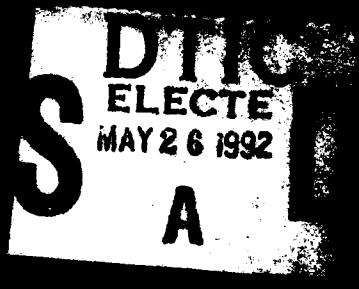
36th OHOLO CONFERENCE:

MULTIDISCIPLINARY APPROACHES TO CHOLINESTERASE FUNCTIONS



April 6-10 1992, Eilat, Israel.

36th OHOLO CONFERENCE:

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April 6-10 1992, Eilat, Israel.

Organized by:
Israel Institute for Biological Research
Ness-Ziona Israel.

Co-Chairpersons: Avigdor Shafferman and Baruch Velan

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Scientific Program

LECTURES

MONDAY, APRIL 6, 1992.

OPENING SESSION:

20:15-21:30 TAYLOR, P. (U.C. San Diego. U.S.A.) - Impact of Recombinant DNA Technology and Structural Studies on Past and Future Research of the Cholinesterase.

TUESDAY, APRIL 7, 1992

SESSION I - POLYMORPHISM AND STRUCTURE

08:30-09:00 BRODBECK, U. (U. of Bern - Bern. Switzerland) - Subunit Assembly and Glycosylation of Mammalian Acetylcholinesterases.

09:00-09:30 SILMAN, I. (Weizmann Inst. of Science. Rehovot. Israel) - Structural and Functional Studies on the GPI-Anchored Form of Acetylcholinesterase.

09:30-10:00 INESTROSA, N.C. (Catholic U. Chile. Santiago. Chile) - Binding of A_{12} AChE to C_2 Muscle Cells and to CHO Mutants Defective in Glycosaminoglycan Synthesis.

10:00-10:30 COFFEE BREAK

10:30-11:00 MASSOULIE, J. (E.N.S. C.N.R.S. Paris. France) - Synthesis of Acetylcholinesterase Molecular Forms in Transfected Cells.

11:00-11:30 TOUTANT, J.P. (I.N.R.A. Montpellier. France) - Nematode Acetylcholinesterases: Several Genes and Molecular Forms of their Products.

11:30-12:00 SUSSMAN, J.L. (Weizmann Inst. Rehovot. Israel) - Three Dimensional Structure of Acetylcholinesterase.

12:00-12:30 CYGLER, M. (Bio. Tech. Research. Inst. Montreal. Canada) - Structural Role of Highly Conserved Amino Acids in Esterase/Lipase Fold Family.

BREAK

SESSION II - GENE ORGANIZATION AND EXPRESSION

15:30-16:00 LOCKRIDGE, O. (U. Nebraska Med. Cent. Omaha. U.S.A.) - SV-40 Transformed Cell Lines, for Example COS-1 but Not Parental Untransformed Cell Lines, Express Butyrylcholinesterase (BCHE).

16:00-16:30 VELAN, B. (IIBR Ness-Ziona. Israel) - Molecular Organization of Recombinant Human AChE in High-Level Expression Systems.

17:00-17:30 ROTUNDO, R.L. (U. Miami. Miami. U.S.A.) - Regulation of AChE Expression in Skeletal Muscle.

17:30-18:00 FOURNIER, D. (I.N.R.A. Antibes, Cedex. France) - Drosphila Acetylcholinesterase: Analysis of Structure and Sensitivity to Insecticides by in vitro Mutagenesis and Expression.

18:00-18:30 TAYLOR, P. (U.C. San Diego. U.S.A.) - Gene Structure and Regulation of Expression of Acetylcholinesterase.

19:00-20:00 DINNER

20:00-22:00 POSTER SESSION A (Including short oral presentation by: Beeri, R., Chatonnet A., Fisher M., Malcolm, C.A., Masson P., Primo-Parmo, SL.)

WEDNESDAY, APRIL 8, 1992

SESSION III - CATALYTIC MECHANISMS AND STRUCTURE FUNCTION RELATIONSHIP - A.

08:30-09:00 HUCHO, F. (Freie. U. Berlin. Germany) - Binding Sites and Subsites of Acetylcholinesterase from torpedo and cobra.

09:00-09:30 SHAFFERMAN, A. (IIBR. Ness Ziona. Israel) - Acetylcholinesterase Function - Protein Engineering Studies Guided by Amino Acid Sequence Conservation and 3-D Structure.

09:30-10:00 SOREQ, H. (Hebrew U. Jerusalem. Israel) - Molecular Dissection of Functional Domains in Human Cholinesterases Expressed in Microinjected Xenopus Oocytes.

10:00-10:30 COFFEE BREAK

10:30-11:00 ROSENBERRY, T.L. (Case Western. Res. U. Cleveland. Ohio). - Acetylcholine Binding to a Peripheral Anionic Site on Acetylcholinesterase is an Important Feature of the Catalytic Pathway.

11:00-11:30 HIRTH, C. (U. Louis Pasteur. Strasbourg. France) - Structural Analysis of Acetylcholinesterase Ammonium Binding Sites.

11:30-12:00 BERMAN, H.A. (Fac. of Hlth. Sci. Buffalo. NY. U.S.A.) - Electrostatic Control of Acetylcholinesterase Topography: Role of the Peripheral Anionic Site.

FREE AFTERNOON

19:00-20:00 DINNER

20:00-22:00 POSTER SESSION B (Including short oral presentation by: Amitai, G., Barak, D., Hjalmarsson K., Magazanik, L.G., Sherman, K., Williamson M.S.)

THURSDAY, APRIL 9, 1992

SESSION III - CATALYTIC MECHANISMS AND STRUCTURE FUNCTION RELATIONSHIP - B.

08:30-9:00 ZHOROV, B.S. (Pavlov Inst. St. Petesburg. Russia) - Relationships Between Accessibility of Phosphorus Atom as Estimated by Molecular Mechanics Calculations and Anti-Acetylcholinesterase Activity of Organophosphorus Inhibitors.

09:00-09:30 ASHANI, Y. (IIBR. Ness-Ziona. Israel) - Elucidation of the Structure of Organophosphoryl Conjugates of Butyrylcholinesterase by 31P-NMR Spectroscopy.

09:30-10:00 QUINN, D.M. (U. Iowa. Iowa City. U.S.A.) - Cryptic Catalysis and Cholinesterase Function.

10:00-10:30 WEINSTOCK, M. (Hebrew U. Jerusalem. Israel) - Acetylcholinesterase Inhibition by Novel Carbamates: A Kinetic and Nuclear Magnetic Resonance Study.

10:30-10:45 COFFEE BREAK

SESSION IV - PHYSIOLOGICAL AND DEVELOPMENTAL FUNCTIONS

10:45-11:15 SKETELJ, J. (Inst. of Pathophys. Ljubljana. Slovenia) Regulation of Acetylcholinesterase in Fast and Slow Skeletal Muscles.

11:15-11:45 ANGLISTER, L. (Hebrew U. Jerusalem. Israel)- Synaptic Acetylcholinesterase at Intact and Damaged Neuromuscular Junctions.

11:45-12:15 LAYER, P.G. (Max. Plank. Inst. Tubingen. Germany) - Towards a Functional Analysis of Cholinesterases in Neurogenesis: Histological Molecular, and Regulatory Features of BCHE from Chicken Brain.

12:15-12:45 GREENFIELD, S.A. (U. Dept. of Pharmacology. Oxford. England) - A Non-Cholinergic Function of Acetylcholinesterase in the Brain and its Relation to the Generation of Movement.

BREAK

SESSION V - CLINICAL IMPLICATIONS

15:30-16:00 BRIMIJOIN, S. (Mayo Clinic Rochester. U.S.A.) - Experimental Acetylcholinesterase Autoimmunity.

16:00-16:30 ENZ, A. (Sandoz Pharma. Basle. Switzerland) - Influence of Different Acetylcholinesterase Inhibitors on Molecular Forms G1 and G4 Isolated from Alzheimer's Disease and Control Brains.

16:30-17:00 ZAKUT, H. (Wolfson Hosp. Holon. Israel) - Clinical Implications of Cholinesterase Aberrations in Syndromes of Hemopoietic Cell Division.

17:00-17:30 DOCTOR, B.P. (WRAIR, WRAMC. Washington D.C., U.S.A.) - Acetylcholinesterase: a Pretreatment Drug for Organophosphate Toxicity.

FRIDAY, APRIL 10, 1992

09:00-10:30 Round Table Discussion

10:30 End of Conference.

POSTER SESSION A

TUESDAY APRIL 7, 1992 - 20:00-22:00

A1.- Andres Christian, Mustapha El Mourabit, Jean Mark and Albert Waksman. - Anchoring of Rat Brain Acetylcholinesterase to Membranes.

A2.- Anselmet Alain, Mireille Fauquet, Jean-Marc Chatel, Yves Maulet, Jean Massoulie and Francois-Marie Vallette - Evolution of Acetylcholinesterase Expression in Developing Central Nervous System of the Quail.

A3.- Yann Fedon, Jean-Pierre Toutant and Martine Arpagaus - characterization of an Esterase Gene Located on Chromosome 5 in Caenorhabditis elegans.

A4.- Bank, Gina - Cloning and Analysis of the Acetylcholinesterase Gene of Ceratitis capitata (medfly)

A5.- Bartels, C., T. Zelinski, O. Lockridge - Histidine 322 to Asparagine Mutation in Human Acetylcholinesterase (AChE) Associated with the Rare YT2 Blood Group Antigen.

A6.- * Beeri, R., Averell Gnatt, Yaron Lapidot-Lifson, Dalia Ginzberg, Moshe Shani, Haim Zakut and Hermona Soreq - Amplification of human Butyrylcholinesterase cDNA and its Impaired Transmission Studied in Transgenic Mice.

A7.- Dickie, B.G.M. and S.A. Greenfield,- "On Line" Recording of Acetylcholinesterase from Substantia Nigra: A Comparison of Stimulus- and 5-Hydroxytryptamine (5HT) - Evoked Release.

A8.- Dolginova, Elena A., Esther Roth, Israel Silman and Lev M. Weiner - Chemical Modification of Torpedo Acetylcholinesterase with Disulfides: Reversible Modification and Irreversible Inactivation.

A9.- Eichler Jerry, Lilly Tocker and Israel Silman - Effect of Heat Shock on Acetylcholinesterase Activity in Chick Muscle Cultures.

A10.- Eichler Jerry, Israel Silman and Lili Anglister - G_2-Acetylcholinesterase is Presynaptically Localized in Torpedo Electric Organ.

A11.- * Fisher Meir and Marian Gorecki - Expression and Isolation of Biologically Active Human Acetylcholinesterase from E. Coli.

* Short oral presentation

A12.- Harel, M., I. Silman and J.L. Sussman - A Model of Butyrylcholinesterase based on the X-Ray Structure of Acetylcholinesterase Indicates Differences in Specificity.

A13.- *Jbilo, O., Cousin X., Toutant J.P., Chatonnet A. and Lockridge O. - Tissue Distribution of Human BCHE Transcripts. Comparative Study of the 5' Regions of Human and Rabbit BCHE Genes.

A14.- Kerem Anat, Chanoch Kronman, Baruch Velan, Avigdor Shafferman and Shoshana Bar-Nun - Postranslational Modifications of Human Acetylcholinesterase in Transfected 293 Cells.

A15.- Kronman Chanoch, Baruch Velan, Yehoshua Gozes, Moshe Leitner, Yehuda Flashner, Tamar Sery, Arye Lazar, Haim Grosfeld, Hermona Soreq and Avigdor Shafferman - Establishment of Stable Cell Lines which Produce and Secrete High Levels of Recombinant Human AChE.

A16.- Kronman Chanoch, Moshe Leitner, Yehuda Flashner, Dana Stein, Gila Friedman, Tamar Sery, Avigdor Shafferman and Baruch Velan - Contribution of the Three N-Glycosylation Sites to Productivity, Catalytic Activity and Molecular Heterogeneity of Recombinant Human AChE Expressed in Human Cells.

A17.- Lazar, A., Kronman C., Silberstein, L., Reuveny., S., Velan B., and Shafferman A. - Production of Human Recombinant Acetylcholinesterase - Comparison of Anchorage-Dependent Cell Propagation Systems.

A18.- Liao, J., V. Mortensen, C. Koch, B. Norgaard-Pedersen and U. Brodbeck - Production and Characterization of Monoclonal Antibodies Specific for Mammalian Brain Acetylcholinesterases.

A19.- *S. Rooker, A. Edwards, L.M.C., Hall, D. Heckel. R. Drown, P. Mason, A. Devonshire, J. Wierenga and C.A. Malcolm - Use of the PCR Method to Rapidly Generate Homologou s DNA Probes and Sequence for Acetylcholinesterase Genes in Non-Dipteran Pest Insects.

A20.- *Masson Patrick, Steve Adkins, Philippe Pham-Trong, and Oksana Lockridge - Expression and Refolding of Functional Human Butyrylcholinesterase from E.coli.

A21.- *Primo-Parmo, SL., Bartels C., Hidaka K., Lightstone H., van der Spek A. and La Du B.N. - Heterogeneity of the Silent Phenotype of Human Butyrylcholinesterase - Identification of Ten New Mutations.

A22.- Edwards, S. Rooker, C.A. Malcolm, N. Pasteur, M. Raymond and L.M.C. Hall - Sequence Analysis of Genes Coding for Insecticide Insensitive Acetylcholinesterase in Mosquitoes.

* Short oral presentation

A23.- Roshchina, V.V. - Plant Cholinesterases.

A24.- Seidman Shlomo, Revital Ben-Aziz Aloya, Yael Loewenstein, Robert Goldstein, Mitchell Weiss, Baruch Velan, Chanoch Kronman, Moshe Leitner, Avigdor Shafferman and Hermona Soreq - Transient Expression of Recombinant Human AChE in Developing Embryos of Xenopus Laevis.

A25.- Szegletes, T., O. Kufcsak, G. Lang, J. Nemosok - Fish AChE Molecular Forms and Their Biochemical Characterization as a Biomonitoring Tool of Aquatic Environment.

A26.- Tarrab-Hazdai R., B. Espinoza, N.J. Bolton, R. Arnon, I. Silman, and A. Agnew - Comparison of the Acetylcholinesterases of Three Species of Schistosome.

POSTER SESSION B

WEDNESDAY APRIL 8, 1992 - 20:00-22:00

B1.- *Amitai, G. - I. Rabinovitz, G. Zomber, G. Cohen and L. Raveh. Efficacy of Oximes as Antidotes against Organophosphorus Poisoning and its Relation to AChE Reactivation.

B2.- Yacov Ashani and Bhupendra P. Doctor - Studies on the Mechanism of Inhibition of Cholinesterases by Huperzine A.

B3.- *Barak Dov, Naomi Ariel, Yacov Ashani, Baruch Velan and Avigdor Shafferman - Construction of Molecular Models for Human AChE and for its Phosphonylation Products by Enantiomers of Isopropyl Methylphosphonates (IMP)

B4.- Gentry, Mary K., Ashima Saxena, Yacov Ashani and Bhupendra P. Doctor - Characterization of Anti-Acetylcholinesterase Inhibitory Monoclonal Antibodies.

B5.- Soren Andersen, Karine Pecorella, Patrick Masson, Jean-Pierre Toutant and Grassi Jacques - Colorimetric Determination of Cholinesterase Activity. New Methods Leading to the Formation of Soluble or Insoluble End-products.

B6.- Hajos, M. and S. Greenfield - Non-Cholinergic Action of AChE: Subcellular Target on Substantia Nigra Neurones.

B7.- Hawkins, C.A. and S.A. Greenfield - Non-Cholinergic Action of Acetylcholinesterase in the Rat Substantia Nigra: Behavioural Effects.

B8.- *Goran Bucht, Hjalmarsson Karin, Briua Haggstrom, and Annika Osternam - Structurally Important Residues in the Region Ser91 to Asn98 of Torpedo Acetylcholinesterase.

B9.- Jones, S.A., K. Ostergaard, S.A. Greenfield and J. Zimmer - Acetylcholinesterase (AChE) in Organotypic Slice Cultures of Ventral Mesencephalon and Striatum.

B10.- Klegeris, A., L.G. Korkina and S.A. Greenfield - A Direct Action of Acetylcholinesterase on Dopamine Oxidation.

B11.- Lammerding-Koppel, M. and U. Drews - Embryonic Cholinesterase as Part of an Embryonic Muscarinic System.

B12.- Lev-Lehman Efrat, Dalia Ginzberg, Averell Gnatt, Asher Meshorer, Haim Zakut and Hermona Soreq - Differential Transcriptional Control of Cholinesterase Genes in Developing Megakaryocytes.

* Short oral presentation

B13.- Loewenstein Yael, Michel Denarie, Haim Zakut and Hermona Soreq - Differential Inhibition of Various Cholinesterases by N-Methyl Carbamates Predicts Differences in Active Site Groove.

B14.- * Magazanik, L.G., J. Molgo, F. Bosch, J.M. Hermel, J. Stinnakre, E. Karlsson - Common and Specific Actions of Acetylcholinesterase Inhibitors on Endplate Currents

B15.- A.P. Breskin, A.E. Khovanskikh, B.N.Kormilitsyn, L.I. Kugusheva, Maizel, E.B., S.N. Moralev, K.D. Mukanova, A.A. Abduvakhabov, B.N. Babaev, D.N. Dalimov - Comparative Investiga- tion of Interaction of some Hydrophobic Organophosphorous Inhibitors with Cholinesterases of Spring Grain Aphid and Warm-Blooded Animals.

B16.- Masson Patrick, Frederique Renault, Marie-Therese Froment, Corinne Ducourneau and Oksana Lockridge - Characterization of Pseudomonas Fluorescens Cholinesterase.

B17.- O'Callaghan, J.F. and S.A. Greenfield - Is the Non-Cholinergic Effect of Acetylcholinesterase in the Substantia Nigra Mediated by Dopamine?

B18.- Ordentlich Arie, Haim Grosfeld, Chanoch Kronman, Moshe Leitner, Baruch Velan, and Avigdor Shafferman - Modulation of Catalytic Activity of Human Acetylcholinesterase by Mutation of ASP74.

B19.- Raveh Lily, Jacob Grunwald, Ephraim Cohen, Dino Marcus, Yoel Papier, Eran Gilat, Nahum Allon and Yacov Ashani - Human Butyrylcholinesterase: A Universal Prophylactic Antidote Against Organophosphate Poisoning.

B20.- Richter ED., I. Orun, J. Ronen, WQ Lu, Y. Yodfat, F. Grauer, J. Marzouk, M. Gordon - Cholinesterase Revisited

B21.- Lucie Zemach, Dina Segal and Shalitin Yechiel - Melittin Inhibits Cholinesterases.

B22.- * Sherman, Kathleen A. - Novel Mechanism of Brain Acetylcholinesterase Inhibition by a Piperidine, E2020.

B23.- Sketelj, J., Crne-Finderle N., Sket D., Dettbarn W-D and Brzin M. - Rapid Postdenervation Decrease of the A12 Acetylcholinesterase Form in the Motor Endplates Is Not Due to Muscle Inactivity.

B24.- Sket, D., Cucek D. and Brzin M. - The Functional Link between Acetylcholinesterase Activity and Dopaminergic Function of the Striatum.

* Short oral presentation .

B25.- Sketelj, J., Cucek D., Brzin M. - Early Postnatal Acetylcholinesterase Focalization and Differentation of Subsynaptic Sarcolemma in the Absence of Innervation.

B26.- Webb C.P. and S. Greenfield - Is the Non-Cholinergic Effect of Acetylcholinesterase in the Substantia Nigra Mediated by an Atp-Sensitive Potassium Channel?

B27.- * Williamson Martin S., Graham D. Moores and Alan L. Devonshire - Altered Forms of Acetylcholinesterase in Insecticide-Resistant Houseflies (Musca domestica).

B28.- Wolfe A.D., B.P. Doctor, Chiang and H. Leader - The Effect of the Monoclonal Antibody (MAB) AE-2 on Inhibition of Fetal Bovine Serum Acetylcholinesterase (FBS AChE) by Organophosphates (OPS) and Carbamates (CBS).

* Short oral presentation .

Opening Session

IMPACT OF RECOMBINANT DNA TECHNOLOGY AND STRUCTURAL STUDIES ON PAST AND FUTURE RESEARCH ON ACETYLCHOLINESTERASE STRUCTURE

P. Taylor. Department of Pharmacology, University of California, San Diego, La Jolla, CA 92093-0636.

In the past six years studies on cholinesterase molecules have shown a quantum advance with the elucidation of primary structures, the study of evolutionary relationships within a large family of proteins and ultimately the three dimensional crystal structure. Recombinant DNA techniques also enable us to manipulate both acetylcholinesterase gene and protein structure at will. Hence tools are available for delineating fine details in gene structure, examining regulation of gene expression and delineating the structure of the gene product and its ligand complexes at atomic-level resolution. While these approaches invite innovative approaches to future endeavors, they also enable us to re-examine work done prior to having a detailed structural base. Hence, major developments in cholinesterase research will be reviewed and interpreted in the light of the new structural information that has recently emerged.

