and had no effect on adenylyl cyclase activity in membranes prepared from EL4 cells. IL1α (1nM) had no significant effects either on high affinity GTPase activity or on the rates or extent of [^{35}S] GTP binding to membranes. Furthermore, IL1α did not alter [^{32}P] ADP-ribosylation of a 40kd substrate for pertussis toxin in membranes from control or IL1-treated cells. Interestingly, however, guanine nucleotides altered the kinetics of [^{125}I]IL1α binding to sites present in EL4 membranes. In conclusion our results are not consistent with a mechanism for IL1 action involving stimulation of cyclic AMP levels in EL4 cells; on the contrary, elevation of cyclic AMP seems to inhibit IL1 responsiveness. Direct coupling of IL1 receptors to "classical" G-protein(s) seems unlikely. However, alternative mechanisms for guanine nucleotide-dependent regulation of receptor function may exist.

G 117 **α_2-ADRENERGIC AGONISTS STIMULATE DNA SYNTHESIS IN CHINESE HAMSTER LUNG FIBROBLASTS TRANSFECTED WITH A HUMAN α_2-ADRENERGIC RECEPTOR GENE.** Klaus SEUWEN, Catherine KAHAN, Isabelle MAGNALDO, Brian K. KOBILKA*, Marc G. CARON*, John W. REGAN*, Robert J. LEFKOWITZ* and Jacques POUYSSEGUR, Centre de Biochimie du CNRS, Faculté des Sciences, Parc Valrose 06034 Nice - France. (*) Department of Medicine, Howard Hughes Medical Institute Duke University Medical Center, Durham, NC 27710, USA.

In Chinese Hamster Lung fibroblasts (CCL39 cell line), serotonin stimulates DNA synthesis through a receptor negatively coupled to adenylate cyclase (*Nature* 335, 254-256, 1988). In an attempt to generalize this observation we have expressed a human α_2-adrenergic receptor (α_2-C10) in CCL39 cells and studied the effects of α_2-agonists on reinitiation of DNA synthesis in quiescent cells. We report that the α_2-agonists epinephrine and clonidine stimulate [^{3}H]-thymidine incorporation in synergy with fibroblast growth factor and the α_2- antagonist yohimbine efficiently inhibits this response. Epinephrine- and clonidine- stimulated DNA synthesis is completely blocked by pertussis toxin and correlates well with the inhibition of prostaglandin E_1-stimulated adenylate cyclase. Serotonin- and epinephrine- stimulated DNA synthesis reinitiation are not additive, suggesting that both agents act through a common pathway. Interestingly, α_2-agonists also induced release of inositol phosphates, indicating that α_2-adrenergic receptors can interact both with the adenylate cyclase and phospholipase C messenger system. The level of PLC activation, however, seems insufficient to trigger a mitogenic response as comparative studies on CCL39 cells expressing functional 5-HT1c receptors have shown. Our data show that α_2-adrenergic receptors can regulate cell proliferation in an appropriate context and support the hypothesis that inhibition of adenylate cyclase or the activation of a so far undefined effector system by a Gi protein plays an important role in mediating the effects of growth factors acting through G protein coupled receptors.

G 118 FUNCTIONAL EXPRESSION OF β2ADR/STE2 RECEPTOR HYBRIDS IN YEAST, Paul Sheppard, Anne Bell and Andrzej Z. Sledziewski, ZymoGenetics, Inc., 4225 Roosevelt Way NE, Seattle, WA 98105

Like mammalian cells, yeast have cell surface receptor systems coupled to G protein signal transduction pathways. It may be possible, using hybrid receptors, to functionally link some mammalian receptors to yeast signal transduction pathways. This might facilitate functional dissection of components of signal regulation and transmission, as well as promoting further understanding of local membrane structure using yeast genetics. In addition, this model might be useful in screening for new ligands for the mammalian receptor of interest. We have chosen to construct hybrids between human β2 adrenergic receptor (β2ADR), and the mating type pheromone receptor (STE2) from *Saccharomyces cerevisiae*. Both of these receptors are functionally coupled to G protein signal transduction pathways and share similar structural characteristics.

A variety of tools are available in our yeast system which allow us to follow ligand binding and signal transmission. These include strains which are deficient in STE2 and receptor associated proteins, and functional assays to determine the extent of signal transmission. Early work indicates the STE2/β2ADR hybrids can be expressed in yeast and show partial activity in the mating pathway in response to yeast pheromone. In response, we have also expressed full-length *β2ADR* cDNA in yeast, and are currently defining saturation binding levels and ligand binding site competition.

We are also constructing β2ADR/STE2 hybrids. We hope these hybrids will increase expression levels and functionally couple the STE2 G protein binding domain with the β2ADR ligand binding domain(s). Using functional assays we will be looking for morphological and metabolic changes in the yeast host cells after challenge with yeast mating pheromone or catecholamines.

G 119 G-PROTEIN - EFFECTOR COUPLING: INTERACTION OF RECOMBINANT INHIBITORY γ-SUBUNIT AND ITS MUTANT FORMS WITH TRANSDUCIN AND CATALYTIC SUBUNITS OF THE cGMP PHOSPHODIESTERASE, N. P. Skiba, I. P. Udovichenko, M. Yu. Natochin, V. A. Bondarenko, H. G. Muradov, A. A. Yurovskaya, E. V. Telnykh, V. M. Lipkin. Branch of Shemyakin Institute of Bioorganic Chemistry, USSR Academy of Scienses, Pushchino, 142292, USSR.

The light activated cGMP Phosphodiesterase (PDE) of photoreceptor outer segments is a key enzyme in the transduction process of rod photoreceptors. Enzyme consists of two similar large subunits - α and β, and small γ-subunit, which is the internal inhibitor of its activity. The cDNA encoding of the PDE γ-subunit was inserted into pGEM-2 plasmid downstream from its SP6 RNA polymerase promoter. After transcription the cDNA with SP6 RNA polymerase, functionally active recombinant γPDE was synthezised using enzymatic machineries present in rabbit reticulocyte lysates and wheat germ extracts. The bifunctional reagent, maleimidobenzoyl-N-hydroxysuccinimide ester, was used for chemical cross-linking of membrane-bound PDE subunits. Complexes αγ, βγ and also β_2, $\beta_2\gamma$, $\alpha_2\gamma$ were obtained. These results suggest that both catalytic subunits directly interact with γ-subunit whereas not interacting with each other. By means of site-directed mutagenesis single amino acid substitutions were introduced into central region of the inhibitor (24-45), rich in Arg and Lys, and also into C-terminal part of the molecule. Such alterations allowed determination of functionally essential sites of the γPDE polypeptide chain involving into binding with transducin and catalytic subunits.

G 120 EXPRESSION OF THE ALFA SUBUNIT OF THE GTP-BINDING REGULATORY PROTEIN Go IN THE RAT 1 FIBRBLASTS. V.Z.Slepak, A.N.Pronin, E.I.Frolova and V.M.Lipkin, Shemyakin Institute of Bioorganic Chemistry, USSR Academy of Sciences, Miklucho-Maklaya 16/10 Moscow USSR.

The RAT 1 cells were transfected by an expressing vector pPS-3-neo carrying Goα cDNA derived from the bovine cerebellum cDNA library. Several positive clones were celected by the immunoblot analisys using the anti-Goα rabbit antisera. The quantity of the protein determined both by means of immunoblot and ^{35}S GTPγS binding and the GTP-ase activity varied in the range of 0,01-0,05 % of the total protein in different clones. The study of enzymatic properties as well as data obtained in the nucleotide-dependent limited proteolysis proved that the protein is expressed in the native conformation. The expressed α-subunit was detected only in the membrane fraction of the cells. It is found that the expression of Goα has no effect to content of the β-subunits in the cells. Basal and GTP-stimulated adenylate cyclase activity in the Goα expressing cells is found to be 2 fold higher than in control cells, while forscolin - stimulated activity was unchanged.

G 121 PHOSPHOLIPASE C REGULATES A GLYCOSYL-PHOSPHATIDYLINOSITOL ANCHORED SERINE PROTEASE IN THE HUMAN MALARIA PARASITE *Plasmodium falciparum* , Snewin V. A., Langsley G. and Braun-Breton C., Unité de Parasitology Expérimentale, Institut Pasteur, Paris, France.

A developmentally regulated glycosyl-phosphatidyinositol specific phospholipase C (GPI-PLC) solubilises a membrane bound serine protease in *Plasmodium falciparum* (1, 2). GPI-PLC cleavage of a GPI anchor liberates diacyl glycerol and an inositol-phosphate glycan (3). This system may be comparable to a G-protein mediated PI-PLC signal transduction system (4). To detect the existence of G-proteins in *P. falciparum* merozoites, which may be linked to the GPI-PLC, two approaches have been undertaken. Biochemical studies have been complemented by molecular cloning, whereby a number of clones have been isolated using a G-protein α-subunit consensus sequence oligonucleotide probe. Initial characterisation of these putative parasite G-proteins will be reported.

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(3) Low G. *et al* . (1986) TIBS 11 212-215.
(4) Cockcroft S. and Stutchfield J. (1988) Phil. Trans. R. Soc. Lond. B320 247-265.

G 122 GTP-BINDING PROTEINS IN PLASMA MEMBRANES OF THE ENTOMOPATHOGENIC FUNGUS METARHIZIUM ANISOPLIAE, Richard C. Staples, Raymond J. St.Leger, and Donald W. Roberts, Boyce Thompson Institute, Cornell University, Ithaca, NY 14853. Metarhizium anisopliae is an entomopathogenic fungus which responds to touch and chemical stimuli by producing an appressorium. Ca^{+2} and cAMP dependent phosphorylation events are involved in this differentiation. To date, however, there has been no demonstration or analysis of the GTP-binding proteins of M. anisopliae, and information on GTP-binding proteins in other fungi is sparse. Plasma membranes of M. anisopliae contain a multiplicity of GTP-binding proteins in at least six separate families. Two proteins resemble mammalian G_n proteins (18.4 and 24 kDa) in their being toxin insensitive, binding [α-^{32}P]-GTP on nitrocellulose blots of SDS-PAGE gels and also in their immunological properties. Three other toxin insensitive bands (18.6, 18.8, and 24 kDa) are novel proteins antigenically related both to mammalian G-proteins and ras gene products. An additional 23 kDa pertussis toxin substrate (the major G-protein in a crude mycelial extract) reacted strongly with antisera to G-proteins but not with anti-ras serum. There were also a cholera toxin substrate (18.2 kDa), which reacted strongly with both the G-protein and ras p21 antisera, and a botulinum toxin G-protein substrate (43 kDa). Although unknown, the localization of these proteins on the plasmalemma implies an involvement in transmembrane signaling reactions, the only role so far ascribed to them in mammalian systems.

G 123 STRUCTURE AND FUNCTION OF *GPA1*, A G_α PROTEIN IN YEAST, David E. Stone and Steven I. Reed, Research Institute of Scripps Clinic, La Jolla, CA 92037

The mating response of yeast is initiated by the binding of mating pheromone to surface receptors—Ste2 in *MATa* cells, and Ste3 in *MATα* cells. Pheromone binding causes cells to arrest late in the G_1 phase of the cell cycle, and induces mating specific genes whose products are necessary for cellular fusion. Transduction of the signal from membrane to nucleus is mediated by G proteins similar to those found in animal cells. The yeast genes *GPA1*, *STE4*, and *STE18* encode proteins that are structurally and functionally similar to the alpha, beta, and gamma subunits, respectively, of mammalian G proteins. To better understand the relationship between structure and function of G_α proteins, we sought to identify inactivating mutations in *GPA1*—i.e., mutations that prevent the release of beta-gamma in response to pheromone treatment, and hence transduction of the mating signal. A plasmid containing *GPA1* was treated with hydroxylamine, and mutations that conferred pheromone resistance in a *GPA1* wildtype background were selected. Eight pheromone unresponsive *gpa1* (*gpa1^{phun}*) alleles were identified: G50D (a gly to asp change at postion 50), G321S, G322E, G322R, E355K, E364K, G470D, and a E364K/G470D double mutant. These mutations all lie within large regions that are highly conserved between Gpa1 and four other G_α proteins: Transducin, G_s, G_i, and G_o. By analogy with RAS and G_s models of structure and function, five of our *gpa1^{phun}* mutations are located in domains with proposed functions. The gly 50 to asp50 mutation is in the phosphate box, a region that is thought to interact with GDP and GTP. This position is analogous to that of the activating (oncogenic) RAS mutation, val12. G321S, G322E, and G322R mark the first two positions of the five residue S box, thought to affect the conformational shift induced in G_α proteins by the binding of GTP, and the gly to asp change at postion 470 is in a putative receptor binding domain. The remaining mutations, E355K and E364K, are in conserved regions of unknown function. The pheromone response of yeast cells carrying these *gpa1^{phun}* alleles suggests that the changes at position 322 result in a Gα protein that is unable to release beta-gamma, whereas mutations at the other positions appear to confer pheromone resistance by activating Gpa1 and thereby stimulating desensitization.

G 124 IMMUNOLOCALIZATION OF STABLY-EXPRESSED G-PROTEIN αi-2 AND αi-3 SUBUNIT GENE PRODUCTS TO DISTINCT MEMBRANE DOMAINS IN LLC-PK_1 CELLS. Jennifer L. Stow, Dennis A. Ausiello and Louis Ercolani, Renal Unit & Pathology Dept. Massachusetts General Hospital & Harvard Medical School, Boston, MA 02114.
We have previously demonstrated in polarized renal epithelial cells that basolateral membrane adenylyl cyclase and apical membrane Na^+ channels are regulated by pertussis-toxin sensitive αi subunits of G-proteins. To define the location and function of different αi subunits in the renal epithelial cell line, LLC-PK_1, we have utilized cDNA transfection, immunolocalization with specific antibodies and assay of adenylyl cyclase. A eukaryotic plasmid expression vector containing a mouse metallothionein I promoter and cDNAs encoding the translated regions of the αi-2 or αi-3 genes in sense (αi-2+,αi-3+) or antisense (αi-2-, αi-3-) orientations were stably transfected into LLC-PK_1 cells. The addition of 2-10μM $CdCl_2$ to these cell lines increased the content of the chimeric new transcript for αi-2 at least 10-fold accompanied by a significant increase in αi-2 protein associated with membranes as shown by Western blot. The αi-2 protein was found on basolateral but not apical domains of the plasma membrane and in vesicles throughout the cytoplasm of normal and transfected LLC-PK_1 cells stained by immunofluorescence with specific antibodies. Further analysis of its polarized distribution by confocal microscopy revealed that αi-2 is found primarily on the lateral membranes of the cells and appears to be concentrated at points of cell-cell contact. The amount of basolateral staining in αi-2+ cells but not in αi-2- cells increased after a 12 hr induction with 5mM $CdCl_2$. The functional significance of the increase in basolateral αi-2 was demonstrated by its ability to inhibit vasopressin and forskolin-stimulated adenylyl cyclase in the presence of GTP. In contrast, αi-3 subunits were not found associated with plasma membrane in normal LLC-PK_1 cells but were localized in perinuclear vesicles in the region of the Golgi complex. In αi-3+ cells induced with 5mM $CdCl_2$ the amount of immunofluorescent staining in the Golgi region increased and a small amount of apical plasma membrane staining was also seen. These data demonstrate 1) the production of stably-transfected LLC-PK_1 cell lines in which the overexpression of αi-2 and αi-3 subunits can be induced, 2) that the αi-2 subunit is found only on basolateral membranes where it appears to regulate adenylyl cyclase, and 3) that the αi-3 subunit is found intracellulary, associated with the Golgi complex and when overexpressed is also found on apical membranes where it may regulate the Na^+ channel.

