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13. ABSTRACT (Maximum 200 words)

This is a final report submitted to effect a change of institution by the author. The report reflects progress at Baylor College of Medicine on the project which is being continued at The University of Texas Medical Branch.

The human neuronal GABA transporter, hGAT-1, has been cloned and functionally expressed in mouse fibroblasts. The cloned transporter exhibits all of the properties anticipated based on studies done with the rodent transporter. Chemicals that inhibit GABA transport are potent anticonvulsants that many have utility as counter measures against the convulsive effects of organophosphorus anticholinesterases.

In order to elucidate the molecular mechanism of GABA transport and pharmacological inhibition of the transporter, we have constructed mutations to probe structural features thought to be important to function. These include the hydrophilic portions of the amphipathic helices, evolutionarily conserved peptide sequences, and sites of N-linked glycosylation.

The effect of these mutations is currently being studied by expression in mouse fibroblasts. Early results indicated that Ser-543 and Tyr-551 of the transmembrane helix XII are important to function. Elimination of potential N-linked glycosylation sites at positions 176, 181, and 184 does not totally abolish transport function.

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STRUCTURE-FUNCTION RELATIONSHIPS IN THE GABA TRANSPORTER: Studies on a Presynaptic Anticonvulsant Binding Site

FINAL REPORT

AUTHOR: Dr. Steven C. King

April 8, 1994

U.S. ARMY RESEARCH OFFICE

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1. FOREWARD

The following is an abbreviated "final report" prepared in order to effect a change of institution by the investigator (Dr. Steven C. King) who has moved from Baylor College of Medicine to the University of Texas Medical Branch where the research project is ongoing. This report is referenced to Funding Document DAAL03-92-G-0285 for the period September 30, 1992 through Semptember 29, 1993. The report is a summary of results obtained by Dr. Steven C. King during first year of funding by the ARO Young Investigator Program (30274-LS-YIP).

2. TABLE OF CONTENTS none

3. LIST OF APPENDIXES, ILLUSTRATIONS, AND TABLES none

4. BODY OF REPORT

A. STATEMENT OF PROBLEM STUDIED. Synaptic neurotransmission can be mediated by many low molecular weight neurotransmitters (e.g., serotonin, norepinephrine, dopamine, glycine, glutamate, GABA) which upon release from presynaptic nerve terminals diffuse across the synapse to activate postsynaptic receptors (Kanner & Schuldiner, 1987). Communication across the synapse is terminated via high-affinity neurotransmitter reuptake proteins found in the plasma membrane of presynaptic nerve teminals. The availability of inhibitors specific for many of these transporters affords the opportunity to increase the flow of information across synapses utilizing these transmitters.

GABA (γ-aminobutyric acid) is the major inhibitory neurotransmitter in the mammalian brain. Augmentation of GABAergic inhibitory pathways via blockade of the high-affinity GABA transporter is known to be efficacious for the treatment of chemically-induced seizures (Braestrup et al., 1990). Seizures are an important manifestation of central nervous system toxicity following exposure to organophosphorous cholinesterase inhibitors (Dunn & Sidell, 1989). Thus, in the context of developing countermeasures against organophosphorous intoxication, the GABA transporter is of interest as novel anticonvulsant target. Drugs acting to inhibit the GABA transporter are fundamentally different from other anticonvulsants in that they are neither general membrane stabilizing agents nor ligands that mediate global activation of a postsynaptic receptors. Instead inhibitors of GABA transport are expected to exert their actions only on selected synapses at which there is endogenous GABA release. Thus, the toxicity and spectrum of limiting side-effects are likely to differ from conventional anticonvulsant agents.