Session I:

Polymorphism and Structure

SUBUNIT ASSEMBLY AND GLYCOSYLATION OF MAMMALIAN ACETYLCHOLINESTERASES

U. Brodbeck, Institute of Biochemistry and Molecular Biology, University of Bern, CH-3012 Bern, Switzerland

G_2-AChE from mammalian erythrocytes and a number of other sources consists of two disulfide linked catalytic subunits which are membrane bound through a glycosylphosphatidylinositol (GPI) moiety covalently attached to the C-terminus of each subunit. Mammalian acetylcholinesterase (AChE) exists in brain as tetrameric globular enzyme (G_4 form) of which approximately 80% are amphiphilic, membrane bound through a structural subunit linked by disulfide bridges to one pair of the four catalytic subunits. G_2-and G_4-AChE thus not only differ in their subunit assembly but also in the way they are membrane anchored. In order to obtain information about the forces holding the subunits together, monomerization studies were carried out with both forms of AChE. While G_2-AChE is readily monomerized by reduction and alkylation, this treatment alone did not result in monomerization of brain AChE. After reduction and alkylation, catalytically inactive monomers were obtained in the presence of SDS suggesting the presence of hydrophobic inter-subunits contact areas. On the other hand, catalytically active monomers could be obtained from G_4-AChE by selective tryptic cleavage near the C-terminus. This treatment not only released the hydrophobic TID-label from the catalytic subunit but also the TID-labelled 20 kDa anchor. Our results confirm the notion that the cystein residue situated nearest to the C-terminal, is involved in inter-subunit disulfide bonding as well as in the attachment of AChE to its membrane anchor. Furthermore, the C-terminal region in the primary structure of G_4-AChE provides the area of hydrophobic contact between the different subunits and the membrane anchor. The C-terminal region of G_2-AChE is some 38 amino acids shorter. G_2-AChE thus lacks the hydrophobic inter-subunit contact area and consequently, hydrophobic bonding does not contribute significantly to the oligomer assembly in G_2-AChE.

G_2- and G_4-AChE further differ in the extent of N-glycosylation. G_2-AChE from human and bovine red cell membranes is more heavily N-glycosylated than human and bovine G_4-form. Bovine AChE (both G_2- and G_4-forms) are more heavily N-glycosylated than the corresponding human forms. N-Acetylgalactosamine could neither be detected in bovine brain nor in bovine erythrocyte AChE indicating that both enzyme forms contain no O-linked carbohydrates. Novel monoclonal antibodies against AChE were raised wich recognized G_4-AChE from brain but not G_2-AChE from red cells. A subset of them specifically reacted with N-linked carbohydrates of G_4-AChE but not with the G_2-form indicating that G_2- and G_4-AChE undergo different post-synthetic modifications leading to different subsets of N-linked carbohydrates (For details on mAbs see abstracts by J. Liao *et al.* and B. Nørgaard-Pedersen *et al.*).

STRUCTURAL AND FUNCTIONAL STUDIES ON THE GPI-ANCHORED FORM OF ACETYLCHOLINESTERASE

Israel Silman, Department of Neurobiology, The Weizmann Institute of Science, Rehovot 76100, Israel

In electric organ tissue of the electric fish, *Torpedo*, a substantial amount of the acetylcholinesterase (AChE) is a membrane-bound hydrophobic dimer. Its membrane-anchoring domain is provided by the diacylglycerol moiety of a single phosphatidylinositol (PI) residue which is covalently attached, via an intervening oligoglycan, to the COOH-terminus of each of the two catalytic subunit polypeptides. This form of AChE can be selectively solubilized from electric organ tissue of *Torpedo californica* by a PI-specific phospholipase C (PIPLC) of bacterial origin, and subsequently purified by affinity chromatography. The selective solubilization by PIPLC provides a novel approach to the immunocytochemical localization of this form of the enzyme. The AChE so purified from electric organ tissue of *Torpedo californica* provides a convenient preparation for structural and functional studies, viz. chemical modification and identification of residues involved in catalytic activity, studies on folding and unfolding of the native protein and chemical characterization of the membrane-anchoring domain. Results in these various areas will be presented and discussed.

BINDING OF A_{12} AChE TO C_2 MUSCLE CELLS AND TO CHO MUTANTS DEFECTIVE IN GLYCOSAMINOGLYCAN SYNTHESIS

[1]Inestrosa, N.C. [2]Gordon, H., [3]Esko, J.D. and [4]Hall, Z.W.

[1]Molecular Neurobiology Unit, Catholic Univ. of Chile, Santiago, CHILE, [2]Univ. Arizona Tucson, AZ 85721, [3]Univ. Alabama, Birmingham, AL 35294 and [4]UCSF, CA 94143, USA.

It has been postulated that the asymmetric acetylcholinesterase (A_{12} AChE) binds to cell surface proteoglycans (PGs). In the present study, we have directly evaluated the binding of purified A_{12} AChE to cultured wild-type and mutant C_2 muscle cells, as well as Chinese hamster ovary (CHO) cell mutants defective in glycosaminoglycan synthesis.

A time-dependent, saturable and specific binding of A_{12} AChE to C_2 myotubes was demonstrated. The interaction required an intact collagenous tail. A single class of binding sites was involved and a dissociation constant of 0.65×10^{-7} M was calculated. The binding was partially blocked by heparan sulfate (HS), and preincubation of C_2 cells with heparinase led to a 50% reduction in AChE binding. In mutant C_2 myotubes deficient in cell surface PGs (chondroitin sulfate (CS) and HS) the binding of A_{12} AChE was reduced to 66% of wild-type cells.

In the CHO parent line K1, the binding of AChE was partially blocked by preincubation with heparinase (60%). The 677 mutant (excess of CS and deficient in HS) caused a 2 fold decrease in binding, and the 606 mutant (defective in N-sulfotransferase activity) resulted in a 4-5 fold decrease in binding.

The results suggest that both heparan and also CS-dermatan PGs mediate the anchorage of A_{12} AChE to the cell surface.

Synthesis of acetylcholinesterase molecular forms in transfected cells

Suzanne Bon, Françoise Coussen, Nathalie Duval, Eric Krejci
Claire Legay and Jean Massoulié

Laboratoire de Neurobiologie, CNRS UA 295, Ecole Normale Supérieure, 46 rue d'Ulm, 75005 Paris, France

COS cells were transfected with CDM8 vectors encoding the T and H subunits of *Torpedo* acetylcholinesterase (AChE), corresponding respectively to collagen-tailed and glycolipid-anchored forms, and with a truncated T subunit (TΔ), which retained only 4 aminoacids of the T C-terminal peptide. All subunits yielded active AChE, but only when transfected cells were transferred from 37 to 27°C. The truncated TΔ subunit produced only non amphiphilic monomers. The H subunits produced glycolipid-anchored dimers, as *in vivo*. The T subunit produced non amphiphilic tetramers (G_4^{na}), and also amphiphilic monomers and dimers (G_2^a and G_2^a) of type II. Similarly, COS cells transfected with the rat AChE T subunit produced mostly G_4^{na} and G_1^a (type II). Amphiphilic forms of type II exist in *Torpedo* tissues, other than electric organs, and are abundant in muscles and nervous tissue of higher vertebrates. They are not glycolipid-anchored, but the nature of their hydrophobic domain is not yet known.

Asymmetric forms were produced in COS cells, when they expressed the *Torpedo* collagen-tail subunit (Q) together with T subunits of either *Torpedo* or rat AChE, but not with the H subunit or with the truncated TΔ subunit. This shows that : 1) the T peptide is essential for assembly of A forms ; 2) the collagenic and T subunits can associate in non specialized cells, so that the restricted expression of A forms in differentiated muscle and nerve cells simply results from the synthesis of both subunits ; 3) the complementarity between the T peptide and the collagenic subunit is conserved in vertebrates.

Using an antiserum directed against the C-terminal non-collagenic region of the C subunit, we showed that this region can be removed by collagenase and is thus located at the distal end of the tail. We confirmed that the N-terminal domain of the C subunit is able to bind AChE T subunits, by constructing a chimeric protein in which this domain was linked to the H peptide of the *Torpedo* AChE H subunit : its co-expression with AChE T subunit yielded glycolipid-anchored tetramers. The structure of these artificially engineered molecules is similar to that of the hydrophobic-tailed G_4^a forms from mammalian brain, which consists of T subunits linked to an hydrophobic 20 kDa subunit.

Thus, the T and H subunits, appear to account for all the known forms of acetylcholinesterase.

NEMATODE ACETYLCHOLINESTERASES:

Several Genes and Molecular Forms of their Products.

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Vertebrate and *Drosophila* AChEs are encoded by a single gene. At variance, *Caenorhabditis elegans* possesses three genes coding for three AChEs that differ in their catalytic properties (Johnson and Russell, 1983; Johnson *et al.*, 1988). We have first characterized the products of the two major genes (ace 1 and ace 2) in *Steinernema carpocapsae* (a Rhabditidae nematode close to *C. elegans* but with a higher AChE activity).

The two types of AChE were distinguished by their different sensitivity to eserine. Ace 1 codes for an amphiphilic catalytic subunit of about 65 kDa (4S) that associates into a membrane-bound dimeric form (G2a for amphiphilic) sedimenting at 7S. A PI-PLC treatment converts the G2a form into a hydrophilic G2 form (G2h) indicating that this type of catalytic subunit possesses a glycolipidic domain that mediates the membrane attachment. Ace 2 codes for a protein of approximately 90 kDa that is found under a major amphiphilic 14S form. This molecular form likely associates, by disulfide bonds, a tetramer of hydrophilic catalytic subunits to one (or two?) non-catalytic (structural) hydrophobic component(s). Ace 2 product is also found as hydrophilic 7 and 12S forms that likely correspond to hydrophilic dimers and tetramers of subunits. A complete scheme of molecular forms of *Steinernema* AChEs is presented. It is remarkable that nematode AChEs present the two types of membrane association previously identified:

-in amphiphilic G2 forms of vertebrate and *Drosophila* AChEs (glycolipid-anchored dimers)

-in amphiphilic G4 forms of AChE in mammalian brains (hydrophobic structural component, see review in Massoulié and Toutant, 1988).

A parallel study of *C. elegans* AChEs molecular forms is also presented: they are similar to those in *Steinernema*. In *C. elegans* however we have demonstrated directly the relationships of each gene with the corresponding molecular forms by an analysis of AChE in the following mutants: ace1, ace2, ace3 and ace1/ace2.

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Three Dimensional Structure of Acetylcholinesterase

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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, for the first time, at atomic resolution, a protein binding pocket of the neurotransmitter ACh. We found that the active site consists of a catalytic triad (S_{200}-H_{440}-E_{327}) 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 W_{84}.

A variety of AChE inhibitors have been synthesized and characterized pharmacologically, due to the fact that symptomatic treatment of diseases, whose etiology involves depletion of ACh levels, can be achieved by controlled inhibition of AChE. Inhibition of the catalytic activity of AChE with anticholinesterase agents is thus of therapeutic importance in countering the effects of diseases such as glaucoma and myasthenia gravis, and the possible management of Alzheimer's disease. In order to study the interactions of AChE with these agents, in detail, we have soaked into crystals of AChE a series of different inhibitors, and recently determined the 3-D structure of AChE:edrophonium and AChE:tacrine. Edrophonium is a drug used in treatment of myasthenia gravis, it contains a quaternary ammonium group and acts at neuromuscular junctions. Tacrine is an AChE inhibitor lacking a quaternary ammonium group; hence it can penetrate the blood brain barrier and act on the central nervous system and is currently being evaluated as a drug for the management of Alzheimer's disease. The crystal structures of both of these complexes are in good agreement with our model building of the ACh bound in the active site of AChE and indicate the interactions of these two drugs with the enzyme.

This work was supported by the U.S. Army Medical Research and Development Command under Contract DAMD17-89-C9063, 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.

STRUCTURAL ROLE OF HIGHLY CONSERVED AMINO ACIDS IN ESTERASE/LIPASE FOLD FAMILY.

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The recently determined X-ray structures of *Torpedo californica* acetylcholinesterase and *Geotrichum candidum* lipase show a high degree of similarity in their three-dimensional fold extending throughout the whole length of their polypeptide chains. Three-dimensional superposition of their structures allowed us to improve the alignment of the family of nearly 30 homologous esterases and lipases. There are nearly 25 invariant residues in all the sequences and ~70 other that are highly conserved. Based on the two X-ray structures it is possible to find for many of these residues a clear structural basis for their preservation.

Session II:

Gene Organization and Expression

SV-40 TRANSFORMED CELL LINES, FOR EXAMPLE COS-1, BUT NOT PARENTAL UNTRANSFORMED CELL LINES, EXPRESS BUTYRYLCHOLINESTERASE (BCHE).

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Until now no cultured cell lines have been reported that have BChE activity. We have found that SV40 transformed cell lines including COS-1 and COS-7 monkey kidney cell lines, and WI38 VA13 and MRC-5 SV40 human lung embryonal cells have significant levels of BChE both in the cell lysate and secreted into the culture medium. In contrast, the nontransformed parental cell lines CV-1, WI38, and MRC-5 have little or no BChE. Polyacrylamide gradient gel electrophoresis and Ferguson plot analysis showed that most of the activity secreted by COS-1 formed polydisperse aggregates of pI 6.0. In addition COS-1 secreted a monomer of 85 to 90 kD. The enzyme produced by the SV40 transformed cells was identified as BChE on the basis of the following: 1) the enzyme hydrolyzed benzoylcholine and butryrylthiocholine, 2) activity was inhibited by diisopropylfluorophosphate, iso-OMPA and eserine, but not by BW284C51, 3) metabolic labeling with S35-methionine followed by immunoprecipitation yielded an intense band of 85 kD, the correct size for the subunit monomer of BChE, 4) polyA+mRNA from the SV40 transformed cells hybridized with a BCHE cDNA probe on Northern blots. The mRNA band size of 2.55 kb was the same as the most intense band in human liver. The nontransformed parental cell lines showed no BCHE mRNA. BChE is one of only 15 proteins whose level increases more than 3 fold following transformation by SV40 virus. Transfection of COS-1 cells with a plasmid containing human BCHE cDNA increased the activity of secreted BChE from 0.001 units/ml (5 ng/ml) to 0.002 units/ml (10 ng/ml). This suggests that the COS-1 system is not useful for studying transient expression of BChE mutants whose activity is low compared to wild-type BChE. Supported by US Army Medical Research Development Command DAMD17-91-Z-1003.

MOLECULAR ORGANIZATION OF RECOMBINANT HUMAN ACETYLCHOLINESTERASE IN HIGH-LEVEL EXPRESSION SYSTEMS.

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Comparative analysis of several engineered expression vectors and different transfected mammalian cells allowed establishment of cell lines secreting high levels of recombinant human acetylcholinesterase (the soluble globular species). Production levels as high as 5-25 pg enzyme per cell per 24 hours were obtained in lines derived from transfected 293 human embryonal cells.
rHuAChE is secreted from the recombinant lines in the oligomeric form (dimers and tetramers), displaying heterogeneity both in glycosylation level and in signal peptide processing. The heterogeneity in N-terminus processing is consistent with presence of two typical targets for signal-peptidase cleavage on the nascent polypeptide. Analysis of secretion kinetics indicates that dimerization of the rHuAChE subunits occurs early within the endoplasmic reticulum (ER). Dimerization is followed by transport through the Golgi apparatus, terminal glycosylation and secretion from the cells, thus implying that monomers are not exported from the ER and therefore, not secreted.
Substitution of the cysteine at position 580 by alanine (C580->A) resulted in impairment of interchain disulfide bridge formation and block of HuAChE oligomerization, yet allowed generation of an active mutant monomer that was secreted from the cells. The mutation did not affect the efficiencies of HuAChE synthesis and secretion. This was demonstrated not only in 293 cells but also in transfected human neuroblastoma (SK-N-SH) cells. Furthermore, the mutation did not lead to increased degradation of intracellular AChE or to change in transport rate. It appears therefore, that while AChE dimerization precedes the export of wild-type enzyme, oligomerization is not a prerequisite for secretion.
Site directed mutagenesis was also used to analyse the involvement of glycosylation in secretion of rHuAChE. N-glycosylation was found to be essential for efficient production; mutagenesis of each of the three putative N-glycosylation-site aspargine residues resulted in a significant decrease in AChE levels. Substitution of all three aspargines led to dramatic decrease in enzyme production. (Supported by US Army Medical Research Development Command Contract DAMD 17-89-C-9117).

REGULATION OF ACHE EXPRESSION IN SKELETAL MUSCLE

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Normal development of cholinergically-innervated skeletal muscle fibers requires that all necessary synaptic components be expressed, assembled, and localized to the appropriate regions of the plasma membrane. Skeletal muscle fibers are large multinucleated cells arising from fusion of mononucleated myoblasts. Because of their size they can exhibit regionalized differences in the expression of several muscle-specific genes including those encoding synaptic components. We have previously shown that skeletal muscle fibers are compartmentalized with respect to the expression and translation of AChE mRNAs, and that the polypeptide chains are assembled in the vicinity of the nucleus of origin. The appearance of individual AChE oligomeric forms is regulated, at least in part, by the activity state of the muscle and involves multiple controls at the transcriptional, translational, and post-translational levels. Using tissue-cultured mosaic quail-mouse skeletal muscle fibers and species-specific antibodies we now show that AChE oligomeric forms expressed within a nuclear compartment are localized to the plasma membrane overlying the nucleus of origin, thus giving rise to specialized cell surface nuclear domains. In vivo, AChE is highly concentrated at the neuromuscular junction. This specialized region of the plasma membrane overlies a characteristic accumulation of specialized fundamental myotube nuclei. Using a quantitative mRNA PCR technique to quantitate AChE transcript levels in single cells we demonstrate that innervated regions of individual muscle fibers exhibit a several-fold increase in the amount of AChE mRNA compared to adjacent nerve-free segments. Together these results suggest that regulation of AChE at the neuromuscular junction includes spatial as well as temporal factors involving both neural input and muscle activity. This research supported by grants from the NIH and MDA to RLR.

Drosophila acetylcholinesterase : analysis of structure and sensitivity to insecticides by *in vitro* mutagenesis and expression.

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In Insects, AChE is composed of a single molecular form which is a glycosylated dimer anchored to the membrane via a glycolipid. We identified the amino-acids involved in the glycosylation and in the dimerization. Monomeric and non-glycosylated enzymes are active but more sensitive to protease degradation. Deficiency in glypiation results in the sequestration of the protein inside the cell. The last maturation that we analyzed is the cleavage of the 70 kDa precursor into two polypeptides, 55 and 16 kDa. This cleavage occurs in several places inside a small hydrophilic peptide. It is related to the secretion and does not correspond to an activation of the protein.

Resistance to insecticides is quantitatively and qualitatively determined. Sensitivity to insecticides depends on the amount of AChE in the central nervous system : flies bearing an extracopy of the gene are more resistant than wild-type while rescued flies which express only low levels of AChE are more susceptible. In Insects, AChE is restricted to the CNS. We ectopically expressed a soluble AChE, secreted in the haemolymph. The gene was amplified by several steps of transposition / recombination. We obtained highly resistant strains showing that AChE present outside the CNS acts as a scavenger protein. Besides the dose effect of AChE inside or outside the CNS, resistance is also qualitatively determined. Sequences of resistant strains producing altered AChE revealed several mutations of amino-acids close to the active site. Each mutation is responsible for weak resistance however combinations of these mutations yielded high levels of resistance.

GENE STRUCTURE AND REGULATION OF EXPRESSION OF ACETYLCHOLINESTERASE

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A single gene exists for acetylcholinesterase with defined chromosomal locations in mouse and human, and expression of the molecule is precisely regulated in neurons, muscle and hematopoietic cells. In fact, the regulation of gene expression associated with development and synaptogenesis often shares common properties with receptors and channels which typically are encoded by polygenic systems. The expression of acetylcholinesterase appears to be controlled at three levels. Transcription is affected by the presence of alternative cap sites and alternative mRNA splicing in the 5' non-translated region. Alternative exons corresponding to the very carboxy terminus of the protein encode differences in sequence. The sequence differences yield monomeric enzyme species, a sulfhydryl-containing carboxy terminus allowing for disulfide linkages to structural subunits and a unique hydrophobic sequence allowing for attachment of a glycophospholipid. The 3'-untranslated region also contains alternative polyadenylation signals which affect mRNA stability. Studies in muscle cells show that enhanced mRNA stability is critical to the increased expression seen with muscle cell differentiation. RNase protection, primer extension, transfection and expression of minigene constructs will be used to show the various control points for AChE expression. Supported by grants from USPHS and MDA.

Session III:

Catalytic Mechanisms and Structure Function Relationship (part A)

BINDING SITES AND SUBSITES OF ACETYLCHOLINESTERASE FROM *TORPEDO* AND *COBRA*

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Acetylcholinesterase is being investigated as a model for proteins involved in acetylcholine-mediated signal transmission. Although the primary structures of, e.g., the acetylcholinesterase and the acetylcholine receptor exhibit no homologies, it is interesting to compare the acetylcholine binding sites of these otherwise very different proteins. We are investigating the ligand binding sites of both the esterase and the receptor by affinity labelling and protein chemical methods. We used the cholinium analog N,N-dimethyl-2-phenyl-aziridinium (DPA) for localizing both the active site and the peripheral "anionic" centers of the esterase from *Torpedo californica*. Protecting these sites against affinity labelling with site-specific drugs (propidium, edrophonium) we were able to differentiate sequences involved in forming the peripheral and active sites, respectively. The sequence KPQELIDVE (positions 270-278) was identified as a peripheral anionic subsite peptide; DLFR (positions 217-220) and SGSEMWNPN (positions 79-87) are active site peptides. The label was localized at the hydrophobic position W 84. The corresponding sequence in the cobra enzyme is GAGMWNPN. These findings are discussed in the context of the X-ray analysis by Sussman et al. (Science 253, 872-879, 1991). Furthermore, an FTIR-spectroscopic analysis of the acetylcholinesterase is presented. This is also discussed on the basis of the three-dimensional structure.

ACETYLCHOLINESTERASE FUNCTION - PROTEIN ENGINEERING STUDIES GUIDED BY AMINO ACID SEQUENCE CONSERVATION AND 3-D STRUCTURE.