G 125 *Characterization of G proteins from the Green Alga* Dunaliella salina, Deirdre O'Conner, Guy A. Thompson, Jr. and David L. Herrin, Department of Botany, University of Texas, Austin, TX 78713.

Dunaliella salina has been shown to contain phosphatidylinositol-4,5-bisphosphate (PIP_2), inositol-trisphosphate (IP_3), and a PIP_2-specific, GTPγS-stimulated phospholipase C. Within 2 min. after submitting this halotolerant alga to hypoosmotic stress a transient 30% loss of PIP_2 was observed. A search for GTP-binding proteins which may be involved in phospholipase C activation has revealed two candidates in isolated plasma membrane. One is a 38 kDa protein which cross reacts on Western blots with a G αβ antibody from rabbit provided by S. Mumby and A. Gilman. A second protein of 27 kDa binds $GT^{32}P$ on Immobilon membranes and can be ADP-ribosylated in the presence of pertussis toxin. Further characterization of putative G_α proteins of D. salina is underway.

G 126 SUPPRESSION OF THE MATING RESPONSES IN YEAST BY YEAST-MAMMALIAN Gα HYBRIDS, D.J. Tipper, J. Kane, and M. Zhang, Department of Molecular Genetics and Microbiology, University of Massachusetts Medical School, Worcester, MA 01655.

Current models for the mechanism of transduction of the mating signal in Saccharomyces cerevisiae suggest that a tripartite Gαβγ protein, comprising the products of the SCG1/GPA1, STE4, and STE18 genes, respectively, interacts directly with receptors encoded by STE2 and STE3. Genetic analysis shows that βγ positively controls G1 arrest and expression of genes for cell fusion. Negative control of βγ by α(GDP) is presumably relieved when pheromone/receptor catalyses GDP-GTP exchange, producing α(GTP). α(GTP) may also play a role in desensitization/adaptation. We have demonstrated that rat Gα proteins and hybrid yeast-rat proteins can rescue haploid scg1-null spore segregants and dominantly suppress α factor response and mating (Yoon-Se Kang, J. Kane, J. Kurjan, J. Stadel and D.J. Tipper, In preparation). We have now quantitated the effects of expression of these constructs in exponential growth by following cell number and morphology in MATa bar1 cells treated with α factor, and in scg1-null ste5-3^{ts} and $STE4^{HPL}$ ste5-3^{ts} strains shifted from 34 to 22^{o}. Over-expression of SCG1 suppresses mating, the response to α factor, and the G1 arrest phenotype of $STE4^{HPL}$. SCG1-Gαi2 and SCG1-Gαo hybrids are at least as effective, while an SCG1-Gαs hybrid is less effective. Gαs itself has moderate activity, Gαi2 slight activity, and activity of Gαo is undetectable. The results are consistent with current models of Gα domain functions and with a single point of interaction of foreign Gα proteins with the yeast pheromone response pathway: suppression of STE4/18 functions as analogs of Scg1p. Downstream effects cannot, however, be ruled out.

G 127 FLUORESCENT PROBES OF SUBUNIT INTERACTIONS IN THE TRANSDUCIN-cGMP PHOSPHODIESTERASE CASCADE, Theodore G. Wensel and Latha Ramdas, Department of Biochemistry, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030.

Fluorescent tags at specific sites on subunits involved in G-protein-effector interactions provide useful tools for monitoring these interactions. We have used emission anisotropy to monitor binding of the inhibitory subunit of the rod outer segment cGMP phosphodiesterase (PDE) to the catalytic subunits, with a single fluorescent group attached to cysteine 68 [Wensel, T.G. and Stryer, L. (1989) Biochemistry, in press]. The kinetics of binding and dissociation can be followed continuously, providing insight into the activation mechanism. In addition, we have been exploring the use of ADP-ribose groups introduced by cholera and pertussis toxins as specific sites for labelling the α subunit of transducin. The cholera toxin site can be labelled with retention of PDE activation activity. The pertussis toxin site is also an attractive target for fluorescent labelling; we have found that its ADP-ribosylation occurs without significant effect on spontaneous nucleotide exchange kinetics, and does not block stimulation of PDE by the ADP-ribosylated transducin, once it is activated by AlF_4^-.

Pathways, Signals and Channels

G 200 EFFECT OF ADENYLATE CYCLASE ACTIVATION ON T CELL RECEPTOR (TCR)- OR GUANOSINE 5'-O-(3-THIOTRIPHOSPHATE) (GTPgammaS)-STIMULATED INOSITOL PHOSPHOLIPID (InsPL) HYDROLYSIS IN INTACT OR PERMEABILIZED MURINE T HELPER (Th) LYMPHOCYTES. M. A. Alava, K. E. DeBell, T. Hoffman, M. S. Taplits, and E. Bonvini. Lab. Cell Biology, DBBP, CBER, Bethesda, MD 20892. To obtain information on the regulation of InsPL-specific phospholipase C (PLC) in immune cells, we studied the effect of treatment with adenosine 3':5'-cyclic monophosphate (cAMP) inducers on InsPL hydrolysis in a murine Th lymphocyte clone. Intact Th cells produced maximal amounts of cAMP (approximately 8 pmoles/10^6 cells) in response to 1 ug/ml cholera toxin (CTx), 100 uM forskolin (FSK), or 1 uM prostaglandin E_2 (PGE_2). cAMP generation reached plateau after 3 min of treatment with FSK or PGE_2, and 60 min with CTx. Th cells, permeabilized with bacterial lysins, showed maximal cAMP production (approximately 800 pmoles/10^6 cells) after a 20 min incubation in the presence of 5 mM ATP and 300 uM GTPgammaS, a non-hydrolysable analog of GTP that directly activates guanine nucleotide-binding proteins (G-proteins). In addition to cAMP generation, GTPgammaS also stimulated InsPL hydrolysis. Preincubation of intact Th cells with 100 uM FSK or 1 uM PGE_2 (15 min at 37°C), or 1 ug/ml CTx (60 min at 37°C), completely inhibited InsPL hydrolysis induced by TCR perturbation with an anti-TCR MoAb (145-2C11 Ab, directed against the CD3 epsilon chain of the TCR). Preincubation of intact Th cells with FSK or CTx, followed by permeabilization, also resulted in a 50% inhibition of TCR-induced InsPL hydrolysis. However, GTPgammaS-induced InsPL hydrolysis was not affected by pretreatment of intact cells with cAMP inducers. These results suggest that cAMP-mediated inhibition of TCR-induced InsPL hydrolysis is exerted at a level proximal to PLC or G-protein activation. The ability of GTPgammaS to induce InsPL hydrolysis, despite the considerable level of cAMP generated, may also be attributable to the loss of soluble effector molecule(s) upon permeabilization. This was supported by the absence of inhibition of TCR-induced InsPL hydrolysis when permeabilized cells were preincubated with 100 uM 8-Br-cAMP, a phosphodiesterase-resistant cAMP analog.

G 201 PREGNANCY SUPPRESSES G-PROTEIN COUPLING TO PHOSPHOINOSITIDE PHOSPHOLIPASE C ACTIVATION IN GUINEA PIG MYOMETRIUM. Stephen J. Arkinstall and Colin T. Jones. Institute of Molecular Medicine, University of Oxford, Oxford, OX3 9DU. UK.

The regulatory mechanisms controlling uterine contractile activity during pregnancy remain unclear although pathways modulating intracellular Ca2+ and prostaglandin production play an important role. Since excitatory hormones increase phospholipase C-dependent phosphoinositide hydrolysis, regulation of G-protein functional coupling could be a key site for control. Using myometrial membranes containing prelabelled phosphoinositides and prepared from nonpregnant guinea pig uterus, GTP[S] stimulated a 5-fold increase in [3H]IP and [3H]IP2 formation with an ED50 of 8.11±1.91nM (n=5), an effect specific for guanine nucleotides, blocked by 0.5mM GDP[S] and dependent upon the presence of Mg2+. Together with an Al3+-dependent 5-fold stimulation by NaF (AlF4-) (ED50=2.0±0.46mM (n=5)), this indicates a direct stimulation of a G-protein coupled to phosphoinositide phospholipase C activation. At 53-60 days and 66-69 days of pregnancy (full term 67±2 days) GTP[S]- and AlF4- stimulated [3H]inositol phosphate formation was diminished by 60-80% and moreover, the EC50 for guanine nucleotide activation was increased by up to 38-fold. In contrast, [3H]IP2 production in response to maximally effective concentrations of Ca2+ (3mM) displayed only a limited reduction (28%), an observation consistent with a 28% fall in levels of 60kda phosphoinositide-specific phospholipase C as measured by quantitative immunoblotting. This data indicates a pregnancy-dependent selective suppression of G-protein functional coupling to phospholipase C-dependent phosphoinositide hydrolysis, a change likely to limit considerably uterine contractile activity at this time.

G 202 CHARACTERIZATION OF A NOVEL SIGNAL TRANSDUCING GLYCOPROTEIN ON NK AND T CELLS. Gregory Bell, John Imboden, and William Seaman, Univ. of CA & VAMC, San Francisco, CA. In order to identify cell surface molecules on natural killer (NK) cells that can stimulate the polyphosphoinositide (PPI) pathway, hybridomas were constructed from the spleens of BALB/c mice that had been immunized with RNK-16 cells (a rat leukemia line with NK activity). The resulting mAb were screened for their ability to stimulate PPI turnover in RNK-16. Three separate mAb reacted with a 33-40 kD glycoprotein which has been designated gp35. gp 35 is expressed on granulocytes, monocytes, T and B cells however, only 5% of thymoctes express gp35. mAb to gp35 elicit sustained increases in the concentration of cytoplasmic free Ca^{2+} within RNK-16 cells, inhibit the cytolytic activity of RNK-16 cells and, in soluble form, are mitogenic for freshly isolated rat splenic T cells. Immunoprecipitation studies of ^{125}I surface labelled RNK-16 cells lysed in CHAPS indicate that gp35 is noncovalently associated with a 50-60kD glycoprotein. These studies demonstrate that gp35 can function as a signal transducing molecule on NK cells and T cells and suggest that gp35 may be a component of an oligomeric receptor complex.

G 203 DEPLETION OF INTRACELLULAR CALCIUM STORES BY THE TUMOR PROMOTOR THAPSIGARGIN INHIBITS CALCIUM CHANNEL OPENING IN ACTIVATED HUMAN T LYMPHOCYTES, G. Bismuth, H. Gouy, S.B. Christensen and P. Debré, Laboratoire d'Immunologie Cellulaire et Tissulaire, U.A. CNRS 186, C.H. Pitié-Salpêtrière, Paris, France and Department of Chemistry, Royal Danish School of Pharmacy, Copenhagen, Denmark.
The relationship between mobilization of Ca^{2+} from intracellular stores and opening of Ca^{2+} channels has been studied in a human antigen-specific T cell clone following stimulation by monoclonal antibodies (mAbs) specific for the CD3 or the CD2 cell surface molecules. To adress the question whether the initial mobilization of intracellular Ca^{2+} (still observed in Ca^{2+} free medium) plays a role in the opening of Ca^{2+} channels, we have used the sesquiterpene lactone thapsigargin which has been shown in other cell systems to release Ca^{2+} from intracellular stores independently of the hydrolysis of phosphoinositides. In 1 mM Ca^{2+} medium, previous depletion of intracellular Ca^{2+} stores by thapsigargin supressed the response of Fura 2-loaded T cells to CD3 or CD2-specific mAbs. Moreover studies performed in Ca^{2+} free medium clearly showed that when added after thapsigargin the mAbs did not trigger Ca^{2+} channel opening, while this opening was still evident when the mAbs were added before thapsigargin. In addition, thapsigargin by itself was able to induce Ca^{2+} channel opening although its effect on Ca^{2+} influx was less pronounced than the one observed after T cell stimulation via the CD3 or the CD2 molecules. These results strongly suggest that depletion of intracellular Ca^{2+} pool(s) has a prominent role in the opening of Ca^{2+} channels in stimulated human T lymphocytes.

G 204 α_1-ADRENOCEPTORS MODULATE A TRANSIENT OUTWARD K^+ CURRENT VIA A PERTUSSIS TOXIN-INSENSITIVE G-PROTEIN IN RABBIT ATRIAL MYOCYTES, A.P. Braun*§, D. Fedida*, M.P. Walsh§ and W.R. Giles*, Depts. of Medical Physiology* and Biochemistry§, University of Calgary, Calgary, Canada T2N 4N1
Recently, it has been reported that a reversible decrease of a time- and voltage-dependent transient outward K^+ current(I_t) occurs following stimulation of cardiac α_1-adrenoceptors. Using whole-cell voltage clamp of isolated rabbit atrial myocytes, inclusion of the non-hydrolyzable GTP analogues, GTPγS and Gpp(NH)p (3-5 mM), in the recording micropipette led to an irreversible decrease in I_t following exposure to the α_1-agonist methoxamine (200 μM). GDPβS (10 mM), did not block the agonist induced decrease in I_t. Pretreatment of atrial myocytes with 0.1 μg/ml pertussis toxin (PT) for 8-9 hours at 30-36°C did not prevent the decrease in I_t caused by methoxamine. Using ^{32}P-NAD as the *in vitro* assay substrate, in control myocyte membranes, PT was shown to catalyze the ^{32}P-ADP-ribosylation of at least two substrates, with M_rs of ≈ 39 and 41 kDa. These correspond to the α subunits of the GTP-binding proteins G_o and G_i, respectively. In membranes prepared from PT-pretreated myocytes, the degree of PT catalyzed ^{32}P-ADP-ribosylation of these two bands was decreased ≈70% compared to control. These results thus demonstrate that α_1-adrenoceptors mediate I_t via a PT-insensitive G-protein in rabbit atrial myocytes.

G 205 CARBACHOL AND GUANOSINE 5'-O-(3-THIOTRIPHOSPHATE) STIMULATION OF EXOGENOUS PHOSPHOINOSITIDE BREAKDOWN IN RABBIT BRAIN CORTICAL MEMBRANES: EFFECT OF BIOLOGICAL DETERGENTS, **Helen R. Carter, Michael A. Wallace and John N. Fain,** Dept. of Biochemistry, University of Tennessee, 800 Madison Avenue, Memphis, TN 38163

Rabbit cortical membranes hydrolyse exogenous phosphatidylinositol (PI) and phosphatidylinositol 4,5-bisphosphate (PIP_2) in a carbachol and guanine nucleotide (GTPγS) dependent manner (Claro et al., J. Biol. Chem. in press, 1989). It is possible to extract 60% of the membrane protein by washing with hypertonic buffer (2M KCl) and yet retain a fully coupled receptor~G protein~phospholipase C complex. In order to observe optimal carbachol and GTPγS stimulated phospholipase C activity it was necessary to include sodium deoxycholate in the assay. Stimulation with carbachol and GTPγS did not appear to result in the specific release of phospholipase C nor an inhibitor of phospholipase C from the membranes. We investigated the effect of various frequently used detergents on this system. Low detergent concentrations, insufficient to solubilize membrane proteins, uncoupled the hormone response without affecting basal levels of activity. This indicates a loss of receptor or G protein regulated stimulation rather than loss of control over endogenous phospholipase C activity. These observations are of importance for future work upon the isolation and reconstitution of the components involved in hormonal and neurotransmitter regulation of phospholipase C.