The GABA transporter has in recent years been cloned from rat, mouse, and human sources (Guastella et al., 1990). Cloning has enabled us to study structural aspects of the human GABA transporter by site-directed mutagenesis. The long-term goals of this research are 1) to identify those amino acid residues which constitute the binding site for anticonvulsants, and 2) to identify amino acid residues that are crucial to transport function. Inasmuch as the anticonvulsants and/or substrates (GABA, sodium, and chloride) contain charged or polar groups, it is anticipated that the binding pockets for these ligands would likewise be composed of polar residues able to hydrogen bond to the ligands and essentially "solubilize" them during transit across the nonpolar environment of the lipid bilayer. We have thus targeted for mutagenesis those polar residues of the GABA transporter which are predicted to reside within the hydrophobic (transmembrane) domain. Through this approach it should be possible to initially distinguish "unimportant" residues from those potentially playing a critical role in GABA transport and anticonvulsant binding.

B. SUMMARY OF MOST IMPORTANT RESULTS.

Fundamental Properties. Starting with a cDNA library prepared from the substantia nigra region of human brain, we have isolated and determined the sequence of full length clone (hGAT-1) which encodes a human GABA transporter. This hGAT-1 cDNA has been stably expressed under control of a glucocorticoid response element. GABA transport activity is inducible by growth in medium containing dexamethasone. The non-induced mouse L-M fibroblast host cells are spared any metabolic stress associated with constitutive expression in long term culture. One such inducible cell line, hΩGAT-1, was cloned and characterized.

Following induction with dexamethasone, the GABA transport activity of hΩGAT-1 was found to be of high-affinity ($K_m = 3.5 \pm 0.097$ μM). GABA transport activity depended upon the presence of both Na^+ and Cl^-. The interaction with Na^+ exhibited positive cooperativity, whereas the Cl^- interaction was hyperbolic.

The GABA transporter in hΩGAT-1 was sensitive to a range inhibitors including NO-711, nipecotic acid, and THPO; their respective IC_{50} values were 0.2 μM, 10 μM, and 500 μM. Additionally, it was observed that the mechanism of NO-711 could be converted from competitive to non-competitive by preincubation of cells with this compound. The potency of NO-711 was also increased 10-fold by preincubation.

Although these properties taken as a whole are substantially in accord with previous literature concerning the rat brain GABA transporter, the present work contributes several novel observations: i) stable fibroblast cell lines engineered to bring transport under control of an inducible glucocorticoid response element affords a homogeneous and highly reproducible model system for analysis of drug mechanism and potency against human GABA transporter; ii) the Na^+:GABA stoichiometry appears to be greater than unity which contrasts with previous work utilizing human brain synaptosomes; iii) the pharmacology of the human GABA transporter is consistent with its being of neural origin; and iv) non-competitive inhibition of GABA transport by substrate analogs is probably an intrinsic property of the carrier rather than an artifact arising from synaptosomal heterogeneity.

Thus the fibroblast expression system appears to be ideally suited to the purpose of assaying the effect of mutagenesis on the functional properties of the human neuronal GABA transporter, hGAT-1.

Amphipathic Helix XII. The GABA transporter must contain within it a pathway for the transmembrane passage of GABA, Na^+, and Cl^-. We have concentrated our mutagenesis efforts on those residues judged to have the greatest potential to participate in channel formation.

In order to identify amino acid residues involved in forming the putative transmembrane solute conduit of the human GABA transporter we have begun to systematically engineer isosteric replacements for the hydrophillic amino acid side chains on amphipathic helices—i.e., those helices having both a polar and a nonpolar surface. Such helices would have maximum stability at the interface between bilayer lipid and water. The following briefly describes the effect of eliminating the polar hydroxyl groups from amphipathic helix XII of the human GABA transporter.

Putative transmembrane alpha helix XII consists of 20 amino acid residues running from Val-536 to Phe-556. All of the residues except four—Ser-543, Ser-544, Tyr-551, and Tyr-554—are nonpolar and are thus incapable of participating in the kinds of hydrogen bonding interactions that are anticipated to be of importance for the binding and/or translocation of highly polar groups found on molecules such as GABA and its anticonvulsant analogs. In order to ask whether these four hydroxyl groups of Helix XII are of mechanistic significance, we have engineered for separate mutations in which serine residues have been replaced by alanine and tyrosine residues have been replaced by phenylalanine. These substitutions eliminate the hydroxyl function while preserving much of the side chain bulk.

