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Assembly, Stability and Secretion of Acetylcholinesterase in Skeletal Muscle Fibres

by

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Abstract

Acetylcholinesterase (AChE) serves a vital role in modulating cholinergic neurotransmission by its function of acetylcholine hydrolysis. The AChE gene encodes three mRNAs; R, H and T differing in their choice of C-terminal exons. Of these, $AChE_T$ is exclusively accumulated in the synaptic compartment of adult skeletal muscle fibres. The mechanisms underlying the tissue-specific expression of the T forms as well the targeting of AChE to the neuromuscular junction have yet to be elucidated. To this end, we examined factors influencing the expression of AChE by transfection of C_2C_{12} muscle cells and direct gene transfer in rodent hindlimb muscles.

Transfection of C_2C_{12} muscle cells with cDNAs encoding H ($AChE_H$) and T ($AChE_T$) resulted in high levels of enzyme activity. The H non-muscle subunit was preferentially expressed, several-fold more than the T subunit. Furthermore, transfection of myoblasts with a cDNA containing all the sequences necessary for expression of all AChE splice variants resulted only in expression of T subunits. This indicated that selective expression in muscle fibres is due to exclusive splicing of T mRNA from the primary transcript.

Tetramers of "tailed" AChE subunits are known to associate with a triple helical collagenic structural subunit (tQ1) and are anchored in the synaptic basal lamina. In our experiments, interaction of $AChE_T$ subunits with tQ1 increased enzyme activity in both C_2C_{12} cells and in vivo. Overexpression of tQ1 subunits allowed for the assembly and secretion of A12 asymmetric forms typically found at the neuromuscular junction. Furthermore, the increased AChE activity resulted from the incorporation of newly

synthesized enzyme arising from the transfected $AChE_T$. In addition the increase in AChE activity occurred even when the tQ1 subunit was truncated from the C-terminal region and failed to form a triple helix. These results indicated that the production and secretion of stable forms of AChE is limited by the availability of the collagenic structural subunit.

Co-injection of cDNAs encoding $AChE_T$ and the collagenic structural subunit resulted in overexpression of AChE in the extrasynaptic compartment of rodent muscle fibres. AChE was localized to the periphery of transduced muscle fibres. This indicated that assembly, processing, and externalization of asymmetric forms can occur outside the synaptic compartment of muscle fibres, and does not require the specialized junctional Golgi apparatus.

Dedication

This Thesis is dedicated to my parents Ahmad and Ihsanne who have given me so much love, encouragement and support over the course of my life. They have sacrificed so many things in their own lives to ensure that my siblings and I had the same opportunities enjoyed by our peers. I thank my parents for the lessons they have taught me over the years; to be just, to be generous and to work hard no matter what the task. None of my accomplishments, including this Thesis, would be possible without their strength, wisdom and patience.

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List of Abbreviations

A	Asymmetric
ACh	Acetylcholine
AChE	Acetylcholinesterase
AChR	Acetylcholine Receptor
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
CGRP	Calcitonin gene-related peptide
CNTF	Ciliary neurotrophic factor
COLQ	Rat collagenic structural subunit (asymmetric forms)
EDL	Extensor digitorum longus
Egr-1	Early growth response gene
G	Globular
GPI	Glycophosphatidylinositol
GTP	Guanosine triphosphate
H	Hydrophobic
HSPG	Heparan sulfate proteoglycan
JAK-STAT	Janus kinase-signal transducer and activator of transcription
kb	Kilo bases
kDa	Kilo Dalton
MAP	Mitogen activated protein

MEL	Murine erythroleukemia
mRNA	Messenger ribonucleic acid
P	Hydrophobic structural subunit (G_4^a)
PI-PLC	Phosphatidylinositol-specific phospholipase C
PRAD	Proline-rich attachment domain
Q	Torpedo collagenic structural subunit (asymmetric forms)
R	Readthrough
RT-PCR	Reverse transcription-polymerase chain reaction
T	Tailed
TA	Tibialis anterior
TGN	Trans Golgi network

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First and foremost, I would like to thank my supervisor, Dr. Bernard Jasmin for believing in me over the last four years. I am ever grateful that he agreed to take a chance on a naive and immature Lebanese kid who came knocking on his office door one cold winter morning, despite my academic shortcomings at the time. Dr. Jasmin's leadership and scientific genius provided an excellent environment for learning the merits of the scientific method, which I like to think he has taught me to apply to more than just science. The completion of this Thesis is a direct result of his advice, patience and insight.

I would also like to thank the members of the Jasmin lab, Tony, Céline, Roxanne, Lindsay, Julie, Jun, Feisal, Andrea and last but not least John Lunde who let me play in his boat at John Lunde's cottage. These individuals made my stay in the lab productive and more importantly, entertaining. Most notably, I thank John, Tony and Céline, who were paramount in the completion of this work from day one. I also thank my *cousins* Wadih and George for their continued support and friendship.

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Scholarship for providing me with a studentship. This work was supported by grants from the Medical Research Council of Canada and the Association Française Contre les Myopathies to Dr. B.J. Jasmin.

Chapter 1

Introduction

Formation of the neuromuscular junction requires the targeted deposition of several synaptic proteins allowing for precise control of muscle contraction. Acetylcholinesterase (AChE), is an integral component of this synaptic compartment. This enzyme is responsible for termination of cholinergic transmission by the rapid hydrolysis of acetylcholine (ACh) released from the nerve terminal. From the initial pioneering work of Dale (1914) who postulated the existence of AChE, its characterization has been the focus of much research. These include the enzyme kinetics involved in ACh hydrolysis, the polymorphism AChE displays in various tissues, its plasticity under varying stimuli and its accumulation at the neuromuscular junction. The advent of molecular biology has further shed a light on the regulation of AChE expression and has formed a basis even for the study of possible non-cholinergic functions.

1.1 Cellular and Molecular Biology of AChE

1.1.1 Molecular Forms of AChE

AChE is a glycoprotein defined by its function of choline ester hydrolysis. It is expressed as a catalytic monomer and can occur as a variety of oligomeric forms. These range from simple AChE monomers to complex multi-subunit molecules consisting of catalytic subunits and non-catalytic structural proteins (see Massoulié et al., 1993 for a comprehensive review). Specifically, AChE molecular forms fall into two main classes: Globular (G) forms, which include monomers (G_1), dimers (G_2) and tetramers (G_4) of catalytic subunits; and asymmetric forms (A), which consist of one (A_4), two (A_8) or three

(A_{12}) tetramers attached to a non-catalytic structural protein (Figure 1). Although there is a great degree of variation in the structure of these different molecular forms, the catalytic activity of each subunit remains unchanged regardless of the complexity of the molecule (Vigny et al., 1978).

1.1.1a Globular Forms

A variety of AChE globular forms have been identified (Figure 1). These globular forms can be sub-classified depending on the tissue in which they are expressed. Type I glycophosphatidylinositol-anchored dimers (GPI-G_2) are predominantly expressed in cells of hematopoietic origin (Bon et al., 1988; Rotundo et al., 1988) whereas type II G_1, G_2 and G_4 molecules occur in excitable tissues such as nerve and muscle (Li et al., 1991; Li et al., 1993).

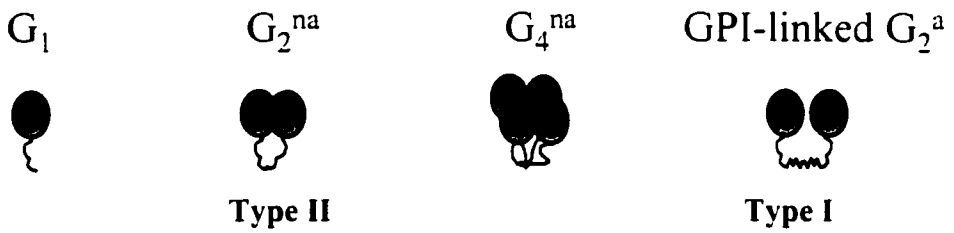
The type I dimers are distinct in their attachment to the cell surface. They are inserted hydrophobically into the plasma membrane by the diacylglycerol moiety of a GPI-anchor located at the C-terminal of each catalytic subunit of the dimer (Silman and Futerman, 1987). They are expressed predominantly on the surface of mammalian erythrocytes as well as in *Torpedo* electric organ and Xenopus muscle (Massoulie et al., 1993). The G_2 GPI signal is composed of two elements, a C-terminal hydrophobic sequence and an upstream cleavage/attachment domain. Shortly after synthesis of the AChE polypeptide chains, the C-terminal fragment is cleaved and a pre-assembled glycolipid is linked covalently by an amide bond to the last amino acid residue of the

Figure 1. Quaternary structures of AChE molecular forms

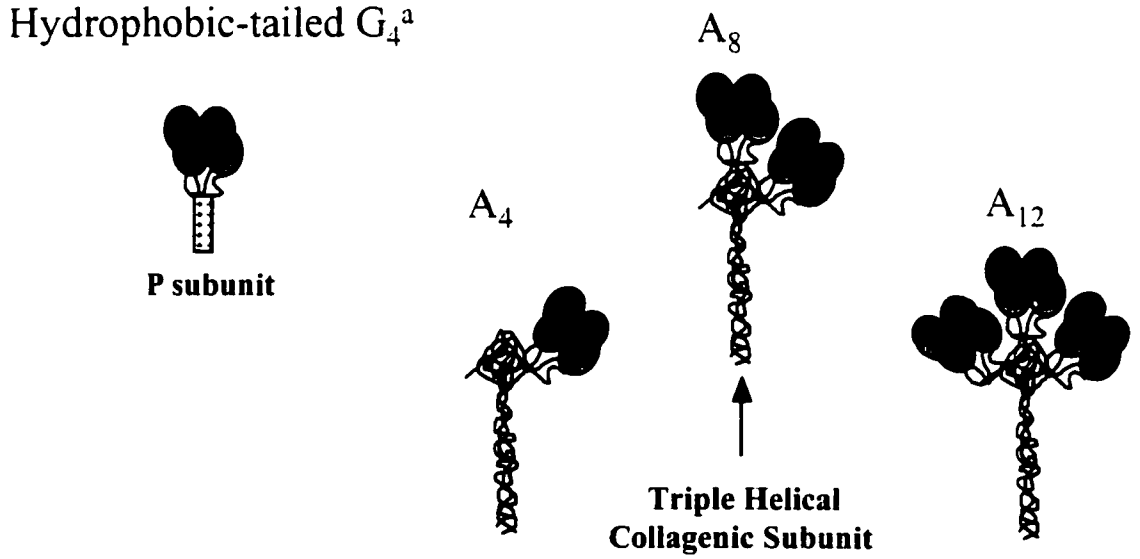
Schematic representations of the molecular forms generated by the mammalian AChE gene. Figure A represents the globular (G) forms, which are sub-classified as type I GPI-linked dimers found in erythrocytes and type II molecules found in brain and muscle. Figure B represents the P-subunit associated AChE tetramers and the asymmetric (A) forms of AChE which are associated with the triple helical collagenic structural subunit. The nomenclature bestowed on AChE molecular forms depends on the number of catalytic subunits, the type of structural subunit and the ability of the molecules to form hydrophobic interactions.

Quaternary Structures of AChE Molecular Forms

(A) Homomeric Forms :



(B) Heteromeric Forms :



catalytic subunit (Moran and Caras, 1991). The GPI-anchor allows for insertion of these molecules directly into the lipid bilayer and can be cleaved by Phosphatidylinositol-specific phospholipase C (PI-PLC) to release the catalytic subunits from the cell surface (Duval et al., 1992).

Alternatively, type II AChE exists as soluble monomers (G_1), dimers (G_2) and tetramers (G_4) in mammalian nerve and muscle cells. Oligomerization of the G_2 and G_4 molecules occurs through an essential cysteine residue in the C-terminal of the catalytic subunit that when mutated results only in production of soluble monomers (Velan et al., 1991). The type II globular forms are thought to be the precursors of the more complex hetero-oligomers localized at the neuromuscular junction.

1.1.1b P-Anchored AChE

The AChE tetramers (G_4) found in muscle and nerves are designated as globular but can also be sub-classified based on the presence of an anchoring protein termed the hydrophobic P subunit (see Figure 1). The P subunit is about 20kDa in size. It interacts with two catalytic subunits of the G_4 molecule via disulfide bridges formed between the two cysteine residues in its sequence, and the C-terminal of each of the catalytic subunits (Boschetti and Brodbeck, 1996). The hydrophobic or amphiphilic P-subunit associated tetramers (G_4^a) are the predominant forms inserted in neuronal cell membranes where they account for 80% of the total brain enzyme (Gennari et al., 1987; Inestrosa et al., 1987).

1.1.1c Asymmetric Forms

The asymmetric forms are the second main subtype of AChE and are found in brain and muscle. They consist of one, two or three tetramers of the muscle-specific AChE catalytic subunits linked to individual strands of a triple helical collagenic structural subunit (Q) resulting in A_4, A_8 and A_{12}, respectively (see Figure 1). It has long been known that the asymmetric forms, specifically A_{12}, are highly concentrated in the basal lamina at the neuromuscular synapse of skeletal muscle fibres where they interact with polyanionic components of the extracellular matrix such as glycosaminoglycans (Hall, 1973; Hall and Kelly, 1971; McMahan et al., 1978). Asymmetric forms exist in muscle and nervous tissue of all vertebrates but do not seem to be present in invertebrates (Massoulie et al., 1993).

It is clear that a great deal of heterogeneity exists in expression of AChE resulting in a multitude of molecular forms. Although the significance of this polymorphism is unclear, it is believed that it allows for divergence in physiological function at different cellular sites. For example, the asymmetric forms located in the synaptic basal lamina are thought to be responsible for the rapid termination of synaptic transmission whereas the G_4 tetramers known to be anchored perijunctionally in the sarcolemma may prevent the diffusion of acetylcholine from the synapse (Gisiger and Stephens, 1988; Massoulie et al., 1993).

1.1.2 Molecular Biology of AChE

A single gene directs expression of all the observed molecular forms of AChE in vertebrates. This gene was first identified in *Torpedo* electric organ (Schumacher et al., 1986; Sikorav et al., 1987) and in quail (Rotundo et al., 1988) but has since been cloned and analyzed in a variety of mammalian species including man (Getman et al., 1992), mouse (Rachinsky et al., 1990; Li et al., 1991) and rat (Legay et al., 1993).

The mammalian gene for AChE is 4.5-4.7 kb in size and is comprised of six exons, of which exons 2-6 contain the coding sequence (Chan et al., 1999; Getman et al., 1995; Soreq et al., 1990). Transcription of the AChE gene is initiated at one of two CAP sites of which the downstream site is used more frequently (Li et al., 1991). These CAP sites are believed to contribute to the efficiency of AChE transcription in mammalian cells (Getman et al., 1995; Sikorav et al., 1987). Sequence analysis of the mammalian AChE promoter revealed that this G-C rich region contains several E-boxes to which muscle-specific transcriptional factors are known to bind as well as consensus sequences for transcription factors such as Egr-1, Sp1, and AP2 similar to the mouse and human AChE genes (Ben Aziz-Aloya et al., 1993; Chan et al., 1999; Li et al., 1993). Although the promoter sequence lacks TATA and CAAT boxes, it does contain an initiator element (Getman et al., 1995) believed to activate transcription. Interestingly, genes devoid of TATA and CAAT boxes were believed to only express housekeeping proteins. However, several highly regulated genes (including AChE) as well as genes involved in signal transduction have been shown to have TATA-less and CAAT-less promoters (Luskey, 1987).

The molecular weight of the AChE translated gene product is generally in the range of 70-80 kDa, except in avians in which two main allelic variants of the gene, α and β result in functionally identical 100-110 kDa peptides (Rotundo et al., 1988). The primary structure of AChE consists of a common catalytic domain of 535 amino acids encoded by exons 2, 3 and 4, followed by one of three variable short C-terminal peptides. The choice of C-terminal peptide is determined by alternative splicing of the primary transcript which allows for the expression of three known mRNAs. These have been termed the R, H and T transcripts (see Figure 2). The existence of multiple transcripts was first postulated by Sikorav and colleagues (1984) who showed that in vitro translation of *Torpedo* mRNA resulted in the production of two precursor proteins. Direct sequencing studies also showed that the catalytic subunits of asymmetric molecules and GPI-anchored dimers of AChE differed in their C-terminal regions (Bon et al., 1986). These studies suggested that the production of different AChE precursors is a result of differentially encoded mRNAs.

The three AChE mRNA splice variants have been shown to distribute in a tissue-specific manner. The T transcript, named so because its expression results in "tailed" AChE, is spliced from exon four to six. This mRNA encodes the predominant form of the enzyme found in muscle and brain. The T transcript varies in size depending on the use of polyadenylation sites which can result in mRNAs of size 2.4 and 3.2 kb (Li et al., 1991). Although the functional importance of this differential modification is not completely understood, the differences in the length of the poly-A tail have been suggested to play a role in mRNA stability (Legay et al., 1993; Taylor, 1991). The protein product of the T transcript is hydrophilic in nature and retains an important cysteine residue near the C-terminal deemed important for quaternary associations (Li et al., 1991; Legay et al., 1993;

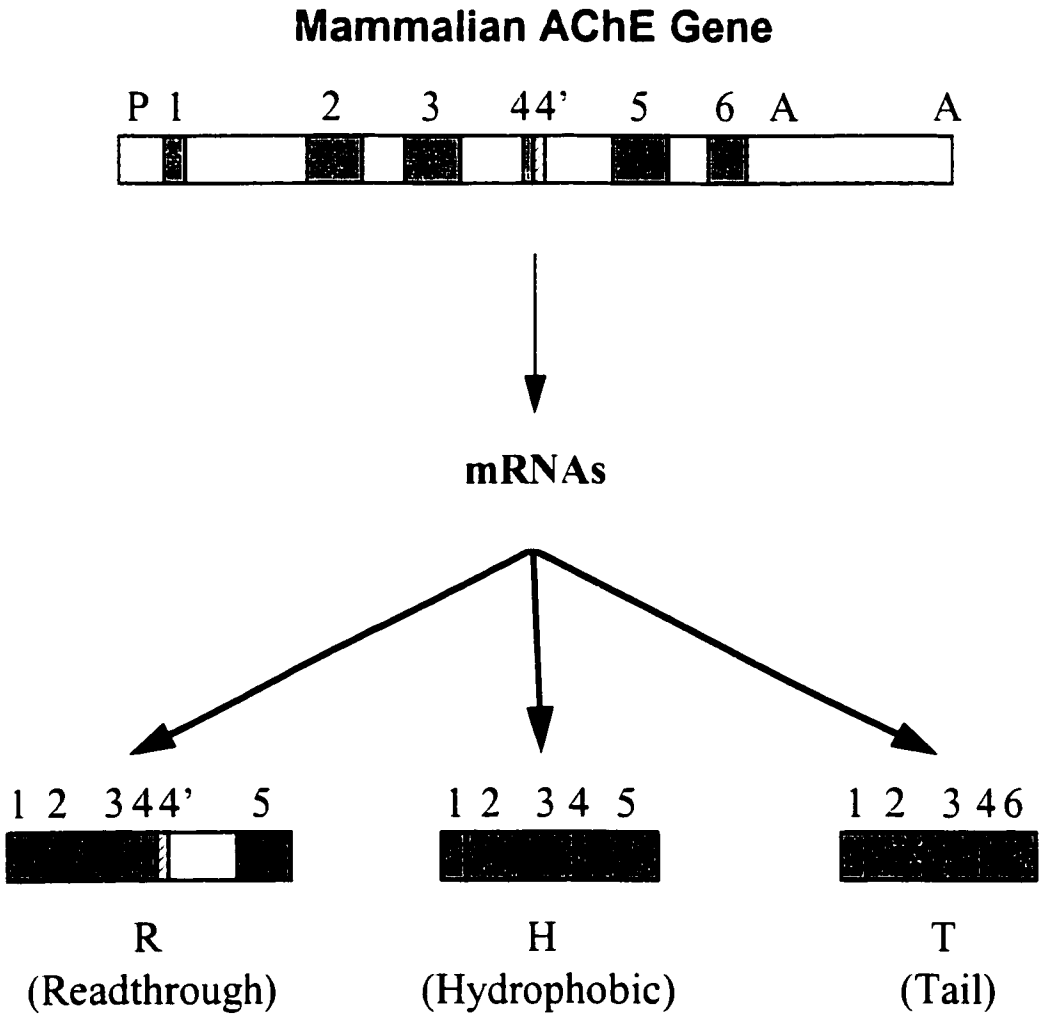
Li et al., 1993). Specifically, this cysteine residue is essential for the formation of disulfide-linked dimers of the catalytic subunit as well as disulfide linkages to structural subunits such as P or Q.

