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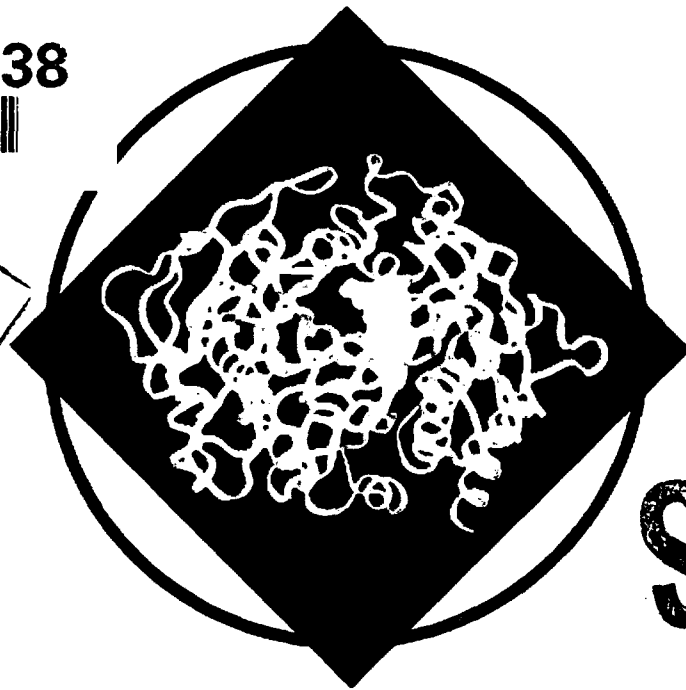
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Fifth International Meeting on Cholinesterases

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September 24-28, 1994
Taj Coromandel Hotel
Madras, India

A Satellite Meeting of
The International Congress of
Biochemistry and Molecular Biology

94-29867



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The principal role played by acetylcholinesterase in neurotransmission within the central and peripheral nervous systems commands the interest of chemists, biochemists, cell biologists, pharmacologists, clinical scientists, neuroscientists, and toxicologists. The field has a very large, multidisciplinary, international following. Recent rapid advances in the understanding of molecular properties and structure of the cholinesterases, in genes encoding the enzymes, and pharmacological properties of cholinesterase inhibitors and reactivators provide an opportunity for an unparalleled account of our knowledge of this rapidly evolving field. These developments have arisen from the molecular cloning and characterization of the cholinesterase genes and the elucidation of the three-dimensional structure of acetylcholinesterase.

Cholinesterases (acetylcholinesterase and butyrylcholinesterase) are of more than simple enzymological interest. They play important roles in coordinating central nervous systems regulation in cholinergic synapse development. Altered expression have been implicated in other physiological and pathological processes in the CNS, e.g., myasthenic defects, and the pathogenesis of Alzheimer's disease. Moreover, the potent toxicity of the organophosphate family of anticholinesterase insecticides and nerve agents has led to their use as structure-function relationships. Analysis of the three-dimensional structure of the enzyme and some of its compounds to an atomic level of resolution is opportune for the first time. Clearly, the timing of this new information. New molecular perspectives on this enzyme and its gene should formulate research directions for the next decade. The cholinesterases have defined a family of homologous proteins of widely varying functions and a second family without sequence homology but with a common α , β hydrolase fold. Much can be learned from comparative analyses of related proteins. Finally, recent advances in molecular and structural arenas have brought novel insights into the development of improved antidotes and scavenging agents.

Major current approaches and new directions in these fields are programmed for discussion at this meeting on cholinesterases.

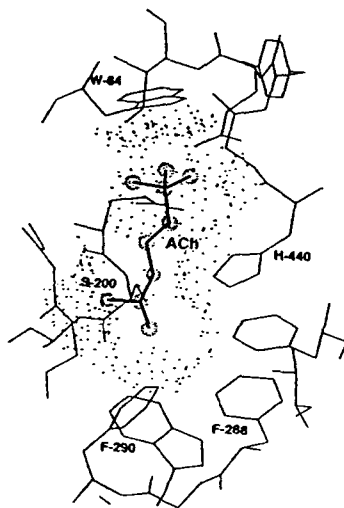


Illustration courtesy of Dr. Joel Sussman, The Weizmann Institute of Science, Rehovot, Israel

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CONFERENCE PROGRAM AND COORDINATING COMMITTEE

Prof. A. S. Balasubramanian
Dept. of Neurological Sciences
Christian Med. College & Hospital
Vellore - 632 004
Tamil Nadu, INDIA
Tel: 91-416-22102, x2018
Fax: 91-416-22103

Dr. B. P. Doctor
Division of Biochemistry
Walter Reed Army Inst. of Research
Washington, DC 20307-5100 USA
Tel: 202-576-3001
Fax: 202-576-1304

Prof. Jean Massoulié
Laboratoire de Neurobiologie
Ecole Normale Supérieure
CRNS-URA 295, 46 rue d'Ulm
75230 Paris Cedex 05 FRANCE
Tel: 33-1-44-323744
Fax: 33-1-44-323887

Dr. Avigdor Shafferman
Israel Institute for Biological Research
P.O.B. 19
70450 Ness-Ziona, ISRAEL
Tel: 972-8-381518
Fax: 972-8-401404

Prof. Israel Silman
Dept. of Neurobiology
Weizmann Institute of Science
76100 Rehovot, ISRAEL
Tel: 972-8-343649
Fax: 972-8-344131

Prof. Palmer Taylor
Dept. of Pharmacology, 0636
School of Medicine
Univ. of California at San Diego
La Jolla, CA 92093-0636 USA
Tel: 619-534-1366
Fax: 619-534-9842

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PROGRAM AND SCHEDULE

Friday, September 23, 1994

Afternoon 1:00 - 6:00 Registration
Evening 6:30 - 8:30 Welcoming Reception

Saturday, September 24, 1994

Morning 8:30 - 12:30 Session A: Gene Structure and Expression of Cholinesterases
Afternoon 2:00 - 5:30 Session B: Polymorphism and Structure of Cholinesterases
Evening 8:00 - 10:00 Poster Session and Discussion

Sunday, September 25, 1994

Morning 8:30 - 12:30 Session C1: Mechanism of Catalysis of Cholinesterases
Afternoon 2:00 - 5:30 Session C2: Mechanism of Catalysis of Cholinesterases
Evening 8:00 - 10:00 Poster Session and Discussion

Monday, September 26, 1994

Morning 8:30 - 11:30 Session D: Cellular Biology of Cholinesterases
Afternoon and Evening Mahabalipuram Tour, Gala Dinner, and Indian Dance Program

Tuesday, September 27, 1994

Morning 8:30 - 12:30 Session E: Structure-Function Relationship of Anticholinesterase Agents: Nerve Agents and Reactivators
Afternoon 2:00 - 5:30 Session F: Structure-Function Relationship of Anticholinesterase Agents: Pesticides and Therapeutic Agents; Non-cholinergic Function of Cholinesterases

Wednesday, September 28, 1994

Morning 8:30 - 12:00 Session G: Pharmacological Utilization of Anticholinesterases

POSTERS WILL BE DISPLAYED THROUGHOUT THE MEETING

Saturday, September 24, 1994

MORNING

Session A: Gene Structure and Expression of Cholinesterases

- 8:30 - 8:45 Review of Topic, Chairman
- 8:45 - 9:10 LIGAND SPECIFICITY AND GENE EXPRESSION OF ACETYLCHOLINESTERASE AND THE NICOTINIC RECEPTOR: A TALE OF DIVERGENCE BETWEEN TWO CHOLINERGIC PROTEINS. Palmer Taylor. Department of Pharmacology, University of California, San Diego, La Jolla, CA 92093-0636
- 9:10 - 9:35 TRANSGENIC OVEREXPRESSION, MOLECULAR GENETICS AND "ANTISENSE" INHIBITION OF CHOLINESTERASE VARIANTS. Hermona Soreq. Dept. of Biological Chemistry, The Hebrew University, Jerusalem 91904, Israel
- 9:35 - 10:00 EXPRESSION OF ACETYLCHOLINESTERASE DURING MYOGENESIS AND SYNAPTOGENESIS OF HUMAN MUSCLE IN VITRO: *IN SITU* HYBRIDIZATION AND ENZYME HISTOCHEMISTRY. Zoran Grubič. Institute of Pathophysiology, School of Medicine, University of Ljubljana, Slovenia
- 10:00 - 10:20 Coffee Break
- 10:20 - 10:45 PROMOTER AND TRANSCRIPTION START SITE OF THE HUMAN AND RABBIT BUTYRYLCHOLINESTERASE GENES. Oksana Lockridge. Eppley Institute, University of Nebraska Medical Center, Omaha, USA
- 10:45 - 11:10 *ACE-1*, THE GENE ENCODING ACETYLCHOLINESTERASE OF CLASS A IN THE NEMATODE *CAENORHABDITIS ELEGANS*. Jean-Pierre Toutant. Départements de Zoologie et de Physiologie Animale, Institut National de la Recherche Agronomique, Centres D'Antibes et de Montpellier, France
- 11:10 - 11:35 MOLECULAR FORMS OF ACETYLCHOLINESTERASE: STRUCTURE AND INTERACTIONS. Jean Massoulié. Neurobiologie Cellulaire et Moléculaire, URA CNRS 1857, Ecole Normale Supérieure, 46 rue d'Ulm, 75005 Paris, France

Session A: Gene Structure and Expression of Cholinesterases

LIGAND SPECIFICITY AND GENE EXPRESSION OF ACETYLCHOLINESTERASE AND THE NICOTINIC RECEPTOR: A TALE OF DIVERGENCE BETWEEN TWO CHOLINERGIC PROTEINS.
Palmer Taylor, Department of Pharmacology, University of California, San Diego, La Jolla, CA 92093-0636.

The availability of the genes encoding acetylcholinesterase (AChE) and the nicotinic receptor (nAChR) and of atomic ($\sim 2\text{\AA}$) and molecular ($\sim 10\text{\AA}$) resolution structures of the respective proteins enable one to compare mechanisms of gene expression and the determinants of ligand specificity for these two proteins. In muscle cell differentiation nAChR expression is transcriptionally controlled with the E-box elements exerting a dominant influence, whereas AChE expression is controlled by mRNA stability. Differentiation causes stabilization of a labile AChE mRNA and superinduction can be seen by inhibiting synthesis of proteins presumably contributing to mRNA instability. Calcium availability appears to be a dominant factor since antagonism of the ryanodine receptor-L channel complex inhibits differentiation induced stabilization of the AChE mRNA without affecting production or levels of AChR mRNA.

The acetylcholine recognition site on AChE is nearly centrosymmetric to one of the subunits, whereas cooperativity demands that and mutagenesis and chemical modification studies show that the acetylcholine recognition site on the receptor resides at a subunit interface. Several other characteristic differences are evident but of particular interest is the observation that the cobra α -toxins interact with the acetylcholine recognition site of the receptor, while the homologous three fingered peptide toxin, fasciculin, has evolved to recognize a peripheral site on the lip of the AChE gorge. Glycosylation appears to be an important mechanism in conferring resistance of the receptor to the snake α -toxins while the differential glycosylation of BuChE and AChE is not the basis of BuChE's low affinity for fasciculin. Complementary structural variation of the ligand and substitution of side chains in the respective proteins have proven most useful when primary processes of catalysis or receptor function are correlated with structure and the respective mutation.

Session A: Gene Structure and Expression of Cholinesterases

TRANSGENIC OVEREXPRESSION, MOLECULAR GENETICS AND "ANTISENSE" INHIBITION OF CHOLINESTERASE VARIANTS. Hermona Soreq. Dept. of Biological Chemistry, The Hebrew University, Jerusalem 91904, Israel.

To study the biological functions of variant human cholinesterase forms in different tissue and cell types, we overexpress these heterologous proteins in transgenic *Xenopus laevis* embryos and mouse pedigrees and in transfected cultured glioma cells. Alternatively, we employ "antisense" oligonucleotides to induce targeted destruction of cholinesterase mRNA transcripts *in vivo* and *ex vivo*. Natural mutations and haplotype frequencies in the ACHE and BCHE genes and their incidence in distinct ethnically isolated populations are being studied by genotype tests. Last, but not least, we use *Xenopus* oocytes as an expression system for the production of recombinant cholinesterases translated from natural and site-direct variants of the human ACHE and BCHE genes. The outcome of these experiments is evaluated by RT-PCR amplification of the corresponding mRNAs, by cytochemical staining of their resultant protein products, by morphometric measurements on optic and electron micrographs and by catalytic activity evaluations of the affected enzymes, following their enrichment by absorption onto selective monoclonal antibodies.

Establishment of the transgenic expression systems revealed an evolutionary conservation which enables human ACHE to effectively accumulate at heterologous synapses, where it induced complex feedback responses of host genes. Antisense destruction of ACHEmRNA interfered with hematopoietic differentiation while inducing stem cell expansion. PCR amplification of genomic DNA demonstrated considerable population diversity in the incidence of specific ACHE and BCHE variants. That such diversity could reflect a genetic predisposition for variable drug responses was indicated from kinetic measurements of catalytic activities of the recombinant forms of these different mutant enzymes. Our findings thus demonstrate involvement of cholinesterases in synapse development and in the differentiation of glial and hemopoietic cells and reveal biochemical and/or pharmacological distinctions between acetylcholinesterase, butyrylcholinesterase and their natural and engineered variants.

Session A: Gene Structure and Expression of Cholinesterases

EXPRESSION OF ACETYLCHOLINESTERASE DURING MYOGENESIS AND SYNAPTOGENESIS OF HUMAN MUSCLE IN VITRO: *IN SITU* HYBRIDIZATION AND ENZYME HISTOCHEMISTRY. Zoran Grubič¹, Winsome F. Walker², Rado Komel³ and Armand F. Miranda⁴. Institutes of Pathophysiology¹ and Biochemistry³, School of Medicine, University of Ljubljana, Slovenia and the Dpts of Neurology² and Pathology⁴, Columbia University, College of Physicians and Surgeons, New York, NY, USA.

Skeletal muscle differentiation from mononuclear myoblasts to mature, multinucleate, functionally innervated fibers proceeds through several morphologically and functionally distinct steps. This process also involves a developmentally controlled redistribution of AChE. The functional importance and the molecular mechanisms that regulate the alterations in the patterns of AChE localization during myogenesis are not known. There is evidence that AChE mRNAs remain close to and are translated in the vicinity of the nucleus of origin. This suggests that differentiation-induced alterations in AChE localization patterns are controlled at the transcriptional level. To test this hypothesis, we analyzed the localization of, both, AChE mRNA and AChE activity at distinct stages of myogenesis and synaptogenesis: we performed *in situ* hybridization and enzyme histochemistry in differentiating cultures of human muscle, innervated *de novo* by fetal rat spinal cord neurons. We observed the following:

1. Developmental redistribution of AChE is most probably regulated at the mRNA level since the localizations of both AChE and its message were altering in parallel at each stage of differentiation studied.
2. There was nonuniformity of AChE mRNA expression amongst individual nuclei, supporting earlier evidence that transcription proceeds intermittently. These data further suggest that transcriptional regulation of individual nuclei is autonomous and asynchronous.
3. During myoblast fusion, intense AChE mRNA staining was present around all nuclei, suggesting that, at this developmental stage, transcriptional asynchrony is overridden by generally acting factors, and that mRNA expression is upregulated in all nuclei. Transcriptional asynchrony is also overridden at the onset of muscle contractions, when the message is downregulated extrajunctionally, becoming restricted to just a few nuclei near the neuromuscular junction.

Session A: Gene Structure and Expression of Cholinesterases

PROMOTER AND TRANSCRIPTION START SITE OF THE HUMAN AND RABBIT BUTYRYLCHOLINESTERASE GENES. Omar Jbilo^{1,2}, Jean-Pierre Toutant¹, Arnaud Chatonnet¹, and Oksana Lockridge². 1 Différenciation cellulaire et Croissance, INRA Montpellier, France; 2 Eppley Institute, University of Nebraska Medical Center, Omaha, USA.

Two kilobase segments of the 5' untranslated regions of the human and rabbit butyrylcholinesterase (BCHE) genes were characterized. The sequences shared extensive identity except for a 333 base pair (bp) Alu repeat present only in human BCHE. One single transcription start site was found in both genes with the techniques of primer extension, amplification of the 5' end of mRNA, and RNase protection. Cap sites in human and rabbit BCHE genes were found in strictly homologous positions. In human BCHE, the transcription start site was found 157 bp upstream of Met -28, the translation start site. Potential regulatory elements in both promoters included one AP1 site and multiple sites for topoisomerase, Oct-1 and PEA-3. Transient expression of BCHE-reporter gene constructs showed that a 194 bp fragment of the 5'-flanking region of human BCHE and a 570 bp fragment of rabbit BCHE were sufficient for promoting chloramphenicol acetyltransferase activity in HeLa cells. No consensus TATA and CAAT boxes were found. However the sequence around the transcription start site exhibited homology with initiator elements found in other TATA-less promoters in developmentally-regulated genes.

Supported by *Ministère de la Recherche et de la Technologie* (91-T-0439), from *Institut National de la Recherche Agronomique* and *Association Française contre les Myopathies* (MNM93-1250) and US Army Medical Research and Development Command Grants DAMD17-91-Z-1003 and DAMD17-94-J-4005.

Session A: Gene Structure and Expression of Cholinesterases

ACE-1, THE GENE ENCODING ACETYLCHOLINESTERASE OF CLASS A IN THE NEMATODE CAENORHABDITIS ELEGANS. Martine Arpagaus, Yann Fedon, Vincenzo Talesa, Emmanuel Culetto, Nathalie Schirru, Didier Fournier, Jean-Baptiste Bergé & Jean-Pierre Toutant. Départements de Zoologie et de Physiologie animale, Institut National de la Recherche Agronomique, Centres D'Antibes et de Montpellier, France.

Three genes, *ace-1*, *ace-2* and *ace-3*, encode three classes of acetylcholinesterase (A, B and C) in the nematode *C. elegans*¹. A fragment of genomic DNA was amplified by a polymerase chain reaction using degenerate oligonucleotides based on sequences EDCLYLN and FGESAG. This fragment mapped to chromosome X at a position that perfectly matched the location of *ace-1* on the genetic map¹. Comparison of genomic and cDNA sequences showed that the open reading frame was interrupted by eight introns with only one splice site conserved in other cholinesterases genes. Northern blot analysis showed that a single transcript of 2.6kb was produced throughout development with maxima at L1 and L3 larval stages. *Ace-1* mRNAs were usually trans-spliced by the short leader SL1 although some non-trans-spliced RNAs were also found. ACE-1, the product of *ace-1*, possessed 620 aminoacids and presented 42% identity with *Torpedo* and human AChEs, 41% with human butyrylcholinesterase, and only 35% with *Drosophila* AChE². The overall structure of cholinesterases was conserved in ACE-1 as indicated by the conserved sequence positions of Ser-216, His-468 and Glu-346 as components of the catalytic triad, of Trp-99 (84), a critical side chain in the choline binding site, and of the six Cys that form three conserved intrachain disulfide bonds. Twelve out of the fourteen aromatic residues lining the active site gorge in *Torpedo* AChE were conserved in *C. elegans* AChE. The C-terminus was hydrophilic with a series of aromatic residues WxxxYxxxWxxxFxxY conserved in other T subunits of cholinesterases. *Spodoptera* Sf9 cells were infected by a recombinant baculovirus containing *ace-1* cDNA. The secreted enzyme was active and existed as hydrophilic 5 and 11.5S molecular forms (likely monomer and tetramer). It hydrolyzed acetylthiocholine and butyrylthiocholine and was inhibited by acetylthiocholine above 10mM. The molecular forms synthesized in the baculovirus-Sf9 cells system appeared to lack non-catalytic components that explain the diversity of molecular forms produced *in vivo* by *ace-1*. A mutation in *ace-1* was identified in null mutants. A similar amount of 2.6kb *ace-1* transcript was present in mutants, but a point mutation Trp99(TGG)---->TGA(Stop) prevented the production of enzyme. For the study of promoter activity, a 1.2kb fragment of *ace-1*, upstream the initiator ATG, has been cloned and sequenced. It is being used for transfection of CAT constructs into 10T1/2 cells.

(1) see references in Johnson et al., 1988, *Neuron* 1, 165-173.

(2) Arpagaus et al., 1994, *J. Biol. Chem.* 269, 9957-9965.

Session A: Gene Structure and Expression of Cholinesterases

MOLECULAR FORMS OF ACETYLCHOLINESTERASE: STRUCTURE AND INTERACTIONS. Jean Massoulié. Neurobiologie Cellulaire et Moléculaire, URA CNRS 1857, Ecole Normale Supérieure, 46 rue d'Ulm, 75005 Paris, France.

In vertebrates, the catalytic subunits of acetylcholinesterase (AChE) are generated from a single gene: the catalytic domain, encoded by common exons, is associated with C-terminal peptides, arising from alternative splicing. The R ("readthrough") transcript, which retains the genomic sequence following the last common exon, is clearly present in some tissues, particularly in murine embryonic liver and muscle, as well as in adult spleen and hematopoietic cells. The corresponding catalytic subunits have not been characterized *in vivo*, and their potential physiological role is problematic, especially since the deduced sequences are widely different among species. The C-terminal peptide encoded by the H ("hydrophobic") transcript contains a cysteine residue involved in inter-subunit disulfide linkage and a signal peptide for cleavage/addition of a GPI anchor. It thus produced GPI-anchored dimers, which constitute the major form of AChE in *Torpedo* muscles, and are found on the surface of blood cells in mammals. In the mature form, the subunits encoded by the T ("tailed") transcript retain their 40-aminoacids C-terminal peptide, which also contains a cysteine residue involved in homologous or heterologous inter-subunit disulfide bonds. They produce soluble tetramers, as well as amphiphilic monomers and dimers (called type II, as opposed to the GPI-anchored type I molecules). The hydrophobic interactions of these molecules may be explained by the structure of the C-terminal peptide, organized as an amphiphilic α helix. T subunits also participate in hetero-oligomeric collagen-tailed and hydrophobic-tailed molecules, in which tetramers are associated with anchoring subunits. When co-expressed in transfected COS cells, T subunits and collagenic subunits form collagen-tailed AChE. *Torpedo* collagenic subunits also associate with rat T subunits, as well as with the human butyrylcholinesterase T subunits, showing a remarkably conserved complementarity. The physiological importance of collagen-tailed forms is dramatically demonstrated in human congenital myasthenia associated with end-plate AChE deficiency, in which these forms are specifically lacking.

Saturday, September 24, 1994

AFTERNOON

Session B: Polymorphism and Structure of Cholinesterases

- 2:00 - 2:15 Review of Topic, Chairman
- 2:15 - 2:40 3-D STRUCTURE AND FUNCTION OF ACETYLCHOLINESTERASE. Joel Sussman, Department of Structural Biology, The Weizmann Institute of Science, Rehovot 76100 Israel
- 2:40 - 3:05 CHARACTERIZATION OF REACTION INTERMEDIATES IN *CANDIDA RUGOSA* LIPASE, A CLOSE RELATIVE OF ACETYLCHOLINESTERASE. Mirosław Cygler, Biotechnology Research Institute, Natl. Res. Council, Montréal, Quebec H4P 2R2, Canada
- 3:05 - 3:30 COMPUTER MODELLING OF ACETYLCHOLINESTERASE AND ACETYLCHOLINESTERASE-LIGAND COMPLEXES. Michael K. Gilson. Chemistry Department, University of Houston, 4800 Calhoun Road, Houston, TX, USA, 77204-5641
- 3:30 - 3:55 STRUCTURES OF COMPLEXES OF ACETYLCHOLINESTERASE WITH COVALENTLY AND NON-COVALENTLY BOUND INHIBITORS. Michal Harel. Dept. of Structural Biology, Weizmann Institute of Science, Rehovot, Israel
- 3:55 - 4:15 Coffee Break
- 4:15 - 4:40 STRUCTURAL ANALYSIS OF THE OLIGOSACCHARIDES OF CHOLINESTERASES. Ashima Saxena. Division of Biochemistry, Walter Reed Army Institute of Research, Washington, D.C., USA
- 4:40 - 5:05 PRESSURE EFFECTS ON STRUCTURE AND ACTIVITY OF CHOLINESTERASE. Patrick Masson. Centre de Recherches du Service de Santé des Armées, Unité de Biochimie, 24, avenue des Maquis du Grésivaudan, 38702 - La Tronche Cédex, France
- 5:05 - 5:30 FTIR-SPECTROSCOPIC INVESTIGATIONS OF THE STRUCTURE AND TEMPERATURE STABILITY OF THE ACETYLCHOLINESTERASE FROM *TORPEDO CALIFORNICA*. Ferdinand Hucho. Institut für Biochemie, Freie Universität Berlin, Thielallee 63, 14195 Berlin, Germany
- 5:30 - 5:55 CHARACTERIZATION OF PARTIALLY UNFOLDED STATES OF ACETYLCHOLINESTERASE. Israel Silman. Dept. of Neurobiology, Weizmann Institute of Science, Rehovot, Israel

Session B: Polymorphism and Structure of Cholinesterases

3-D STRUCTURE AND FUNCTION OF ACETYLCHOLINESTERASE. Joel Sussman, Department of Structural Biology, The Weizmann Institute of Science, Rehovot 76100 ISRAEL.

The principal biological role of acetylcholinesterase (AChE) is termination of impulse transmission at cholinergic synapses by rapid hydrolysis of the neurotransmitter acetylcholine (ACh). Based on our recent X-ray crystallographic structure determination of AChE from *Torpedo californica* (Sussman *et al.*, *Science* 253, 872-879, 1991), we can see, at atomic resolution, a protein binding pocket of the neurotransmitter ACh. We found that the active site consists of a catalytic triad (S200-H440-E327) which lies close to the bottom of a deep and narrow gorge, that is lined with the rings of 14 aromatic amino acid residues. Despite the complexity of this array of aromatic rings, we suggested, on the basis of modelling which involved docking of the ACh molecule in an all-trans conformation, that the quaternary group of the choline moiety makes close contact with the indole ring of W84. A variety of AChE inhibitors have been soaked into crystals of AChE and their 3-D structures of these complexes have been determined showing a image remarkably similar to this model. The inhibitors studied include edrophonium, tacrine and decamethonium (Harel *et al.*, *PNAS* 90, 9031-9035, 1993) and others.

This enzyme generates a strong electrostatic field that can attract the cationic substrate, acetylcholine (ACh), to the active site (Ripoll *et al.*, *PNAS* 90, 5128-5132, 1993). However, the long and narrow active site gorge appears inconsistent with the enzyme's high catalytic rate (turnover number 20,000/sec). A molecular dynamics simulation of AChE, in water, which has revealed a transient opening of a short channel, large enough to pass a water molecule thorough a thin wall of the active site near W84. This suggests that substrate, products, and/or solvent, could move through the *back door*, in addition to the route suggested by the crystallographic structure (Gilson *et al.*, *Science* 263, 1276-1278, 1994; Axelsen *et al.*, *Protein Science* 3, 188-197, 1994). These calculations show a strong electrostatic field at the *back door*, oriented to attract cations, such as the substrate & one of the reaction products, choline, and to repel anions, such as the other reaction product, acetate. These studies suggest a novel hypothesis that thermal motion of an enzyme may cause it to have multiple routes of access to the active site.

This work was supported by the U.S. Army Medical Research and Development Command under Contract DAMD17-93-C3070, the Association Franco-Israélienne pour la Recherche Scientifique et Technologique, the Minerva Foundation, Munich, Germany and the Kimmelman Center for Biomolecular Structure and Assembly, Rehovot.

Session B: Polymorphism and Structure of Cholinesterases

CHARACTERIZATION OF REACTION INTERMEDIATES IN *CANDIDA RUGOSA* LIPASE, A CLOSE RELATIVE OF ACETYLCHOLINESTERASE. Mirosław Cygler, Biotechnology Research Institute, Natl. Res. Council, Montréal, Quebec H4P 2R2, Canada.

Candida rugosa lipase (CRL) and acetylcholinesterase (AChE) belong to a large family of enzymes showing significant amino acid homology and similarity of their three-dimensional structures. Both enzymes belong to the α/β hydrolase fold superfamily and ~75% of their C α atoms superimpose with r.m.s. of 1.9Å, including the catalytic apparatus composed of a Ser-His-Glu triad. Yet the two enzymes hydrolyze very different substrates. While lipase breaks down water insoluble triglycerides, the acetylcholinesterase hydrolyzes the water soluble neurotransmitter, acetylcholine. Two conformations have been observed for the lipase: the 'closed' conformation, in which the active site is inaccessible from the solvent and is covered by a long loop called the flap, and an 'open' conformation in which the flap is found in a different conformation opening the access to the catalytic triad. The active site in AChE is located at the bottom of long gorge and is accessible from the solvent.

AChE is a very fast enzyme; lipase, on the other hand, hydrolyzes the substrate at a much slower rate. The lipase undergoes a conformational rearrangement from the inactive state in solution to an active state at the lipid/water interface. In CRL, the rearrangement involves the movement of the flap. An analogous loop also exists in AChE, but it is shorter by a few residues. Molecular dynamics simulations (Gilson *et al.*, *Science* 263, 1276 (1994)) indicated that this loop may play an important role in the catalytic mechanism of AChE.

I will compare the two structures near the active site and describe the reaction intermediates for lipase catalyzed hydrolysis of fatty acyl esters based on CRL-inhibitor complexes and will discuss the relevance of these findings for the understanding of AChE catalysis.

Session B: Polymorphism and Structure of Cholinesterases

COMPUTER MODELLING OF ACETYLCHOLINESTERASE AND ACETYLCHOLINESTERASE-LIGAND COMPLEXES. Michael K. Gilson, Jan Antosiewicz, J. Andrew McCammon, and Stanislaw T. Wlodek. Chemistry Department, University of Houston, 4800 Calhoun Road, Houston, TX, USA, 77204-5641.

The current status of our efforts in computer modelling of acetylcholinesterase (AChE) and its complexes with tacrine and 6-chlorotacrine is presented. The pKa values of all ionizable groups have been theoretically determined with a recently developed titration procedure [1]. The possible role of monovalent cations located in the active site of AChE on enzyme activation is discussed. The most likely ionization state of the enzyme at pH 7, derived from the above electrostatic model, is used in multiconfiguration thermodynamic integration [2] analysis of the binding free energy of the drugs tacrine and 6-chlorotacrine.

1. J. Antosiewicz, J. A. McCammon and M. K. Gilson, *J. Mol. Biol.* (1994), in press.
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Session B: Polymorphism and Structure of Cholinesterases

STRUCTURES OF COMPLEXES OF ACETYLCHOLINESTERASE WITH COVALENTLY AND NON-COVALENTLY BOUND INHIBITORS. Michal Harel*, Israel Silman† & Joel Sussman*. *Dept. of Structural Biology, †Dept. of Neurobiology, Weizmann Institute of Science, Rehovot, Israel.

The 2.8Å refined structure of acetylcholinesterase (AChE) (1) with a decamethonium molecule (DECA) in its active site gorge (2) and the refined X-ray structure of the AChE complex with edrophonium (EDR) and tacrine (3) have left a major question unanswered as to the enzyme properties. Namely, how can the substrate reach the active site at the bottom of the deep and narrow gorge (which is filled with water molecules) and the reaction products leave it through the same path at such a fast rate? Here we present the refined X-ray structures of three more complexes of AChE with inhibitors of interest. The complex of AChE with the bisquaternary inhibitor BW251 refined to 2.85Å, shows the interaction of one quaternary nitrogen group with the inner-gorge Trp84 and its outer benzene ring stacking against the outer-gorge Trp279. The bulkier BW251 molecule, occupies a different path along the gorge than DECA. The complex of AChE with pralidoxime (2-PAM) was refined to 2.7Å, and shows the flat 2-PAM interacting with the carboxyl group of Glu199. The complex of AChE with a trifluoroacetophenone inhibitor (TMTFA), refined at 2.8Å, shows a the inhibitor covalently bound to Ser200 Oγ. The oxygen is found in the oxyanion hole, and the quaternary nitrogen group nestles against Trp84 in an identical position to that seen in EDR. The AChE structure in all complexes studied so far, does not show any major conformational changes. The major puzzle of AChE activity rate still remains unsolved.

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This work was supported by the U.S. Army Medical Research and Development Command under Contract DAMD17-93-C3070, the Association Franco-Israélienne pour la Recherche Scientifique et Technologique, the Minerva Foundation, Munich, Germany and the Kimmelman Center for Biomolecular Structure and Assembly, Rehovot.

