SEPARATION OF TRANS-MEMBRANE PEPTIDES BY CAPILLARY ELECTROPHORESIS - EFFORTS AND ACHIEVEMENTS [MAS]

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Integral membrane proteins are required for cellular signaling, chemotaxis and other fundamental cellular functions. Because it has only been possible to crystallize very few of these proteins, very little is known about their folding structure. At the same time, structural information is required to fully understand function and to make possible more efficient development of inhibitors and other targeted drugs. Mass spectrometry can be used to obtain structural information on integral membrane proteins, for example, on the amino acids lining the active sites by affinity labeling. To obtain this information, the labeled protein is proteolytically digested and the structures of the resulting peptides obtained by tandem mass spectrometry. However, it has proven extremely difficult to achieve the required purification of these very hydrophobic peptides by (reverse-phase) BLC. Here, we describe efforts to separate cyanogen-bromide digests of bacteriorhodopsin by capillary electrophoresis (CE). Capillary electrophoresis is an extremely promising method for this application due to its high separation efficiency and its proven ability to break up non-covalent complexes. We have used detergents to minimize absorption of peptides on the capillary wall and in an effort to limit aggregation of the hydrophobic peptides. Results are shown for several different detergents, including SDS, octylglucoside, dodecyl-D-maltoside, under varying CE experimental conditions.

IDENTIFICATION OF TRANSMEMBRANE TRYPTIC PEPTIDES FROM THE INTEGRAL MEMBRANE PROTEIN RHODOPSIN USING MASS SPECTROMETRY [MAS]

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Rhodopsin was used as a model integral membrane protein for the development of a mass spectrometric technique designed to identify hydrophobic peptides generated by enzymatic digests. Affinity purified rhodopsin, as well as rhodopsin in retinal rod membranes, were digested with trypsin. Tryptic peptides were separated using a modified reverse phase HPLC technique with the detergent octyl-pglucoside in the mobile phase. The fractionated peptides were analyzed by matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry using a-cyano-4- hydroxy cinnamic acid (ACHCA) as the matrix. The putative transmembrane regions of rhodopsin contain six tryptic peptides. Four of the six tryptic peptides, ranging in mass from 3,259Da to 6,528Da, were

identified by their molecular weight and by the amino acid sequence for five of their N-ternimal residues found by Edman micro-sequencing. In addition, heterogeneity in the glycosylation of the N-terminal tryptic peptide of rhodopsin was also identified using this method without modifying the carbohydrate prior to analysis by MALDITOF mass spectro-metry. This study demonstrates a new utility for mass spectrometry in the analysis of integral membrane protein structure.

Biochemical Characterization, Regulation and Potential Function of a New gd T Cell Lineage-specific Marker (GD3.5Ag) MAS

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We describe GD3.5, a new lineage-specific gd T cell marker that is distinct from TCR, and WC1. Nonreducing Western blot analysis and immunoprecipitation experiments revealed a single 220-240kD glycoprotein recognized by GD3.5 compared to two IL-A29 reactive bands at 200kD and 300kD. Cross-immunoprecipitation experiments demonstrated that GD3.5 antigen and WC1 could be immunoprecipitated from lysates cleared of IL-A29/WC1 and GD3.5Ab/ GD3.5Ag complexes, respectively. The GD3.5 antigen and WC1 possess disparate sensitivity to PNGase F, O-glycoprotease, and neuraminidase, indicating differences in N- and O-linked sugars and the presence of sialic acid residues. Surface expression of GD3.5 antigen, but not WC1, was reduced by very low-dose chymotrypsin treatment. Treatment of gd T cells with PHA or Con A resulted in differential regulation of GD3.5Ag and WC1 providing clues to their function. Additionally, *in vitro* shear-dependent adhesion assays suggest that GD3.5 antigen may play a role in gd T cell adhesion to Pselectin. Therefore, our data suggest that GD3.5 antigen is a previously uncharacterized, lineage-specific gd T cell antigen that may by involved in P-selectin mediated adhesion.

The Use of Dual Energy X-Ray Absorbtiometry to Study the Effects of Low-dose Methotrexate on Bone Density MAS

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A study at Deaconess Research Institute was initiated to determine the contribution of low-dose Methotrexate to the development of osteopenia. Findings are based upon analysis of Dual Energy X-ray Absorptiometry (DEXA) scans for bone density and bone mass. Scans were performed 18 to 24 mo apart for 5 of 11 patients currently undergoing low-dose Methotrexate therapy for the treatment of rheumatoid arthritis. The results suggest there is no correlation between low-dosage Methotrexate and osteopenia/ osteoporosis and that therapeutic doses seem to pose little threat of significant bone loss.