The H transcript, named so because its protein product is anchored to the membrane by "hydrophobic" interactions, is spliced from exon four to five. This mRNA codes for a catalytic subunit with a signal that results in cleavage and addition of a hydrophobic GPI-anchor (Duval et al., 1992; Li et al., 1991). These transcripts produce dimers which in mammals, are found predominantly inserted in the membranes of erythroid cells (Li et al., 1991; Li et al., 1993; Rachinsky et al., 1990). A minor fraction of H transcripts is evident in developing mammalian muscle fibres but disappears at birth (Legay et al., 1995).

The R or "readthrough" transcript contains the entire genomic sequence immediately downstream of the last common exon, exon four. Although, a protein product has yet to be identified for R, its sequence predicts that it would produce a secreted soluble monomer because of a premature stop codon in intron four. This stop codon precludes the cysteine residue or hydrophobic regions in the distal exons required for oligomer formation or anchoring, respectively (Li et al., 1993). Experiments using mouse diaphragm muscle revealed that the R transcript can be detected in muscle from E14 until birth but that only the T transcript is present in adult muscle (Legay et al., 1995). The R transcript was also shown to be expressed in murine erythroleukemia (MEL) cells (Chan et al., 1998) and in the brains of stressed mice (Kaufer et al., 1998).

Figure 2. Structure of the mammalian AChE gene

Schematic representation of the mammalian AChE gene and the three alternatively spliced transcripts it encodes. These transcripts are defined by their variable C-terminal regions. The readthrough (R) transcript contains the entire genomic sequence immediately downstream of the last common exon. The hydrophobic (H) transcript is spliced from exon 4 to exon 5 which includes a signal for cleavage and GPI-addition. The tailed (T) transcript is spliced from exon 4 to exon 6 which contains cysteine residues important for association with structural subunits.


Mammalian AChE Gene
P 1 2 3 4 4' 5 6 A A
mRNAs
1 2 3 4 4' 5
1 2 3 4 5
1 2 3 4 6
R
(Readthrough)
H
(Hydrophobic)
T
(Tail)

1.1.3 Biosynthesis of AChE

The biosynthesis of AChE mirrors that of other glycosylated membrane and secreted proteins. AChE is initially synthesized as an inactive peptide at the level of the rough endoplasmic reticulum and is translocated into the lumen where an N-terminal signal peptide is cleaved and the enzyme becomes functional (Rotundo, 1988). In an elaborate cell hybrid experiment, Rotundo (1990) has shown that homo-oligomerization of AChE, occurs in the endoplasmic reticulum in the vicinity of the nucleus of origin. The folding and oligomerization of AChE is believed to be mediated by heat shock proteins as suggested by experiments in which chick myotubes transferred from 37°C to 45°C showed a sharp drop in activity followed by rapid and spontaneous recovery that did not involve de novo protein synthesis (Eichler et al., 1991). Calnexin and BiP/GRP78, two heat shock proteins found in the endoplasmic reticulum of mammalian cells are likely candidates for this function (Hammond et al., 1994).

Newly synthesized inactive AChE has been shown to be a part of a rapidly turning over pool in which a large portion of the peptides are degraded by proteolysis shortly after synthesis in chick skeletal muscle (Rotundo, 1988). Specifically, all newly synthesized polypeptide chains are believed to be transported from the rough endoplasmic reticulum to the Golgi apparatus where the majority (80%) do not acquire endo H resistance, a marker for successful transit through the Golgi apparatus. The remaining active molecules are believed to act as precursors for the more complex forms of AChE found at the surface of muscle and nerve cells. Indeed, an inactive pool of AChE has been identified in chick brain and skeletal muscle (Chatel et al., 1993). This pool accounts for 40% of the total

enzyme in embryonic stages of development (E11) and decreases progressively to 20% of the total enzyme level at birth. As expected the inactive AChE in brain and skeletal muscle corresponds specifically to G1 and G2 forms. G4 and A12 forms are composed exclusively of active molecules (Chatel et al., 1993).

Although an inactive pool of AChE has been characterized in chick skeletal muscle, its existence in mammalian cells is still questioned. Analysis of human brain extracts by enzyme-linked immunosorbent assay showed no evidence for an inactive pool of AChE (Hammond and Brimijoin, 1988). In sharp contrast to these experiments, Schober and colleagues (Schober et al., 1997) showed that a deficit in AChE activity fails to correspond to any overt changes in AChE protein levels in mammalian preganglionic sympathetic neurons and chromaffin cells, suggesting that inactivation of AChE occurred in these cells. Hence, the existence of an inactive pool of AChE is still an area of debate. Furthermore, the functional significance of such an inactive pool has yet to be addressed.

AChE molecules that are not degraded become oligomerized in the endoplasmic reticulum. Cleavage and addition of the hydrophobic GPI-anchor has been shown to occur before the molecules leave this compartment (Rotundo, 1988). The modified AChE peptides are subsequently shuttled through the intermediate compartment to the Golgi apparatus where they gain 10-15% of their total mass as carbohydrate by glycosylation (Velan et al., 1993). N-linked glycosylation, which occurs in the Golgi apparatus has been shown to be important for stability and secretion of AChE. Molecules mutated in their N-glycosylation sites undergo inefficient biogenesis and result in the accumulation of unstable, mis-folded molecules (Velan et al., 1993). Despite the importance of glycosylation in AChE biosynthesis, abrogation of N-glycosylation has no detectable effect

on activity of human AChE molecules (Velan et al., 1993). Interestingly, unlike human AChE, the chick enzyme has been shown to require glycosylation for acquisition of catalytic activity (Rotundo, 1988). The functional homomeric globular forms assembled in the endoplasmic reticulum are believed to associate with structural subunits depending on the availability of the latter in the secretory pathway. To determine the spatial and temporal location of assembly of asymmetric molecules, Rotundo (1984) used specific lectins to show that the assembly of AChE tetramers with Q subunits likely occurs in the trans-Golgi compartment approximately 90 minutes post-translation. The overall transit of AChE molecules from the point of synthesis through the cell requires approximately 2.5 hours at which time they are secreted or anchored on the cell surface (Lazar et al., 1984; Rotundo and Fambrough, 1980). Although the mechanism of P subunit-AChE assembly has yet to be elucidated, it is believed that this process, similar to assembly of asymmetric forms, occurs in the trans-Golgi compartment (Massoulie et al., 1993).

1.1.4 Assembly of Asymmetric Forms

A significant fraction of AChE occurs as asymmetric forms clustered in the basal lamina of the neuromuscular synapse. The importance of the collagenic structural subunit (Q), an essential component of these asymmetric forms, in AChE expression is evidenced by studies implicating mutations in Q with end-plate AChE deficiency. Specifically, mutations in the C-terminal of Q were shown to result in congenital myasthenic syndrome (CMS), a rare autosomal recessive disease in which the AChE gene was excluded as a potential culprit (Donger et al., 1998). These experiments confirm the physiological

importance of Q in AChE expression.

The Q subunit has previously been cloned from *Torpedo* electric organ (Krejci et al., 1991). Dissection of its primary sequence shows that it comprises a signal peptide, an N-terminal domain containing a pair of adjacent cysteines believed to associate with the catalytic subunit (Camp et al., 1995), a central collagen domain flanked by two pairs of cysteines and a C-terminal domain. In a collagen-tailed A_{12} molecule, the three Q subunits are disulfide linked together and each can associate with a catalytic tetramer via disulfide bonds with the two internal catalytic subunits. These internal dimers then associate with the two peripheral catalytic dimers via quaternary interactions (Anglister and Silman, 1978; Rosenberry and Richardson, 1977). Duval and colleagues (1992) have defined the specific region of the Q subunit that associates with the catalytic subunits. They showed that $AChE_T$ subunits truncated at the C-terminal could not associate with Q subunits. Furthermore, expression of cDNAs encoding the N-terminal of the collagenic tail linked to a signal for GPI-anchoring from exon 5 of the *Torpedo* AChE gene (Q_NH_C), resulted in assembly of $AChE_T$ subunits into tetramers that were efficiently inserted into the membrane of transfected COS cells (Duval et al., 1992), thus confirming that the T peptide at the C-terminal of $AChE_T$ associates with the N-terminal of Q to form asymmetric AChE.

The sequence of the *Torpedo* Q subunit has been shown to be highly conserved between species. In fact, experiments have shown that the *Torpedo* Q subunit can associate with $AChE_T$ subunits of *Torpedo*, rat or human to form asymmetric molecules in transfected COS cells (Camp et al., 1995; Krejci et al., 1991; Legay et al., 1993). Similar experiments have shown that rat basoleukemia cells (RBL-2H3) also produced asymmetric molecules when co-transfected with AChE and Q cDNAs. However, subtle yet significant

differences exist between these two expression systems. In contrast to COS cells which produced all asymmetric species, the RBL-2H3 cells which possess a well developed secretory pathway, only produced A_4 molecules suggesting that a differential assembly pathway exists in these cells (Coussen et al., 1995). Nonetheless, the ability of the *Torpedo* Q subunit to bind to AChE catalytic subunits in several species suggests that the sequence of Q is highly conserved, emphasizing the importance of its function in vivo.

Although it is well documented that AChE catalytic subunits associate with the N-terminal of the Q subunit, the specific interaction between these two molecules remained rudimentary until recently. In a detailed analysis of the N-terminal region of the collagenic tail using a series of deletions and point mutations, Bon and colleagues, (Bon et al., 1997; Bon and Massoulie, 1997) determined that a proline-rich attachment domain (PRAD) in this region is required for binding of Q to AChE tetramers. They also found that the cysteine residues located in the N-terminal of the Q subunit play minor peripheral roles in this association. Additionally, they showed that synthetic polyproline peptides alone can induce tetramerization of AChE in vitro (Bon and Massoulie, 1997) emphasizing the importance of this sequence in linking with AChE catalytic subunits.

Recently, the rat collagenic structural subunit was cloned and sequenced (Krejci et al., 1997). This mammalian Q subunit termed COLQ, was shown to be encoded by a single gene homologous to the *Torpedo* Q subunit. Transfection experiments verified that COLQ, like its *Torpedo* counterpart, was able to recruit AChE into asymmetric forms in COS cells. Furthermore, COLQ was shown to associate with butyrylcholinesterase, another choline esterase with similar quaternary interactions as AChE. These interactions were demonstrated by sedimentation analysis using antibodies against the COLQ subunit

(Krejci et al., 1997). Cloning of the COLQ subunit also allowed for characterization of the function of this protein in vivo. Inactivation of the COLQ gene in mice by homologous recombination resulted in a loss of AChE accumulation at the neuromuscular junction, demonstrating that the collagenic structural subunit is necessary for this accumulation (Feng et al., 1999). Asymmetric forms of AChE probably do not play an important role in early synaptogenesis, since the neuromuscular junctions of newborns lacking COLQ were initially robust and the lack of AChE was not fatal to the animals. The animals lacking COLQ however, failed to thrive and most died before reaching maturity.

1.1.5 Tissue-Specific Expression of Acetylcholinesterase

In addition to its synaptic localization, AChE exists in several non-neuronal and non-muscle cell types including epidermal and hematopoietic cells. The mechanisms underlying tissue-specific distribution of AChE mRNAs are currently under investigation. For instance, transfection of a mouse erythroleukemia cell line with recombinant DNA encoding the tissue-specific mRNA species of the mouse AChE gene resulted in gene products having the same properties expected for the respective enzyme forms found in different tissues (Li et al., 1993). Specifically, cDNAs representing the H form resulted in expression of GPI-anchored dimers whereas cDNAs encoding the R form produced hydrophilic secreted monomers. Interestingly, transfection of mouse erythroleukemia cells with a cDNA encoding the human R form did not result in expression of mRNA encoding the hydrophilic secreted monomers suggesting that differential splicing between mouse and human genes is intrinsic to the coding sequence and is not dependent on trans-acting

factors unique to cells of different tissues.

Recent experiments, using a unique in vivo model for expression of alternatively spliced human AChEs in transiently transgenic embryos of Xenopus laevis, (Ben Aziz-Aloya et al., 1993) showed that cDNAs carrying the alternative exon 6 directed expression of AChE in muscle cells, nerve terminals and neuromuscular junctions of 2 and 3 day old embryos. Overexpression of this cDNA also resulted in features of enhanced development of the neuromuscular junction such as intense AChE staining and increased synaptic size. Follow-up experiments using a cDNA that potentially encodes the remaining two alternative mRNAs resulted in staining of epidermal cells and secretion of AChE into the external medium. Furthermore, these alternatively spliced forms were absent from muscle and did not affect development of the neuromuscular junction (Seidman et al., 1995). These experiments were done using a pan-active CMV (cytomegalovirus) promoter to direct expression of AChE thus bypassing transcriptional regulation of tissue-specific expression conferred by the native AChE promoter. The above findings suggested that the 3'-terminal exons encoding the various AChE subtypes played important roles in their muscle or epidermal localization. More importantly, they suggested that tissue-specific accumulation of alternatively spliced AChE is mediated by posttranscriptional and/or posttranslational management of their mRNAs and protein products.

A novel mechanism suggesting that introns influence AChE pre-mRNA splice selection was also recently examined (Luo et al., 1998). In these experiments, deletion of introns two and three of an AChE gene transfected into differentiating skeletal muscle cells resulted in production of R and H transcripts that were not expressed when the introns were left intact. Furthermore, control of these downstream splicing events was shown to

be a result of correct upstream splicing and not the secondary structure of the AChE pre-mRNA. Interestingly, the presence of alternative intron four was found to control the efficiency of upstream splicing. In contrast to Seidman and colleagues (Seidman et al., 1995) who suggest that all transcripts are expressed and then selectively stabilized, these data indicate that selective splicing is governed by the interaction of tissue-specific factors with conserved cis-elements in introns 5' of the splice region which act to produce T transcripts exclusively during myogenesis.

1.1.6 Expression of AChE in Transfected Cells

cDNAs encoding H and T subunits have been cloned and transfected into a variety of cells allowing for detailed analysis of AChE structure and function. Expression in Xenopus oocytes (Soreq and Seidman, 1992) and human kidney "293" cells (Kronman et al., 1992; Randall, 1994; Shafferman et al., 1992; Velan et al., 1991) showed that AChE could be expressed and folded correctly for secretion in systems that do not possess high levels of endogenous enzyme. COS cells transfected with the cDNAs encoding the H subunit produced dimers and showed intense surface labelling suggesting that these molecules were efficiently processed for GPI-anchoring and were successfully targeted to the cell membrane (Duval et al., 1992; Gibney and Taylor, 1990). Cells transfected with cDNAs encoding the T catalytic subunit of *Torpedo*, rat, mouse and human (Duval et al., 1992; Gibney and Taylor, 1990; Velan et al., 1991) produced all the known globular forms except the GPI-anchored dimers (type I). As expected, cells transfected with the T subunit were able to assemble G_4^{na} hydrophilic tetramers secreted into the media and G_4^{a}

hydrophobic tetramers which remained cell-associated. COS cells transfected with cDNAs encoding the R splice variant produced secreted hydrophilic monomers (Li et al., 1993) supporting the view that cysteine residues lacking in the C-terminal of R are critical for oligomeric assembly and anchoring of AChE.

1.2 Expression of AChE in Muscle

1.2.1 Developmental Expression of AChE in Muscle

During development, AChE activity levels fluctuate and spatial localization is altered within muscle fibres. AChE activity can be detected, albeit at low levels in the cytoplasm of mononucleated precursor muscle cells. These low levels of activity correspond to globular forms G_1, G_2 and G_4. Upon fusion of these myoblasts into multinucleated myotubes, AChE activity and protein levels increase dramatically both in vivo (Tennyson et al., 1973) and in tissue cultured muscle cells (Inestrosa et al., 1983). Although aneurally grown myotubes show species specific differences in AChE localization such as diffuse distribution in rat and patchy accumulations in mouse (C_2C_{12} myotubes) and quail (Inestrosa et al., 1982; Rotundo and Fambrough, 1982), upon innervation, all myotubes show a preferential concentration of AChE at the neuromuscular junction (Massoulie et al., 1993). These molecules can only be extracted with collagenase confirming that they are exclusively asymmetric forms (Inestrosa et al., 1982). In embryonic and early postnatal rat muscles, asymmetric forms are present along the entire length of the fibres but become restricted to the endplate after the first month in fast muscles (Sketelj and Brzin, 1980).

Alternatively, the asymmetric forms found in slow soleus muscles remain present at low levels in extrajunctional regions (Crne et al., 1991). Interestingly, human skeletal muscle is distinct from the muscle of other mammals in that extrajunctional regions contain the same proportion of asymmetric forms as junctional regions (Carson et al., 1979).