Session B: Polymorphism and Structure of Cholinesterases

STRUCTURAL ANALYSIS OF THE OLIGOSACCHARIDES OF CHOLINESTERASES. Ashima Saxena and B.P. Doctor, Division of Biochemistry, Walter Reed Army Institute of Research, Washington, D.C., USA.

Cholinesterases are serine esterases that hydrolyze choline esters faster than other substrates. They are highly glycosylated proteins with up to 24% of their molecular weight constituted of carbohydrates. Here we report the results of our studies on the glycosylation of fetal bovine serum acetylcholinesterase (FBS AChE) and horse serum butyrylcholinesterase (Eq BChE). Analysis of the monosaccharide content of the two enzymes indicated that FBS AChE contained 520 nmoles of monosaccharide/mg protein, as compared to 1290 nmoles/mg protein for Eq BChE. Both enzymes contained mannose, galactose, N-acetylglucosamine and sialic acid, and the relative amount of these monosaccharides was the same in both cases. Fucose was present in FBS AChE only and not in Eq BChE. The relatively high content of mannose and the absence of N-acetyl-galactosamine suggested that the carbohydrate in these enzymes was present in the form of N-linked oligosaccharides only. The total oligosaccharide pool consisted of 28% neutral and 72% acidic oligosaccharides for FBS AChE as compared to 19% neutral and 81% acidic oligosaccharides for Eq BChE. The structures of the two major oligosaccharides from FBS AChE and one major oligosaccharide from Eq BChE were determined and found to be very similar except that one of the oligosaccharides from FBS AChE contained a galactose α 1-3 galactose β 1-4-determinant which has been identified as a potentially immunogenic oligosaccharide determinant.

Session B: Polymorphism and Structure of Cholinesterases

PRESSURE EFFECTS ON STRUCTURE AND ACTIVITY OF CHOLINESTERASE. Patrick Masson.
Centre de Recherches du Service de Santé des Armées - Unité de Biochimie, 24, avenue des Maquis du Grésivaudan, 38702 - La Tronche Cédex - FRANCE.

Pressure as a physical variable has long been disregarded in biochemistry in spite of the fact that a diversified life thrives in abyssal zones. High-pressure biochemistry emerged only recently. Yet, unlike temperature, pressure affects weak interactions exclusively, with no change in the total energy of the system under study. Hence, information gained through high pressure studies are important for understanding catalytic mechanisms of enzymes and factors that determine the conformational stability of macromolecules (1,2).

Due to difficulties inherent in implementation of massive equipment, design of experiments and interpretation of results, few high-pressure studies on cholinesterases have been undertaken. However, although results of the pioneering works (3) have to be reinterpreted in the light of the active site structure of AChE as revealed from X-ray diffraction analysis and that results on BuChE are still sketchy (4,5), high pressure may be regarded as a powerful tool for investigating structure and functions of cholinesterases.

Here we present results on the effects of pressure upon binding properties, kinetic behavior and stability of human BuChE. Although microscopic descriptions are still premature, the main conclusions that can be drawn are: a) binding and catalytic steps are accompanied by large volume changes that are dominated by rearrangements of water molecules; b) non linearity of the plots of $\ln k$ vs p indicates differences in compressibility between activated complexes and reactants that may be ascribed to a pressure effect on the enzyme structure rather than on the reactions; c) pressure-induced inactivation and subsequent "denaturation" of the enzyme is a slow process leading to partially unfolded tetramer that resembles the "molten globule" state.

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Session B: Polymorphism and Structure of Cholinesterases

FTIR-SPECTROSCOPIC INVESTIGATIONS OF THE STRUCTURE AND TEMPERATURE STABILITY OF THE ACETYLCHOLINESTERASE FROM *TORPEDO CALIFORNICA*. Ferdinand Hucho, Dieter Naumann¹, and Ute Görne-Tschelnokow. Institut für Biochemie, Freie Universität Berlin, Thielallee 63, 14195 Berlin, Germany; ¹ Robert Koch-Institut des Bundesgesundheitsamtes, 13353 Berlin, Germany.

FTIR spectroscopy of acetylcholinesterase (AChE) isolated from *Torpedo* electric tissue by phospholipase C treatment resulted in an estimate of secondary structure elements (1) which agrees satisfactorily with the X-ray structure of the crystalline enzyme (2). Thermal inactivation of the enzyme occurred above 35°C and reached its maximum at 40°C. Little change of secondary structure was observed in this temperature range. The temperature increase seems to cause only local structural changes, while the gross structure of the polypeptide backbone remains intact. Significant changes in the absorption bands representing secondary structure elements were observed only above 50°C, and especially between 55°C and 60°C. α -Helix and β -structures disappeared. On the other hand, bands at wave numbers 1622 and 1683 cm⁻¹, already present in the spectra of native AChE, increased considerably. These bands were interpreted as representing " β -aggregation".

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- (2) Sussman, J.L., Harel, M., Frolow, F., Oefner, C., Goldman, A., Toker, I., and Silman, I. (1991) Science 253, 872-879.

Session B: Polymorphism and Structure of Cholinesterases

CHARACTERIZATION OF PARTIALLY UNFOLDED STATES OF ACETYLCHOLINESTERASE. David I. Kreimer, Elena A. Dolginova, Israel Silman & Lev Weiner. Depts. of Neurobiology & Organic Chemistry, Weizmann Institute of Science, Rehovot, Israel.

Chemical modification, by a repertoire of thiol reagents, of the non-conserved Cys²³¹ residue of *T. californica* AChE, results in inactivation, even though Cys²³¹ is not involved in catalysis. Modification by disulfides and alkylating agents produces partial unfolding of AChE to a state resembling a molten globule (MG). Although modification by disulfides is reversed by reduced glutathione (GSH), the native (N) conformation is not restored, and no catalytic activity is recovered. AChE so demodified is a partially unfolded species stable for many hours without aggregating. In contrast, modification by mercurials produces a N-like state, viz. a conformational state much closer to N, although also devoid of catalytic activity. Upon demodification with GSH of AChE freshly modified with mercurials, up to 85% of initial AChE activity is recovered, with full restoration of the spectroscopic characteristics of the N state. However, the N-like state produced by mercurials is not stable; it unfolds spontaneously, with $t_{1/2}$ ca. 1 hour, to a non-reactivable, partially unfolded form, similar to that produced by disulfides and alkylating agents. Since these stable states display many of the features of the MG, we also produced such states of AChE by 'traditional' methods, e.g. by exposure to 1.2 M guanidinium chloride (Gu).

In 1.2-2.1 M Gu AChE is in a MG state, but the N $\rightarrow$ MG transition is irreversible; upon removing Gu, no enzymic activity is detected, and the spectroscopic characteristics of N are not recovered. At 5 M Gu, AChE is in an unfolded state (U), in reversible equilibrium with MG. EPR, using a spin-labeled organomercurial, showed that MG and U co-exist in the transition region. We thus demonstrated, for the first time, that the MG $\leftrightarrow$ U transition is two-state. Whereas the MG $\leftrightarrow$ U transition of spin-labeled AChE is completely reversible over a wide range of [Gu], no reverse transition from MG to N occurs upon removing Gu.

The energetic and structural relationships of these partially unfolded states to each other and to N, and the possible reasons for irreversibility of the N $\rightarrow$ MG transition will be discussed.

Sunday, September 25, 1994

MORNING

Session C: Mechanism of Catalysis of Cholinesterases - I

- 8:30 - 8:45 Review of Topic, Chairman
- 8:45 - 9:10 DETERMINANTS OF THE CATALYTIC MACHINERY AND ALLOSTERIC REGULATION OF CHOLINESTERASES. A. Shafferman. Israel Institute for Biological Research, Ness-Ziona, Israel
- 9:10 - 9:35 INSECT ACETYLCHOLINESTERASE AND RESISTANCE TO INSECTICIDES. D. Fournier. Lab. d'entomologie, Université Paul Sabatier, 118 route de Narbonne, 31062 Toulouse, France
- 9:35 - 10:00 MUTATION OF HUMAN BUTYRYLCHOLINESTERASE GLYCINE 117 TO HISTIDINE PRESERVES ACTIVITY BUT CONFERS RESISTANCE TO ORGANOPHOSPHOROUS INHIBITORS. C.A. Broomfield. U.S. Army Medical Research Institute of Chemical Defense, APG, MD 21010-5425
- 10:00 - 10:20 Coffee Break
- 10:20 - 10:45 INHIBITION OF WILDTYPE AND MUTANT ACETYLCHOLINESTERASES WITH FASCICULIN. Zoran Radić. Department of Pharmacology, University of California San Diego, La Jolla, CA 92093-0636
- 10:45 - 11:10 AMINO ACID RESIDUES THAT CONTROL MONO- AND BISQUATERNARY OXIME-INDUCED REACTIVATION OF O-ETHYL METHYL-PHOSPHONYLATED CHOLINESTERASES. Yacov Ashani. Israel Institute for Biological Research, Ness-Ziona, Israel
- 11:10 - 11:35 STRUCTURAL DETERMINANTS OF FASCICULIN SPECIFICITY FOR ACETYLCHOLINESTERASE. Pascale Marchot. Department of Pharmacology, University of California, San Diego, La Jolla, CA 92093-0636 and CNRS URA 1455, Faculté de Medecine-Secteur Nord, Université d'Aix-Marseille, France
- 11:35 - 12:00 THE FUNCTION OF ELECTROSTATICS IN ACETYLCHOLINESTERASE CATALYSIS. D.M. Quinn. Department of Chemistry, University of Iowa, Iowa City, IA.

Session C: Mechanism of Catalysis of Cholinesterases - I

DETERMINANTS OF THE CATALYTIC MACHINERY AND ALLOSTERIC REGULATION OF CHOLINESTERASES. A. Shafferman, D. Barak, A. Ordentlich, C. Kronman, M. Leitner, Y. Flashner, S. Cohen, D. Stein, N. Ariel, S. Reuveny, A. Lazar, D. Marcus, Y. Segall, A. Bromberg and B. Velan. Israel Institute for Biological Research, Ness-Ziona, Israel.

Development of efficient expression systems of recombinant human acetylcholinesterase (HuAChE) were utilized to generate over 80 site directed mutants of HuAChE. A combination of immunological and binding assays, molecular modelling and kinetic studies of these mutants with various substrates and inhibitors revealed that: a. Ser203, His447 and Glu334 constitute the catalytic triad as predicted by the 3D-structure of *Torpedo californica* AChE. b. Trp86 is the anionic subsite involved in π -cation interactions with the quaternary ammonium group of ACh. c. Phe295 and Phe297 constitute the acyl pocket and determine specificity towards alkyl moiety of different substrates and organophosphorous inhibitors. d. Glu202 and Glu450 at the bottom of the "gorge" modulates the catalytic activity towards charged and noncharged substrates and reactivity towards organophosphorous inhibitors. These residues are also involved in catalysis of the "aging" process of OP-AChE conjugates. Accordingly "aging" resistance mutant were generated. e. The aromatic amino acids Tyr72, Tyr124, Trp286, and Tyr341 located close to the rim of the active site gorge together with Asp74 constitute degenerated peripheral anionic sites which allosterically modulate the catalytic activity via a cross talk with residues (Trp86 and Tyr337) at the active center of HuAChE. f. Replacement of up to 7 acidic residues vicinal to the rim of the gorge had no effect of the turnover rates of the mutants, even though calculation shows a gradual shrinking of the electrostatic potential. Thus electrostatic attraction by surface charge does not contribute to the catalytic efficiency of acetylcholinesterase.

This work was supported by the U.S. Army Research and Development Command, Contracts DAMD17-89-C-9117 and DAMD17-93-C-3042.

Session C: Mechanism of Catalysis of Cholinesterases - I

INSECT ACETYLCHOLINESTERASE AND RESISTANCE TO INSECTICIDES. D. Fournier¹, M. Maturano², L. Gagnoux³, P. Ziliani⁴, C. Tertuy³, M. Pralavorio⁴, J.M. Bride⁴, A. Klæbe² and P. Masson³. 1- Lab. d'entomologie, Université Paul Sabatier, 118 route de Narbonne, 31062 Toulouse, France; 2- Lab. IMRCP, groupe de Chimie organique biologique, 118 route de Narbonne, 31062 Toulouse, France; 3- Unité de biochimie, CRSSA 24 Av. des maquis du Guésivaudan BP87 38702 La Tronche cedex, France; 4- Lab. de biologie des invertébrés, INRA, BP 2078 06606 Antibes, France.

Extensive utilization of pesticides against insects provides us a good model for studying the adaptation of an eukaryotic genome submitted to a strong selective pressure. A mechanism of insect resistance to insecticides consists in the alteration of acetylcholinesterase, the molecular target for organophosphates and carbamates. The sequence analysis of the *Ace* gene in several resistant field-strains of *Drosophila melanogaster* resulted in the identification of five point mutations associated with reduced sensitivities to insecticides. Interestingly, some of them were found to be identical in other insects.

Knowledge of wild type acetylcholinesterase being a prerequisite for studying the effect of each mutations found in resistant insects, we first studied the catalytic behavior of the enzyme for substrates. We found that hydrolysis of thioesters deviated from simple Michaelis-Menten model. Kinetics was triphasic, displaying complexities of both BuChE and AChE, i.e., at 25°C and pH7, K_m for acetylthiocholine was 11 μ M, activation at high substrate concentration ($K_{m1} = 0.1$ mM) was followed by inhibition at higher substrate concentration ($K_{m2} = 38$ mM).

Among the mutations found in resistant insects, Ile199(Val129) was mutated to Val or Thr depending on the resistant strain. To study the effect of this mutation, we mutated this residues and we found a positive correlation between the bulkiness of the residue side chain and the sensitivity to insecticide. This allowed us to produce mutant enzymes more sensitive to insecticide than the wild type. As resistance originates from a decrease of the side chain size, we tentatively designed antiresistant compounds by increasing the steric hindrance of the leaving group. With some coumarin derivatives, antiresistant effects have been observed.

Phe368(Phe288) was found to be mutated in Tyr in some resistant strains. We mutated this residues and we found that the amino acid at this position is important for the affinity of the enzyme towards the organophosphate.

In most resistant strains, several of these mutations were found to be present in the same protein leading to different resistance patterns. Combinations of several mutations in the same protein, gave high resistant enzymes. This result suggests that recombination between resistant alleles preexisting in natural populations is a mechanism by which insects rapidly adapt to new selective pressures.

Session C: Mechanism of Catalysis of Cholinesterases - I

MUTATION OF HUMAN BUTYRYLCHOLINESTERASE GLYCINE 117 TO HISTIDINE PRESERVES ACTIVITY BUT CONFERS RESISTANCE TO ORGANOPHOSPHOROUS INHIBITORS. C.A. Broomfield, C.B. Millard, O. Lockridge, and T.L. Caviston. U.S. Army Medical Research Institute of Chemical Defense, APG, MD 21010-5425 and University of Nebraska Medical Center, Eppley Cancer Institute, Omaha, NE 68198-6805.

Our goal is to design, express and characterize mutants of cholinesterases that resist or hydrolyze the organophosphorous (OP) nerve agents, especially soman. Our initial studies have been concentrated on human serum butyrylcholinesterase (BuChE; EC 3.1.1.8) because of its relatively open active site region. By computer-aided molecular modeling based upon the crystal structure of acetylcholinesterase, several residues were selected for site-specific replacement with histidine. We reasoned that introducing an appropriately-positioned imidazole group could promote general base catalysis to hydrolyze the phosphorylated active site serine. The approach was oligonucleotide-directed mutagenesis in M13mp19 and subsequent stable expression in both CHO and human 293 cells by using a cytomegalovirus promoter and the geneticin drug resistance gene. One of the histidine mutants, G117H, was found to retain butyrylthiocholine (BuSCh), acetylthiocholine and benzoylcholine (Bz) activity at pH 7.4 with a $K_{m2} = 0.23 \pm 0.017$ mM for BuSCh. Unmutated, recombinant BuChE had a $K_{m2} = 0.20 \pm 0.016$ mM for BuSCh. Using BuSCh to measure activity, we found the inhibition rates for the BuChE G117H mutant were altered for soman, sarin, DFP and echothiophate (EcSH). For soman, sarin and DFP inhibition, unmutated BuChE showed an apparent, first-order $k_i > 2.8 \text{ min}^{-1}$ and for EcSH a k_i of 0.23 min^{-1} . However, for G117H the k_i was 0.02 min^{-1} for soman, 0.016 min^{-1} for sarin and no inhibition was found for up to 4 hr in the presence of excess ($0.24 \text{ }\mu\text{M}$) EcSH. Assays of the mutant inhibited by DFP produced curved lines that indicated rapid spontaneous reactivation and obviated determination of an accurate inhibition rate constant. By developing a competitive ELISA for BuChE, we estimated the specific Bz activity of G117H to be $180 \pm 20 \text{ U/mg}$, which compares favorably to pure serum BuChE. We conclude that G117H is an active cholinesterase with unusual resistance to nerve agent inhibition.

Session C: Mechanism of Catalysis of Cholinesterases - I

INHIBITION OF WILDTYPE AND MUTANT ACETYLCHOLINESTERASES WITH FASCICULIN. Zoran Radic^{1*}, Daniel M. Quinn², Daniel C. Vellom¹, Shelley Camp¹ and Palmer Taylor¹. ¹ Department of Pharmacology, University of California San Diego, La Jolla, CA 92093-0636; ² Department of Chemistry, University of Iowa, Iowa City, IA 52242.

Fasciculin (FAS), a potent peptide inhibitor with picomolar affinity for acetylcholinesterase (AChE) (EC 3.1.1.7) was used to inhibit recombinant mouse AChEs: wild type and three mutants of tryptophan 86: W₈₆Y, W₈₆F and W₈₆A. These mutations in the AChE choline binding site decreased fasciculin affinity for the enzyme only slightly resulting in one order of magnitude higher K_i's. However, none of the enzymes was inhibited completely even at nanomolar or higher FAS concentrations after prolonged (overnight) incubation of enzyme with FAS. Residual activity ranged from 0.1 % to 200 % of control activity and was dependent on mutation, substrate concentration and whether acetylcholine or neutral substrates phenylacetate or p-nitrophenylacetate were used in the assay of enzyme activity. Substrate concentration dependence measurements yielded K_m and k_{cat} values for these activities. Inhibition by neutral organophosphate paraoxon was largely unaffected by presence of bound FAS, while rates of the cationic organophosphate echothiophate were only up to an order of magnitude slower. These results indicate that AChE active serine is readily accessible to substrates and inhibitors in the FAS-AChE. Fasciculin inhibition appears to occur in part by restricting the access of substrates to the active site and by allosterically affecting the catalytic constants. Part of the latter mechanism appears to be mediated by a linkage between the FAS binding site at the rim of the gorge and W₈₆.

* Visiting Fellow from the Institute for Medical Research and Occupational Health, University of Zagreb, 41000 Zagreb, Croatia

Session C: Mechanism of Catalysis of Cholinesterases - I

AMINO ACID RESIDUES THAT CONTROL MONO- AND BISQUATERNARY OXIME-INDUCED REACTIVATION OF *O*-ETHYL METHYLPHOSPHONYLATED CHOLINESTERASES. Yacov Ashani^{¶§}, Bhupendra P. Doctor[§], Zoran Radić[‡], Daniel C. Vellom[‡], Natilie A. Pickering[‡], Daniel M. Quinn ^{#‡}, and Palmer Taylor[‡]. [§]Division of Biochemistry, Walter Reed Army Institute of Research, Washington, DC 20307-5100 and [‡]Department of Pharmacology, University of California at San Diego, La Jolla, CA 92093.

We have examined the role of specific amino acid side chains in the kinetics of dephosphonylation of cholinesterases. Recombinant mouse acetylcholinesterase (rMoAChE) mutants were inhibited with racemic MEPQ, and the resulting mixture of two stereo adducts, $\text{CH}_3\text{P}_{\text{sR}}(\text{O})(\text{OC}_2\text{H}_5)\text{-AChE}$ ($\text{EMP}_{\text{sR}}\text{-AChE}$) were subjected to reactivation with monoquaternary (P_2S) and bisquaternary (HI-6) monooximes. The nucleophilicity of the oximate anions of P_2S and HI-6 was determined with respect to their reaction with MEPQ. Kinetic analysis of the reactivation profiles revealed a biphasic behavior with approximately 1:1 mixture of two reactivable components. Biochemical constants of the reactivation (oxime affinity to $\text{EMP}_{\text{sR}}\text{-AChE}$, K'_{ox} ; unimolecular rate constant, k'_{max} ; bimolecular rate constant, $k_i = k'_{\text{max}}/K'_{\text{ox}}$) were compared with those obtained for wild-type rMoAChE, tissue-derived *Torpedo* AChE and human plasma butyrylcholinesterase (HuBChE). Substitution of key amino acid residues at the entrance to the active site gorge had a greater influence on k_i of HI-6 compared with P_2S . Residues deep within the gorge, such as F295, E202, and W86, have distinctive influence on reactivation. These results together with energy minimization experiments enabled us to (a) delineate surfaces that orient the oximate nucleophiles toward the organophosphonyl-bound moiety, (b) provide an explanation for differences in the reactivation potency of P_2S and HI-6, (c) identify amino acid residues that are important for substrate hydrolysis but are not required for the reactivation process, and (d) explain the stereoselectivity of displacement of EMP_{sR} -bound moiety.

[¶] Visiting scientist from Israel Institute for Biological Research, Ness-Ziona, Israel.

[#] Visiting scientist from Department of Chemistry, University of Iowa, Iowa City, IA.

Session C: Mechanism of Catalysis of Cholinesterases - I

STRUCTURAL DETERMINANTS OF FASCICULIN SPECIFICITY FOR ACETYLCHOLINESTERASE.

Pascale Marchot^{1,2}, Zoran Radić¹, Shelley Camp¹, Pierre E. Bougis² and Palmer Taylor¹. ¹ Department of Pharmacology, University of California, San Diego, La Jolla, CA 92093-0636 and ² CNRS URA 1455, Faculté de Médecine-Secteur Nord, Université d'Aix-Marseille, France.

The fasciculins are a family of three fingered, 6500 Da peptides, derived from snake venoms and homologous to the α -toxins and cardiotoxins, that bind to acetylcholinesterase (AChE) with dissociation constants between 1-10 pM. The portion of their binding site that is unique to AChE has been defined through site-specific mutagenesis and found to be near the rim of the gorge. Irreversible inhibitors such as DFP can still enter the gorge of the fasciculin-AChE complex to react with the active site serine. To examine this interaction further, we have taken a multifaceted approach. First, we have developed comparative assays for inhibition of catalytic activity by fasciculin and for direct radiolabeled fasciculin binding to soluble AChE. Second, we have examined fasciculin interactions with mutant enzyme species of AChE in which high affinity binding occurs yet inhibition of catalysis is limited. Third, we have created a synthetic gene for fasciculin 2 and expressed recombinant fasciculins in mammalian cells. This approach is allowing us to examine in detail the site and mechanism of inhibition of this peptide. Fasciculin appears to exert a bimodal inhibition mechanism. By associating with the lip of the active center gorge, it performs a partial gating function affecting substrate entry. Second, it has a dramatic allosteric influence on the catalytic constants which appears mediated between the peripheral site and active center.

Session C: Mechanism of Catalysis of Cholinesterases - I

THE FUNCTION OF ELECTROSTATICS IN ACETYLCHOLINESTERASE CATALYSIS. D.M. Quinn, J. Seravalli, H.K. Nair, R. Medhekar. Department of Chemistry, University of Iowa, Iowa City, IA. Z. Radić, D. Vellom, N. Pickering, P. Taylor. Department of Pharmacology, University of California-San Diego, La Jolla, CA.

The function of the electrical field of *T. californica* AChE has been studied by measuring the effects of ionic strength on transition state analog binding and substrate turnover. Michaelis-Menten parameters for ATCh hydrolysis were measured in H₂O and D₂O. The solvent isotope effect on k_{cat}/K_m increases from -1.2 at zero ionic strength to > 2.0 at infinite ionic strength, while k_{cat}/K_m decreases ~15-fold. These trends are interpreted in term of a model wherein, as ionic strength increases, k_1 decreases ~20-fold and k_2 increases ~2 to 3-fold; k_1 and k_2 are the respective rate constants for ATCh binding and release. As ionic strength increases the affinity of AChE for the transition state analog *m*-(N,N,N-trimethylammonio)trifluoroacetophenone, TMTFA, decreases. As observed for ATCh turnover, the ionic strength effect on k_{on} was more pronounced than on k_{off} . Site-directed mutagenesis of Glu¹⁹⁹ to Gln reduces k_{cat}/K_m for ATCh turnover by 100-fold. Proton inventories of this reaction in H₂O-D₂O mixtures indicate that in the Gln mutant the chemical step in the acylation stage of catalysis is slowed 200-fold. This effect on chemical catalysis is matched by a 300-fold loss of binding affinity of AChE for TMTFA. These results indicate that the function of anionic residues in AChE is to maximize forward flux through the acylation stage of catalysis. Glu¹⁹⁹ does this by specifically stabilizing the tetrahedral intermediate, which by a Hammond effect stabilizes the acylation transition state. The overall electrical field of AChE maximizes forward flux by accelerating diffusional substrate encounter with and decelerating substrate release from the active site.

Sunday, September 25, 1994

AFTERNOON

Session C: Mechanism of Catalysis of Cholinesterases - II

- 2:00 - 2:15 Review of Topic, Chairman
- 2:15 - 2:40 MODULATION OF CATALYSIS AND INHIBITION OF ACETYLCHOLINESTERASE BY MONOCLONAL ANTIBODIES. Bhupendra P. Doctor. Walter Reed Army Institute of Research, Washington, DC 20307-5100 USA
- 2:40 - 3:05 ANTI-ACHE MONOCLONAL ANTIBODIES BEHAVE AS STRONG INHIBITORS BY BINDING TO THE PERIPHERAL SITE OF THE ENZYME. Jacques Grassi. C.E.A., Service de Pharmacologie et d'Immunologie, DRIPP, C.E Saclay, 91191 Gif sur Yvette cedex, France
- 3:05 - 3:30 NEUROPATHOLOGY AND PHYSIOLOGY OF DYSAUTONOMIA INDUCED BY ACETYLCHOLINESTERASE ANTIBODIES. S. Brimijoin. Department of Pharmacology, Mayo Clinic, Rochester, MN 55905
- 3:30 - 3:50 Coffee Break
- 3:50 - 4:15 ORGANOPHOSPHONATE REACTIONS WITH ACETYLCHOLINESTERASE. Harvey Alan Berman, Department of Biochemical Pharmacology, SUNY at Buffalo, Buffalo, New York 14260
- 4:15 - 4:40 SITE-DIRECTED LABELING STUDIES ON CHOLINESTERASES. Maurice Goeldner. Laboratoire de Chimie Bio-Organique, URA 1386 CNRS, Faculté de Pharmacie, Université Louis Pasteur Strasbourg, BP 24, 67401 Illkirch cedex, France
- 4:40 - 5:05 INTERACTIONS BETWEEN THE ACHE PERIPHERAL AND ACTIVE SITES. Terrone L. Rosenberry. Department of Pharmacology, Case Western Reserve University, Cleveland, OH 44106
- 5:05 - 5:30 COMPUTATIONAL STUDIES OF THE CATALYTIC APPARATUS OF ACETYLCHOLINESTERASE AND TRYPSIN. Ildiko M. Kovach. The Catholic University of America, Washington D. C. 20064

Session C: Mechanism of Catalysis of Cholinesterases - II

MODULATION OF CATALYSIS AND INHIBITION OF ACETYLCHOLINESTERASE BY MONOCLONAL ANTIBODIES. Bhupendra P. Doctor, Yacov Ashani, Mary K. Gentry, and Ashima Saxena. Division of Biochemistry, Walter Reed Army Institute of Research, Washington, DC 20307-5100 USA.

Monoclonal antibodies (MAbs) generated against FBS AChE and several organophosphate (OP)-inhibited forms of FBS AChE were analyzed for their ability to modulate catalytic activity of AChE. Six MAbs were found to inhibit from 60 to 98% of the catalytic activity of FBS AChE. Four of the six MAbs (25B1, 6H9, 5E8, and 4E5) bound to both the monomeric and tetrameric forms of the enzyme and caused >98% inhibition of enzyme activity. The two remaining MAbs (2A1 and 13D8) appeared to bind and inhibit the tetrameric form of the enzyme more efficiently than the monomeric form. The MAbs that inhibited AChE activity >98% also remarkably slowed the binding of DFP and other OPs to the AChE:MAB complex. Displacement of DFP from DFP:AChE:MAB complexes by oxime, complete in 24 hr in the case of MAbs 25B1 and 13D8 and less than 50% displacement for the other four MAbs, indicated that the oxime was able to reach the Ser-OP moiety of the enzyme. MAbs bound to the native form of AChEs and in some cases to the denatured form also. The monomeric form of FBS AChE appeared to form single complexes with all six MAbs, as judged by sucrose density gradient centrifugation. The tetrameric form of enzyme, however, formed two types of AChE:MAB complexes. Single complexes with tetrameric subunits were formed with MAbs 6H9, 5E8, and 2A1, whereas MAbs 25B1, 13D8, and 4E5 formed multimeric complexes between tetrameric subunits. The rate of complex formation between all the MAbs and the tetrameric form was not affected by edrophonium, indicating that these MAbs do not bind at the catalytic site. Only MAb 2A1 showed decreased binding to the monomeric form in the presence of edrophonium. The reduction in the rate of complex formation by propidium in all cases suggests that the epitope for these MAbs lies in the vicinity of the peripheral anionic site of the molecule. These findings indicate that changes in conformation caused by interaction with MAbs at a site remote from the catalytic site result in modulation of the catalytic activity of FBS AChE.

Session C: Mechanism of Catalysis of Cholinesterases - II

ANTI-ACHE MONOCLONAL ANTIBODIES BEHAVE AS STRONG INHIBITORS BY BINDING TO THE PERIPHERAL SITE OF THE ENZYME. Marie-Hélène Remy*, Yveline Frobert and Jacques Grassi. C.E.A., Service de Pharmacologie et d'Immunologie, DRIPP, C.E. Saclay, 91191 Gif sur Yvette cedex, France.
*Institut de Pharmacologie et Biologie Structurale, CNRS, UPR 8221, 205 route de Narbonne, 31077 Toulouse cedex, France.

Anti-Electrophorus AChE antibodies have been raised in mice by immunizing with MPT-treated enzyme (G4 form). Three monoclonal antibodies (Elec 403, Elec 408 and Elec 410), presenting inhibitory properties have been isolated and characterized. All Mabs bind to AChE with high affinity and behave as strong non competitive inhibitors (apparent $K_i \leq 10^{-10}M$) with regard to charged and neutral substrates. One of them (Elec 403) appeared particularly effective since only one percent of residual activity is observed in presence of excess antibodies. When used together with Mab Elec 408, which bind to another epitope, a cumulative effect is observed leading to a quite total inactivation of AChE. By the means of radioimmunological measurements performed with radioiodinated AChE we could demonstrate that the binding of Elec 403 and Elec 410 can be antagonized by known enzyme inhibitors including: propidium, tacrine, BW 284 c51 and fasciculine. These results strongly suggest that these antibodies allosterically modulate the enzyme activity by binding to the "peripheral site" of Electrophorus AChE. We think that a good understanding of Mab induced inhibition could allow to define new sites of action for anti-cholinesterase drugs.

Session C: Mechanism of Catalysis of Cholinesterases - II

NEUROPATHOLOGY AND PHYSIOLOGY OF DYSAUTONOMIA INDUCED BY ACETYLCHOLINESTERASE ANTIBODIES. S. Brimijoin, Z. Rakonczay, G.N. Tyce, P. Hammond. Department of Pharmacology, Mayo Clinic, Rochester, MN 55905.

Systemically injected AChE antibodies selectively destroy preganglionic sympathetic neurons in adult rats and AChE-bearing fibers in brain and spinal cord of newborn rats. Several lines of evidence indicated that these neural lesions depend on a humoral immunologic mechanism, primarily complement-activation. For example immunocytochemistry of ganglia soon after antibody injection revealed punctate deposits of murine immunoglobulin G and complement component C3, whereas heavy infiltrations of inflammatory cells were never seen. Nerve cell bodies resisted the direct effects of AChE antibodies but morphometric analysis of preganglionic neurons in the intermediolateral nucleus of the spinal cord showed progressive loss of cholinergic perikarya over several months. The delayed neuronal death, possibly by apoptosis, resulted in permanent dysautonomia. To characterize the dysautonomia, catecholamines were measured. AChE-antibodies caused no loss of adrenaline or noradrenaline in peripheral tissues, nor any marked change of basal levels in plasma and urine. On the other hand, the massive catecholamine release that normally accompanies forced immobilization was almost completely blocked. These results are consistent with previous evidence of widespread and persistent antibody-mediated damage to the preganglionic sympathetic system. Taken together, the findings suggest that experimental AChE-autoimmunity is a good model for the human neurologic disease known as Multiple System Atrophy.