DETERMINATION OF THE SITES OF INTERACTION BETWEEN THE FORMYL PEPTIDE RECEPTOR AND ITS LIGAND FORMYL-MET-LEU-PHE[MAS]

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Membrane receptors are involved in a number of critical functions of cells, especially signal transduction. However, X-ray crystallography and NMR have generally failed with very few exceptions to determine the structures of integral membrane proteins. As such, the understanding of the structure and function of these proteins remains a major unsolved problem in biochemistry. Thus, new alternative methods, which provide structural information on these proteins, are needed. We are developing a method that we call site directed photoaffinity scanning. G protein-coupled receptors belong to a receptor super family based on conserved amino acids and a conserved pattern of hydrophobic residues that are thought to be responsible for their unique transmembrane topology of seven membrane-spanning domains. The phagocyte chemotactic receptors, the formyl peptide receptor (FPR), C5a receptor (C5aR), platelet-activating factor receptor (PAF-R), and interleukin-8 receptor (IL8-R) are all G protein-coupled receptors. The best characterized of these is neutrophil FPR which binds N-formyl peptides, such as N-formyl-met-leu-phe (*f*MLF). We have synthesized several photoaffinity analogues of *f*MLF that will place photoaffinity labels at different positions on the molecule. 1. Phenylalanine was substituted with the photoaffinity analogue benzoylphenylalanine. 2. The leucine was replaced with benzoylphenylalanine. 3. The formyl group was replaced by a diazopyruvyl group. The analogues were derivatives of *f*MLFYK (1 or 2) or *f*MLFNleYC (3). The lysine was tagged with fluorescein hexanonate and the cysteine with fluorescein iodoacetamide. All three analogues bind to FPR with high affinity and are displaced by *f*MLF. *f*MBenzoylPheYK-Fluorescein can be specifically photocrosslinked to a protein in CHO cells expressing wild type FPR that elutes just before rhodopsin on a HPLC gel filtration column. Labeling is inhibited in the presence of 10 μM*f*MLF. Tandem anion exchange (AX500) with the gel filtration column provides a much cleaner preparation of the *f*MBenzoylPheYK-fluorescein labeled protein and the peak fractions immunoblot with anti-FPR antibodies. Approximately 80 pMoles of photocrosslinked FPR was obtained from $2x10^6$ CHO transfectants. This should be sufficient for determining the site of photocrosslinking of proteolytic or CNBr cleavage fragments of FPR by MALDI mass spectroscopy. Placing 10 pMoles of *f*MBenzoylPheYKfluorescein bound to antifluorescein Sepharose directly in the mass spectrometer has allowed MALDI mass spectroscopy and post source decomposition sequencing. Thus, it should be possible to use these antibodies to isolate the crosslinked proteolytic or CNBr fragments of FPR followed by direct analysis of these fragments bound to antifluorescein Sepharose. Carboxypeptidase treatment of these fragments will also be used to confirm the crosslinking site.

Does Trimethyltin Induce Nitric Oxide Synthase in Rat C6 Glioma?[MAS]

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The inducible form of nitric oxide synthase (iNOS) is responsible for the sustained production of the biological free radical gas nitric oxide (NO). The iNOS is induced by various stimuli such as bacterial endotoxins and cytokines. This induction of iNOS and sustained production of NO is thought to contribute to such pathological conditions as excitotoxicity, demyelinating disorders, and septic shock. In our laboratory, trimethyltin (TMT) has been shown to increase dichlorofluorescein (DCF) fluorescence, indicating generation of reactive oxygen species (ROS). Other investigators have shown that TMT exposure releases cytokines. Based on these two observations we investigated the effects of TMT on iNOS induction. Rat C6 glioma cells were grown to confluence in 12 well plates and either coadministered phorbol-12-myristate-13-acetate, (PMA) and the bacterial endotoxin lipopolysacchride (LPS), or treated with either 2.5 uM or 5.0 uM TMT. Additional wells were given the above treatments with the inclusion of 10 mM aminoguanidine (AG), a selective iNOS inhibitor. Cells from each treatment were analyzed cytochemically for the presence of NADPH diaphorase (NPD), which colocalizes with iNOS. Treatment with PMA/ LPS or TMT appeared to increase the amount of NPD staining. Media aspirated from the cultures was also analyzed for the presence of NO. NO levels were assessed by the presence of N02- in the extracellular fluid as determined by the Griess reaction. We found that the PMA/LPS treatment produced a significant ($p<0.05$) and sustained doubling of NO production from 16 to 72 hours. Pre-incubation of the cultures for 30 minutes with AG alone had no effect on NO production, but AG inhibited the increase seen with the PMA/LPS treatment by up to 50%. Treatment with TMT did not significantly increase NO levels. We concluded that the TMT generated increase of ROS in the C6 cell line is not due to the induction of iNOS, and subsequent production of NO.