Expression of AChE mRNA mirrors that of AChE protein in differentiating muscle cells. Northern blot analysis and RNAase protection assays showed marked increases in AChE transcripts upon differentiation of C_2C_{12} myoblasts into myotubes (Fuentes and Taylor, 1993). Nuclear run-on experiments showed that AChE transcription rate does not increase suggesting that the increase in mRNA level occurs via transcript stabilization and not up regulation of transcriptional activity. Furthermore, cycloheximide inhibition of protein synthesis results in a superinduction of AChE mRNA in undifferentiated C2C12 myoblasts without affecting transcription rates suggesting the presence of proteins that destabilize AChE in undifferentiated myoblasts (Fuentes and Taylor, 1993). The mRNAs present in developing muscles correspond mainly to T transcripts (Legay et al., 1995). Although low amounts of R transcripts and H transcripts (1% and <1% of total, respectively) exist in myogenic cell lines and in embryonic mouse diaphragm from E14 until birth, only the T transcript can be detected in adult muscle (Legay et al., 1995).

1.2.2 Distribution of AChE in Skeletal Muscle

In skeletal muscle, AChE is localized at the neuromuscular synapse which occupies less than 0.1% of the total surface of muscle fibres. Asymmetric forms are anchored in the synaptic basal lamina and hydrophobic G_4 forms are present along the presynaptic and postsynaptic membranes, including the junctional folds (Schatz and Veh, 1987). Asymmetric AChE is localized in the vicinity of the site of ACh release and of the receptor-rich postsynaptic membrane allowing for rapid hydrolysis of ACh and prevention of multiple binding to receptors. The density of asymmetric AChE molecules in the synaptic basal lamina of mouse muscle can reach up to 600 sites/μm^2 (Anglister et al., 1994) as compared to the nicotinic AChR, which can reach levels of up to 10 000/μm^2. In fast skeletal muscles, the hydrophobic-tailed (P subunit) G_4^a molecules are abundant in the perijunctional compartment surrounding the synapse (Gisiger and Stephens, 1988) and may function in preventing diffusion of ACh from the synapse. Smaller globular molecules, specifically G_1, G_2 and hydrophilic G_4^{na} are found in the endoplasmic reticulum and cytoplasm of muscle fibres and are believed to serve as precursors for the more complex forms found on the surface (Rotundo, 1988).

1.2.3 Association of Asymmetric AChE with Muscle Basal Lamina

Electron microscopy studies have determined that the distal region of the collagenic subunit is responsible for attachment of asymmetric forms to the synaptic basal lamina (Bon et al., 1978). Several lines of evidence suggest that these asymmetric forms bind to

heparan sulfate proteoglycan (HSPG) molecules. HSPGs, although found throughout the extracellular matrix, are highly concentrated in the synaptic basal lamina (Anderson and Fambrough, 1983; Sanes et al., 1986). Furthermore, asymmetric forms are known to bind specifically to heparin and sulfated glycosaminoglycans (Bon et al., 1978; Brandan et al., 1985; Vigny et al., 1983) and have been shown to be solubilized from muscle by heparin (Barat et al., 1986; Torres and Inestrosa, 1983) or other polyanions (Perez-Tur et al., 1991). In a detailed study using quail skeletal muscle cultures, heparin blocked the accumulation of newly synthesized asymmetric forms on the myotube surface (Rossi and Rotundo, 1993; Rossi and Rotundo, 1996). Although heparin was capable of solubilizing a subset of asymmetric AChE from skeletal muscles, a significant fraction already present in the synaptic basal lamina could not be detached (Rossi and Rotundo, 1993). These experiments suggest that HSPGs are involved in the initial association of AChE to the specialized synaptic basal lamina via ionic bonds but once localized, more permanent mechanisms, such as covalent bonds are involved in securing this attachment.

Recently, Peng and colleagues (Peng et al., 1999) have proposed a model to describe the binding partners of asymmetric AChE in the synaptic basal lamina. Using fasciculin-2, a snake α-neurotoxin that binds the AChE catalytic subunit, they determined the acceptor proteins involved in clustering AChE at the neuromuscular junction. Specifically, they showed that AChE collagen tailed forms bind perlecan, a modular HSPG, by an interaction between the C-terminal of the collagen tail and the heparin sulfate chain of perlecan. Perlecan which is an extracellular matrix protein, binds to dystroglycan, a component of the synaptic transmembrane protein complex that interacts with molecules in the extracellular matrix as well as the cytoskeleton (Henry and Campbell, 1996; Peng

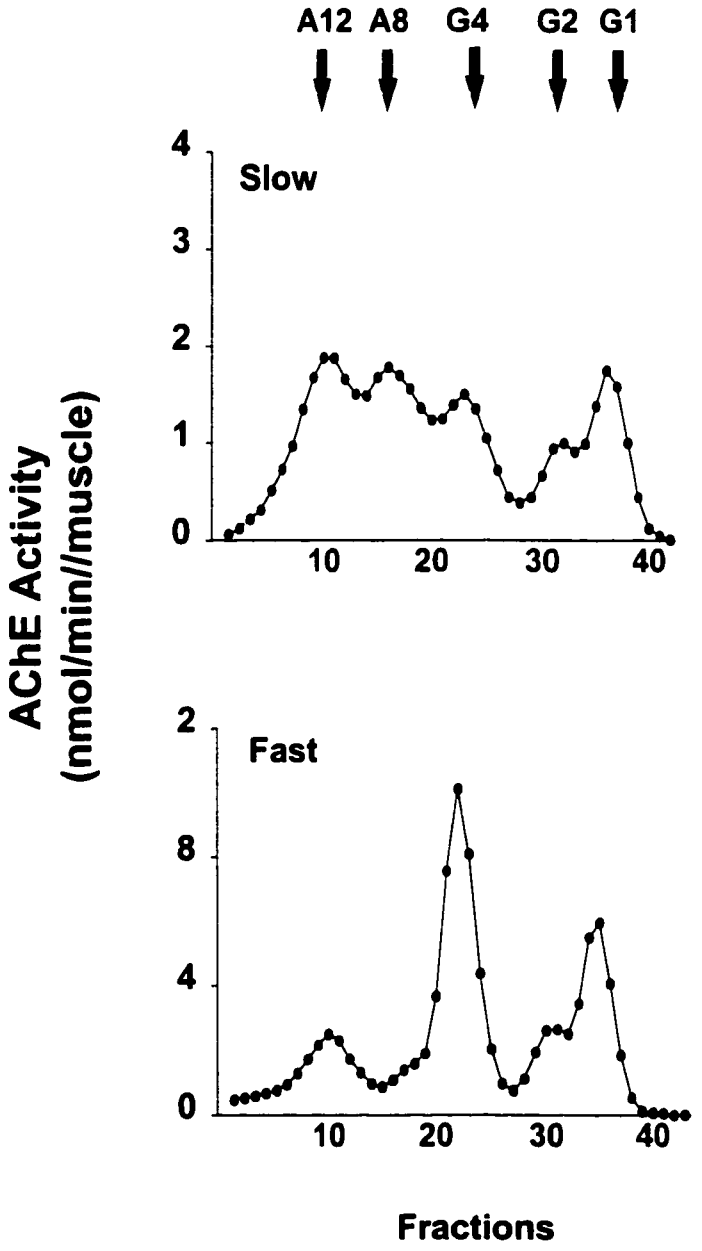
et al., 1998). This interaction is believed to allow migration of AChE in the plane of the membrane until the molecules become fixed in the synaptic cleft. This model is similar to that of Kidokoro and Brass (1985) who describe a mechanism for clustering AChR at the neuromuscular junction via migration through the cell membrane mediated by components of the cytoskeleton.

1.2.4 AChE in Fast and Slow Skeletal Muscle

The molecular forms of AChE can vary depending on the nature of skeletal muscle. Fast twitch muscles such as the extensor digitorum longus (EDL) contain high levels of A_{12}, G_4 and G_1, whereas slow muscles such as the soleus contain a more complex distribution of all forms including distinct peaks of A_8, A_4 and G_2 and a markedly decreased level of G_4 as compared to the EDL (Figure 3) (Groswald and Dettbarn, 1983). These differences are evident early on in post-natal animals. For example, rats show differences in the molecular form profiles of EDL and soleus muscles at four days after birth, before the stimulation pattern of the two muscles diverges into the characteristic phasic and tonic patterns of adult muscles (Sketelj et al., 1991). These data suggest that slow and fast muscles originate from distinct pools of myoblasts that are programmed to express specific patterns of AChE molecular forms. Studies by Boudreau-Lariviére and colleagues (1997) however, have shown that inactive, though still innervated fast muscles resulted in a molecular form profile similar to that of slow muscles. Hence in these experiments, fast and slow muscles display one primordial basic AChE molecular form profile in which G_4 levels are modulated depending on activity and not muscle fibre type.

Figure 3. AChE molecular form profiles of slow versus fast skeletal muscles

This figure compares AChE molecular form profiles of slow and fast skeletal muscle fibres obtained from velocity sedimentation of whole muscle extracts. Fast fibres contain high levels of A_{12}, G_4 and G_1, whereas slow muscle typically displays a more complex distribution of all forms including A_8, A_4 and G_2.


A12
A8
G4
G2
G1
Slow
Fast
AChE Activity
(nmol/min//muscle)
Fractions
0
1
2
3
4
8
10
20
30
40

Heterogeneous expression of AChE in fast versus slow muscle is not limited to molecular form distribution. Overall levels of AChE protein and mRNA are significantly higher in fast as compared to slow muscles, possibly to accommodate the functional burden faced by fast twitch muscle fibres (Cresnar et al., 1994). The levels of AChE in fast and slow muscles are modulated by the nerve induced pattern of muscle activation. Chronic low-frequency stimulation of fast twitch fibres results in a decrease in the abundance of transcripts to levels comparable to slow muscles (Sketelj et al., 1998). The importance of this polymorphism in AChE expression has yet to be elucidated, however the vast array of AChE molecular forms as well as differences in total AChE levels must be involved in the fine tuning of cholinergic transmission at neuromuscular junctions, depending on the specific task of each individual muscle type.

1.2.5 Plasticity of AChE mRNA expression

The precise localization of AChE transcripts has been suggested to result from a number of converging mechanisms. Nerve-evoked activity plays a significant role in maintaining transcript levels in the junctional compartment as shown by experiments in which animals exposed to protocols of disuse such as denervation, displayed dramatic decreases in AChE mRNA (Cresnar et al., 1994; Michel et al., 1994). Alternatively, animals subjected to enhanced neuromuscular activation such as exercise training or compensatory hypertrophy showed marked increases in AChE transcript levels which lead to prominent changes in the levels of various molecular forms (Jasmin and Gisiger, 1990; Sveistrup et al., 1995). Furthermore, integrity of the nerve terminal and axonal transport

of trophic molecules to the synapse have been implicated in the maintenance of transcript levels in the synaptic compartment of muscles (Boudreau-Lariviere et al., 1996; Michel et al., 1994). The idea that neurotrophic factors can modulate transcriptional activity of the AChE gene upon release from the nerve terminal was examined by Boudreau-Lariviére and colleagues (1996, 1999) who demonstrated that calcitonin gene-related peptide (CGRP) and ciliary neurotrophic factor (CNTF) can regulate AChE gene expression in muscle cells. Although the signaling pathways resulting in induction of AChE gene expression are not yet known, it is believed that the action of CNTF is mediated through the Janus kinase-signal transducer and activator of transcription (JAK-STAT) or the mitogen-activated protein (MAP) kinase pathway (Curtis and DiStefano, 1994) and the action of CGRP is mediated by stimulation of the adenylyl cyclase pathway, triggering cAMP synthesis and activating various kinases (Duclert and Changeux, 1995; Ray et al., 1995). Our laboratory has also shown that mechanical stress alone can affect transcript levels in developing myotubes (Hubatsch and Jasmin, 1997) and that this action is mediated in part by calcium influx through L-type channels in the sarcolemma.

1.3 Origin of AChE at the Neuromuscular Synapse

The presence of nerve, muscle and glial cells in the environment encompassing the neuromuscular junction poses the question as to the origin of AChE localized to the synaptic basal lamina. The asymmetric forms found at the neuromuscular junction are known to originate predominantly from the muscle fibre (Di Giamberardino and Couraud, 1978). This was shown by experiments in which denervated rat soleus muscles from

which asymmetric forms had disappeared were reinnervated at ectopic sites. Reinnervation induced the reappearance of asymmetric molecules at the original site of nerve contact where the nerve was no longer present (Weinberg and Hall, 1979). Furthermore, using co-cultures of rat muscle and chicken spinal cord neurons and species-specific antibodies, De la Porte and colleagues were able to verify that AChE clusters appearing at the nerve-muscle contacts always originated from the muscle (De La Porte et al., 1986). Nonetheless, a small amount of asymmetric AChE at the neuromuscular junction has been shown to originate from nerve and schwann cells. This was shown by experiments in which newly synthesized AChE became associated with frog basal lamina preparations associated with motor neurons after muscle fibres were induced to degenerate and become permanently removed (Anglister, 1991).

1.3.1 Synapse-Specific Expression of AChE

The exclusive localization of AChE and other synaptic proteins at the neuromuscular junction has prompted an intense search for the molecular mechanisms involved in regulating this unique distribution. Several theories have been postulated, including synapse-specific expression of AChE transcripts, stabilization of transcripts and proteins in the synaptic compartment or shuttling of gene products from extrajunctional regions into the synaptic compartment. Recent studies have examined the molecular basis underlying the accumulation of AChE at both avian (Jasmin et al., 1993) and mammalian (Michel et al., 1994) adult neuromuscular synapses. In these studies, quantitative RT-PCR experiments revealed that AChE transcripts were 10 fold higher in

the junctional compartment of quail and rodent muscle fibres as compared to extrajunctional regions. These results were confirmed by in situ hybridization experiments which showed that AChE transcripts were localized in the sarcoplasm directly beneath the nerve terminal (Grubic et al., 1995; Legay et al., 1995; Michel et al., 1994). These experiments confirmed the importance of transcript accumulation in AChE localization at the neuromuscular junction.

Transcript stability may be an important mechanism responsible for the accumulation of AChE in the synaptic compartment. Although no direct evidence exists to show that transcript stability results in preferential accumulation of AChE at the synapse, this hypothesis is supported by the finding that AChE transcription is not activated during muscle differentiation when a marked increase in AChE transcripts and proteins occurs (Fuentes and Taylor, 1993). Interestingly, transcript stability also accounts for the increased AChE enzyme activity and transcript levels that occurs during neuronal and hematopoietic cell differentiation as shown by nuclear run on assays and half life experiments (Coleman and Taylor, 1996; Chan et al., 1998).

Alternatively, the accumulation of AChE transcripts in the junctional compartment of muscle fibres may be due to local transcriptional activation of the AChE gene in myonuclei located within the postsynatpic sarcoplasm. Recently, cis regulatory elements were identified in the 5' untranslated regions of genes encoding several synaptic proteins that may account for this localized expression. Specifically a short stretch of nucleotides termed the N-box was identified in the promoter region of several genes including utrophin and various components of the AChR, known to be localized to the neuromuscular junction (Duclert and Changeux, 1995; Koike et al., 1995; Gramolini et al., 1997). The N-box is

believed to bind transcription factors of the Ets family that activate expression of genes in the junctional compartment of muscle fibres (Schaeffer et al., 1998). Hence, the question arose as to whether AChE was capable of such fine tuned transcriptional regulation.

The recent cloning of a 5.3 kb 5' flanking DNA fragment located 600 bp upstream of the initiation codon corresponding to the promoter of the rat AChE gene revealed that this sequence does indeed contain several transcription binding motifs including four N-boxes, two of which are in the first intron (Chan et al., 1999). To determine the contribution of the N-box motifs in targeting AChE to the neuromuscular junction, our laboratory performed gene transfer experiments using rat AChE promoter-reporter constructs. These experiments suggested that the AChE promoter does confer synapse-specific expression that could be abolished by mutation of the N-box closest to the initiator element in intron I. We also noted that deletion of 800 bp from the 3' end of this DNA fragment corresponding to a region in the first intron, resulted in a decrease in the activity of the promoter suggesting that an enhancer is also present in the first intron (Chan et al., 1999). Hence, these experiments suggest that local transcription by nuclei in the synaptic compartment plays a significant role in the accumulation of AChE at the neuromuscular junction.

Others have also shown evidence to suggest that preferential expression of AChE transcripts by synaptic nuclei occurs in muscle fibres. Indeed, in situ hybridization experiments revealed that although AChE transcripts generally have a Gaussian distribution in association with most nuclei of the myotube, some heavily labelled nuclei do exist suggesting that preferential transcription of the AChE gene does occur (Tsim et al., 1992). It is important to note that AChE transcripts are not as skewed in their

distribution as transcripts encoding the α-subunit of the AChR of which high levels of transcript labelling are associated with a small percentage of nuclei (Tsim et al., 1992).

1.3.2 Molecular Parking Lot Hypothesis

Although transcriptional and post-transcriptional mechanisms may play an important role in AChE accumulation at the synaptic cleft, it is also possible that receptors for asymmetric forms of AChE are exclusively localized in the synaptic basal lamina. Thus, the availability of these receptors may be an important factor limiting attachment of externalized AChE. Using species-specific monoclonal antibodies against AChE, Rotundo and colleagues (Rotundo et al., 1997) showed that frog muscles incubated with purified quail collagen-tailed AChE resulted in exclusive insertion of the quail molecules at previously established frog neuromuscular junctions. This suggests that specific attachment sites or a "parking lot" exists that allows for insertion of collagen-tailed AChE exclusively into the synaptic basal lamina where the molecules can be released, retained or turned over (Rotundo et al., 1997).

1.3.3 Subneural Specialization of the Secretory Pathway in Muscle Fibres

In addition to transcriptional and post-transcriptional regulation, the possibility that post-translational mechanisms contribute to the highly specific localization of AChE at the neuromuscular synapse also exists. In this context, the Golgi apparatus may play a key role in processing and targeting synaptic proteins to their final destination. The Golgi apparatus is an organelle responsible for post-translational regulation of proteins in the secretory pathway. It is involved in glycosylation, phosphorylation, sulfation and proteolysis, protein sorting, and coupling for transportation to intracellular, membrane and extracellular destinations (Mellman and Simons, 1992). The idea of a biochemically differentiated Golgi apparatus in the subneural compartment of skeletal muscle fibres was first emphasized by the finding of a synapse-specific carbohydrate that associates with AChE at the neuromuscular junction correlating with the localization of N-acetylgalactosaminyl transferase (Scott et al., 1988; Scott et al., 1990).