Session C: Mechanism of Catalysis of Cholinesterases - II

ORGANOPHOSPHONATE REACTIONS WITH ACETYLCHOLINESTERASE. Harvey Alan Berman, Department of Biochemical Pharmacology, SUNY at Buffalo, Buffalo, New York 14260.

Organophosphonate reactions with *Torpedo* AChE can be analyzed with respect to three independent covalent criteria: *inhibition*, a bimolecular reaction between enzyme and organophosphonate, *oxime reactivation*, which is thought to occur as a bimolecular reaction between covalently modified enzyme and oxime, and *aging*, a unimolecular C-O bond scission, sometimes termed *dealkylation*. Inhibition rates reflect the initial orientation of the organophosphonate with respect to reactive residues within the active center. Rates of reactivation and aging, in contrast, provide an indication of orientation achieved by the phosphonate *after* inhibition, and as such reflect the degree to which pseudorotation of the organophosphonyl-enzyme serves to position the different groups within the active center. Comparative analysis of these three reactions therefore offers insight into steric, electronic, and nonbonded interactions that govern the ligand recognition in the primary nonbonded complex and stability and fate of the subsequent covalent conjugate.

With this rationale in mind, we examined the inhibitory activity of a large family of racemic cycloheptyl *n*-alkylphosphonofluoridates and *n*-alkylphosphonyl thiocholines; these agents differ in the steric and hydrophobic contributions of the alkyl groups surrounding phosphorus, and the electrostatic charge of their leaving groups. An additional family of agents that differ in the degree of *branching* at the α -carbon adjacent to phosphorus allows direct estimation of any steric hindrance in ligand approach to the reactive serine. Covalent reactions to form the organophosphonyl conjugate and the fate of the covalent conjugate are found to be dependent on the nature of the initial leaving group. These observations direct attention to the tetrahedral configuration of organophosphonates, the trigonal bipyramidal geometry of their transition states and intermediates, the multiplicity of reaction mechanisms through which such molecules undergo reaction, and the importance of orientational freedom of the organophosphonate within the active center cleft. The latter consideration, in turn, raises questions about the occurrence of productive and nonproductive complexes, on the one hand, and physical determinants of chiral preference, on the other.

Session C: Mechanism of Catalysis of Cholinesterases - II

SITE-DIRECTED LABELING STUDIES ON CHOLINESTERASES. Isabelle Schalk, Christine Loeb, Florian Nachon, Laurence Ehret-Sabatier and Maurice Goeldner. Laboratoire de Chimie Bio-Organique - URA 1386 CNRS, Faculté de Pharmacie - Université Louis Pasteur Strasbourg, BP 24 - 67401 Illkirch cedex - France.

Aryldiazonium salts represent affinity and photoaffinity labels of the cholinergic enzymes by interacting with the enzyme quaternary ammonium binding sites. Initially, [³H]DDF, paradimethylamino benzene diazonium fluoro-borate, was described as a powerful photoaffinity label of *Torpedo* acetylcholinesterase which allowed us to probe the quaternary ammonium binding site at the active site (Phe330 and Leu 332)^[1,2] as well as from the peripheral site (Trp 279)^[2,3]. The use of a newly synthesized coumarine diazonium derivative led to an efficient and specific affinity labeling of the peripheral site of acetylcholinesterase. The candidate nucleophilic residues from the enzyme were identified as Tyr 70 or Tyr 121. From site-directed mutagenesis studies (Collaboration S. Bon, J. Massoulié E.N.S. Paris), it was possible to demonstrate the involvement of Tyr 70 in this covalent reaction. In particular, a correlation between the nucleophilic character of the mutated residue and the rate of alkylation with the coumarin diazonium probe was established. Finally, photoaffinity labeling studies on human serum butyrylcholinesterase will be presented, first using the DDF probe and secondly, using an aromatic ester diazonium derivative, substrate of the enzyme.

^[1] B. Kieffer, M. Goeldner, C. Hirth, R. Aebersold, J.-Y. Chang (1986) FEBS Lett. 202, 91-95.

^[2] M. Harel, I. Schalk, L. Ehret-Sabatier, M. Goeldner, C. Hirth, P. Axelsen, I. Silman and J.L. Sussman (1993) Proc. Natl. Acad. Sci. USA 90, 9031-9035.

^[3] I. Schalk, L. Ehret-Sabatier, F. Bouet, M. Goeldner, C. Hirth (1994) Eur. J. Biochem. 219, 155-159.

Session C: Mechanism of Catalysis of Cholinesterases - II

INTERACTIONS BETWEEN THE AChE PERIPHERAL AND ACTIVE SITES. Terrone L. Rosenberry, Jean Eastman, and Robert Haas. Department of Pharmacology, Case Western Reserve University, Cleveland, OH 44106.

The three-dimensional crystal structure of AChE shows the active site to be a 20 Å gorge lined with aromatic residues. Certain cationic ligands bind to a peripheral anionic site near the mouth of the gorge and alter catalytic activity. We are determining whether these peripheral site ligands act primarily by sterically blocking access to the active site or by conformationally altering the active site structure. Two specific peripheral site reagents are chloro(2,2':6',2''-terpyridine)platinum(II) chloride (Pt(terpy)Cl), which reacts covalently with H285 in the peripheral site of human AChE, and fasciculin 2, a polypeptide neurotoxin that produces long-lasting fasciculations in mice. The rate constants k_{on} and k_{off} for fasciculin 2 binding were $2.7 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ and $2.9 \times 10^{-4}\text{s}^{-1}$ respectively. Propidium acted as a strict competitive inhibitor of fasciculin binding, decreasing only k_{on} and altering k_{off} by less than 25%. This observation indicates strongly that propidium and fasciculin 2 bind competitively to the same site. In contrast, the active site inhibitor edrophonium formed a ternary complex with AChE and fasciculin 2 in which the k_{on} for fasciculin was decreased only about 25% while k_{off} was increased 5 fold. The increase in k_{off} indicated a modest conformational interaction between the sites. In contrast to (Pt(terpy)Cl), which inhibits acetylthiocholine but not phenyl acetate hydrolysis, fasciculin 2 was a noncompetitive inhibitor of both substrates, decreasing k_{cat} for both substrates by about a factor of 30. In a key test of whether this decrease was predominantly a steric effect of fasciculin 2 binding on substrate access or a conformationally mediated decrease in k_{cat} in the ternary complex, the D₂O effects on k_{cat} and k_{cat}/K_{app} for both substrates at saturating concentrations of fasciculin 2 were measured. Kinetic models predicted that a steric effect would give a decrease in the D₂O effects on these parameters while a conformational effect would give an increase. A decrease in all the parameters was observed, indicating that fasciculin 2 acts predominantly to alter the conformation of the active site in the ternary complex so that steps involving general acid-base catalysis are slowed. The observations that Pt(terpy)Cl has almost no effect on phenyl acetate hydrolysis while fasciculin 2 blocks phenyl acetate hydrolysis just as well as acetylthiocholine hydrolysis indicate that these two peripheral site blockers affect the active site in somewhat different ways.

Session C: Mechanism of Catalysis of Cholinesterases - II

COMPUTATIONAL STUDIES OF THE CATALYTIC APPARATUS OF ACETYLCHOLINESTERASE AND TRYPSIN. Ildiko M. Kovach. The Catholic University of America, Washington D. C. 20064.

Molecular mechanics and molecular dynamics simulations were performed with trypsin and acetylcholinesterase (AChE) from *Torpedo californica* for purposes of comparison of the mechanisms of catalysis of the two enzymes. X-ray crystallographic coordinates for the enzymes were obtained from the Brookhaven Protein Data Bank. The structures were prepared for molecular mechanics calculations in the YETI (V5.3) environment. Hydrogens were assigned to heteroatoms including solvate water and additional solvate water was generated by a module of YETI. The entire protein was then refined to convergence criteria, 0.025 kcal mol⁻¹ deg⁻¹ for torsional RMS first derivatives, to 0.050 kcal mol⁻¹ deg⁻¹ for rotational RMS first derivatives, and to 0.75 kcal mol⁻¹ Å⁻¹ for translational RMS first derivatives. The dynamics simulation was carried out with program AMBER as implemented in MacroModel. Low energy conformations were identified for the catalytic His and the oxyanion hole region in each enzyme.

Minimum energy structures of the serine esters of a number of alkoxy alkylphosphonic acids were generated with MNDO as implemented in MOPAC 6.0. Mulliken point charges and ESP charges were also computed for the fragments. Each enantiomer of the serine ester of the appropriate alkoxy alkylphosphonate was then incorporated into the structure of the enzyme to be studied and the entire structure was again minimized. The conformational mobility of the catalytic His at the active site has been reevaluated for the adducts. Energy partitioning of the resulting diastereomeric enzyme adducts showed that the potential for hydrogen bonding to the P=O group in the oxyanion hole and the various interactions at the binding site for the evolutionarily anticipated natural substrate provide distinction between the enantiomers of an inhibitor. The contribution of the hydrogen bonding interactions in the oxyanion hole in different hydrolases and different inhibitors will be analyzed.

Monday, September 26, 1994

MORNING

Session D: Cellular Biology of Cholinesterases

- 8:30 - 8:45 Review of Topic, Chairman
- 8:45 - 9:10 RESTRICTED REGULATION OF ACHE TRANSCRIPTION, TRANSLATION, AND LOCALIZATION: INDIVIDUAL NUCLEI RESPOND TO LOCALLY-GENERATED SIGNALS IN MULTINUCLEATED SKELETAL MUSCLE FIBERS. Richard L. Rotundo. Dept. of Cell Biology and Anatomy, University of Miami School of Medicine, Miami, Florida 33136
- 9:10 - 9:35 ACETYLCHOLINESTERASE AT NEUROMUSCULAR JUNCTIONS: DENSITY, CATALYTIC TURNOVER AND MOLECULAR FORMS WITH MODELING AND PHYSIOLOGICAL IMPLICATIONS. L. Anglister. Dept. Anatomy, Hebrew Univ. Med. Sch., Jerusalem 91120, Israel
- 9:35 - 10:00 RESIDUES IN *TORPEDO CALIFORNICA* ACETYLCHOLINESTERASE REQUIRED FOR GLYCOPHOSPHOLIPID ANCHORING. Göran Bucht. National Defense Research Establishment (Dept. of NBC Defense, S-901 82 Umeå, Sweden)
- 10:00 - 10:20 Coffee Break
- 10:20 - 10:45 ACETYLCHOLINESTERASE ADAPTS TO VOLUNTARY WHEEL RUNNING BY A MASSIVE G_4 INCREASE IN FAST MUSCLES AND AN A_{12} DECLINE IN SOLEUS. V. Gisiger. Département d'Anatomie, Université de Montréal, Québec, Canada
- 10:45 - 11:10 NEURAL REGULATION OF ACETYLCHOLINESTERASE IN SLOW AND FAST MUSCLES OF THE RAT. J. Sketelj. Institute of Pathophysiology, 61105 Ljubljana, Slovenia
- 11:10 - 11:35 POST TRANSLATION PROCESSING OF ACETYLCHOLINESTERASE - CELLULAR CONTROL OF BIOGENESIS AND SECRETION. B. Velan. Israel Institute for Biological Research, Ness-Ziona, Israel

Session D: Cellular Biology of Cholinesterases

RESTRICTED REGULATION OF AChE TRANSCRIPTION, TRANSLATION, AND LOCALIZATION: INDIVIDUAL NUCLEI RESPOND TO LOCALLY-GENERATED SIGNALS IN MULTINUCLEATED SKELETAL MUSCLE FIBERS. Richard L. Rotundo, Susana G. Rossi, Rosely O. Godinho, Ana E. Vazquez, and Bhavya Trivedi. Dept. of Cell Biology and Anatomy, University of Miami School of Medicine, Miami, Florida 33136.

The highly localized accumulation of AChE at vertebrate neuromuscular junctions, and the paucity of this enzyme in extrasynaptic regions, requires that expression be differentially regulated in the innervated and non-innervated compartments. In skeletal muscle fibers, AChE mRNA and protein are concentrated at sites of nerve muscle contact (Jasmin et al., 1993), where a portion of the expressed AChE is tightly attached to the synaptic basal lamina (Rossi and Rotundo, 1993). In contrast, AChE transcripts are almost undetectable in non-innervated regions where enzyme levels are less than 3-5% of those found at the synapse. Primary quail myoblasts initially express low levels of AChE transcripts and protein which subsequently increase prior to the time of myoblast fusion. The transcripts accumulate adjacent to the myoblast nuclei, detected by *in situ* hybridization, and the vast majority of nuclei in newly formed myotubes are bracketed by patches of AChE mRNA. As differentiation proceeds, and the myotubes begin to spontaneously contract, a dramatic decrease in the number of nuclei exhibiting accumulations of AChE mRNA occurs, in parallel with the decrease in mRNA levels measured by Northern blot analysis. It thus appears that the decrease in AChE mRNA results from a decrease in the fraction of nuclei expressing the gene rather than a synchronous decrease by all myotube nuclei. Preliminary studies with tetrodotoxin-treated (TTX) myotubes indicate that blocking spontaneous contraction increases total AChE mRNA with most nuclei surrounded by transcripts, whereas sodium channel agonists such as veratridine or scorpion toxin (ScTx) reduce transcript expression and the number of nuclei with detectable AChE mRNA. Using microchambers (Campenot, 1981) to physically isolate segments of individual myotubes, exposure of individual nuclei to either TTX or ScTx results in a localized increase or decrease of AChE indicating that each nucleus responds to signals generated on the overlying plasma membrane. These signals are generated by combinations of membrane depolarization and production of second messengers such as DAG which increases A₁₂ AChE expression by activating protein kinase C, or increases in cAMP which specifically blocks assembly of A₁₂ AChE. Together, these molecular events provide the foundation for local control of AChE expression in synaptic and extra-synaptic regions of skeletal muscle fibers.

Session D: Cellular Biology of Cholinesterases

ACETYLCHOLINESTERASE AT NEUROMUSCULAR JUNCTIONS: DENSITY, CATALYTIC TURNOVER AND MOLECULAR FORMS WITH MODELING AND PHYSIOLOGICAL IMPLICATIONS. L. Anglistter, J.R. Stiles*, B. Haesaert, J. Eichler and M.M. Salpeter*. Dept. Anatomy, Hebrew Univ. Med. Sch., Jerusalem 91120, Israel. * Section of Neurobiology and Behavior, Cornell Univ., Ithaca, NY 14853.

Acetylcholinesterase (AChE) is concentrated at vertebrate neuromuscular junctions, where being interposed between presynaptic release sites and postsynaptic receptors (AChR's), it hydrolyzes acetylcholine and terminates synaptic transmission. Knowledge of the junctional AChE and AChR concentrations is crucial for understanding transmission at this synapse. However, although AChR density has been measured in endplates of several species and shows little variability, AChE concentration has been determined only in mouse endplates. We determined AChE site density at the neuromuscular junctions of frog by electron microscope autoradiography and biochemistry to be ~ 600 sites/ μm^2 of post-synaptic surface area, a value ~ 4 -fold lower than previously determined for mouse endplates. On the other hand, frog AChE hydrolytic turnover number was relatively high, $9,500\text{ s}^{-1}$, but well within the range determined for AChE in rat and lizard muscles, and *Electrophorus* and *Torpedo* electric organs ($2,000$ - $16,000\text{ s}^{-1}$). To investigate the physiological implications of our experimental results, we performed Monte Carlo computer simulations of miniature endplate currents for the different frog and lizard neuromuscular junction geometries. The modeling and simulations showed that for vertebrate neuromuscular junctions with different morphologies, an AChE density of ~ 400 sites/ μm^2 and a turnover number of only $\sim 1,000\text{ s}^{-1}$ are sufficient for normal quantal currents. Above these critical lower limits, miniature endplate currents were essentially insensitive to AChE density and turnover number values up to $5,000$ sites/ μm^2 and $16,000\text{ s}^{-1}$, respectively, providing additional component to the safety factor for this synapse.

(Supported by Israel - US Binational Science Foundation).

Session D: Cellular Biology of Cholinesterases

RESIDUES IN *TORPEDO CALIFORNICA* ACETYLCHOLINESTERASE REQUIRED FOR GLYCOPHOSPHOLIPID ANCHORING. Göran Bucht, Lena Lindgren, and Karin Hjalmarsson. National Defense Research Establishment (Dept. of NBC Defense, S-901 82 Umeå, Sweden).

The requirements for GPI-modification of acetylcholinesterase, (AChE), has been studied by transient expression of wild type and mutant enzymes in COS-1 cells. The C-terminus in the precursor form of AChE contain a stretch of 28 amino acids, which constitute a signal peptide, (GPIsp), for GPI-modification. The modification takes place in the endoplasmatic reticulum, following or at the same time as the removal of the GPIsp. Expression of mutant enzymes have identified residues that are important for recognition and processing to a membrane anchored enzyme.

Analysis of truncated variants indicate that a deletion of 6 amino acids from the C-terminus, results in a non-modified and inactive intracellular product. An active enzyme was produced and secreted, in high amounts, into the medium when 16 and 21 amino acids were deleted. No enzyme activity was detected on or inside these cells. A slightly decreased efficiacy in dimerization was also found in these two mutants. When all 28 amino acids, the complete GPIsp, was deleted leaving Cys 537 as the C-terminal amino acid, high amounts of the polypeptide was found in the cell culture medium. This mutant enzyme was only produced as a monomer lacking enzyme activity, probably due to the unstability of the monomeric form.

The target site(s) for the glycopospholipid modification was mapped by single and multiple amino acid replacements. Postulated attachment sites was analysed by amino acid replacements into amino acids not allowed in the cleavage and modification site. When the attachment site in native *Torpedo californica* AChE, Cys 537, and neighbouring amino acids were replaced, the mutant enzymes were surprisingly still found anchored to the membrane. These results indicate the existence of differences in the mode of GPI-modification between different species (monkey vs. electric ray).

Session D: Cellular Biology of Cholinesterases

ACETYLCHOLINESTERASE ADAPTS TO VOLUNTARY WHEEL RUNNING BY A MASSIVE G_4 INCREASE IN FAST MUSCLES AND AN A_{12} DECLINE IN SOLEUS. V. Gisiger*, M. Bélisle and P.F. Gardiner. Départements d'Anatomie* et d'Education Physique, Université de Montréal, Québec, Canada.

Chronic enhancement of neuromuscular activity by walking, running and swimming training programs results in selective G_4 adaptations in fast muscles of the rat, with only minor and non-selective AChE changes in the slow-twitch soleus. In order to shed further light on the physiological significance of the AChE adaptations to physical activity, we turned to a voluntary exercise model, much closer to natural activity and exempt of the limitations inherent to coercive training. The impact of 5 days and 4 weeks of voluntary wheel cage running on AChE molecular forms was examined in 4 hindlimb fast muscles and the soleus. Rats, placed in live-in wheel cages, exercised spontaneously for distances which, while varying widely among individual animals, progressively increased from about 0.5-10 km/day, at the 5th day, to about 2-20 km/day at the end of the 4th week. Fast muscles responded by massive G_4 increases (up to 420%) with almost no changes in A_{12} , so that by the 4th week the tetramer became the main AChE component of these muscles. The additional G_4 was composed primarily of amphiphilic molecules, suggesting a membrane-bound state. The G_4 content of fast muscles, which varied extensively among individual animals, was highly correlated with the distance covered by each rat during the last 5 days ($r = 0.850 - 0.879$, $P < 0.001$ in 3 muscles). In opposition to its marginal AChE response to training programs, the soleus muscle adapted to wheel cage activity by a marked selective reduction of its asymmetrical forms, up to 45% in the case of A_{12} . This A_{12} decline, already maximal by the 5th day of wheel running, showed no relationship with the distance covered. These adaptations appear difficult to reconcile with the classical concept limiting the role of muscle AChE to the rapid inactivation of just-released ACh. The present results strengthen the proposal that muscle AChE controls additional functional parameters of the neuromuscular junction, including endplate excitability. With the support of FCAR.

Session D: Cellular Biology of Cholinesterases

NEURAL REGULATION OF ACETYLCHOLINESTERASE IN SLOW AND FAST MUSCLES OF THE RAT. J. Sketelj. Institute of Pathophysiology, 61105 Ljubljana, Slovenia.

Acetylcholinesterase (AChE) regulation in the slow soleus muscle of the rat differs from that in the fast muscles in two respects: a) activity of the globular molecular forms of AChE in the slow muscle is significantly lower than that in the fast muscles which is probably due to much lower level of mRNA for the catalytic AChE subunit in the slow muscles; b) the asymmetric molecular forms of AChE continue to be produced extrajunctionally in the mature slow muscle but not in the fast. Denervation decreases both junctional and extrajunctional AChE activity in all rat muscles. Junctional AChE falls rapidly mostly due to proteolytic degradation of the preexistent enzyme. Extrajunctional globular forms of AChE in fast muscles drop in parallel with a heavy decrease in the steady state level of the mRNA of the catalytic subunit of AChE. This decrease, observed both in junctional and extrajunctional regions, is probably induced by lack of electromechanical activity in muscles after denervation since it can be exactly mimicked by botulinum toxin produced muscle paralysis. In respect to the extrajunctional AChE, differences between slow and fast muscles remain also after denervation. Immediately after denervation, G₄ AChE form stays high in the fast muscles but not in the slow soleus muscle. Several weeks later, the asymmetric AChE forms increase extrajunctionally in the denervated slow muscle but not in the fast. Non-innervated immature (regenerating) rat muscles exhibit high AChE activity all along the muscle fibers, which is in great contrast to denervated mature muscles. If innervated, the regenerating muscles need about a week to start behaving like mature rat muscles in respect to AChE regulation after subsequent denervation.

Immediately after reinnervation, previously denervated slow soleus muscle transiently reverts to an immature type of AChE regulation very similar to that observed during the early postnatal period. Activity of the extrajunctional asymmetric AChE forms increases tremendously for about 10 days and then decreases again. This phenomenon is probably not regulated by a specific neural stimulation pattern since it occurs also in reinnervated disused soleus muscles in which the neural stimulation pattern is changed from tonic to phasic. No such reversion to an immature state occurs in fast muscles in which the synthesis of the asymmetric AChE forms in the extrajunctional regions seems to be suppressed completely and also irreversibly.

Session D: Cellular Biology of Cholinesterases

POST TRANSLATION PROCESSING OF ACETYLCHOLINESTERASE - CELLULAR CONTROL OF BIOGENESIS AND SECRETION. B. Velan, C. Kronman, A. Ordentlich, and A. Shafferman. Israel Institute for Biological Research, Ness-Ziona, Israel.

The interrelationship between polypeptide-folding, subunit-assembly, cellular retention and active secretion of AChE was examined in HEK-293 cells expressing recombinant human enzyme. Enzyme mutants defective in their ability to undergo various post-translation modification processes, as well as molecules carrying specific signals for cellular sorting were generated for monitoring AChE biosynthesis in the cell. Secretion from the cell can be abrogated through various mechanisms: Mutations affecting formation of essential salt-bridges lead to intracellular degradation of the polypeptide; mutations at the of N-glycosylation signals lead to cellular accumulation of malformed inactive molecules; and mutations which introduce the KDEL endoplasmic reticulum (ER) retention signal lead to accumulation of large quantities of fully active molecules (only in monomeric forms). Subunit-assembly does not appear to be a prerequisite for AChE secretion, even though distinct preferential secretion of homodimers over unassembled monomer can be observed. The restriction on monomer secretion does not depend on monomer binding to ER retention receptors nor on pre-golgi degradation of unassembled subunits, and appears therefore to be mediated through other mechanisms. These studies uncover a series of quality control processes which ensure the secretion of properly-folded, oligomerized AChE.

This work was supported by the U.S. Army Research and Development Command, Contract DAMD17-89-C-9117 and DAMD17-93-C-3042.

Tuesday, September 27, 1994

MORNING

**Session E: Structure-Function Relationship of Anticholinesterase Agents:
Nerve Agents and Reactivators**

- 8:30 - 8:45 Review of Topic, Chairman
- 8:45 - 9:10 COMPARISON OF ANTIDOTE PROTECTION AGAINST ORGANOPHOSPHORUS COMPOUNDS BY HI-6, PYRIDOSTIGMINE, AND ACETYLCHOLINESTERASE. D.M. Maxwell. US Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD 21010-5425
- 9:10 - 9:35 PHARMACOLOGICAL EVALUATION OF ANTIDOTES TO NERVE AGENTS. S.N. Dube. Defence Research and Development Establishment Gwalior-474002, India
- 9:35 - 10:00 BISQUATERNARY OXIMES AS ANTIDOTES AGAINST TABUN POISONING: ANTIDOTAL EFFICACY IN RELATION TO IN VITRO AND IN VIVO CHOLINESTERASE REACTIVATION. G. Amitai. Dept. of Pharmacology, IIBR, P.O. Box 19, Ness Ziona 70450, Israel
- 10:00 - 10:25 TOXICOKINETICS OF INHALED SOMAN AND SARIN IN GUINEA PIGS. Hendrik P. Benschop. TNO Prins Maurits Laboratory, P.O. Box 45, 2280 AA Rijswijk, The Netherlands
- 10:25 - 10:45 Coffee Break
- 10:45 - 11:10 MEDICAL PROTECTION AGAINST ORGANOPHOSPHORUS TOXICITY. S. Das Gupta. Defence Research and Development Establishment, Gwalior-4745002, India
- 11:10 - 11:35 EVALUATION OF THE DIRECT ACTIONS OF HI-6 IN REVERSING SOMAN-INDUCED TETANIC FADE. Michael Adler. Neurotoxicology and Biochemical Pharmacology Branches, U.S. Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD 21010-5425 U.S.A
- 11:35 - 12:00 ORGANOPHOSPHATE-SENSITIVE CHOLINERGIC RECEPTORS: POSSIBLE ROLE IN MODULATION OF ANTICHOLINESTERASE-INDUCED TOXICITY. C. Pope. Sch. Pharm., Northeast Louisiana Univ., Monroe, LA, USA
- 12:00 - 12:25 EFFECT OF PHYSICAL/CHEMICAL STRESSORS ON CHOLINERGIC SYSTEM IN RAT. Satu M. Somani. Southern Illinois University School of Medicine, Department of Pharmacology, P.O. Box 19230, Springfield, IL 62794-9230 USA

Session E: Structure-Function Relationship of Anticholinesterase Agents: Nerve Agents and Reactivators

COMPARISON OF ANTIDOTE PROTECTION AGAINST ORGANOPHOSPHORUS COMPOUNDS BY HI-6, PYRIDOSTIGMINE, AND ACETYLCHOLINESTERASE. D.M. Maxwell. US Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD 21010-5425.

Pyridostigmine in combination with atropine, HI-6 in combination with atropine, and fetal bovine serum acetylcholinesterase (FBS-AChE) were compared as examples of carbamate, oxime and enzyme scavenger approaches to protection against organophosphorus (OP) compounds. Each antidotal regimen produced approximately equal maximal protection against the lethal effects of the highly toxic OP compound soman in mice. FBS-AChE was better than either pyridostigmine + atropine or HI-6 + atropine in reducing postexposure incapacitation from soman as measured by motor dysfunction and behavioral tests in mice. Similar results were obtained in rhesus monkeys when protection against the behavioral toxicity of soman by either FBS-AChE or equine butyrylcholinesterase was assessed by the serial probe recognition task or the primate equilibrium platform task. A lower dose of pyridostigmine (566 nmol/kg) or FBS-AChE (1150 nmol/kg) was required to protect against 968 nmol/kg ($8 \times$ Control LD_{50}) of soman than was required for HI-6 (200,000 nmol/kg) in mice. The *in vivo* biological half-life in mice of FBS-AChE (1550 min) was much greater than the biological half-lives of either pyridostigmine (48 min) or HI-6 (11 min). The superiority of FBS-AChE with regard to its prevention of behavioral deficits, stoichiometry, and biological half-life suggests a clear advantage for the enzyme scavenger approach to protection against OP compounds. A major obstacle to general applicability of the enzyme scavenger approach to protection against a wide range of OP compounds is the 1:1 limitation on OP/enzyme stoichiometry of present enzyme scavengers. Current efforts to increase the OP/enzyme stoichiometry include combining enzyme pretreatment with oxime reactivation and the development of AChE mutants that are more easily reactivated.

Session E: Structure-Function Relationship of Anticholinesterase Agents: Nerve Agents and Reactivators

PHARMACOLOGICAL EVALUATION OF ANTIDOTES TO NERVE AGENTS. S.N. Dube, R. Bhattacharya, K. Husain and A.K. Sikder. Defence Research and Development Establishment Gwalior-474002, India.

The organophosphorus (OP) compounds are highly toxic irreversible inhibitors of acetylcholinesterase (AChE) and cause variety of route specific physiological effects. The therapy against OP compounds usually involves co-administration of a cholinolytic and an oxime along with adjuncts while prophylaxis is accomplished by a carbamate pretreatment. In order to develop an effective antidote against OP poisoning, some new series of mono and bispyridinium oximes were synthesised and evaluated for their therapeutic efficacy against sarin and soman intoxication in rodents. The new oximes produced significant beneficial effects as evident by protection index, reactivation of peripheral and central AChE enzyme and on neuromuscular functions. The reactivation of AChE in cerebral cortex by these oximes, in spite of their being quaternary salts, is a notable feature. Pretreatment with a carbamate viz. pyridostigmine produced a dose dependent efficacy against soman poisoning on the recovery of neuromuscular blockade using rat phrenic nerve diaphragm preparation and on the *in vivo* protection in rats and guinea pigs.

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BISQUATERNARY OXIMES AS ANTIDOTES AGAINST TABUN POISONING: ANTIDOTAL EFFICACY IN RELATION TO IN VITRO AND IN VIVO CHOLINESTERASE REACTIVATION. G. Amitai*, G. Cohen, I. Rabinovitz, G. Zomber, R. Adani and L. Raveh. Dept. of Pharmacology, IIBR, P.O. Box 19, Ness Ziona 70450, Israel.

Reactivation of OP-inhibited AChE by bisquaternary oximes is the main mechanism by which these compounds exert their antidotal efficacy in cases of OP poisoning. We have compared the antidotal efficacy of five oximes: AB-8, AB-13, Toxogonin (TOX), HI-6 and HLo-7 against tabun poisoning in mice, guinea pigs, dogs and monkeys. Antidotal efficacy was studied in mice and guinea pigs in combination with atropine and benactyzine either with or without pyridostigmine (PYR) as pretreatment. Relatively high protection ratios were obtained for TOX, HLo-7 and AB-13 even without PYR in guinea pigs (8.6-21.7). These oximes reactivated tabun-inhibited FBS AChE at rates which are consistent with the *in vivo* data (k_r values: 157, 18.7 and 12.5 M⁻¹ min⁻¹, respectively). HLo-7 enhanced by 2-fold the initial reactivation rate of AChE inhibited by both PYR and tabun as compared to reactivation rate obtained after inhibition by tabun only. Using TOX as a reference oxime, a calculated equivalent dose (CED) was defined as the dose equal to the ratio between its minimal toxic dose and the therapeutic ratio of TOX. The CED approach enabled us to compare the antidotal efficacy of oximes in large animals on an equivalent effective dose basis. Antidotal efficacy of oximes was evaluated in dogs and monkeys together with atropine based on the following parameters: survival rate, clinical symptoms and the recovery process during 7 days. Antidotal mixture comprised of atropine and 0.3, 1 or 3x CED of each oxime was used against 5xLD₅₀ tabun in dogs and monkeys. All oximes except for HI-6 exhibited good antidotal efficacy against tabun in dogs. However, HI-6 (3x CED) displayed moderate antidotal efficacy against tabun poisoning in monkeys although it is a poor reactivator of tabunyl-AChE *in vitro*. The best antidotal efficacy in monkeys was obtained for AB-13 (100% survival) and HLo-7 (84% survival) at 3x CED. Antidotal efficacy in both dogs and monkeys was markedly decreased at 0.3x CED except for TOX. *In vivo* reactivation of blood ChE in dogs and monkeys which were exposed to tabun usually correlated well with the *in vivo* recovery process following treatment with oximes and atropine.

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TOXICOKINETICS OF INHALED SOMAN AND SARIN IN GUINEA PIGS. Hendrik P. Benschop and Johannes P. Langenberg. TNO Prins Maurits Laboratory, P.O. Box 45, 2280 AA Rijswijk, The Netherlands.