G 206 MODULATION OF ADENYLATE CYCLASE BY PHOSPHATIDYLINOSITOL (PI) SIGNALLING SYSTEMS IN BRAIN and NCB-20 CELLS. Dermot M.F. Cooper and Christine L. Boyajian. Department of Pharmacology, University of Colorado Health Sciences Center, Denver, CO 80262.

Individual signal transduction systems within cells interact at a number of levels. We have been studying the interplay between Ca^{2+}-mobilizing and cAMP-generating systems at the level of signal generation. In most brain regions, Ca^{2+} concentrations corresponding to those achieved upon membrane depolarization or activation of the PI system, stimulate adenylate cyclase activity via calmodulin. This permits synergistic stimulation of neurotransmitter synthesis and release in response to a single stimulus. However, in a number of peripheral systems and cultured cell lines (including platelets, NCB-20 and GH3 cells) these concentrations profoundly inhibit (~45%) plasma membrane adenylate cyclase activity. This effect is independent of calmodulin, although highly cooperative for Ca^{2+} ions. It requires a stimulated activity state of adenylate cyclase, and is not sensitive to the actions of pertussis toxin. In intact NCB-20 cells, bradykinin, which stimulates PI hydrolysis and Ca^{2+}-mobilization, causes a significant inhibition of cAMP production, which is not due in any part to stimulation of cAMP phosphodiesterase activity. Blockade of the mobilization of Ca^{2+}, by the intracellular chelator, MAPTAN, diminishes the effects of bradykinin. These data indicate that Ca^{2+} plays diverse roles in modulating the responsiveness of cAMP-generating systems, which may vary between cell types to accommodate specific physiological demands.

G 207 CONTROL OF THE IgE RECEPTOR-ACTIVATED Ca^{2+} TRANSPORT SYSTEM OF RBL-2H3 MAST CELLS BY A G PROTEIN AND Ca^{2+}/CALMODULIN. G.G. Deanin, B.S. Wilson and J.M. Oliver, Department of Pathology, University of New Mexico School of Medicine, Albuquerque, NM 87131.

In RBL-2H3 rat basophilic leukemia cells, antigens that crosslink IgE-receptor complexes stimulate the turnover of inositol phospholipids, the mobilization of Ca^{2+} from intra- and extracellular sources, and the release of serotonin and other inflammatory substances from granules. Ca^{2+} influx and secretion are inhibited by mycophenolic acid (MPA), which depletes cellular GTP, and are enhanced by cholera toxin (CTX), implicating a G protein in the control of the antigen-stimulated calcium transporter of RBL-2H3 cells (Wilson et al, J. Immunol. *143:* 259, 1989; Narisimhan et al., J. Biol.Chem. *263:* 19626, 1988). Both previous studies concluded that the antigen-induced activation of PLC, that leads to the release of intracellular Ca^{2+} stores (the Ca^{2+} transient), most likely occurs by a GTP-independent pathway. We report that cholera toxin alone does not stimulate Ca^{2+} influx and secretion. However, secretion is induced in cells treated with CTX in the presence of low levels of the Ca^{2+} ionophore, ionomycin, that do not alone stimulate significant secretion. We also report that treatment of IgE-primed RBL-2H3 cells with a seies of calmodulin inhibitors impairs secretion and $^{45}Ca^{2+}$ uptake induced by antigen, ionomycin and ionomycin or antigen plus CTX. The calmodulin antagonists do not impair the antigen and ionomycin-induced release of intracellular Ca^{2+}. These results suggest that there are at least two requirements for the stimulation by antigen of the Ca^{2+} transport system of RBL-2H3 cells: the activation by receptor crosslinking of a cholera toxin-sensitive G protein and the activation by the GTP-independent release of Ca^{2+} stores of a Ca^{2+}/calmodulin-dependent event. Cholera toxin in combination with MPA yields supra-optimal antigen-stimulated secretion, suggesting that an additional, cholera toxin-insensitive inhibitory G protein is involved in the control of IgE receptor-mediated secretion.

G 208 MUSCARINIC INHIBITION OF PHOSPHOLIPASE C ACTIVITY IN THYROID CELLS. A POSSIBLE DIRECT MECHANISM INVOLVING AN INHIBITORY G PROTEIN, Maria Di Girolamo, Cinzia Bizzarri and Daniela Corda, Istituto di Ricerche Farmacologiche "Mario Negri", Consorzio Mario Negri Sud, 66030 Santa Maria Imbaro (Chieti), Italy

The role of the cholinergic system in the regulation of thyroid functions has been analyzed in a continuous line of rat thyroid cells (FRTL5). In these cells we have previously demonstrated the functional coupling of the thyrotropin and α1 adrenergic receptors to phospholipase C (PLC) and phospholipase A2 (PLA2) and the role of these enzymes in the regulation of iodide efflux. Recently, we have been able to show the presence of two muscarinic receptors in FRTL5 cells. A M1 receptor is coupled to the activation of PLA2, as shown by the carbachol-induced (Cch) and pirenzepine-sensitive increase (80±8%) in arachidonic acid release. A M2 receptor (pirenzepine insensitive) inhibits PLC, based on the following evidences. Cch decreased the cytosolic Ca^{++} level induced by norepinephrine (NE) by 30±3%. A similar inhibition could be detected evaluating the total inositol phosphates (IPs) accumulation. Moreover, Cch decreased the basal levels of inositol 1,4,5 trisphosphate (IP3) by 47±3%. This inhibition does not involve known second messengers, since Ca^{++}, cAMP and PMA were not able to mimick or prevent the Cch effect. A further indication that the coupling between the M2 receptor and the PLC regulation could be direct was obtained in FRTL5 cells permeabilized by streptolysin O. In this system NE increased the IP3 production by 135±17% over the basal. The addition of Cch inhibited this effect by 50%. Both effects required the presence of GTP. Pertussis toxin (PT) completely abolished the effect of Cch on both cytosolic Ca^{++} levels and IPs production. Taken together these data suggest that a PT sensitive GTP binding (G) protein provides a direct inhibitory coupling between the M2 receptor and PLC.

G 209 ENHANCEMENT BY POTASSIUM IONS OF CARBACHOL-STIMULATED INOSITOL PHOSPHOLIPID BREAKDOWN IN THE RAT BRAIN. POSSIBLE INVOLVEMENT OF G-PROTEINS,
Christopher J. Fowler[1] and Gunnar Tiger[2], Astra Research Centre AB, S-151 85 Södertälje[1] and Department of Pharmacology, University of Umeå, S-901 87 Umeå[2], Umea[2], Sweden.

Raising the assay potassium ion concentration from 6 to 18 mM greatly enhances the inositol phospholipid breakdown response to the muscarinic agonist carbachol in rat brain miniprisms. This effect is selective for the M1-type muscarinic receptor-mediated response. The enhancement is distal to effects on the muscarinic acceptor site, and may thus involve an action of K^+ upon a specific G-protein coupling the M1-type muscarinic receptors to phospholipase C. To explore this possibility, the effects of NaF upon inositol phospholipid breakdown have been studied in rat cerebral cortical miniprisms. NaF concentration-dependently increased basal inositol phospholipid breakdown by similar extents at 6 and 18 mM K^+. Thus, 19 mM NaF increased the breakdown by means of 0.060 and 0.063 (values as InsP/(Lipid + InsP)) at 6 and 18 mM K^+, respectively. In the absence of NaF, carbachol (1000 μM) increased inositol phospholipid breakdown by means of 0.052 and 0.176 at 6 and 18 mM K^+, respectively. In the presence of 19 mM NaF, the increases produced by carbachol (over those produced by NaF alone) were 0.014 and 0.011, respectively. A similar pattern was observed for frontal cortical, hippocampal and striatal miniprisms. Thus, activation of G-proteins by NaF reduces the inositol phospholipid breakdown response to carbachol and blocks the enhancement of this response by K^+ ions. Evidence will also be presented to show that NaF inhibits the dephosphorylation to [^{3}H]myo-inositol of the [^{3}H]inositol phosphates.

G 210 CHOLERA TOXIN (CT)-MEDIATED INHIBITION OF INTRACYTOPLASMIC Ca^{2+} INCREASE IN STIMULATED HUMAN T LYMPHOCYTES IS INDEPENDENT OF CT EFFECT ON cAMP PRODUCTION, H. Gouy, I. Theodorou, P. Debré and G. Bismuth, Laboratoire d'Immunologie Cellulaire, CNRS U.A. 186, C.H. Pitié, Paris, France. In human T lymphocytes, CT induces a sustained increase of intracellular cAMP via ADP ribosylation (ADPr) of G_{sa} subunit which in turn activates adenylyl cyclase. CT also inhibits the mobilization of Ca^{2+} from intracellular stores and the opening of Ca^{2+} channels observed in response to various T cell-specific agonists. To adress the question whether these inhibitory effects of CT may be distinct from the previously described immunomodulatory action of cAMP on the normal T cell activation process, we have selected clones of the Jurkat T cell line resistant to the growth inhibitory effect of CT. Two types of resistant clones were obtained. In type one, the response of the adenylyl cyclase pathway to either CT, PGE2 or forskolin was almost totally abolished; ADPr of G_s by CT was undetectable in membrane fractions; CT no more inhibited the intracellular Ca^{2+} increased triggered by CD3 or CD2-specific antibodies. In type two, the cAMP response to CT, PGE2 and forskolin was unaltered together with a conserved ADPr of G_s by CT. Mainly, mobilization of Ca^{2+} via the CD3 or the CD2 T cell activation pathway was not affected by CT. Together with the finding that the Ca^{2+} response in PGE2-desensitized Jurkat cells was still completely inhibited by CT, these results demonstrate that CT may affect the initial steps of the human T cell activation process independently of cAMP.

G 211 INTERACTION OF FLUORIDE WITH G PROTEINS IN RAT PANCREATIC ACINI .

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Guanylnucleotides modulate the exocrine secretion from rat pancreas . In order to investigate the regulatory site involved, the effect of fluoride (F) , a general activator of G protein, was tested . F, at a 10 mM concentration, maximaly stimulated amylase release (5-fold). Higher concentration induced infra-maximal stimulation . A similar decrease was observed when F was combined with a maximal (100 µM) carbamylcholine (Cb) concentration. By itself , F did not significantly affect cyclic AMP levels, but inhibited by 50% the response to Forskolin and Secretin . F stimulated the release of inositolphosphates from prelabelled acini . This effect was probably secondary to Phospholipase C (PC) activation since it was confirmed by HPLC analysis that inositol 1,4,5 trisphosphate increased in response to F . A similar activation of PC was observed with 100 µM Cb, but while the response to the muscarinic agent was already noticeble after 15 sec. , the response to F exhibited a 3-5 min lag phase . Similary F increased ^{45}Ca efflux after 5 min. experiment . The activity of inositol monophosphatase was measured in crude homogenate: Lithium was a non competitive antagonist while F competitively inhibited the activity of the enzyme , with a Ki around 10^{4}µM. In conclusion, F is a pancreatic secretagogue because it stimulates the G protein activating the PC . F also stimulates the G protein inhibiting the adenylate cyclase . Itmight also activate the G protein coupled to low affinity muscarinic receptor and involved in the inhibition of amylase secretion . Finaly , F is a competitive inhibitor of the inositol monophosphatase .

G 212 THE CHARACTERISTICS OF IgE-RECEPTOR MEDIATED CALCIUM INFLUX IN RBL-2H3 CELLS,

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IgE-receptor-mediated secretion in mast cells and the related RBL-2H3 cultured cell line is associated with accelerated influx of Ca^{2+} ions that promotes and sustains elevated levels of free Ca^{2+} in the cytosol ($[Ca^{2+}]_i$). The influx of Ca^{2+} into RBL-2H3 cells has the following characteristics: 1) It operates only when the surface membrane remains polarized and becomes inoperative when cells are depolarized with high external K^+; 2) it may be regulated by a cholera-toxin sensitive G-protein as the antigen-stimulated influx and rise in $[Ca^{2+}]_i$ are significantly enhanced in cholera toxin-treated cells; 3) it is non-selective. Indeed, ions such as $La^{3+}>Zn^{2+}>Mn^{2+}>Sr^{2+}$ block entry of Ca^{2+} by competing with Ca^{2+} either at the exterior portion of the channel (i.e. La^{3+} or Zn^{2+}) or by co-passage through the channel (i.e. Mn^{2+} or Sr^{2+}). These properties permitted the following manipulations of antigen-induced responses. Secretion, but not hydrolysis, of inositol phospholipids could be blocked by low concentrations (<50 µM) of La^{3+} and Zn^{2+}, whereas both responses were blocked by high concentrations of the metal ions. Replacement of Ca^{2+} with Mn^{2+} or Sr^{2+}, permitted antigen-stimulation of inositol phospholipid hydrolysis and some secretion. The influx of Ca^{2+} ions, which appears to be necessary for amplification of the stimulatory and secretory responses in RBL-2H3 cells, is mediated by a non-selective, voltage-independent, influx mechanism that is possibly regulated by a G-protein. The ability to block the entry of Ca^{2+} with low concentrations of Zn^{2+}(10-50 µM) could account for the therapeutic properties of zinc oxide in topical preparations.

G 213 THE EFFECTS OF cAMP ON PHOSPHOLIPID TURNOVER DURING THE RESUMPTION OF MEIOSIS IN THE BOVINE OOCYTE, Sheryl T. Homa, Department of Zoology, Arizona State University, Tempe, AZ 85287. The resumption of oocyte meiotic maturation is triggered by luteinizing hormone (LH) in vivo. Under specified culture conditions, this process will resume spontaneously in the absence of hormone. Classically, LH has been shown to interact with receptors linked to adenylate cyclase. However, oocyte meiotic arrest is known to be maintained by cAMP. More recently, an increase in phospholipid turnover has been associated with the action of hormones, including LH, which are known to induce meiotic maturation. This study was carried out to investigate phospholipid turnover in oocytes during meiotic resumption, and to determine whether this turnover can be modulated in the presence of high levels of cAMP. Bovine oocytes were cultured in medium conducive to spontaneous maturation containing [^{32}P]orthophosphate, in the presence or absence of the cAMP-phosphodiesterase inhibitor, isobutylmethylxanthine (IBMX). Total lipids were extracted at hourly intervals and phospholipids were separated by thin layer chromatography. The results reveal that oocytes undergo selective changes in phospholipid turnover as the cells become committed to mature, even though they do not exhibit any chromosomal changes at this time. This is demonstrated by a rapid decline in labeled phosphatidic acid and an elevation of phosphatidylcholine and phosphatidylinositol, which is independent of IBMX. There is a surge in labeled phosphatidylethanolamine and phosphatidylcholine which accompanies germinal vesicle breakdown 2h later. This effect is significantly inhibited upon treatment with 5mM IBMX which inhibits spontaneous meiotic maturation. The results suggest that cAMP formation and phospholipid turnover may be intimately related in signalling germinal vesicle breakdown.