Unlike the Golgi apparatus in most eukaryotic cells which displays a polarized organization in close proximity to the nucleus, the Golgi apparatus in skeletal muscle undergoes a distinct change in distribution during myo- and synaptogenesis (Antony et al., 1992; Jasmin et al., 1995; Jasmin et al., 1989). Using specific anti-Golgi antibodies, directed against a 210 kDa protein, a 160 kDa sialoglycoprotein, the rab6p GTP-binding protein, α-mannosidase II and TGN38, this group showed that the Golgi apparatus of mononucleated myoblasts, like most eukaryotic cells, shows a polarized juxtanuclear localization. Upon fusion of myoblasts to multinucleated myotubes, its distribution changes

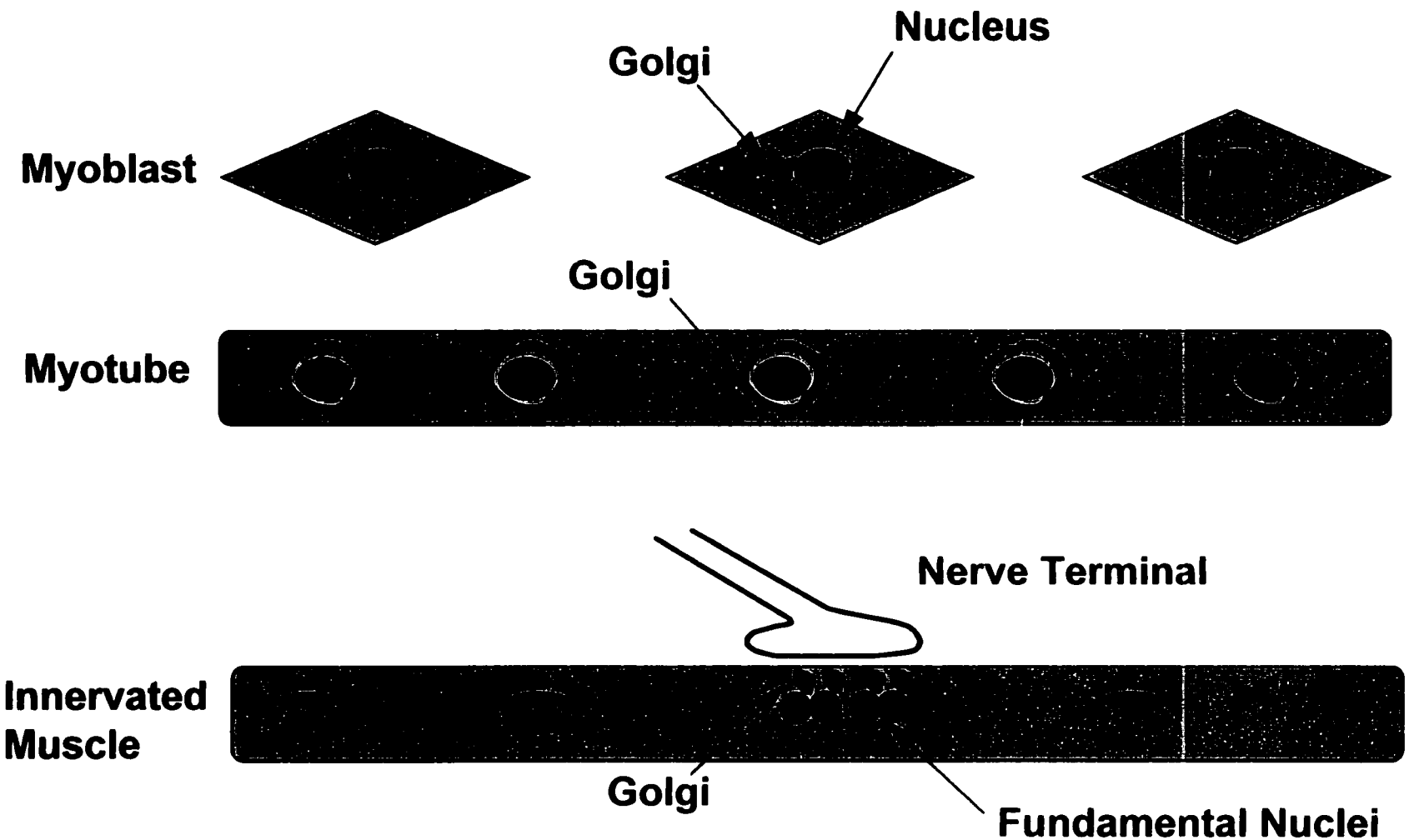
from polarized to perinuclear, surrounding each nucleus of the myotube. This is followed by a striking subneural compartmentalization of the Golgi apparatus when the muscle fibre is innervated (see Figure 4). This restriction of Golgi markers to the myonuclei of the postsynaptic sarcoplasm was found to occur at embryonic day 17 of development, correlating to 2-3 days after innervation (Antony et al., 1992). Furthermore, a lack of immunoreactivity with the TGN38 and α-mannosidase II antibodies in innervated muscle fibres was found by Jasmin and colleagues (1995), suggesting a biochemical differentiation of the subneural Golgi apparatus in adult muscle fibres.

This redistribution of the Golgi apparatus specifically to junctional nuclei upon innervation, the biochemical differentiation, and the presence of a differentiated subset of microtubules at the synapse (Jasmin et al., 1990) suggest a possible function in maturation and targeting of proteins bound for the synapse by the specialized Golgi apparatus. Furthermore, colchicine, an inhibitor of microtubule polymerization, was shown to block secretion of AChE and resulted in a cytoplasmic build-up of the enzyme in neurons suggesting that these microtubules may be involved in targeting AChE to the cell surface and ultimately, to the synapse. In addition, clathrin coated vesicles have been shown to be involved in targeting asymmetric and P subunit associated AChE to the cell surface (Hodges-Savola et al., 1989; Rotundo et al., 1989). Thus, in skeletal muscle fibres, it appears that the highly differentiated subsynaptic Golgi apparatus and the secretory pathway in general is responsible for assembly, maturation and targeting of asymmetric forms of AChE as well as other synaptic proteins.

Figure 4. Golgi redistribution during muscle development

Representation of Golgi apparatus redistribution during muscle development. Immature myoblasts contain Golgi stacks displaying a juxtanuclear association, typical of most eukaryotic cells. Upon fusion into multinucleated myotubes, the Golgi apparatus shows a perinuclear association with each nucleus of the myotube. Innervation of these myotubes results in a synapse-specific localization of the Golgi apparatus, specifically, with the morphologically distinct "fundamental" nuclei beneath the nerve terminal.

Golgi Redistribution During Muscle Development



1.4 Objectives of Study and Hypothesis

Although expression and localization of AChE at the neuromuscular junction has been studied extensively at the transcriptional and post-transcriptional level (Jasmin et al., 1993; Michel et al., 1994), the contribution of post-translational regulatory mechanisms to this synapse-specific accumulation has yet to be elucidated. The recent evidence for a synapse-specific secretory pathway in muscle fibres, including a specialized Golgi apparatus (Antony et al., 1992; Jasmin et al., 1995; Jasmin et al., 1989), distinct microtubules (Jasmin et al., 1990) and AChE carrying clathrin-coated vesicles (Hodges-Savola et al., 1989; Rotundo et al., 1989) provide strong support for a likely involvement of post-translational mechanisms to the targeting of AChE as well as other synaptic proteins to their final destination at the neuromuscular junction. In the first part of this study, we examined the tissue-specific expression, assembly, stability and secretion of overexpressed AChE in cultured myotubes. Secondly, we examined the targeting of overexpressed AChE in rodent muscle fibres. Based on previous findings detailed in the literature, we hypothesized that: 1) The T form of AChE would be exclusively expressed in cultured myotubes. 2) Co-expression of AChE with collagenic structural subunits would result in increased levels of AChE that would be specifically secreted into the media and attached to the muscle basal lamina. 3) Lastly, that expression of AChE in rodent muscle fibres would be limited exclusively to the synaptic compartment of muscle fibres.

We employed two models of gene transfer to examine these hypotheses. The first model involved transfection of cDNAs encoding AChE and the *Torpedo* collagenic structural subunit, all of which were under the control of strong constitutive promoters, into

C_2C_{12} myoblasts followed by examination of AChE expression by activity assays and immunofluorescence labelling. Secondly, we employed gene transfer techniques to examine targeting of overexpressed AChE in rodent hindlimbs. Similar to the culture system, detection of gene products in muscle sections was accomplished by AChE histochemical and immunofluorescence labelling.

Chapter 2

Methods

2.1 Cultures of C_2C_{12} mouse Myotubes

C_2C_{12} cells originate from hindlimb muscles of mice (Yaffe and Saxel, 1977) and serve as an ideal model system to study a variety of skeletal muscle characteristics. We obtained C_2C_{12} myoblasts (ATCC; Rockville, MD) frozen in Dulbecco's modified Eagle medium (DMEM):fetal calf serum:dimethylsulfoxide (Sigma Chemical Co., St. Louis, MO) (7:2:1). Cells were thawed quickly at 37°C and pelleted at 350 X g for 10 min at 4°C. They were subsequently washed 3X at room temperature in DMEM and plated on 35 mm plates coated with Matrigel™ (Collaborative Biomedical Products; Bedford, MA). Cells were grown in a proliferation medium consisting of DMEM with 20% horse serum, 10% fetal calf serum, 1% L-glutamine and 1% penicillin/streptomycin. They were maintained at 37°C in a water-saturated environment containing 5% CO_2. To stimulate differentiation, the growth media was replaced with a low-serum, differentiation media consisting of 5% horse serum, 1% L-glutamine and 1% penicillin/streptomycin in DMEM. All tissue culture reagents were purchased from Gibco BRL (Burlington, ON) unless otherwise indicated.

C_2C_{12} myoblasts were passaged when they reached approximately 75% confluence. The growth media was removed and replaced with 1 ml of a DMEM solution containing 0.25% trypsin and 1 mM EDTA. The mixture was incubated at 37°C for 10 min or until all cells were detached at which point the trypsin/EDTA solution was neutralized with 2 ml of the original growth media. Culture dishes were then washed 2X with DMEM. This DMEM was then added to the previous cell suspension and the mixture was centrifuged at 350 X g for 10 min at 4°C. The pellet was resuspended in growth media and the cells replated on Matrigel™-coated plates.

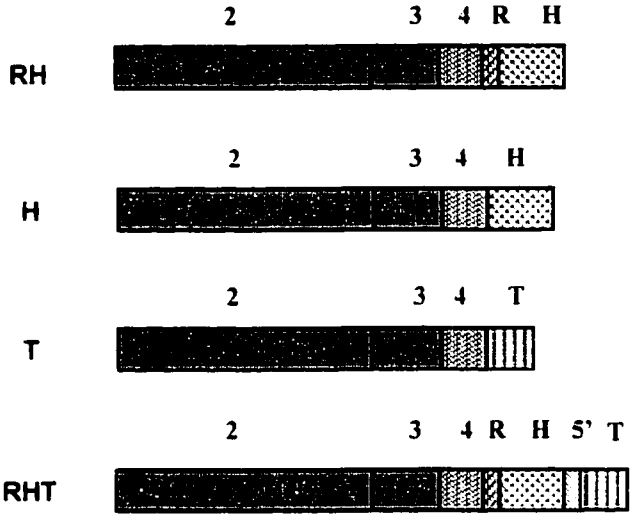
2.2 Expression Vectors

1) ***AChE***. The rat $AChE_T$ and $AChE_H$ cDNAs used were cloned by Legay and colleagues (Legay et al., 1993; Legay et al., 1993). $AChE_{RH}$ and $AChE_{RHT}$ cDNAs were obtained by RT-PCR using total RNA isolated from rat spleen. The RH construct contains the 4 common exons followed by intron 4 and exon 5 and thus allows for expression of both the R and H catalytic subunits. The $AChE_{RHT}$ cDNA, allows for the expression of all three AChE alternatively spliced forms. It contains the 4 common exons followed by intron 4, exon 5 and exon 6 (see Figure 5a). None of these constructs contain a 3' untranslated sequence. All constructs have 12 bp of the 5' untranslated sequence GTCCTGGCAGTC preceeding the initiation codon. In addition, all constructs contain the ACCATGG Kozak sequence flanking the ATG start codon. This is the optimal sequence for initiation on eukaryotic ribosomes and it has been shown to increase the translation efficiency of eukaryotic genes (Kozak, 1986). All of the above cDNAs were inserted at the Xba1 restriction sites of the pEF-BOS plasmid polylinker region (Mizushima and Nagata, 1990) under the control of the elongation factor-1α (EF-1α) promoter.

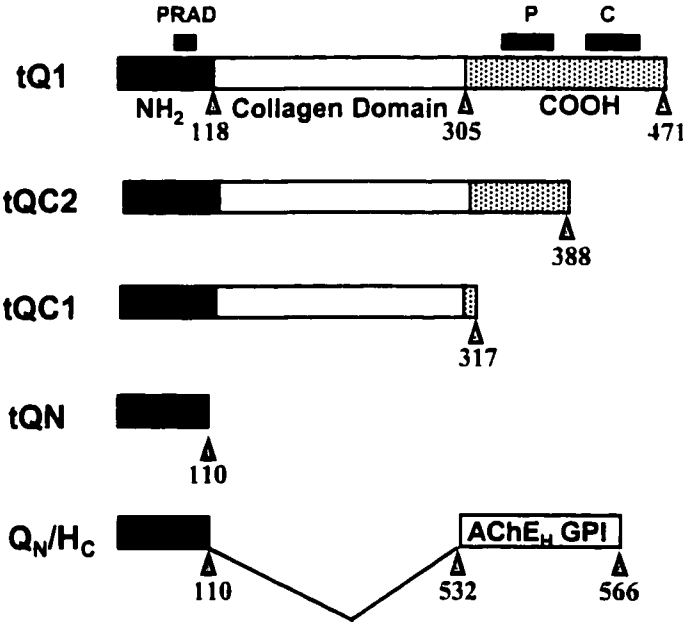
Figure 5. AChE catalytic and structural subunit cDNAs

Schematic representations of the cDNAs encoding the splice variants of the AChE gene (Figure A) and the *Torpedo* collagenic structural subunit mutants (Figure B) used in C_2C_{12} and in vivo transfections. All cDNAs are controlled by the constitutive pEF BOS promoter.

(A) AChE Catalytic Subunit cDNA Constructs



(B) Collagenic Subunit cDNA Constructs



PRAD = Proline-Rich Attachment Domain (70-96 aa)
P = Proline-Rich Region (318-385 aa)
C = Cysteine-Rich Region (388-443 aa)

2) ***Collagenic structural subunits***. A pEF-BOS vector containing the collagenic structural subunit cDNA (tQ1) from *Torpedo marmorata* (Krejci et al., 1991) was created by PCR. Mutants of this cDNA were also created using a forward common oligonucleotide and various mutagenized 3' oligonucleotides (see Figure 5b). The tQC2 and tQC1 mutants code for truncated collagenic subunits, lacking 83 and 154 amino acids of the C-terminal, respectively. Another mutant, tQN, codes for a collagenic subunit lacking the entire collagenic domain. Its sequence contains only 110 amino acids of the N-terminal. The Q_H/N_C collagen tail mutant, was modified to express a chimeric protein in which the above N-terminal domain of the collagenic subunit (tQN) was linked to the GPI-anchor signal located in the C-terminal of the $AChE_H$ cDNA (Duval et al., 1992). Each of these constructs also contained the Kozak sequence. Finally, we also used a cDNA encoding the full length rat collagenic structural subunit (COLQ) inserted into a pcDNA plasmid (Krejci et al., 1997).

3) ***CMV-LacZ***. A pCMVβ reporter vector (Clonetech Laboratories, Palo Alto, CA) designed to express β-galactosidase from the human cytomegalovirus immediate early gene promoter was also used. This plasmid contains the immediate early gene promoter/enhancer, an intron (splice donor/splice acceptor), a polyA signal from SV40 and the full length E. coli β-galactosidase gene with eukaryotic translation initiation signals.

2.3 Transfection of C_2C_{12} cells

Plasmid DNA was prepared using the Qiagen Mega-Prep kit (Qiagen Inc., Chatsworth, CA) according to the manufacturer's recommendations. DNA pellets were dissolved in 0.1 M phosphate-buffered saline (PBS) or H_2O for in vivo and in vitro experiments, respectively. At 50% confluence, C_2C_{12} cells were transfected by using the calcium phosphate method as described previously (Ekstrom et al., 1993) using the Profection Mammalian Transfection System (Promega, Madison, WI), according to the manufacturer's recommendations. The growth media was replaced with fresh media 3 hrs before transfection. Cells were then incubated for 6 hrs with a solution containing 5 µg of each of the appropriate cDNAs in a solution of 2 M $CaCl_2$ and 2X Hepes-buffered saline. Following this incubation, cells were washed with DMEM and treated with 15% glycerol in DMEM for 3 min to increase uptake of plasmid DNA. After washing 3X with DMEM, cells were then incubated in proliferation media until they reached 90% confluence, at which point, the proliferation media was replaced with differentiation media for a period of two days followed by serum-free media for the third day. The three day time period was sufficient for myotubes to form on the entire surface of the plate. Although we were not able to determine what proportion of the myoblasts fused to form myotubes, few myoblasts were visible on the surface of the plates prior to analysis. Three µg of the plasmid encoding CMV-LacZ was included in the DNA mixture used to transfect tissue cultured muscle cells. This allowed for the normalization of AChE activity to β-galactosidase activity to account for transfection efficiency.

2.4 AChE Extraction and Biochemical Analysis

C_2C_{12} myotube cultures were washed with 0.1 M cold PBS and then scraped with a rubber policeman (Sarstedt Inc., Newton, N.C.) in 400 µl of a high-salt detergent buffer containing protease inhibitors: 10 mM Tris-HCl (pH 7); 10 mM EDTA; 1 M NaCl; 1% Triton X-100 (BDH Inc., Toronto, ON); 1 mg/ml bacitracin (Sigma); 2.5 mg/ml aprotinin (Boehringer Mannheim, GmbH, Germany). Cells were then homogenized on ice for 30 seconds with a Polytron (Kinematica; Littan, Switzerland) set at 2. Homogenates were centrifuged for 15 min at 20 000 x g at 4°C. The resulting pellets and supernatants were frozen at -80°C for further analysis. A 600 µl aliquot of media was also taken to analyse AChE secretion.

Total AChE activity was determined by the spectrophotometric method of Ellman (1961) as previously described (Gisiger and Stephens, 1983; Jasmin and Gisiger, 1990). Aliquots of 25 µl from the homogenates were incubated in 1 ml of a 0.2 M phosphate-buffered solution (pH 7) containing 7.5 x 10^{-4} M acetylthiocholine iodide (Sigma) as substrate, 5 x 10^{-4} M dithionitrobenzoic acid (DTNB, Sigma) also known as the Ellman's Reagent and 10^{-5}M of the butyrylcholinesterase inhibitor tetraisopropylpyrophosphoramide (iso-OMPA, Sigma). Non-specific hydrolysis was measured in the presence of both iso-OMPA and the AChE specific inhibitor 5-bis(4-allyldimethylammoniumphenyl)pentanone dibromide (BW, Sigma). Hydrolysis was measured at 412 nm using a Beckman DU 640 spectrophotometer (Palo Alto, CA).