The inhalation toxicokinetics of C(±)P(±)-soman and (±)-sarin were studied in anesthetized, atropinized guinea pigs. An apparatus was constructed for continuous generation of nerve agent vapor in air and nose-only exposure. During exposure the respiratory minute volume (RMV) and respiratory frequency (RF) were monitored. Blood samples were taken for gas chromatographic analysis of the concentrations of nerve agent stereoisomers, and to measure the progressive inhibition of acetylcholinesterase (AChE). The animals were exposed for 4-8 min to 0.4-0.8 LC₅₀ of C(±)P(±)-soman or (±)-sarin. Concentrations of the toxic C(±)P(-)-isomers increased rapidly during exposure, up to several ng/ml blood. The absorption phase of C(+)P(-)-soman lagged behind that of the C(-)P(-) isomer. The measured progression of AChE inhibition was approximately in accordance with the observed blood levels of C(±)P(-)-soman. There were no soman-related effects on RMV and RF. Upon exposure for 5 h to 0.02 ppm of C(±)P(±)-soman, blood levels of C(±)P(-)-isomers were gradually build up, with maximum concentrations of ca. 40 pg/ml. Correspondingly, blood AChE was gradually inhibited up to ca. 95%. Intravenous infusion of C(±)P(±)-soman appeared to be a suitable substitute for the absorption phase of the technically involved inhalation exposure, unlike intramuscular bolus injection.

This research was supported in part by the US Army Medical Research and Development Command under Grant No. DAMD17-90-Z-0034 and in part by the Directorate of Military Medical Services of the Ministry of Defence, The Netherlands

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MEDICAL PROTECTION AGAINST ORGANOPHOSPHORUS TOXICITY. S. Das Gupta*. Defence Research and Development Establishment, Gwalior-4745002, India.

Organophosphorus compounds (OP) elicit their effects by inhibiting the enzyme acetylcholinesterase (AChE). The therapy against these agents is based on co-administration of a cholinolytic to antagonize the effects of endogenously accumulated acetylcholine and oximes that reactivate inhibited AChE. Among the oximes, pralidoxime, toxogonin and trimedoxime are well known cholinesterase reactivators. The controversy on the cholinesterase reactivating drug which should be preferred is still open. In the process of selecting essential drugs, a founded decision on the 'drug of choice' is only possible on the basis of an experimental evaluation under strictly comparable conditions. For tropical countries this is of special importance. The present work will focus on the different aspects of medical protection against toxic OP compounds based on studies carried out in animals for the last fifteen years. The data will be presented on comparative evaluation of newly synthesised oximes, actions and interactions of various cholinolytics together with cholinesterase reactivators in the treatment of acute OP toxicity. Prophylactic therapy was given in the form of pretreatment using reversible AChE inhibitors viz pyridostigmine, physostigmine against sarin aerosols and soman intoxication. In all these studies measurement of the enzyme AChE in blood and different parts of the brain has been used as a marker. Besides efficacy of various adjuncts like diazepam and bronchodilators (α -adrenoceptor agonists) in the therapy against OP toxicity, the noncholinergic action of oximes using electrophysiological technique will be presented.

* Present address: Indian Science Congress Association, 14 Dr Biresh Guha Street, Calcutta: 700017, India.

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EVALUATION OF THE DIRECT ACTIONS OF HI-6 IN REVERSING SOMAN-INDUCED TETANIC FADE. Michael Adler, Donald M. Maxwell and Sharad S. Deshpande. Neurotoxicology and Biochemical Pharmacology Branches, U.S. Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD 21010-5425 U.S.A.

The actions of the bispyridinium oxime HI-6 ([[(4-aminocarbonyl)pyridino]-methoxy)methyl]-2-[(hydroxyimino)methyl]-pyridinium dichloride) were investigated *in vitro* on rat phrenic nerve-hemidiaphragm preparations. Isometric twitch and tetanic tensions were elicited at 37°C with supramaximal nerve stimulation at frequencies of 20 and 50 Hz. To approximate normal respiration patterns, trials consisting of 30 successive 0.55 s trains were alternated with 1.25 s rest periods. Under control conditions, the above stimulation pattern generated tensions that were well maintained at both frequencies. In contrast, a marked depression of muscle tension was observed in diaphragms removed from rats administered 339 µg/kg soman (3 LD₅₀) and tested *in vitro*. Addition of HI-6, 4 h after soman exposure, led to a nearly complete recovery of muscle tension at 20 Hz. At 50 Hz, muscle tensions still declined especially when trains were elicited at 1.25 and 3 s intervals. The recovery by HI-6 observed in this study appears to be mediated by mechanisms unrelated to acetylcholinesterase reactivation since no increase of enzymatic activity was detected and the effect was reversed by a brief washout in oxime-free physiological solution. The results suggest that the direct action of HI-6 may play a role in restoring soman-induced diaphragmatic failure but this effect would be significant primarily under low use conditions.

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ORGANOPHOSPHATE-SENSITIVE CHOLINERGIC RECEPTORS: POSSIBLE ROLE IN MODULATION OF ANTICHOLINESTERASE-INDUCED TOXICITY. C. Pope, J. Chaudhuri and T. Chakraborti. Sch. Pharm., Northeast Louisiana Univ., Monroe, LA, USA.

The general mechanism of toxicity for organophosphate (OP) pesticides is *via* irreversible inhibition of acetylcholinesterase (AChE). It appears, however, that other biochemical factors can modify the clinical expression of toxicity caused by AChE inhibition. Several investigations in our laboratory have demonstrated that exposure to the common OP pesticides chlorpyrifos (CPF) and parathion (PS) can produce similar levels of brain and peripheral AChE inhibition but marked differences in toxicity. Changes in various functional indicators of toxicity (e.g., SLUD, motor activity, arousal) are highly correlated with the degree of AChE inhibition following PS treatment but not after CPF exposure. Several laboratories (including our own) have shown that some OPs bind directly *in vitro* to a subset of cholinergic receptors (labeled by the high affinity muscarinic agonist [³H]cis-methyldioxolane). We have determined that this subset of OP-sensitive brain muscarinic receptors is differentially up-regulated and down-regulated 2-7 days following exposure to high doses of CPF and PS, respectively. Additionally, these OP-sensitive receptors in cardiac tissue are down-regulated following PS exposure but not affected by CPF administration. Similar degrees of AChE inhibition by CPF and PS are therefore associated with chemical-specific and tissue-specific changes in central and peripheral muscarinic receptor subpopulations. We hypothesize that these differentially-regulated receptors are autoreceptors which modify the synaptic release of acetylcholine. Preliminary *ex vivo* release studies suggest that both chlorpyrifos and parathion exposures alter brain (striatal) acetylcholine release, apparently through modification of autoreceptor function. Comparison of alterations in acetylcholine release with changes in OP-sensitive muscarinic receptor populations following different OP exposures should clarify the role of these receptors in the expression of anticholinesterase-mediated toxicity.

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EFFECT OF PHYSICAL/CHEMICAL STRESSORS ON CHOLINERGIC SYSTEM IN RAT. Satu M. Somani and Shashi N. Dube. Southern Illinois University School of Medicine, Department of Pharmacology, P.O. Box 19230, Springfield, IL 62794-9230 USA and Defence Research and Development Establishment, Tansen Road, Gwalior, India.

Alterations in central neurotransmitter systems due to physical exercise and/or chemical stressors have not received much attention. We have examined whether physostigmine (phy) and exercise or the combination of the two elicit adaptive changes in the synthetic cholineacetyl transferase (ChAT) and degradative-acetylcholinesterase (AChE) enzymes of acetylcholine in the brain regions of rat. ChAT and AChE activities in corpus striatum were significantly decreased due to Phy as well as Phy + exercise. This suggests that corpus striatum is affected by chemical stressors but more so by the combination of chemical and physical stressors. The brainstem is the only region which showed inhibition of ChAT activity due to exercise. Subacute Phy also inhibited brainstem ChAT activity. The hippocampus showed significant decrease in ChAT activity due to Phy + exercise but not due to Phy alone. These results suggest that the brain regions involved with control of motor, autonomic and cognitive functions were affected by subacute Phy and exercise. These data are consistent with the hypothesis that the responsiveness of these brain regions to different stressors is a function of the level of ongoing cholinergic activity and that elevations in ACh levels due to AChE inhibition may have long-term effects on the regulation of ChAT and AChE activities through a negative feedback mechanism. Our results also showed that Phy and exercise has significant effect on ChAT and AChE enzymes in active EDL muscle. Exercise has prolonged the inhibitory effect of Phy on ChAT and AChE activities both in active EDL and passive soleus muscles. This study showed that Phy + exercise modified the functional activity of cholinergic system in EDL and soleus muscles.

Whether the pharmacodynamics of physostigmine (Phy) [rate of decarbamylation of cholinesterase (ChE) enzyme (K_d)] is altered due to acute and/or trained exercise in brain and various tissues of rat has also been addressed. Acute exercise (AE) + Phy increased, whereas endurance training (ET) + Phy decreased ChE activity in brain, red blood cells (RBC), and various tissues as compared to Phy alone. The K_d of heart ChE was significantly decreased by ET + Phy as compared to Phy alone. The K_d of diaphragm ChE was significantly increased in AE + Phy and decreased in ET + Phy as compared to Phy alone. The K_d of muscle ChE significantly decreased by AE + Phy as compared to Phy alone, but ET + Phy did not affect the K_d in muscle. These results suggested that AE and ET have opposite effects on K_d after Phy administration.

Tuesday, September 27, 1994

AFTERNOON

**Session F: Structure-Function Relationship of Anticholinesterase Agents:
Pesticides and Therapeutic Agents;
Noncholinergic Function of Cholinesterases**

- 2:00 - 2:15 Review of Topic, Chairman
- 2:15 - 2:40 SECRETION OF ACETYLCHOLINESTERASE AS A NON-CLASSICAL FORM OF SIGNALLING IN THE BRAIN. Susan A. Greenfield. University Dept. of Pharmacology, Mansfield Rd., Oxford OX1 3QT U.K.
- 2:40 - 3:05 GLYCOSYLATED INACTIVE FORMS OF CHICKEN BUTYRYLCHOLINESTERASES AND THEIR POSSIBLE FUNCTIONS. P. G. Layer. Faculty of Biology, University of Technology Darmstadt, 64287 Darmstadt, Germany
- 3:05 - 3:30 EVIDENCE FOR CHOLINERGIC AND NON-CHOLINERGIC ACTIONS OF TRANSIENTLY EXPRESSED ACETYLCHOLINESTERASE IN DEVELOPMENT OF THALAMOCORTICAL PROJECTIONS. R.T. Robertson. University of California, Irvine, CA 92717
- 3:30 - 3:55 NON-CHOLINERGIC FUNCTION OF CHOLINESTERASE. K.M. Kutty. Janeway Child Health Centre, Department of Pathology and Pediatrics, Faculty of Medicine, Memorial University of Newfoundland Coffee Break
- 3:55 - 4:15 Coffee Break
- 4:15 - 4:40 CHLORPYRIFOS TREATMENT INCREASES BRAIN ACETYLCHOLINESTERASE CONTENT. S. Padilla. Neurotox. Div., US EPA, Research Triangle Park, NC 27711 USA
- 4:40 - 5:05 INHIBITION OF FISH BRAIN ACETYLCHOLINESTERASE BY CADMIUM AND MERCURY: INTERACTION WITH SELENIUM. Shelley Bhattacharya. Environmental Toxicology Laboratory, Department of Zoology, Visva Bharati University, Santiniketan 731235, India
- 5:05 - 5:30 CLINICAL BLOOD CHOLINESTERASE MEASUREMENTS FOR MONITORING PESTICIDE EXPOSURES. Barry W. Wilson. University of California, Davis, CA

Session F: Structure-Function Relationship of Anticholinesterase Agents: Pesticides and Therapeutic Agents; Noncholinergic Function of Cholinesterases

SECRETION OF ACETYLCHOLINESTERASE AS A NON-CLASSICAL FORM OF SIGNALLING IN THE BRAIN. Susan A. Greenfield. University Dept. of Pharmacology, Mansfield Rd., Oxford OX1 3QT U.K.

Acetylcholinesterase (AChE) is secreted from the dendrites of dopaminergic neurons within the substantia nigra. This phenomenon is completely independent of cholinergic transmission and appears instead to have a more modulatory, non-enzymatic role. Recent observations on the characteristics of both the secretion and subsequent action of AChE have provided clues for advancing our understanding of its possible non-cholinergic functions.

Secretion of AChE can be evoked by activation of certain afferent pathways to the substantia nigra including those from the raphe nucleus and subthalamus, which use serotonin and glutamate respectively. Although these transmitters are completely different substances operating via distinct receptors, they have in common an action of increasing calcium influx into the neuron, within the substantia nigra. By contrast selective activation of a sub-population of glutamate (AMPA) receptors that opens sodium in preference to calcium channels, does not evoke secretion of AChE. In addition, AChE secretion is dramatically reduced by treatment with alpha methyl-paratyrosine. Hence we can deduce that two important factors regulating secretion of AChE are calcium influx into the neuron and *de novo* synthesis of dopamine.

Once secreted into the extracellular space electrophysiological evidence suggests that AChE can itself enhance further calcium entry into the neuron. In turn these subsequently higher calcium levels could trigger a cascade of events that mediate long term changes in the excitability of nigral neurons, thus accounting for long term changes in movement seen following infusion of AChE into the substantia nigra. Such an action might also underscore trophic effects of AChE observed in neonatal nigral neurons: the protein significantly enhances neurine outgrowth independent of cholinergic transmission. In addition, AChE can also activate macrophages again independent of its conventional catalytic site but dependent on the presence of calcium. In both the *in vivo* and *in vitro* substantia nigra, AChE appears to be endocytosed in an energy dependent manner. It is possible therefore that uptake of the protein serves as a means of terminating any or all of the above actions.

Secretion of AChE could thus represent a non-classical form of signalling from neuron to neuron and neuron to glia, in both the mature and developing substantia nigra. Since it is the dopaminergic nigral neurons that are lost in Parkinson's disease, this phenomenon could be of eventual relevance to identifying new targets for an improved drug therapy in that disorder.

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Noncholinergic Function of Cholinesterases**

GLYCOSYLATED INACTIVE FORMS OF CHICKEN BUTYRYLCHOLINESTERASES AND THEIR POSSIBLE FUNCTIONS. P. G. Layer, T. Weikert and E. Willbold. Faculty of Biology, University of Technology Darmstadt, 64287 Darmstadt, Germany.

Inactive homologues of butyrylcholinesterase (BChE) with 75, 62 and 54 kD subunit size have been isolated from adult chicken serum. While the active BChE from serum with a subunit size of 81 kD forms tetramers, the 75 kD protein is isolated as monomer. The homology of the 75 kD protein with active BChE is shown by immunoreactivity with BChE-specific monoclonal antibodies, by coisolation with the active BChE, and by their identical first six N-terminal amino acids. By deglycosylation of these proteins and by their lectin binding, we show that the active BChE is an N-glycosylated protein, while the inactive 75 kD protein is O-glycosylated. Interestingly, the HNK-1 epitope is absent from active serum BChE, but is strongly expressed on all inactive serum BChE's and on active brain BChE. These data show for the first time the existence i) of multiple inactive forms of BChE, ii) of secreted inactive cholinesterases, since they are found in serum, iii) of an O-glycosylated cholinesterase, and iv) show that all inactive, but not all active BChE's contain the HNK-1 epitope. Since cholinesterases have been shown by us and others to regulate neurite growth in vitro by a non-enzymatic mechanism, these data strongly support the notion that both inactive and active forms of BChE may be involved in noncholinergic communication.

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EVIDENCE FOR CHOLINERGIC AND NON-CHOLINERGIC ACTIONS OF TRANSIENTLY EXPRESSED ACETYLCHOLINESTERASE IN DEVELOPMENT OF THALAMOCORTICAL PROJECTIONS. R.T. Robertson, R.S. Broide, J. Yu and F.L. Leslie. University of California, Irvine, CA 92717.

Recent work in this laboratory has demonstrated transient expression of acetylcholinesterase (AChE) activity in thalamocortical neurons and their axonal projections to cerebral cortex of developing rodent brains. AChE expression occurs during the time when thalamocortical axons are growing into cortex and forming synapses with cortical neurons. The function of transiently expressed AChE is unknown. Three sets of studies investigated possible functions of transiently expressed AChE activity in the ingrowth of thalamocortical axons into cortex. (1) *In situ* hybridization studies using an oligonucleotide probe for AChE mRNA confirmed that thalamocortical neurons have the message for AChE. (2) AChE esterase activity was inhibited in newborn rat pups by unilateral implants of Elvax sheets containing phospholine iodide (PI), an irreversible non-competitive AChE inhibitor. After three days of exposure, histochemical evidence demonstrated a marked reduction of AChE histochemical activity in the PI treated cerebral cortex; AChE positive staining of afferent axons was virtually eliminated. However, thalamocortical axons, demonstrated by HRP histochemistry of DiI fluorescent microscopy, displayed normal laminar and areal patterns of terminations. These data indicate that esterase activity of AChE is not necessary for ingrowth of thalamocortical axons. (3) *In situ* hybridization studies demonstrated that message for the $\alpha 7$ subunit of the nicotinic receptor is distributed transiently in developing cortex in regional and temporal patterns that appear identical to those patterns of transiently expressed AChE, raising the possibility that transiently expressed AChE may be associated with cholinergic functioning. Supported by NIH grant NS 30109.

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NON-CHOLINERGIC FUNCTION OF CHOLINESTERASE. K.M. Kutty, V. Prabhakaran and A.R. Cooper.
Janeway Child Health Centre, Department of Pathology and Pediatrics, Faculty of Medicine, Memorial
University of Newfoundland.

Cholinesterase (ChE) otherwise known as pseudocholinesterase has survived evolution and this is an enigma. Although a very efficient catalyst in hydrolysing choline esters such as butyrylcholine *in vitro* and succinylcholine *in vivo*, no such substrates have been demonstrated either in humans or animals. If this is so, the question is what might be the true biological function of ChE? This is not the only enzyme exhibiting such an exception in terms of a biological role. For example no substrate or biological function has been associated with either alkalinephosphatase or the red cell membrane acetylcholinesterase.

Despite the uncertainty about the biological role of ChE, this protein has been found to be consistently elevated in the serum of patients who are obese, diabetic and hyperlipoproteinemic. Animal models also show increased ChE activity with similar pathology.

Initial studies with Hep-G2 cells appear to indicate that there is a correspondence between the increased induction of ChE with the induction of triglyceride synthesis.

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CHLORPYRIFOS TREATMENT INCREASES BRAIN ACETYLCHOLINESTERASE CONTENT. S. Padilla¹, S. Chiappa², C. Koenigsberger², V.C. Moser¹, and S. Brimijoin². ¹Neurotox. Div., US EPA, Research Triangle Park, NC 27711; ²Dept. of Pharmacol. Mayo Clinic, Rochester, MN 55905, USA.

O,O 'Diethyl-*O*-[3,5,6-trichloro-2-pyridyl]-phosphorothionate (chlorpyrifos; Dursban®), a common insecticide, causes an unusually lengthy dose-dependent fall in the activity of brain acetylcholinesterase (AChE, EC 3.1.1.7) when given to rats (Pope *et al.*, *Toxicol.* 1991, 68:51; Bushnell *et al.*, *J. Pharm. Exp. Therap.* 1993, 266:1007). In order to determine whether the slow recovery involves impaired AChE resynthesis, experiments were designed to measure AChE activity, immunoreactive AChE protein (AChE-IR) and AChE mRNA. Male, Long-Evans rats, maintained at 350±5 gm, were dosed (sc) weekly for 4 weeks with 0, 15, 30 or 60 mg/kg chlorpyrifos in peanut oil. Brain tissue was harvested 1, 3, 5, 7 and 9 weeks after treatment began. AChE activity was measured by Ellman assay, and AChE-IR was estimated by 2-site ELISA using monoclonal antibodies to rat brain AChE. While AChE activity fell significantly at all times and doses, AChE-IR increased at 3 and 5 weeks, but only at the two higher dosages. Larger increases of AChE-IR were observed after higher dosages of chlorpyrifos were administered orally 5 day/wk for 4 weeks. Northern blots quantified with reference to cyclophilin were consistent with stable levels of AChE mRNA. We conclude that (1) chronically reduced brain AChE activity after chlorpyrifos treatment reflects sustained enzyme inhibition, not loss of enzyme protein or suppression of AChE message and (2) that sustained AChE inhibition may actually increase the rate of AChE synthesis or decrease AChE breakdown, thus stabilizing regional brain AChE activity.

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INHIBITION OF FISH BRAIN ACETYLCHOLINESTERASE BY CADMIUM AND MERCURY: INTERACTION WITH SELENIUM. S. Sen, S. Mondal, J. Adhikari, D. Sarkar, S. Bose, B. Mukhopadhyay, and Shelley Bhattacharya. Environmental Toxicology Laboratory, Department of Zoology, Visva Bharati University, Santiniketan 731235, INDIA.

Acute poisoning by Se causes CNS defects although it has a negligible effect on ChEases. It is also reported that Se reduces human cancer death rates. Such contradictory reports prompted us to study the role of Se in the inhibition of fish brain AChE caused by Cd and Hg at I_{50} concentrations of 2×10^{-5} M and 6.2×10^{-8} M respectively. Since Se showed no I_{50} at a lower (3.1×10^{-6} M) and a higher (5.7×10^{-5} M) concentration was used.

Positive interaction of Se was clear when Hg was added either before or after Se, or together, where 50% inhibition was completely abolished and 100% activity was recorded. This effect of Se was observed at both the test concentrations. Interaction of Se and Cd was remarkably different recording significant inhibition at all test systems. The activation of Hg-inhibited AChE by Se may be due to acceleration of enzyme deacetylation. In presence of Cd, Se probably binds to the active site to effect a conformational change thereby decreasing the AChE activity.

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CLINICAL BLOOD CHOLINESTERASE MEASUREMENTS FOR MONITORING PESTICIDE EXPOSURES. Barry W. Wilson¹, Stephanie Padilla², James R. Sanborn³, John D. Henderson¹ and Joseph E. Billitti¹. ¹University of California, Davis, CA; ²US Environmental Protection Agency, Research Triangle Park, NC, ³California Environmental Protection Agency, Sacramento, CA.

Acetylcholinesterases (AChEs) in red blood cells (RBCs) and plasma of man and other animals have been used to monitor exposure to organophosphate and organocarbamate pesticides. One of the most common assays uses acetylthiocholine hydrolysis and the color reagent DTNB as described by Ellman et al. (1963).

A Round Robin test on rat blood exposed to selected pesticides was carried out by several research and clinical laboratories focusing on conditions recommended for automated assays. A commercial automated assay of AChE from rats treated with pesticides resulted in higher values than expected for RBCs but not for brain and plasma. Parameters that affected the results were substrate concentration, pH, wavelength and use of appropriate blanks.

In addition, the conditions specified for human RBCs in two commercial kits and those recommended by Ellman were studied. The pH and substrate conditions of the kits were not optimal for human RBCs, yielding lower activities for untreated samples.

Both studies lead to the conclusion that standardization of clinical assays to monitor exposure is needed to guarantee both the accuracy of the results and reproducibility between laboratories. A round robin test of clinical laboratories monitoring farmworkers in California is planned.

Wednesday, September 28, 1994

MORNING

Session G: Pharmacological Utilization of Anticholinesterases

- 8:30 - 8:45 Review of Topic, Chairman
- 8:45 - 9:10 NOVEL APPROACHES TO ALZHEIMER DISEASE TREATMENT WITH
CHOLINESTERASE INHIBITORS. E. Giacobini. Dept. Pharmacology, Southern
Illinois Univ. Sch. Med., P.O. Box 19230, Springfield, IL 62794-9230 USA
- 9:10 - 9:35 SYNTHETIC AND BIOLOGICAL STUDIES OF HUPERZINE A AND ITS
ANALOGUES: A NATURAL PRODUCTS BASED APPROACH TO ALZHEI-
MER'S DISEASE. Alan P. Kozikowski, Trophix Pharmaceuticals, Inc., 40
Cragwood Road, South Plainfield, New Jersey 07080
- 9:35 - 9:55 Coffee Break
- 9:55 - 10:20 CHOLINESTERASES IN ALZHEIMER'S DISEASE. Marsel Mesulam, M.D.,
Center for Behavioral and Cognitive Neurology, Departments of Neurology and
Psychiatry, Northwestern University Medical School, Chicago, IL 60611, USA
- 10:20 - 10:45 ACETYLCHOLINESTERASE PROMOTES NEURITE OUTGROWTH FROM
CHICK SYMPATHETIC NEURONS IN CULTURE: IMPLICATIONS FOR
ALZHEIMER'S DISEASE. David H. Small. Dept. of Pathology, University of
Melbourne, Parkville, Victoria 3052, Australia

Session G: Pharmacological Utilization of Anticholinesterases

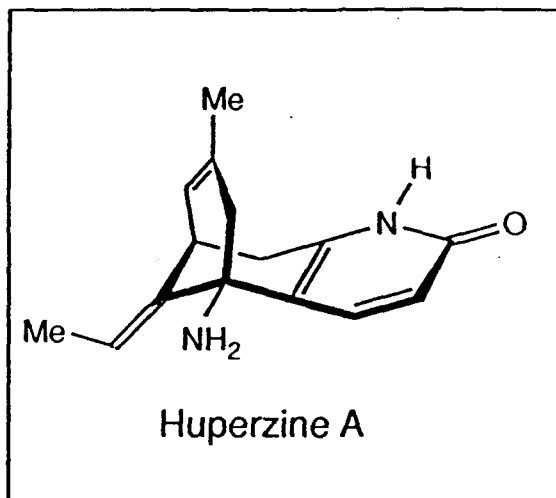
NOVEL APPROACHES TO ALZHEIMER DISEASE TREATMENT WITH CHOLINESTERASE INHIBITORS. E. Giacobini. Dept. Pharmacology, Southern Illinois Univ. Sch. Med., P.O. Box 19230, Springfield, IL 62794-9230 USA.

Several new cholinesterase inhibitors have been designed, studied preclinically and tested clinically. Clinical studies demonstrate that cholinesterase inhibitors (ChEI) are the only drugs that demonstrate, so far, clinical efficacy in the treatment of Alzheimer disease (AD) patients. The therapeutical effect seems to be related to the compounds' ability to produce long-lasting, steady-state and non-toxic elevations of acetylcholine (ACh) in brain. Using a modified microdialysis technique, we have demonstrated that this ACh increase is not directly related to acetylcholinesterase (AChE) inhibition in brain, RBC or plasma. We have shown that ChEI interact with both cholinergic and aminergic systems and that ACh and norepinephrine are mutually regulatory in cortex. Better characterization of these mechanisms provides new approaches in the design of ChEI and in therapeutic strategies in AD treatment. (Supported by National Institute on Aging #P30 AG08014)

Session G: Pharmacological Utilization of Anticholinesterases

SYNTHETIC AND BIOLOGICAL STUDIES OF HUPERZINE A AND ITS ANALOGUES: A NATURAL PRODUCTS BASED APPROACH TO ALZHEIMER'S DISEASE. Alan P. Kozikowski, Trophix Pharmaceuticals, Inc., 40 Cragwood Road, South Plainfield, New Jersey 07080.

Huperzine A, a potent acetylcholinesterase inhibitor isolated from the club moss *Huperzia serrata*, has found use in China for the treatment of Alzheimer's dementia. As a consequence of the promise of huperzine A as a safe, orally active cholinesterase inhibitor exhibiting a long duration of action, we have been engaged in efforts to make this molecule and its analogues available for continued pharmacological and behavioral testing. In order to better understand the structure-activity profile for this molecule, with the aim toward discovering analogues of improved efficacy, we have investigated the possible binding sites for huperzine A within the acetylcholinesterase structure through use of the Sysdoc program. These computer-based modeling studies together with the chemical synthesis efforts and results from the enzyme inhibition assays will be presented. The relevance of the modeling studies to the discovery of a more potent analogue of huperzine A will be discussed.



Session G: Pharmacological Utilization of Anticholinesterases

CHOLINESTERASES IN ALZHEIMER'S DISEASE. Marsel Mesulam, M.D., Center for Behavioral and Cognitive Neurology, Departments of Neurology and Psychiatry, Northwestern University Medical School, Chicago, IL 60611, USA.

Butyrylcholinesterase (BChE) and an altered form of acetylcholinesterase (AChE) accumulate in the plaques and tangles of Alzheimer's disease (AD). Our recent observations indicate that AChE and BChE activities with pH preferences and inhibitor selectivities identical to those of plaque and tangle-bound cholinesterases are found in the astrocytes and oligodendrocytes of control and AD brains. Preliminary experiments also indicate that the immunological characteristics of glial AChE differ from those of neuronal AChE. In control brains, the glial cholinesterases appear confined to the intracellular space whereas in AD they decorate plaques and tangles as well. In comparison to age-matched control specimens, AD brains had a significantly higher density of BChE glia and a lower density of AChE glia in entorhinal and inferotemporal regions but not in the primary somatosensory or visual areas. There were no age-related differences in the density or distribution of cholinesterase-positive glia.

The AD as well as control brains contained cortical β A4 amyloid plaques. However, a much larger area of the β A4 deposit was BChE-reactive in AD than in controls. These results suggest that glia constitute a likely source for the cholinesterase activity of plaques and tangles, that BChE glia may play a permissive or causative role in the neuropathology of AD and that BChE reactivity may lead to the maturation (or pathogenicity) of amyloid plaques.

Session G: Pharmacological Utilization of Anticholinesterases

ACETYLCHOLINESTERASE PROMOTES NEURITE OUTGROWTH FROM CHICK SYMPATHETIC NEURONS IN CULTURE: IMPLICATIONS FOR ALZHEIMER'S DISEASE. David H. Small, Gullveig Reed, Bryony Whitefield & *Victor Nurcombe. Depts. of Pathology and *Anatomy and Cell Biology, University of Melbourne, Parkville, Victoria 3052, Australia.

Acetylcholinesterase (AChE) is commonly found in association with amyloid plaques and neurofibrillary tangles in the brains of patients with Alzheimer's disease. The distribution of AChE in amyloid plaques does not reflect the topography of cholinergic innervation, suggesting that the presence of cholinesterases in plaques and tangles is unrelated to the classical action of AChE in cholinergic neurotransmission. In amyloid plaques, high levels of AChE are seen in the dystrophic neurites which typically surround the amyloid plaque cores, suggesting that AChE could play a role in the generation of dystrophic neurites.

In early development, transient expression of AChE is associated with neurite outgrowth. For this reason, we examined the effect of acetylcholine and AChE on neurite outgrowth from chick sympathetic neurons in culture. High levels of AChE are found in the growth cones of sympathetic neurites in culture. We found that acetylcholine inhibited neurite outgrowth from dissociated chick sympathetic neurons in culture. The effect of acetylcholine was mediated by both nicotinic and muscarinic receptors. The drug tacrine and other AChE inhibitors enhanced the effect of acetylcholine. We also found that substrate-bound AChE stimulated neurite outgrowth. The action of AChE in stimulating neurite outgrowth was dependent on the presence of heparan sulfate proteoglycans (HSPG). As catalytically inactive AChE also supported the neurite outgrowth effect, the results suggest that the neurite outgrowth-promoting action of AChE maps to a domain distinct from the active site. Most interestingly, the effect of AChE on neurite outgrowth was very similar to that found for the amyloid protein precursor (APP) of Alzheimer's disease, which is also a promoter of neurite outgrowth in culture (Small et al., J. Neuroscience 14, 2117-2127, 1994).

The results suggest that during early development, AChE expressed on the surface of growing neurites promotes neurite outgrowth. We speculate that AChE found in dystrophic neurites around amyloid plaques in Alzheimer's disease could have a similar function and that the reported therapeutic benefits of the drug tacrine are a consequence of inhibition of this non-classical function.

POSTERS

Session A: Gene Structure and Expression of Cholinesterases

POSTER NO. 1: PATIENTS WITH CONGENITAL MYASTHENIA ASSOCIATED WITH END-PLATE ACETYLCHOLINESTERASE DEFICIENCY SHOW NORMAL SEQUENCE, SPLICING AND ASSEMBLY OF CATALYTIC SUBUNITS. Shelley Camp¹, Suzanne Bon², Ying Li¹, Damon K. Getman¹, Andrew G. Engel³, Jean Massoulié² and Palmer Taylor¹. ¹ Department of Pharmacology, University of California, San Diego. ² Laboratoire de Neurologie, Ecole Normale Supérieure, Paris. ³ Mayo Clinic, Rochester, MN.