G 214 ANTAGONIST-RECEPTOR-G PROTEIN INTERACTIONS?,
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G proteins are known to change the binding characteristics of agonists to receptors. In addition, agonist binding to receptors changes G protein activity. Conversely, it has been commonly assumed that antagonist effects are limited to receptor binding. However, there have been several reports suggesting the occurence of antagonist-receptor-G protein interactions. We have examined the effect of AlF^-_4 (NaF and $AlCl_3$) on muscarinic antagonist (^{3}H-QNB) binding to rat parotid membranes, as well as the effect of the muscarinic antagonist atropine on receptor independent, AlF^-_4 mediated, $[Ca^{2+}]_i$ mobilization of dispersed rat parotid acinar cells (RPAC). The binding of ^{3}H-QNB (Kd 85±12 pM, Bmax 488±36 fmol/mg protein) was competetively inhibited (Kd 150±11 pM) by 10 mM NaF and 10 uM $AlCl_3$. This effect was [NaF]- dependent with a half- maximal inhibition at ~3.5 mM. When the reciprocal situation was examined, atropine (10 uM) was found to both delay and blunt the AlF^-_4 mediated increase in $[Ca^{2+}]_i$ in RPAC. Atropine, however, was without effect on AlF^-_4 mediated $[Ca^{2+}]_i$ changes in a fibroblastic cell line (B82) lacking muscarinic receptors. Our data suggest that muscarinic antagonist binding to RPAC is influenced by G protein activation. In turn, G protein activity is influenced by muscarinic antagonist binding.

G 215 CYTOKININ STIMULATES CALMODULIN GENE EXPRESSION AND ALTERS CALMODULIN-DEPENDENT PROTEIN KINASE ACTION IN PLANTS,
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Cytokinin (CK) is a major plant growth regulator that stimulates cell division and morphogenesis. We have studied the CK signal transduction pathway in *Nicotiana plumbaginifolia* cells in culture and in doing so have cloned cDNA representing mRNAs which are induced upon addition of CK. CK stimulates the accumulation of a mRNA encoding a calmodulin (CAM)-like protein. To study the possible involvement of CAM-like proteins in the cytokinin response, we have characterized CAM-dependent protein kinase (PK) in extracts from *N. plumbaginifolia* cells. PK activity has been observed which phosphorylates a target set of proteins *in vitro* of 46 kD (pp46 a & b). The PK activity was judged to be CAM-dependent because the onset of phosphorylation activity occurs at 0.1 μM Ca++ and is inhibited by trifluoperazine. The proteins pp46 a & b are also phosphorylated *in vivo* in *N. plumbaginifolia* cells. It has been observed that the *in vivo* phosphorylation of pp46 a & b (during a pulse of $^{32}PO_4$) declines over several hours upon addition of CK to the culture. The decline could be due to changes in cytosolic Ca++ concentrations, reduction in the amount of target proteins or availability of unphosphorylated sites.

G 216 T CELL ANTIGEN RECEPTOR REGULATES A DISTINCT PATHWAY FOR PHOSPHATIDYLINOSITOL 4-5-BISPHOSPHATE PRODUCTION.
S. Inokuchi and J.B. Imboden, Univ. of CA & VAMC, San Francisco, CA. Stimulation of the human T cell line, Jurkat, by the addition of monoclonal antibodies (mAb) reactive with the T cell antigen receptor (TCR) leads to sustained hydrolysis of polyphosphoinositides (PPI) and increased levels of inositol 1,4,5-trisphosphate for >2min. To investigate the possibility that the production of PPI is regulated during TCR stimulation, we studied Jurkat cells whose inositol phospholipids had been labeled to steady state with [^{3}H]inositol or nonequilibrium labeled with [^{32}P]orthophosphate. Within 60s of TCR stimulation, [^{3}H]phosphatidylinositol bisphosphate (PIP_2)and [^{3}H]phosphatidylinositol phosphate (PIP) decreased by 65% and 35%, respectively. The decrease in [^{3}H]PIP_2 was sustained for >20min, but, after 5min, the levels of [^{3}H]PIP were comparable in stimulated and unstimulated cells. To examine the rate of flux through PPI, we measured the change in the ratio ^{32}P cpm/^{3}H cpm in each inositol phospholipid. TCR stimulation led to accelerated fluxes through PIP_2 and phosphatidylinositol (PI) that continued for >20min. In contrast, after the initial 30s, the flux through PIP was equal to that in unstimulated cells despite the fact that the ^{32}P-specific activity of PIP was never greater than 12% of the γ-phosphate of ATP. This observation suggested that, during TCR stimulation, production of PIP_2 from PI might occur via a small pool of PIP with a very high turnover. The existence of such a pool was established by determining that, in stimulated cells, the ^{32}P-specific activity of the 1-position phosphate of PIP_2 was greater than that of PIP; the specific activities for PIP and PIP_2 were, respectively, 32 and 200 cpm/pmole at 10 min, and 328 and 1708 cpm/pmole at 20 min. We conclude that, during the initial 60s of TCR stimulation, there is substantial depletion of cellular PIP and PIP_2. Thereafter, the generation of PIP_2 from PI continues for >20min via a TCR-regulated pathway which utilizes a small but highly labile pool of PIP.

G 217 THROMBOXANE-UNRESPONSIVE DOG PLATELETS: A CONSEQUENCE OF AN ABNORMAL THROMBOXANE A_2/PROSTAGLANDIN H_2 RECEPTOR-LINKED G_p? G.J. Johnson, P.C. Dunlop and L.A. Leis, VA Medical Center and University of Minnesota, Minneapolis, MN 55417

Platelets obtained from most mongrel dogs form thromboxane A_2 (TXA_2) from exogenous arachidonate, but they fail to aggregate or secrete in response to TXA_2 although they respond appropriately to other agonists. However, some dogs have platelets that are TXA_2-responsive (TXA_2+) as are human platelets. To evaluate the mechanism responsible for TXA_2-unresponsive (TXA_2-) platelets, we compared **TXA_2/PGH_2 receptor binding, consequences of phospholipase C** activation and GTPase activity in dog TXA_2-, TXA_2+ and human platelets. The TXA_2/PGH_2 receptor antagonist, [^{3}H] SQ29548, bound to a comparable number of sites on **dog TXA_2- (870 ± 10) and human (711 ± 17) platelets and** slightly more sites (1033 ± 15) on dog TXA_2+ platelets. **Both types of dog platelets had greater affinity (Kd** = 2.2 ± 0.8, 2.2 ± 0.6) than human platelets (Kd = 3.1 ± 0.5). **Biochemical consequences of phospholipase C activation** (phosphatidic acid (PA) formation, rise in cytoplasmic ionized calcium ($[Ca^{2+}]_i$) and 47 kDa protein phosphorylation) were reduced 40-50% in dog TXA_2- platelets compared to human or dog TXA_2+ platelets following stimulation with the TXA_2 mimic, U46619. G protein stimulation with GTPγS also resulted in 40-50% less PA formation and rise in $[Ca^{2+}]_i$ in TXA_2- platelets. Basal GTPase activity was higher in platelet membranes from TXA_2- dogs (22.2 ± 6.7 pmoles/min/mg; n=14) and TXA_2 dogs (22.6 ± 7.1 pmoles/min/mg; n=11) than humans (15.5 ± 4.0 pmoles/min/mg; n=7). However, stimulation with 715 nM U46619 resulted in a significantly greater ($p<0.03$) rise in GTPase activity in platelet membranes from human (40.9 ± 10%; n=6) and TXA_2+ dogs (25.5 ± 9.9%; n=4) than TXA_2- dogs (10.8 ± 6.8%; n=5). Therefore, TXA_2- dog platelets have defective stimulus-response coupling in response to TXA_2 that appears to involve the TXA_2/PGH_2 receptor linkage to phospholipase C, possibly due to a dysfunctional G_p.

G 218 N-FORMYL-METHIONYL-LEUCYL-PHENYLALANINE AND PAF- INDUCED PROTEIN KINASE C TRANSLOCATION IN GUINEA-PIG ALVEOLAR MACROPHAGES. **Chakir Kadiri, Gisèle Cherqui, Joelle Masliah, Tania Rybkine, Brigitte Hermelin, Jacqueline Etienne, and Gilbert Bérézíat.** Laboratoire de Biochimie, URA CNRS 1283, 27 rue de Chaligny, Paris cedex 12, France. Two agonists stimulating the release of arachidonic acid in guinea-pig alveolar macrophages, fMLP (N-formyl-methionyl-leucyl-phenylalanine) and PAF (Platelet Activating Factor), were proved able to activate protein kinase C activity :both of them induced a transient translocation of PKC from cytosol to the membranes. By contrast, the translocation induced by 12-O-tetradecanoyl phorbol 3 acetate increased and led after 5hours to a 65% activity, indicating that PKC from alveolar macrophages could be effectively but not totally down-regulated by the phorbol ester. The effect of various inhibitors were measured on PKC activity, validating their use in this cell type.

G 219 A PERTUSSIS TOXIN SENSITIVE SUBSTRATE MEDIATES α_1-ADRENERGIC RECEPTOR REGULATION OF cAMP IN NEONATAL RAT VENTRICULAR MYOCYTES, Joel S. Karliner, Sondra Barrett and Norman Honbo, Cardiology Section, VA Medical Center and CVRI, University of California, San Francisco. Cyclic AMP stimulated by the β-agonist (-)-isoproterenol [ISO] (100 nM) was diminished when either the α-agonist (-)-phenylephrine (100 nM) or the mixed α + β agonist (-)-norepinephrine [NE] (100 nM) was coincubated with (-)-ISO suggesting that α-agonism regulates cAMP by its effect on cAMP synthesis. Evidence for α_1-receptor mediation of this response was enhancement of (-)-NE-stimulated cAMP by the selective α_1-antagonist terazosin (10 nM). The α_1-receptor mediated depression of (-)-ISO-stimulated cAMP and adenylyl cyclase activation was prevented by pertussis toxin pretreatment, suggesting involvement of a guanine nucleotide regulatory protein in this process. Evidence for depressed synthesis by α_1-agonism was inhibition by the mixed α + β agonist (-)-NE of β-agonist mediated cAMP generation in the presence of phosphodiesterase (PDE) inhibition by IBMX. Occupation of the α_1-receptor by (-)-NE did not accelerate the rate of cAMP breakdown in the absence of PDE inhibition. There was no enhancement of total PDE activity by (-)-NE in the presence of (-)-propranolol. By contrast, pertussis toxin pretreatment accelerated cAMP breakdown and also augmented PDE activity. Neither pertussis toxin nor (-)-NE increased cAMP egress. We conclude that in rat neonatal cardiac myocytes agonist occupation of the α_1-receptor modulates β-adrenergic stimulated cAMP accumulation by coupling to a guanine nucleotide inhibitory protein.

G 220 INSULIN INTERACTS WITH THE GUANINE NUCLEOTIDE-BINDING PROTEIN SYSTEM IN BC3H-1 MYOCYTES. E. Kilgour, L. Luttrell and G. Romero, Department of Pharmacology, University of Virginia School of Medicine, Charlottesville, Virginia, 22908.
Treatment of Murine BC3H-1 myocytes with Bordetella pertussis toxin attenuates some of the metabolic effects of insulin (Luttrell et al, 1988, J. Biol. Chem. 263:6134). We now provide evidence of direct effects of insulin on the functioning of guanine nucleotide-binding proteins (G-proteins). Insulin treatment of myocyte membranes increased, by 30-40%, high-affinity GTPase activity. Insulin also induced a two to three-fold increase in [^{32}P]-GTPγS binding to the membranes. In addition 100 μM GTP, or its non-hydrolyzable analogue GTPγS, reduced by 60-70% binding of [^{25}I]-iodoinsulin to myocyte membranes and this effect was mimicked by pertussis toxin. Scatchard analysis showed that this resulted from a loss of high-affinity binding with little change in low-affinity insulin binding. None of these effects appears to be correlated to tyrosine phosphorylation of G protein α-subunits. We conclude that the insulin signalling mechanism involves a direct interaction of the hormonal receptor with the regulatory G-protein system.

G 221 Go-α TRIGGERS MATURATION IN XENOPUS OOCYTES.
Spencer Kroll, Gilla Omri, Emmanuel M. Landau and Ravi Iyengar, Departments of Pharmacology and Psychiatry, Mount Sinai School of Medicine and The Bronx VA Medical Center, N.Y. One Gustave Levy Place, New York, NY 10029 The heterotrimeric G protein, G_O, can serve as the signal transducer in receptor regulated phospholipase C activation in the Xenopus oocyte. Activation of this pathway produces diacylglycerol, a second messenger that can serve to activate protein kinase C. We have microinjected a purified, activated form of the α subunit of G_O into Xenopus oocytes. Microinjection of this activated α_O subunit results in germinal vesicle breakdown (GVBD), which seems to be morphologically similar to the GVBD observed when oocytes are exposed to progesterone. Microinjection of a peptide encoding the pseudosubstrate region, a.a. 19-36, in the regulatory domain of protein kinase C, prevents GVBD elicited by activated α_O subunit. This peptide is a potent and specific inhibitor of protein kinase C. The peptide does not inhibit the progesterone stimulated GVBD. External calcium is not required by the oocyte for GVBD elicited either by progesterone or activated α_O subunit. Furthermore, incubation of oocytes with cycloheximide prior to microinjection of activated α_O subunit or addition of progesterone prevents the initiation of GVBD. These results suggest that persistent activation of protein kinase C activation is a mechanism for reentry into the cell cycle, causing oocyte maturation, and may represent a pathway independent of progesterone induced maturation. These independent pathways, however, may intersect at some point where protein synthesis is required for maturation.

G 222 REDUCTION OF ADENYLYL-CYCLASE ACTIVITY BY CHOLERA TOXIN IN MYELOID CELLS: DOWN-REGULATION OF Gsa SUBUNITS DURING CT TREATMENT.
M. LANOTTE AND S. HERMOUET , Centre G. Mayem, Hôpital St Louis, 75010 Paris, France.

The rat promyelocytic leukemia cell line IPC-81 is highly sensitive to cholera toxin (CT). A CT-resistant (CTr) clone has been isolated. CT-sensitive (CTs) and CTr cells are equally sensitive to cAMP inducers other than CT. In the presence of CT, CTr adenylyl-cyclase activity is decreased, as is the amount of Gsa (both 42 and 47 kDa subunits) in the membrane. Gsa proteins are undetectable in CTr cells after only 4hr CT exposure, while a 15 h CT exposure is necessary in CTs cells. The Gsa decrease by CT is not mediated by cAMP but requires ADP-ribosylation. Coincubation of membrane extracts from CTr cells treated by CT and cyc-S49 membranes does not restore cyclase activity, whereas following a similar coincubation of extracts from either untreated CTs or CTr cells with cyc- membranes, one observes cyclase activity. Following CT treatment, CTr cells require 20 to 30 days to recover normal Gsa levels and cyclase activity. No evidence for alterations in other G proteins (Gi, Go) was found. This may explain the resistance to CT previously observed in some myeloid cells.