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A system for recombinant human acetylcholinesterase (HuAChE) expression in a human cell line was employed for site directed mutation analysis. The mutant HuAChEs were evaluated by a combination of immunological quantitation assays and extensive kinetic studies which allowed to diffrentiate between activity-related and production/ folding-related effects. Evidence for the involvement of Ser203, His447 and Glu334 in the catalytic triad of HuAChE was provided by substitution of these amino acids by alanine residues. Mutations at these positions abolished enzymatic activity (less than 0.0003 of wild type), yet allowed proper production, folding and secretion. These studies demonstrate the involvement of glutamate in a hydrolase triad and supporting the X-ray crystal data on the T. californica AChE (Sussman et al., 1991, Science 253:872). Similar characteristics (loss of enzyme activity but effecient productivity) were associated with the Trp86->Ala mutant. This is consistent with the previously suggested involvement of this aromatic amino acid in binding the quarternary amine moiety of the substrate (Sussman et al., ibid.; Kreienkamp et al., 1991, PNAS 88:6647). Mutations at positions Glu84, Asp95, Asp333, Asp 349 and His447, all conserved among ChEs, did not result in detectable alteration of AChE, though polypeptide productivity of the Asp95->Asn and His447->Ala mutants was considerably lower. In contrast, complete absence of secreted HuAChE polypeptide was observed when Asp175 or Asp404 were substituted by Asn. These two residues are conserved in the entire cholinesterase/ thyroglobulin family and appear to play a role in generating and/or maintaining the folded state of the polypeptide. Substitution of other anionic and aromatic amino acids lining the active-site gorge led to pleotropic effects on the reactivity of the enzyme with various ligands. These studies reveal non-catalytic binding site(s) which are able to transduce signals to distantly located amino acids in the active-site gorge. (Supported by US Army Medical Research Development Command Contract DAMD 17-89-C-9117).

Molecular Dissection of Functional Domains in Human Cholinesterases Expressed in microinjected Xenopus oocytes

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Xenopus oocytes microinjection was employed in search for key amino acid residues participating in interactions between cholinesterases(CHEs) and their ligands and inhibitors. For this purpose 19 normal, site-directed and natural mutants of human acetyl- and butyrylcholinesterase (ACHE, BCHE) and combinations thereof were expressed from transcription constructs. Inhibition profiles, Km and Ki values were compared for the resultant recombinant enzymes with selected choline esters, procaine derivatives, toxic glycoalkaloids, organophosphorous (OP) agents and carbamates.

The biochemical properties of recombinant normal ACHE and BCHE were found to be similar to their counterpart natural serum BCHE and erythrocyte membrane ACHE. Mutations in BCHE were classified into 4 principal subgroups, according to functions disrupted by them:
I. Interference with catalysis- 5 substitutions of Ser198 (into Cys, Asp, Gln, His or Thr) completely abolished catalysis. The double substitution of the water binding domain Glu441->Gly and Glu443-> Gln reduced hydrolysis by > 95%, demonstrating that both the catalytic triad serine and binding of a key water molecule are essential for substrate hydrolysis. II. Disturbance of ligand entrance into the active site groove- 7 different mutants carrying the Asp 70 -> Gly natural substitution (alone or with Tyr114-> His, Phe561 -> Tyr, Ser 425 -> Pro, and 3 other triple combinations thereof) displayed essentially normal catalysis yet interfered with the entrance of several choline esters, toxic glycoalkaloids, OP agents and carbamates. III. Inhibition of ligand binding- 2 other substitutions conferred no changes in either catalysis or ligand entrance, but interfered with the binding of OP (Tyr440->Asp) or of procaine derivatives (Ser425 -> Pro and Asp70 -> Gly) as apparent from their 3- dimensional location and >10- fold increased IC50 values. The Tyr440 -> Asp mutation exemplifies the complexity of binding sites in that one may alter an OP binding site without affecting general substrate catalysis. IV. Ameliorating substitutions- 2 substitutions, apparently ineffective on their own, restored catalytic activity over the otherwise deficient (25% activity of normal) Asp70 -> Gly mutant (correcting for up to >95% for Tyr114->His and up to 75% for Phe561-> Tyr), suggesting that various regions influence the ability of ligands to reach the active site gorge.

Our findings imply that the functionally effective residues in CHEs are mostly localized in an area of about 12 Angstroms surrounding the catalytic site triad and the rim of the ChE gorge and include highly conserved charged and hydrophobic residues involved in ligand attraction and binding, in addition to the catalytic process itself.

ACETYLCHOLINE BINDING TO A PERIPHERAL ANIONIC SITE ON ACETYLCHOLINESTERASE IS AN IMPORTANT FEATURE OF THE CATALYTIC PATHWAY

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The binding of certain cationic ligands to peripheral anionic sites on acetylcholinesterase (AChE) has long been known to result in inhibition of AChE catalytic activity. The pioneering studies of Taylor and Lappi (Biochemistry 14, 1989-1997, 1975) introduced propidium as a prototypic peripheral site ligand and showed that propidium binding was virtually unaffected by the binding of edrophonium to the AChE active site. In contrast, the binding of both propidium and edrophonium to AChE is blocked by *bis*-quaternary cations like decamethonium that appear to block both sites by bridging between them. A new breakthrough recently has been provided by determination of the three-dimensional structure of AChE by X-ray crystallography (Sussman *et al.*, Science 253, 872-879, 1991), which shows the active site to be a deep and narrow gorge lined with aromatic residues. The structure suggests that cationic ligands which bind to peripheral anionic sites near the mouth of the gorge may partially block entrance to the active site. This suggestion is supported by steady-state inhibition patterns obtained during AChE-catalyzed hydrolysis of acetylthiocholine and phenyl acetate substrates. Hydrolysis of both substrates is completely blocked by edrophonium binding to the active site. In contrast, with propidium bound to the peripheral site, hydrolysis of the neutral substrate phenyl acetate occurs at about 10% of the rate obtained with the free enzyme. This trend is even more pronounced with a new peripheral site affinity reagent that we introduce here, chloro(2,2':6',2"-terpyridine)platinum(II) chloride. Formation of an initial AChE complex by this reagent is blocked by propidium and decamethonium, but not by edrophonium. The initial complex forms a covalent conjugate with an AChE histidine residue with a rate constant of about 0.1 min^{-1}. In both the initial complex and the covalent conjugate, phenyl acetate hydrolysis can proceed at about 50% of the rate obtained with the free enzyme. However, the acetylcholine hydrolysis rate is reduced to less that 5% of that obtained with the free enzyme. We conclude from this and other data that acetylcholine binding to an AChE peripheral site is an obligatory step on the catalytic pathway for acetylcholine but that neutral substrates bypass this site in gaining access to the AChE active site.

STRUCTURAL ANALYSIS OF ACETYLCHOLINESTERASE AMMONIUM BINDING SITES

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Two para-dialkylaminobenzene diazonium salts, the dimethylamino (A) and dibutylamino (B) derivatives, were presented as structural probes for acetylcholinesterase.

Through the use of a specific photolabelling method [1], *Torpedo* acetylcholinesterase was inactivated, up to 70 %, by both radioactive probes. [^{3}H]-A was shown to exclusively label the catalytic active site of this enzyme while [^{3}H]-B, in the presence of edrophonium, acted as a selective probe of the peripheral site [2]. Labelled acetylcholinesterase was submitted to proteolysis and the resulting radioactive peptides were purified by HPLC and sequenced.

Phenylalanine 330 was identified as being part of the catalytic site of *Torpedo* acetylcholinesterase, in agreement with our previous results on *Electrophorus* enzyme[3]. Others amino-acid residues belonging to ammonium binding sites will be presented.

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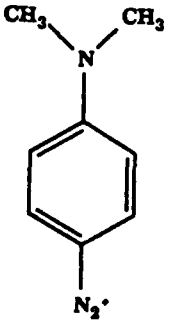

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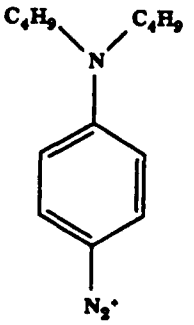

B

ELECTROSTATIC CONTROL OF ACETYLCHOLINESTERASE TOPOGRAPHY: ROLE OF THE PERIPHERAL ANIONIC SITE, by Harvey Alan Berman, Department of Biochemical Pharmacology, SUNY at Buffalo, Buffalo, New York 14260.

This paper addresses two salient features of AchE that have come under recent discussion concerning interaction between the peripheral anionic site and the active center. The first feature notes that substrate hydrolysis at the active center occurs in a manner that is relatively independent of ionic composition of the surrounding medium; such behaviour stands in contrast to the marked ionic strength dependence of ligand occupation of the peripheral anionic site. Second, the capacity of peripheral site ligands to alter substrate hydrolysis is critically dependent on the electrostatic nature of the substrate. That is, peripheral site ligands cause *linear* inhibition of hydrolysis of cationic substrates, *nonlinear* inhibition of uncharged substrates, and antagonize inhibition by *uncharged* methylphosphonates only incompletely. On this basis, we have concluded that the peripheral anionic site interacts with the active center principally through an electrostatic mechanism. This conclusion prompts the proposal that, by virtue of the electrostatic nature of the interaction, the peripheral anionic site serves as an *electrostatic sensor* of the immediate ionic environment, providing one means for maintaining the catalytic efficiency of the enzyme. Such a proposal requires that upon transfer of AchE to media of differing ionic composition, the enzyme conformation undergoes distinct alterations while substrate hydrolysis remains relatively constant. This paper examines this requirement by comparing the influence of ionic composition of the bulk medium on rates of substrate hydrolysis and conformation of AchE. Enzyme conformation is assessed through utilization of (NBD)aminoalkyl methylphosphonofluoridates. These fluorescent probes are of advantage in that when bound to the surface of a protein their fluorescence is governed by static quenching mechanisms. As a consequence, small changes in orientation of the probe with respect to the enzyme surface are predicted to result in measurable changes in fluorescence intensity, providing a sensitive indicator of ion-induced changes in protein conformation.

Session III:

Catalytic Mechanisms and Structure Function Relationship (part B)

Relationships between Accessibility of Phosphorus Atom as Estimated by Molecular Mechanics Calculations and Antiacetylcholinesterase Activity of Organophosphorus Inhibitors

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Phosphorylation of serine hydroxyl in acetylcholinesterase (AChE) active center is the important step of action of irreversible organophosphorus inhibitors (IOPI). The phosphorus atom of IOPI may be attacked by the serine hydroxyl from the sides opposite to the breaking bond and phosphoryl oxygen, the first way being more probable [1]. Therefore, the efficiency of the reaction may depend on the steric accessibility of phosphorus atom for the attacking agent. To test this suggestion, we have calculated all minimum-energy conformations of 12 IOPI with nitrophenyl and thiomercaptoethyl leaving groups and varied structure of phosphoryl group. Conformational energy was calculated as in work [2]. Steric accessibility of phosphorus atom in each conformer was calculated according to [3].

It was found that IOPI have conformers with the phosphorus atom accessible from the side opposite to the breaking bond, while their non-active analogs with cyclic phosphoryl group have no such conformers. The corresponding conformers were considered to be productive. A correlation between the population of productive conformers and bimolecular inhibition constant was revealed, the correlation coefficient being equal to 0.94. These data together with the results of our earlier investigation of conformation-activity relationships of AChE substrates [2] indicate that interaction of AChE with both IOPI and substrates is effective if these ligands approach the serine hydroxyl of the enzyme active center with the electrophilic atom located on plane or convex rather than concave face of the molecules.

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ELUCIDATION OF THE STRUCTURE OF ORGANOPHOSPHORYL CONJUGATES OF BUTYRYLCHOLINESTERASE BY 31P-NMR SPECTROSCOPY.

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To characterize the substituents attached to the P atom of an aged organophosphoryl(OP)-ChE conjugate, butyryl-cholinesterase (BChE) purified from horse serum was inhibited by DFP, allowed to undergo aging and 31P-nmr chemical shifts were determined at 18-23C (pH 6-10). In contrast to OP conjugates of chymotrypsin (Cht), the native form of DFP-inhibited and aged BChE produced a broad , low-intensity 31P-nmr signal. Unfolding in 6M guanidine gave rise to a sharp signal (10-15 Hz) with a chemical shift similar to that observed for DFP-inhibited and aged Cht in 6M guanidine. These observations are consistent with an assumed location of the active site serine of BChE near the bottom of a deep and narrow gorge as has been shown for AChE from Torpedo (Sussman et.al., 1991, Science, 253, 872-879). Transfer of DFP-inhibited and aged BChE to 0.1N NaOH resulted in a relatively rapid release of a phosphate ester which was identified as monoisopropylphosphoric acid using a 31P-nmr spectroscopy . In the case of soman-inhibited BChE, methylphosphonic acid was released following an incubation in 0.1N NaOH. Thus, it is proposed that **(isoPrO)P(O)(O^-)-** and **CH_3P(O)(O^-)-BChE** constitute the structures of aged OP conjugates of BChE which were obtained by use of DFP and soman, respectively. To our best knowledge this is the first direct evidence for the presence of a **P-O^-** bond in aged OP conjugates of ChE. Modeling of binding of **(isoPrO)P(O)(O^-)** and the corresponding reactivatable (non-aged) residue, **$(C_2H_5O)_2$P(O)**, to Ser200 of Torpedo AChE suggests, that the detachment of the phosphoryl-bound moiety, if occurs at all, could proceed via an in-line nucleophilic displacement both for the aged and the non-aged OP conjugates, whereas in the case of Cht, similar analysis implies an adjacent displacement for both derivatives . The latter appear to require a pseudorotation process which may not occur readily. Results may help to explain (a) the unusual resistance of aged OP conjugate of ChEs and Cht to reactivation and (b) differences between the stability of the non-aged forms of OP-ChEs and OP-Cht to oxime reactivators.

CRYPTIC CATALYSIS AND CHOLINESTERASE FUNCTION

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Acetylcholinesterase (AChE) possesses the tremendous catalytic power of an evolutionarily perfect enzyme when operating on the physiological substrate acetylcholine (ACh) or the surrogate substrate acetylthiocholine (ATCh). Unfortunately, this perfection conceals the chemically interesting internal reaction dynamics of the AChE catalytic cycle. However, alternate substrates of varying reactivity can be employed to expose internal transition states as rate determining. Consequently, we have investigated the AChE-catalyzed hydrolyses of ATCh, propanoylthiocholine (PrTCh), and butanoylthiocholine (BuTCh). The relative acylation rate constants (i.e. k_{cat}/K_m values) decrease in the order 1:0.03:0.001 for ATCh:PrTCh:BuTCh. The mechanisms of the AChE-catalyzed turnovers of these substrates were investigated by measuring pH-rate profiles and solvent isotope effects. These investigations yield the following general conclusions: 1) the active site catalytic triad S200-H440-D327 does not operate as a charge-relay catalyst, as long proposed for serine proteases. 2) E199 likely functions as the anionic locus of the active site that, along with W84, binds the quaternary amino function of choline esters. 3) As the reactivity of the substrate decreases, the AChE mechanism shifts from nucleophilic attack by S200, assisted by general acid-base catalysis by H440, to general base catalysis by E199 of attack of water on the substrate. Therefore, AChE displays a heretofore unexpected mechanistic diversity that is clearly revealed by variation of substrate reactivity.

ACETYLCHOLINESTERASE INHIBITION BY NOVEL CARBAMATES: A KINETIC AND NUCLEAR MAGNETIC RESONANCE STUDY

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The aim of this study was to measure the kinetic parameters of inhibition of human erythrocyte acetylcholinesterase (AChE) by novel mono- and disubstituted m-(N,N-dimethylaminoethyl) phenyl carbamates. Enzyme activity was measured at 37°C by the spectroscopic method of Ellman et al. We also investigated a possible relationship between the extent of doublebond character about the C-N bond of the amide, as measured by dynamic NMR spectrometry, and the affinity of the carbamate to AChE. In both mono- and disubstituted derivatives, the change from methyl to ethyl resulted in a 100-fold decrease in affinity, which was restored in mono substituted analogs, as the size increased from propyl to hexyl. Replacement of the hydrogen on the amide N by an alkyl group reduced affinity to AChE. In disubstituted derivatives, a close correlation was found between enzyme affinity and the degree of restriction of rotation about the C-N bond. The latter enhances the nucleophilic character of the C-atom of the carbonyl and favors the association between the carbamate and the enzyme. Additional substituents, or branching of the alkane chain interfered with the fit of the inhibitor to the site. However, in contrast to the reduction in affinity to the enzyme, such changes in structure increased the durability of the drug-enzyme complex, by slowing the rate of hydrolysis. A close correlation was also found between these parameters of *in vitro* kinetics and the potency and duration of brain AChE inhibition *in vivo*.

Session IV:

Physiological and Developmental Functions

REGULATION OF ACETYLCHOLINESTERASE IN FAST AND SLOW SKELETAL MUSCLES

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The patterns of AChE molecular forms in slow soleus (SOL) and fast extensor digitorum longus (EDL) muscles of the rat differ significantly. Major differences in AChE regulation are present especially in the extrajunctional regions: the asymmetric AChE forms are absent in the EDL because their production was completely repressed soon after birth, but they persist in the SOL. Differences are still present at least six weeks after muscle denervation. Therefore, they are not directly due to different neural stimulation patterns in both muscles. Dramatic loss of G1 AChE form and temporary increase of G4 form observed early after denervation in the EDL but not in the SOL are due to muscle inactivity since identical changes occur in muscles paralysed by botulinus toxin.

We presume that some intrinsic properties of fast and slow muscles, conveyed by their myogenic stem cells, differ in respect to AChE regulation and are, therefore, at least in part responsible for the above mentioned differences between these muscles. This hypothesis was tested during the process of muscle regeneration in disused and cross-transplanted SOL and EDL muscles of the rat, regenerating after ischemic-toxic injury.

Leg immobilization causes drastic reduction of the neural stimulation of the SOL and the pattern changes from tonic to phasic. However, immobilization did not convert the pattern of AChE molecular forms in the regenerating SOL muscle into the EDL type. In 6-week old cross-transplanted regenerates, the muscle of origin and not the reinnervating motor nerve largely determined the pattern of AChE molecular forms. In more mature 13-week old cross-transplanted regenerates, the AChE pattern was modified to become quite similar but not identical to the pattern of the muscle in place of which it had grown. The results corroborate the assumption that myogenic cells (satellite cells) in fast and slow muscles differ. Their intrinsic properties predominantly affect the regulation of AChE molecular forms during early period of muscle regeneration. Later on, however, the pattern of neural stimulation decisively modifies the regulation of AChE in muscle regenerates. It seems that both intrinsic muscle properties and neural influences are important for AChE regulation in muscles.

SYNAPTIC ACETYLCHOLINESTERASE AT INTACT AND DAMAGED NEUROMUSCULAR JUNCTIONS.

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In skeletal muscle acetylcholinesterase (AChE) is concentrated at the neuromuscular junctions, where it is found in the synaptic cleft. A major fraction of the enzyme at the synapse is associated with the synaptic portion of the myofiber's basal lamina. Muscle contains several molecular forms of AChE. The amount of AChE, its distribution and its molecular forms in muscle in general, and at the junctions in particular, are highly dependent on the integrity of synaptic structure and function. We used the well characterized neuromuscular junction in frog muscle to study the distribution and forms of the synaptic AChE in vivo, and its regulation following damage to muscle or motor nerve.

Quantitative EM-autoradiography was employed to determine the density of AChE active sites at the junctions. We demonstrate that the density of AChE sites at the frog neuromuscular junction was much lower than at the mammalian enplate. These findings were correlated with the specific activity of frog AChE and implications with respect to synaptic function will be discussed. Further biochemical analysis was carried out to determine the molecular forms of AChE that are concentrated at the junctions and are associated with synaptic basal lamina. A major contribution of globular AChE was observed.

Several surgical procedures were used to cause specific damage to muscle, and to selectively remove in vivo each of the cellular components of the neuromuscular junctions. These preparations were used to examine the individual roles of nerve, muscle and basal lamina in producing and directing the accumulation of AChE at synaptic sites. The molecular forms of AChE associated and maintained at synaptic basal lamina following damage and regeneration of the neuromuscular synapse were determined. The results demonstrate direct contribution of nerve terminals to synaptic AChE, and also a neuronal influence on synaptic AChE from muscle origin.

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TOWARDS A FUNCTIONAL ANALYSIS OF CHOLINESTERASES IN NEUROGENESIS: HISTOLOGICAL, MOLECULAR, AND REGULATORY FEATURES OF BCHE FROM CHICKEN BRAIN.

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Long before synapses are formed in the neural tube of vertebrates, acetylcholinesterase (AChE) is expressed in young postmitotic neuroblasts that are about to extend the first long tracts. AChE histochemistry can thus be used to map primary steps of brain differentiation. Preceding AChE in avian brains, butyrylcholinesterase (BChE) spatially foreshadows the course of their axons; e.g. during migration of neural crest cells in the head, AChE is highest in migrating cells, whereas BChE is strongly expressed in early Schwann cells and in ectodermal placodes. Biochemically, we have isolated the minute amounts of BChE from chicken brain. Whereas the BChE core proteins both from brain and from serum consist of 59 kDa, their glycosylation patterns are very different. A significant fraction of the brain BChE is membrane-bound. It is releasable by proteases, but not by phosphoinositol-specific phospholipase C (PIPLC). Both molecules from brain and serum bear the HNK-1 epitope. This correlates with our histochemical observation that BChE and HNK-1 are coexpressed, possibly indicating that BChE is linked with cell adhesion phenomena. Reaggregation experiments of embryonic retinal cells suggest histogenetic roles of cholinesterases, but also allow us to study the interdependence of their expression. The relevance of these findings for neuronal development and for cholinesterase functioning will be discussed.