A congenital myasthenic condition was described in 1977 which is characterized by a deficiency in end-plate acetylcholinesterase (AChE). Since then, several additional patients with a similar disorder have been identified. The characteristic form of AChE in the end-plate has the catalytic subunits disulfide linked to a tail unit of partial collagenous structure that associates with the basal lamina. To ascertain the genetic basis of the disease, we have examined the structure of the gene encoding the catalytic subunits of AChE deficient patients and controls. Southern analysis revealed no differences between patient and control DNA. PCR amplification of genomic DNA yielded clones covering exon 4 and the alternatively spliced exons 5 and 6; this region was analyzed by nuclease protection and sequencing. While allelic differences were detected between two of the controls, we found no differences in exonic and intronic areas that might give rise to distinctive splicing patterns in patients and control individuals. The *ACHE* gene was cloned from genomic libraries generated from one of the patients and a control. Expression of the cloned gene following transfection into HEK and COS cells revealed identical patterns of mRNA production and species of expressed AChE. Cotransfection of the genes expressing the catalytic subunits from the two individuals with a cDNA from *Torpedo* encoding the tail unit yielded asymmetric species (16 + 14 S) which require assembly of catalytic subunits and tail unit. Thus, the catalytic subunits of AChE expressed in the congenital myasthenia syndrome appear identical in sequence, arise from similar splicing patterns and assemble normally with a tail unit to form a heteromeric species. These findings point to the genetic abnormality likely existing in the tail unit or in a protein responsible for assembly of the catalytic subunit with the tail subunit.

POSTER NO. 2: REGULATION OF *AChE* GENE EXPRESSION IN NEURONALLY INDUCED MOUSE P19 CELLS. B.A. Coleman and P. Taylor. Dept. of Pharmacology, UCSD School of Med., La Jolla, CA 92093.

Mouse p19 embryonic carcinoma cells are uncommitted, pluripotent cells which can be induced to terminally differentiate along the neuroectodermal lineage. Neurons, glia and astrocytes develop after free-floating aggregates, previously cultured with retinoic acid (RA), are plated onto tissue culture dishes. Neuronal cultures up to 90% purity can be obtained by treatment with mitotic inhibitors. We have used this cell line to examine the developmental and tissue-specific transcriptional regulation of acetylcholinesterase (AChE). AChE activity is not expressed in undifferentiated or aggregated cells, but can be detected within 24 hr of plating neuronally-induced cultures. The level of AChE activity increases during neuronal differentiation. This correlates with the increase in cells exhibiting neuronal morphology. Histochemical and immunohistochemical results indicate that AChE activity is associated with neuron-like cells, but not with glia-like cells. Splicing of AChE mRNA is consistent with that observed in mouse brain as demonstrated by Northern blot and message protection analyses. Although AChE activity is only detected in cultures containing neurons, run-on transcription assays demonstrate that the *AChE* gene is actively transcribed at a similar rate in undifferentiated monolayer cells, aggregates, and neurons. During morphological differentiation into neurons, *AChE* mRNA and enzyme activity show parallel increases. However, aggregates maintained for a long period of time in the presence of retinoic acid contain high levels of *AChE* message but little to no activity. Transcription inhibition experiments indicate that *AChE* mRNA decays more quickly in aggregates than in differentiated neurons, suggesting a role for mRNA stabilization in expression of the enzyme. AChE has commonly been used as a marker for terminal differentiation of neurons and has been suggested to play a role in neuronal development. P19 cells appear to provide a useful model for examining AChE regulated expression during neuronal development (Supported by USPHS GM18360, 24437 and American Heart Association Postdoctoral Fellowship No. 93-58)

POSTERS

Session A: Gene Structure and Expression of Cholinesterases

POSTER NO. 3: REGULATION OF HUMAN ACETYLCHOLINESTERASE GENE EXPRESSION. Damon Getman, Ken Inoue and Palmer Taylor. Department of Pharmacology 0636, University of California San Diego, La Jolla, CA 92093.

Acetylcholinesterase in human cells is encoded by a single gene, *ACHE*, located on chromosome 7q22. We find the major DNA promoter elements controlling the expression of this gene reside immediately upstream of the first untranslated exon, and are characterized by GC rich sequences containing consensus binding sites for several transcription factors, including SP1, EGR-1, and AP2. RNase protection analysis of mRNA isolated from human NT2/D1 teratocarcinoma cells demonstrates two closely spaced transcription cap sites. Both cap sites are located at a consensus initiator element similar to that found in the terminal transferase gene initiator element. Transient transfection experiments reveal that removal of portions of this initiator sequence reduce promoter activity 70% in NT2/D1 cells. *In vitro* transcription studies and transient transfection of a series of 5' deletion mutants of the *ACHE* promoter linked to a luciferase reporter show one of the SP1 sites at -70 is essential for promoter activity. Purified SP1 protein protects this site from DNase cleavage during *in vitro* footprinting experiments. SP1 protein also protects adjacent promoter elements from DNase cleavage in a synergistic fashion with other transcription factors. Retinoic acid treatment of NT2/D1 cells induces AChE enzyme activity greater than 30 fold and mRNA levels 50 fold, while the rate of gene transcription remains unchanged. These data indicate that *ACHE* transcription is constitutively activated by SP1 or similar factors, while mRNA levels are controlled by changes in mRNA stability. (Supported by USPHS GM18360).

POSTER NO. 4: RABBIT ACHE AND BCHE: TISSUE DISTRIBUTION IN THE ADULT AND DURING DEVELOPMENT. Omar Jbilo, Yann L'Hermite, Vincenzo Talesa, Jean-Pierre Toutant and Arnaud Chatonnet. Physiologie animale INRA, place Viala, 34060 Montpellier cedex France.

Expression of *ACHE* and *BCHE* genes was studied in adult rabbit tissues and during development by a correlation of Northern blot analysis and assay of enzymatic activities. Brain and skeletal muscle were the richest source of AChE whereas brain, lung and heart showed the highest BCHE expression. An eserine resistant esterase active on butyrylcholine was present in serum liver and lung.

During development BCHE transcripts were detected as early as day 10 postcoitum, whereas *ACHE* transcripts were only apparent in Northern blots on day 12.

Differences in the expression pattern of AChE and BChE in adult tissues and during development may correspond to the effect of different cis-acting factors which control the promoter of the two genes. Indeed the promoter region of BCHE, although lacking TATA and CAAT boxes and possessing an INR (initiator element) very homologous to the *ACHE* promoter, is widely different in base composition and putative transcription factors binding sites. BCHE 5' region can promote reporter CAT gene expression when transfected in mammalian cells. Different factors which may modulate the basal activity are under test. This work was supported by *Association Française contre les Myopathies* (MNM 93-1250) and from *Ministère de la recherche et de la technologie* (91-T-0439). O.J. was supported by a grant from *Institut National de la recherche Agronomique* and V.T. by an EEC grant (Human capital and mobility).

POSTERS

Session A: Gene Structure and Expression of Cholinesterases

POSTER NO. 5: DEVELOPMENTAL REGULATION OF ACETYLCHOLINESTERASE mRNA IN THE MOUSE DIAPHRAGM: ALTERNATIVE SPLICING AND FOCALIZATION. Claire Legay*, Monique Huchet§, Jean Massoulié* & Jean-Pierre Changeux§. *Neurobiologie Cellulaire et Moléculaire, CNRS URA 1857, Ecole Normale Supérieure, 46 rue d'Ulm, 75005 Paris, France. §Neurobiologie Moléculaire, CNRS URA 1284, Institut Pasteur, 25 rue du Dr Roux, 75015 Paris, France.

A single gene generates distinct catalytic subunits of acetylcholinesterase (AChE, EC 3.1.1.7), by alternative splicing of exons encoding C-terminal peptides¹. The T ("tailed") subunits produce a variety of molecular forms, including collagen-tailed molecules and hydrophobic-tailed tetramers, the H ("hydrophobic") subunits produce membrane-bound dimers, and the R ("readthrough") subunits have only been inferred from the existence of cDNAs which retain the intervening sequence following the common exon in the genome². In adult mammals, only mRNAs encoding the T subunit have been detected in muscles and brain³. We have used *in situ* hybridization and polymerase chain reaction (PCR) to analyze the nature and distribution of AChE mRNAs along myofibers in the mouse diaphragm, during development. While adult muscle only contains the T transcript, expressed by junctional nuclei⁴, we were surprised to find that, from embryonic day 13 (E₁₃) until birth, the diaphragm contains the three types of AChE mRNAs: T is already predominant at this stage, R represents a few percent of mRNA and H is detectable in still lower proportion. This is the first report of the existence of the R transcript in muscle, and we obtained similar results with the C2 myogenic cell line, both as myoblasts and differentiated myotubes. As early as E₁₃, T mRNAs preferentially accumulate in the midline, where the first neuromuscular contacts are forming.

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POSTER NO. 6: REGULATION OF ACETYLCHOLINESTERASE GENE EXPRESSION DURING MYOGENESIS. Annick Muter, Shelley Camp, Ying Li, and Palmer Taylor. Dept. of Pharmacology, University of California, San Diego, La Jolla, CA 92093.

Unlike other muscle-specific proteins that are transcriptionally activated during terminal differentiation, AChE is actively transcribed in the myoblast and its increased expression in myotubes is due to the stabilization of a labile mRNA. We have studied the transcriptional regulation of mouse AChE during early determination into the muscle lineage using the C3H10T1/2 cell line. In contrast to the nicotinic acetylcholine receptor, we find that transcription of AChE is active in this undifferentiated fibroblastic stage and is not enhanced when these cells are committed to myoblasts upon expression of myogenin. We have studied the role of elements present in the promoter region on gene transcription. Upstream E-box-like sequences were found not to be active. Deletion analysis in the promoter region revealed the presence at -105 to -59 bp of a GC-rich enhancer region containing binding sites for the transcription factors Sp1 and EGR1. Mutation of the Sp1 sites dramatically reduces the promoter activity while mutation of the Egr-1 sites has little effect. We show that Sp1 and Egr-1 compete for binding to overlapping sites and that an increase in Egr-1 decreases the expression of the AChE gene. We have also characterized an AP2-like binding site immediately upstream of the main Cap site that is inhibitory, possibly by interfering with the binding of elements to the polymerase site. We find that AP2 is associated with this site in cells not committed to the muscle phenotype but not in myoblasts or myotubes.

POSTERS

Session A: Gene Structure and Expression of Cholinesterases

POSTER NO. 7: ALTERNATIVE EXON 6 DIRECTS SYNAPTIC LOCALIZATION OF RECOMBINANT HUMAN ACETYLCHOLINESTERASE IN NEUROMUSCULAR JUNCTIONS OF *XENOPUS LAEVIS* EMBRYOS. Meira Sternfeld, Shlomo Seidman, Revital Ben Aziz-Aloya, Michael Shapira, Rina Timberg, Daniela Kaufer and Hermona Soreq. Dept. of Biological Chemistry, The Hebrew University of Jerusalem, Israel.

We have expressed and characterized catalytically active recombinant human acetylcholinesterase (rHACHE) produced in microinjected oocytes of *Xenopus laevis*. However, the highly specialized, single-cell nature of the oocyte limits its usefulness in addressing questions regarding tissue-specific processing and the biological roles of cloned nervous system proteins. Therefore, to study the role of 3' alternative splicing in regulating tissue-specific expression of the human ACHE gene encoding AChE, we established an *in vivo* model in transiently transgenic *Xenopus* embryos. Following injection into *in vitro* fertilized *Xenopus* eggs, whole-mount cytochemistry and electron microscopy revealed that ACHE DNA bearing the alternative 3' exon E6 (ACHE-E6) induced prominent overexpression of catalytically active AChE in myotomes of 2- and 3-day-old embryos, including neuromuscular junctions (NMJs). NMJs from ACHE-E6-injected embryos displayed, on average, 4-fold greater AChE-stained areas (SA) and 80% increased post-synaptic lengths (PSL) compared to age-matched uninjected controls. Perhaps more significantly, ACHE-E6 overexpression stimulated the appearance of a class of large NMJs (PSL > 4 μ m) rarely observed in control embryos, apparently at the expense of small (PSL < 3 μ m) NMJs. Homogenates prepared from these embryos demonstrated increased binding of biotinylated α -bungarotoxin, indicating enhanced expression of the endogenous *Xenopus* acetylcholine receptor and suggesting coordinated regulation of cholinergic proteins in the developing NMJ. When exon E6 was replaced by ACHE DNA encoding the pseudointron I4 and 3' alternative exon E5, overexpressed rHACHE accumulated in epidermal cells, but not in muscle or NMJs. These findings, therefore, attribute an evolutionarily conserved NMJ-targeting role for the C-terminal peptide encoded by exon E6 and provide *in vivo* evidence for tissue-specific management of alternative AChEs.

POSTER NO. 8: STUDIES ON THE EXPRESSION OF *DROSOPHILA* ACETYLCHOLINESTERASE IN THE YEAST *SACCHAROMYCES CEREVISIAE*. K.C.A. Stopps, S.A. Khalawan, C.A. Malcolm, B.P.G. Curran*, L.M.C. Hall**. *School of Biological Sciences, QMW College, University of London, Mile End Road, London, E1 4NS; ** Department of Medical Microbiology, London Hospital Medical College, Turner Street, London, E1 2AD.

To date the heterologous expression of insect acetylcholinesterase (AChE) for the study of insecticide resistance has been in *Xenopus* oocytes or baculovirus systems (Fournier *et al.*, 1992; Mutero and Fournier, 1992; Mutero *et al.*, 1992). The advantages of a yeast expression system are convenience, well characterised genetics, and the possibility of direct mutagenesis. A *Drosophila* cDNA (Hall and Spierer, 1986) was subcloned into a constitutive expression vector under the powerful yeast glyceraldehyde phosphate dehydrogenase promoter and assayed for expression of active AChE (Hestrin, 1949; Sabine, 1955). Protoplasts were made from separate overnight cultures of yeast carrying the expression vector with the gene in both the correct and incorrect orientations in addition to further control cultures of the host *Saccharomyces* strain minus the expression plasmid. Significant levels (up to 50% of substrate hydrolysed over the incubation period of 45 minutes) of active AChE were detected from lysed protoplasts carrying the expression plasmid and gene in the correct orientation. Southern blots of yeast genomic DNA from experimental groups indicated that the expression vector, a high copy number episomal plasmid, is not down regulated during expression. The ultimate aim is to utilize a yeast expression system for the study of mosquito insensitive AChEs.

POSTERS

Session B: Polymorphism and Structure of Cholinesterases

POSTER NO. 9: SUBUNIT ASSOCIATION AND STABILIZATION OF BUTYRYLCHOLINESTERASE (BChE). Renee M. Blong and Oksana Lockridge. Eppley Institute and Dept. of Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, NE.

The goal of this project was to determine what parts of the BChE protein are important for its tetrameric organization. In human serum the BChE molecule has a dimer of dimers structure. Dimers are covalently linked by an interchain disulfide bond between the cysteines at amino acid 571. To study the effect the disulfide bond has on tetrameric structure and stability, a recombinant mutant was engineered replacing cysteine 571 with alanine. The mutant was expressed in Chinese hamster ovary cells. Elimination of the interchain disulfide bond at amino acid 571 decreased the heat stability of the enzyme but preserved its tetrameric structure. A second mutant in which 40 amino acids were deleted from the carboxy terminus was also expressed. This mutant gave an active monomer, thus suggesting that the region involved in dimer and tetramer contact is the carboxy terminus. Additional mutations, engineered by PCR (Polymerase Chain Reaction) site directed mutagenesis, will allow further characterization of the tetrameric stabilization of BChE.

Supported by U.S. Army Medical Research and Development Command Grant DAMD17-94-J-4005.

POSTER NO. 10: EXPANDED ALIGNMENT OF CHOLINESTERASES, ESTERASES, LIPASES, AND RELATED PROTEINS. Mary K. Gentry and B. P. Doctor. Division of Biochemistry, Walter Reed Army Institute of Research, Washington, DC 20307-5100 USA.

We previously presented an amino acid alignment of acetyl- and butyrylcholinesterases (Gentry and Doctor, Proceedings of the Third International Meeting on Cholinesterases, 1991, pp. 394-398.). This alignment was improved by a realignment based on x-ray structures of *Torpedo californica* acetylcholinesterase and *Geotrichum candidum* lipase (Cygler *et al.*, *Protein Science*, 2, 366-382, 1993.) and augmented by the inclusion of sequences of lipases, carboxylesterases, esterases, and neurotactin. We have now further enlarged the group with 23 newly-published amino acid sequences.

POSTER NO. 11: HYDROPHOBICITY ON ESTERASE ACTIVITY OF HUMAN SERUM CHOLINESTERASE. L. Jaganathan, K. Padmalatha, G. Revathi & R. Boopathy. Department of Bio-Technology, Bharathiar University, Coimbatore - 46, Tamil Nadu.

Studies using Triton X-100 on Human Serum Cholinesterase at various stages of its purification, suggested the presence of two distinct type of hydrophobic domain/region(s) involved in esterase activity. The domain/region(s) sensitive to low concentrations of Triton X-100 facilitated an activation of the enzyme, while the domain/region(s) sensitive to higher concentrations stabilize the enzyme activity. Proteolytic digestion with trypsin and papain decreased the enzyme activity, concomitant to a decrease in hydrophobicity of the protein as evident by its retention time and elution pattern from Phenyl-Sepharose chromatography. Deglycosylation by oxidation with Sodium metaperiodate decreased the esterase activity, but increased the relative hydrophobicity of the protein, as indicated by the fluorescence probe 8-Anilino-1-Naphthalene Sulfonic acid. Desialylation by neuraminidase with low concentrations caused a decrease, while higher concentrations caused an increase in esterase activity. On prolonged incubation at both concentrations of neuraminidase, the change in activity was brought back to near normal. However, desialylation does not alter the hydrophobicity of the protein. Lipophilization (addition of fatty acid to lysine residues) in view to increase the hydrophobicity of the protein, using caproylchloride resulted in an inhibition of the esterase activity. Further, hydrophobicity of protein in relation to esterase activity is discussed

POSTERS

Session B: Polymorphism and Structure of Cholinesterases

POSTER NO. 12: DENATURATION OF RECOMBINANT HUMAN ACETYLCHOLINESTERASE. M. Lebleu, C. Clery and P. Masson. Centre de Recherches du Service de Santé des Armées - Unité de Biochimie, 38702 - La Tronche Cédex - FRANCE. S. Reuveny, D. Marcus, B. Velan and A. Shafferman. Israel Institute for Biological Research, Ness-Ziona - 70450 ISRAEL.

The study of unfolding process of proteins has been a subject of growing interest during recent years. The availability of recombinant cholinesterases in large amounts permits now to use biophysical tools for gaining information about folding and stability of these enzymes.

The stability of recombinant human acetylcholinesterase (rHuAChE) produced by human embryonic kidney cell line (293) in a fixed-bed reactor (1) was investigated at pH 8.0 by thermal, pressure, urea and guanidinium chloride denaturation. The thermal unfolding was studied by differential scanning calorimetry (DSC) in the temperature range from 20° to 80°C. The pressure effects were studied by following tryptophan fluorescence under pressure at 15°C up to 4 kbar, by measuring remaining enzyme activity after pressure release and by electrophoresis under pressure at 10°C (construction of Ferguson plots (2)). Denaturation by urea and guanidinium chloride under equilibrium conditions was studied by monitoring tryptophan fluorescence *versus* denaturant concentration at 25°C in the presence and absence of tris (2-carboxyethyl) phosphine (TCEP) as a reducing agent.

Apparent $\Delta G_{H_2O/D}$ values calculated from urea and guanidinium chloride transition curves, assuming the two-state reversible unfolding (3), were found to be of the order of 20 kJ.mol⁻¹. These values lower than values found for globular proteins, suggest that unfolding of rHACHe does not obey the two-state model.

Thermal denaturation cannot be described by thermodynamical equilibrium. Using DSC we showed that denaturation is an irreversible process under kinetic control. Indeed, a) the midpoint transition temperature is dependent on the scan rate (e.g. $T_m = 59.2^\circ\text{C}$ with $v = 1^\circ\text{C}/\text{min}$); b) there is no release of heat upon decreasing temperature. The ratio $\Delta H_{cal}/\Delta H_{V_{max}/H_{off}} = 2.5$ ($\Delta H_{cal}/O = 1500$ kJ.dimer mol⁻¹, as expected for a stable compact globular protein) also showed that thermal denaturation is an irreversible multistep process. The energy of activation, calculated according to several methods (4) is 450 kJ.mol⁻¹.

Hydrostatic pressure, up to 4 kbar, led to partially but irreversibly denatured states followed by their subsequent aggregation.

The structural basis of the stability of cholinesterases is still unresolved. What is clear from the present work is that the denaturation reaction of rHACHe dimer proceeds through several steps and that final denatured states depend on both the external conditions and the protein structure. Complexity may arise from the imperfect packing of aminoacids at subunit interface and from the existence of several independent subdomains in each structural domain (monomer).

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POSTERS

Session B: Polymorphism and Structure of Cholinesterases

POSTER NO. 13: NEW INHIBITORS OF CHOLINESTERASES DESIGNED FOR RAPID PHOTOCHEMICAL RELEASE OF CHOLINE. Ling Peng and Maurice Goeldner. Laboratoire de Chimie Bio-Organique - URA 1386 CNRS, Faculté de Pharmacie - Université Louis Pasteur Strasbourg, BP 24 - 67401 Illkirch cedex - France.

The location of acetylcholinesterase active site together with the rapid turnover of this enzyme as well as electrostatic characteristics raise the question of the dynamics of the enzymatic product (choline). In particular the exit of choline from the active site remains undefined. The possibility of a rapid and discrete conformational change requires, to be studied, appropriate tools for which the caged choline inhibitors might be proposed.

In a first approach a series of ortho-nitrobenzyl choline derivatives were synthesized and tested for their reversible binding properties on acetyl- and butyrylcholinesterase. The ortho-nitrobenzyl group has been used as photochemical protectors for several biological ligands (1) and has been studied here for the photochemical release of choline. The photolytic release of choline was first established and the kinetic parameters will be determined after flash photolysis. Photochemical release of choline from enzyme-inhibitor complexes was undertaken and analyzed.

Finally the search for pseudoirreversible inhibitors is under investigation to allow a mimic of the enzymatic reaction and the conformational change to occur after photochemical release of choline. These pseudosubstrates as well as the inhibitors would constitute valuable candidate probes for photochemical studies on the crystalline enzyme [2] and further time-resolved crystallographic studies (collaboration J. Sussman, I. Silman - Weizmann Institute).

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POSTER NO. 14: LONDON DISPERSION INTERACTIONS IN MOLECULAR RECOGNITION BY ACETYLCHOLINESTERASE. D.M. Quinn, H.K. Nair, J. Seravalli, K. Lee, T. Arbuckle. Department of Chemistry, University of Iowa, Iowa City, IA. Z. Radić, D. Vellom, N. Pickering, P. Taylor, Department of Pharmacology, University of California-San Diego, La Jolla, CA.

Interaction of the aromatic residues W84, Y130 and F330 (or Y330) of *T. californica* and mouse AChEs with the quaternary ammonium function of ligands and substrates has been probed by QSAR and site-directed mutagenesis studies. For a series of ten *meta*-substituted aryl trifluoroketone inhibitors, *m*-XC₆H₄COCF₃ (X = H, Me, CF₃, Et, *i*Pr, *t*Bu, NO₂, NH₂, NMe₂, Me₃N⁺), inhibitor potency (i.e. pK_i) is not correlated with substituent hydrophobicity, but is well described by a three-dimensional correlation with the molar refractivity (MR) and σ_{π} of the substituents. MR depends on surface area and polarizability, and thus is a measure of London dispersion interactions between ligands and the aromatic residues of the quaternary ammonium binding locus of the active site. Of the 10⁷-fold range of inhibitor potency, 10⁵ arises from the MR sensitivity, and the corresponding linear subcorrelation indicates that all substituents share a common binding locus and interaction mechanism. A linear correlation of pK_i versus amino acid MR values for a series of mutants of mouse AChE indicates that about half of the binding free energy comes from interaction with W84. Similar correlations for substrate turnover support the importance of dispersion interactions in AChE catalysis. These studies also indicate that the aromatic residues in the quaternary ammonium binding locus of the active site do not function as anionic sites.

POSTERS

Session B: Polymorphism and Structure of Cholinesterases

POSTER NO. 15: ELECTRON PARAMAGNETIC RESONANCE REVEALS ALTERED MICRO-TOPOGRAPHY AT THE ACTIVE SITE GORGE AFTER MONOCLONAL ANTIBODY BINDING TO THE BOVINE ACETYLCHOLINESTERASE PERIPHERAL ANIONIC SITE. Marjeta Šentjura¹, Zoran Grubič², Anton Štalc², Slavko Pečar³, Mary K. Gentry⁴ and Bhupendra P. Doctor⁴. Jožef Stefan Institute¹, Ljubljana, Slovenia; Institute of Pathophysiology², School of Medicine, University of Ljubljana, Slovenia; Dpt. of Pharmacy³, Faculty of Science and Technology, University of Ljubljana, Slovenia; and Division of Biochemistry, Walter Reed Army Institute of Research⁴, Washington, DC, USA.

Using electron paramagnetic resonance (EPR), we studied and compared the effects of binding of Fab fragments of monoclonal antibody mAb 25B1 and propidium on the microtopography of the active site gorge of fetal bovine serum AChE (FBS AChE). As revealed in previous experiments both ligands bind to the peripheral anionic site of FBS AChE. However, their effects on the motional freedom of spin labelled molecule attached to the active site serine were not the same. No conformational alterations or protection against heat denaturation could be detected after propidium binding. On the other hand narrowing and/or closing of the gorge as well as stabilization against heat denaturation were observed after incubation of spin labelled FBS AChE with Fab fragments of mAb 25B1. Absence of propidium effects on the motional freedom of the spin labelled molecule might be due to prevention of propidium binding due to the steric hindrances exerted by relatively long and bulky spin labelled organophosphate molecule (binding of Fab 25B1 seems to be less affected by these steric hindrances). Also, conformational stabilization of the active site by binding of spin labelled organophosphate molecule to the active site serine might interact with propidium effects. We are demonstrating EPR as a tool enabling studies of the microtopography of the active centers of cholinesterases.

POSTER NO. 16: INTERACTION OF ACETYLCHOLINESTERASE IN A MOLTEN GLOBULE STATE WITH THE LIPID BILAYER. Irina Shin, Israel Silman & Lev Weiner. Depts. of Neurobiology and Organic Chemistry, Weizmann Institute of Science, Rehovot, Israel.

We have recently shown that *Torpedo* acetylcholinesterase (AChE) can exist for several hours in a stable, partially unfolded state ("molten globule", MG) under physiological conditions, without undergoing aggregation. The ability of AChE in this MG state to interact with lipid bilayer was studied. It was shown that native AChE does not insert into dimyristoylphosphatidylcholine liposomes. In contrast, in the MG state it incorporates into such liposomes within minutes, as monitored by a flotation gradient technique. NaCl (1M) does not significantly decrease the protein-lipid interaction, indicating that electrostatic forces are not predominant in this process. Use of negative staining and freeze-fracture electron microscopy allowed us to observe that aggregation of liposomes was stimulated by the MG.

Interaction of the MG with small unilamellar vesicles causes weak leakage of fluorescent (calcein) or spin (TEMPO-choline) probes which had been pre-loaded into the liposomes. Rates of tryptic digestion of AChE in the MG state in solution and incorporated into the liposomes were studied by EPR.

The physicochemical characteristics of the MG-phospholipid interaction and its possible biological significance will be discussed.

POSTERS

Session C: Mechanism of Catalysis of Cholinesterases

POSTER NO. 17: ELECTROSTATIC ATTRACTION BY SURFACE CHARGE DOES NOT CONTRIBUTE TO THE CATALYTIC EFFICIENCY OF ACETYLCHOLINESTERASE. D. Barak*, A. Ordentlich*, C. Kronman*, R. Ber*, T. Bino*, N. Ariel*, R. Osman, B#. Velan* and A. Shafferman*. #Mount Sinai School of Medicine, CUNY, New York, NY 10029, USA; *Israel Institute for Biological Research, Ness, Ziona, 70450, Israel.

Acetylcholinesterases (AChEs) are characterized by a high net negative charge and by an uneven surface charge distribution, giving rise to a negative electrostatic potential extending over most of the molecular surface. To evaluate the contribution of these electrostatic properties to the catalytic efficiency, 20 single and multiple site mutants of human AChE, were generated by replacing up to 7 acidic residues, vicinal to the rim of the active center gorge by neutral amino acids. Progressive simulated replacement of these charged residues results in a gradual decrease of the negative electrostatic potential which is essentially eliminated by neutralizing 6 or 7 charges. In marked contrast to the shrinking of the electrostatic potential, the corresponding mutations had no significant effect on the apparent bimolecular rate constants of hydrolysis for charged and noncharged substrates, or on K_i value for a charged active center inhibitor. Moreover the k_{cat} values for all the 20 mutants, are essentially identical to that of the wild type enzyme and the apparent bimolecular rate constants show a moderate dependence on the ionic strength, which is invariant for all the enzymes examined. These findings suggest that the surface electrostatic properties of AChE do not contribute to the catalytic rate, that this rate is probably not diffusion controlled and that long-range electrostatic interactions play no role in stabilization of the transition states of the catalytic process.

This work was supported in part by the U.S. Army Research and Development Command, Contract DAMD17-93-C-3042.

POSTER NO. 18: AMINO ACIDS DETERMINING SPECIFICITY TO OP-AGENTS AND FACILITATING THE "AGING" PROCESS IN HUMAN ACETYLCHOLINESTERASE. A. Ordentlich, C. Kronman, D. Stein, N. Ariel, S. Reuveny, D. Marcus, Y. Segall, D. Barak, B. Velan, and A. Shafferman. Israel Institute for Biological Research Ness-Ziona, Israel.

Recombinant human acetylcholinesterase (HuAChE) and several selected active site gorge mutants were studied with respect to catalytic activity towards charged and noncharged substrates, phosphorylation by organophosphorus (OP) inhibitors and subsequent aging of the resulting OP-conjugates. On the basis of these studies we have identified some of the critical elements in the active center that determine specificity to various OP-agents. We have shown that most of the residues participating in the acylation reaction affect also the rates and stereochemistry of phosphorylation processes. Some of the mutations have generated biomolecules whose OP-conjugates are practically resistant to "aging". From kinetic studies of aging and molecular modeling we begin to elucidate the underlying structure-function determinants of this chemical phenomenon that is quite unique for cholinesterases. In addition such engineered novel biomolecules should be advantageous for treatment against OP poisoning or decontamination of environmentally harmful OP agents.

This work was supported by the U.S. Army Research and Development Command, Contract DAMD17-93-C-3042.

POSTERS

Session C: Mechanism of Catalysis of Cholinesterases

POSTER NO. 19: PERIPHERAL ANIONIC SITE OF WILD-TYPE AND MUTANT HUMAN BUTYRYL-CHOLINESTERASE. P. Masson¹, M.T. Froment¹, C. Bartels² and O. Lockridge². 1 - Centre de Recherches du Service de Santé des Armées, Unité de Biochimie B P 87 - 38702, La Tronche Cédex, France - 2 - Eppley Institute, University of Nebraska Medical Center, Omaha, NE 68198-6805, USA.

Human butyrylcholinesterase (BChE) was mutated in several residues: A277(279)W/H, Q119(121)Y, G283(285)D and D70(72)G; the double mutant A277W/G283D was also constructed. Wild-type (wt) and mutated BChE genes were expressed in stably transfected human embryonal 293 kidney cells. Kinetics of recombinant wtBChE and its mutants was examined in 67mM phosphate pH 7.0 at 25°C with butyrylthiocholine (BSCh) and o-nitrophenylacetate as substrates. Reversible inhibition was analyzed with two peripheral anionic site (PAS) ligands of AChE: propidium and fasciculine-2-, and with an inhibitor which binds to the active site gorge: dibucaine. Progressive inhibition of wtBChE and D70G mutant was performed with paraoxon and reactivation using 2-PAM.

The wtBChE shows: a) activation at high BSCh concentration, b) biphasic reactivation by 2-PAM after phosphorylation by paraoxon, and c) complex non linear inhibition of o-nitrophenylacetate hydrolysis by dibucaine (1), suggesting that it possesses some components of PAS. The D70G mutant displays a michaelian behavior with respect to BSCh hydrolysis and 2-PAM-reactivation of paraoxon-phosphorylated enzyme, indicating that D70 is involved in activation phenomena. On the other hand, the other mutants retain substrate activation. Thus, residues neighboring the rim of the active site gorge (in particular #277) are not involved in this kinetic complexity.