Functional Effects. The functional effect of these amino acid substitutions was assessed by measuring GABA transport parameters in stably transfected mouse L-M fibroblasts. The L-M

fibroblast, being non-neuronal, has a very low level of endogenous GABA transport activity. Transfection of the L-M host cell with human GABA transporter cDNA cloned into the expression vector pJ5Ω leads to the appearance of a high-affinity GABA transport activity having an apparent K_m value of 4 μM. and V_{max} value of 220 pmoles/min/mg protein. Kinetic analysis of the mutant transporter suggested that the hydroxyl groups on Ser-544 and Tyr-554 are essential for activity. Cells transfected with plasmids harboring these mutants failed to transport GABA any better than the host L-M fibroblasts.

Removal of the hydroxyl function at either Ser-543 or Tyr-551 results in a defective level of GABA transport activity. Kinetic analysis suggested that the mutant cell lines have a defective V_{max} value, approximately 20 percent that of the parent. Although the V_{max} value is sufficient to explain the defect in these cell lines, it is worth noting that these mutants display increased "affinity" for the substrate, GABA (K_m values around 1 μM). These mutant may therefore reveal in the GABA transporter an inverse relationship between catalytic turnover and substrate binding affinity. The sacrifice of substrate binding energy to achieve higher turnover numbers is a characteristic feature of many catalytic systems; the present data are thus consistent with the possibility that nature has adopted a similar strategy to optimize rate of neurotransmitter accumulation.

Evolutionarily Conserved Amino Acids. It has become clear that the neuronal GABA tranporter (i.e., GAT-1) is one member from a large family of neurotransmitter cotransport proteins. There is a particular amino acid sequence (VWRFPYLCGKNGGGAFLIPY) that has been conserved in the GABA, norepinephrine, dopamine, and serotonin transporters. This peptide connects transmembrane helices I and II of the human neuronal GABA transporter (GAT-1). It is possible that this peptide plays a crucial role in the orientation of these helices relative to one another. If so, we would anticipate that the proline residues would have a direct role. Accordingly, we have engineered substitutions (Glycine) for the proline residues in this peptide. We expect to examine the effects of this substitution in the following year.

Sites for N-linked Glycosylation. The GABA transporter is a glycoprotein. Glycosylation is found most frequently on asparagine residues that occur in either of two motifs, Asn-X-Ser or Asn-X-Thr. Potential sites for N-linked glycosylation occur at Asn-176, Asn-181, and Asn-184 in the human GABA transporter (GAT-1). It is known that GABA transport is impaired when cells are treated with either glycosidases or with inhibitors of glycosylation. These treatments could be effective either i) because GABA transporter glycosylation is essential to function or ii) because the global effects of these treatments affect a cellular process required for functional expression of GABA transport activity. Use of mutagenesis to genetically remove glycosylation sites will provide a means for highly selective deglycosylation as well as very specific information regarding the sites of sugar attachment to the transporter. We have created three separate mutations which replace either Asn-176, Asn-181, or Asn-184 with Gln.

Functional Effects of Deglycosylation. None of these mutations appear to completely abolish [^{3}H]GABA transport as assessed in transfected mouse fibroblasts. Transport appears to be about 20%-50% of normal . The results must be considered preliminary until biochemical studies can be done to determine the extent to which any of these mutations may have "deglycosylated" the transporter. It will be necessary to determine whether more than one of the N-linked glycosylation sites is actually utilized and whether there might also be other sites of glycosylation (e.g., O-linked sugars). It should be possible to do this by comparing the mobility (SDS-PAGE) of enzymatically deglycosylated transporter to that of untreated mutant transporters. We anticipate being able to perform these studies in the next year.

C. LIST OF PUBLICATIONS. none

D. LIST OF PARTICIPATING SCIENTIFIC PERSONNEL. Steven C. King

5. REPORT OF INVENTIONS

There have been no inventions

6. BIBLIOGRAPHY

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7. APPENDIXES

There is no appended material