A NON-CHOLINERGIC FUNCTION OF ACETYLCHOLINESTERASE IN THE BRAIN AND ITS RELATION TO THE GENERATION OF MOVEMENT

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In the substantia nigra, acetylcholinesterase (AChE) is present in large amounts and may have a novel non-cholinergic function:(1) there are disproportionately low levels of choline acetyltransferase, (2) a soluble form of AChE is secreted specifically from dopaminergic nigrostriatal neurons, (3) this secretion is not modified by either cholinergic agonists or antagonists. Unlike classic synaptic transmission, AChE is secreted in a fashion unrelated to somatic discharge, primarily from the distal dendrites of nigrostriatal neurons. Rather, secretion of the protein is evoked by selective local afferents, activation of which probably occurs during multimodal sensory stimulation, including proprioception: indeed movement enhances secretion of nigral AChE.

Once released, AChE has an action on dopaminergic nigral neurons independent of hydrolysis of acetylcholine: (1) butyrylcholinesterase is inefficacious, (2) the action persists during irreversible inhibition of the catalytic site, (3) potency of the effect is related much more to purity of the AChE preparation than to activity towards its normal substrate. This non-cholinergic action of AChE is manifest as a selective opening of a certain potassium channel linked to neuronal metabolism. The ensuing hyperpolarization deinactivates a dendritic calcium conductance which increases cell activity and could be triggered by incoming synaptic inputs: hence secreted AChE effectively enhances the sensitivity of the dopaminergic neuron to incoming synaptic inputs. This increased activation of the nigrostriatal pathway leads to subsequently enhanced movement. Dendritic secretion of AChE in the substantia nigra thus reflects activation of afferent sensory inputs and subsequently acts to enhance the response of dopaminergic neurons to such inputs. In this way, the secretion of AChE in a non-cholinergic capacity could be an important factor in sensory-motor integration, in a brain area critical for the generation of movement.

Session V:

Clinical Implications

EXPERIMENTAL ACETYLCHOLINESTERASE AUTOIMMUNITY

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Rats injected systemically with monoclonal antibodies to acetylcholinesterase (AChE) experience selective damage to specific subsets of cholinergic neurons (Brimijoin and Lennon, PNAS 87: 9630, 1990). In adults, experimental AChE autoimmunity (EAA) rapidly destroys preganglionic sympathetic terminals but spares postganglionic neurons, motor and parasympathetic neurons, and other AChE-rich cells. Severe dysautonomia ensues, with hypotension, bradycardia, ptosis and miosis. Stress-induced release of catecholamines is depressed, but postganglionic adrenergic neurons still respond to direct electrical and chemical stimulation. The immunologic mechanism of EAA is a complement-mediated lysis of neural membranes. Within 12 hr of treatment, deposition of antibody and complement (component C3) at synaptic sites in sympathetic ganglia is demonstrated by immunocytochemistry. Over the next few days there follows: 1) removal of AChE from ganglionic neuropil; 2) near-total loss of ganglionic choline acetyltransferase (ChAT); and 3) degeneration of ganglionic synapses. The structural, functional, and biochemical reactions are blocked when the complement-cascade is inactivated by cobra venom factor. Otherwise, immunologic lesions of preganglionic sympathetic neurons appear within hours and persist for weeks after one injection of AChE-antibodies. In superior cervical ganglia there is little recovery during the life of the animal. Stellate ganglia show partial recovery, but biochemical and functional deficits remain indefinitely. These long lasting abnormalities reflect progressive death of up to 70% of the preganglionic sympathetic perikarya in the upper thoracic, intermediolateral spinal cord. The mechanism of delayed neuronal death has not been elucidated, but it does not involve direct, antibody-attack on nerve cell bodies, which are protected by the blood-CNS barrier in adult animals.

EAA has quite different characteristics in newborns. The dysautonomia is much milder than in adults. However, when injected i.p. on the first postnatal day, AChE-antibodies gain access to the brain and combine selectively with externally anchored oligomeric forms of AChE. Central access depends critically on timing. If antibodies are not injected until postnatal day 6, they do not complex with brain AChE because the blood-brain barrier has matured. In rat pups injected 24 hr after birth, brain AChE and ChAT activities are depressed for up to 2 weeks. Furthermore, for at least 1 week, AChE-positive fibers cannot be visualized histochemically in the cortex or in basal ganglia. This model shows promise for studies of the role of AChE in neuronal development. (Supported by grant NS29646)

INFLUENCE OF DIFFERENT ACETYLCHOLINESTERASE INHIBITORS ON MOLECULAR FORMS G1 AND G4 ISOLATED FROM ALZHEIMER'S DISEASE AND CONTROL BRAINS.

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In human brain, different molecular forms (G1 and G4) of acetylcholinesterase (AChE) exist. During aging and more dramatically in Alzheimer's disease (AD) an increase of the enzyme activity ratio G1/G4 is well documented. Several AChE-inhibitors are currently under clinical evaluation as a therapeutic treatment in this disease. We examined the potencies of tacrine (THA), physostigmine, heptylphysostigmine and the novel brain region selective inhibitor ENA 713 with respect to inhibition of the G1 and the G4 enzymes isolated from AD and control brains. The preferential inhibition of the G1 activity by ENA 713 and heptylphysostigmine is in contrast to the unspecific action of THA and physostigmine. Possible clinical implications will be discussed.

Clinical implications of cholinesterase aberrations in syndromes of hemopoietic cell division

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Various aberrations in cholinesterases (CHEs) were found to be correlated with impaired cell division control in hemopoiesis. Amplification of both the acetyl- and butyrylcholinesterase genes (ACHE;BCHE) was shown to occur in parallel to several oncogene amplifications (c-raf, c-fes/fps and c-myc) in 3 patients with acute myeloblastic leukemia(AML), and in one patient with the preleukemic blood cell proliferation syndrome Polycythemia vera. In another patient, with severe platelet reduction (thrombocytopenia) due to the autoimmune disease Lupus erythematosus, the ACHE and BCHE genes amplified together with the c-raf, v-sis, and c-fes/fps oncogenes, altogether suggesting a correlation between CHE gene amplifications and abnormal control of hemopoietic DNA replication.

Both the ACHE and the BCHE proteins include the S/T-P-X-Z peptide motif, which makes them potential substrates for phosphorylation by cdc_2 kinases, controllers of the cell cycle. To investigate this putative linkage in hemopoietic development, antisense phosphorothioate oligodeoxynucleotides (AS-oligos) were designed to interrupt the expression of BCHE as well as of two human cdc_2 kinases expressed in bone marrow, the yeast homolog 2Hs and the larger CHED protein, recently cloned in our research group. The efficient uptake of such AS-oligos into cultured bone marrow cells, their ability to interact with their target mRNAs and induce their destruction and their stability in the cells were directly examined by RNA-PCR amplification. Thus, AS-CHED significantly reduced the ratio between CHED mRNA and Actin mRNA within 1hr of its administration to cultures, an effect which persisted for 4 days. Therefore, the biological outcome of AS-oligos interference with CHED, BCHE and 2Hs production was examined. AS-2Hs blocked bone marrow cell proliferation in general without altering the cell type composition in surviving colonies. In contrast, both AS-BCHE and AS-CHED reduced colony counts and selectively inhibited megakaryocyte development but did not prevent other hemopoietic pathways, evidenced by increasing numbers of macrophages.

These findings demonstrate the efficacy of AS-oligos as a research tool to study the biological role(s) of CHEs and reinforce the connection between CHEs and cell division control in hemopoiesis. The aberrations observed in the above patients could hence be causally related with defects in the ACHE and BCHE genes and/or their transcription products, particularly in cases of platelet abnormalities.

ACETYLCHOLINESTERASE;
A PRETREATMENT DRUG FOR ORGANOPHOSPHATE TOXICITY

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We have pursued a novel, extremely effective approach for protection against organophosphate (OP) toxicity. This approach reverses the target for treatment. The multiple drug treatment presently in use focuses on OP as anti-ChE where as the present approach focuses on ChEs as anti-OP. Accordingly, ChEs and other enzymes have been used successfuly as scavengers to remove OP before they reach their physiological targets. The biochemical approach underlying prophylaxis by exogenous esterases such as ChEs is established and has been tested in small animals. We demonstrated that pretreatment of rhesus monkeys with fetal bovine serum AChE (FBS AChE) provided complete protection against 2.75 LD_{50} without any signs of soman toxicity or performance decrements as measured by serial probe recognition (SPR) tests. Also, administration of FBS AChE or horse serum BChE to monkeys completely protected them against up to $5xLD_{50}$ of soman again without any signs of soman toxicity or performance decrements as measured by Primate Equillibrium Platform (PEP) performance. Although such use of enzyme as a single pretreatment drug for OP toxicity is sufficient to provide complete protection, a relatively large (stoichiometric) amount of enzyme will be required to *in vivo* detoxify OPs. However, when mice were pretreated with sufficient amounts of FBS AChE and a reactivator, e.g., HI-6, and then challenged with repeated doses of sarin, the AChE continously reactivated (preventing the "aging" of AChE) and sarin was continously detoxified as long as the molar concentration of sarin was less than that of AChE at that time. The *in vitro* stoichiometry of sarin:AChE was as high as 400:1 and *in vivo* in mice it was as high as 65:1. Addition of reactivator such as HI-6 to pretreatment of mice with enzyme appears to multiply the effectivness of exogenous AChE.

Poster Session A

ANCHORING OF RAT BRAIN ACETYLCHOLINESTERASE TO MEMBRANES

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Rat brain globular acetylcholinesterase (AChE, EC 3.1.1.7) exists as three major size isomers, i. e. monomers (G_1), dimers (G_2) and tetramers (G_4). Each size isomer can be found either in a salt-soluble (SS) fraction or in a detergent-soluble (DS) fraction. The localization and function of these forms is not univoque, since at least a part of SS rat brain globular AChE is amphiphilic, as shown by detergent dependency of enzymatic activity and binding to liposomes, and DS forms remain in solution in absence of detergent (Andres et al., 1990, Neurochemical Research, 15, 1065-1072). In view to get a better understanding of this polymorphism and its physiological signification, we studied the interaction of purified rat brain globular AChE with artificial liposomes (phosphatidylcholine-cholesterol-stearylamine) and attempted to identify the hydrophobic domain(s) involved in membrane binding. Binding to liposomes was possible for both SS and DS-AChE, and was temperature dependant, maximal above the phase transition temperature of the liposomes. Binding was not dependant on the ionic strength and was partially influenced by the composition or the electrostatic global charge of the liposomes. Proteinase K and papain treatment transformed SS-AChE and DS-AChE into forms that, in absence of detergent, no longer aggregated nor bound to liposomes. In contrast, phosphatidylinositol-specific phospholipase C had no effect on these properties. Labelling DS-AChE with 3 - (trifluoromethyl) - 3 - (m - (^{125}I) - iodophenyl) diazirine (^{125}I-TID) (a photoactivable reagent which covalently binds to hydrophobic domains) revealed, by polyacrylamide gel electrophoresis (PAGE) under reducing conditions, one single band of 69 kD apparent molecular mass. Proteinase K treatment transformed the 11 S ^{125}I-TID labelled AChE into a 4 S form which no longer showed ^{125}I radioactivity and was unable to bind to liposomes. The ^{125}I labelled domain migrated after proteinase K treatment on PAGE close to the front of the gel with an apparent mass of less than 3 kD. These results are compatible with the existence of a hydrophobic segment not linked to the catalytic subunits by disulfide bounds in contrast to the 20 kD non-catalytic subunit described by Inestrosa et al. for bovine caudate nucleus G_4 DS-AChE (1987, J. Biol. Chem. 262, 4441-4444). Moreover, this ^{125}I labelled hydrophobic segment could be common to DS-AChE and to amphiphilic SS-AChE and could be a part of the different size isomers. Chemical characterization is now necessary to confirm our model of globular AChE hydrophobic domain.

Evolution of acetylcholinesterase expression in the developing central nervous system of the quail

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We studied the expression of acetylcholinesterase (AChE) in different regions of the brain and in the neuroretina, during the embryonic development of the quail. We analyzed AChE mRNAs and protein by Northern and Western blots, as well as AChE activity and molecular forms.

A partial cDNA clone, encoding quail AChE, was identified by hybridization with a *Torpedo* AChE probe. Its identity as a cholinesterase clone was demonstrated by homology with other known sequences. The fact that it corresponds to acetyl- rather than butyrylcholinesterase was established by the high content of (G+C) in the third bases of the codons. Exon III (according to the *Torpedo* nomenclature) is highly homologous to exon III_T of other species, corresponding to the type T polypeptide, which is known to correspond to the catalytic subunit of collagen-tailed and hydrophobic-tailed forms, as well as of soluble tetramers.

A 650 bp coding sequence, including a part of exon I and the totality of exons II and III, was amplified by PCR and used as a probe in Northern blots. We observe three major bands around 4 kb, 4.8 kb and 6.4 kb, the 4.8 kb band being sometimes very faint. AChE mRNAs develop first in the brain stem, then in the optic lobes and the neuroretina, and later in the cerebellum, in agreement with the evolution of AChE specific activity.

At all stages of development, the major molecular forms of AChE are G_4 and G_1 in the quail brain, instead of G_4 and G_2 in the chick brain. The proportion of G_4 increases continuously between E4 and E16 and reaches 80% of the total activity just before hatching (E16).

In Western blots, the catalytic subunits appear as 100-110 kDa bands. For a constant enzymatic activity, the staining intensity of these bands increases between E8 and E16, indicating an increased contribution of inactive AChE protein. The proportion of inactive AChE follows the order: brain stem > optic lobes > cerebellum > neuroretina. After birth, it continues to increase for some time in the cerebellum. The adult level is, however, lower than the E16 level. The possibility that this inactive AChE component plays a role in the developing brain is intriguing.

Characterization of an esterase gene located on chromosome 5 in *Caenorhabditis elegans*.

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In our effort in cloning the three different genes coding for acetylcholinesterases in the nematode *C. elegans*, we obtained from Dr. Waterston's lab (Saint Louis, Missouri) a partially-sequenced cDNA clone (cm06b1) that presented certain homologies with *Drosophila* AChE.

This cDNA was entirely sequenced and maps to chromosome 5 as indicated by hybridization to the YAC polytene grid. Therefore it does not correspond to any of the three AChE genes already identified (ace1, ace2 and ace3 located on chromosome X, 1 and 2 respectively[a]). We purchased four cosmids (average size 40kb) covering the interesting region of chromosome 5 from the *Caenorhabditis* Genetics Center (Saint Louis). From these, only one (T25D4) hybridized with a cmo6b1 probe. This cosmid allowed the characterization of the gene.

Both sequences (genomic and cDNA) are presented. Analysis of genomic clones showed the existence of six short introns (48, 95, 50, 134, 76 and 46 bp) interrupting the coding sequence. The cDNA codes for a 557 amino acids polypeptide and presents the trans-spliced leader SL1 at the 5' end. There is no obvious sequence for a signal peptide. If the initiator methionine is removed, the N-terminal sequence becomes GGFLSHLT: a sequence suggestive of a possible N-myristoylation. The whole coding sequence was aligned with known cholinesterases and other related proteins. Of the twelve Cys only two are found in conserved position and may be involved in a intrachain disulfide bond homologous to the first S-S bond of cholinesterases. A sequence FQESAG is found in homologous position to the active site FGESAG sequence of cholinesterases and certain other esterases. The sequence FQESAG (obtained by directed mutagenesis G-->Q in *Torpedo* AChE) was shown to preserve 50% of cholinesterase activity and abolished excess substrate inhibition[b]. The most related proteins to cm06b1 product are the rat cholesterolesterase and pancreatic lysophospholipase (BLASTX scores 258 and 257).

a Johnson, C. L. (1991) in "Cholinesterases" ACS, Washington

b. Gibney, G. *et al.* (1990) Proc. Natl. Acad. Sci USA 87, 7546-7550

ABSTRACT FOR THE 36th OHOLO CONFERENCE

CLONING AND ANALYSIS OF THE ACETYLCHOLINESTERASE GENE OF *Ceratitis capitata (medfly)*

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Sequence data has been determined for the acetylcholinesterase (AChE) gene from *Ceratitis capitata* (medfly), the most serious fruit fly pest in the Mediterranean region. AChE is the target site for both organophosphate and carbamate insecticides and an altered form causes broad spectrum resistance in many insects. Resistance has, as yet, not been reported in the field despite heavy insecticidal pressure.

A 311 base pair fragment of medfly genomic DNA called M_3 was obtained by the polymerase chain reaction using oligonucleotides designed on the basis of AChE sequences from *Drosophila melanogaster* and *Anopheles stephensi*. The amino acid similarity of M_3 to the counterpart of *D. melanogaster* was 86% and the nucleic acid similarity, 67%. Using M_3 as a probe a 13kb clone named M_{16} was isolated from an EMBL3 genomic library. An EcoR I digest of M_{16} produced a 1.8kb fragment which was homologous to M_3 by hybridisation. This EcoR I fragment called $M_{16}R$ was subcloned into the sequencing vector M_{13} and complementary subclones called M_8 and M_4 were isolated which contain the 1.8kb fragment in opposite orientations.

Sequence data has shown that $M_{16}R$ contains the active-site of the medfly AChE gene. A restriction map of the clone is presented. Sequence analysis of coding regions of the medfly AChE clone reveals up to 90% amino acid similarity to the homologous regions of *D. melanogaster* and 80% to the mosquito *An. stephensi*. The M_{16} clone and $M_{16}R$ are currently being used as probes to define the cytological location of the medfly AChE gene on larval salivary gland polytene chromosomes by *in situ* hybridisation.

HISTIDINE 322 TO ASPARAGINE MUTATION IN HUMAN ACETYLCHOLINESTERASE (AChE) ASSOCIATED WITH THE RARE YT2 BLOOD GROUP ANTIGEN

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Spring & Anstee (Transfusion Medicine Vol. 1, Suppl 2 1991, p. 42) provided evidence from immunoprecipitation with monoclonal antibodies and SDS gel electrophoresis that the human YT blood group is identical to red cell acetylcholinesterase. The YT allele frequencies in Europeans and Canadians are 0.9564 for YT1 and 0.0436 for YT2. Following multiple transfusions, anti-YT1 antibodies have been found in patients with the YT:-1,2 phenotype (homozygous for YT2). In these patients survival of donor red cells having the common phenotypes YT:1,-2 (homozygous for YT1) or YT:1,2 (heterozygous) may be significantly decreased. To determine whether the YT2 blood group antigen represents a genetic variation in the acetylcholinesterase protein, we sequenced genomic DNA from one person homozygous for YT2 and one person homozygous for YT1. Genomic DNA was amplified by the polymerase chain reaction (PCR) using primers for human ACHE. PCR was carried out several times on each sample as a check for Taq polymerase errors. Sequencing of amplified DNA showed that ACHE from a person with the YT2 blood group had a nucleotide substitution which changed Histidine 322 (CAC) to Asparagine (AAC). Amino acids are numbered so that the N-terminal of the mature protein, Glu, is number 1. Histidine 322 of human AChE is analogous to Lys 315 of Torpedo AChE. Lys 315 is located on the surface in the crystal structure of Torpedo AChE (Sussman et al Science 253: 872-879, 1991), suggesting that this position is accessible to antibodies and could be responsible for the antigenic properties of the YT blood group. The YT blood group locus has been mapped to human chromosome 7q22 (Zelinski et al Genomics 11: 165-167, 1991) by linkage analysis. These results imply that an amino acid substitution in red cell AChE probably results in antibody formation in patients who receive red cells of incompatible ACHE genotype. This is the first report of a genetic variation in human acetylcholinesterase; we propose that the formal name for the variant be ACHE*322N. Supported by US Army Medical Research Development Command DAMD17-91-Z-1003 (OL) and Grant MT3391 from MRC Canada (TZ).

Amplification of human butyrylcholinesterase cDNA and its impaired transmission studied in transgenic mice

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Aberrations in cholinesterase (CHE) genes and their protein products are common both in tumor tissues and as inherited variations. In contrast, autosomal gene amplifications in general are abundant in tumor tissues, yet not known to be heritable. One exception is the human butyrylcholinesterase (BCHE) gene, amplified in a parathion-exposed father and son. The BCHE gene, together with the acetylcholinesterase (ACHE) gene and several oncogenes, also amplifies in hemopoietic and ovarian tumors. To study whether the BCHE gene coding sequence alone suffices to induce the appearance and heritability of this gene amplification, we constructed transgenic mice carrying human BCHEcDNA. Blot hybridization of tail DNA samples revealed that somatic transgene amplifications occurred in 31% (11/35) of the FII generation in two independent pedigrees of these transgenic mice but decreased to 4% (2/51) in the FIII generation. However, 10-100-fold amplifications of BCHE, ACHE and c-raf appeared in testes and epididymis DNA from >80% of FIII mice, carrying no somatic gene amplifications. Furthermore, the observed gonadal DNA amplifications were accompanied by drastic, selective reductions in the ratio between testes BCHE mRNA and actin mRNA, as detected by direct PCR amplification of testes cDNA, and apparently resulted in infertile sperm. These findings demonstrate that the BCHE gene is transcriptionally active in mammalian testes, present a transgenic mice model for a potentially heritable germline amplification of the human BCHE coding sequence and suggest that the induction of subsequent spermatogenic amplifications of other genes in these mice lead to the selective preclusion of this potentially stable gene amplification from the pool of inherited genetic material. Gonadal gene amplifications may therefore provide a general explanation for the limited transmission of amplified autosomal genes.

"ON LINE" RECORDING OF ACETYLCHOLINESTERASE FROM SUBSTANTIA NIGRA: A COMPARISON OF STIMULUS- AND 5-HYDROXYTRYPTAMINE (5HT)- EVOKED RELEASE.