Protein concentration was determined using the Bicinchoninic acid protein assay reagent kit (Pierce, Rockford, Il) as described by the manufacturer. β-galactosidase activity was determined using the β-galactosidase Enzyme Assay System (Promega,

Madison, WI). Briefly, 50 µl of the homogenate was incubated for 1hr in the 2X Assay buffer solution provided in the kit. Samples were then read at 420 nm using the Beckman spectrophotometer. AChE activity was divided by the values obtained for protein content and β-galactosidase activity to obtain activity values normalized for cell number and gene transfer efficiency.

In separate experiments, the various AChE molecular forms were separated by velocity sedimentation as described in detail elsewhere (Jasmin and Gisiger, 1990). One hundred µl aliquots of the myotube extracts were layered onto 5-20% sucrose gradients prepared in a high salt tris-HCl buffer. Samples were centrifuged in a Beckman SW41 rotor at 40 000 rpm for 16 hr at 4°C. Approximately 45 fractions were collected from the bottom of the tubes and assayed for AChE activity using the Ellman method. Molecular forms were designated according to their sedimentation coefficients using the accepted nomenclature (Bon et al., 1979).

2.5 Histochemistry

Transfected myotubes were grown for three days after differentiation was induced. They were then fixed for 15 min with 2% paraformaldehyde, 2 mM $MgCl_2$, 1.25 mM EDTA in PBS and washed in 1X PBS at room temperature. They were subsequently stained for 30 min using the AChE histochemical procedure of Karnovsky and Roots (1964) which results in a reddish-brown stain due to the activity of AChE.

2.6 Immunofluorescence

Immunofluorescence experiments were performed to identify AChE expression in myotubes transfected with the various plasmids. AChE was labelled using the A63 rabbit anti-rat AChE polyclonal antibody (Marsh et al., 1984). Myotubes were first fixed for 15 min at room temperature with the paraformaldehyde solution described above and washed 2 X 5 min with buffer A which contains 0.5% bovine serum albumin and 0.15% glycine in 0.1 M PBS. In some cases, 0.1% Triton X-100 (BDH) was included in the wash buffer to permeabilize the cells. Non-specific labelling was then blocked by incubating the myotubes with 5% normal goat serum in buffer A for 15 min. The myotubes were then incubated with the A63 antibody, diluted 1:300 in buffer A for 1 hr at room temperature. Following thorough washing with buffer A, myotubes were incubated with an anti-rabbit secondary antibody labelled with FITC (1:500 in buffer A, Jackson Immunoresearch Laboratories; Mississauga,ON) for 1 hr and subsequently washed 3 x 15 min with 1 X PBS. Cultures were then mounted in Citifluor (Canterbury, UK) to prevent fading and visualized using a Zeiss Axiophot fluorescence photomicroscope (Oberkochen, Germany). Photographs were taken with Kodak Tmax-100 black and white film.

2.7 Animal Care and Surgical Procedures

Four-week old female C57BL/6J mice and adult female Sprague Dawley rats were purchased from Charles River laboratories (St. Constant, P.Q.) and were used for all direct gene transfer experiments. Care and treatment of the animals were rigorously monitored

by the Animal Care Committee of the University of Ottawa to meet the guidelines established by the Canadian Council on Animal Care.

2.8 In Vivo Gene Transfer

In vivo gene transfer experiments were done as described elsewhere (Gramolini et al., 1997). Animals were first anaesthetized in a plexiglass chamber using gaseous Halothane. Both hindlimbs were then shaved and 50 µl of the appropriate plasmid solution (see Table 1) was injected directly into each of the tibialis anterior (TA) muscles through the skin, as the needle was slowly pulled out of the muscle. A 3/10cc syringe with a 29 Ga. x 1/2" needle was used to perform these injections. Seven days later, the animals were anaesthetized and their TA muscles were removed quickly, and frozen in melting isopentane cooled in liquid nitrogen. They were stored at -80°C until further analysis.

Table 1

Composition of Plasmid Cocktails Used In Injection Experiments

cDNA type and Concentration (µg/µl)	Volume	# of Animals
Control LacZ (2.5)	50µl	5
$AChE_T$ (2.5) + LacZ (2.5)	40µl + 10µl	5
$AChE_T$ (2.5) + tQ1 (2.5) + LacZ (2.5)	20µl + 20µl +10µl	12
$AChE_T$ (2.5) + Q_H/N_C (2.5) +LacZ (2.5)	20µl + 20µl +10µl	8
$AChE_T$ (2.5) + COLQ (2.5) + LacZ (2.5)	20µl + 20µl +10µl	8

2.9 Identification of Gene Products In Vivo By Histochemistry or Immunofluorescence

Longitudinal serial muscle sections, 12 µm thick, were cut with a cryostat. Serial sections were placed on sets of three Superfrost slides. The first slide of each set was stained for β-galactosidase using a modification of the method of Dannenberg and Suga (1981). These muscle sections were fixed for 15 min at 4°C in 1% glutaraldehyde in PBS and washed 3 x 5 min in PBS at 4°C. They were then incubated overnight at 37°C in a solution containing X-gal at 400 mg/mL, 1 mM $MgCl_2$, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide in 0.1 M PBS to label β-galactosidase activity enabling identification of the sections where plasmid uptake and expression by muscle fibres was most efficient. To detect the presence of AChE derived from injected plasmids and to localize the neuromuscular junctions, the second slide was fixed in 2% paraformaldehyde, washed 2x with buffer A and analyzed for immunofluorescence using the A63 AChE antibody as previously described for tissue culture experiments. All slides labelled for immunofluorescence were mounted in Citifluor (Canterbury, UK) to prevent fading. To ensure that the AChE observed in the immunofluorescence experiments was indeed active, the third slide was fixed with 1% glutaraldehyde in PBS and stained for AChE activity. Sections were visualized using the Zeiss Axiophot fluorescence photomicroscope (Oberkochen, Germany). Photographs were taken using Kodak Gold 100 colour film or the Sony 3CCD Digital Colour Video Camera in conjunction with Northern Eclipse software (Empix Imaging Inc., Mississauga, ON).

2.10 Statistics

To evaluate the effects of the experimental conditions, student's t-test were performed. Paired student's t-tests were performed to examine the effects of the in vitro transfection protocols versus control conditions. The level of significance for all tests was set at $p < 0.05$. The AChE molecular form profiles displayed in the figures are representative examples.

Chapter 3

Results

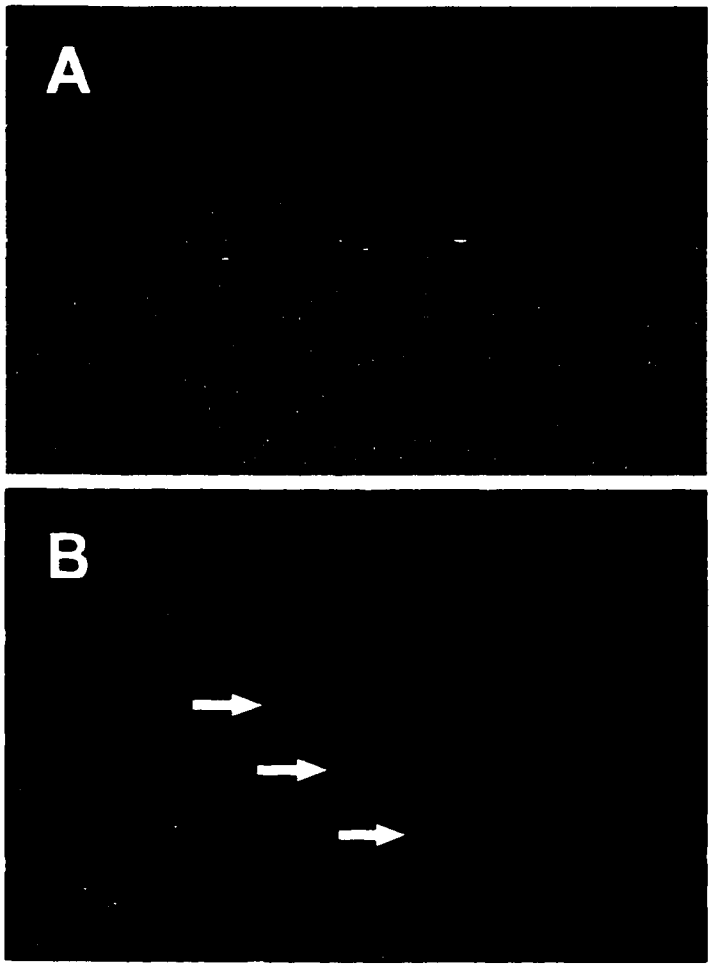
C_2C_{12} myoblasts required approximately 72 hours of incubation in a low-serum media to develop into myotubes after they reached 100% confluence (see Figure 6 for morphology). Hence, this was the time period we allowed before analysing the cells for AChE expression. Control myotubes contained extremely low levels of AChE activity in C_2C_{12} cell extracts and little to no activity in the media as determined by the Ellman assay (Figure 7). Velocity sedimentation analysis showed that the low activity level occasionally observed in myotubes, corresponded to G_1 and G_2 molecules (Figure 8). In agreement with recent studies using C_2C_{12} cells, control myotubes did not express asymmetric forms of AChE (Boudreau-Larivière and Jasmin, 1999; Luo et al., 1998). This was confirmed by histochemical and immunofluorescence staining of the myotubes which showed no surface and very little intracellular AChE activity or immunolabelling, respectively (data not shown).

3.1 Expression of AChE Splice Variants in Cultured Mouse Myotubes

C_2C_{12} muscle cells were transfected using the calcium phospate method with cDNAs encoding the alternatively spliced forms of AChE (see Figure 5). These experiments showed that all splice variants of AChE were efficiently expressed in transfected myotubes resulting in varying levels of activity depending on the construct used. In comparison to control non-transfected myotubes, muscle cells transfected with the different cDNAs resulted in a marked increase ($p<0.05$) in AChE activity (Figure 7). For example, the increase in total activity (including cell extract and secreted AChE) was six and thirty times higher in cells transfected with $AChE_T$ and $AChE_H$ constructs,

Figure 6. Morphology of C_2C_{12} cultured myoblasts and myotubes

Figure A shows C_2C_{12} immature muscle precursor cells termed myoblasts which originate from mouse hindlimb muscles. Incubation of these myoblasts in a low serum media results in fusion and formation of multinucleated myotubes (indicated by arrows) made visible in Figure B by Hoechst staining (Mag.=50x and 200x, respectively).


A
B

as compared to non-transfected myotubes. In our experiments, C_2C_{12} cells transfected with any of the AChE cDNAs showed no morphological difference from controls.

Using velocity sedimentation analysis, we determined the molecular forms assembled in myotubes transfected with the various constructs. Myotubes transfected with $AChE_T$ resulted in activity corresponding to the G_1 and G_4 globular forms, in decreasing order of abundance (Figure 8). The corresponding media contained G_1 and G_4 in similar amounts along with a lower proportion of G_2 (Figure 8).

Myotubes transfected with $AChE_H$ showed the highest level of AChE activity. We determined by velocity sedimentation, that this activity corresponded exclusively to G_2 molecules (Figure 8). In order to determine that these molecules were indeed GPI-anchored AChE dimers, cell extracts were incubated with phosphatidyl-inositol specific phospholipase C (PI-PLC). PI-PLC is an enzyme known to specifically cleave GPI-anchors from proteins inserted into the cell membrane, and treatment with this enzyme resulted, in our experiments, in a shift of the G_2 molecular form peak (Figure 8). This shift is characteristic of a change in sedimentation resulting from cleavage, thus verifying that these dimers were indeed associated with GPI-anchors. Immunofluorescence experiments using a polyclonal antibody (A63) on non-permeabilized myotubes showed that the high levels of AChE enzyme produced in cells transfected with $AChE_H$ were in fact anchored to the cell surface (Figure 9).

Figure 7. All splice variants of AChE are expressed in transfected C_2C_{12} muscle cells

AChE activity associated with the cell and media fraction of myotubes transfected with cDNAs encoding each of the various alternatively spliced forms of the AChE gene are shown. The AChE activity values were corrected for β-galactosidase activity to account for gene transfer efficiency. All transfected cDNAs resulted in increased levels of AChE activity in both cell associated and secreted fractions (paired t-test; $p<0.05$, $n=5$).

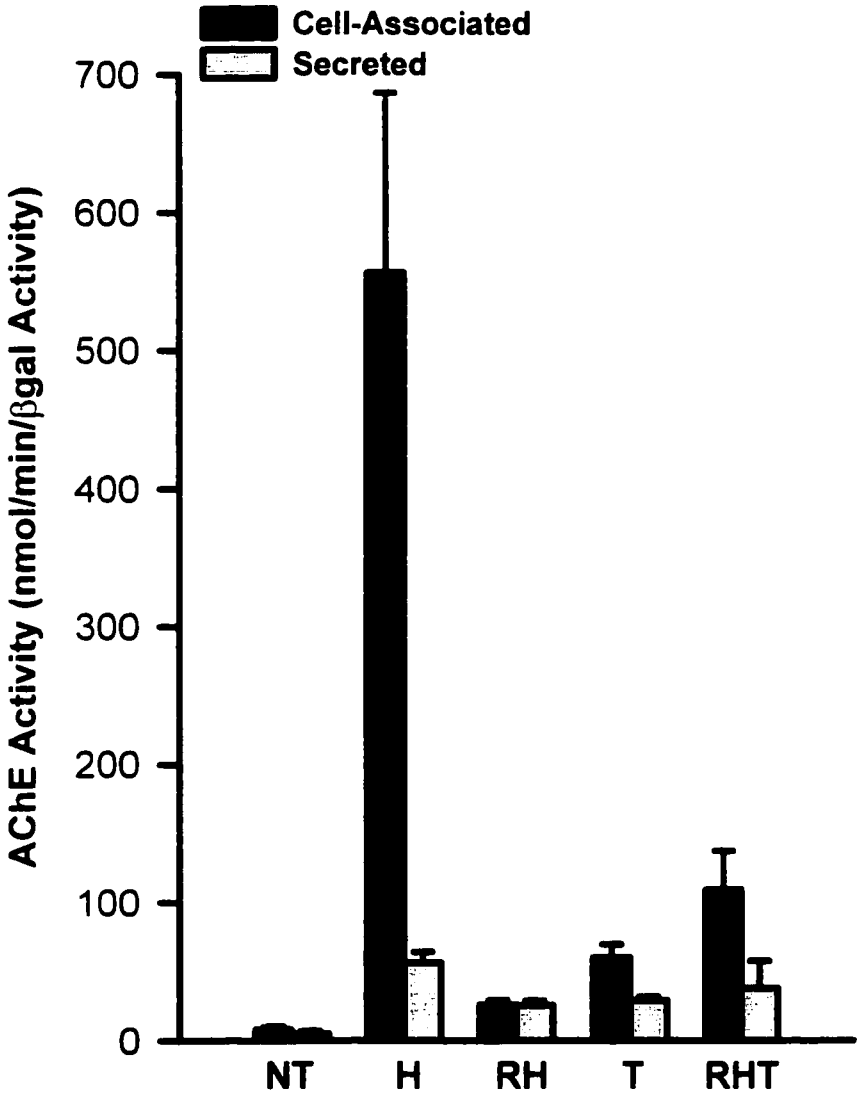
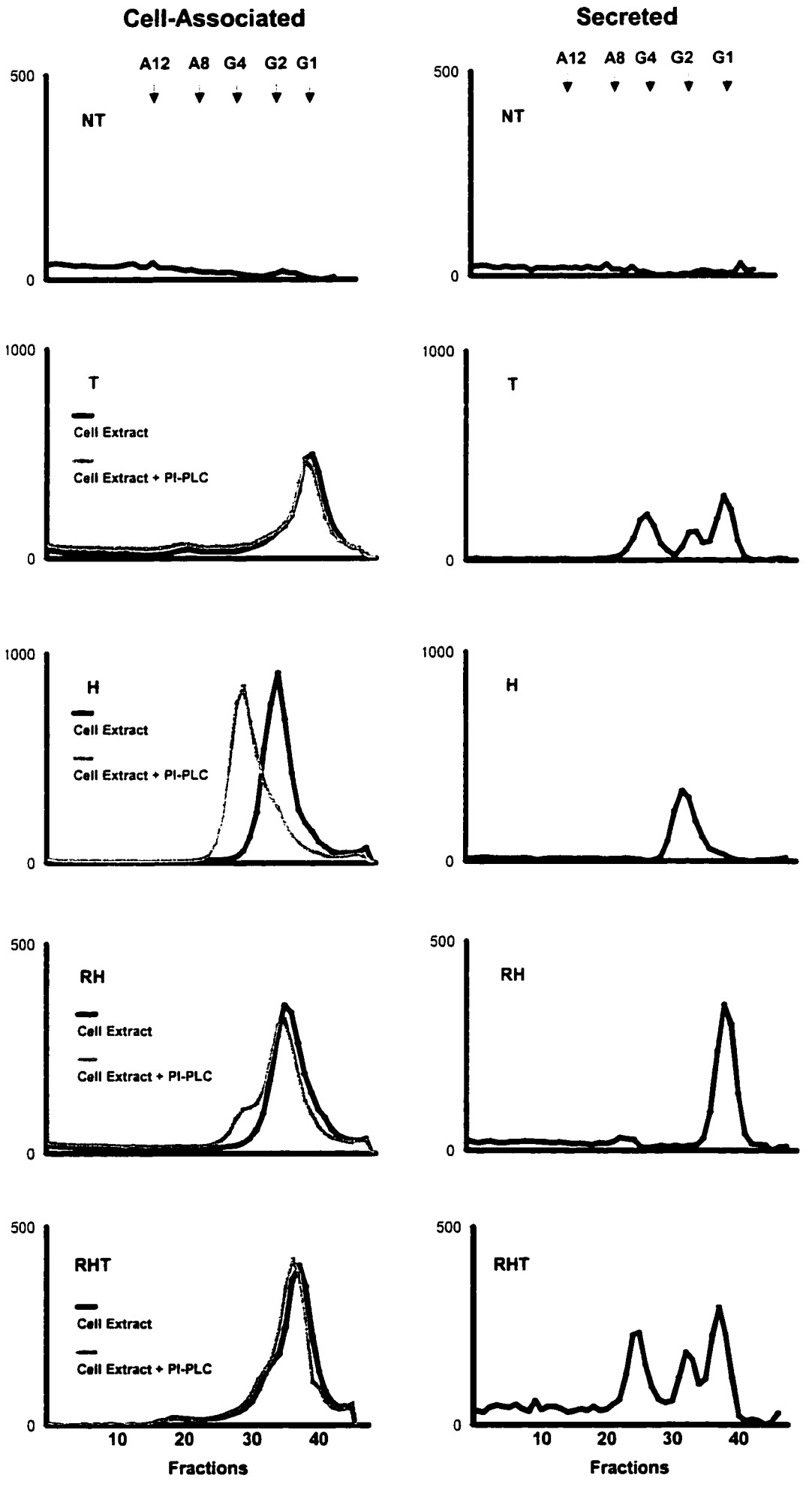

Cell-Associated
Secreted
AChE Activity (nmol/min/βgal Activity)
700
600
500
400
300
200
100
0
NT
H
RH
T
RHT

Figure 8. Molecular form profiles of myotubes transfected with the AChE splice variants

This figure shows the AChE molecular form profiles in the cell associated and secreted fractions of myotubes transfected with the above cDNAs. These profiles are determined by velocity sedimentation of extracted enzyme in a 5-20% sucrose gradient. The nomenclature assigned to the peaks seen in these figures are designated according to (Bon et al., 1979).