The inhibitory effect of propidium depends on both BSCh concentration [S] and enzyme type. Inhibition of wtBChE is competitive ($K_i = 4.5 \mu\text{M}$ at low [S], $18 \mu\text{M}$ at high [S]), that of D70G mutant is very weak and non competitive ($K_i \approx 100 \mu\text{M}$). Inhibition of A277W mutant is of mixed type at low [S] ($K_i = 1.5 \mu\text{M}$, $\alpha K_i = 4.3 \mu\text{M}$) like human AChE (2), it is competitive at high [S] ($K_i = 1.5 \mu\text{M}$), indicating that propidium interacts with both D70 and W277. Inhibition of Q119Y mutant is of mixed type ($K_i = 1.6 \mu\text{M}$, $\alpha K_i = 4.7 \mu\text{M}$ at low [S] and $K_i = 3.9 \mu\text{M}$, $\alpha K_i = 6.3 \mu\text{M}$ at high [S]), that of double mutant is of mixed type at low [S] ($K_i = 1.3 \mu\text{M}$, $\alpha K_i = 2.5 \mu\text{M}$) and parabolic at high [S], suggesting that 2 molecules of propidium bind and that D283 may be an element of the 2nd propidium binding site.

Inhibition of wtBChE, by fasciculine-2 is of mixed type at low BSCh concentration ($K_i = 1.4 \mu\text{M}$, $\alpha K_i = 2.3 \mu\text{M}$) and non competitive at high BSCh concentration ($K_i = 2 \mu\text{M}$). Inhibition of mutants except D70G is of mixed type, greater than that of wtBChE, but far weaker than that of mammalian AChE, indicating that tight inhibition needs interaction with several residues located in the PAS area (3).

The PAS is partly present in wtBChE because D70 was identified as an element of this site. Mutations on residues 119, 277 and 283 increase affinity for PAS ligands and change inhibition mechanisms, but do not alter the catalytic mechanism of BSCh hydrolysis. Thus, unlike AChE, D70 is the only one PAS residue responsible for non michaelian behavior of BChE.

References

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POSTERS

Session C: Mechanism of Catalysis of Cholinesterases

POSTER NO. 20: ORGANOPHOSPHATE SPECIFICITY OF ACYL POCKET CHOLINESTERASE MUTANTS. Natilie A. Pickering†, Palmer Taylor†, and Harvey Berman*. †Department of Pharmacology, University of California, San Diego, La Jolla, Ca 92093-0636, USA; *Department of Biochemical Pharmacology, State University, New York, Buffalo, New York, 14260, USA.

Organophosphate (OP) compounds potently and irreversibly inhibit cholinesterases by phosphorylating the active site serine. Their tetrahedral geometry about the phosphate, in contrast to the trigonal carbonyl carbon of carboxyl or carbamoyl ester substrates, adds another dimension to substituent substitution. Therefore, size and geometric constraints as well as stereospecificity can be examined. As reported in Berman & Leonard (JBC 264(7), 3942-3950, 1989), *Torpedo* acetylcholinesterase (AChE) has marked stereospecificity and substituent size specificity to OP's with the differences in reaction rates up to 200 fold between enantiomers. This specificity is suspected to be due to steric hindrance as found for substrate specificity between AChE and butyrylcholinesterase (BuChE) (Vellom et al., *Biochemistry* 32, 12-17, 1993). The side chains of F295 and F297 have been shown in several studies to form the steric constraints on acyl pocket size, however these residues will be shown to have distinct influences on substrate catalysis and substrate inhibition for carbonyl substrates. In the case of alkylphosphates, a more discriminating analysis of structure function is possible where we have examined acylation by the two enantiomers of a series of alkyl methyl phosphonyl thiocholines in relation to acyl pocket substituents. Such studies provide a data base for modeling the orientation of substituent groups in the transition state and the acyl enzyme. (Supported by USPHS grant GM18360 & DAMD17-9-C1058)

POSTER NO. 21: CATALYTIC PROPERTIES OF HUMAN SERUM CHOLINESTERASE PHENOTYPES IN THEIR REACTION WITH SUBSTRATES AND INHIBITORS. Vera Simeon-Rudolf, Mira Škrinjarčić-Špoljar and Elsa Reiner. Institute for Medical Research and Occupational Health, POB 291, 41001 Zagreb, Croatia.

Serum samples identified to belong to the U, FS or A cholinesterase (EC 3.1.1.8) phenotypes were analyzed with acetylthiocholine (ATCh) and propionylthiocholine (PTCh) as substrates. For all three phenotypes, the measured activities did not follow the Michaelis-Menten kinetics. The K_m evaluated at low substrate concentrations (0.02-0.25 mM) was about 0.05 mM, and at high substrate concentrations (1-10 mM) about 1.0 mM, for both substrates and all three phenotypes. The V_m for the U phenotype was three times higher than for the A; the V_m for the FS phenotype was twice as that for the A. It was shown previously (M. Škrinjarčić-Špoljar, V. Simeon, J. Enz. Inhib., 1993) that the U and A phenotypes have about the same affinity (K_i) for reversible inhibition by two positively charged oximes (HI-6 and PAM-2), but also the same affinity for the non-charged 4,4-bipyridine. However, rate constants of phosphorylation (k_i) by positively charged compounds were higher for the U than A phenotype, while non-charged compounds had the same k_i for both. It seems that binding of the studied compounds (expressed in terms of K_m or K_i) is not influenced by a positive charge, while rates of phosphorylation and acylation/deacylation by charged compounds (expressed in terms of k_i and V_m) differ for the two phenotypes.

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Session D: Cellular Biology of Cholinesterases

POSTER NO. 22: TRANSGENIC EXPRESSION OF HUMAN ACETYLCHOLINESTERASE IN MOUSE CENTRAL NERVOUS SYSTEM. Christian Andres, Rachel Beeri, Efrat Lev-Lehman, Moshe Shani and Hermona Soreq. Dept. of Biol. Chem., The Hebrew Univ. of Jerusalem, 91904, Israel. Dept. of Genetic Engineering, The Inst. of Animal Sci., Agric. Res., 906, Beit Dagan 50250, Israel.

To modulate cholinergic neurotransmission in a mammalian *in vivo* model, we established stably transgenic mice expressing human acetylcholinesterase (AChE) under the control of the human AChE promoter. The transgene was found to RT-PCR to yield hAChEmRNA in adult animals in the central nervous system, but not in adrenal glands, muscle or bone marrow. *In situ* hybridization with the hAChEmRNA and immunodetection with alkaline phosphatase demonstrated normal transcription patterns in brain sections of the transgenic mice as compared with the controls. Total AChE activity was enhanced two-fold in basal forebrain, and 1.10 to 1.20 fold in the other regions. The human transgenic enzyme displayed with selective monoclonal antibodies identical immunochemical interactions to those of the corresponding human brain protein. This allowed the detection of the human transgenic enzyme in the central nervous system in accordance to the mRNA distribution. Transgenic activity represented 50% of total AChE activity in basal forebrain and 30 to 40% in the other regions of the central nervous system. Because of the variable enhancement levels in total enzyme activities, this suggests differential expression of the transgene and adaptation of the endogenous enzyme levels depending on the region. Human AChE was further detected in muscle homogenates, which in absence of any hAChEmRNA detectable in this tissue suggests axonal transport of the human transgenic protein from the spinal cord motoneurons to the neuromuscular junction. Sucrose gradient sedimentation profiles revealed correctly assembled transgenic monomers, dimers and tetramers in salt-soluble and detergent-extractable brain and muscle fractions. The transgenic animals were apparently normal, showing no developmental or morphological differences. These results suggest that the AChE coding sequence and/or its transcription and translation products are sufficiently conserved between humans and rodents to allow specific expression and assembly. Furthermore, the differential quantitative expression patterns of the transgenic and the endogenous enzymes show region-specific feedback responses which could be linked with different functional compartments of the cholinergic system.

POSTER NO. 23: DEVELOPMENTAL REGULATIONS OF ACETYLCHOLINESTERASE AT THE MOLECULAR LEVEL IN THE CENTRAL NERVOUS SYSTEM OF THE QUAIL. Alain Anselmet, Jean-Marc Chatel and Jean Massoulié. Laboratoire de Neurobiologie Cellulaire et Moléculaire, URA 1857 C.N.R.S., Ecole Normale Supérieure, 46 rue d'Ulm, 75005 Paris - France.

We have analyzed the evolution of acetylcholinesterase (AChE) transcripts and molecular forms during development in the central nervous system of the quail. We found that several aspects are developmentally regulated: 1) the production of multiple mRNA species; 2) the production of enzymatically active and inactive AChE molecules; 3) the production of amphiphilic and nonamphiphilic AChE forms; 4) the proportions of tetrameric G4, dimeric G2 and monomeric G1 forms.

Anselmet, A., Fauquet, M., Chatel, J.-M., Maulet, Y., Massoulié, J. & Vallette, F.-M. (1994) *J. Neurochem.* **62**, 2158-2165.

POSTERS

Session D: Cellular Biology of Cholinesterases

POSTER NO. 24: EFFECT OF ACUTE IRREVERSIBLE INHIBITION OF ACETYLCHOLINESTERASE ON NERVE STIMULATION-EVOKED CONTRACTILE PROPERTIES OF RAT MEDIAL GASTROCNEMIUS. R. Panenic, P. Gardiner, and V. Gisiger*. Départements d'Éducation Physique et d'Anatomie*, Université de Montréal, Montréal, Québec, Canada, H3C 3J7.

The study was conducted to determine the effect of decreased muscle acetylcholinesterase (AChE) on the expression of muscle contractile properties during indirect stimulation *in situ*. In anesthetized Sprague-Dawley rats, the medial gastrocnemius was surgically isolated, and isometric forces and muscle EMG were measured in response to a protocol of sciatic nerve stimulation. Contractions examined included twitches, and responses to trains (800 ms) at 25, 50, and 200 Hz. Fatigue resistance was also determined by stimulating with trains (100 ms) at 75 Hz, once every 1.5 seconds, for 3 minutes. In one group, AChE was irreversibly inhibited before the protocol by bathing the muscle for 25 minutes with a solution of methanesulfonyl fluoride (MSF, 1 mg/ml in saline) followed by repetitive rinsing with saline. This resulted in at least 95% inhibition of AChE, as measured in muscle homogenates following experiments. In MSF-treated muscles, peak forces for twitch and 25 Hz contractions were potentiated (96% and 40%, respectively), while force at 200 Hz was depressed (by 36%), compared to controls. Thus, the ratio of twitch/maximum tetanic force was 50% in MSF-treated muscles, compared to 16% in controls. The pattern of force decline during the fatigue protocol was similar in control and MSF preparations, although the latter generated significantly less contractile force throughout the 3 minutes. During trains, forces declined dramatically from peak force early in the train to a force plateau in MSF-treated muscles. Interestingly, these force plateaus increased with increasing frequencies of stimulation in MSF-treated muscles. This finding, as well as the lack of effect of MSF-treatment on the pattern of force decrement during intermittent trains, would appear inconsistent with an increased desensitization of ACh receptors due to elevated ACh accumulation at higher frequencies of stimulation. Funded by NSERC Canada.

POSTER NO. 25: REVERSAL OF SIGNAL-MEDIATED CELLULAR RETENTION BY SUBUNIT ASSEMBLY OF HUMAN ACETYLCHOLINESTERASE. C. Kronman, Y. Flashner, A. Shadkhan, and B. Velan. Israel Institute Biological Research, Ness-Ziona, 70450, Israel.

The interrelationship between signal-mediated ER retention and control of subunit assembly in secreted complex proteins was examined in recombinant 293 cells expressing human acetylcholinesterase (HuAChE). The function of putative signals within the C-terminal tetrapeptide CSDL of HuAChE was examined by site directed mutagenesis. The CSDL tetrapeptide carries the free cysteine (cys-580) involved in subunit assembly, yet fails to function as a KDEL-type retention signal. This was demonstrated by mutations that increase similarity to the canonical retention signal (substitution of CSDL by KSDL) or those that deviate from it (substitution to CSAL). Cells expressing both types of mutants exhibited cell-associated HuAChE levels identical to that of wild-type enzyme. Appendage of an engineered KDEL retention signal to a dimerization-impaired HuAChE subunit (the C580A mutant) resulted in intracellular retention of large amounts of fully active enzyme, not prone to proteolytic degradation. On the other hand, attachment of KDEL to a native, dimerization-competent HuAChE polypeptide did not lead to intracellular retention, and allowed efficient secretion of enzyme to the cell growth medium. Yet, appendage of KDEL to the native HuAChE led to some retardation in the transport of enzyme molecules through the Golgi apparatus, as manifested by increase in cellular population of endo H-resistant dimers, as compared to WT-enzyme. Taken together, these results indicate that: a. Subunit dimerization mediated by the C-terminal cysteine of HuAChE can reverse the signal-mediated retention by masking recognition of KDEL by its cognate receptor. b. The native sequences of the AChE subunit polypeptide do not appear to function as a coupled retention/dimerization signal in the control of secretion of assembled enzyme molecules.

This work was supported by the U.S. Army Research and Development Command, Contract DAMD17-93-C-3042.

POSTERS

Session D: Cellular Biology of Cholinesterases

POSTER NO. 26: THE GRAIN APHID CHOLINESTERASES. B. Leszczynski, T. Bakowski, *A.F.G. Dixon and H. Matok. Agricultural & Pedagogic University, Department of Biochemistry, ul. Prusa 12, PL-08110 Siedlce, Poland. *University of East Anglia, School of Biological Sciences, Norwich NR4 7TJ, United Kingdom.

Cholinesterases are important enzymes in chemical interactions between insects and their host plants. The activity of two cholinesterases: acetylcholinesterase [E.C. 3.1.1.7] and butyrylcholinesterase [E.C. 3.1.1.8] was found in homogenates from whole grain aphid, *Sitobion avenae* (F.). The acetylcholinesterase was mostly associated with mitochondria and microsome membranes, instead about 55% of the activity of the butyrylcholinesterase occurred in soluble cytosolic fraction. Within studied morphs of the aphid, winged apterous adults had higher activity of the acetylcholinesterase instead larvae higher activity of the butyrylcholinesterase. The EDTA extracts and EDTA phloem exudates from modulatory resistant and susceptible wheat cultivars showed a various modulatory effect on the activity of the grain aphid cholinesterases. The modulation of the enzymes activity was also observed when effect of the cereal allelochemicals: phenolic compounds, hydroxamic acids and indole alkaloids was tested *in vitro*.

POSTER NO. 27: ACETYLCHOLINESTERASE ACTIVITY IN PLANTS. S. Madhavan and Gautam Sarath. Department of Biochemistry, University of Nebraska, Lincoln, NE 68583-0718, USA.

The presence of acetylcholine in various plant tissues has been well demonstrated. Earlier studies have suggested that acetylcholine (ACh) acts as a second messenger in various cellular processes, primarily in the regulation of membrane permeability to ions.

Acetylcholinesterase (AChE) activity has also been detected in leaves of many plant species. We report in this study the detection of AChE in extracts of guard cell protoplasts (GCP) from *Vicia faba* L., *Nicotiana glauca* Graham and *Kalanchoe diargremontiana*. A pair of guard cells with the enclosed aperture constitute the stoma and several such stomata occur in leaf epidermal layers. Mechanisms that control ion fluxes which not only regulate gas exchange, but also control water status and carbon assimilation in plants, are presumably highly developed in guard cells.

AChE, from crude homogenates of *Vicia faba* GCP, showed a 10-fold greater specific activity for acetylthiocholine hydrolysis when compared to either the whole leaf or mesophyll cell protoplast homogenates. Well known active site specific inhibitors of mammalian AChE, neostigmine and tacrine, inhibited 85-90% of GCP AChE activity. Furthermore GCP AChE, like animal AChE showed an identical order of preference of substrates for its hydrolysis, viz. acetylthiocholine > propionylthiocholine > butyrylthiocholine. GCP AChE was also partially purified using either a tacrine-sepharose-6B or procainamide-sepharose 4B affinity column. The affinity purified GCP AChE was comparable to the affinity purified AChE from both bush bean and pea roots. Immunoblots of the crude GCP homogenates and the affinity purified GCP AChE when probed with Bovine serum AChE antibodies, further conformed the identity of AChE in guard cells. Purification of GCP-AChE to its homogeneity and its further characterization are in progress in our laboratory.

Epidermal peels of *Vicia faba* leaves when treated with acetylcholine (10 μ M to 1 mM) induced a closure of their stomata. However, application of either butyrylcholine and propionylcholine, induced only a minimal closure of stomates. Both eserine and tacrine, potent inhibitors of mammalian AChE, when applied to the epidermal peels also induced a partial closure of stomates. Results from these studies suggest that guard cells in plants have a true AChE and that ACh has a significant physiological role in stomatal movements

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Session D: Cellular Biology of Cholinesterases

POSTER NO. 28: MOLECULAR POLYMORPHISM OF ACETYLCHOLINESTERASE(S) IN *HIRUDO MEDICINALIS*. Vincenzo Talesa^{1,2}, Marta Grauso¹, Elvio Giovannini¹, Gabriella Rosi¹ and Jean-Pierre Toutant². 1: Department of Experimental Medicine, Division of Cell and Molecular Biology, University of Perugia, Italy. 2: Différenciation cellulaire et Croissance, INRA, Montpellier, France.

Two acetylcholinesterases (AChE) differing in molecular forms, in substrate and inhibitor specificities have been characterized in the leech *Hirudo medicinalis*. A spontaneous soluble portion of AChE activity (SS-AChE) was recovered from tissues dilacerated in Low-Salt (LS) buffer, and a Detergent-Soluble fraction (DS-AChE) was solubilized after extraction in LST buffer (1% Triton-X100). Both enzymes were purified to homogeneity by affinity chromatography on Edrophonium- and Concanavalin A-sepharose columns. Purified enzymes were studied by SDS-PAGE in reducing conditions. SS-AChE gave two bands at 30 and 66 kDa, while only one band was seen at 66 kDa for DS-AChE. Sephadex G200 chromatography indicated Mr of 66.000 and 130.000 for native SS- and DS-AChEs. Sedimentation analysis of crude and purified preparations was performed with or without Triton X-100 in the sucrose gradient. SS-AChE showed a single peak sedimenting at 5.0S in both conditions suggesting that it was a hydrophilic monomer (G1). DS-AChE sedimented as a single 6.5S peak in presence of Triton X-100 and aggregated in the absence of detergent. A treatment with PI-PLC suppressed aggregation and gave a 7S peak. DS-AChE was thus an amphiphilic glycolipid-anchored dimer. That hydrophilic monomer in SS-AChE was not a degradation product of the amphiphilic dimer was shown by kinetic analyses and inhibitor specificities. Substrate specificities were studied using p-nitrophenyl-acetate, -propionate and -butyrate and corresponding thiocholine-esters as substrates. SS-AChE displayed marked similarity in Km values with charged and uncharged substrates, suggesting a reduced influence of electrostatic interactions in the enzyme substrate affinity. At variance, DS-AChE displayed higher Km values with uncharged than with charged substrates. Differences in eserine and DFP sensitivity were also found between SS and DS enzymes. SS-AChE was more sensitive to eserine and DFP (IC50 = 10⁻⁷ and 10⁻⁸ M respectively) than DS enzyme (IC50 = 10⁻⁶ and 10⁻⁵ M). The present results suggest the existence of two different acetylcholinesterases in *Hirudo*.

POSTER NO. 29: A FRACTION OF ACETYLCHOLINESTERASE GLOBULAR FORMS FROM *OCTOPUS VULGARIS* (CEPHALOPODA) IS SPECIFICALLY SOLUBILIZED BY HIGH SALT OR HEPARIN. Vincenzo Talesa^{1,2}, Marta Grauso¹, Elvio Giovannini¹, Gabriella Rosi¹ and Jean-Pierre Toutant². 1: Department of Experimental Medicine, Division of Cell and Molecular Biology, University of Perugia, Italy. 2: Différenciation cellulaire et Croissance, INRA, Montpellier, France.

Octopus acetylcholinesterase (AChE) is found as two molecular forms: an amphiphilic dimeric form (G2) sensitive to phosphatidylinositol phospholipase C and a hydrophilic tetrameric (G4) form. G2 and G4 forms belong to a single pharmacological class of AChE. Thus they likely result from a post-transcriptional or post-translational processing of a single AChE gene. Sequential solubilizations reveal that a significant portion of both G2 and G4 forms can be recovered only in a High Salt-Soluble fraction (1M NaCl, no detergent). Heparin (2mg/ml) was able to solubilize G2 and G4 forms with the same efficiency than 1M NaCl. The solubilizing effect of heparin was concentration-dependent and was reduced by protamine (2mg/ml). This suggests that heparin operates through the dissociation of ionic interactions existing *in situ* between globular forms of AChE and cellular or extracellular polyanionic components.

POSTERS

Session D: Cellular Biology of Cholinesterases

POSTER NO. 30: REVERSAL OF GLYCEROL ETHER-STIMULATED ACETYLCHOLINESTERASE ACTIVITY BY *c-fos* ANTISENSE OLIGONUCLEOTIDE IN PRIMARY NEURONAL CULTURES. J.R. Dave, E. Fasnacht, F.C. Tortella, J.M. Best, B.P. Doctor and H.S. Ved. Divisions of Neuropsychiatry and Biochemistry, Walter Reed Army Institute of Research, Washington, DC 20307-5100, U.S.A.

We have previously reported that dodecylglycerol (DDG), an alkyl glycerol lipid similar to platelet activating factor (PAF), stimulates differentiation in primary neuronal cultures obtained from fetal rat cerebral cortex, hippocampus and cerebellum. The neuronal differentiation was evident both morphologically as development of axon-like extensions and biochemically as an increase in neuron specific enzyme activity. We also reported that DDG mediated neuronal differentiation resulted in a transient increase in *c-fos* mRNA levels. The objectives of present study were to determine if *c-fos* antisense oligonucleotide (ASO) would reverse DDG-stimulated acetylcholinesterase (AChE) and choline acetyltransferase (ChAT) activities, and neuronal differentiation. Primary enriched neurons derived from embryonic (E-17) rat cerebral cortex, cerebellum or hippocampus were treated in a serum-free medium for 24 hrs with either vehicle, DDG (4 μ M), or DDG with either *c-fos* antisense oligonucleotide (ASO) (5-20 μ M), *c-myc* ASO or non-sense ASO. Treatment with DDG produced a maximal stimulation in AChE and ChAT activities and outgrowth of neuronal processes in cultures obtained from cerebellum and minimum effects in those obtained from cerebral cortex. The percent stimulation in AChE activity following DDG treatment in these neuronal cells was approximately 200%, 150% and 65% above the control levels in cerebellum, hippocampus and cortex, respectively. The percent stimulation in ChAT activity following DDG treatment in neuronal cells was approximately 260%, 220% and 45% above the control levels in cerebellum, hippocampus and cortex, respectively. Pretreatment with *c-fos* ASO partially inhibited DDG-stimulated AChE and ChAT activities and outgrowth of the neuronal processes; however, *c-myc* ASO and non-sense ASO had no significant effect. The inhibitory effect of *c-fos* ASO on DDG-stimulated AChE and ChAT activities and neuronal differentiation was greatest in neurons obtained from cerebellum and hippocampus (100%-52% inhibition). These results suggest a causative role of *c-fos* proto-oncogene in DDG-mediated neuronal differentiation and further suggest a direct role of *c-fos* gene product on modulation of AChE and ChAT enzyme activities in the neuronal cells.

POSTERS

Session E: Structure-Function Relationship of Anticholinesterase Agents: Nerve Agents and Reactivators

POSTER NO. 31: PROTECTION OF GUINEA PIGS AGAINST SOMAN INHALATION BY PRETREATMENT ALONE WITH HUMAN BUTYRYLCHOLINESTERASE. Nahum Alon, Lily Raveh, Eran Gilat, Jacob Grunwald, E. Cohen*, and Yacov Ashani. Israel Institute for Biological Research, Ness Ziona, Israel and *Faculty of Agriculture, The Hebrew University, Rehovot, Israel.

Human butyrylcholinesterase (HuBChE) was evaluated by us both *in vitro* and *in vivo* as a single prophylactic antidote against the lethal effects of nerve agents. Remarkable protection could be demonstrated in HuBChE-treated mice, rats, and monkeys following an iv exposure to lethal doses of sarin, soman, VX, and tabun. Inhalation challenge is the most realistic simulation of exposure to nerve agents. The following protocol was designed to address the question whether HuBChE could be used as a single prophylactic antidote against inhaled soman vapor in guinea pigs. Awake animals caged in whole body plethysmograph designed and built in our laboratory, were exposed for 45 to 75 sec to 417-460 $\mu\text{g/L}$ soman. Five out of 8 animals containing 28 to 45 nmol circulating HuBChE/animal were completely protected against 1.3-2.4XLD₅₀ doses of inhaled soman. Two animals displayed slight tremors and ataxia and one guinea pig exposed to 2.6XLD₅₀ died. A linear correlation was established between nmols of inhaled soman and the reduction in the levels of circulating HuBChE. A group of 3 guinea pigs pretreated with 10 mg/animal (119 nmol) HuBChE was exposed to soman vapor, in three successive sessions at 1 h time intervals as described above. Animals were protected against a single dose of as high as 3XLD₅₀ soman and accumulated dose of up to 5.5XLD₅₀. Compared to prophylaxis with HuBChE alone, the protective ratio in control animals pretreated with pyridostigmine and administered conventional post exposure therapy was estimated at 2.3; however, in contrast to HuBChE-treated guinea pigs, all surviving animals showed severe toxic symptoms. Of all antidote regimens tested in our laboratory, prophylaxis alone with HuBChE afforded an almost perfect protection against inhalation of soman.

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POSTER NO. 32: STRUCTURE-ACTIVITY OF PYRIDINIUM OXIMES AS ANTIDOTES TO ORGANO-PHOSPHORUS ANTICHOLINESTERASE AGENTS. A.K. Sikder, D.K. Jaiswal, S.N. Dube*, K. Husain*, R. Bhattacharya* and S. Das Gupta*. Synthetic Chemistry Division and *Pharmacology & Toxicology Division, Defence Research and Development Establishment, Gwalior-474002, India.

In an attempt to develop effective antidotes against organophosphorus inhibited acetylcholinesterase enzyme (AChE, EC. 3.1.1.7), some new series of asymmetrically substituted bis-pyridinium monooximes bridged by 2-oxopropane and propane group and 1-alkyl-2-(and 4-)hydroxyiminomethyl pyridinium bromides were synthesised and characterized by spectral data and acid dissociation constant (pKa). The value of dissociation constant decreases with the increase of lipophilicity of oximes. The compounds with 2-oxopropane link were stronger inhibitors and weak reactivators than corresponding propane derivatives. Compounds with 'E'-configuration of the active hydroxyiminomethyl group (-CH=NOH) enhanced the antidotal property. No significant correlation was observed between physico-chemical and biological parameters. Changing substituents in the pyridine rings or altering linking groups between pyridine rings did not appreciably improve antidotal properties. However, the present series of pyridinium salts are effective antidotes against diisopropyl fluorophosphate (DFP) and isopropyl methylphosphono fluoridate (SARIN) poisoning in rodents as evidenced by AChE reactivation in cerebral cortex and blood as well as *in vitro* AChE reactivation potency.

POSTERS

Session E: Structure-Function Relationship of Anticholinesterase Agents: Nerve Agents and Reactivators

POSTER NO. 33: EVALUATION OF THE ANTIDOTAL EFFICACY OF OXIMES IN SOMAN-POISONED ANIMALS: CLINICAL SYMPTOMS AND BLOOD CHOLINESTERASE ACTIVITY. G. Amitai, I. Rabinovitz*, G. Zomber, G. Cohen, R. Adani and L. Raveh. Department of Pharmacology, IIBR, P.O. Box 19, Ness Ziona, Israel.

Bisquaternary oximes such as toxogonin and TMB-4 exert their antidotal efficacy against organophosphorus (OP) poisoning mainly by serving as potent reactivators of inhibited acetylcholinesterase (AChE). However, in the case of soman poisoning the rapid aging of somanyl-AChE renders the antidotal treatment with oximes and atropine notoriously difficult. We have compared the antidotal efficacy of a group of oximes: AB-8, AB-13, toxogonin, HI-6 and HLo-7 in combination with atropine against soman poisoning in beagle dogs and baboon monkeys. Oximes were administered at doses which are equivalent fractions of their respective minimal toxic dose (MTD). Using toxogonin as a reference oxime, a calculated unit of equivalent dose (CED) was defined as the oxime dose equal to the ratio between its MTD and the therapeutic ratio of toxogonin. The antidotal evaluation was carried out during seven days post exposure and treatment with atropine and oxime (at 0.3, 1 and 3xCED). Antidotal efficacy was based on the following criteria: survival rate, dynamics of clinical symptoms, cumulative mean score of clinical condition and kinetics of recovery of blood ChE activity. The highest survival rate (100, 84 and 84%) and lowest cumulative clinical score (16.7, 29.2, 31.2) were obtained for AB-8, HI-6 and HLo-7, respectively, against 5xLD₅₀ soman in dogs (n=6, 1xCED). Time of recovery from soman poisoning was the shortest (4h) following treatment with AB-8. In contrast, dynamics of clinical symptoms in dogs following treatment with either HI-6 or HLo-7 exhibited a relapse at 1-3 days post exposure and recovery was not complete even after 7 days. Toxogonin and AB-13 provided 33 and 50% survival rate, respectively, in dogs and no survival was obtained with these oximes against 3xLD₅₀ of soman in monkeys. In contrast, marked antidotal efficacy of AB-8, HI-6 and HLo-7 was observed in monkeys exposed to 3xLD₅₀ soman (n=6, 1x and 3xCED). In vitro reactivation of somanyl-AChE (FBS) with AB-8, AB-13, HI-6 and HLo-7 (0.5 mM) displayed within 1 h a recovery of 10, 20, 60 and 80%, respectively. Blood ChE measured 1 hour following soman poisoning in dogs (and treatment with all five oximes) did not exceed 5% of its normal activity. Furthermore, the kinetics of ChE recovery obtained for all oximes, except for toxogonin, showed a 15-30% recovery 1-3 days post exposure and was independent of the oxime dose. Similar kinetic patterns of blood ChE recovery which were obtained for all oximes administered at various doses indicate de-novo synthesis of ChE's rather than reactivation of inhibited ChE. These results are consistent with the notion that the progress of alleviation in clinical symptoms following soman poisoning, and treatment with oximes and atropine, is not directly related to the rate of recovery of blood ChE.

POSTERS

Session E: Structure-Function Relationship of Anticholinesterase Agents: Nerve Agents and Reactivators

POSTER NO. 34: EFFICACY OF PROPHYLAXIS WITH HUMAN BUTYRYLCHOLINESTERASE AGAINST SOMAN AND VX POISONING: A COMPARATIVE ANALYSIS. Lily Raveh, Jacob Grunwald, E. Cohen*, and Yacov Ashani. Israel Institute for Biological Research, Ness Ziona, Israel and *Faculty of Agriculture, The Hebrew University, Rehovot, Israel.

Prophylaxis with human butyrylcholinesterase (HuBChE) confers excellent protection against soman and VX without the need for post exposure therapy. Despite its ability to dramatically increase survival of mice, rats and monkeys challenged with these nerve agents (NA), minor but significant differences were observed between the rates of sequestration of soman and VX in blood of animals. The reaction between circulating HuBChE and NA was completed within less than 20 sec for soman, whereas 60 to 120 sec were required to sequester VX. Similar results were obtained from *in vitro* experiments with animal blood. These findings are consistent with HuBChE to NA molar ratios of 0.5 and 1.2 that were needed to completely prevent onset of post exposure symptoms caused by soman and VX, respectively. The differences observed are likely to result from either the differences in the bimolecular rate constant of the scavenging of soman and VX by HuBChE, to different pharmacokinetic and pharmacodynamic properties of the two NA, or to a combination of both factors.

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POSTER NO. 35: INTERLEUKIN 6 MODULATES ACHE ACTIVITY OF CNS NEURONS. D. Clarençon, E. Multon, M. Galonnier, M. Estrade, C. Fournier, J. Mathieu, J.C. Mestrias and M. Falôme. CRSSA Émile Pardé, 3P 87, 38702, La Tronche, France.