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A soluble form of acetylcholinesterase (AChE) has been shown to be liberated from the dendrites of dopamine-containing neurones in the substantia nigra. This phenomenon would appears to be unrelated to hydrolysis of acetylcholine, but rather represents a distinct, non-cholinergic neuromodulatory role. The substantia nigra receives serotoninergic innervation from neurones whose cell bodies lie within the dorsal raphe nuclei. Indeed, a 5HT-induced enhancement of AChE release from the substantia nigra of anaesthetised guinea pigs has been previously demonstrated by fluorometric analysis of fractions of perfusate obtained via push-pull cannulae. The more recent development of a sensitive chemiluminescent assay, however, has permitted the continuous recording of AChE levels *in vivo*. This 'on-line' measurement of AChE activity has been used in the present experiments to monitor levels of the protein in anaesthetised guinea pigs and to compare the effects of direct application of 5HT with stimulation of raphe dorsalis neurones. Exogenous 5HT enhanced AChE levels in the perfusate by an order similar to that recorded by the conventional assay, and in a dose-dependent fashion. Electrical stimulation of serotoninergic raphe dorsalis neurones similarly enhanced release of AChE, the amount of the liberated protein correlating roughly with the strength of stimulus applied. By comparison of the levels of 5HT and stimulus-evoked AChE it would appear that perfusate levels of 5HT must reach at least $5x10^{-8}M$ before significant changes in AChE release can be observed. These phenomena are currently being investigated in the freely moving animal to determine a possible causal relationship between 5HT, AChE and motor behaviour. By enhancing the release of AChE, the raphe nuclei could play a crititcal role in neuromodulatory events in a key brain area involved in motor control.

Chemical Modification of *Torpedo* Acetylcholinesterase with Disulfides: Reversible Modification and Irreversible Inactivation

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Modification of *Torpedo californica* acetylcholinesterase (AChE) by the disulfides, bis(2,2,5,5-tetramethyl-3-imidazoline-1-oxyl-4-il) disulfide (biradical) and 4,4'-dithiopyridine, via a thiol-disulfide exchange reaction, was monitored by EPR and optical spectroscopy, respectively. Incubation with both these reagents led to a complete loss of enzymic activity which, in both cases, occurred concomitantly with reaction of one mole of disulfide per mole of catalytic subunits, most likely with the single free thiol group of Cys231. Both chemical modification and loss of activity displayed pseudo-first-order kinetics. The EPR spectrum of AChE modified with biradical was typical of an immobilized nitroxyl radical. Treatment with glutathione of AChE modified by either of the two disulfides led rapidly to release of the bound reagent with concomitant regeneration of the single free thiol group of the enzyme. However, no concomitant recovery of catalytic activity was observed.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, in the absence of reducing agent, showed that both the modified and demodified enzyme retained their structure as a disulfide-linked dimer. Circular dichroism revealed, however, that the disulfide-modified enzyme differs significantly in secondary structure from native acetylcholinesterase. Modification by the disulfide agents led to a complete disappearence of the ellipticity in the near UV region and to a decrease of the ellipticity in the far UV region. The observed changes in secondary structure were similar to those produced by low concentrations (0.6-1M) of the denaturing agent, guanidine hydrochloride. The changed circular dichroism spectrum was retained upon demodification with glutathione.

One possible explanation for our observations might lie in a rearrangement of intrachain disulfide bonds triggered by the disulfide reagents. We are currently exploring this possibility.

EFFECT OF HEAT SHOCK ON ACETYLCHOLINESTERASE ACTIVITY IN CHICK MUSCLE CULTURES

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The effect of heat shock was studied on the acetylcholinesterase activity of chick muscle primary cultures. In cultures transferred from 37°C to 45°C, a sharp drop in activity was followed by rapid spontaneous recovery. The time of onset of recovery resembled the time needed for expression of heat shock proteins. In cultures exposed to heat shock at 45°C and allowed to recover at 37°C, reappearance of acetylcholinesterase activity did not involve *de novo* protein synthesis since it was not prevented by cycloheximide. Our data raise the possibility of a role for heat shock proteins as molecular chaperones in rescuing heat-denatured acetylcholinesterase.

G_2-ACETYLCHOLINESTERASE IS PRESYNAPTICALLY LOCALIZED IN *TORPEDO* ELECTRIC ORGAN

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In *Torpedo* electric organ, much of the acetylcholinesterase (AChE) is a globular dimer (G_2), anchored to the plasma membrane via covalently attached phosphatidylinositol and selectively solubilized by a bacterial phosphatidylinositol-specific phospholipase C.

While the structure of this form of the enzyme is well-established, the ultrastructural localization of G_2-AChE is still unclear. Selective solubilization with phosphatidylinositol-specific phospholipase C was, therefore, combined with immunocytochemistry at the electron microscope level, in order to localize G_2-AChE in electric organ of *Torpedo. ocellata*. Thin sections of electric organ were labelled with antibodies raised against *Torpedo* AChE, followed by gold-conjugated second antibodies, before or after exposure to the phospholipase. For comparison, the location of AChE was examined using histochemical methods. We show that: (1) immunolabelling is concentrated in the synaptic clefts between nerve terminals and the innervated face of the electrocyte; (2) this labelling co-localizes with AChE histochemical reaction products; (3) prior exposure to the phospholipase causes a decrease in AChE-associated labelling. Quantitative analysis of immunolabelling in the synaptic clefts shows that the phospholipase treatment had reduced primary labelling at or adjacent to the presynaptic membrane. Together with our previously presented biochemical and immunofluorescent evidence, these results support our previous assignment of a neuronal and synaptic localization for G_2-AChE in *Torpedo* electric organ.

EXPRESSION AND ISOLATION OF BIOLOGICALLY ACTIVE HUMAN ACETYLCHOLINESTERASE FROM E. COLI

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Authentic human acetylcholinesterase was expressed in Escherichia coli under regulation of the constitutive deo promoters or the thermoinducible P_L promoter and cII ribosomal binding site derived from bacteriophage λ. To facilitate the expression, cDNA was modified at the N-terminus by site-directed mutagenesis in order to replace some of the GC-rich regions by AT. These modifications did not alter the amino acid sequence but resulted in ample production of the protein. Production of recombinant AChE (rAChE) was induced by a temperature shift from 32° to 42°C. The protein accumulated for a period of 2 hrs and reached a level of 10% of total bacterial proteins. A partially purified inactive recombinant protein was recovered from inclusion bodies. AChE was obtained after solubilization, folding and oxidation. An analog of hAChE in which the C-terminus cysteine was replaced by serine resulted in a 20- to 40-fold increase in the recovery of active enzyme relative to that obtained with the wild type. The reconstituted enzyme was indistinguishable from native AChE isolated from erythrocytes in terms of substrate specificity and inhibitor selectivity.

A MODEL OF BUTYRYLCHOLINESTERASE BASED ON THE X-RAY STRUCTURE OF ACETYLCHOLINESTERASE INDICATES DIFFERENCES IN SPECIFICITY

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In vertebrates, two principal enzymes are known which hydrolyse acetylcholine (ACh) rapidly. Acetylcholinesterase (AChE) is associated primarily with nerve and muscle synaptic contacts while butyrylcholinesterase (BChE) is synthesized in the liver and found primarily in the blood serum. While the principal role of AChE is the termination of impulse transmission at cholinergic synapses by hydrolysis of ACh, the physiological function of BChE is not yet known. Whereas AChE hydrolyzes ACh much faster than the bulkier substrate butyrylcholine, BChE hydrolyzes both these substrates well.

There is a high degree of sequence similarity (54% identity) between the 575 residues of *Torpedo californica* AChE and the 574 residues of human BChE. This suggested that it would be possible to use the three-dimensional atomic structure of *Torpedo* AChE, which we recently determined at 2.8 Å resolution, to model human BChE.

We changed the amino-acid sequence of AChE to that of BChE and then energy-minimized this structure to obtain a model of the BChE structure. The BChE model clearly shows where the bulkier butyryl moiety might fit into the active site as a result of two side-chain substitutions from aromatic to non-aromatic ones in the vicinity of the active site S200, i.e., F288L and F290V. In addition, substitutions of W279A and F330A in BChE, are a likely reason for differences in affinity between BChE and AChE, since refined X-ray structures of AChE-inhibitor complexes show not only a direct interaction of the choline-like moieties with W84, but also that conformational changes of W279 and F330 occur upon binding of inhibitors to AChE.

TISSUE DISTRIBUTION OF HUMAN BCHE TRANSCRIPTS. COMPARATIVE STUDY OF THE 5' REGIONS OF HUMAN AND RABBIT BCHE GENES.

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mRNAs from different human tissues were probed on Northern blots with very high specific activity probes made by PCR from either exon 2 or exon 4 of human BCHE cDNA. mRNAs were detected in all tissues except placenta. Liver was the richest source of BCHE mRNA followed by lung, brain, heart, muscle, pancreas and kidney. The major transcript in all tissues was 2.5 kb long, a length consistent with the distance between the transcription start and the polyadenylation site. Other transcripts were found at 1kb, 1.4kb, 1.8 kb, 6.7 kb and 15 kb. 1.4, 1.8 and 6.7 kb transcripts hybridized with exon 2 but not with exon 4 whereas 1, 2.5 and 15 kb transcripts hybridized with both. The 6.7 kb is interesting as it might be alternatively-spliced to encode a novel BChE protein.
A genomic clone containing exon 1 of rabbit BChE was found using an homologous human probe (Arpagaus *et al.*, 1990, Biochemistry 29,124-131). The homologies extend over 1.3 kb upstream of the first coding nucleotide of the mature human BChE protein. The sequence is rich in A and T with repeats of up to 16 T. Different consensus sites of fixation of transcription factors are found in the region (Mef 1, MyoD 1). Primer extension showed a transcription start in human BCHE located 106 nucleotides in front of ATG = Met -69 which is the first ATG in phase with the coding sequence of BChE. In rabbit BCHE the transcription start was 315 nucleotides in front of the same ATG. We could not find sequences matching the consensus TATA or CAT box but the sequences TAATA and CAAT were found 21b and 43b upstream of the transcription start site in the human BCHE gene. In the rabbit gene, no such sequences were found in the proximity of the transcription start site. Portions of the 5' region of human and rabbit BCHE genes are being tested for promotor activity with a CAT reporter gene in NIH3T3 cell line. This work was supported by grants DAMD 17-91-Z-1003 from the US Army Medical Research and Development Command to O.L. and by the French MRT (91T0439).

POSTRANSLATIONAL MODIFICATIONS OF HUMAN ACETYLCHOLINESTERASE IN TRANSFECTED 293 CELLS

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Transfected 293 embryonal kidney cells (R11), which express high levels of soluble secreted recombinant human acetylcholinesterase (rHuAChE) enable the investigation of its intracellular transport and assembly. Cells were pulsed, chased and the radiolabeled rHuAChE was immunoprecipitated from lysed cells and from the medium. The newly synthesized intracellular monomers were assembeled into dimers within 1-2 hours and the HuAChE was secreted, at high recovery, in the oligomeric form (dimers and tetramers). Dimer formation takes place in the endoplasmic reticulum (ER). This is indicated by analysis with brefeldin-A, which blocks export of proteins from the ER, and nocodazole which inhibits the brefeldin A - induced retrograde transport of the Golgi enzymes into the ER. In their route to secretion, dimers of rHuAChE travel through the Golgi complex were they acquire GlcNAc in the medial Golgi and galactose in the trans-Golgi. Immunoprecipitated rHuAChE dimers from cell lysates display binding of concavalin A to terminal mannose residues and of wheat germ agglutinin (WGA) to terminal GlcNAc residues. Only a minute fraction of intracellular rHuAChE bound Ricinus Communis agglutinin (RCA) to galactose, whereas this lectin was heavily bound to the secreted rHuAChE. This suggests that the transport and processing steps prior to the trans-Golgi galactolysation compartment are relatively rate-limiting, while the following movement to secretion is rapid. From the trans-Golgi, rHuAChE travels through the trans-Golgi network (TGN), where it is sialylated. This was indicated by acquisition of endo-H resistance and reduction in the electrophoretic mobility of the secreted rHuAChE as compared to the intracellular forms. Intracellular monomers were exclusively endo H-sensitive and did not bind WGA or RCA, while intracellular dimers were both sensitive and resistant to endo H. The analysis of the processing of the carbohydrate moieties of rHuAChE further supports: (a) the formation of dimers in the ER, prior to the terminal glycosylation events, and (b) the rapid transport of dimeric rHuAChE through the TGN en route to secretion. (Supported in part by US Army Medical Research Development Command Contract DAMD 17-89-C-9117).

ESTABLISHMENT OF STABLE CELL LINES WHICH PRODUCE AND SECRETE HIGH LEVELS OF RECOMBINANT HUMAN AChE

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A series of multicistronic expression vectors containing the coding region of the human AChE hydrophilic subunit (Soreq et al. 1990, Proc. Natl. Acad. Sci. USA. 87: 9688-9692) were introduced into various mammalian cell lines to test human AChE expression and secretion. AChE levels were highest in the extracellular medium of transiently transfected cells when the huAChE coding region was linked to a CMV promoter or an RSV LTR unit. Highest levels were obtained with the human 293 embryonal kidney cell line. To facilitate selection of stably transfected cells exhibiting high levels of huAChE expression, the various expression vectors were provided with the bacterial $\underline{neo}^r$ gene linked to a weak eukaryotic promoter (H_2L^d, mouse histocompatability gene) and transfected cells were grown in the presence of the neomycin analogue, G418. The weakly functioning promoter should restrict cell growth in the presence of G418 to those cells which have integrated the vector into an actively transcribed region within the host chromosome. By this method we have been able to isolate cell clones which secrete up to 150 units of biologically active human acetylcholinesterase per 10^6 cell per 24 hours. Southern blot analysis revealed that the high level expressor cells contain a low copy no. of the human AChE CDNA suggesting that the high level of expression is indeed due to specific integration into transcriptionally favored sites within the chromosome. The enzyme secreted by high expression clones displayed a high turnover number (k_3 = 3.9×10^5 min^{-1}) as well as substrate specificity, selective sensitivity to inhibitors and inhibition by high substrate concentrations, all similar to other known acetylcholi- nesterases, thus confirming its authenticity and integrity. (Supported by US Army Medical Research Development Command Contract DAMD 17-89-C-9117).

CONTRIBUTION OF THE THREE N-GLYCOSYLATION SITES TO PRODUCTIVITY, CATALYTIC ACTIVITY AND MOLECULAR HETEROGENEITY OF RECOMBINANT HUMAN AChE EXPRESSED IN HUMAN CELLS.

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Purified recombinant HuAChE secreted by established cloned 293 cell lines resolved into two bands of 67 and 70 kD under reducing conditions. When these preparations were treated with N-Glycanase, a single discrete band of 62 kD was discerned, suggesting that the 67 kD and 70 kD bands represent two differentially N-glycosylated versions of the human AChE monomer unit. To test this hypothesis and to study the possible involvement of the three potential glycosylation (Asn-X-Thr/Ser) sites we employed site directed mutagnesis analysis. HuAChE mutated at either one, two or three of the Asn residues (position 265, 350, 464) within the glycosylation consensus signals were substituted by Gln or Ala residues. Following transient transfection of 293 cells with the various glycosylation mutant vectors, secreted AChE was determined by both enzyme activity and ELISA. While mutations of a single glycosylation site reduced AChE polypeptide production to ~20% of wild type, double mutants exhibited 3-5% of wild type levels. Removal of one or two glycosylation sites had no effect on the catalytic activity (specific activity) of the secreted enzyme. When all three potential N-glycosylation sites were eliminated, no secretion of AChE polypeptide was detected. These results suggest that in the rHuAChE molecule the carbohydrate moieties play a role in molecular stability and subsequent export of the protein. To further address the possible role of each specific glycosylation site to the unique glycosylation pattern of the rHuAChE, we generated stable 293 cell clones expressing each of the three single site mutants (Asn464 ->Gln; Asn350->Gln; Asn265->Gln). Cells were pulsed with ^{35}S-methionine and chased over various periods of time, followed by immunoprecipitation of intracellular and extracellular rHuAChE. The polypeptides of all three single site glycosylation mutants resolve into at least two species, all having similar migration profiles. The highest MW band of the secreted glycosylation mutants comigrates with the highest MW band of the secreted wild type rHuAChE. Upon treatment of the single site glycosylation mutants with N-Glycanase, a discrete 62 kD band was generated as in wild type rHuAChE. These results may be interpreted to suggest that: a) none of the three N-glycosylation consensus sequences show preferential glycosylation potential and b) probably only two glycosylation sites are utilized in wild type rHuAChE expressed in 293 cells.(Supported by US Army Medical Research Development Contract DAMD 17-89-C-9117).

PRODUCTION OF HUMAN RECOMBINANT ACETYLCHOLINESTERASE - COMPARISON OF ANCHORAGE-DEPENDENT CELL PROPAGATION SYSTEMS

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Three main cell propagation systems, surface propagator, fixed-bed reactor and stirred microcarrier cultures were evaluated for large-scale production of recombinant human acetylcholinesterase (AChE) by a human embryonal kidney cell line (R11). The recombinant cell line is capable of producing and secreting 5-25 micrograms AChE/10^6 cells/24 hours in lab-scale cultures. Bench-scale cultivations were carried out with modified fetal bovine serum or low-protein serum substitutes containing intrinsic bovine AChE levels below 0.5 nanograms/mL. In the surface propagator (multitray system), 4.5 mg AChE/L/day were produced continuously for 15 days. In the fixed-bed system (polyurethane-based packed-bed reactor), 4.2 mg AChE/L/day were produced for culture period of 10-15 days. The product yield in the microcarrier system varied considerably depending on the carrier type. Four types of microcarriers were studied: Diethylaminoethyl (DEAE)-dextran beads (Cytodex-1); collagen-coated dextran beads (Cytodex-3); polystyrene beads (Biosilon); and gelatin macroporous beads (Cultisphere-G). System productivities with Cytodex-1, Cytodex-3 and Biosilon microcarriers were 6.6, 3.3 and 3 mg/L/day respectively for a time period of 15 days. During cell growth, aggregates of multilayered cells and beads were formed and maintained for about 15 days, followed by migration of the cells to the center of the aggregates and ending up in detachment of the cell clumps from the beads. With the macroporous Cultisphere-G beads, cells were maintained in the aggregates for longer culture periods resulting in system productivity of 5 mg/L/day for 22 days. This system was selected for further evaluation on a larger scale in a 4-liter controlled microcarrier bioreactor system. (Supported by US Army Medical Research Development Command Contract DAMD 17-89-C-9117).

PRODUCTION AND CHARCTERIZATION OF MONOCLONAL ANTIBODIES SPECIFIC FOR MAMMALIAN BRAIN ACETYLCHOLINESTERASES

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The non-inhibitory monoclonal antibodies, mAb 111-2 (IgG_1 with kappa chain), raised against native detergent-soluble tetrameric form of acetylcholinesterase (AChE, EC 3.1.1.7) from human brain and mAb 132-5 (IgG_1, with kappa chain), raised against heat denatured detergent-soluble tetrameric form of acetylcholinesterase (AChE, EC 3.1.1.7) from human brain, were found to be specific for mammalian brain AChE. The properties of the two mAbs towards different cholinesterases (ChEs) were studied by enzyme antigen immunoassay (EAIA) and solid phase immunoassay. EAIA showed that the reaction of mAb 111-2 with human brain G_4-AChE (both of DS-form and SS-form) is about 115 times stronger than with human erythrocyte G_2-AChE. However in solid phase immunoassay and ELISA mAb 111-2 gave almost the same response towards human G_2- and G_4-AChE, indicating that upon surface denaturation of human G_2-AChE the epitope for mAb 111-2 became exposed. Binding experiments showed that native human G_2-AChE in solution bound only weakly to mAb 111-2 even after incubation for 24 hours at room temperature, while the reaction of 111-2 with human brain G_4-AChE was very strong. Heat denaturation and SDS-treatment of brain AChE abolished binding of mAb 111-2 indicating that mAb 111-2 targets a discontinuous epitope. mAb 111-2 proved to be useful in the determination of G_4-AChE from aminotic fluid as blood contamination did not influence the results even when the aminotic fluid samples were heavily contaminated by red cell G_2-AChE.

The results obtained by mAb 132-5 was even more specific than mAb 111-2 for mammalian brain AChE as it reacted with native as well as with heat and SDS denatured antigens indicating that it targeted a continuous epitope. However the reaction with native brain was weaker than that of mAb 111-2. Again no reaction of mAb 132-5 was obtained with G_2-AChE from erythrocytes nor with BtChE from human serum. Results from western blots showed that mAb 132-5 reacted much stronger with tetramer, heavy dimers and heavy monomers of mammalian brain AChE than with light dimers and light monomers, suggesting that the 20 kDa hydrophobic anchor may be part of the epitope.

Use of the PCR method to rapidly generate homologous DNA probes and sequence for acetylcholinesterase genes in non-dipteran pest insects

The polymerase chain reaction has been successfully employed to generate homologous DNA probes for three mosquito species and Medfly (see posters by Edwards *et al.* and Banks *et al.* this meeting). Many pest insects, in addition to the medically important mosquito species, have developed insecticide resistance mechanisms involving insensitive acetylcholinesterase (AChE). This approach involving the PCR was extended to orders other than Diptera. A range of degenerate oligonucleotide primers were available from regions across most of the AChE gene. One set termed F and G, which flank an intron and a particularly heterologous region in dipteran AChE genes, were found to be particularly efficient across a variety of insect species. Initial PCR reactions with total genomic DNA from the tobacco budworm *Heliothis virescens* generated a prominent 0.3kb fragment. Preliminary confirmation that this was from the AChE gene, was obtained by hybridisation to a cDNA clone of the *Drosophila melanogaster* AChE gene. A further four species of moth were then examined: the diamondback moth *Plutella xylostella*, the velvetbean caterpillar *Anticarsia gemmatalis*, the corn earworm *Helicoverpea zea* and the Imperial moth *Eucles imperialis*; each produced a prominent band on electrophoresis of approximately 0.3kb. Similar results were obtained with the grain storage beetle *Oryzaephilus surinamensis*, the aphid *Myzus persicae*, the two-spotted spider mite *Tetramychus urticae* and the Colorado Potato Beetle *Leptinotarsa decemlineata*. Each of the results were consistent with dipteran species with respect to the presence of a relatively small intron. Sequence analysis is being performed on species known to possess insecticide insensitive AChEs. Comparative DNA sequence data will be presented.