G 223 FLUORIDE STIMULATES BRAIN MEMBRANE POLYPHOSPHOINOSITIDE (PPI) HYDROLYSIS BY A NOVEL MECHANISM, Li, P.P., Sibony, D. and Warsh. J.J. Section of Biochemical Psychiatry, Clarke Institute of Psychiatry, University of Toronto, Toronto, Ontario, M5T 1R8, Canada.
The effects of NaF and guanosine 5'-O-thiotriphosphate (GTPγS) on PPI hydrolysis was examined in rat brain cortical membranes prelabeled with [^{3}H]-inositol. GTPγS and NaF increased [^{3}H]-inositol monophosphate (InsP) with half-maximal stimulation at 1 μM and 2 mM; and maximal response at 30 μM and 10 mM, respectively. The maximal increases of InsP induced by GTPγS and NaF were 55 ± 4% and 340 ± 10 % of basal controls. The stimulatory effect of GTPγS (30 μM) on [^{3}H]InsP production was insensitive to Ca^{2+} (10^{-7} to 10^{-4} M), wheras NaF-evoked [^{3}H]InsP formation was dependent on Ca^{2+} concentrations. GDPβS (0.5 mM) significantly attenuated GTPγS- but not NaF-stimulated [^{3}H]InsP production. Co-incubation of GTPγS (30 μM) and submaximal concentrations of NaF (1 or 3 mM) stimulated [^{3}H]InsP formation which was nearly additive with that produced by either drug alone. However, the resultant accumulation of [^{3}H]InsP in the presence of maximally effective concentrations of GTPγS and NaF was not different from that produced by NaF alone. Incubation of cortical membranes with GTPγS and NaF for 1 min stimulated the accumulation of [^{3}H]-inositol bisphosphate ($InsP_2$) but not [^{3}H]InsP. [^{3}H]$InsP_2$ production elicited by GTPγS but not NaF was markedly enhanced by carbachol. Our findings of different characteristics of GTPγS- and fluoride-activation of PPI hydrolysis suggest that separate regulatory mechanisms are involved in these two modes of stimulation in brain membranes. While GTPγS-stimulated PPI hydrolysis in CNS is mediated by G-protein activation, the fluoride effect may be mediated by a direct stimulation of polyphosphoinositide phosphodiesterase or by activating a putative G-protein at a locus distinct from the GTP-binding site (i.e. not required GDP-GTP exchange).

G 224 **Reconstitution of Membrane G_p and Phospholipase C from Solubilized GH_3 Cell Membrane Components**, Thomas F.J. Martin and Judith A. Kowalchyk, Department of Zoology, University of Wisconsin, Madison, WI 53706
In GH_3 pituitary cells, thyrotropin-releasing hormone activates a membrane-associated phospholipase C (PL C) via a GTP-dependent mechanism that is insensitive to functional modification by pertussis or cholera toxins. To further characterize the putative G_p and PL C that mediate hormonal activation, efforts have been directed at establishing in vitro assays for G_p-PL C coupling. Several simple observations provided a format for the separation and subsequent functional reconstitution of crude preparations of G_p and PL C: 1. Coupled G_p-PL C in the membrane is N-EtMal-insensitive whereas uncoupled PL C is N-EtMal-sensitive; 2. Membrane PL C is reversibly salt (0.5-2.0M KCl)-extractable whereas G_p is not; 3. Membrane G_p is cholate (1%)-extractable. Hence, a solubilized G_p-free PL C was prepared by salt extraction of membranes. PL C-free G_p was prepared by cholate extraction of N-EtMal-treated, salt-extracted membranes and reconstituted by dialysis into PtdSer/PtdEt liposomes. Functional reconstitution of G_p with PL C was detected as GTP[S]-stimulated PtdIns-P_2 hydrolysis in reactions which required soluble PL C and G_p-containing liposomes in addition to [^{3}H]PtdIns-P_2 substrate. PL C activity was stimulated by .01-1 uM GTP[S] but not by other nucleoside di- or triphosphates. Purification of the components responsible for apparent reconstitution will be required to fully characterize this system.

G 225 N-FORMYL-METHIONYL-LEUCYL-PHENYLALANINE AND PAF-INDUCED ARACHIDONIC ACID RELEASE IN GUINEA-PIG ALVEOLAR MACROPHAGES. INVOLVEMENT OF A G-PROTEIN, ROLE OF PROTEIN KINASES. **Joelle Masliah, Chakir Kadiri, Tania Rybkine, Jacqueline Etienne and Gilbert Bereziat.** Laboratoire de Biochimie, URA CNRS 1283, 27 rue de Chaligny, Paris cedex 12, France. The chemotactic peptide fMLP (N-formyl-methionyl-leucyl-phenylalanine) and PAF (Platelet Activating Factor) stimulate arachidonic acid release from purified alveolar macrophages. Various pharmacological effectors were used to investigate the mechanism of arachidonic acid release by these two agonists. The fMLP- and PAF-induced arachidonic acid release was (1) mimicked by aluminium fluoride and inhibited by Pertussis toxin; (2) mimicked by A 23187, but not reduced by the calmodulin inhibitor R 24571; (3) mimicked by TPA (4) markedly decreased when protein kinase C had been down regulated or inhibited by sphingosine. These results suggest the participation of a G protein- and of protein kinase C in the regulation of the release of arachidonic acid The fMLP and PAF-stimulated arachidonic acid release was inhibited by cholera toxin and was potentiated by N-(2 methylamino-ethyl 5-isoquinoline-sulfonamide dihydrochloride) (H8), an exclusive protein kinase A inhibitor, suggesting a negative regulation by protein kina

G 226 MUSCARINIC STIMULATION OF Mn^{2+} ENTRY IN RAT PAROTID ACINAR CELLS,
Lawrence M. Mertz, Bruce J. Baum and Indu S. Ambudkar, CIPCB, NIDR, NIH, Building 10, Room 1N-113, Bethesda MD 20892.
Muscarinic stimulation of rat parotid acini results in both mobilization of internal Ca^{2+} stores and influx of extracellular Ca^{2+}. However, the point at which Ca^{2+} entry commences during stimulation remains poorly defined. In the presence of extracellular Mn^{2+}, activation of divalent cation (Mn^{2+}) entry can be monitored by assessing intracellular fura 2 fluorescence quenching at the calcium-insensitive wavelength (360 nm). With Mn^{2+} (50 uM) present in the cell medium, an increase over the basal fluorescence quenching rate commences 10-15 s after carbachol (10 uM) addition to acini. This stimulation is atropine-sensitive (0.1 uM) and does not appear to correlate temporally with the initial onset of intracellular Ca^{2+} release. Moreover, this stimulation in quench rate is transient since the basal quench rate is regained within 250 s after carbachol addition. Muscarinic stimulation of fluorescence quench rate is Mn^{2+} dose-dependent and is due to stimulation of Mn^{2+} entry into acinar cells, as determined by DTPA (100 uM) addition to the cell medium. Our observations suggest that Mn^{2+} may be used as a tool to assess activation of Ca^{2+} entry in these cells.

G 227 MAPPING OF β-ADRENOCEPTOR-G_S COUPLING DOMAINS BY SITE-SPECIFIC SYNTHETIC PEPTIDES.
Gerald Münch, Mirko Hekman, Christian Dees and Dieter Palm, Department of Physiological Chemistry, University of Würzburg Medical School, D-8700 Würzburg, Fed. Rep. Germany
The components of the β-adrenoceptor-mediated adenylate cyclase system, which consist of the β-adrenergic receptor (βAR), stimulatory G-protein (G_S) and adenylate cyclase (C), have been purified and reconstituted into lipid vesicles. The assignment of functional domains involved in receptor-G_S interaction was based as yet on site-directed mutagenesis and is still under investigation. In the present study, the βAR-G_S interaction has been probed using synthetic peptides corresponding to the known amino acid sequence of the turkey erythrocyte βAR. The peptides tested correspond mainly to the conserved regions of the intracellular loops and were able to influence the βAR-G_S interactions. Peptides covering parts of the second and third loop and the N-terminal part of the C-terminus inhibited hormone-induced cyclase activity in a concentration-dependent manner with an IC_{50} value of 15-20 μM. By synthesizing overlapping peptide fragments, contact domains in these regions could be defined precisely. In contrast, peptide T284-295 (part of loop i3) increased G_S-mediated cyclase activity even in the absence of hormone, suggesting a key role for this region in G_S activation. No effects were observed with peptides corresponding to extracellular parts or with unrelated peptides of similar length and charge. A possible influence of receptor peptides on the coupling of G_S to adenylate cyclase was tested by using AlF_4^--activated G_S. There was no significant inhibition of cyclase activity by receptor peptides up to 100 μM concentrations.

G 228 THROMBIN AND PARATHYROID HORMONE UTILIZE DISTINCT PATHWAYS TO MOBILIZE INTRACELLULAR CALCIUM IN RAT OSTEOSARCOMA CELLS, Robert A. Nissenson, Michael Babich, and Hester Choi, Endocrine Unit, Veterans Administration Medical Center and Departments of Medicine and Physiology, University of California, San Francisco, CA 94121.
UMR 106 rat osteosarcoma cells retain phenotypic properties of osteoblasts including receptors for the bone resorbing agents parathyroid hormone (1-34) (PTH) and human α-thrombin (T). We have isolated a subclone (UMR 106-H5) that displays increased intracellular calcium (Ca_i^{2+}) in response to PTH and to T, assessed by indo-1 fluorescence. The objective of the present study was to compare the cellular pathways utilized by these two agents. PTH and T each produced a spike-phase Ca_i^{2+} response that returned to baseline within 75 seconds. Neither agent elicited a sustained increase in Ca_i^{2+}, and the magnitude of the spike-phase response to maximal concentrations of PTH or T (3-to-4 fold over basal) was unaffected by the prior acute addition of excess EGTA or 16-hour exposure to pertussis toxin (100 ng/ml). Maximal concentrations of PTH and T produced additive effects on Ca_i^{2+} in the presence or absence of extracellular calcium. Analysis of Ca^{2+} using interactive laser-based image analysis confirmed that PTH and T acted in an additive fashion on individual UMR 106-H5 cells. T elicited a rapid increase in inositol phosphate (IP_2 and IP_3) production in UMR 106-H5 cells labeled with [^{3}H]myo-inositol. T-stimulated IP production was insensitive to pertussis toxin, but was inhibited by GDPβS (100 μM) in saponin-permeabilized cells. In contrast to T, PTH produced little or no increase in IP production in UMR 106-H5 cells, and failed to potentiate the IP response to T. These results indicate that: 1) T mobilizes Ca_i^{2+} in UMR 106-H5 cells by activating PI-specific phospholipase C via a pertussis toxin-insensitive G-protein; 2) PTH mobilizes a distinct cellular pool of calcium, apparently by a mechanism other than the classical PI turnover pathway.

G 229 PURIFICATION AND PARTIAL CHARACTERIZATION OF ARG-SPECIFIC ADP-RIBOSYLTRANSFERASE FROM SKELETAL MUSCLE MICROSOMAL MEMBRANES. J.E. Peterson, J.S.A. Larew, and D.J. Graves, Department of Biochemistry and Biophysics, Iowa State University, Ames, IA 50011. Integral membrane-associated arginine specific mono-ADP-ribosyl-transferase was purified from rabbit skeletal muscle microsomes. The ADP-ribosyltransferase was solubilized from the 100,000 x g pellet with 0.3% sodium deoxycholate and purified to $\geq$ 95% homogeneity by successive DE-52, concanavalin A agarose and 3-amino-benzamide agarose chromatography steps in the presence of detergents. Two molecular weight forms of the enzyme were isolated and partially characterized. The apparent M_r of the α-form of the enzyme purified to $\geq$ 95 homogeneity was about 39,000 $\pm$ 500 as estimated on silver-stained sodium dodecyl sulfate polyacrylamide gel electrophoresis. The M_r of the β-form purified to $\geq$ 50% homogeneity was 38,500 $\pm$ 500. The rapid procedure results in a 2382-fold purification with a 0.1% yield for the α-form and 1.6% yield of activity for the β-form, relative to the homogenate. Positive identification of the enzyme was confirmed by utilizing a zymographic in situ gel assay and by gel slice incubations with a guanylhydrazone substrate HPLC assay. The specificity of the mono-ADP-ribosyltransferase zymographic assay was characterized by hydroxylamine sensitivity, 3-amino-benzamide inhibition, and histone dependence.

G 230 PROLACTIN STIMULATED MITOGENESIS OF LYMPHOID NB2 CELLS IS G PROTEIN MEDIATED, J.T. Phillips, K.A. Meyers and L. A. Krupicki, Department of Neurology, University of Texas Southwestern Medical Center, Dallas, TX 75235-9036
The NB2 rat lymphoma cell line is dependent on exogenous prolactin (PRL), present in serum, for growth. This model system affords the opportunity to define hormone mediated events which lead directly to cellular proliferation. Although NB2 proliferation is known to be sensitive to pertussis and cholera toxins, suggesting G protein involvement, little else is known about PRL signal transduction mechanisms in this or other PRL dependent systems. Our studies indicate that guanine nucleotides GTP and GTPγS (10fM - 100μM) decrease the binding of radioiodinated rat PRL to the NB2 PRL receptor. Analysis of equilibrium binding data from NB2 membrane preparations shows the GTP effect is due to an increase in the PRL receptor Kd from 5 nM to 40 nM in the presence of 100 μM GTP or GTPγS. Similar concentrations of GDP, GMP, ATP and ATPγS are without effect. Furthermore, kinetic studies indicate a marked increase in the binding dissociation rate in the presence of 100 μM GTPγS, but no effect of identical concentrations of GDP, GDPβS, GMP, ATP or ATPγS. These studies provide compelling evidence consistent with G protein regulation of PRL receptor interactions and links these events to subsequent NB2 cellular proliferation.

G 231 REGULATION OF ADENYLYL CYCLASE IN HETEROLOGOUS DESENSITIZATION BY cAMP-DEPENDENT PROTEIN KINASE, Richard T. Premont and Ravi Iyengar, Department of Pharmacology, Mount Sinai School of Medicine, New York, NY 10029.
Chick hepatocytes in primary culture undergo both homologous and heterologous desensitization of adenylyl cyclase in response to glucagon. Heterologous desensitization is characterized by a rapid loss of NaF-stimulated adenylyl cyclase activity which is rapidly reversed upon removal of the agonist, and is associated with loss of Gs activity in treated cell membranes. Purified erythrocyte Gs added to desensitized cell membranes, over a range of concentrations, is unable to restore NaF stimulation to control levels, indicating some loss in the ability of the desensitized adenylyl cyclase to respond to Gs. 8-Br-cAMP treatment of hepatocytes induces a small loss of NaF-stimulated adenylyl cyclase activity. Gs added to 8-Br-cAMP-treated membranes is also unable to restore control levels of activity. Membranes incubated in vitro with cAMP-dependent protein kinase demonstrate a reduction in NaF-stimulated adenylyl cyclase activity which can be blocked by WIPTIDE, a protein kinase A pseudosubstrate. Heterologous desensitization in chick hepatocytes thus appears to result from both loss of Gs activity and from cAMP-induced loss in the ability of adenylyl cyclase to respond to Gs. This second component is consistent with cAMP-induced phosphorylation of the catalyst of adenylyl cyclase. Incubation of S49 kin- cell membranes with protein kinase A similarly induces a loss in NaF-stimulated adenylyl cyclase activity, indicating that protein kinase A phosphorylation of adenylyl cyclase may lead to a reduction in the ability of Gs to stimulate adenylyl cyclase in many tissues.