Classically, radiation injuries result in a peripheric inflammatory process, and another team of our department observed an early systemic cytokine release following irradiation (Hérodin et al.). Among many mediators playing a role in the early stage of inflammation, it is well documented that interleukin 6 (IL-6) plays a major role in the development of inflammation. Besides, we demonstrated an early decrease of rat brain AChE activity following a whole-body gamma exposure. The object of the present study is to find possible IL-6 systemic effects on the central nervous system.

We used a microspectrophotometric method to measure *in vivo* AChE rat brain activity (Testylier et al., 1987). Two groups of rats, anesthetized with chloral hydrate (80 mg.kg⁻¹.h⁻¹ IV), received either saline or IL-6 (870 µg.kg⁻¹) IV injections. AChE activity was recorded every 30 min. We observed a significant decrease of AChE (15 to 20%) 105 min after IL-6 injection when compared to saline. No recovery of AChE activity was observed, up to two hours after administration.

Then, we studied the *in vitro* effect of IL-6 on isolated mouse neurons. The activities of both membrane-bound and released enzyme were not modified up to four hours after exposure to IL-6 (5 to 500 ng.ml⁻¹). These results suggest that IL-6 could induce an AChE decrease by an indirect mechanism.

Very few reports refer to a relationship between cytokines and cholinergic systems. If IL-1 is known to decrease extracellular ACh in the hippocampus, no report mentions IL-6 effects on AChE activity in live animals. Our *in vivo* data suggest that circulating IL-6 could act, through a mechanism still to be found, by modulating neuronal AChE activity after radiation exposure.

- Hérodin, F. et al., *Blood*, 1992, 80, 3, 688-695.

- Testylier, G. and Gourmelon, P., *Proc. Natl. Acad. Sci. USA*, 1987, 84, 8145-8149

POSTERS

Session E: Structure-Function Relationship of Anticholinesterase Agents: Nerve Agents and Reactivators

POSTER NO. 36: INDUCTION OF CARBOXYLESTERASE ISOENZYME AND ALTERED ACETYLCHOLINESTERASE DURING OP RESISTANCE IN MOSQUITO *CULEX QUINQUEFASCIATUS*. N. Gopalan, S. Prakash, K.M. Rao and B.K. Bhattacharya. Defence Research and Development Establishment, Gwalior-474002, India.

Mechanism of development of malathion resistance in mosquitoes has been a subject of intense study. Altered sensitivity of target enzyme acetylcholinesterase (AChE) as well as enhanced activity of detoxifying scavenger carboxylesterase (CaE) have been reported. We studied the development of malathion resistance in *Culex quinquefasciatus* in laboratory. This mosquito species is a vector for filariasis and nuisance mosquito worldwide. LC_{50} value increased by nearly 400 folds in resistant larvae. Though total AChE level did not change, LC_{50} values of several inhibitors i.e., paraoxon, diisopropylfluorophosphate, N,N' diisopropyl phosphorodiamidic fluoride, propoxur, etc for AChE increased in resistant mosquitoes showing a lowered inhibitor sensitivity of AChE while change in inhibitors constant was marginal. Partially purified CaE showed differential specificities for naphthyl esters substrates, with increased affinities for α -naphthyl esters compared to β -naphthyl esters. K_m values obtained for different substrates showed maximum affinity for propionate with two distinct Michaelis constants. Arrhenious plots using different substrates showed a lowered transition temperature for CaE obtained from resistant larvae showing an adaptation phenomenon. Native PAGE of CaE showed a distinct B2 isoenzyme with naphthyl butyrate as substrate which may be used as a marker for detection of malathion resistance in field mosquito samples.

POSTER NO. 37: REACTIVATION OF PHOSPHORYLATED HUMAN ACETYLCHOLINESTERASE. Chun-Yuan Luo, Feng-Zhen Li, Man-Ji Sun, Jin-Sheng Yang. Institute of Pharmacology and Toxicology, Beijing 100850, China.

A 2360-fold purified human brain acetylcholinesterase (AChE, EC 3.1.1.7) with a specific activity of 532 IU/mg, and washed human red blood cells were used throughout the experiments. Amounts of tabun, sarin, VX, soman and DFP causing more than 90% inhibition of the AChE activity were added. The phosphorylated AChE were reactivated by oximes (2-PAM, TMB₄, LuH6 and HI-6). Reactivation rates were calculated according to Gilbert's formula. Inhibition and reactivation were carried out at 37°C for 10 and 30 min respectively in phosphate buffer (pH 7.2). In order to avoid rapid aging of soman-AChE, inhibition was performed in ice for 10 min and reactivated at 4°C for 6h. The results showed that all the four oximes were quite effective in reactivate soman-AChE to the extent of 28-35%, but not in cases of tabun and DFP. 2-PAM did not significantly reactivate tabun and soman-AChE, however TMB₄ and LuH6 exhibited better in reactivating tabun- and DFP-AChE.

acetylcholinesterase
reactivation
nerve agents
oximes
inhibition

POSTERS

Session E: Structure-Function Relationship of Anticholinesterase Agents: Nerve Agents and Reactivators

POSTER NO. 38: TIME DEPENDENT PROTECTION BY CARBAMATES AGAINST INHALED SARIN AEROSOLS IN RATS. R. Vijayaraghavan, K. Husain, Pravin Kumar, K.S. Pandey and S. Das Gupta. Defence Research and Development Establishment, Gwalior - 474 002, INDIA.

The comparative efficacy of carbamates physostigmine (Phy) and pyridostigmine (Pyr) pretreated at various time intervals (-50, -35, -20 and -5 min) prior to exposure to 51.2 mg.m^{-3} sarin aerosols in rats was carried out. A symptom free dose of Phy (0.1 mg.kg^{-1} , i.m.) given 20 min prior to sarin exposure reduced significantly the inhibition of cholinesterase (ChE) by sarin in cerebral as well as peripheral tissues, and significantly increased the survival time. A symptom free dose of Pyr (0.75 mg.kg^{-1} , i.m.) given 20 min prior, reduced inhibition of cholinesterase only in peripheral tissues and the increase in the survival time was less than with Phy. Phy given 5 min prior to sarin significantly protected the lung ChE and increased the survival time whereas Pyr did not. Phy given 35 min prior to sarin significantly protected the blood and lung ChE, while Pyr protected only the blood ChE. The protection of blood ChE by Pyr was significantly enhanced, but it failed to elevate the survival time when compared to Phy. Pretreatment with Phy or Pyr, 50 min prior to sarin exposure did not protect the inhibition of ChE by sarin in all the tissues and there was no significant change in the survival time. The study demonstrates that pretreatment with Phy gives better protection than Pyr which may be due to its protection of the central cholinergic receptors or due to their differential pharmacokinetics. Pretreatment, 20 min prior to exposure is optimal for protection against the lethality of inhaled sarin.

POSTERS

Session F: Structure-Function Relationship of Anticholinesterase Agents: Pesticides and Therapeutic Agents; Noncholinergic Function of Cholinesterases

POSTER NO. 39: THE INACTIVATION OF HUMAN SERUM BUTYRYLCHOLINESTERASE, ARYL ACYLAMIDASE AND PEPTIDASE ACTIVITIES BY METAL CHELATORS. C.D. Bhanumathy and A.S. Balasubramanian. Neurochemistry Laboratory, Department of Neurological Sciences, Christian Medical College and Hospital, Vellore - 632 004, India.

Purified human serum butyrylcholinesterase (BChE) exhibiting cholinesterase, aryl acylamidase (AAA) and peptidase activities was inactivated by the metal chelators 1,10 phenanthroline and TPEN (N,N,N',N'-tetrakis (2-pyridyl-methyl)ethylene diamine). Both BChE and AAA activities were inactivated to a similar extent using the two metal chelators. The inhibition by TPEN of BChE and AAA was reversed by addition of the transition metal salts of Zn^{2+} , Co^{2+} , Mn^{2+} and Ni^{2+} , but not by Ca^{2+} or Mg^{2+} . EDTA inhibited the peptidase activity exclusively without affecting the BChE and AAA activities. The purified BChE pretreated with EDTA could bind to metal chelate-Sepharose columns and could be eluted with EDTA. Diethylpyrocarbonate treatment (which modified histidine residues) of the EDTA treated enzyme prevented its binding to the metal chelate-Sepharose column. It is suggested that more than one metal binding sites are present on BChE and they selectively influence the catalytic activities in BChE.

POSTER NO. 40: ACTIVATION OF PERITONEAL MACROPHAGES BY ACETYLCHOLINESTERASE IS INDEPENDENT OF CATALYTIC ACTIVITY. A. Klegeris, T.C. Budd and S.A. Greenfield. University Dept. of Pharmacology, Mansfield Road, Oxford, OX13QT, England, UK.

To date non-cholinergic effects of acetylcholinesterase (AChE) in the central nervous system has been associated with its action on neuronal cells. Two distinct modes of non-classical activity of AChE has been suggested: first, that AChE is a neuromodulatory agent modifying neuronal activity (Greenfield, 1991, *Cell. Mol. Neurobiol.* 11, 55). Secondly, that AChE is playing the role of a cell adhesion molecule during the development and differentiation of the central nervous system (Layer et al., 1993, *Cell Tissue Res.* 273, 219). Although these hypotheses imply involvement of different cellular mechanisms, a common assumption for both is an interaction between AChE and the neuronal cell membrane in a fashion that is currently unknown.

In our previous study (Klegeris et al., 1994, *Cell. Mol. Neurobiol.* 14, 87) we used rat peritoneal macrophages, as an accessible model system for central neurons, to investigate interactions of AChE with the cell membrane. We found that purified Electric Eel AChE was capable of inducing a 'respiratory burst' in macrophages that did not involve the catalytic activity of AChE and was mannose and divalent cation dependent. This effect suggested that there may be two possible mechanisms involved: either a mannose-fucose receptor (MFR) on the macrophage membrane, which would bind the surface sugars of AChE, or a lectin-like sugar binding site embedded in the AChE molecule itself.

Here we report a series of experiments, which attempts to tackle each of these proposed mechanisms separately. A lectin-like activity of AChE was tested by affinity chromatography on immobilized monosaccharides, gel-diffusion and erythrocyte agglutination. To test the involvement of the MFR, inhibition studies with specific ligands of this receptor (neoglycoproteins), were performed. We found no evidence for lectin-like activity of AChE. On the other hand the activation of macrophages by AChE was specifically inhibited in presence of mannosylated BSA. Furthermore human recombinant AChE, which is unglycosylated, failed to activate peritoneal macrophages. We conclude that the interaction between Electric Eel AChE and peritoneal cells is mediated by macrophage MFR. Our studies also indicate that several other proteins are capable of activating macrophages (peroxidase, mannose-BSA). Therefore AChE activation of these cells could be an example of more general phenomenon of signalling between neurons and brain macrophages.

POSTERS

Session F: Structure-Function Relationship of Anticholinesterase Agents: Pesticides and Therapeutic Agents; Noncholinergic Function of Cholinesterases

POSTER NO. 41: ORGANOPHOSPHATE-SENSITIVE CARBOXYLESTERASE ISOZYME(S) IN THE RAT LIVER. Subramanya Karanth and T. Shivanandappa. Toxicology Unit, Infestation Control and Protectants Department, Central Food Technological Research Institute, Mysore - 570 013, India.

It is not clearly known if molecular forms (isozymes) of Carboxylesterase (CE) show differential sensitivity to organophosphorus (OP) compounds. The present study was aimed at studying the isozymes of carboxylesterase of rat liver and their *in vivo* and *in vitro* sensitivity to selected OP compounds. 12,000g supernatant of the rat liver was subjected to 10% polyacrylamide gel electrophoresis (PAGE) and enzymes were located by using a-naphthyl acetate as the substrate and Fast Blue B as the coupling agent. Six well defined bands of enzyme activity are present of which the first band (from anode end) is the major band and the last one, a minor band. To study the *in vivo* sensitivity of the enzyme bands to OPs, a single oral dose (1/5 of LD₅₀) was given to albino rats and sacrificed after 24 hours. The gel pattern in treated rats showed differential sensitivity of the bands to OPs. The first major band was found to be more sensitive than the other bands to Bromophos (LD₅₀ = 3000 mg/kg b.w) where as the last (minor) band seems to be the target for Monocrotophos (LD₅₀ = 20 mg/kg b.w). *In vitro* studies showed equisensitivity of all the bands to OPs. The first major band of rat liver is likely to play a key role in the detoxication of at least some OPs and its selective inhibition may be one of the reasons that underlie the differential toxicity of certain OP compounds to mammals.

POSTER NO. 42: PATTERN OF INHIBITION OF ACETYLCHOLINESTERASES IN DIFFERENT REGIONS OF THE BRAIN BY TWO ORGANOPHOSPHORUS HOMOLOGUES, IN RELATION TO THEIR DIFFERENTIAL NEUROTOXICITY. P. Santhosh Kumar, Subramanya Karanth and T. Shivanandappa. Toxicology Unit, ICP Dept., CFTRI, Mysore - 570 013, India.

Bromophos and Ethylbromophos are the two structurally homologous organophosphorus (OP) insecticides which show distinct differences in their toxicity. Ethylbromophos is about 25-fold more toxic [LD₅₀ (rat) - 125 mg/kg b.w] than Bromophos and produces characteristic tremors and lacrimation which are absent in the case of the latter. In order to understand the differential neurotoxic effects of these compounds, pattern of Acetylcholinesterase (AChE) inhibition in different regions of the brain has been studied. Albino rats were intubated with single equitoxic doses (1/5 LD₅₀) and the time-course of ChE inhibition in the whole brain and in different regions of the brain (viz. cortex, cerebellum, striatum, hippocampus, thalamus, stem, pons and optic chiasma) was measured. The degree of AChE inhibition in the whole brain varied greatly. Maximum inhibition of AChE was seen at 12 h and 24 h time points for Ethylbromophos and Bromophos respectively. The response of different areas of the brain to the OP homologues differed significantly. The brain stem was least sensitive to Bromophos but most sensitive to Ethylbromophos. Hippocampus was most inhibited while the cortex was least inhibited by both OPs. The pons was most affected among the brain regions by Bromophos. The recovery from *in vivo* AChE inhibition in brain was faster in case of Ethylbromophos (12 days) whereas it was slow (>16 days) in case of Bromophos. The relative rate of metabolism of the OP homologues may cause the differential inhibition of AChE in the brain regions, which may underlie the pattern of cholinergic symptoms.

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Session F: Structure-Function Relationship of Anticholinesterase Agents: Pesticides and Therapeutic Agents; Noncholinergic Function of Cholinesterases

POSTER NO. 43: ISOLATION OF A 155 KDA PEPTIDE WITH NEUROPATHY TARGET ESTERASE ACTIVITY. Christopher E. Mackay, Bruce D. Hammock and Barry W. Wilson. University of California, Davis, CA, USA, 95616.

A method is described for the isolation of a 155 kDa protein that possesses phenyl valerate esterase activity in the presence of paraoxon and the absence of mipafox, the functional definition of neuropathy target esterase (NTE). Microsomes isolated from 18-day chicken embryos were treated with phospholipase A2 to solubilize the NTE activity. The extract was combined with polyoxyethylene W1 detergent and resolved by gel filtration chromatography to yield an active fraction with an apparent mass of 200 kDa. This fraction was purified further by preparative IEF and native electrophoresis to yield a 155 kDa protein with NTE activity. This was confirmed by affinity chromatography using 3-(9'-mercaptiononylthio)-1,1,1-trifluoropropan-2-one (MNTFP) bound to sepharose. This represents the first report of the isolation of NTE in its active form and helps to confirm the 155 kDa protein as the most likely candidate for NTE.

POSTER NO. 44: EVIDENCE FOR A PUTATIVE ACETYLCHOLINESTERASE UPTAKE MECHANISM WITHIN THE SUBSTANTIA NIGRA. T.C. Budd, B.G.M. Dickie, and S.A. Greenfield. University Dept. of Pharmacology, Oxford, OX1 3QT, UK. D. Vaux, Sir William Dunn School of Pathology, OX1 3RE, UK.

There is a paucity of cholinergic innervation within the substantia nigra. However, a soluble form of acetylcholinesterase (AChE) is secreted from the dendrites of nigral dopaminergic neurones in a K^+ evoked and Ca^{2+} dependent manner that is insensitive to cholinergic agonists and antagonists (Greenfield, 1991). These observations suggest that secreted AChE may serve another function; indeed, *in vivo* studies have demonstrated that AChE has long term functional consequences (Hawkins and Greenfield, 1992a,b). This long term action may possibly be due to the AChE released being incorporated back into neurones as already shown in the periphery (Jessen *et al.*, 1978). To test this hypothesis, AChE (2-20 pM) was perfused via push-pull cannulae into substantia nigra of conscious and anaesthetised guinea pigs. The amount of AChE recovered in the effusate from the substantia nigra was significantly reduced in both conscious and anaesthetised animals, but not in animals where the cannulae were deliberately placed in extra-nigral sites. This reduction in AChE was not apparent when the experiments were repeated immediately *post mortem* or when the medium perfused into the substantia nigra was cooled to approximately 4°C. This would suggest that retention of AChE within the substantia nigra involves an active uptake mechanism which is absent, or non functional, in the extra nigral sites. Moreover, perfusion of enzymatically denatured (boiled) AChE elevates basal levels of endogenous AChE detected in the effusate from the substantia nigra but not from extra-nigral sites. This may be due to competition between the boiled exogenous and secreted endogenous AChE for a putative membrane binding site.

In a further series of experiments, substitution of AChE with biotinylated AChE (b-AChE), enabled uptake of AChE into nigral cells to be visualised directly by fluorescence microscopy. Deposits of b-AChE were observed (with an avidin fluorochrome conjugate) only in sections taken from around the tops of cannulae placed in the substantia nigra; but not at the tops of cannulae located extra-nigally. B-AChE immunofluorescence was observed in both tyrosine hydroxylase-immunopositive (dopaminergic) and -negative cells within the substantia nigra. These results suggest that within the substantia nigra, secreted AChE may be subject to a temperature and energy dependent uptake mechanisms. It is therefore possible that this process may underlie the long term functional effects of AChE.

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POSTERS

Session F: Structure-Function Relationship of Anticholinesterase Agents: Pesticides and Therapeutic Agents; Noncholinergic Function of Cholinesterases

POSTER NO. 45: NEUROTROPHIC FUNCTION OF CIRCULATING ACETYLCHOLINESTERASE IN *APLYSIA*. M. Srivatsan and B. Peretz. Department of Physiology, University of Kentucky Medical Center, Lexington, KY USA 40536-0084

Hemolymph of *Aplysia*, a marine gastropod, is rich in true acetylcholinesterase (AChE) which is linked to neuronal function (Peretz and Srivatsan, Soc. Neurosci. Abstr., 19:569, 1993). Our behavioral and electrophysiological studies have shown that circulating AChE has both a cholinergic and a non-cholinergic modulatory function on *Aplysia* neurons (Peretz, 1994; Srivatsan and Peretz, 1994). Now we have evidence for a neurotrophic function of hemolymph AChE.

The hemolymph of sexually mature adult *Aplysia* contains a neurotrophic factor (Schacher and Proshansky, J. Neurosci. 3:2403, 1983), which is yet to be identified. The age-related changes in AChE activity levels in the hemolymph (Srivatsan et al., J. Comp. Physiol. B. 162:29, 1992) prompted us to investigate if AChE is the neurotrophic factor. The neurons from the central ganglia of young *Aplysia* were dissociated and maintained for four days in cell culture on poly-L-lysine coated glass coverslips in Leibowitz medium (L-15) supplemented with salts to raise the osmolality to that of *Aplysia* serum. Neurons from the same animal were maintained in four different culture conditions as follows: 1) L-15 by itself (control); 2) equal proportions of L-15 and hemolymph; 3) equal proportions of L-15 and hemolymph with its AChE inhibited by BW284c51, a specific, membrane impermeable and reversible inhibitor that blocks both the catalytic and the peripheral sites of AChE and 4) equal proportions of L-15 and hemolymph with its AChE inhibited by edrophonium chloride which blocks the catalytic site of AChE. The influence of each condition on cell survival, attachment, neurite numbers and growth was recorded at 12, 24, 48 and 72 hrs after plating. Length of neurites was measured from photographs. The number and length of neurites increased significantly in neurons cultured in L-15 medium with the added hemolymph when compared to those cultured in L-15 alone. Medium containing hemolymph with its AChE activity inhibited by BW284c51 led to poor attachment of the cells to the substratum and impaired neurite growth. Hemolymph with its AChE inhibited by edrophonium chloride did not alter the neurotrophic influence of hemolymph. Also carbachol, an analogue of acetylcholine, when added to the culture medium did not interfere with neurite growth. Thus *Aplysia* hemolymph AChE has a non-cholinergic neurotrophic influence on adult neurons in vitro. The mechanism by which AChE promotes neurite regeneration and growth is being investigated.

POSTERS

Session F: Structure-Function Relationship of Anticholinesterase Agents: Pesticides and Therapeutic Agents; Noncholinergic Function of Cholinesterases

POSTER NO. 46: FENTHION TREATMENT PRODUCES TISSUE, DOSE- AND TIME-DEPENDENT DECREASES IN MUSCARINIC SECOND MESSENGER RESPONSE IN THE ADULT RAT CNS. P. Tandon¹, C.N. Pope², S. Barone, Jr.³, W. Boyes⁴, H.A. Tilson⁴, and S. Padilla⁴. ¹Dept. Biology, Boston College, Boston, MA; ²Toxicol. Program, NLU, Monroe, LA; ³ManTech Environ. Tech., Inc.; ⁴Neurotox. Div., US Environ. Prot. Ag., Res. Tri. Pk., NC, USA.

Fenthion (dimethyl 3-methyl-4-methylthiophenyl phosphorothionate) belongs to a class of organophosphate compounds which are used as insecticides throughout the world. Exposure to fenthion has been reported to produce permanent ocular degeneration in laboratory animals and visual dysfunction in humans. We have reported that a single, moderate (100 mg/kg) dose of fenthion produces long-term changes in muscarinic receptor density and function in the rat retina in the absence of overt pathology (Tandon *et al.*, *Toxicol. Appl. Pharmacol.*, 125:271-280, 1994). The present series of experiments investigates the effects of lower fenthion dosages on muscarinic receptor function in the retina and the cortex either using a single or repeated dosing regimen. Fenthion was administered as a single dose in corn oil at 0, 10, 50 or 100 mg/kg (sc) at day 0 to adult, male, Long-Evans rats. The animals were killed 4, 14 or 56 days after dosing. For the repeated dosing regimen, animals received 0, 10, 25, or 50 mg/kg fenthion (sc) 2 days/wk for 13 weeks. Cholinesterase (ChE) activity, muscarinic receptor (mChR) density and carbachol-stimulated release of inositol-phosphates (IP) were measured in the retina and frontal cortex. In the acute dosing experiment, although both ChE activity and mChR density decreased with increasing dose, there appeared to be no correlation among the depression in ChE activity, down regulation of mChR and depression of IP release. Moreover, carbachol-stimulated IP release was depressed only in the retina; fenthion (50 or 100 mg/kg) produced a decrease in retinal IP release which was still evident 56 days after the single fenthion dose. In the repeated dosing regimen, muscarinic receptor response was depressed at all doses in both tissues by 45 days into the dosing period; however, by 104 days after the last fenthion administration, the cortical carbachol stimulated IP release was normal whereas the retinal IP release remained depressed in the animals which received the 25 or 50 mg/kg dosage. We conclude that (1) a single exposure to fenthion produces a dose-dependent decrease in muscarinic receptor function specifically in the rat retina; whereas (2) repeated dosing with fenthion depressed muscarinic receptor response both in the retina and cortex; (3) only the retina showed persistent muscarinic receptor dysfunction.

POSTER NO. 47: ROLES OF BUTYRYLCHOLINESTERASE DURING LAMINAR RETINOGENESIS OF THE CHICK EMBRYO. Elmar Willbold and Paul G. Layer. Technische Hochschule Darmstadt, Institut für Zoologie, Schnittpahnstrasse 3, 64287 Darmstadt.

During chicken neurogenesis, butyrylcholinesterase (BChE) and acetylcholinesterase (AChE) are expressed in specific patterns between final cell proliferation and differentiation. Since both cholinesterases are also able to regulate neurite growth *in vitro*, a more general regulatory role of the enzymes can be assumed. We investigated the effects of inhibition of BChE on laminar histogenesis in *retinospheroids* that arise from dissociated embryonic chicken retinal cells in rotation culture. The addition of the irreversible BChE inhibitor tetraisopropylpyrophosphoramidate (iso-OMPA) fully inhibits BChE expression. Unexpectedly, iso-OMPA also suppresses the expression of AChE to 35 - 60%. Histochemically, this inhibition is most pronounced in fibrous regions. The release of AChE into the media is inhibited by iso-OMPA to more than 85%. Control experiments show that AChE suppression by the BChE inhibitor cannot be explained by a direct cross inhibition of iso-OMPA on AChE. Morphologically, the number of spheroids/dish is increased, and their diameter is decreased by about 20%, corresponding to about 50% volume size. Concomitantly, the temporal course of histotypical differentiation is dramatically accelerated. Thus, as a consequence of BChE inhibition both organisation of nuclear cell layers and of plexiform-like (neuropile) areas is significantly accelerated.

POSTERS

Session G: Pharmacological Utilization of Anticholinesterases

POSTER NO. 48: ACETYLCHOLINESTERASE GENE SEQUENCE AND COPY NUMBER ARE NORMAL IN ALZHEIMER'S DISEASE PATIENTS TREATED WITH ORGANOPHOSPHATES. C.F. Bartels, P.L. Moriearty*, R.E. Becker* and O. Lockridge. Eppeley Institute, University of Nebraska Medical Center, Omaha, NE 68198-6805; *Dept. of Psychiatry, Southern Illinois Univ School of Medicine, USA.

Metrifonate, an organophosphate drug, has been used in clinical studies to inhibit AChE in patients with Alzheimer's disease (AD) in the hope that it would stop or inhibit progression of the disease.

Gene amplification can occur with drug application. Gene amplification has been suggested for acetylcholinesterase and butyrylcholinesterase following accidental exposure to organophosphorous insecticides. We looked for ACHE and BCHE gene amplification in 18 patients with AD who received metrifonate for 3 to 24 months. No gene amplification was detected in Southern blots hybridized with human ACHE and BCHE probes.

Several differences between AChE from normal brains and AChE from AD brains have been published: differences in optimal pH for activity, differences in inhibition by BW284C51, tacrine, physostigmine, indoleamines and proteases, and a difference in banding patterns on isoelectric focusing gels.

To see if these differences were due to mutations within the acetylcholinesterase molecule itself, we sequenced white blood cell DNA from two patients with Alzheimer's disease and compared the sequences to a control sample. There were no mutations that caused amino acid changes to distinguish patients with Alzheimer's disease from the control.

Supported by U.S. Army Medical Research and Development Command Grant DAMD17-94-J-4005.

POSTER NO. 49: HUMAN BUTYRYLCHOLINESTERASE AS PROPHYLAXIS TREATMENT AGAINST SOMAN: BEHAVIORAL TESTS IN RHESUS MONKEYS. Ettie Grauer, Lily Raveh, Jacob Grunwald, E. Cohen*, and Yacov Ashani. Israel Institute for Biological Research, Ness Ziona, Israel and *Faculty of Agriculture, The Hebrew University, Rehovot, Israel.

Pretreatment of rats with human butyrylcholinesterase (HuBChE) has been shown by us to prevent soman-induced behavioral deficits, with no need for post exposure therapy. The present study in rhesus monkeys was designed to address two questions: (a) Can HuBChE ameliorate soman-induced behavioral toxicity? (b) Are there untoward behavioral side effects induced by HuBChE itself? Animals were assigned to one of three treatments: 1. HuBChE alone (13 mg/kg, iv, n=3). 2. Pretreatment with pyridostigmine followed by exposure to soman (0.9-3.3xLD₅₀, iv, n=4) and post treatment with TMB/atropine/benactyzine (TAB). 3. Prophylactic administration of HuBChE (21-26 mg/monkey) followed by exposure to soman (3.3-3.6xLD₅₀, iv, n=4). The behavioral test selected was an originally designed paradigm of spatial discrimination that allowed the simultaneous evaluation of several behavior parameters, with specific emphasis on various memory processes. Two of the monkeys treated with HuBChE alone showed no behavioral alteration, whereas the third animal displayed a transient increase in some of the error parameters immediately following the injection and a day later. Variable levels of behavioral deficits, some lasting for about two weeks following soman exposure, were seen in animals of group 2 (the 'positive control'). Although complete protection, i.e., prevention of general and behavioral toxicity, was demonstrated in one animal of group 3 treated prophylactically with HuBChE, the enzyme was unable to completely counteract toxic symptoms in other animals. This is probably because it was administered in insufficient quantities. No prediction could be made about the severity of the ensuing behavioral deficits. Further experiments can help in the understanding of the quality of the 'partial' protection conferred by HuBChE following exposure to multiple lethal doses of soman.

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POSTERS

Session G: Pharmacological Utilization of Anticholinesterases

POSTER NO. 50: HORSE SERUM BUTYRYLCHOLINESTERASE DOES NOT DISRUPT PASSIVE AVOIDANCE LEARNING OR SPONTANEOUS MOTOR ACTIVITY IN RATS. Raymond F. Genovese, Averi R. Roberts, William E. Fantegrossi, Roberta W. Larrison and Bhupendra P. Doctor. Walter Reed Army Institute of Research, Washington, DC, USA.

We have previously shown that single or repeated administration of horse serum butyrylcholinesterase (HS-BChE) (500 U, 5000 U) does not produce performance deficits on schedule-controlled behavior in rats. We further investigated the possibility that enzyme administration alone might degrade performance. Rats were trained on a passive avoidance procedure where traversal from a lighted compartment into a dark compartment produced a single electric shock stimulus. Acquisition and retention of avoidance learning was evaluated by the traversal times during testing 24h, 72h, and 168h after training. Traversal times of rats treated with 5000 U BChE (IP) before training did not differ from rats receiving vehicle. In contrast, rats receiving atropine (10 mg/kg) showed significantly shorter traversal times than rats receiving vehicle, when tested 168h after training. In an additional study, spontaneous motor activity was monitored in rats continuously, using photodetectors mounted in the home cages. Approximately 80% or more of the total daily activity counts for all rats occurred during the dark period (i.e., circadian pattern). When animals were examined daily for 10 days following injection, HS-BChE (7500 U, IP) or vehicle did not affect total daily activity or the circadian pattern of activity. These results confirm and extend earlier reports demonstrating that HS-BChE does not disrupt cognitive or motor performance in rats.

POSTER NO. 51: PREVENTION OF BRAIN DAMAGE AND BEHAVIORAL PERFORMANCE CHANGES FOLLOWING AN IV INJECTION OF SOMAN AND VX IN RATS PRETREATED WITH HUMAN BUTYRYLCHOLINESTERASE. Tamar Kadar, Lily Raveh, Rachel Brandeis, Jacob Grunwald, E. Cohen*, and Yacov Ashani. Israel Institute for Biological Research, Ness Ziona, Israel and *Faculty of Agriculture, The Hebrew University, Rehovot, Israel.

We have demonstrated by using the Morris Water Maze task that pretreatment of rats with human butyrylcholinesterase (HuBChE, ~42 nmol/rat) prevented completely the severe soman (~97 nmol/rat)-induced deficits in cognitive functioning (i.e., learning, memory, and reversal learning). Similar protection was observed following exposure to VX (~20 nmol/rat); however, VX-induced memory deficits were only partially ameliorated. Following the behavioral tests, at 2 weeks and one month post-exposure, animals were sacrificed and their brains were processed for histology (10-12 rats/group). A severe brain damage was observed in most of the nonprotected animals following an iv injection of 0.9-1.1XLD₅₀ of either soman (~65 nmol/rat) or VX (~12 nmol/rat). The injuries were found mainly in the piriform cortex, hippocampal CA1 layer and thalamus, and differ in their distribution and severity among animals. Pretreatment with HuBChE prevented in all the animals (n=10) the histopathological damage observed in nonprotected rats following exposure to either soman or VX. Comparison of the individual histological findings with the behavioral scores revealed a reasonable correlation between the severity of brain injury and performance. Some nonprotected rats that showed toxic symptoms following iv injection of either soman or VX did not display brain damage nor performance decrements. This is consistent with reports on the variability among animals in their response to nerve agent toxicity. Results clearly demonstrated that exogenously administered HuBChE confers protection in rats from the toxic effects of both soman and VX in terms of the integrity of brain tissues and behavioral performance, with no need for post exposure therapy.