Cell-Associated
Secreted
A12 A8 G4 G2 G1
500
0
NT
1000
T
Cell Extract
Cell Extract + PI-PLC
H
RH
RHT
10 20 30 40
Fractions

Total AChE activity associated with myotubes transfected with $AChE_{RH}$ was significantly lower ($p<0.05$) than those transfected with $AChE_T$. However, both cell-associated and secreted AChE activity were markedly higher ($p<0.05$) than in control myotubes (Figure 7). In comparison to myotubes transfected with $AChE_T$, an increased proportion of activity derived from the $AChE_{RH}$ construct, was secreted as soluble non-amphiphilic monomers (Figure 8) suggesting that these molecules originated from R transcripts derived from the $AChE_{RH}$ cDNA. Velocity sedimentation of the cell extract also revealed the presence of a large peak of monomers. Concomitantly, the $AChE_{RH}$ construct allowed for expression of a small percentage of AChE dimers in the cell extract, likely corresponding to GPI-anchored molecules. This was in fact verified by PI-PLC treatment which resulted in a shift of the G_2 peak observed in these transfections (Figure 8). These dimers were likely produced by an alternative splicing event in the transcript of the $AChE_{RH}$ cDNA which resulted in production of the H peptide.

Myotubes transfected with $AChE_{RHT}$ showed a marked increase ($p<0.05$) in AChE activity as compared to controls. Interestingly, although the total AChE activity in $AChE_{RHT}$ transfected myotubes was significantly higher ($p<0.05$) than in those transfected with $AChE_T$ (Figure7), the molecular form profiles (Figure 8) were identical. In these cells, the sedimentation profiles were not affected by treatment of the cell extracts with PI-PLC suggesting that the G_2 peaks observed in cells transfected with $AChE_{RHT}$ did not correspond to GPI-anchored molecules but more likely corresponded to AChE derived from the T transcript (Figure 8).

Figure 9. Production of surface-anchored AChE in C_2C_{12} myotubes transfected with $AChE_H$

Immunofluorescence staining of non-permeabilized myotubes transfected with the $AChE_H$ cDNA using the A63 anti-AChE polyclonal antibody resulted in intense labelling of the cell surface. This verifies that the AChE molecules produced by this splice variant were able to efficiently recruit GPI-anchors for insertion into the sarcolemma of C_2C_{12} myotubes (Mag.=200x).

$AChE_H$

3.2 Association of $AChE_T$ Catalytic Subunits with the Collagenic Structural Subunit (Q)

To analyse the influence of Q subunits on AChE expression, we transfected C_2C_{12} muscle cells with cDNAs encoding the rat $AChE_T$ subunit, alone, or together with the *Torpedo* collagenic structural subunit, tQ1. tQ1 has already been shown to associate with *Torpedo* and rat catalytic subunits in other cell culture systems (Duval et al., 1992; Krejci et al., 1991; Legay et al., 1993). Myotubes co-transfected with $AChE_T$ and tQ1 displayed a significant increase ($p<0.07$) in total AChE activity as compared to cells transfected with T alone (Figure 10) indicating that a fraction of the enzyme became stabilized by associating with the collagenic subunit. As expected, velocity sedimentation analysis showed that a substantial amount of the enzyme produced in these cells was recruited by tQ1 and assembled into asymmetric molecules of which A_{12} was the predominant form (Figure 11). High levels of G_1 and G_4 were also evident in the cell-associated fraction. Asymmetric forms were also shown to be efficiently secreted into the media at high levels. Interestingly, very little if any G_1 was secreted into the media (Figure 11).

Co-transfection of $AChE_T$ with Q_N/H_C, a cDNA encoding a chimeric structural subunit containing the N-terminal of the *Torpedo* collagenic structural subunit linked to the GPI-signal from the C-terminal of the H subunit, also resulted in a significant increase ($p<0.06$) in total AChE activity as compared to transfection with $AChE_T$ alone (Figure 10). Unlike co-transfection with the wild-type tQ1 subunit however, this activity was predominantly cell-associated, with only a small fraction secreted into the media (Figure 10).

Figure 10. Association of $AChE_T$ catalytic subunits with collagenic (tQ1) and chimeric (Q_H/N_C) structural subunits resulted in increased AChE activity in myotubes

AChE activity in the cell associated and secreted fractions of myotubes co-transfected with $AChE_T$ and either the collagenic structural subunit (tQ1) or the chimeric structural subunit (Q_H/N_C) are shown. Both co-transfections resulted in a large increase in activity as compared to cells transfected with $AChE_T$ alone (paired t-test; $p < 0.07$ for tQ1 and $p<0.06$ for Q_H/N_C, n=6).

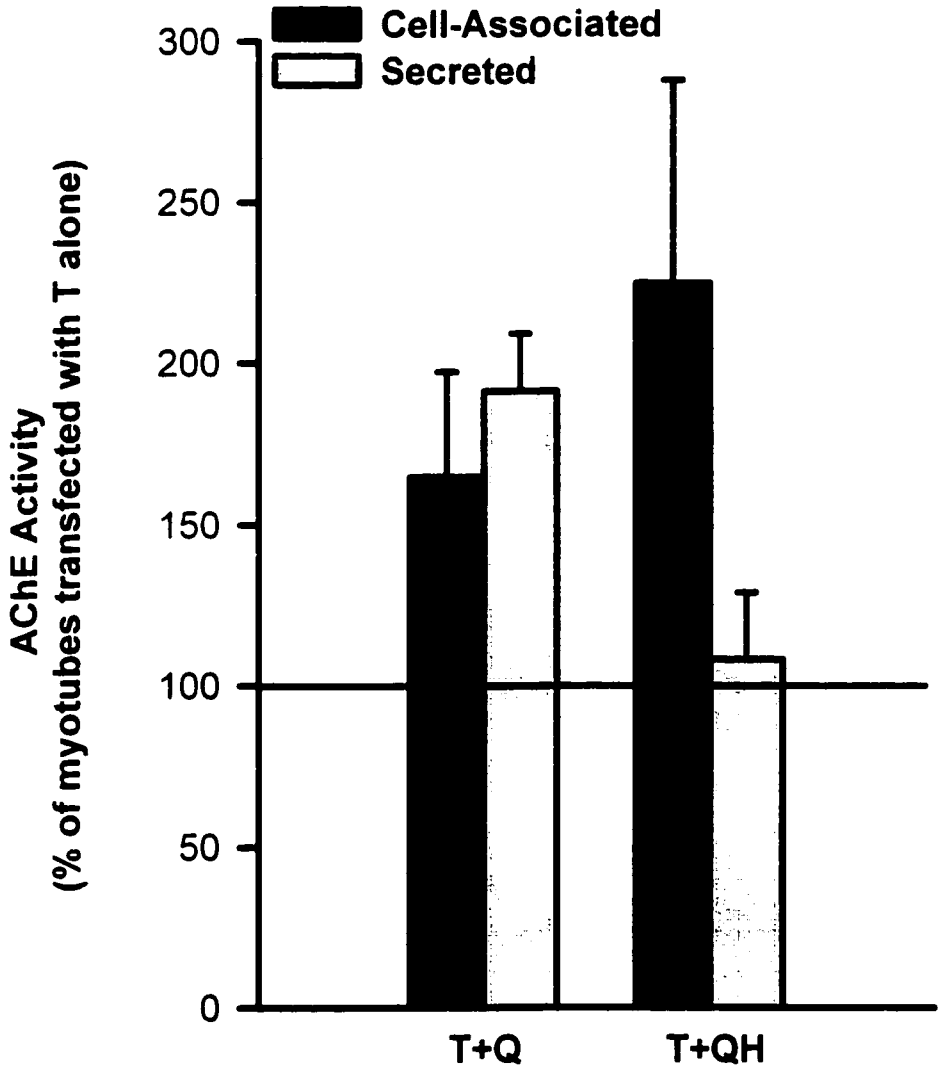
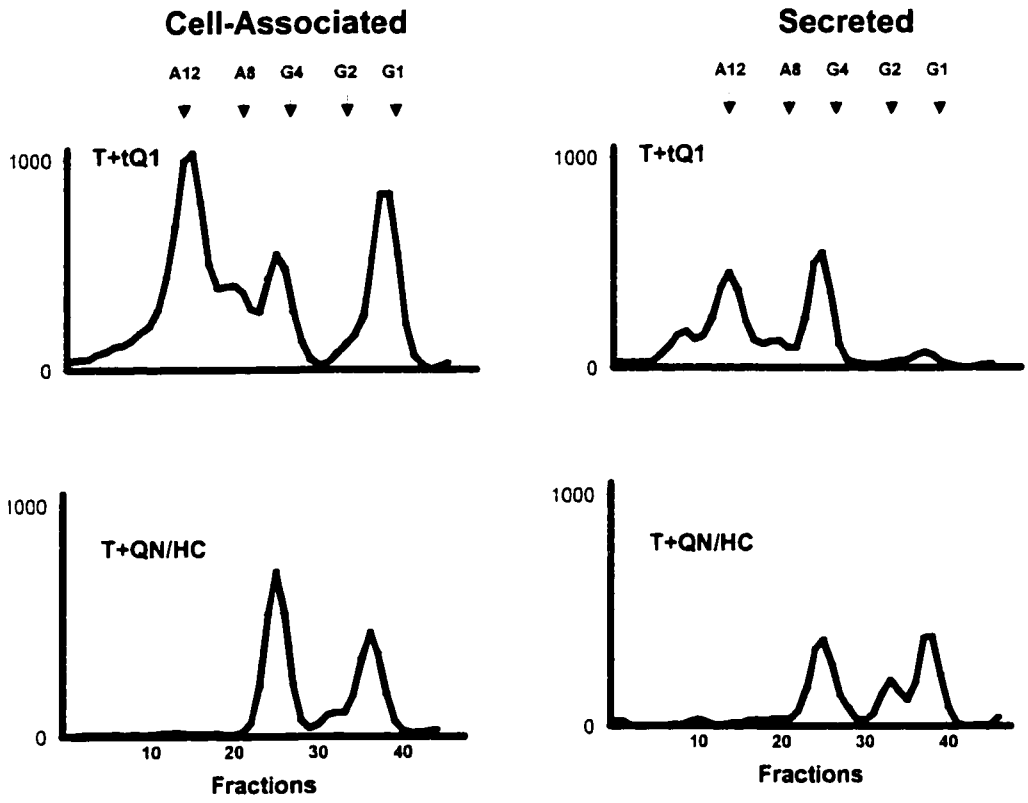

Cell-Associated
Secreted
AChE Activity
(% of myotubes transfected with T alone)
300
250
200
150
100
50
0
T+Q
T+QH

Figure 11. Molecular form profiles of myotubes co-transfected with cDNAs encoding AChE and collagenic structural subunits

Shown are the molecular form profiles of myotubes co-transfected with $AChE_T$ and tQ1 or Q_H/N_C. This figure highlights the substantial amount of asymmetric molecules, specifically A_{12} produced in myotubes co-transfected with $AChE_T$ and the wild type tQ1 subunit as well as the high levels of cell associated tetramers associated with myotubes co-transfected with $AChE_T$ and Q_H/N_C.


Cell-Associated
Secreted
A12
A8
G4
G2
G1
1000
T+tQ1
0
T+QN/HC
10
20
30
40
Fractions

Sedimentation analysis showed that the Q_N/H_C subunit was able to recruit AChE catalytic subunits into tetramers, most of which were cell-associated due to anchoring via GPI-linkage to the sarcolemma (Figure 11).

We also performed immunofluorescence experiments on transfected C_2C_{12} cells using the A63 antibody directed against rat AChE. Transfection with $AChE_T$ alone resulted in low levels of immunolabelling, most of which was only visible when the myotubes were permeabilized (Figure 12). This suggested that most of the overexpressed AChE remained intracellular. Co-transfection of $AChE_T$ with tQ1 resulted in patchy labelling of AChE protein on the cell surface, likely reflecting the association of asymmetric forms with the basal lamina surrounding each myotube. Permeabilization of these cells revealed that an even greater level of immunolabelling was localized intracellularly in co-transfected myotubes (Figure 12).

Myotubes co-transfected with $AChE_T$ and Q_N/H_C resulted in a very intense labelling of AChE along the entire surface of non-permeabilized myotubes as shown by immunolabelling using the A63 antibody (Figure 13). Localization of these molecules to the surface of myotubes suggested that they were efficiently assembled with GPI-anchors and inserted into the sarcolemma. To confirm that these molecules were indeed active, we also stained co-transfected myotubes with the histochemical stain of Karnovsky and Roots (1964). These experiments resulted in intense staining of myotubes co-transfected with $AChE_T$ and either tQ1 or Q_N/H_C (Figure 14) confirming the biochemical activity data (compare Figures 10 and 14)

Figure 12. Association of AChE with the basal lamina of myotubes co-transfected with $AChE_T$ and tQ1

Immunofluorescence experiments using the anti-AChE polyclonal antibody (A63) were performed to localize exogenous AChE expression in myotubes. C_2C_{12} myotubes transfected with $AChE_T$ showed no labelling on the surface (A), but when permeabilized with Triton X-100, low levels of AChE were seen intracellularly (B). However, when myotubes were transfected with $AChE_T$ and tQ1, patchy surface immunolabelling was observed (C) along with high levels of intracellular AChE as seen in permeabilized myotubes (D) (Mag.=200x).

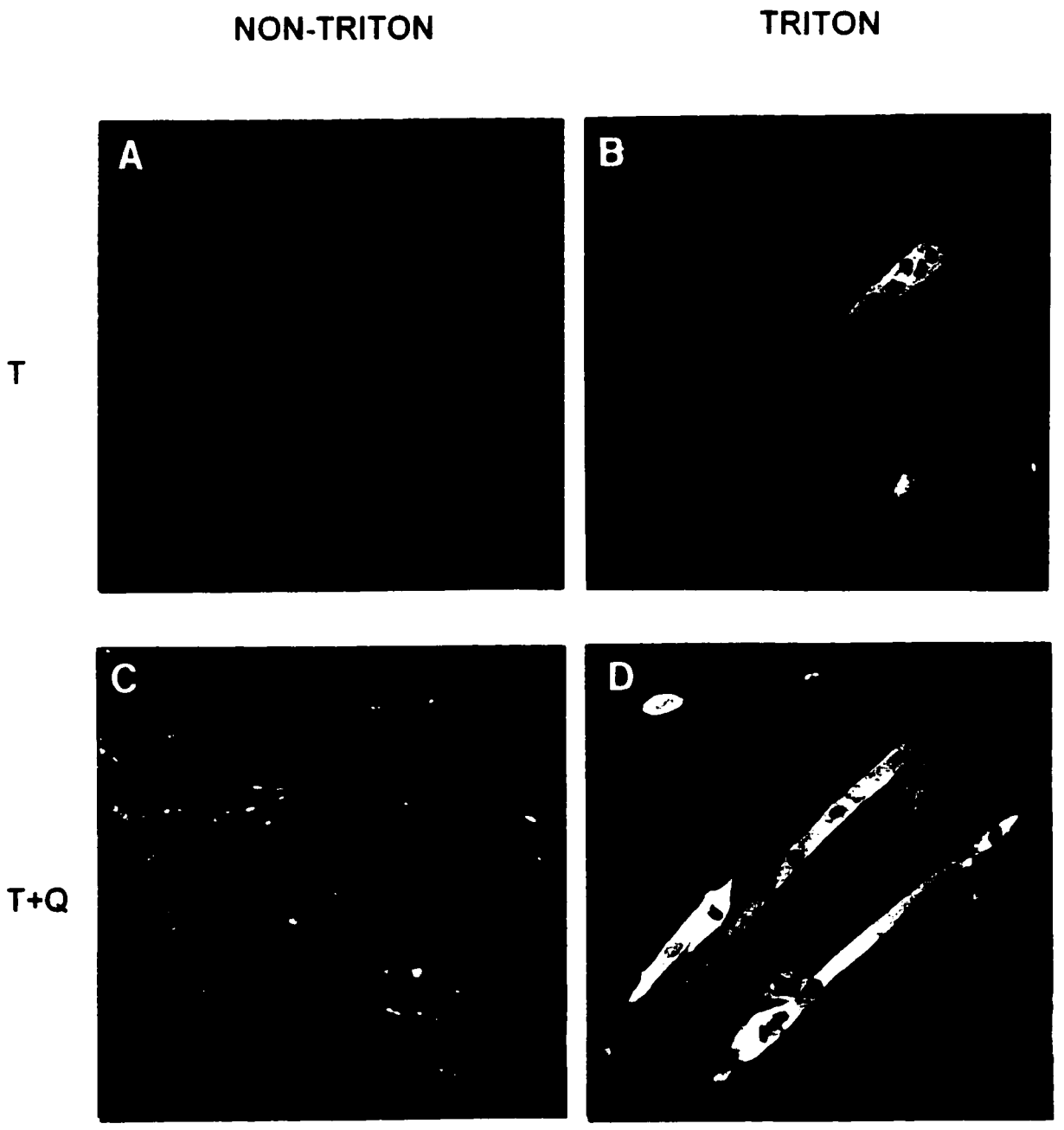

NON-TRITON
TRITON
A
B
T
C
D
T+Q

Figure 13. Production of surface-anchored AChE in C_2C_{12} myotubes transfected with $AChE_T$ and Q_H/N_C

Immunofluorescence experiments using the A63 anti-AChE antibody were performed to localize the GPI-anchored tetramers assembled in myotubes co-transfected with $AChE_T$ and the chimeric Q_H/N_C subunit. Intense AChE labelling was observed on the surface of transfected myotubes (Mag.=200x).