G 232 FLOW CYTOMETRIC ANALYSIS OF CALCIUM MOBILIZATION BY MUSCARINIC RECEPTORS ON A PC12 SUBCLONE ESTABLISHED BY POSITIVE SELECTION OF ACETYLCHOLINE INDUCED CALCIUM RESPONSIVENESS, John Ransom, Holly Cherwinski, Ron Delmendo, Jack Dunne, Naj Sharif and Richard Eglen, Institutes of Molecular Immunology, Immunology and Experimental Pharmacology, Syntex Research, Palo Alto, CA 94304.

Single cell calcium (Ca) mobilization was studied by flow cytometry using indo-1 loaded PC12 cells. Due to their heterogeneity in terms of morphology and muscarinic responsiveness, cells which gave large Ca responses were singly sorted, cultured and re-analysed. Muscarinic responses of a stable subclone, ACH2, were studied further. 70% of ACH2 cells respond to 10 uM acetylcholine (Ach). All cells were inhibited by 10 nM atropine or 10 uM pirenzipine. Binding studies indicate the presence of a single population of binding sites characteristic of M4 receptors. For either bradykinin (BK), Substance-P (SP) or Ach stimulation, an identical number of cells mobilize Ca whether Ca is present in the medium or chelated by EGTA. Response to Ach, but not to BK or SP, is blocked by pertussis toxin. In Ca free media prestimulation of the cells with Ach does not affect a subsequent response to BK or SP, but prestimulation with BK or SP strongly inhibits a subsequent muscarinic response. Thus, the response can be desensitized by either BK or SP. Results suggest that this receptor is coupled to intracellular Ca mobilization by a pertussis toxin sensitive GTP-binding protein.

G 233 LIPOPEPTIDE INDUCED LEUKOCYTE ACTIVATION - TRANSMEMBRANE SIGNALLING MECHANISMS AND ROLE OF G-PROTEINS. P. Scheipers, W. G. Bessler, and S. Hauschildt, Institut für Immunbiologie der Universität, D-7800 Freiburg, FRG

Synthetic lipopeptide analogues of bacterial lipoprotein constitute potent macrophage and B-lymphocyte activators. In vitro they induce B-lymphocyte proliferation and differentiation. In macrophages they initiate the release of interleukin 1, of prostaglandin E and F2, and induce tumor cytotoxicity (1, 2). The signal transduction mechanisms utilized by lipopeptides are still poorly understood. In B-lymphocytes internal Ca^{2+} concentrations raise upon treatment with lipopeptides. Lipopeptide fails to induce PI degradation, PKC-translocation and the generation of the second messengers cAMP and cGMP (3). To test whether G-proteins are involved in lipopeptide induced signal transduction we investigated the effect of lithiumchlorid, pertussistoxin and choleratoxin on B-lymphocyte proliferation. Choleratoxin reduced the proliferation rate but only at high concentrations (0.5 µg/ml), while pertussistoxin had no effect on B-lymphocyte proliferation. In the macrophage cell line P 388 D_1, upon lipopeptide stimulation, PI turnover is enhanced (accompanied by PKC translocation from the cytosol to the plasma membrane), but not in bone marrow derived macrophages. The role of G-proteins in the macrophage system is currently being investigated.

1. W. G. Bessler et al., J. Immunol. 153, 1900 (1985). 2. P. Hoffmann et al., Biol. Chem. Hoppe-Seyler 370, 575 (1989). 3. U. Steffens et al., Mol. Imunol. 26, 897 (1989).

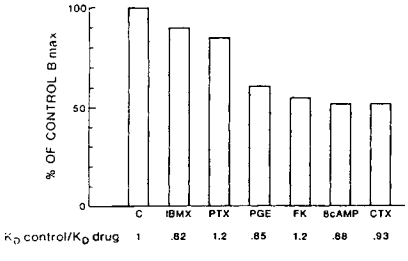
G 234 INVOLVEMENT OF A PTX-SENSITIVE G PROTEIN IN DIHYDROPYRIDINE MODULATION OF VOLTAGE DEPENDENT CALCIUM CHANNELS IN PC12. Schettini G., Meucci O., Grimaldi M., Florio T., Landolfi E., Scorziello A., Ventra C. Inst.of Pharmacology, II School of Medicine, University of Naples, Via S. Pansini 5, 80131 Naples ITALY.

Recently it has been reported that a G protein is involved in dihydropyridine (DHP) modulation of voltage dependent calcium channels (VDCC). In the present study we evaluated the effect of PTX pretreatment on agonist and antagonist DHP derivatives modulation of both maitotoxin (MTX), a marine toxin which increases intracellular calcium levels, likely through the activation of VDCC, and K^+-activated calcium channels in PC12 cells. In these cells PTX pretreatment does not influence K^+ or MTX-induced calcium rise, measured by Fura 2 fluorescent probe, and it has also no effect on nicardipine inhibition of K^+-activated calcium channels. Conversely the dose dependent nicardipine inhibition of MTX-induced calcium flux is significantly reduced by PTX treatment. Bay-k 8644 potentiates K^+-evoked calcium influx with a maximal efficacy at 100nM. The addition of Bay-k 8644 right after K^+ is able to prolong the depolarization-dependent calcium flux. Bay-k 8644 potentiation of K^+-activated calcium channels is reduced by PTX pretreatment. These results suggest that a PTX-sensitive G protein may be involved in DHP modulation of VDCC in PC12 cells.

G 235 MECHANISM OF ACTION OF GLYCOPROTEIN HORMONES: NATURE OF HUMAN CHORIOGONADOTROPIN-LUTROPIN RECEPTOR GTP-BINDING PROTEIN INTERACTION, Prem K. Seth and Om P. Bahl, Department of Biological Sciences, State University of New York at Buffalo, Buffalo, NY 14260. The biological function of glycoprotein hormones involves the interaction of the specific receptor and GTP binding protein, regardless whether it is medicated by cAMP or IP3. Therefore, this investigation was undertaken to study the nature of receptor-G protein interaction. It is not known whether the receptor as such or in its aggregated form interacts with G protein (specifically GS), covalently or non-covalently. The possibility of covalent linkage emerged from our earlier observation of the presence of free -SH groups in human choriogonadotropin/lutropin (hCG/LH) receptor and G proteins. Preliminary evidence based on the following experiments suggests a direct interaction of hCG/LH receptor and Gs protein. (i) The extract of rat ovarian membranes treated with hCG showed a major peak of Gs protein which elutes at ve/vo of 1.3 (Peak I) of Biogel A.5m column. However, in the control extracts Gs protein elutes at lower molecular weight position of ve/vo = 1.6 (Peak II). Similar pattern was seen for hCG/LH receptor as judged by binding of ^{125}I-hCG to the column fractions. (ii) The binding of ^{125}I to hCG/LH receptor present in Peak I was inhibited by GTP. However, GTP did not inhibit the ligand binding to the hCG/LH receptor present in Peak II. (iii) Preincubation of the crude receptor with hCG enhanced the binding of hCG/LH receptor with GTP-Sepharose by about 30%. (iv) An affinity column of hCG-Sepharose binds with Gs protein, in addition to hCG/LH receptor. All these experiments suggest that hCG induces the formation of hCG/LH receptor and Gs protein complexes which elute in the higher molecular weight Peak I. The biochemical nature of these complexes is being further investigated by experiments using purified hCG/LH receptor and Gs protein in artificial membrane, liposomes. Supported by U.S.P.H.S. Grant #RO1-08766.

G 236 ACTIVATION OF PROTEIN KINASE C (PKC) INHIBITS INTRACELLULAR (IC) Ca^{2+} MOBILIZATION AND UPTAKE, INOSITOL TRISPHOSPHATE ($InsP_3$) FORMATION, AND PARATHYROID HORMONE (PTH) RELEASE IN BOVINE PARATHYROID CELLS (PTCs). Dolores Shoback and Tsui-Hua Chen. Endocrine Unit, VA Hospital, University of California, San Francisco, CA 94121.
Low extracellular (EC) Ca^{2+} stimulates, and high EC Ca^{2+} inhibits PTH release. The effects of PKC activation on PTH secretion depend on the EC Ca^{2+}. PKC agonists reduce PTH release at low EC Ca^{2+} and block high EC Ca^{2+}-induced inhibition of secretion. Since raising EC Ca^{2+} increases intracellular free Ca^{2+} ($[Ca^{2+}]_i$) and $InsP_3$ in PTCs, we examined the effects of phorbol myristate acetate (PMA) on high EC Ca^{2+}-induced $InsP_3$ formation and Ca^{2+} mobilization and of the combination of PMA (10^{-6} M) + the Ca^{2+} ionophore ionomycin (Iono) on PTH secretion. We measured $[Ca^{2+}]_i$ with Fura 2, InsPs after labeling with ^{3}H-myoinositol, and PTH by RIA. With raising EC Ca^{2+} from 0.5 to 2 mM, $[Ca^{2+}]_i$ rose from 260±11 to 713±48 (peak,P) and 574±45 nM (sustained,S) in control cells and from 262±11 to 475±11* (P) and 397±17 nM (S) in PMA-treated cells (*p<.02). IC Ca^{2+} responses to Iono were also reduced in PMA-treated cells with 550±32*/394±16* nM vs 738±49/501±30 nM in controls for P/S responses at 0.5 mM EC Ca^{2+}, respectively. At "0" EC Ca^{2+} (no added Ca^{2+}+2 mM EGTA), Iono increased $[Ca^{2+}]_i$ to 461±19/166±6 nM (P/S) in controls but only to 323±15*/157±15 nM (P/S) in PMA-treated cells. High EC Ca^{2+}-induced increments in $InsP_3$, $InsP_2$, and $InsP_1$ within 5 seconds of raising EC Ca^{2+} from 0.5 to 3 mM were substantially inhibited in cells after ≥5 minutes of exposure to PMA. The addition of Iono to PMA-treated PTCs at high EC Ca^{2+} did not restore the expected inhibition of secretion. We conclude that PKC activation decreases IC Ca^{2+} mobilization and membrane Ca^{2+} uptake potentially via changes in $InsP_3$ but that other second-messenger systems are required for high Ca^{2+}-induced inhibition of PTH secretion.

G 237 CYCLIC AMP-DEPENDENT REDUCTION OF [^{3}H]BATRACHOTOXININ (BTXB) BINDING TO THE CARDIAC SODIUM CHANNEL, Mohammed Taouis, Roger J. Hill, Robert S. Sheldon, Leslie M. Wilson, Henry J. Duff, Division of Cardiology, Department of Medicine, Calgary, Alberta T2N4N1 Canada. Long-term exposure of myocytes to agents that increase intracellular cAMP induce sodium channel upregulation*. We determined if this cAMP effect is similar following a 30 minute exposure in freshly isolated cardiac myocytes. This was done by assessing the effect of agents that increase intracellular cAMP [8 Bromo cAMP(8cAMP), forskoline(FK), cholera toxin(CTX), pertussis toxin(PTX), isobutylmethylxanthine(IBMX), prostaglandineE1(PGE)] using a sodium channel specific radioligand BTXB. The histogram represents the percentage of control specific binding (Bmax) and the ratio of the affinity(KD). Conclusions: agents which increased cAMP levels induced a reduction of sodium channel density without any modification of BTX affinity. Thus this short term effect of cAMP is in contrast to upregulation observed following chronic exposure. This suggests that acute effects of cAMP on the cardiac sodium channel may be mediated by a cAMP-dependent kinase



*Sherman et al., J. Neurosc., 5:1570, 1985

G 238 MODULATION OF G PROTEINS FOLLOWING CHRONIC κ OPIATE AGONIST TREATMENT IN RAT SPINAL CORD-DORSAL ROOT GANGLION COCULTURES. Z. Vogel, D. Saya, S.Y. Nah. and B. Attali. Dept. of Neurobiology, Weizmann Institute of Science, Rehovot, Israel. κ-Opiate agonists inhibit the activity of adenylate cyclase and of the dihydropyridine sensitive voltage-dependent Ca^{2+} channels in rat spinal cord-dorsal root ganglion cocultured cells. Following chronic exposure of the cells to κ agonists, there is a decreased ability of κ receptors to inhibit adenylate cyclase activity and Ca^{2+} influx. Interestingly, this desensitization process was found to be heterologous, since in these κ opiate-treated cultures the α_2 adrenergic agonist clonidine and the muscarinic agonist carbachol exhibited a lower potency for inhibiting both cyclase activity and Ca^{2+} channels, as compared to paired control cultures. We explored for possible impairment at the GTP-binding protein level. We found that chronic opiate treatment leads to a marked reduction (>50%) in the susceptibility of $G\alpha_i$ and $G\alpha_o$ subunits to pertussis toxin-catalyzed ADP-ribosylation. No significant differences were found in the cholera toxin-induced ADP-ribosylation of a 45 kDa substrate protein. Using selective polyclonal antibodies, we observed a marked down-regulation of the $G\alpha_i$ subunit (including $G\alpha_{i\ .1}$). No significant changes in the immunoreactive levels of the α_o and β subunits were detected. It is suggested that the alterations in G protein transduction system may account for the opiate-induced desensitization and heterologus desensitization. *Supported by the Israel Ministry of Health and the German-Israel Foundation for Scientific Research and Development.*

G 239 ULTRASTRUCTURAL CORRELATES OF MEIOTIC MATURATION AND cAMP-DEPENDENT MEIOTIC ARREST IN BOVINE OOCYTES, Scott D. Webster, Department of Zoology, Arizona State University, Tempe, AZ 85287. Meiotic resumption in bovine oocytes is characterized physiologically by germinal vesicle breakdown, apparent under the light microscope by 9-10 hr after release from the follicle. Prior to this is a commitment period beginning at 7 hr during which no changes in the germinal vesicle are observable by light microscopy but the onset of breakdown cannot be prevented. It has been demonstrated that meiotic arrest in mammalian oocytes is maintained in the presence of an active adenylate cyclase. Thin sections of plastic-embedded bovine oocytes were examined with the electron microscope to compare oocyte ultrastructure in the presence or absence of high levels of cAMP. Immature oocytes were mass-harvested from ovaries and cultured in medium with or without isobutylmethylxanthine (IBMX), an inhibitor of cAMP-phosphodiesterase. Oocytes were examined for evidence of ultrastructural changes associated with the commitment period which were prevented by IBMX. After 7 hr in culture changes in the nucleus, including the disappearance of electron-dense granules at the site of the nuclear membrane and apparent convolutions of the nuclear envelope, were observed in control oocytes (-IBMX) but not in oocytes cultured in medium containing 5 mM IBMX. This suggests that signal transduction via cAMP has effects at the level of the nuclear membrane, which may contribute to the mechanism of the previously characterized inhibition of meiotic resumption by enhanced adenylate cyclase activity.