Purification, Characterization, and PCR Cloning of a Wound Induced Tomato Leaf Protein[MAS]

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Tomato plants respond to wounding caused by herbivorous insects with the induction of certain defensive genes. This wound response is mediated by systemin, an 18 amino acid polypeptide derived from a larger precursor called prosystemin. Transgenic plants that over express the systemin precursor exhibit a constitutive wound response allowing the accumulation of high levels of proteins produced by these defensive genes. Reported here is the

purification of one of these defense related proteins from transgenic plant extracts by polyacrylamide gel electrophoresis. The N-terminal amino acid sequence was obtained after blotting to PVDF- membranes. An oligonucleotide corresponding to the amino acid sequence was then designed and used as a primer in the polymerase chain reaction (PCR). Three specific PCR products were obtained. The largest one (900 bp) was used to screen a tomato leaf cDNA library. A cDNA clone of 2.1 kb was retrieved. Preliminary sequence data has identified the clone as a ketol-acid reductoisomerase. The second largest PCR product (500 bp) was cloned via a plasmid vector and preliminary sequence data has identified it as an acyl-CoA binding protein. The role that these proteins play in the systemic wound-response remains to be found.

Release of Leukotriene D_4-Dipeptidase from Activated Macrophages [MAS]

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The activation of macrophages by bacterial lipopolysaccharide (LPS) or other signals results in the production of a mixture of lipid mediators derived from arachidonic acid (eicosanoids), which have potent biological activities. Among these are the peptido-leukotrienes (leukotriene C_4, LTD_4, and LTE_4) which are major mediators of the asthmatic response and other hypersensitivity reactions. The leukotrienes differ in potency (LTD_4 > LTC_4 >> LTE_4); therefore, the intensity and duration of a leukotriene mediated reaction will be determined by the type, and relative amount of the different leukotrienes present in the tissue. In most biological assays the conversion of LTD_4 to LTE_4 by LTD_4-dipeptidase results in a 10 to 100 fold reduction in activity, such as smooth muscle contraction. In some tissue, such as sheep lung, LTD_4-dipeptidase is present as a phosphatidylinositol anchored protein which is released from the cells (and activated) by treatment with PI-specific phospholipase C. In this study, treatment of mouse peritoneal macrophages with LPS or phorbol myristate acetate (PMA) at concentrations of 1 to 10 ng/ml resulted in the release of LTD_4-dipeptidase within 10 to 20 minutes. The LPS and PMA concentrations and the kinetics of release of LTD_4-dipeptidase were almost identical to the LPS and PMA concentrations and the kinetics of release of eicosanoids, including leukotrienes, from mouse macrophages. Treatment directly with LTD_4, however, did not cause release of LTD_4 dipeptidase. The activation of cells to release LTD_4dipeptidase by the same signals which cause the release LTD_4 suggest that this enzyme plays a role in the modulation of the leukotriene mediated response.

NEUROSCIENCES

SEROTONERGIC RECEPTOR BINDING ACTIVITY OF *PETIVERIA ALLIACEA* [MAS]

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Petiveria alliacea (PA) is a woody herb found from the Southern U.S. to Argentina. It has been studied for its anti-tumor properties and has ethnopharmacologic uses including the treatment of headache. Migraine headache is a severe neurovascular disorder in which the pathology is thought to center on serotonergic (5HT) systems. Acutely acting anti-migraine drugs appear to bind to a number of 5HT1 receptors, while prophylactic anti-migraine drugs are hypothesized to act via one or more 5HT2 receptors. Samples of PA were collected in Peru in May 1995 and again in November 1995 at different locations. Seventy thousand ethanol extracts of *Petiveria alliacea* were used to displace known radiolabeled ligands from 5HT1a and 5HT2a receptors. *Petiveria alliacea* is inactive at 5HT1a receptors. However, at 5HT2a receptors a 1/100 dilution produces about 90k displacement of 3H-ketanserin. This effect is concentration-dependent over the range 1/100 to 1/1000. *Petiveria alliacea* is now undergoing HPLC fractionation in an attempt to isolate and identify drugs that may be candidates as anti-migraine prophylactics.

PHARMACOLOGY AND TOXICOLOGY

MONTANA HIGHWAYS: SAFE AT ANY SPEED - BUT NOT AT ANY LEVEL (BETTER DRIVING THROUGH CHEMISTRY-NOT) [MAS]

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This project was undertaken to determine whether or not MCA 61-8-402 , paragraph (1); prohibitive DUID legislation should be amended. Statistical data including age, gender, race, blood alcohol concentration (BAC), and drug levels were compiled from 744 subjects over a two (2) year period. These results were compared to a parallel study over a six month period in which data from 376 subjects were compiled regardless of the BAC. The current study indicates an approximate 40% positive drug detection in suspected DUI cases compared to an approximate 39% positive drug rate in drivers where the BAC > 0. 10gm/dl in the six month study. Other than ethanol, the most common drugs detected in drivers were THC (marihuana), followed by amphetamines, benzodiazepines, and opiates. It is the author's contention that the additive effects of drugs in drivers, where the BAC is greater than 0.10 gm/dl, make those drivers as (or far more) dangerous and should not preclude them from drug testing.