$AChE_T$
$+ Q_H/N_C$

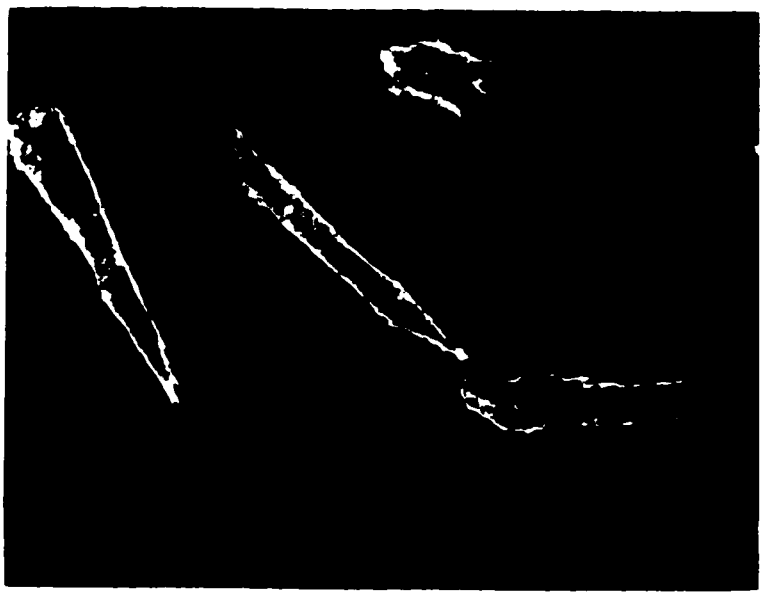
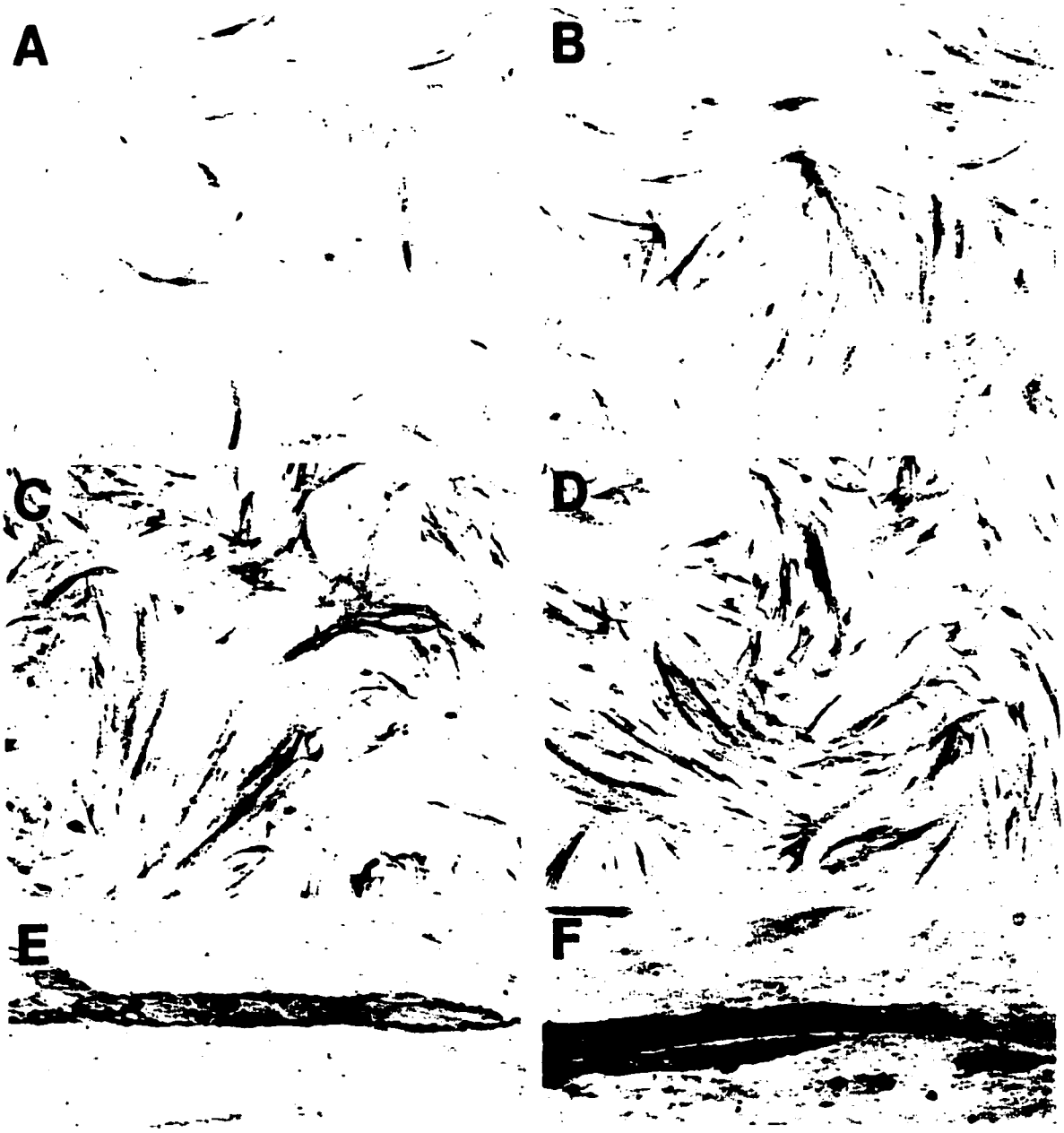


Figure 14. Catalytically active AChE is produced in myotubes co-transfected with cDNAs encoding catalytic and structural subunits

(A) Low levels of AChE activity are detected in myotubes transfected with $AChE_T$ alone as shown by histochemical staining using the method of Karnovsky and Roots. (B) Co-expression of the $AChE_T$ with the collagenic structural subunit tQ1 resulted in markedly higher activity levels. (C and D) Transfection with $AChE_H$ cDNAs or co-transfection with T catalytic subunits and Q_H/N_C respectively, resulted in the most dramatic increase in the intensity of staining. (E) Higher magnification of an individual myotube transfected with $AChE_T$ and tQ1. (F) Myotube transfected with $AChE_T$ and Q_H/N_C resulted in GPI-anchored molecules that are visible in the periphery of the myotubes (Mag.=25x).


A
B
C
D
E
F

3.3 Domains of the Collagenic Structural Subunit Involved in Assembly of Asymmetric AChE

The collagenic structural subunit consists of three main domains: 1) the N-terminal domain containing the PRAD region known to associate with AChE tetramers (Bon et al., 1997; Bon and Massoulie, 1997); 2) the collagenic domain which associates with other collagenic strands via disulfide bonds to assemble the triple helical structure observed in A_{12} molecules; and 3) the C-terminal domain which associates with the basal lamina of muscle fibres (Figure 5). Using cDNAs encoding $AChE_T$ and mutants of the *Torpedo* collagenic structural subunit generated by deletions at specific sites in the C-terminal (Figure 5), we examined the contribution of the various domains of tQ1 on the expression, assembly and targeting of asymmetric forms of AChE in myotubes. Specifically, we used cDNAs encoding a mutant of tQ1 that results in a protein lacking a small portion of the C-terminal domain including the cysteine-rich domain (tQC2), a mutant lacking the entire C-terminal domain including both the cysteine- and proline-rich domains (tQC1) and lastly, one lacking the C-terminal and collagenic domains, and containing only the N-terminal region (tQN).

Similar to myotubes co-transfected with $AChE_T$ and the wild-type tQ1 (Figure 10), activity in myotubes co-transfected with $AChE_T$ and either one of these three mutant structural subunits, was significantly higher ($p<0.05$) than in myotubes transfected with T alone (Figure 15). The highest level of total AChE activity was observed when the $AChE_T$ cDNA was co-transfected with the wild type tQ1 whereas the lowest amount was detected with co-transfection of tQN.

Sedimentation analysis revealed that, in contrast to the similar increase in total AChE activity, the molecular form profiles seen following transfection with $AChE_T$ and the tQ1 mutants were clearly distinct. The QC2 construct was the only mutant able to recruit catalytic subunits into complex asymmetric forms similar to tQ1 (Figure 16). Furthermore, only co-transfection with tQC2 led to secretion of A_{12} and A_8 molecular forms into the media (Figure 16). Alternatively, although co-transfection of myotubes with $AChE_T$ and either tQC1 and tQN resulted in higher activity levels ($p<0.05$) than in myotubes transfected with $AChE_T$ alone (Figure 15), velocity sedimentation analysis revealed that A_{12} and A_8 asymmetric molecules were not assembled (Figure 16). These experiments suggest that the tQC1 and tQN subunits are not capable of forming the triple helical structural subunit necessary for complex hetero-oligomeric interactions. The increased activity seen in myotubes co-transfected with $AChE_T$ and either tQC1 or tQN corresponded to a dramatic increase in the abundance of tetramers in both the cell-extract and media (Figure 16) indicating that these tetramers must be A_4 as opposed to soluble G_4 which were not abundantly expressed in myotubes transfected with $AChE_T$ alone (Figure 8). These experiments suggest that the association of the N-terminal of tQ1 with AChE catalytic subunits is important for stabilizing AChE tetramers resulting, in this case, in high levels of A_4.

Figure 15. AChE activity in myotubes co-transfected with $AChE_T$ and the truncated tQ1 subunits

Cell associated and secreted fractions of AChE activity in myotubes transfected with $AChE_T$ and the tQ1 subunit mutants truncated at their C-terminals are shown. Similar to myotubes co-transfected with $AChE_T$ and wild type tQ1, activity in myotubes co-transfected with $AChE_T$ and either one of the three mutant structural subunits (tQC2, tQC1 and tQN), was higher than in myotubes transfected with T alone (paired t-test; $p<0.05$, $n=6$).

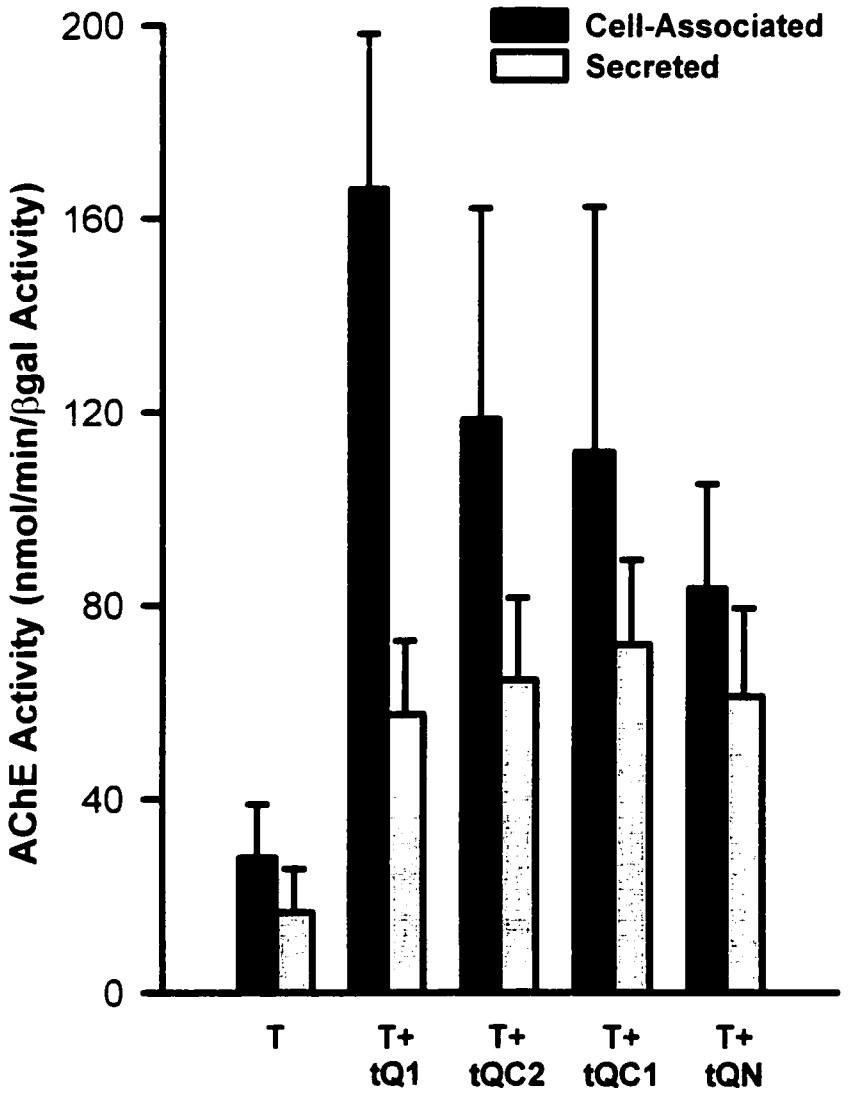
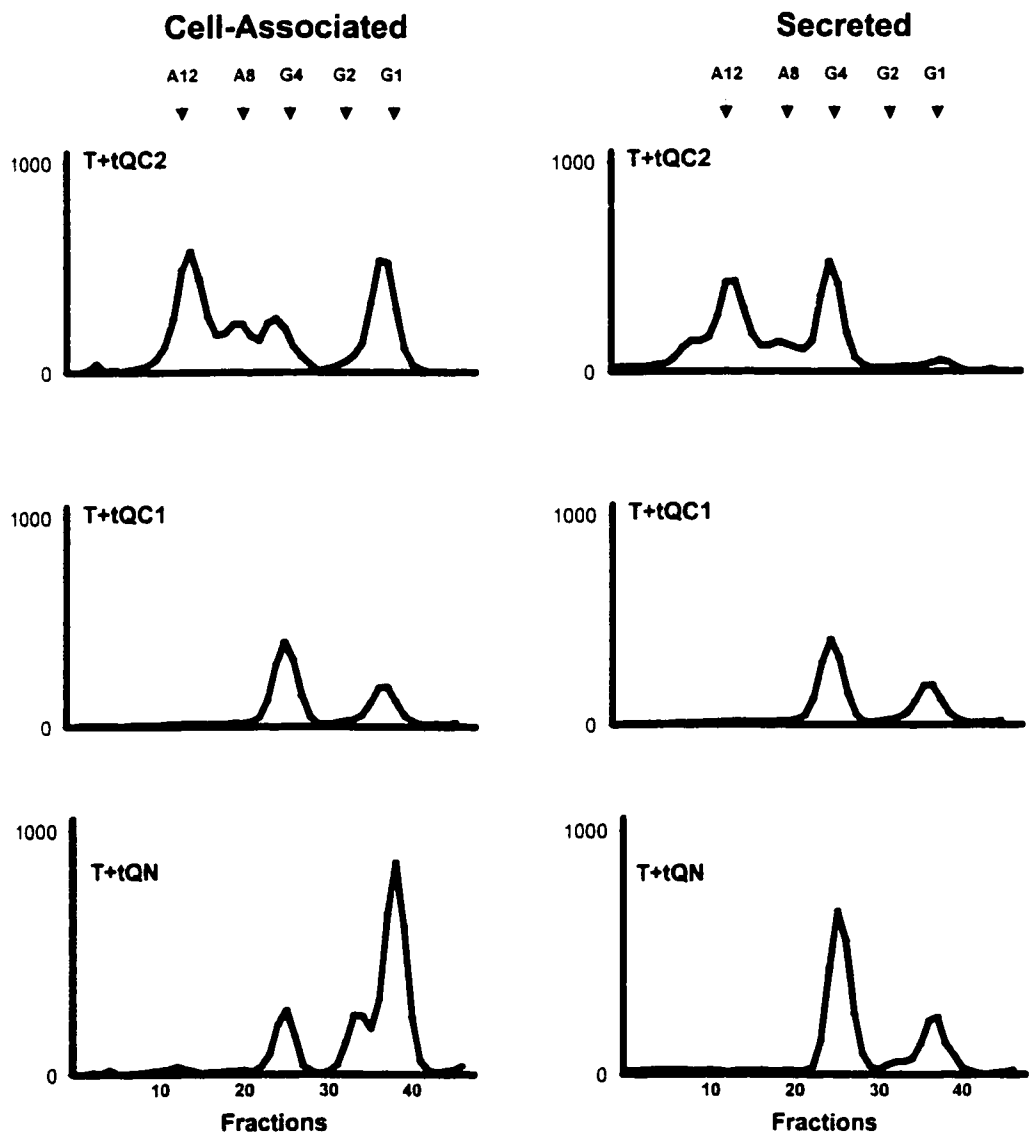

Cell-Associated
Secreted
AChE Activity (nmol/min/βgal Activity)
200
160
120
80
40
0
T
T+ tQ1
T+ tQC2
T+ tQC1
T+ tQN

Figure 16. Molecular form profiles of myotubes co-transfected with $AChE_T$ and the truncated tQ1 subunits

Shown are the AChE molecular forms associated with transfections of cDNAs encoding AChE and the mutated collagenic structural subunits. Most notably, this figure shows that QC2 was the only mutant able to recruit catalytic subunits into complex asymmetric forms (similar to tQ1). Furthermore, only co-transfection with tQC2 lead to secretion of high order (A12, A8) molecular forms. Lastly, this figure shows that tQC1 and tQN were able to associate with AChE catalytic subunits and resulted in high levels of secreted and cell associated tetramers but no high order asymmetric molecules (A12 and A8).


Cell-Associated
Secreted
A12
A8
G4
G2
G1
1000
0
T+tQC2
T+tQC1
T+tQN
10
20
30
40
Fractions

3.4 Structural Subunits Associate with Newly Synthesized AChE

We transfected C_2C_{12} muscle cells with cDNAs encoding either tQ1 and Q_N/H_C subunits alone to determine if these structural subunits could recruit an inactive or rapidly degraded endogenous pool of AChE precursor molecules that may be present in differentiating myotubes. Total AChE activity, including cell-associated and secreted fractions, did not increase to levels higher than those seen in non-transfected controls (Figure 17). Furthermore, transfection with structural subunits did not result in the assembly of asymmetric forms (Figure 18). This suggests that the increased AChE activity observed in co-transfection experiments resulted from the incorporation of newly synthesized enzyme arising from the transfected $AChE_T$ construct.

3.5 Expression of AChE in Skeletal Muscle Fibres In Vivo

We conducted in vivo gene transfer experiments in which cDNAs encoding the rat $AChE_T$ subunit were injected alone or with cDNAs encoding either the *Torpedo* Q_N/H_C subunit, the Torpedo tQ1 subunit or the rat COLQ subunit directly into TA muscles of young rodents. TA muscles were excised seven days after injection and immediately frozen in melting isopentane. The muscles were then sectioned on a cryostat and analysed for AChE expression by histochemistry and immunofluorescence. Control rodent TA muscle sections histochemically stained or immunolabelled for AChE, displayed a distinct pattern of AChE expression in which neuromuscular junctions were heavily labelled and extrajunctional regions showed no labelling (Figure 19).

Figure 17. Increased AChE activity in transfected C_2C_{12} myotubes is due to newly synthesized enzyme

Activity levels in myotubes transfected with either the tQ1 or Q_H/N_C structural subunits alone remained low, comparable to non-transfected controls in both cell associated and secreted fractions (paired t-test; $p>0.05$, $n=6$).