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POSTERS

Session G: Pharmacological Utilization of Anticholinesterases

POSTER NO. 52: GENETIC PREDISPOSITION FOR VARIABLE RESPONSE TO ANTICHOLINESTERASE THERAPY ANTICIPATED IN CARRIERS OF THE BUTYRYLCHOLINESTERASE "ATYPICAL" MUTATION. Y. Loewenstein¹, M. Schwartz¹, D. Glick¹, B. Nogaard-Pedersen², H. Zakut³, and H. Soreq¹.
¹ Dept. of Biol. Chem., The Hebrew Univ. of Jerusalem, Israel; ² Dept. of Clinical Biochem., Statens Seruminstitut, Division of Biotechnol., Copenhagen, Denmark; ³ Dept. of Obst. Gyn., The Edith Wolfson Medical Ctr., The Sackler Faculty of Medicine, Tel Aviv Univ., Israel.

Anticholinesterases were recently approved for treating patients suffering from Alzheimer's disease (AD) in an attempt to balance their cholinergic system. These drugs are targeted at acetylcholinesterase (AChE) but also inhibit butyrylcholinesterase (BuChE), known for its numerous genetic variants. The most common of these is the "atypical" phenotype created through a replacement of Asp70 by Gly (D70G) due to a point mutation. The "atypical" enzyme causes prolonged postanesthesia apnea following succinylcholine administration for muscle relaxation and displays a considerably reduced sensitivity to various other inhibitors. The allelic frequency of "atypical" BuChE was studied in different populations and revealed distinct patterns particular to various ethnic groups. Recently, a relatively high allelic frequency of 0.06 was found in a population of Georgian Jews, differing by up to 4-fold from the incidence in other populations (Ehrlich *et al.*, Genomics, in press). This implies that in groups of AD patients from diverse ethnic origins, a significant fraction of carriers of at least one allele of this mutation should be expected. To predict their responsiveness to anticholinesterase treatment, we examined the susceptibility of AChE, as compared to that of BuChE and the "atypical" BuChE variant towards several anticholinesterases in use for AD treatment. IC₅₀ value and rate constants reflecting inhibitor susceptibilities were calculated for various recombinant human cholinesterases produced in *Xenopus* oocytes and immobilized on microtiter plates through selective monoclonal antibodies. The reversible amino acridinium compound Tacrine, currently in use for AD therapy, displayed a 300-fold higher IC₅₀ for the "atypical" enzyme than for BuChE (1mM BtCh as substrate). Pseudo first order rate constants for inhibition of BuChEs by the carbamates heptylphysostigmine (0.139min⁻¹, 10nM inhibitor), phsostigmine (0.3min⁻¹, 1μM inhib.) and SNZ-ENA713 (0.139min⁻¹, 10μM inhib.) were found to be higher than or equal to those of AChE, suggesting that BuChE serves as a second primary target for these drugs. Moreover, the "atypical" variant of BuChE displayed considerably slower inactivation rates to these drugs (0.01min⁻¹, 0.025min⁻¹, and 0.01min⁻¹, respectively) as compared with the wild type BuChE. These findings predict that carriers of the D70G BuChE mutation would vary from other patients in their susceptibility to the above drugs, which potentially contributes to the wide variability of responses observed in clinical trials. Screening patients for D70G carriers should therefore precede anticholinesterase treatment.

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Session G: Pharmacological Utilization of Anticholinesterases

POSTER NO. 53: COMPARATIVE INHIBITION OF ACETYLCHOLINESTERASE BY TACRINE, PHYSOSTIGMINE AND HUPERZINE IN THE ADULT RAT BRAIN. H.S. Ved, J.M. Best, J.R. Dave and B.P. Doctor. Divisions of Biochemistry and Neuropsychiatry, Walter Reed Army Institute of Research, Washington, D.C. 20307, USA.

Alzheimer's disease (AD) is the fourth largest crippling disease in the world. Current therapeutic strategies for the treatment of AD aim mainly to alleviate associated cognitive deficits by activating the defective cholinergic transmission. There has been considerable interest in the development of acetylcholinesterase (AChE) inhibitors, such as velnacrine, tacrine, amiriden, E-2020 acid, huperzine-A, etc., as potential therapeutic drugs. Huperzine A (Hup A) was recently shown to be a potent, reversible inhibitor of AChE (Ashani *et al*; 1992: *BBRC* 184:719-26). The fairly long half-life ($T_{0.5}$ = 35 min) for AChE-Hup A complex is in marked contrast to the rapid on/off rates that characterize other reversible inhibitors of AChE with similar potency (Taylor and Radić; 1994: *Annu Rev Pharmacol Toxicol* 87:281-320). In the present studies, we have observed the *in vitro* differences in the ability to inhibit AChE by Hup-A, tacrine and physostigmine in homogenates of various anatomical regions of the adult rat brain. The findings show that while all these compounds inhibited AChE in a dose-dependent manner, the degree of inhibition varied from inhibitor to inhibitor. The rank order of potency for the inhibition of whole rat brain AChE was physostigmine > Hup-A > tacrine. The AChE inhibition varied in the different brain regions. Hup-A was most effective in the cortex > hypothalamus > cerebellum > hippocampus. In contrast, tacrine was most effective in the cerebellum > hypothalamus > cortex > hippocampus, whereas physostigmine's rank order of inhibition was cerebellum > cortex > hippocampus > hypothalamus. Further enzyme kinetic studies show that these compounds are uncompetitive inhibitors at the effective inhibitory concentrations, whereas they act as competitive inhibitors at lower concentrations. Moreover, the enzyme kinetic parameters (K_m , K_i , $K_{0.5}$ and $K_{0.5m}$, etc.) for each of the compounds are different in the different regions of the rat brain. These findings suggest that different AChE inhibitors may have varying degree of effectiveness, which could partially be explained by their differential interaction with AChE in the distinct anatomical regions of rat brain.

Late-Arriving Poster, Session A

POSTER NO. 54: REDUCTION OF ACETYLCHOLINESTERASE (AChE) mRNA IN DYSGENIC MOUSE SKELETAL MUSCLES LACKING L-TYPE CALCIUM CHANNEL RECEPTORS. Z. Luo, M. Pincon-Raymond* and P. Taylor. Department of Pharmacology-0636, University of California, San Diego, La Jolla, California 92093, USA; *Institut National de la Sante et de la Recherche Medicale, U. 153, 17, Rue du Fer-a-Moulin, 75005 Paris, France.

Treatment of C2-C12 cells in cultures with ryanodine and L-type, but not N-type, Ca^{2+} channel antagonists blocks the differentiation-induced increase in AChE expression indicating that ryanodine-sensitive Ca^{2+} channels in sarcoplasmic reticulum and L-type Ca^{2+} channels in T-tubules of skeletal muscle link to play important roles in regulation of AChE mRNA during myogenesis (Luo *et al.* J. Biol. Chem., submitted). Measurements of transcription rates using run-on transcription and reporter gene expression, as well as the capacity for superinduction, show the increased mRNA associated with differentiation to be due to stabilization of a labile mRNA rather than enhanced transcription. Ryanodine and the L-type Ca^{2+} channel blockers do not influence muscle fusion or the enhanced expression of the nicotinic acetylcholine receptors (nAChR) associated with fusion. To confirm the importance of this signaling pathway in regulation of AChE expression in intact skeletal muscle, we examined mRNA levels of AChE in skeletal and cardiac muscles from muscular dysgenic mice lacking the skeletal type, but not the cardiac type, L-type Ca^{2+} channel receptors. Results from RNA protection experiments indicated 50-80% reductions in AChE mRNA levels in leg muscles from new born and day 18 embryonic mutant mice as compared to control mice. Similar reductions in AChE activity were also observed. In contrast to AChE transcripts, mRNA of γ -subunit of nAChR was increased in mutant mice. However, mRNA levels and AChE activity were not altered in cardiac tissues from mutant mice. These findings provide further evidence that L-type Ca^{2+} channels play an important role in regulation of AChE expression in intact skeletal muscle. The reciprocal regulation of mRNA levels of AChE and nAChR suggests distinct mechanisms of regulation controlled by L-type Ca^{2+} channels in intact skeletal muscles (Supported by GM 18360 & GM 24437).

Late-Arriving Poster, Session G

POSTER NO. 55: ACETYLCHOLINESTERASE INHIBITORS (AChE-I) AS A POTENTIAL USE FOR ALZHEIMER'S DISEASE (AD) THERAPY: DIFFERENCES IN MECHANISMS OF ENZYME INHIBITION. Albert Enz. Preclinical, Sandoz Pharma Ltd., Basel Switzerland.

The rationale underlying utilization of AChE inhibitors in treatment of AD is based on the assumption that inhibition of AChE results in reduced rates of removal of ACh from the synaptic cleft, thereby increasing the probability for effective encounter between neurotransmitter and the reduced numbers of muscarinic receptors in this disease. In the history of experimental clinical trials, compounds exerting different mechanisms of cholinesterase inhibition were investigated. From the experience with clinically used AChE inhibitors in AD several problems can be encountered, possible related with the intrinsic individual properties of these drugs:

- i.). Non-selective inhibitors have a low therapeutic index, and the inhibition of peripheral cholinesterases in heart, muscle and plasma contribute to adverse peripheral effects.
- ii). Fast and multiple (complicated) drug metabolism have the potential to lead to organ toxic effects and can influence an efficient long-lasting AChE inhibition.
- iii). The desired enzyme inhibition both in terms of duration and selectivity is dependent on the mechanism of inhibition.

Regarding the different inhibition mechanisms, the compounds can be divided into three main classes: reversible inhibitors (like aminoacridines), pseudo-irreversible inhibitors (carbamates) and irreversible inhibitors (organophosphorus compounds).

AChE inhibitors, experimentally used in clinical trials will be compared based on their mechanism of enzyme inhibition and in relation to their known toxic potential.

In more detail the mechanism of the carbamate SDZ ENA 713 will be presented regarding the brain selectivity and the human safety parameters.

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T

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V

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W

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Y

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Z

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Zomber, G.	55, 94

LIST OF PARTICIPANTS

Jaya Adhikari
ETL, Dept. of Zoology, Visva Bharati Univ.
Santiniketan 731235, West Bengal, India
Tel: 03463-52751, x88 Fax: 91-03463-52672

Gabi Amitai
Div. of Chemistry
Israel Institute for Biological Research
P. O. Box 19, Ness-Ziona 70450, Israel
Tel: 972-8-381506 Fax: 972-8-401404

Lili Anglister
Dept. of Anatomy & Embryology, Hebrew University
Hadassah Medical School, Jerusalem 91120, Israel
Tel: 972-2-758450 Fax: 972-2-757451

Yacov Ashani
Israel Institute for Biological Research
P. O. Box 19, Ness-Ziona 70450, Israel
Tel: 972-8-381455 Fax: 972-8-401404

Dov Barak
Israel Institute for Biological Research
P. O. Box 19, Ness-Ziona 70450, Israel
Tel: 972-8-381529 Fax: 972-8-401404

Hendrick P. Benschop
TNO Prins Maurits Laboratory
PO Box 45, 2280 AA Rijswijk, The Netherlands
Tel: 31-15-843529 Fax: 31-15-843991

Dhanasekaran Bhanumathy
Dept. of Neurological Sciences
Christian Medical College and Hospital,
Vellore - 632 004, India
Tel: 91-416-22102 Fax: 91-416-22103

Shelley Bhattacharya
ETL, Dept. of Zoology, Visva Bharati University
Santiniketan-731 235, West Bengal, India
Tel: 091-03463-52751, x88 Fax: 091-03463-52672

Rathanam Boopathy
DST Scheme, Department of Bio-Technology
Bharathiar University
Coimbatore - 641 046, Tamil Nadu, India
Tel: 91-422-42222-258 Fax: 91-422-42387

Michael Adler
Neurotoxicology Branch, ATTN: SGRD-UV-YN
U.S. Army Research Institute of Chemical Defense
Aberdeen Proving Ground, MD 21010-5425, USA
Tel: 410-671-1913 Fax: 410-671-1960

Christian Andres
The Hebrew University of Jerusalem
Dept. of Biological Chemistry, Institute of Life Sciences
Jerusalem 91904, Israel
Tel: 972-2-585450 Fax: 972-8-666804

Alain Anselmet
Laboratoire de Neurobiologie Moléculaire et Cellulaire
CNRS URA 1857, Ecole Normale Supérieure
46 rue d'Ulm, 75005 Paris, France
Tel: 33144323749 Fax: 33144323887

A. S. Balasubramanian
Neurochemistry Lab., Christian Medical College and Hospital
Vellore 632 004, Tamil Nadu, India
Tel: 91-416-22102 Fax: 91-416-22103

Cynthia Bartels
University of Nebraska Medical Center, Eppley Institute
600 S. 42nd St., Box 986805, Omaha, NE 68198-6805, USA
Tel: 402-559-6014 Fax: 402-559-4651

Harvey A. Berman
Dept. of Biochemical Pharmacology, School of Pharmacy
SUNY at Buffalo, 335 Hochstetter Hall,
Buffalo, NY 14260, USA
Tel: 716-645-2859

B.K. Bhattacharya
Defence Research & Development Establishment
Tansen Road
Gwalior-474002, India
Tel: 91-751-340354 Fax: 91-751-341148

Renee Blong
University of Nebraska Medical Center, Eppley Institute
600 S. 42nd St., Box 986805, Omaha, NE 68198-6805, USA
Tel: 402-559-6014 Fax: 402-559-4651

Cassian Bon
Unité des Venins, Institut Pasteur
25 rue du Dr. Roux - 75724 Paris Cedex 15, France
Tel: (33-1) 45688685 Fax: (33-1) 40613057

Shambhunath Bose
ETL, Dept. of Zoology, Visva Bharati University
Santiniketan-731 235, West Bengal, India
Tel: 091-03463-52751, x88 Fax: 091-03463-52672

Clarence A. Broomfield
Biochemical Pharmacology Branch
U.S. Army Research Institute of Chemical Defense
Aberdeen Proving Ground, MD 21010-5425, USA
Tel: 410-671-2626

Timothy Budd
Dept. of Pharmacology, Oxford University
Mansfield Road, Oxford OX1 3QT, UK
Tel: 0865-271627 Fax: 0865-271853

Arnaud Chatonnet
Différenciation Cellulaire et Croissance, INRA
place Viala 34060, Montpellier Cedex 1, France
Tel: 33-676126677 Fax: 6754694

Ephraim Cohen
Dept. of Entomology, Faculty of Agriculture
The Hebrew University, Rehovot 76100, Israel
Tel: 972-8-481118 Fax: 972-8-466768

Austin Cooper
Janeway Child Health Centre
St. Johns, Newfoundland, A1A1R8, Canada
Tel: 709-778-4530

Miroslaw Cygler
Biotechnology Research Institute, NRCC
6100 Royalmount Ave.
Montreal, Quebec H4P 2R2, Canada
Tel: 514-496-6321 Fax: 514-496-5143

Iroudayanodir Delamanche
Biologie Generale, Centre D'études Dubouchet BPN 3
91710 Vert-Le-Petit, France
Tel: 33-69908258 Fax: 33-64935266

S. N. Dube
Pharmacology & Toxicology Division
Defence Research & Development Establishment
Tansen Road, Gwalior-474002, India
Tel: 91-751-340354 Fax: 91-75-1341148

Albert Enz
Preclinical Research, Sandoz Pharma. Ltd., Bldg. 386 762
CH-4002 Basel, Switzerland
Tel: 61-324-4705 Fax: 61-324-4787

Marie-Thérèse Froment
CRSSA, Biochemistry Unit
BP 87 - 38702 La Tronche Cedex, France
Tel: 33-76-63-59-59 Fax: 33-76-63-69-61

William S. Brimijoin
Department of Pharmacology, Mayo Clinic
200 First Street S.W., Rochester, Minnesota 55905, USA
Tel: 507-284-8165 Fax: 507-284-9111

Göran Bucht
National Defence Research Establishment
Dept. of NBC Defense
S-901 82 Umeå, Sweden
Tel: 46-90106672 Fax: 46-90106800

Shelley Camp
Dept. of Pharmacology, 0636
UCSD, 9500 Gilman Rd., La Jolla, CA 92093-0636, USA
Tel: 619-534-1367 Fax: 619-534-8248

Didier Clarençon
CRSSA Émile Pardé, 3P 87
38702, La Tronche Cedex, France
Tel: 33-76-36-69-00 Fax: 33-76-63-69-01

Barbara Coleman
Dept. of Pharmacology, 0636
UCSD, 9500 Gilman Rd., La Jolla, CA 92093-0636, USA
Tel: 619-534-1367 Fax: 619-534-8248

Francoise Coussen
Laboratoire de Neurobiologie Moléculaire et Cellulaire
CNRS URA 1857, Ecole Normale Supérieure
46 rue d'Ulm, 75005 Paris, France
Tel: 33144323748 Fax: 33144323887

Jitendra Dave
Dept. of Medical Neurosciences
Walter Reed Army Institute of Research
Washington, DC 20307-5100, USA
Tel: 202-576-0534 Fax: 202-576-1910

Bhupendra P. Doctor
Div. of Biochemistry, Walter Reed Army Institute of Research
Washington, DC 20307-5100
Tel: 202-576-3001 Fax: 202-576-1304

Heinrich Egghart
USARDSC-UK European Research Office
223 Old Marylebone Rd., London NW1 5TH, UK
Tel: 44-71-514-4423 Fax: 44-71-724-1433

Didier Fournier
Lab. d'Entomologie, Université Paul Sabatier
118 route de Narbonne, 310162 Toulouse, France
Fax: 33-61-55-60-00

John George
Dept. of Zoology, University of Guelph
Guelph, Ontario, Canada N1G 2W1
Tel: 519-824-4120, x347 Fax: 519-767-1656

Damon Getman
Dept. of Pharmacology, 0636
UCSD, 9500 Gilman Rd., La Jolla, CA 92093-0636, USA
Tel: 619-534-1367 Fax: 619-534-6833

Kurt Giles
Dept. of Pharmacology, Oxford University
Mansfield Road, Oxford OX1 3QT, UK
Tel: 44-865-271857 Fax: 44-865-271853

Maurice Goeldner
Laboratoire de Chimie Bio-Organique, URA 1386 CNRS
Faculté de Pharmacie, ULP Strasbourg
BP 24-67401 Illkirch cedex, France
Tel: 3388676991 Fax: 3388678891

Patrick Gourmelon
ISPN - DPJD BP n
6 P-92269 Pontenay aux Rosde Cedex, France
Tel: 33 1 46 54 77 54 Fax: 33 1 46 54 46 10

Susan A. Greenfield
University Department of Pharmacology
Mansfield Road, Oxford OX1 3QT, England
Tel: (0)865-271628

Raj Gupta
U.S. Army Medical Research & Development Command
Fort Detrick, Frederick, MD 21703-5012, USA
Tel: 301-619-7567 Fax: 301-619-2416

Brennie E. Hackley, Jr.
U.S. Army Research Institute of Chemical Defense
Aberdeen Proving Ground, MD 21010-5425, USA
Tel: 410-671-3276 Fax: 410-671-1960

Ferdinand Hucho
Freie Universitat Berlin, Institut für Biochemie
Thielallee 63, 14195 Berlin, Germany
Tel: 49-30-838-5545 Fax: 49-30-838-3753

Subramanya Karanth
Toxicology Unit IC & P Dept.
CFTRI, Mysore - 570 013, India
Tel: 091 0821 37150 Fax: 091 821 27697 2447

Ildiko Kovach
Dept. of Chemistry, The Catholic University of America
Washington, DC 20064, USA
Tel: 202-319-6550 Fax: 202-319-5381

Eric Krejci
Laboratoire de Neurobiologie, Ecole Normale Supérieure
46 rue d'Ulm, 75005 Paris, France
Tel: 33-1-44323748 Fax: 33-1-44323887

Ezio Giacobini
Dept. of Pharmacology
Southern Illinois University School of Medicine
P. O. Box 19230, Springfield, IL 62794-9230
Tel: 217-785-2185 Fax: 217-524-0145

Victor Gisiger
Département d'Anatomie, Université de Montréal, C.P. 6128,
Succursale Centre-Ville, Montréal, Québec H3C 3J7, Canada
Tel: 514-343-5765 Fax: 514-343-2459

N. Gopalan
Defence Research & Development Establishment
Tansen Road, Gwalior-474002, India
Tel: 91-751-340354 Fax: 91-751-341148

Jacques Grassi
Service de Pharmacologie et d'Immunologie, DRIPP
C.E. Saclay, 91191 Gif sur Yvette cedex, France
Tel: 33-1-69-08-28-71 Fax: 69-08-59-07

Zoran Grubić
Institute of Pathophysiology, School of Medicine
Zaloška 4, 61105 Ljubljana, Slovenia
Tel: (386) 61 310 841 Fax: (386) 61 302 272

S. Das Gupta
Indian Science Congress Association
14, Dr Bireesh Guha St., Calcutta, 700017, India
Tel: 91-33-247-4530 Fax: 91-33-402551

Michal Harel
Department of Structural Biology
Weizmann Institute of Science, Rehovot 76100, Israel
Tel: 972-8-342647 Fax: 972-8-344159

Lakshmanan Jaganathan
Dept. of Biotechnology, Bharathiar University
Coimbatore, India Pin Code: 641046
Tel: 91-422-42222-258 Fax: 91-422-42387

Andis Klegeris
University Dept. of Pharmacology
Mansfield Rd., Oxford OX1 3QT, UK
Tel: (0865) 271 627 Fax: (0865) 271 853

Alan Kozikowski
89 Headquarters Plaza, North Tower, 14th Floor
Morristown NJ 07960, USA
Tel: 201-984-1777 Fax: 201-993-1685

David Kreimer
Dept. of Neurobiology, Weizmann Institute of Science
Rehovot 76100, Israel
Tel: 972-8-342128 Fax: 972-8-344131

Chanoch Kronman
Israel Institute for Biological Research
P.O. Box 19, Ness-Ziona 70450, Israel
Tel: 972-8-381522 Fax: 972-8-401404

Dr. Paul G. Layer
Technical University Darmstadt, Faculty of Biology
Schnittspahnstrasse 3, Darmstadt, Germany
Tel: 0049-6151-163800 Fax: 0049-6151-166548

Bogmut Lezczunski
Agricultural & Pedagogic University, Dept. of Biochemistry
ul. Prusa 12, PL-08110 Siedlce, Poland
Tel: 48 25 445959 Fax: 48 25 44 5959

Oksana Lockridge
University of Nebraska Medical Center, Eppley Institute
600 S. 42nd St., Box 986805, Omaha, NE 68198-6805, USA
Tel: 402-559-6032 Fax: 402-559-4651

Patrick Masson
CRSSA Biochemistry Unit
BP87 - 38702 la Tronche cedex, France
Tel: 33-76-63-69-59

Jean Massoulié
Laboratoire de Neurobiologie, Ecole Normale Supérieure
CRNC-URA 295, 46 rue d'Ulm, 75005 Paris, France
Tel: (33)144323891 Fax: (33)144323887

Marcel Mesulam
Dept. of Neurology, Northwestern Univ. Medical Center
233 E. Erie Street, Suite 614, Chicago, IL 60611
Tel: 312-908-8266

David H. Moore
U.S. Army Medical Research & Development Command
Fort Detrick, Frederick, MD 21703-5012
Tel: 301-619-7201 Fax: 301-619-2416

Annick Mutero
Dept. of Pharmacology, 0636, UCSD
9500 Gilman Rd., La Jolla, CA 92093-0636, USA
Tel: 619-534-1367 Fax: 619-534-6833

Stephanie Padilla
Cellular and Molecular Toxicology Branch
Neurotoxicology Division (MD-74B), US EPA
Research Triangle Park, NC 27711, USA
Tel: 919-541-3956

Natalie Pickering
Dept. of Pharmacology, 0636, UCSD
9500 Gilman Rd., La Jolla, CA 92093-0636, USA
Tel: 619-534-4026 Fax: 619-534-6833

Madhavan Kutty
Janeway Child Health Centre, Janeway Place
St. Johns, Newfoundland, Canada A1A1R8
Tel: 709-778-4512 Fax: 709-778-4333

Claire Legay
Laboratoire de Neurobiologie, Ecole Normale Supérieure
46 rue d'Ulm, 75005 Paris, France
Tel: 44323748 Fax: 44323887

Yael Loewenstein
Dept. of Biological Chemistry, Institute of Life Sciences
Jerusalem 91904, Israel
Tel: 972-2-585450 Fax: 972-2-666804

Zhigang Luo
Dept. of Pharmacology, 0636, UCSD
9500 Gilman Rd., La Jolla, CA 92093-0636, USA
Tel: 619-534-1367 Fax: 619-534-6833

Pascale Marchot
Dept. of Pharmacology, 0636
UCSD, 9500 Gilman Rd., La Jolla, CA 92093-0636, USA
Tel: 619-534-1367 Fax: 619-534-6833

Donald M. Maxwell
Biochemical Pharmacology Branch
U.S. Army Medical Research Institute of Chemical Defense
Aberdeen Proving Ground, MD 2101-5425
Tel: 410-671-1315 Fax: 410-671-1960

Shawli Mondal
ETL, Dept. of Zoology, Visva Bharati University
Santiniketan-731 235, West Bengal, India
Tel: 03463-52751, x88 Fax: 91-03463-52672

Banibrata Mukhopadhyay
ETL, Dept. of Zoology, Visva Bharati University
Santiniketan-731 235, West Bengal, India
Tel: 03463-52751, x88 Fax: 91-03463-52672

Arie Ordentlich
Israel Institute for Biological Research
P.O. Box 19, Ness-Ziona 70450, Israel
Tel: 972-8-381442 Fax: 972-8-401404

Ranganathan Parthasarathy
3732 Beaufort Lane
Louisville, KY 40207
Tel: 502-894-6187 Fax: 502-894-6155

Cary Pope
School of Pharmacy, Northeast Louisiana University
Monroe, LA 71209-0470
Tel: 318-342-1723 Fax: 318-342-1686

Daniel M. Quinn
Department of Chemistry, University of Iowa
Iowa City, Iowa 52242, USA
Tel: 319-335-1335 Fax: 319-335-1270

Zoran Radić
Dept. of Pharmacology, 0636
UCSD, 9500 Gilman Rd., La Jolla, CA 92093-0636, USA
Tel: 619-534-4021 Fax: 619-534-6833

Terrone L. Rosenberry
Dept. of Pharmacology, Case Western Reserve University
Cleveland, OH 44106-4965, USA
Tel: 216-368-3494 Fax: 216-368-3395

Richard L. Rotundo
Dept. of Cell Biology and Anatomy (R-124)
Univ. of Miami School of Medicine, 1600 N.W. 10th Ave.
Miami, FL 33136-1015, USA
Tel: 305-547-6940 Fax: 305-545-7166

Debapriya Sarkar
ETL, Dept. of Zoology, Visva Bharati University
Santiniketan-731 235, West Bengal, India
Tel: 03463-52751, x88 Fax: 91-03463-52672

Sutapa Sen
ETL, Dept. of Zoology, Visva Bharati University
Santiniketan-731 235, West Bengal, India
Tel: 03463-52751, x88 Fax: 91-03463-52672

T. Shivanandappa
Toxicology Unit, IC&P Dept.
CFTRI, Mysore - 570 013, India
Tel: 091-0821-37150 Fax: 091-821-27697, 2447

Dr. Israel Silman
Department of Neurobiology, Weizmann Institute of Science
76100 Rehovot, Israel
Tel: 972-8-343649 Fax: 972-8-344161

David H. Small
Department of Pathology, University of Melbourne
Parkville, Victoria 3052, Australia
Tel: 61-3-344-4205 Fax: 61-3-344-4004

Hermona Soreq
Dept. of Chemistry, The Life Sciences Institute
The Hebrew University, 91904 Jerusalem, Israel
Tel: 972 2 585109 Fax: 001 619 534 6833

Malathi Srivatsan
MS 510, Dept. of Physiology
Univ. of Kentucky Medical Ctr., Lexington, KY 40536-0084
Tel: 606-323-5447 Fax: 606-258-1070

I. Rabinovitz
Dept. of Pharmacology, Israel Institute of Biological Research
P. O. Box 19, Ness-Ziona, Israel
Tel: 972-8-381506 Fax: 972-8-401404

Richard T. Robertson
Dept. of Anatomy and Neurobiology, College of Medicine
University of California, Irvine, CA 92717, USA
Tel: 714-856-6050 Fax: 714-856-8549

Susana Rossi
Dept. of Cell Biology and Anatomy (R-124)
P.O. Box 016960, Miami, FL 33101, USA
Tel: 305-547-6940 Fax: 305-545-7166

P. Santhoshkumar
Toxicology Unit, IC & P Dept.
CFTRI, Mysore-570 013, India
Tel: 091-0821-37150 Fax: 091-821-27697, 2447

Ashima Saxena
Div. of Biochemistry, Walter Reed Army Institute of Research
Washington, DC 20307-5100, USA
Tel: 202-576-0087 Fax: 202-576-1304

Avigdor Shafferman
Israel Institute for Biological Research
P.O. Box 19, Ness-Ziona 70450, Israel
Tel: 972-8-381518 Fax: 972-8-401404

A.K. Sikder
Synthetic Chemistry Division
Defence Research and Development Establishment
Gwalior-474002, India
Tel: 91-751-340354 Fax: 91-75-1341148

Janez Sketelj
Institute of Pathophysiology, Zaloska 4
61105 Ljubljana, Slovenia
Tel: 386-61-310-841 Fax: 386-61-302-272

Satu M. Somani
Southern Illinois Univ. School of Medicine
P.O. Box 19230, Springfield, IL 62794-9230
Tel: 217-785-2196 Fax: 217-524-0145

Madhavan Soundararajan
Dept. of Biochemistry, University of Nebraska
East Campus, NE 68583-0718
Tel: 402-472-2939 Fax: 402-472-7842

Meira Sternfield
Dept. of Biological Chemistry
Inst. of Life Sciences, Jerusalem 91904, Israel
Tel: (02) 585 Fax: (02) 666804

Kevin Stopps
School of Biol. Sciences, Queen Mary & Westfield College
Mile End Rd., London, UK E1 4NS
Tel: 071-775-3013 Fax: 081-983-0973

Joel Sussman
Dept. of Structural Biology, Weizmann Institute of Science
Rehovot 76100 Israel
Tel: 972-8-342647 Fax: 972-8-344159

Dr. Palmer Taylor
Dept. of Pharmacology, 0636
UCSD, 9500 Gilman Rd., La Jolla, CA 92093-0636, USA
Tel: 619-534-1366 Fax: 619-534-6833

Latha Unni
Southern Illinois Univ. School of Medicine
Neurochemistry Lab, 801 N. Rutledge, Rm. 4326
Springfield, IL 62702
Tel: 217-782-8103 Fax: 217-782-0988

Haresh Ved
Div. of Biochemistry, Walter Reed Army Institute of Research
Washington, DC 20307-5100, USA
Tel: 202-576-0956 Fax: 202-576-1304

R. Vijayaraghavan
Defence Research and Development Establishment
Gwalior - 474 002, India
Tel: 91-751-340354 Fax: 91-75-1341148

Stanislaw Wlodek
Chemistry Dept., University of Houston
4800 Calhoun Rd., Houston, TX 77204-5614
Tel: 713-743-3320 Fax: 713-743-2709

Sean M. Wilson
Dept. of Avian Science
University of California at Davis, Davis, CA 95616
Tel: 916-752-3519 Fax: 916-752-8960

Man-Ji Sun
Institute of Pharmacology and Toxicology
Tai-Ping Road No. 27, Beijing 100850, China
Tel: Fax: 861-821-1656

Vincenzo Talesa
Unité Différenciation Cellulaire et Croissance, INRA
Place Viala, 34060 Montpellier, cedex 1, France
Tel: 67 61 26 87 Fax: 67 54 56 94

Jean-Pierre Toutant
Physiologie Animale, INRA, Place Viala
34060 Montpellier, France
Tel: (33)67 61 26 87 Fax: (33)67 54 56 94

Lalit Vaya
Torrent Pharmaceuticals Ltd
Torrent House, Off Ashram Rd., Ahmedabad-380 009, India
Tel: 079-405090, 44323 Fax: 079-460048

Baruch Velan
Israel Institute for Biological Research
P. O. Box 19, 70450 Ness-Ziona, Israel
Tel: 972-8-381518 Fax: 972-8-401404

Elmar Willbold
Technische Hochschule Darmstadt, Institut für Zoologie
Schnittspahnstrasse 3, 64287 Darmstadt, Germany
Tel: 49-6151-163800 Fax: 49-6151-166548

Barry W. Wilson
Dept. of Avian Science
University of California at Davis, Davis, CA 95616
Tel: 916-752-3519 Fax: 916-752-8960