G 240 G-PROTEIN TRANSDUCTION IN EXTRACELLULAR ATP MODULATION OF SECRETION FROM CHROMAFFIN CELLS, E.W. Westhead, Maria Diversé-Pierluissi and K.T. Kim, Program in Molecular and Cellular Biology, University of Massachusetts, Amherst, MA 01003
Cultured cells of the adrenal medulla (chromaffin cells), widely used as models of neuronal secretion, secrete 150 mM ATP along with catecholamines. In recent years ATP has been identified as a co-transmitter in several neuronal systems. We have discovered that extracellular ATP, in micromolar concentrations, has three, apparently separate, effects on secretion. Alone, ATP can release Ca^{2+} from internal stores, cause influx of Ca^{2+} and stimulate secretion. Added together with a nicotinic agonist, it will enhance the nicotinic secretory response. Added to cells 30 seconds to 180 seconds prior to nicotinic stimulation, ATP inhibits the nicotinic response. Independent receptors seem to be responsible for these effects because each can be specifically mimicked by an ATP analogue. UTP releases Ca^{2+} from internal stores, ADP produces inhibition and $ATP\gamma S$ enhances secretion. Pertussis toxin specifically blocks the inhibitory effect and cholera toxin specifically blocks the enhancing effect. We have partially identified the G-proteins responsible using a combination of antibodies and labeling with ^{32}P-NAD. The latter is used to post-label membranes from cells that have been toxin-treated in the presence or absence of ADP, ATP or $ATP\gamma S$. Measurements of second-messenger concentrations show little correlation with inhibition or activation, but cytosolic calcium increases do correlate with changes in secretion. The results suggest that ATP activation and inhibition may couple through G-proteins directly to ion channels. Supported by USPHS Grant NS 26606.

G 241 INVOLVEMENT OF A Gs GTP-BINDING PROTEIN IN THE REGULATION OF HUMAN NATURAL KILLER CELLS, Margaret M. Whalen and Arthur D. Bankhurst, Department of Medicine, University of New Mexico School of Medicine, Albuquerque, NM 87131. Membranes from highly purified natural killer (NK) cells were ADP-ribosylated by treatment with cholera toxin (CTX) (20 μg/ml) in the presence of [^{32}P]NAD. This was followed by SDS-PAGE on a 10% gel and autoradiography. CTX treatment resulted in a single band of ^{32}P incorporation at 44,000 Mr. CTX treatment (5 μg/ml) of intact NK cells caused a nine fold increase in cAMP concentrations. Pretreatment of NK cells with CTX diminished their ability to lyse the K562 tumor cell by as much as 79%. Forskolin treatment (10 μM) elevated NK cell cAMP levels eight fold and decreased lysis of K562 cells by as much as 45%. These data suggest that Gs coupled to adenylate cyclase is involved in transducing signals which inhibit NK cell lysis of tumor cells. Pretreatment with CTX diminishes the ability of NK cells to bind to K562 cells (binding is necessary for lysis) by approximately 50%. Inhibition of binding is also seen with forskolin treatment. This suggests that the NK-cell receptor(s) for the tumor cell may be altered as a consequence of cAMP mediated events or by activation of Gs.

G 242 G-PROTEINS IN BRAIN: EFFECTS OF CHRONIC ETHANOL INGESTION, James P. Whelan, Paula L. Hoffman and Boris Tabakoff. Section on Receptor Mechanisms, LPPS, DICBR, National Institute on Alcohol Abuse and Alcoholism, Bethesda, MD 20892.

Chronic ethanol ingestion produces apparent "heterologous desensitization" of receptor-coupled adenylate cyclase in mouse cerebral cortex. The data suggested a qualitative or quantitative change in the stimulatory guanine-nucleotide binding protein (Gs) in cortex and also in hippocampus (Saito, et al., J. Neurochem. 48: 1817, 1987; and Valverius, et al. J. Neurochem. 52: 492, 1989), since there was a decrease in cholera toxin-induced ^{32}P-ADP ribosylation of a protein migrating like Gsα from cortical membranes of ethanol-treated mice (Nhamburo, et al., Substance Abuse 7: 99, 1988). We have quantitated C57BL/6 mouse brain Gsα by Western blotting using an antiserum (obtained from A. Spiegel, NIDDK) that recognizes both forms of brain Gsα (46kD and 52kD). The level of the 52 kD form is similar in cortex, hippocampus, cerebellum and striatum. However, the level of the 46 kD form varies dramatically among brain areas: the 46kD/52kD ratio is 0.8 in cortex, 0.65 in hippocampus, 0.1 in cerebellum and 2.5 in striatum. We are currently measuring guanine-nucleotide stimulation of adenylate cyclase activity in these brain areas to evaluate functional consequences of these differences. Chronic ethanol ingestion did not alter the quantity or the ratio of forms of Gsα in any brain region. Qualitative alteraltions of Gsα are being assessed by 2-D electrophoresis.

Small Molecular Weight G-Protein, SMGs

G 300 ANALYSIS OF G_n PROTEINS BY TWO-DIMENSIONAL POLYACRYLAMIDE-GEL ELECTROPHORESIS: IDENTIFICATION OF SOME OF THE PLATELET G_n27 PROTEINS AS *ral* GENE PRODUCTS. Rajinder P. Bhullar*, Pierre Chardin+ and Richard J. Haslam*, *Department of Pathology, McMaster University, Hamilton, Ontario, Canada L8N 3Z5 and +INSERM U-248, Faculté de Médicine Lariboisière-Saint-Louis, 75010 Paris, France.

G_n proteins represent a family of 23-27 kDa GTP-binding proteins that can be specifically labelled by [α-^{32}P]GTP on nitrocellulose blots of membrane proteins that have been separated by SDS-PAGE (Bhullar & Haslam, (1987) Biochem. J. 245, 617-620). Polyclonal antibodies raised against specific recombinant low molecular mass GTP-binding proteins were tested for their ability to recognize human platelet membrane G_n proteins. Partially purified G_n proteins were separated by SDS-PAGE and transferred electrophoretically to nitrocellulose sheets, which were shaken in solution containing 10 mM Tris-HCl, pH 7.5, 0.154 M NaCl and 1% (w/v) haemoglobin. The blots were then incubated with various antisera and bound antibody was detected using ^{125}I-labelled protein A. An antiserum against simian *ral*A protein recognized a 27 kDa protein with the same apparent molecular mass as the major platelet G_n27 protein. The partially purified G_n proteins were further analyzed by two-dimensional polyacrylamide-gel electrophoresis, which permitted resolution of 12 major G_n protein forms, seven of 27 kDa (G_n27*a*-*g*), one of 26 kDa (G_n26) and four of 24 kDa (G_n24*a*-*d*). The *ral*A antibody reacted strongly with the five most basic G_n27 species (G_n27*a*-*e*), weakly with G_n26 and not at all with G_n27*f*, G_n27*g* or G_n24*a*-*d*. The results demonstrate that multiple GTP-binding polypeptides with a molecular mass of 27 kDa are present in the platelet and that only some of these are products of *ral* genes.

G 301 CHARACTERIZATION OF $p25^{rab3A}$: TISSUE DISTRIBUTION AND REGULATION OF GTPase ACTIVITY BY CELLULAR FACTOR(S), Ethan S. Burstein, Adriana Moscucci, Alan Wolfman, Ann Moss, Ian G. Macara, Environmental Health Sciences Center, University of Rochester, Rochester, New York 14642. We detected a 25 kd guanine nucleotide-binding protein by immunoblotting with an anti-peptide antiserum developed against a sequence near the C-terminus of $p25^{rab3A}$. This protein was observed both in cytosolic and membrane fractions of brain tissue. Western blot analysis of five regions of the rat brain indicated that $p25^{rab3A}$ is most abundant in hypothalamus and hippocampus. Immunostaining of coronal sections of the rat brain diencephalon revealed that $p25^{rab3A}$ is expressed exclusively in neurones. This protein was not detected in other tissues. We have purified $p25^{rab3A}$ to near homogeneity using the method of Kikuchi *et al.* The dissociation constant for GDP was 100 fold smaller in the presence of Mg2+ than in the presence of excess EDTA. GDP binding was not inhibited by N-ethylmaleimide. The intrinsic catalysis rate constant for this protein was 0.012/min. In the presence of detergent solubolized brain tissue, the GTPase activity was increased 2-fold. The GTPase stimulating activity of the cellular factor(s) towards $p25^{rab3A}$ was dose dependent and saturable. We were able to resolve GAP activity towards $p25^{rab3A}$ from GAP activity towards $p21^{ras}$ on a Superose 12 column. Furthermore, recombinant ras-specific GAP had no activity towards $p25^{rab3A}$, suggesting the activity we see is specific for the rab3A protein.

G 302 S. CEREVISIAE'S CDC25 AND SCD25 PROTEINS: PROTOTYPES OF GUANINE NUCLEOTIDES RELEASING FACTOR FOR GTP/GDP-BINDING PROTEINS ? J. H. Camonis, E. Boy-Marcotte, H. Garreau, F. Damak, R. Guilbaud, D. LeRoscouet, J.B. Crechet*, P. Poulet*, M. Yves*, A. Parmeggiani*. Groupe IGD, CNRS URA1354, Bât.400, Université Prais-Sud, 91405 Orsay cedex, France, and Laboratoire de Biochimie, CNRS URA144, Ecole Polytechnique, 91128 Palaiseau cedex, France.

In budding yeast, the CDC25 gene acts through RAS proteins as a positive effector of adenylatecyclase which is required at START, the yeast equivalent of mammalian restriction point during cell cycle.

The $RAS2^{gly->val19}$ allele, and the $RAS2^{thr->ile152}$ allele, found in a spontaneous suppressor of the cdc25 mutation, allow the CDC25 requirement for growth to be bypassed. Biochemical properties of RAS, $RAS2^{val19}$ and $RAS2^{ile152}$ produced in and purified from E. coli show that, although $RAS2^{val19}$ gives rise to active proteins with a reduced GTPase activity, the $RAS2^{ile152}$ product has affinity characteristics for guanyl nucleotides that lead to an accelerated exchange rate. Computer simulations show that this acceleration leads to an increase in the GTP-bound form of the $RAS2^{ile152}$ product. In an in vitro adenylatecyclase stimulation test with GTP present in the buffer, only $RAS2^{ile152}$ was able to stimulate adenylatecyclase when proteins are precharged with GDP-as if this protein was the only one able to avoid the need of an "exchange factor".

By immunodetection, a 200 kd product associated with cellular membranes was detected as the product of CDC25.

Another gene, SCD25, was cloned by the ability of the C-terminal part of its product to suppress the cdc25 mutation. SCD25 and CDC25 proteins share common sequences, especially in their C-terminal ends. However, the SCD25 entire gene, even when expressed from a multicopy plasmid, is unable to suppress the cdc25 mutation. We propose that the C-terminal part of CDC25 and SCD25 contain the prototype of a domain acting as a guanyl nucleotide releasing factor toward GTP/GDP binding proteins. They could be the first members of a family whose relatives have to be identified, in yeast and other species.

The "releasing factor hypothesis" was strenghtened by the capacity of the SCD25 C-terminal domain produced in E. coli to greatly enhance the dissociation rate of GDP-bound, and to a lower extend, of GTP-bound forms of RAS2 proteins and of $p21^{c\text{-}Ha\text{-}ras}$.

G 303 H-RAS p21 ACTIVE SITE AND 'EFFECTOR' DOMAIN MUTANTS: STRUCTURE/FUNCTION INVESTIGATIONS, Sharon Campbell-Burk and Drew E. Van Dyk, E. I. du Pont de Nemours and Company, Central Research and Development Department, Experimental Station, P. O. Box 80328, Wilmington, DE 19880-0328; Channing Der, LaJolla Cancer Research Foundation, LaJolla, CA; David Jameson, University of Hawaii, Oahu, HI.

We have been studying several different mutants with single and double amino acid substitutions in the guanine nucleotide binding domain and/or putative effector domain of ras-p21. The biological and biochemical properties of these mutants are being characterized. We have been using NMR spectroscopy, molecular modeling, and fluorescence spectroscopy to investigate the structural basis for functional differences observed between wildtype and mutant p21 proteins.

The NMR spectroscopic approaches are directed at two levels: 1) assignment of the proton NMR spectrum of H-ras, and 2) use of stable isotopes (^{15}N, ^{13}C) as site specific probes, much like spin label and fluorescence probes, to quickly assess perturbations in the protein as a function of single amino acid substitutions and binding of specific guanine nucleotide substrates.

Two different position 12 mutants have been studied (D12 N-ras, R12 H-ras). Perturbations were observed in the phosphoryl binding domain and the effector domain. The structural basis of these perturbations is currently being investigated. In addition, several ras mutants with single amino acid substitutions were generated in the 'putative' effector or target recognition domain of H-ras p21. This domain has been identified as being critical for biological activity and is believed to be the site of interaction with GAP, a protein capable of stimulating the GTPase activity of ras proteins. Single tryptophan containing proteins were made to aid in biophysical analysis of these proteins. H-ras effector mutants with transforming and non-transforming properties were generated by site-directed mutagenesis and characterized using isotope-edited NMR techniques as well as steady state and time resolved fluorescence spectroscopy. The biological and biochemical properties of these H-ras mutants are currently being characterized. Our preliminary results support a dual role for GAP as both a GTPase activating protein and downstream regulator of ras proteins.

G 304 IDENTIFICATION OF GTP-BINDING PROTEINS AS SUBSTRATES FOR ADP-RIBOSYLATION BY EXOENZYME S. Jenifer Coburn, Larry Feig and D.Michael Gill, Department of Molecular Biology and Microbiology, Tufts University Health Sciences Campus, Boston, MA 02111
Exoenzyme S of *Pseudomonas aeruginosa* is an ADP-ribosyl transferase that has been shown to modify a number of cellular proteins. We have shown that exoS in fact has a limited number of substrates. One of the more abundant substrates is vimentin, which is most readily ADP-ribosylated when disassembled or when cleaved into fragments that cannot form filaments. However, the preferred substrates of exoS are a group of GTP-binding proteins of apparent Mr=23,000-25,000 after ADP-ribosylation (JBC 264:9004-9008). $p21^{c\text{-}H\text{-}ras}$, $p21^{v\text{-}K\text{-}ras}$, and a variety of mutant ras proteins with guanyl nucleotide handling defects are all substrates for exoenzyme S. ADP-ribosylation does not obviously change the GTP binding, GTP for GDP exchange, or GTPase of $p21^{c\text{-}H\text{-}ras}$, nor its interaction with GAP. ADP-ribosylation does, however, cause changes in the electrophoretic mobilities of $p21^{ras}$ proteins that imply a significant conformational change. In all previously known cases ADP-ribosylations by bacterial toxins have caused important changes in the functions of their substrates. Thus we anticipate that exoenzyme S will be useful in determining the functions of its small GTP-binding protein substrates and in identifying cellular proteins that interact with $p21^{ras}$.