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Expression and refolding of functional human Butyrylcholinesterase from *E.coli.*

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A recombinant plasmid was constructed for the expression of human Butyrylcholinesterase in *E.coli* . The vector is pGEM 3 Zf from Promega. Expression of BCHE cDNA is under the control of the lac operon promoter. Transformed *E. coli* strain HB 101 grew at 37°C.

Production of BuChE was induced by 1mM IPTG. Inactive "scrambled" enzyme was recovered from the inclusion body fraction. After Western blotting, the protein was immunodetected with four distinct monoclonal antibodies directed toward human plasma BuChE. On SDS gel electrophoresis in the presence of β -mercaptoethanol a smear was always observed ; the more intense band had a size of 60-67 kD.

Inclusion bodies were denatured by 8 M urea for 30 min at 20°C.Folding BuChE into the correct disulfide-bonded structure was achieved by diluting 10 times the solubilized inclusion bodies in refolding buffers containing 2 mM GSH and 1 mM GSSG. The maximun rate of refolding was at 4°C. Several active forms were found that presumably correspond to monomer, dimer, tetramer (major form) and active aggregates. The pI of the refolded enzyme was found to be 5.2 (pI=3.99 for the plasma enzyme). Theoretical calculation gave a pI value of 5.47 for the nonglycosylated enzyme.

This work demonstrates that a). sugars are not important for enzyme activity ; b). folding to the biologically active enzyme state is thermodynamically controlled.

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HETEROGENEITY OF THE SILENT PHENOTYPE OF HUMAN BUTYRYLCHOLINESTERASE - IDENTIFICATION OF TEN NEW MUTATIONS

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Heterogeneity of the silent phenotype of human butyrylcholinesterase has been shown by several studies. To identify specific DNA alterations leading to different silent alleles we have sequenced the PCR amplified four exons of the *BCHE* gene of individuals known to carry at least one silent allele. Up to the present our laboratory has identified a total of eleven distinctive alleles associated with the silent phenotype (Table below).

Grossly altered versions of the enzyme are coded by the three frame-shift mutations, while a truncated molecule is caused by the nonsense mutation. Amongst the missense mutations one (S-7) codes for an unstable enzyme and another (S-11) causes the loss of the active site serine. There were no mutations in the entire coding regions of the *BCHE* gene in representatives of three different silent pedigrees. These may be the result of mutations in essential regulatory regions.

The large number of distinct silent alleles we have found in the relatively small sample examined, to date, makes it clear that there must be far greater structural heterogeneity within the silent phenotype than had been previously suspected.

Trivial name	Allele[a]	DNA changes		Affected pedigrees to date
S-1	BCHE*FS117[b]	nt 351	GGT→GGAG	3
S-2	BCHE*FS6	nt 16	ATT→_TT	2
S-3	BCHE*500STOP	nt 1500	TAT→TAA (Tyr→STOP)	1
S-4	BCHE*37S	nt 109	CCT→TCT (Pro→Ser)	2
S-5	BCHE*365R[c]	nt 1093	GGA→CGA (Gly→Arg)	3
S-6	BCHE*FS315[c]	nt 944	ACC→AACC	1
S-7	BCHE*115D	nt 344	GGT→GAT (Gly→Asp)	3
S-8	BCHE*471R	nt 1411	TGG→CGG (Trp→Arg)	1
S-9	BCHE*170E	nt 509	GAT→GGT (Asp→Glu)	2
S-10	BCHE*518L	nt 1553	CAA→CTA (Gln→Leu)	1
S-11	BCHE*198G	nt 592	AGT→GGT (Ser→Gly)	1

[a] According to La Du et al (1991) Cell Mol Neurobiol 11:79-88; [b] Nogueira et al (1990) Am J Hum Genet 46:934-942; [c] Hidaka et al (to be published)

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Sequence analysis of genes coding for insecticide insensitive acetylcholinesterase in mosquitoes

Acetylcholinesterase (AChE) is the target site for the inhibitive action of organophosphate and carbamate insecticides. A number of pest species have developed a modified AChE which shows reduced sensitivity to inhibition by these insecticides. Based on AChE gene sequence data for *Drosophila melanogaster* and *Anopheles stephensi* several sets of degenerate oligonucleotide PCR primers were prepared from regions throughout the gene. Most were eventually successful. One pair, termed F and G, was used to prepare homologous DNA probes for the AChE gene in three important mosquito vectors of disease, *Culex pipiens*, *An. albimanus* and *An. gambiae*. The success of which was confirmed by direct sequencing of the PCR products. This sequence included a particularly heterologous region thought to be cleaved out of the mature protein. It also contained an intron and so some variation in fragment size was observed between species. This PCR fragment was used to screen genomic DNA libraries in Lambda DashTM II, from *Cx. pipiens* and *An. albimanus*. A number of positive clones were identified from each. Restriction enzyme mapping indicated that the clones were overlapping. Further analysis utilising PCR and the T7 and T3 promoters indicated that each clone contained the whole AChE gene, totalling approximately 4kb for *Cx. pipiens* and approximately 3kb for An. albimanus. The sequence data generated is being used to design primers for rapid PCR analysis of insecticide sensitive and insensitive alleles of the AChE gene in each species. Comparative DNA sequence data will be presented.

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PLANT CHOLINESTERASES.

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The cholinesterase activity is observed in more than 60 plant species belonging to 20 families. The proteins are isolated from 15 species, partly purified and characterized. All of them are usually inhibited physostigmine, neostigmine and organophosphates. Molecular masses of plant cholinesterases vary from 60 to 80 kDa and are similar with the animal ones. The inhibitory analysis of catalytic center of cholinesterase from Pisum sativum leaves shows that the plant cholinesterase is analogous to cholinesterases from some invertebrata such as starfish Asteroidea. Most studied plant cholinesterases from leaves, seeds and roots as well from fungi Aspergillus niger are acetylcholinesterases, because they hydrolyzed acetylcholine with higher rates than other cholinic esters, and the excess of substrate inhibits the enzyme activity. The proteins from Phaseolus vulgaris roots, Avena sativa seedlings, and flowers of Hippeastrum and Gladiolus are neither inhibited by high concentrations of substrates, no by isoOMPA. Unlike the vertebrata cholinesterases, plant cholinesterases possess lower activity, more sensitive to neostigmine than physostigmine and inhibited by higher concentrations of organophosphates. Cholinesterases are also found both within plant cell (in chloroplasts, nucleus, cytoplasm and plasmalemma) and in cell wall. Role of plant cholinesterases in evolution of cholinesterases is discussed.

Transient Expression of Recombinant Human ACHE in Developing Embryos of Xenopus laevis

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We have undertaken the expression of human ACHEcDNA, under the control of the cytomegalovirus (CMV) major immediate early gene promoter, in microinjected Xenopus oocytes and embryos. Expression of CMV-controlled ACHEcDNA (CMVACHE) in Xenopus oocytes is efficient, yielding catalytically active enzyme capable of hydrolyzing up to 750 nanomoles acetylthiocholine/hr/oocyte. These data represent a 15-20 fold- increase in ACHE activities observed following the microinjection of synthetic ACHEmRNA into oocytes, and approach that displayed by 1 µl human serum.

Microinjected into 2- to 16- cell cleaving Xenopus embryos, CMVACHE directs the biosynthesis of recombinant ACHE (rACHE) at levels similar to those observed in DNA-injected oocytes. Following overnight incubation, at which time embryos have reached the late gastrula stage, endogenous ACHE levels are negligible and rACHE activity may represent a 50 to 100-fold increase over background. Shortly thereafter, endogenous ACHE activity increases rapidly and steadily. Using ecothiophate to distinguish between endogenous frog and heterologous human ACHE, we have observed the persistence of active human enzyme at least up to the swimming tadpole stage, 5 days post-fertilization. At day 5, rACHE accounted for 33% of the total measured activity

Recombinant human ACHE is assembled by Xenopus embryos into monomers and dimers at early stages where endogenous frog enzyme is either undectectable or present exclusively as a 5S peak. Furthermore, some human ACHE appears to be incorporated into the G4, A8, and A12 molecular forms appearing on day 3 of embryonic development, and may persist there through day 5. Significantly, no gross morphological defects have so far been associated with ACHE overexpression and, to date, we have raised apparently normal CMVACHE-injected embryos for up to 4 weeks.

These experiments represent the first successful attempt to express a recombinant cholinesterase in a multicellular organism. They therefore lay the basis for the analysis of spatio-temporal expression and processing of rACHE in a complex, dynamic, biological background. Furthermore, the persistence of rACHE in apparently normal tadpoles with a developed neuromuscular system opens the door to a systematic study of the potential for transiently expressed recombinant cholinesterases to protect a transfected organism from environmental ACHE poisoning.

FISH AChE MOLECULAR FORMS AND THEIR BIOCHEMICAL CHARACTERIZATION AS A BIOMONITORING TOOL OF AQUATIC ENVIRONMENT

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Abstract

Activity and molecular forms of acetylcholinesterase (AChE) were characterized in different tissues of carp (*Cyprinus carpio L.*). Tissue activity was determined in response to the insecticide methidathion (MD). The highest AChE activity was found in the brain (113±12 U/l). In the other organs there was less activity. The ratio of the membrane bound to the cytoplasmatic free AChE molecular forms was increased in the order of brain, trunk muscle and heart. In sera of fishes treated with MD (2 ppm) there was an 80% inhibition of AChE lasting for two weeks. Mimicking the seasonal temperature changes, experiments were carried out at 4 and 20°C. It was concluded that MD has a higher toxic effect at higher temperatures, whereas its relatively low concentrations can have a toxic effect as it is accumulated in fish organs.
Brain and liver contain three different molecular forms: G_1, G_4 and A_{12}. During exposure of fish to 2 ppm MD *in vivo*, the enzyme activity decreased significantly in the tissues investigated. There was a significant decrease in the G_1 form and an increase in the G_4 form in the brain, while in the liver there was a decrease in the G_4 form and increase of about 50% in the G_1 form. The results suggest that fish AChE and its molecular forms might be a good biomonitoring tool in assessing chemical effects and their potential hazards in the aquatic environment.

COMPARISON OF THE ACETYLCHOLINESTERASES OF THREE SPECIES OF SCHISTOSOME

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The enzyme Acetylcholinesterase (AChE) is present in all stages of the life cycle of *S. mansoni* , part of it in muscle tissue and part on the surface of the parasites. Drugs that inhibit AChE have been beneficial in treating schistosomiasis and the location of the enzyme on the parasite's surface facilitates AChE involvement in anti-parasite treatment; therefore the properties, such as localization, molecular composition, serological cross-reactivity, specific activity and inhibitor specificity of AChEs from three schistosome species were compared. In all three species AChE was abundant on the outer surfaces tegument, and muscle tissue of adult worms. Antigenic similarity was also observed, thus, AChE from *Schistosoma haematobium* and *S. bovis* cross-reacted with specific antibodies against AChE from cercaria of *S.mansoni.* This cross-reactivity was demonstrated by complement dependent cytotoxicity towards intact schistosomula and by immunofluorescence microscopy. Moreover, immunoprecipitation with anti-cercarial *S. mansoni* AChE antibodies, of soluble extracts from ^{125}I-labeled adult worms of *S. haematobium , S. mansoni* and *S. bovis* revealed two bands of molecular weight of *110kDa and 76kDa in all three* species. On the other hand, significant differences between the AChE's of of *S. haematobium* and those of the other species were with regard to their specific activities molecular forms and sensitivity to AChE inhibitors. Likewise on sucrose density gradients, the AChE from *S. mansoni* migrated as an 8S form whereas a 6S peak was observed in the AChE from *S. haematobium.* Thus, whereas the localization, subunit structures, and serological reactivity of AChE from various schistosomes species are similar, the sedimentation coefficients, specific activity and sensitivity to AChE inhibitors of AChE varied between species. Since these variations are reminiscent of differences observed in response to certain anti-schistosome treatments in humans, they may explain the differential effects of anti AChE drugs on schistosome species.

Poster Session B

EFFICACY OF OXIMES AS ANTIDOTES AGAINST ORGANOPHOSPHORUS POISONING AND ITS RELATION TO AChE REACTIVATION.

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The use of quaternary oximes such as 2-PAM and toxogonin for the treatment of organophosphorus (OP) poisoning is usually attributed to their ability to reactivate OP inhibited AChE. Poisoning incurred by soman is notoriously difficult for antidotal treatment due to a rapid aging of somanyl-AChE. Nonetheless, certain oximes e.g. HI-6, display antidotal efficacy against soman poisoning. The exact mechanism of action of these oximes is not as yet known. However, some oximes also display antimuscarinic and antinicotinic activity. In order to enhance the anticholinergic activity of oximes we have prepared a series of bisquaternary oximes which contain substituted quinuclidine moiety. Two of these oximes, AB-8 and AB-13, were chosen for further antidotal evaluation.

We have studied in parallel the antidotal efficacy of five oximes: HI-6, HLo-7, AB-8, AB-13 and toxogonin against poisoning by soman and tabun. Protection experiments in pyridostigmine-pretreated mice against soman, using these oximes in conjunction with atropine and benactyzine demonstrated high antidotal efficacy of HI-6, AB-8 and AB-13 with the following protection ratios (PR): 11.6, 8.2 and 6.4, respectively, while toxogonin was relatively limited in its efficacy (PR = 3.2). Toxogonin, HI-6, AB-8 and AB-13 displayed good antidotal efficacy against tabun in mice (PR = 8.0, 12.3, 10.0 and 8.1, respectively). Protection experiments in guinea pigs without pyridostigmine have shown that HI-6 has limited efficacy against tabun poisoning (PR = 3.8). In contrast, HLo-7, toxogonin, and AB-13 were found as efficacious antidotes against tabun in guinea pigs even in the absence of pyridostigmine (PR = 21.7, 21.0, and 9.0, respectively). In the absence of pyridostigmine pretreatment all oximes except HI-6 displayed poor antidotal efficacy against soman in guinea pigs. Pretreatment with pyridostigmine markedly increased the PR values for HI-6, AB-8, AB-13, toxogonin and HLo-7 (20.0, 14.2, 10.6, 10.3 and 7.1, respectively).

Reactivation kinetics of inhibited diethylphosphoryl-AChE yielded the following bimolecular rate constants of reactivation (k_r) for toxogonin, AB-13 and AB-8: 7.2×10^4, 2.5×10^4 and 47.2 $M^{-1} min^{-1}$, respectively. The low k_r value obtained for AB-8 is not reflected in its marked in-vivo antidotal efficacy against soman and tabun. Thus, our data further substantiates the notion that antidotal efficacy against OP poisoning could not be solely attributed to reactivation of AChE.

STUDIES ON THE MECHANISM OF INHIBITION OF CHOLINESTERASES BY HUPERZINE A.

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Huperzine A (HUP-A), a Lycopodium alkaloid isolated from the Chinese plant Huperzia serrata, has been described as an anti-cholinesterase (reversible inhibition, K_I=14 nM) which may be superior to physostigmine and tetrahydroaminoacridine (THA) for the treatment of Alzheimer's disease and other cholinergic-related impairements (Kozikowski et al., 1991, J. Org. Chem. 56, 4636-4645). In experiments using AChE from several sources (human, fetal bovine serum and Torpedo californica) we found that HUP-A inhibited AChE in a time-dependent manner (k_i), and that the complex formed dissociated rather slowly (k_{dis}) following dilution into phosphate buffer to restore enzyme activity. This phenomenon is in marked contrast to the rapid on- and off-rates which characterizes reversible inhibitors of ChEs. Long-term incubation of relatively high concentration of AChE (1-8 μM) with HUP-A did not reveal chemical modifications in either the enzyme or HUP-A. Although all AChEs tested displayed essentially the same behavior in presence of HUP-A, the rate of inhibition and the rate of the spontaneous regeneration differed for each enzyme (for FBS-AChE, k_i=1x10^6 $M^{-1}min^{-1}$ and k_{dis}=0.02 min^{-1}). In contrast to AChE, when BChE from either human or horse serum was used, both the inhibition and dissociation occurred sufficiently rapid to permit categorization of HUP-A as a classical reversible ligand; however, the concentration required to demonstrate such inhibition was 1000-fold higher than the concentration needed to inibit AChE. Results suggest that HUP-A is a slow reversible inhibitor of AChE. Both the rate of approach to steady state and the steady state levels could explain, in part, the longer duration of brain AChE inhibition following administration of HUP-A in rats than values reported for physostigmine. Structurally, the differences in on- and off-rates between AChE and BChE may be due to: a) replacement of six aromatic amino acids in tne catalytic pocket region of AChE by neutral aliphatic amino acids in BChE. b) possible differences in the dimensions of the active site cleft.

CONSTRUCTION OF MOLECULAR MODELS FOR HUMAN AChE AND FOR ITS PHOSPHONYLATION PRODUCTS BY ENANTIOMERS OF ISOPROPYL METHYLPHOSPHONATES (IMP)

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A molecular model for human acetylcholinesterase (HuAChE) was constructed, by methods of comparative modeling, based on the known x-ray structure of TcAChE (Sussman et.al., 1991, Science 253, 872-879), and on sequence homology of the cholinesterases. Stretches of 20-25 amino acid residues were mutated and zone refined by methods of molecular mechanics. The fully mutated structure was further zone refined including addition of hydrogen atoms to the residues comprising the active site gorge. Comparison of this model to the x-ray structure of TcAChE revealed only minor structural changes in the active site region, as could be expected from the near identical enzymatic activity characteristics. This model is currently being used for rationalization of structural and functional effects of site directed mutagenesis on HuAChE and for investigation of the interaction of pharmacophores with the enzyme.

Acetylcholinesterases demonstrate marked chiral selectivity for organophosphorous compounds. The ratio of the bimolecular rate constants for inhibition (k_i) for S_P-IMP vs R_P-IMP is in the range of 2-3 orders of magnitude. No such enantioselectivity was observed for BuChE. In order to define the molecular basis for this stereoselectivity and for its differences from BuChE we modeled the phosphonylation products of AChE with the S_P and R_P diastereomers of IMP. We assumed that for each diastereomer of IMP only one chiral product results, irrespective of the chirality of the leaving group, (Berman et.al., 1989, J. Biol. Chem. 264, 3951-3956) and that the products and the starting IMPs are of oposite chirality (in -line nucleophilic displacement of the leaving group by Ser203 O_γ in HuAChE). Zone refinements around the active site residues included 23 aminoacids. Molecular graphics, energy optimizations by molecular mechanics and atomic charge calculations were performed using the Sybyl software.

Examination of the product structures reveals conformational differences in the phosphonyl moieties and in the sidechains of some of the surrounding amino acids. The S_P-IMP product was found to be energetically more stable than the R_P-IMP product. Most of the energy difference can be attributed to the unfavorable conformation forced upon the phosphonyl moiety, by interactions with phenyl groups of Phe295 and Phe297. The models provide insight into the differential characteristics of chiral phosphonylated cholinesterases namely: stereoselectivity of AChE vs BuChE; influence of chirality upon rate of aging as well as on the rate of reactivation by oxime reactivators.

CHARACTERIZATION OF ANTI-ACETYLCHOLINESTERASE INHIBITORY MONOCLONAL ANTIBODIES

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Monoclonal antibodies (mAbs) generated against (a) Torpedo Californica AChE, (b) DFP-inhibited Torpedo AChE, (c) fetal bovine serum AChE (FBS AChE), (d) DFP-inhibited FBS-AChE, (e) MEPQ-inhibited FBS-AChE, and (f) soman-inhibited FBS-AChE were analyzed for their cross reactivity with AChEs isolated from various species using ELISA, and their ability to inhibit the catalytic activity of the enzyme using a microtiter assay. Eight of these mAbs were found to inhibit the catalytic activity of either Torpedo AChE (mAbs 2C8, 7G4) or FBS-AChE (mAbs 13D8, 25B1, 2A1, 4E5, 5E8 and 6H9). The ability to inhibit serum AChE isolated from several mammalian species by four of the anti FBS-AChE mAbs showed a spectrum of cross-reactivity. In all cases the inhibitory mAbs bound to the native form of AChE. None of the antibodies reacted with human or horse serum BChE. Sucrose gradient centrifugation of mAb-AChE complexes showed two types of profiles, one exhibiting several peaks, indicating a multi-complex formation between tetrameric forms, and the other displaying a single peak, indicating antibody-enzyme complex formation within the tetrameric form. Some of the antibodies blocked the binding of DFP and other organophosphates whereas other did not. The phosphoryl residue of DFP-inhibited FBS-AChE bound to mAbs could be displaced by oximes. Edrophonium, an esteratic site ligand did not retard the rate of inhibition of AChE by mAbs. In contrast, in almost all cases binding of AChE to mAbs was slowed in presence of propidium, a peripheral site-specific ligand. These observations are consistent with the contention (Ogert et.al., 1990, J. Neurochem. **55**:756-763) that mAbs will not interact with the esteratic site which is located deep inside the protein molecule (Sussman et.al., 1991, Science, **253**: 872-879). It is suggested that different mAbs interact with slightly varying regions encompassing the peripheral anionic site. This interaction resulted in an apparent modulation of the catalytic activity of AChE.

COLORIMETRIC DETERMINATION OF CHOLINESTERASE ACTIVITY. NEW METHODS LEADING TO THE FORMATION OF SOLUBLE OR INSOLUBLE END-PRODUCTS.