G 305 EARLY ALTERATIONS IN *ras* p21 PROTEINS IN MEMBRANES OF REGENERATING RAT LIVER, **Jennifer L. Cruise *, Stephanie J. Muga, David Fleischacker*, and George K. Michalopoulos, *Department of Biology, College of St. Thomas, St. Paul, MN 55105, and Department of Pathology, Duke University Medical Center, Durham, NC 27710.** Liquid competition radioimmunoassay of detergent extracts of rat liver membranes indicate that a significant drop in p21 immunoreactivity occurs within the first 2 hr after partial hepatectomy, when the pan-reactive monoclonal antibody (mAb) Y13-259 is used for detection (*J. Cell. Physiol., (1989) 140: 195-201.)* This change has been further characterized by Western blotting of membrane extracts, using both pan-reactive and type-specific mAbs to *ras* proto-oncogene p21s. While increased *ras* gene expression during liver regeneration is a late event, concurrent with DNA synthesis, the finding that p21 proteins undergo early changes suggests that they may be involved in prereplicative signaling, and that observed changes in mRNA levels may reflect recovery of normal p21 levels.

G 306 PURIFICATION OF LOW MOLECULAR WEIGHT GTP-BINDING PROTEINS FROM TYPE II PNEUMOCYTE LAMELLAR BODIES, Burton F. Dickey, Timothy M. Shannon and Jeffrey B. Rubins, Pulmonary Center, Boston University School of Medicine, Boston, MA 02118.
Genetic studies in yeast and studies in mammalian systems using slowly hydrolyzed analogues of GTP suggest a key role for low molecular weight GTP-binding proteins (LMW-GBPs) in processes of vesicular sorting and secretion. Pulmonary type II alveolar epithelial cells package surfactant phospholipids in a unique secretory organelle, the lamellar body, which is exocytosed both constitutively and in response to exogenous ligands. We have previouly found that several LMW-GBP are enriched in purified lamellar bodies as compared to other subcellular fractions. Because lamellar bodies have a highly distinctive sedimentation density, this exocytic compartment from a single cell type can be purified from whole lung homogenate by equilibrium sedimentation in a density gradient. Lamellar bodies were solubilized in cholate and LMW-GBPs were purified by sequential Ultrogel AcA-44, octyl-Sepharose and Mono Q HR 5/5 chromatography, using [35S]GTPγS binding to follow activity. Column fractions were also assayed for [32P]GTP binding after SDS-PAGE and electrophoretic transfer to nitrocellulose, and for GTPase activity. Following this scheme, microgram quantities of four major GTP-binding proteins of molecular masses 27, 25, 24 and 22 kDa were purified essentially to homogeneity. Three of the four [35S]GTPγS-binding proteins also bind [32P]GTP after transfer to nitrocellulose, and all four proteins have intrinsic GTPase activity which is modulated by type II cell cytosol fractions. We are currently microsequencing these proteins to allow their molecular identification, producing antisera to confirm their localization immunologically, and developing an 'in vitro' assay of exocytosis for functional reconstitution.

G 307 THE RHOA,B AND C PROTEINS, IDENTIFIED BY C3-CATALYSED [32P]ADP-RIBOSYLATION. TISSUE DISTRIBUTIONS AND SUBCELLULAR LOCALIZATIONS IMPLY DIFFERENCES IN FUNCTION. Simon Dillon and D. Michael Gill, Department of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, MA. 02111,

Rho proteins are 22K GTP binding proteins that are ADP-ribosylated by exoenzyme C3 of Clostridium botulinum C and D strains. ADP-ribosylation of rho proteins causes actin microfilaments to disassemble, with consequent cell rounding, and this result has suggested that one function of the rho proteins is to control the cytoskeleton (Chardin et al EMBO Journal 8: 1087-1092 1989). Using 3T3 cells expressing mutant rho we could establish the 2D gel positions of ADP-ribosylated rho proteins and hence determine the distributions of rho A, rho B and rho C individually. RhoA and rhoC proteins are mostly soluble while rhoB protein is particulate, presumably membrane-bound. RhoA is the only rho protein present in all of the many mammalian tissues and cell types examined, and its widespread occurence suggests that rhoA may mediate the C3 effect on actin polymerization. RhoC protein is much less abundant than rhoA protein. RhoB protein cannot be detected in some tissues, is present in low amounts in others, and is abundant only in the brain. There is no detectable rhoB protein in C6 glioma cells and little of it in PC12 cells, even when they are induced by nerve growth factor to assume a neuronal morphology. The tissue-specific distribution and membrane localization suggests that rhoB may have a separate function from rhoA.

G 308 ras INDUCED MITOGENIC EFFECTS REQUIRE ACTIVATION OF C-KINASE AND A SERUM RESPONSE FACTOR , Cécile Gauthier-Rouvière, Anne Fernandez and Ned J.C. Lamb,Cell Biology, CRBM-CNRS, INSERM-U249, B.P.5051, 34033 Montpellier Cedex France

It is likely that the ras oncoproteins are regulatory proteins controlling a critical aspect of cell proliferation and we are investigating the molecular mecanisms by which ras oncogene expression causes the malignant transformation of cells. Microinjection of the ras oncogene protein into quiescents fibroblasts induces the rapid expression of c-fos and transiently stimulates DNA synthesis and cell proliferation. It is believed that the 5' region of the c-fos gene contains, among promotor regions, the serum response element (SRE), a short sequence of dyad symmetry which bind a protein factor, SRF, that is required for induction of c-fos transcription by serum, mitogens and activators of protein kinase C. In order to assess if c-fos induction by ras microinjection also required the activation of SRE, double-stranded DNA oligonucleotides corresponding to SRE or mutated SRE were microinjected together with the ras oncogene protein into quiescent cells. We found that cells injected with the ras protein plus SRE showed a very low background level of nuclear fos immunofluorescence similar to that observed in uninjected cells whilst coinjection of mutated SRE did not alter the level of fos expression induced after ras injection. This result indicates that SRE can inhibit c-fos expression induced by ras. In addition, coinjection of the ras protein with SRE into quiescent cells inhibited the incorporation of 5-Br-deoxyuridine normally observed in cells injected with ras alone. Moreover, we demonstrate that such inductive effects of ras are dependent on activation of functional calcium/phospholipid-dependent protein kinase C (C-kinase). By microinjection of an inhibitory peptide which specifically inhibits C-kinase, or anti-C-kinase antiserea, both ras induced c-fos expression and DNA synthesis can be inhibited. These results emphasize the importance of ras protein in the control of proliferation through generation of second(s) messenger(s) that ultimately act within the nucleus to promote changes in gene expression. Indeed, it seems clear that a connection exists between ras activity within cells and c-fos expression through SRE/SRF.

G 309 ROLE OF LOW MOLECULAR WEIGHT G PROTEINS IN CATECHOLAMINE SECRETION FROM BOVINE CHROMAFFIN CELLS, Lung-Sen Kao and Pei-Shan Liu, Institute of Biomedical Sciences, Academia Sinica, Nankang, Taipei, Taiwan, R.O.C. Cultured cells of the adrenal medulla (chromaffin cells) have been shown to contain a complement of the signal transducing G proteins, including Gs, Go and Gi. Little evidence has been obtained that these proteins have a role in secretion or in modulation of the secretory response. Using radioactive GTP, it has been shown that there are also a number of low molecular weight GTP-binding proteins in chromaffin cells. We have found that there are several low molecular weight GTP-binding proteins present in the granule and plasma membrane fractions but not in the cytosol fraction. Rap-1, a 22 kDa GTP-binding protein, has been shown by Laptina et al., to be reversibly phosphorylated, leading to translocation from the platelet plasma membrane to cytosol. Using antibody against rap-1, we have found that rap-1 colocalizes with the 22 kDa GTP-binding protein of chromaffin cells, and we are now studying translocation and phosphorylation of this protein. We will describe the responses of the rap-1-like protein to nicotinic and bradykinin stimulation in the presence and absence of calcium and we will suggest a role for this protein in the secretory response.
(Supported by a grant from National Science Council, NSC78-0412-B001-15)

G 310 INTERACTION OF FLUORESCENT ANALOGUES OF GUANINE NUCLEOTIDES WITH ELONGATION FACTOR Tu FROM E.COLI, David P. Molloy and John F. Eccleston, N.I.M.R., Mill Hill, London NW7 1AA.
Guanine nucleotides modified on the ribose moiety were prepared by either direct acylation with N-methylisatoic anhydride producing 2'(3')-O-(N-methylanthraniloyl)-GTP,(mant-GTP), and the corresponding GDP and GMP.PNP derivatives, or reaction of the 5,1; 5,2 and 6,2 isomers of dansyl chloride with the ethyldiamine carbamate derivatives of the nucleotides. The relative binding constants for mant-GTP, mant-GDP and mant-GMP.PNP binding to EF-Tu were 61, 1.4 and 67 respectively as determined by competitive binding experiments with GDP. An enhancement of fluorescence for all analogues upon binding to EF-Tu was observed, the largest being 80% for Mant-GDP, above that for nucleotide free in solution. Polarisation studies and quenching of fluorescence by acrylamide have yielded information concerning the rotational parameters and solvent accessibility of the fluorophore bound to EF-Tu. Initial studies on the formation of the aminoacyl-tRNA.EF-Tu.analogueGTP ternary complex has indicated a further enhancement of fluorescence. These studies show that the introduction of fluorophores onto the ribose moiety has little effect upon binding to EF-Tu and demonstrate the potential of fluorescence techniques in the study of other G-proteins.

G 311 TEMPERATURE-SENSITIVE LETHAL MUTANT OF ERA, A *RAS*-FAMILY PROTEIN IN *ESCHERICHIA COLI*, Yoshikazu Nakamura, Toshifumi Inada and Donald Court* Institute of Medical Science, University of Tokyo, Tokyo 108, Japan, and *BRI-FCRF, National Cancer Institute, Frederick, Maryland 21701
The *era* gene of *E. coli* encodes a GTP-binding protein which has similarities to elongation factor Tu and yeast *RAS* protein. To investigate its function, mutations affecting *era* were isolated. A mini-Tn*10* insertion, which truncated 22 amino acids from the COOH end of Era, did not affect the cell growth. Using this mini-Tn*10* insert as a co-selectable marker, a temperature-sensitive lethal *era* mutant was isolated by localized mutagenesis. A single base G-to-A change was found at position 23 which substitutes a tyrosine residue for the cysteine-8 residue (*era770*) in addition to the COOH-terminal mini-Tn*10* disruption (*era13*). The genetic reconstitution experiment revealed that both alterations are necessary for the temperature-sensitive phenotype. Thus, Era is essential to growth of *E. coli*. The wild-type and mutant *era* genes were cloned into a λp_L expressing vector and Era proteins were overproduced and purified to homogeneity. The Era770 mutant protein exhibited reduced binding to GTP, whereas the truncated Era13 protein binds GTP as efficiently as wild type. These results indicate that the reduced protein's activity (and stability) may cause the ts lethality of the *era770-era13* strain.

G 312 Dd*rap*1 GENE EXPRESSION DURING *DICTYOSTELIUM* DEVELOPMENT.
S. M. Robbins, V. V. Suttorp, G. B. Spiegelman and G. Weeks. Departments of Microbiology and Medical Genetics, University of British Columbia, Vancouver, B.C. Canada.

Ras and *ras*-related genes comprise a large multigene family that have been found in a wide variety of organisms. We have isolated and sequenced a cDNA clone that hybridized to both of the *Dictyostelium ras* genes, Dd*ras* and Dd*rasG* under low, but not under high stringency conditions. The predicted protein shows a high degree of sequence identity with the human *rap* (*Krev*-1, *smg*21) proteins and thus has been designated Dd*rap*1.

The amount of mRNA that hybridized to the Dd*rap*1 cDNA changed dramatically during development reaching maximum levels at the aggregation stage stage (6-8 hours) and during culmination (18-22 hours). During vegetative and early development a single 1.1 kb mRNA was present, but by aggregation this transcript was no longer detected and two new transcripts of 1.0 and 1.3 kbs were apparent. The maximum levels of the Dd*rap*1 specific mRNA's appeared during developmental stages where the levels of Dd*ras*G and Dd*ras* messages were declining. The reciprocal nature of the Dd*rap*1 gene expression with respect to that of the two *ras* genes suggests the possibility that the *ras* and *rap* gene products in *Dictyostelium* have antagonistic roles.

G 313 EXPRESSION AND CHARACTERIZATION OF RECOMBINANT P23^{RAB2}.
Chung-wen Wei and Ian G. Macara, Departments of Biochemistry and Biophysics, Envinronmental Health Sciences Center, University of Rochester Medical Center, Rochester, NY 14642.
Rab2 is a member of the ras superfamily, that encodes small guanine nucleotide binding proteins. Rab2 can encode a protein of about 23,000 daltons with 38% homology to ras (Touchot et al., PNAS 84, 8210, 1987). However, the protein has not yet been characterized, nor has it been identified in mammalian tissues. We have therefore subcloned a full-length rab2 cDNA, by PCR, into an expression vector (pKK233-3), which was then used to transform E. coli. Induction by IPTG led to the expression of a 23 kD protein, which constituted about 5% of the soluble proteins of the cell. When the soluble proteins were resolved by SDS electrophoresis and transferred to nitrocellulose, a 23 kD band was detected by blotting with α-^{32}P-GDP, indicating that the recombinant p23^{rab2} is functional. The protein has been purified by a two-column procedure and is being used as a substrate for the idnetification of a rab2-specific GAP, and for the preparation of rab2-specific antisera.

G 314 A CYTOSOLIC PROTEIN CATALYZES THE RELEASE OF GDP FROM p21^{ras}, Alan Wolfman and Ian G. Macara, Department of Biophysics and Environmental Health Sciences Center, University of Rochester Medical Center, Rochester, NY 14642.
The ras proteins possess a very slow off-rate for GDP in the presence of physiological concentrations of Mg^{2+}. This property, together with the presence of an intrinsic GTPase activity that is enhanced by GAP, suggest that p21^{ras} would be almost exclusively associated with GDP in vivo, in the absence of a factor for catalyzing GDP release. Such a factor has now been discovered in rat brain cytosol. Using recombinant c-Ha-ras protein loaded with α-^{32}P-GDP, brain cytosol was found to catalyse by many orders of magnitude the release of the guanine nucleotide, even at 4°C. This effect occurred even in the presence of a large excess of Mg^{2+}, but was destroyed by heat, or by incubation of the cytosol for an hour in the absence of phosphatase inhibitors at 37°C. The effect was observed using either v-Ha-ras or c-Ha-ras protein, and could not be mimicked by addition of recombinant ras-GAP. The effect was detectable by either a filter-binding assay or by a spin-column technique, eliminating the possibility that the cytosol was simply interfering with binding of the ras protein to the nitrocellulose filter. By gel filtration chromatography, the factor appears to possess a molecular weight of about 125-150,000 daltons. We propose that this protein (**ras-guanine nucleotide releasing factor, or ras-GRF**) is an exchange factor that activates p21^{ras}.