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We have developed two original methods allowing colorimetric determination of cholinesterase activity. The first method involves a combination of indoxyl acetate (IA) as substrate and NitroBlueTetrazolium (NBT) as secondary chromogen. IA hydrolysis leads to the formation of both indigo and diformazan precipitates (dark blue precipitate). Due to the formation of insoluble end-products, this method allows a localized detection of cholinesterases. It appeared very well suited for detecting enzyme activity either in polyacrylamide gels, or in immunodotting and immunoblotting experiments (experiments performed with AChE-labelled antibodies). The characteristics of the technique have been established using essentially Electrophorus electricus AChE but it appears also suitable for other cholinesterases. When Electrophorus enzyme is used the method is more sensitive than Koelle's, Karnovsky's and Juul's methods. In addition it provides a rapid coloration and is well suited to routine analysis (single step method).

The second method involves the use of acetylthiocholine as substrate and a mixture of cupric ions and Bicinchoninic acid (BCA) as secondary reagents. Acetylthiocholine hydrolysis leads to the formation of thiocholine which reduces Cu^{++} in Cu^{+} that forms a soluble coloured complex with two molecules of BCA. This is a one step method, the formation of the purple complex being monitored by measuring absorbance at 558 nm, it constitutes an interesting alternative to the Ellman's method. Due to a lower extinction coefficient of Cu^{+}/BCA coordinate compared to reduced DTNB (7600 versus 13600), the method is about two fold less sensitive than the Ellman's method. However, it presents the advantage that measurements are made at a higher wavelength (562 nm versus 412 nm) and can thus be performed in biological media presenting high absorbance below 540 nm.

NON-CHOLINERGIC ACTION OF AChE: SUBCELLULAR TARGET ON SUBSTANTIA NIGRA NEURONES

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A soluble form of acetylcholinesterase (AChE) is released from the dendrites of dopaminergic neurons within the substantia nigra (SN). The released protein subsequently modifies the neuronal activity of the same neurons in a non-cholinergic capacity: electrophysiological studies in vitro showed that AChE can hyperpolarize SN dopaminergic neurons and inhibit their spontaneous firing. The sub-set of neurons particularly sensitive to AChE are characterized by functionally active apical dendrites extending into the pars reticulata and generating a powerful calcium conductance ($LTSgCa^{++}$). This study thus attempted to establish directly the importance of these dendrites regarding the action of AChE. Mid-brain slices were obtained from albino guinea-pigs and intracellular recordings were performed from slices cut at the level of the mamillary body, which level is shown to contain dopamine-immunoreactive cell bodies of the A9 group. Individual slices were placed under a dissection microscope and the pars reticulata (containing the apical dentrites) and crus cerebri on one side was cut away. Segregation of the pars compacta from the pars reticulata did not affect the hyperpolarizing effect of AChE on this sub-set of dopaminergic neurons. However, the ionic basis of the hyperpolarization was related to the integrity of the neurons: AChE caused an opening of potassium channels in intact cells. On the other hand when the pars reticulata containing apical dendrites was removed, an action of AChE involving the closure of calcium/sodium channels was revealed. The results demonstrate that the net effect of AChE need not be related to any particular segment of the dopaminergic neurons, whereas the nature of the mechanism underlying that effect depends on the presence, or otherwise, of the apical dendrites.

NON-CHOLINERGIC ACTION OF ACETYLCHOLINESTERASE IN THE RAT SUBSTANTIA NIGRA: BEHAVIOURAL EFFECTS

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In the substantia nigra a non-cholinergic action of acetylcholinesterase has been demonstrated on motor behaviour. At the cellular level, electrophysiological studies have shown not only excitatory actions of AChE, but also inhibitory effects in response to larger amounts of the protein. There were therefore two aims to this study: firstly to investigate the possible dose-dependent effects of AChE in relation to circling behaviour, and secondly to explore the possible biochemical mechanisms that could underlie it.

Both 'ipsiversive' turning (towards side of infusion and indicative of net decreased activity in the nigrostriatal pathway) and 'contraversive' turning (away from side of infusion and indicative of net increased activity) was observed for at least two weeks following a single unilateral AChE infusion. Ipsiversive turning occurred in 10% of animals irrespective of dose. However, the actual number of animals exhibiting contraversive turning increased with increasing dose, whilst those not responding decreased. The most critical factor for direction of response appeared to be related not to dose but cannula placement; infusion of AChE into more posterior regions of the substantia nigra evoked contraversive circling, whereas there appeared to be a discrete site in the anterior nigra in which AChE induced ipsiversive turning.

When animals received a challenge of the direct dopamine agonist apomorphine, no AChE-induced circling was observed: this result suggested that the phenomenon did not entail a down-regulation of striatal dopamine receptors. On the other hand, a challenge of the dopamine uptake inhibitor nomifensine resulted in AChE-induced circling that was indistinguishable from that seen in the presence of amphetamine: hence the circling behaviour seen could be attributable to an AChE-induced increase in availability of extracellular dopamine. In animals where AChE caused contraversive rotation, indicative of an enhanced activity in the nigrostriatal pathway, there was a significant elevation in the dopamine content of the striatum on the treated side.

It is therefore concluded, firstly, that sub-populations of nigrostriatal neurons show differential responsiveness to AChE, and secondly that AChE can chronically enhance the release of dopamine from the nigrostriatal pathway such that motor behaviour is correspondingly modified, but to an extent sufficently modest to avoid compensatory synaptic reversal mechanisms.

Structurally important residues in the region Ser91 - Asn98 of *Torpedo* acetylcholinesterase.

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Amino acid sequence alignments of cholinesterases reveal a high degree of homology in the region Ser91 to Trp100 (numbering of residues taken from acetylcholinesterase from *Torpedo californica*). This region has been postulated to contain the third amino acid of the catalytic triad in cholinesterases, or to play a structurally important role in these enzymes. The former suggestion has been ruled out by the published three-dimensional structure of acetylcholinesterase from *Torpedo* (3).

We have introduced amino acid replacements at positions 92 (Glu to Gln or Leu) and 93 (Asp to Asn or Val) in acetylcholinesterase from *Torpedo californica* by site-directed mutagenesis. The mutations introduced at position 92 resulted in an inactive enzyme. Replacement of Asp93 with Asn resulted in an enzyme with reduced activity, approximately 15% of wildtype activity.

Inspection of the three-dimensional structure of acetylcholinesterase reveals that Glu92 can interact with Arg44. This interaction could stabilize the loop created by the disulfide bond between Cys67 and Cys94. One of the amino acids, Trp84, in this loop has been identified as part of the choline binding site (2). Arg44 can also interact with Leu266, which is located between two regions (Asn251 to His264 and Lys270 to Glu278) identified as part of the peripheral binding site (1, 4). In the three-dimensional structure Asp93 can interact with Tyr96. This interaction could also help in stabilizing the Cys67-Cys94 loop. The effect of the replacements of Glu92 with Gln or Leu and Asp with Asn or Val can be explained by a destabilization of the Cys67-Cys94 loop.

In conclusion, the obtained results indicates that residues Glu92 and Asp93 have important structural roles in acetylcholinesterase. The function of these amino acids could be to stabilize the loop Cys67-Cys94, thereby keeping Trp84 in a correct orientation in the active site.

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ACETYLCHOLINESTERASE (ACHE) IN ORGANOTYPIC SLICE CULTURES OF VENTRAL MESENCEPHALON AND STRIATUM.

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Organotypic slice cultures have been employed to investigate the growth and development of tyrosine hydroxylase (TH) positive neurons of the ventral mesencephalon (consisting of the ventral tegmental area and substantia nigra) in the presence of their target area - the striatum. Previous research by Østergaard et al. 1990 has demonstrated that TH positive fibres extensively innervate the striatum. We have now discovered a donor age-related difference in the ability of the TH positive neurons to innervate the striatum. A more dense innervation of the striatum occurs in co-cultures of ventral mesencephalon (VM) and striatum perpared from newborn than cultures prepared from seven day old rats. Co-cultures prepared from mutual different donor ages result in an intermediate density of innervation.

This *in vitro* nigro-striatal system is suitable for studying the effects of potential trophic factors. One such factor could be AChE. It is likely to play a role in the developing and mature TH positive nigro-striatal pathway *in vivo*: AChE is expressed in these tissues in an age dependent manner and a soluble, secreted form of the protein plays a non-enzymatic role in the mature substantia nigra (Greenfield, 1991).

We have histochemically identified AChE positive cell bodies and fibres in single cultures of both VM and striatum. The possible trophic effect of exogenous AChE was investigated in co-cultures of tissue from combinations of newborn and seven day old donors by adding AChE at activities of 10 or 100mU/ml to the culture medium. In control experiments 100mU/ml butyrylcholinesterase was added to the medium. Cultures were immunocytochemically stained for TH after six days, eleven days and three and a half week culture durations. Results concerning the rate of TH positive fibre outgrowth and the extent of their innervation of the striatum will be presented.

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A DIRECT ACTION OF ACETYLCHOLINESTERASE ON DOPAMINE OXIDATION

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Acetylcholinesterase (AChE) has a non-cholinergic action in the central nervous system. It has been suggested that this action could involve a direct interaction of the protein molecule and neurotransmitter dopamine. Using cell-free systems it has already been shown that dopamine as well as dopamine quinone could bind covalently to the amino acids and tridecapeptides. Molecules of catecholamine dopamine (in dihydroquinone form) in solution could be easily oxidised by a molecular oxygen: the products semiquinone and quinone are subsequently generated. This process of 'autoxidation' leads to the generation of oxygen free radical species, such as superoxide anion and hydrogen peroxide. The effects of AChE have been studied on the process of dopamine autoxidation in basic solutions. For the detection of quinone formation a spectrophotometric method was applied; in addition, a lucigenin-amplified chemiluminescence as well as luminol-peroxide assay were used to register production of superoxide anion and hydrogen peroxide. AChE inhibited the speed of quinone production, but at the same time the superoxide anion generation rates were increased. The analysis of absorption spectra revealed the formation of a new product, which appeared after interaction of AChE and dopamine in neutral pH. These effects were observed for AChE but no for butyrylcholinesterase. Furthermore purified AChE proved more potent than protein from a commercial source. This study demonstrates a clear interaction between dopamine and AChE and provides a possible system for detecting a non-cholinergic action of preparations. The findings also suggest a possible chemical interaction in the living organism. Since AChE and dopamine are coreleased from the cells degenerated in Parkinson's disease (nigrostriatal cells), a direct chemical interaction between these two molecules could have significance for both normal and pathological neuronal functions.

EMBRYONIC CHOLINESTERASE AS PART OF AN EMBRYONIC MUSCARINIC SYSTEM

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By studying histochemical cholinesterase activity in serial sections of the chick embryo and of embryos of various other species we observed a correlation between expression of cholinesterase and morphogenetic movements. We therefore assumed that a cholinergic system was involved in morphogenesis. Our observations were confirmed and extended by others. In addition to cholinesterase and choline acetyltransferase we demonstrated the presence of muscarinic receptors in the embryonic cells and as a biological reaction the induction of intracellular Ca^{++}-mobilization after muscarinic stimulation. A direct link to cellular movement was provided by the observation of a contraction wave in the chick blastoderm after perfusion with muscarinic agonists.

Here we show by immunocytochemistry with the monoclonal antibody M35 that in most of the areas of the embryo with cholinesterase activity also muscarinic receptors are present. In cell suspensions of the whole embryo and of isolated organ anlagen such as limb bud, heart, gonads and isolated primordial germ cells we demonstrate that M35 immunofluorescence and histochemical cholinesterase activity are localized in the same cells.

We assume that embryonic cholinesterase is part of a muscarinic cholinergic system, and that autocrine sjecretion of the transmitter acetylcholine enables the cells to respond to embryonic inducers or chemotactic agents with pulses of cellular movements.

DIFFERENTIAL TRANSCRIPTIONAL CONTROL OF CHOLINESTERASE GENES IN DEVELOPING MEGAKARYOCYTES

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Committed progenitors of bone-marrow megakaryocytes, the producers of blood platelets, undergo several cycles of nuclear multiplications, leading from promegakaryocytes with up to 8 nuclei, through intermedial cells with 16 nuclei to mature, multinucleated platelet-producing cells with up to 64 nuclei. Acetylcholinesterase (ACHE) activity has been found by cytochemical staining throughout megakaryocytopoiesis in the mouse and is accepted as a specific marker of these cells. Furthermore, antisense oligonucleotides blocking butyrylcholinesterase (BCHE) expression were shown to interfere with murine megakaryocytopoiesis. To study the expression of cholinesterase (CHE) genes at the mRNA level, transcription vectors producing human ACHE- and BCHEcRNA (antisense) and ACHE and BCHEmRNA (sense) were constructed and used for the preparation of [^{35}S]-labeled probes for *in situ* hybridization with bone marrow cell layers from *Sabra* mice. Following emulsion autoradiography, computerized image analysis and statistical data management revealed doubling of ACHEmRNA levels from intermediate (average of 46 grains/cell) to mature, multi-nucleated cells (with 100 grains/cell), which displayed a wide variability range in their labeling intensities. BCHEmRNA expression differed from that of ACHEmRNA both in its level and in its developmental pattern, reaching a steady level of 26-27 grains/cell in both intermediary and mature cells. Both ACHE and BCHEmRNA levels were practically negligible in promegakaryocytes. All other cell types in the bone marrow remained unlabeled under the experimental conditions employed for up to six weeks exposure, and labeling with "sense" ACHE and BCHEmRNA was negligible in all cell types, including megakaryocytes. These findings demonstrate differential transcriptional control for the ACHE and BCHE genes in developing megakaryocytes, and suggest that these two enzymes play developmentally important roles at distinct stages in the process of platelet production.

Differential inhibition of various cholinesterases by N-Methyl carbamates predicts differences in active site groove

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The major breakthroughs in the cholinesterases (CHEs) field - molecular cloning data for primary sequence of important members, and the recently resolved 3D structure of Torpedo acetylcholinesterase (ACHE), have enabled studies of structure-function relationships in these enzymes. Carbamate compounds marked for their CHE inhibition are widely used as therapeutics and as insecticides, therefore many carbamate derivatives have been developed. Groups of closely related carbamate molecules, all derived from a single mother compound and having minor differences, provide an important tool in the understanding of the domains responsible for binding these ligands in various members of the enzyme family. In this study we have examined a set of five N-Methyl carbamates, mostly Carbofuran derivatives, differing mainly in the length and branching of their hydrocarbonic chain. Three representative enzymes have been chosen for comparative inhibition profiles: Human erythrocyte ACHE, Human serum butyrylcholinesterase (BCHE) in its normal form or in a mutant form containing the point mutation Asp_{70}->Gly, and Drosophila nervous system CHE. Carbofuran was more toxic to all three CHEs than any of the other derivatives, over a range of IC50 values which differed in some cases by more than 1000 fold. Drosophila CHE appeared to be more sensitive to all of the examined carbamates than either human enzyme, and H. ACHE was consistently more sensitive than H. BCHE. Morever, inhibition efficiency for H. BCHE decreased systematically more effectively than it did for H. ACHE with increased length and complexity of the side chain, indicating less flexible carbamate binding site in BCHE as compared with ACHE. The point mutation Asp_{70}->Gly had no apparent effect on H. BCHE inhibition by N-methyl carbamates, suggesting that the Asp_{70} domain localized at the rim of the active site groove is not important in carbamate binding. Comparison of the IC50 values calculated for each carbamate with published LD50 values characteristic of each compound demonstrates a pronounced correlation between the in vivo toxicity of carbamates and the inhibition of BCHE by these agents, suggesting a biological in addition to scavenging importance for BCHE in mammals. Pinpointing different domains characteristic of carbamate binding in each member of the CHE family can thus shed light on the variable toxicity of these inhibitors to insects and mammals, predict the toxicity of yet untested inhibitor molecules and help in designing novel and improved CHE inhibitors.

Common and specific actions of acetylcholinesterase inhibitors on endplate currents

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Effects of acetylcholinesterase (AChE) inhibitors of different chemical structure and different mode of action (carbamate derivative, neostigmine; acridine derivatives, tacrine and velnacrine; organophosphorous compounds, armin and phospholine; and a peptide isolated from venom of the snake *Dendroaspis angusticeps*, fasciculine-1) have been compared in experiments on isolated frog cutaneous pectoris neuromuscular preparations. Spontaneous (MEPC) and evoked (EPC) postsynaptic currents were recorded by conventional voltage clamp technique. All inhibitors studied induced an increase in the peak amplitude of postsynaptic currents and a prominent prolongation of their decay. However, these changes in amplitude and decay were not the same for MEPC and EPC and differed in their time courses. Marked differences were observed among inhibitors concerning their actions on ACh-activated channel: some of them exhibited little or no channel blocking action while others were potent channel blockers. The ability to produce "giant" MEPC was common for all agents studied. Thus, the presynaptic effects seem to be independent while postsynaptic ones are strongly dependent on the chemical structure of AChE inhibitors studied.

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COMPARATIVE INVESTIGATION OF INTERACTION OF SOME HYDROPHOBIC ORGANOPHOSPHOROUS INHIBITORS WITH CHOLINESTERASES OF SPRING GRAIN APHID AND WARM-BLOODED ANIMALS

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The present paper is the continuation of our previous investigation of characteristic properties of aphid cholinesterases (ChE) [1-4]. We have studied anti-enzymatic potency of 34 newly synthesized organophosphorous inhibitors (OPI) with varying length of alkyl radicals in the phosphoryl (14 compounds) and thioester (20 compounds) parts of molecule with respect to acetyl-ChE (AChE) and butyryl-ChE (BuChE) from spring grain aphid *Schizaphis gramina Rond.* as well as AChE from human erythrocytes and BuChE from horse serum.

All the OPI studied proved to be irreversible inhibitors of ChEs. Their potency was assessed by the value of bimolecular rate constant of the interaction reaction with enzymes (k_a, $M^{-1}min^{-1}$). Two series of dialkylthiophosphorous acid derivatives: $(C_nH_{2n+1}O)_2P(O)SC_6H_5$ (I) and $(C_nH_{2n+1}O)_2P(O)SCH_2\overline{CHOCH_2}$ (II) inhibited human AChE stronger than aphid AChE, and the difference in k_a values reached three orders with n = 4-6. The change of n from 2 to 6 was accompanied for the both series of compounds by decrease of k_a values for aphid AChE and by their increase in the interaction with human AChE. Difference was observed in the response of aphid AChE and human AChE to the replace of radicals of normal structure by corresponding branched ones. It is indicative of great differences in the structure of hydrophobic areas in the esteratic site region of these enzymes. Apparently for aphid AChE it is considerably more narrow. Compounds of the both series were found to be potent inhibitors of BuChE, the compounds of I series stronger inhibit the horse BuChE and the compounds of II series - the aphid one. The size of hydrophobic area in the esteratic site region of aphid BuChE is considerably smaller as compared to horse BuChE.

Compounds of III series $CH_3C(O)OC_2H_4O(CH_3)P(O)SC_nH_{2n+1}$ and of IV series $(CH_3OC_2H_4O)_2P(O)SC_nH_{2n+1}$ were used to study the hydrophobic areas of the anionic site region. The activity of the III series compounds with respect to human AChE increased up to n = 7 and then dropped, and with respect to aphid AChE it practically did not change up to n = 7 and then dropped sharply. The activity of IV series compounds with respect to aphid AChE practically did not vary up to n = 10, and with respect to the human AChE it increased monotonously with the gain of n. From the data obtained it follows that in the anionic site region the both AChEs have hydrophobic areas of similar length, but aphid AChE remains low sensitive to the variations of thioalkyl radical length. The compounds of both the III and IV series demonstrated similar inhibiting activity with respect to the aphid BuChE and horse BuChE, which is the evidence of similar sizes of hydrophobic areas in the anionic site region of these enzymes.

It is suggested that there are only limited possibilities for the investigation of topography of hydrophobic areas by means of OPI series with regularly varying structure. Based on the study of the OPI and ChE interaction mechanism the conception of agreement in the dipole-dipole, ion-dipole, ion-ion and hydrophobic interaction was developed.

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CHARACTERIZATION OF *PSEUDOMONAS FLUORESCENS* CHOLINESTERASE

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Pseudomonas fluorescens (Goldstein' s strain, ATCC 11 150) was cultivated for 48 h at 30°C in the presence of 0.2 % of choline chloride as a cholinesterase inducer. Cholinesterase was purified in three steps by ammonium sulfate precipitation of bacterial proteins, ion-exchange chromatography at pH 7.0 on a DEAE-containing matrix and affinity chromatography on procainamide gel.

The pI of the enzyme was found to be 6.06 and its apparent molecular weight was 45 kD as determined by non-denaturing gel electrophoresis. On SDS-electrophoresis in the presence of β -mercaptoethanol, the [^{3} H] DFP-labeled enzyme had a molecular weight of 40 kD.

The cholinesterase activity at pH 7.0 and 20°C was maximun with propionylthiocholine (PrSCh). Activity toward other thiocholine esters was lower (ASCh > BuSCh). Kinetics of PrSCh hydrolysis showed inhibition at high substrate concentration. Below 0.5 mM the enzyme displayed Michaelian behavior with Km= 20 µM. The enzyme was irreversibly inhibited by organophosphates.

Hybridization experiments of plasmid and chromosome DNA were performed at 41 and 51°C with various ^{32} P-labeled probes (cDNA of *Torpedo* AChE,cDNA of human BuChE, a cDNA fragment of *Geotrichum candidum* lipase, and several oligonucleotides corresponding to strongly conserved peptides). Southern blot analysis did not show clear bands, suggesting that there is little or no DNA sequence homology between *Pseudomonas fluorescens* cholinesterase and the family of cholinesterases and related proteins.

IS THE NON-CHOLINERGIC EFFECT OF ACETYLCHOLINESTERASE IN THE SUBSTANTIA NIGRA MEDIATED BY DOPAMINE?

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Within the substantia nigra acetylcholinesterase (AChE) has non-cholinergic actions. A soluble form of the enzyme is released from the dopaminergic pars compacta cells and is postulated to have a local neuromodulatory role within the nigra. This role may involve an interaction with dopamine: AChE is co-localised with dopamine in similar subcellular storage sites within the nigrostriatal neurones, and both are released within the substantia nigra from the dendrites of the cells. Indeed the dendritic release of dopamine and AChE share many common features, both being released under the same ionic and pharmacological conditions. In addition, both neurochemicals have a hyperpolarising effect on the membrane of the nigrostriatal cells. The aim of this study was to determine whether the action of AChE on cell membrane properties *in vitro* persists following pharmacological depletion of dopamine.

Disruption of dopamine storage was achieved by the administration of reserpine to guinea pigs 16h prior to the experiment, whilst *de novo* synthesis of the transmitter was suppressed by addition of 100uM alpha-methylparatyrosine in the bathing and perfusing solutions. Intracellular recording was performed on coronal mesencephalic slices containing the substantia nigra.

The hyperpolarising action of AChE was found to persist in the absence of dopamine. Six out of eight cells from dopamine-depleted animals responded to AChE: mean response - 21.2mV, average decrease in input resistance approx 50%. These results did not differ significantly from those obtained from controls (n=8). In both test and control cells, the reversal potential obtained from the current-voltage relationship of the cells show the action of AChE to be due to the selective opening of potassium channels.

In conclusion, the presence of dopamine does not seem to be a prerequisite for the electrophysiological action of AChE. However, this does not preclude an interaction between the two neurochemicals in ways not directly influencing membrane properties.

MODULATION OF CATALYTIC ACTIVITY OF HUMAN ACETYLCHOLINESTERASE BY MUTATION OF ASP74

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Human acetylcholinesterase (HuAChE) carries at position 74 an Asp residue which is conserved in all sequenced vertebrate ChEs. Natural mutation of this Asp residue in butyrylcholinesterase (Asp70->Gly) is associated with the atypical phenotype (lower affinity to quarternary amine inhibitors e.g. succinylcholine and dibucaine) (Neville et al.; 1990, J. Biol. Chem. 265:20735, La Du et al.; 1990, Clin. Biochem. 23:423).
Using site directed mutagenesis we substituted the Asp74 in HuAChE with negatively (Glu), neutral (Gly and Asn) and positively (Lys) charged amino acids. These mutations had no significant effect on AChE polypeptide production. The mutated enzymes retained their ability to hydrolyse acetylcholine and exhibited wild type substrate selectivity. However, like atypical BuChE, the generated Asp mutants (with the exception of Asp74->Glu), acquired increased resistance (10-1000 fold) to succinylcholine or dibucaine. Moreover, the Gly, Asn and Lys substitutions led to a decrease in substrate inhibition, lower affinity to acetylcholine, propidium, edrophonium as well as to the selective AChE inhibitor BW284C51 (1,5-bis (4-allyldimethylammoniumphenyl)pentan-3-one dibromide).
The pleiotrophic effects of mutations at position 74 together with the 3-D structural data (Sussman et al., 1991, Science 253:872-879) indicate that the surface exposed Asp74 is not a part of the catalytic anionic site. We propose that this residue is part of a structural element in ChE involved in transducing allosteric signals e.g. substrate inhibition that affect the internal architecture of the active site gorge. (Supported by US Army Medical Research Development Command Contract DAMD 17-89-C-9117).

HUMAN BUTYRYLCHOLINESTERASE: A UNIVERSAL PROPHYLACTIC ANTIDOTE AGAINST ORGANOPHOSPHATE POISONING

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Butyrylcholinesterase purified from human plasma (HuBChE) was evaluated as a single prophylactic drug adequate alone to protect against sarin, soman, tabun and VX in mice, rats, guinea pigs and rhesus monkeys. The second-order rate constants for the inhibition of HuBCHE by these OPs taken together with the stoichiometry established from *in vitro* titrations, indicated that this enzyme is a suitable scavenger capable of rapid sequestration of broad spectrum of nerve agent toxicants. Time course studies following an intravenous and extravascular injections of HuBChE, clearly indicated, in all four species, that 50-80% of the administered dose was recovered in the blood after an im injection of the enzyme. Depending on the species used, the time (t{max}) to reach maximum concentration of HuBChE in the circulation following an im injection was 9-20 hr and the half-life time of the elimination of blood-enzyme activity was 20-60 hr. *In vivo* inhibition of exogenously administered HuBChE by sequential injections of either sarin, soman, tabun or VX gave an almost linear titration curves in all cases. Pretreatment of animals with HuBChE provided excellent protection against iv bolus injections of multiple median lethal doses of all nerve agent tested, without the need for post-challenge treatment. In addition, a remarkable protection against inhalation of soman vapors was conferred to guinea pigs by pretreatment with HuBChE only. In all species studied, the extent of protection could be correlated with the level of circulating HuBChE, suggesting a safe and reliable extrapolation of antidotal efficacy from animals to human. In as much as physiological and immunological compatibilities, reactivity, specificity and pharmacokinetic properties are concerned, HuBChE appears to be a most promising drug capable not only to protect against OP poisoning but also to alleviate post-exposure symptoms.

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Cholinesterase Revisited

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New work using a kit for portable real-time measurements of cholinesterase (ChE) indicate that (1) plasma ChE is more sensitive than Rbc ChE for detecting low level exposures, (2) field supervisors' pre-season estimates of in-season exposures can be extremely reliable, (3) precise information on timetable of exposures of individual workers frequently explains apparent paradoxes and contradictory findings, (4) green house cutters and pickers have greater depressions than sprayers, and may be experiencing more illness, (5) greenhouse workers appear to have a permanent year round depression in ChE, observable even in between spraying periods and (6) complaints (fatigue, headache, dizziness, loss of appetite, and sleep problems) occur in greenhouse workers with depressions in PChE or RBCChe frequently less than 40 50%. Field reports of reduction in toxic effects from exposure in cotton workers appear to result less from personal protection and more from trends towards reduced use of organophosphates, particularly parathion, in the last decade. In moshav greenhouse workers, however, work and exposures appear to involve substantial risks, not only for sprayers and pickers, but migrant workers; the situation with regard to children living closeby needs evaluation. The most important benefits from field real-time ChE testing are: (1) providing infomation on exposure effects when it is needed and (2) activating right-to-know according to both the spirit and letter of the regulation.

MELITTIN INHIBITS CHOLINESTERASES

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Many cationic amphipatic compounds are potent inhibitors of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). It was of interest to study the effect of cationic amphipatic peptides on the enzymes.

To this end we studied the interaction between melittin and AChE (eel) and BChE (bovine serum).

Melittin is a bee venom lytic peptide composed of 26 amino acid residues, the sequence of which is

$$\overset{+}{\text{G}}\text{IGAVLKVLTTGLPALIS}\overset{+}{\text{W}}\overset{+}{\text{I}}\overset{+}{\text{K}}\overset{+}{\text{R}}\text{KRQQ-NH}_2$$

The peptide contains many hydropholic amino acid residues, and 4 neighboring positively charged amino acid residues are located near the C terminus of the molecule.

When melittin was present in the reaction solution of AChE or BChE a pronounced inhibition of reaction was noticed.

Analysis of the reaction kinetics indicated that competitive inhibition took place with K_I = 1.5μM and 2.5μM for BChE and AChE, respectively. When calmodulin was added to the enzyme-melittin solution no inhibition took place. CaM is known to bind melittin very tightly (K_D ~10nM), and so CaM "extracts" melittin from the ChE-melittin complex.

Addition of trypsin to the melittin containing reaction solution eliminated the inhibitory effect of the peptide. As trypsin digests melittin at lysyl and arginyl residues, the results suggests that the integrity of the peptide due to its amphipatic nature is essential for exerting inhibition.

The interaction of other amphipatic peptides with cholinesterases is under investigation.

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NOVEL MECHANISM OF BRAIN ACETYLCHOLINESTERASE INHIBITION BY A PIPERIDINE, E2020. Kathleen A. Sherman, Dept. Pharmacology, Southern Illinois Univ. School of Medicine, Springfield, IL, USA, 62794-9230

E2020 (a piperidine derivative) is a novel type of centrally-active inhibitor which *selectively* acts on acetylcholinesterase (AChE) and prevents its phosphorylation by diisopropyl-fluorophosphate (DFP). Like tacrine (tetrahydroaminoacridine, THA), E2020 binds *reversibly* to AChE; consequently AChE inhibition measured *ex vivo* after systemic drug is diminished proportionately to the log of tissue dilution. Both drugs inhibit AChE by a predominantly non-competitive mechanism at lower substrate concentrations. However, in rats and humans, a marked distinction between E2020 and THA is shown with regard to the selectivity of cholinesterase (ChE) inhibition *in vivo*. In dementia patients, oral E2020 results in dose-dependent antagonism of phosphorylation of red blood cell (RBC) AChE by DFP, which increases markedly as treatment continues to 31.9 ± 3.0% after 1 month on 2 mg/day (range: 20 - 43%); plasma ChE is unaffected. By contrast, acute oral THA (40 mg) markedly inhibits plasma ChE, by 39 ± 2.5% after 1½ to 2 hours (range: 29 -55%). The effect on RBC AChE is less and quite variable (18.7 ± 3%.0, range: 6 - 43%), but closely correlated with plasma inhibition above a threshold of ~22% ChE inhibition. In rats, a dose of E2020 (2 mg/kg, s.c.) producing little sign of peripheral cholinergic hyperactivity, blocks phosphorylation of AChE in cortex by more than 90% for up to 3 hours and markedly slows *ex vivo* ACh hydrolysis (by 70% at 5-fold dilution). Inhibition of ACh hydrolysis in rat plasma is less and almost entirely due to inhibition of AChE (76% reduction of 10 μM BW284C51-sensitive activity vs. 8% decrease of non-specific ChE). E2020 concentrations which produce 60-90% inhibition of homogenate AChE (0.1 - 3.0 μM) dose-dependently potentiate depolarization-induced stimulation of phosphatidylinositol turnover in cortical slices (up to 2-fold), consistent with preservation of released ACh. THA, E2020, and several other candidates for Alzheimer therapy, are distinct from well-known classes of AChE inhibitors which covalently modify the catalytic site (e.g. carbamates), or compete with ACh binding. Yet E2020 is enzyme selective and identification of the binding site by which E2020 inhibits AChE and slows phosphorylation may further understanding of molecular mechanisms of AChE regulation and its distinction from related esterases.

RAPID POSTDENERVATION DECREASE OF THE A12 ACETYLCHOLINESTERASE FORM IN THE MOTOR ENDPLATES IS NOT DUE TO MUSCLE INACTIVITY

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Changes of the pattern of molecular forms of acetylcholinesterase (AChE) in the junctional and extrajunctional regions of rat muscles were followed after the neuromuscular transmission had been blocked by local application of botulinum toxin type A (BoTx). The velocity sedimentation analysis of AChE molecular forms in the fast extensor digitorum longus muscle (EDL) and in the slow soleus muscle (SOL) was carried out on days 4, 8 and 14 after induction of paralysis and compared to the changes observed after denervation of these muscles.

The extrajunctional molecular forms of AChE in the BoTX paralysed muscles displayed virtually identical changes as those in the denervated muscles. A dramatic loss of the 4 S (G1) AChE form and a temporary predominance of the 10 S (G4) form occured in the EDL. On the contrary, no major changes in the pattern of AChE molecular forms were observed in the extrajunctional regions of the SOL muscle except that the activity of all the forms decreased. It seems that muscle activity triggered by phasic neural stimulation pattern critically affects the metabolism of different AChE molecular forms extrajunctionally in the fast EDL muscle, and that the regulation of the same AChE forms in the slow SOL is significantly different.

On the other hand, major differences were observed between the effects of BoTx treatment and nerve section on AChE in the junctional region of both EDL and SOL muscles. The precipitous drop of the asymmetric 16 S (A12) AChE form which occured immediately after denervation in both muscles was much less expressed in the BoTx paralysed muscles. Although the junctional A12 AChE activity per muscle decreased, its specific activity was close to normal even in muscles paralysed by BoTx for two weeks because of increasing muscle atrophy. By irreversibly inhibiting the preexistent AChE at the onset of paralysis we showed, however, that only a minor part of this junctional AChE activity and its A12 form were due to to the enzyme synthesized de novo in the paralysed muscles. The results support the hypothesis that a greater part of junctional A12 AChE pertains to a fraction which is rapidly degraded after denervation but is largely preserved after BoTx produced muscle paralysis, probably due to the fact that the nerve ending, although rendered unfunctional, is not destroyed.

THE FUNCTIONAL LINK BETWEEN ACETYLCHOLINESTERASE ACTIVITY AND DOPAMINERGIC FUNCTION OF THE STRIATUM

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The influence of inhibition and subsequent restoration of enzymic activity and of reappearance of subcellular localization of acetylcholinesterase (AChE) on dopaminergic function of the striatum was studied in rats. Brain AChE was submaximally inhibited with soman (peripheral AChE was partially protected by HI-6) and the effect of inhibition of the enzyme was estimated using behavioral analysis based on amphetamine-induced sterotyped behavior. The onset of inhibition of AChE markedly prolonged the latency of appearance of amphetamine-induced sterotyped sniffing. With a gradual restoration of AChE activity the inhibitory influence on stereotyped sniffing disappeared in three to four days in parallel with only about 15-20% regeneration of AChE activity in striatal homogenates. Furthermore, cytochemistry and electron microscopic analysis of ultrastructural enzyme localization has revealed that the attenuation of the inhibitory effect on amphetamine induced behavior coincides with the reappearance of AChE affiliated with plasmalemma of dendrites and axon terminals of the striatum. This observation suggests that the restoration of AChE on strategic intercellular location may be responsible for the above effect, rather than the recovery of total AChE activity.

EARLY POSTNATAL ACETYLCHOLINESTERASE FOCALIZATION AND DIFFERENTIATION OF SUBSYNAPTIC SARCOLEMMA IN THE ABSENCE OF INNERVATION

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Early in muscle development the neural contact induces permanent changes resulting, among others, in the initial expression and maturation of phenotype characteristics of subsynaptic sarcolemma and focal distribution of AChE. These characteristics are then maintained even in the absence of nerve in denervated adult muscles. However, it is not clear whether the above nerve induced phenotype characteristics become permanently fixed already in immature muscle cells or whether, at that stage, a sustained nerve contact is still essential for their maintenance and further development until final maturation.

Therefore, AChE focalization and the formation of synaptic sarcolemmal specializations was followed in aneurally developing soleus and extensor digitorum longus muscles and compared with innervated controls of the same age. The muscles of newborn rats were denervated two days *post partum* and examined during the next 4 weeks in regard to AChE distribution in whole muscles, its ultrastructural localization and the developmental stage of the subsynaptic sarcolemma.

At the time of denervation, AChE was concentrated at the neuromuscular and myotendinous junctions in both types of muscles. The neuromuscular junctions were still in the initial stages of development exibiting scarce atypical secondary synaptic clefts. Therefore, possible subsequent differentiation of the subsynaptic sarcolemma can be easily observed. The examination of denervated muscles at later stages showed clearly that both the site-selective accumulation of AChE at previous neural contacts and the formation of sarcolemmal invaginations, resembling somewhat atypical secondary postsynaptic clefts, persisted throughout that period in spite of considerable muscle atrophy and degeneration. In contrast, AChE at the myotendinous junctions was lost shortly after denervation. The results suggest the persistence in the immature denervated muscle fibers of nerve induced expression of regulatory mechanisms responsible for AChE transport and focalization at the former subsynaptic sarcolemma and for subsynaptic sarcolemmal differentiation. The loss of AChE at the myotendinous junctions in denervated muscles suggests that this compartment of AChE may be controlled by muscle activity.

IS THE NON-CHOLINERGIC EFFECT OF ACETYLCHOLINESTERASE IN THE SUBSTANTIA NIGRA MEDIATED BY AN ATP-SENSITIVE POTASSIUM CHANNEL?

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In the substantia nigra there are disproportionately large amounts of AChE relative to choline acetyltransferase (ChAT). A soluble form of AChE is released from dopaminergic nigral neurons. Indeed extracellular acetylcholinesterase in this brain region has non-cholinergic actions that can be demonstrated at both behavioural and cellular levels: the aim of this study was thus to explore, in the in vitro guinea pig substantia nigra, the ionic mechanisms which mediate these non-classical phenomena. Acetylcholinesterase had a reversible hyperpolarizing action, via an opening of potassium channels, on a selective population of nigral neurons. These neurons could be characterized by an ability to generate bursts of action potentials and by a sensitivity to amphetamine and a reduction of glucose in the perfusing medium. The acetylcholinesterase-induced hyperpolarization could not be attributed to a contaminant in the exogenous solution, since a highly purified preparation of 125 times less activity towards acetylcholine, was even more potent. Furthermore, enzymatic action of any kind could be eliminated as boiled acetylcholinesterase was equally efficacious. The effect of acetylcholinesterase was not subject to tachyphylaxis and was resistant to blockade of potassium channels with tetraethylammonium. Since both these phenomena are features of the dopamine autoreceptor it seems unlikely that acetycholinesterase is operating on the same target as dendritically released local dopamine. On the other hand, the actions of acetylcholinesterase were enhanced by low glucose and blocked by the sulfonylurea, tolbutamide. In the substantia nigra there is a high density of K^+ channels which are sensitive to reduced ATP and low glucose but blocked by sulfonylureas (the K^+-ATP channel). Hence these results strongly suggest that the non-enzymatic, non-cholinergic action of acetylcholinesterase in the substantia nigra is mediated by these K-ATP channels.

Altered Forms of Acetylcholinesterase in Insecticide-Resistant Houseflies *(Musca domestica)*

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Intensive use of organophosphate (OP) and carbamate insecticides has led to the selection of resistance in many insect species, and this is often associated with altered forms of their target enzyme, acetylcholinsterase (AChE), exhibiting reduced sensitivity to inhibition.

In the housefly, we have identified several distinct forms of AChE with different profiles of insensitivity to a range of OP inhibitors. The AChE-R genotypes of individual insects can be rapidly determined using a colorimetric assay for AChE activity in the presence/absence of specific OP inhibitors with a kinetic microtiter plate reader. Bivariate plots of mean percentage remaining activity with two inhibitors then allows homozygous susceptible and resistant genotypes and their heterozygotes to be easily distinguished. An example of this analysis will be presented.

We are now studying the structural changes responsible for these different forms of insensitivity through the cloning of gene sequences encoding the variant AChE proteins. Using a PCR-based approach, a small fragment (0.5 kb) of the housefly gene was amplified directly from genomic DNA. This fragment was used as a probe to screen a cDNA library from susceptible insects and led to the isolation of a cDNA containing *ca* 80% of the coding sequence for the housefly AChE protein, including the full sequence of the 'catalytic' polypeptide. Primers derived from this sequence are now being used to amplify the corresponding regions of the genes from fly strains with insensitive AChE.

THE EFFECT OF THE MONOCLONAL ANTIBODY (MAB) AE-2 ON INHIBITION OF FETAL BOVINE SERUM ACETYLCHOLINESTERASE (FBS ACHE) BY ORGANOPHOSPHATES (OPS) AND CARBAMATES (CBS)

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The mAb AE-2 forms a complex with FBS AChE, maximally inhibiting the rate of acetylthiocholine (ATC) hydrolysis by 80% while increasing the rate of indophenyl acetate (IPA) hydrolysis 15 fold [Wolfe, BBA 997, 232 (1989)]. The complex is dissociable by dilution, or by incubation with 2-PAM or edrophonium. It was suggested that AE-2 was affecting the active center anionic site [Sorensen et al, Biochim. Biophys. Acta 912, 56, (1987); Wolfe (1989)]. Modulation of substrate hydrolysis by AE-2 was considered non-competitive since the Kms for both ATC and IPA hydrolysis remained constant, in contrast to velocity changes. Treatment of AChE with N-bromosuccinimide, a reagent which preferentially reacts with the aromatic amino acids tyrosine and tryptophan, increased IPA hydrolysis while ATC hydrolysis was reduced. Similar changes in AChE substrate hydrolysis have been shown to occur through treatment of AChE with aziridinium ions [Belleau and Tani, Mol. Pharm. 2, 411 (1966); O'Brien, Biochem. J. 113, 713 (1969)], which modify tryptophan 84 in the amino acid sequence 79-87 of Torpedo californica AChE [Kreienkamp et al, Proc. Nat. Acad. Sci. U.S., 88, 6117, (1991)]. This modification is prevented by edrophonium. Peptide competition experiments had previously suggested that one of the AE-2 binding sites is located between amino acid residues 53-84 (Doctor et al, Proc. 32nd Oholo Conference, 1988). Thus mAbs, amino acid specific reagents and alkylating agents, appear to react with a specific region of AChE to differentially modulate substrate hydrolysis.

The effect of alkylating agents on AChE is not limited to modulation of ATC and IPA hydrolysis, but the potency of OPs and CBs with respect to inhibition of AChE hydrolysis of IPA, in particular, is also altered (O'Brien, 1969]. Thus the bimolecular rate constant for the acid form of amiton, (S-[2-(Diethylamino)ethyl]-phosphorothioic acid, O,O-diethyl ester) was reduced nearly one-thousand fold by alkylation of AChE; inhibition of AChE by other OPs, and CBs as well, was also decreased. We have evaluated the potency of specific OPs and CBs on FBS AChE activity in the presence and absence of AE-2, and have found that AE-2 also modulates enzyme inhibition, principally as a function of the charge on the inhibitor. Thus amiton inhibition of FBS AChE hydrolysis of both ATC and IPA was reduced more than 20 fold by AE-2. AE-2 also significantly reduced inhibition of FBS AChE by both neostigmine and pyridostigmine. Inhibitors lacking a positive charge tended to act more strongly in the presence of FBS AChE:AE-2 than in the presence of FBS AChE alone. Results suggest that AE-2 reduces the effect of a nucleophilic site in the active center of the enzyme in such a manner as to change FBS AChE specificity with respect to positively charged substrates and inhibitors.

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