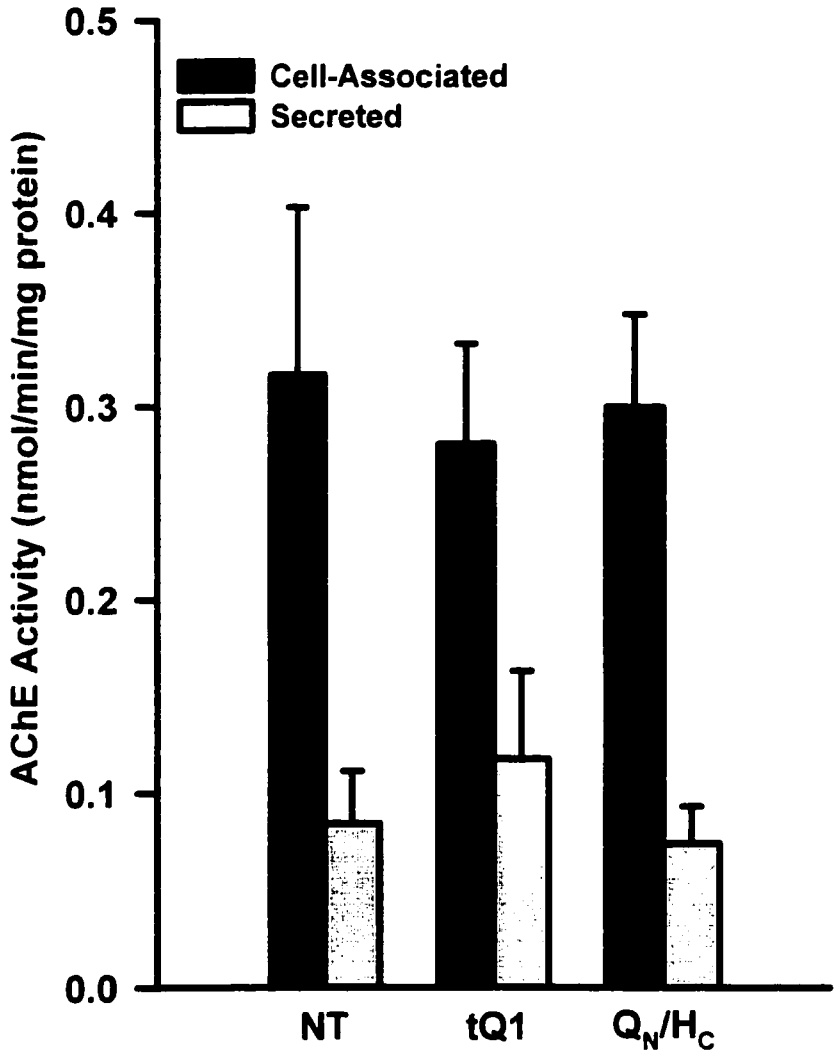
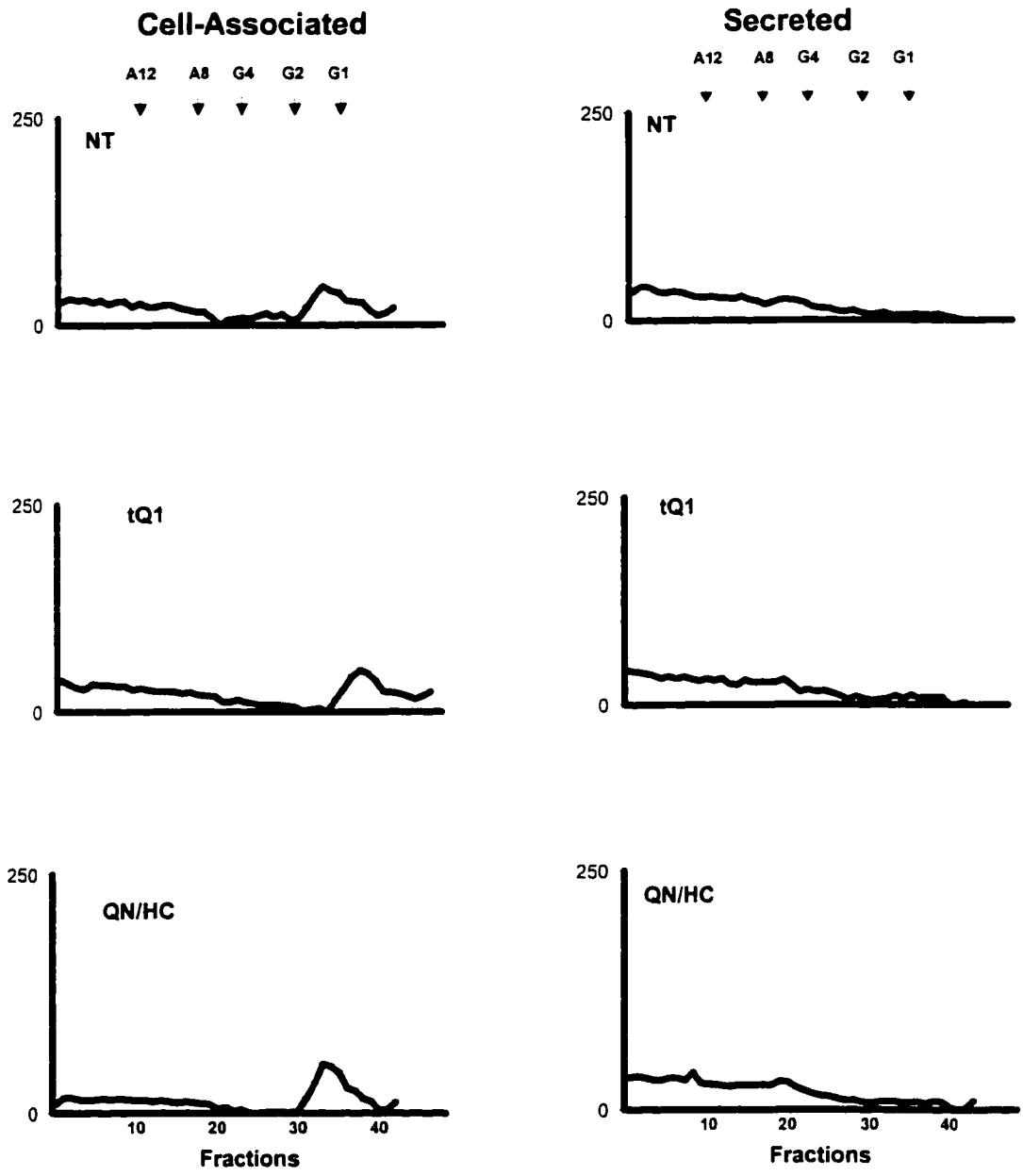

0.5
0.4
0.3
0.2
0.1
0.0
Cell-Associated
Secreted
ACh E Activity (nmol/min/mg protein)
NT
tQ1
Q_N/H_C

Figure 18. Molecular forms of myotubes transfected with tQ1 or Q_H/N_C

Molecular form profiles of myotubes transfected with either tQ1 or Q_H/N_C were similar to controls, showing production of only G1 and G2 molecules.


Cell-Associated
Secreted
A12
A8
G4
G2
G1
250
NT
0
tQ1
QN/HC
10
20
30
40
Fractions

In the first set of experiments, cDNAs encoding $AChE_T$ were co-injected with plasmids encoding β-galactosidase into TA muscles. β-galactosidase activity revealed the site of plasmid uptake and expression and thus highlighted the region of the muscle where AChE expression would most likely occur (Figure 21a). Analysis of adjacent muscle sections for AChE histochemistry and immunofluoresence revealed no differences in AChE localization or activity from that observed in control muscles (data not shown).

3.6 In Vivo Expression of $AChE_T$ and the Chimeric Q_N/H_C subunit

TA muscles were co-injected with $AChE_T$ and Q_N/H_C, since this combination resulted in the highest increase in AChE activity in co-transfected myotubes (Figure 10). Histochemical analysis of longitudinal sections taken from injected muscles, showed an increase in AChE activity as compared to control muscle (Figure 20). As shown, AChE activity occurred predominantly in the periphery of transduced muscle fibres. This staining pattern corresponded to the assembly of catalytically active AChE molecules with chimeric structural subunits and to their subsequent insertion into the sarcolemma of the muscle fibres via GPI-anchors. Interestingly, the AChE activity in these muscle fibres was not restricted to the region of nerve-muscle contacts.

Figure 19. Skeletal muscles are divided into junctional and extrajunctional compartments

This figure shows longitudinal muscle sections taken from 5 week old mice and stained for AChE activity using the method of Karnovsky and Roots (1964). The junctional compartment is identified by the highly accumulated AChE activity which results in a reddish brown stain at neuromuscular contacts (indicated by arrows in Figure A). Extrajunctional regions display no AChE activity (Figure B, bar = 50 μm).


A

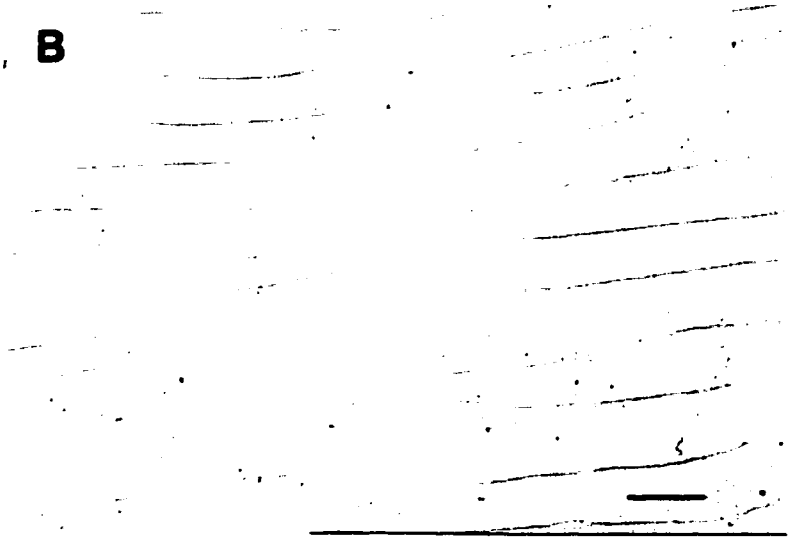

B

Figure 20. Expression of $AChE_T$ with the chimeric Q_H/N_C subunit results in membrane-anchored active enzyme in adult rat TA muscles

A and B both represent longitudinal muscle sections from different regions of a rat TA muscle co-injected with $AChE_T$ and the Q_H/N_C subunit after staining for AChE histochemical activity. As shown, AChE activity occurred predominantly in the periphery of transfected muscle fibres in both A and B. This staining pattern is due to the association of catalytic subunits with chimeric structural subunits and to their subsequent insertion into the sarcolemma of the muscle fibres. AChE activity in these muscle fibres was not restricted to the region of nerve-muscle contacts (bar = 50 μm).

3.7 In Vivo Expression of $AChE_T$ and the Collagenic Structural Subunits

TA muscles were also co-injected with cDNAs encoding $AChE_T$ and either the wild type *Torpedo* tQ1 or rat COLQ subunits. Immunolabelling of cryostat sections taken from $AChE_T$ and COLQ injected muscles with the A63 antibody revealed the presence of high levels of AChE derived from the injected plasmids (Figure 21c). This is in striking contrast to injection of $AChE_T$ alone, in which AChE expression could not be detected at the site of plasmid uptake thus resembling the histochemistry of control sections (Figure 19). Expression of COLQ associated AChE was not limited to junctional regions of the muscle but was rather co-localized with β-galactosidase activity (Compare Figures 21a,b and c) confirming that expression occurred in the proximity of the site of plasmid uptake. The ectopically expressed AChE also appeared to be associated primarily with the periphery of the muscle fibres suggesting that the enzymes were attached to the basal lamina by the collagenic structural subunit (Figure 21b). Histochemical staining of adjacent sections confirmed that the AChE was indeed active in these muscle fibres (Figure 21b). Muscle fibres co-injected with $AChE_T$ and the *Torpedo* tQ1 subunit, resulted in identical expression patterns to those injected with COLQ (data not shown).

Figure 21. Expression of rat $AChE_T$ and COLQ in mouse TA muscles

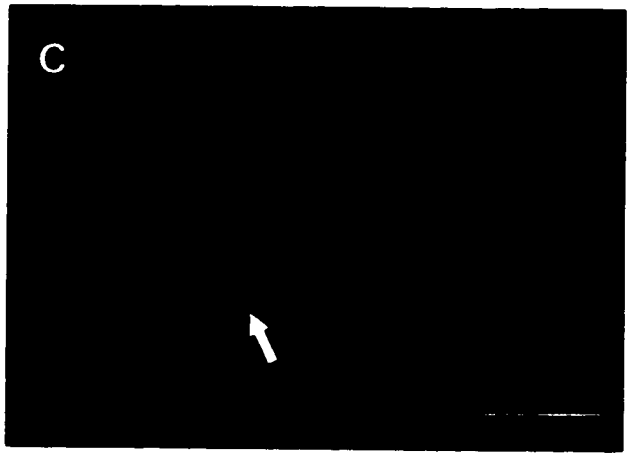
4 week old mice were co-injected with $AChE_T$ and ColQ, a cDNA encoding the recently cloned rat collagenic structural subunit which has been shown to be completely homologous to the *Torpedo* tQ1 subunit (Krejci et al., 1997). Figure A shows the site of injection, identified by β-galactosidase and AChE histochemical activity stain. Figure B shows the next section taken from the muscle, stained for AChE histochemistry alone. This figure shows that the overexpressed AChE was indeed active. Figure C shows the next serial section labelled for AChE using the A63 polyclonal antibody. AChE expression from the exogenously supplied cDNAs was localized to the area of the site of injection in the periphery of muscle fibres. All muscles injected with this combination resulted in the above described AChE expression pattern (bar = 100 μm).

β-gal Activity

B

AChE Activity

AChE



Chapter 4

Discussion

Acetylcholinesterase plays a key role in cholinergic neurotransmission. It is most highly expressed in excitable tissues where it functions in acetylcholine hydrolysis. In skeletal muscle, AChE accumulates in the basal lamina spanning the neuromuscular junction (McMahan et al., 1978). Although there has been some recent progress, the mechanisms involved in the synaptic localization of AChE remain to be fully elucidated. In this study we examined three main aspects of AChE expression in skeletal muscle. First, we focused on the mechanisms involved in the exclusive expression of T transcripts in adult skeletal muscle fibres. To this end, we examined the expression of cDNAs encoding all of the alternatively spliced forms of AChE in C_2C_{12} cultured myotubes. Next, we analyzed the association of AChE with the collagenic structural subunit (tQ1) in myotubes to determine the mechanisms involved in the preferential accumulation of AChE asymmetric forms at the neuromuscular junction. Specifically, we examined the assembly of AChE catalytic subunits with tQ1 as well as the secretion and surface attachment of the asymmetric forms produced by transfected myotubes. Lastly, we examined the contribution of post-translational regulatory mechanisms in the assembly and targeting of AChE asymmetric forms to the neuromuscular junction in vivo. This was accomplished by co-injection of cDNAs encoding AChE and tQ1 under the control of constitutive promoters into rodent hindlimb muscles, followed by identification of the overexpressed gene product by immunofluorescence and histochemistry.

4.1 Expression of AChE Splice Variants in C_2C_{12} Myotubes

As previously described, three transcripts differing in their 3' exon choice have been identified in mammals: 1) AChE found in adult skeletal muscle fibres originates exclusively from the T transcript (Legay et al., 1995); 2) expression of the H transcript is limited to cells of hematopoietic origin and developing muscles (Legay et al., 1993; Li et al., 1993); and 3) the R transcript has been identified in murine erythroleukemia cells (MEL)(Chan et al., 1998; Li et al., 1993), developing muscles (Legay et al., 1995) and in the brains of mice subjected to acute stress (Kaufer et al., 1998).

The mechanisms involved in the accumulation of alternatively spliced transcripts of AChE have been examined by several groups. Microinjection of Xenopus cleaving embryos with cDNAs encoding recombinant AChE containing the invariant exons (1 to 4) spliced to exon 6 (T) or linked to intron 4 and exon 5 (R-H) resulted in tissue-specific expression of the gene products (Seidman et al., 1995). As expected, the product of the T construct was localized to myotomes and neuromuscular junctions. Alternatively, the R-H construct resulted in AChE expression in epidermal cells and secretion into the external medium. The T cDNA also resulted in enhanced neuromuscular junction morphology, whereas the R-H cDNA had no effect on neuromuscular junction development. These experiments suggested that the 3'-terminal exons of alternatively spliced transcripts contain tissue-recognizable elements that account for tissue-specific expression. Specifically, exon 6 is believed to contain an indispensable motif for synaptic accumulation of AChE. Because Seidman and colleagues (1995) used the pan-active CMV promoter to direct expression of AChE, they also proposed that differential post-

transcriptional or post-translational management of AChE mRNAs and/or protein products was responsible for the appropriate expression of AChE in various tissues. Indeed others have demonstrated an important role for mRNA stability in AChE expression during muscle, neuronal and hematopoietic cell differentiation (Fuentes and Taylor, 1993; Coleman and Taylor, 1996; Chan et al., 1998).

Introns have also been shown to be important in the determination of tissue-specific expression of the AChE gene. In fact, excision of introns two and three in a recombinant AChE gene resulted in the loss of exclusive expression of the T transcript in differentiating skeletal muscle cells in culture (Luo et al., 1998). Furthermore, the integrity of exon four was critical for the determination of upstream exon splicing events. These results suggest that tissue-specific expression of AChE in skeletal muscle is determined by exclusive expression of the T transcript due to specialized recognition signals in the 5' introns and not by a post-transcriptional stabilization mechanism as described by Seidman and colleagues (1995).

We transfected cDNAs encoding the alternatively spliced forms of AChE into C_2C_{12} myoblasts and analyzed AChE expression 3 days after fusion of the myoblasts into multinucleated myotubes. Although similar experiments have been performed in other culture systems (Duval et al., 1992; Li et al., 1993; Morel and Massoulie, 1997), they have not yet been performed in skeletal muscle cells. This is critical since myotubes eventually give rise to mature innervated adult muscle fibres and they should therefore contain essential elements important for the regulation of AChE expression in adult muscle. This provides a model in which the contribution of muscle-specific factors involved in the synapse-specific accumulation of T derived AChE can be determined.

Transfection of the cDNAs encoding $AChE_T$, $AChE_H$ and $AChE_{RH.}$ resulted in expression of all the splice variants of AChE in cultured myotubes since dramatic increases in total AChE activity including cell-associated and secreted enzymes, were observed in all cases as compared to controls. This indicated that skeletal muscle cells contain all the components necessary to efficiently express the various AChE splice variants. Similar to the experiments of Luo and colleagues (Luo et al., 1998), these results suggested that the exclusive expression of T transcripts in muscle, relies on regulated alternative splicing events which allow preferential splicing to exon 6 from the primary transcript. To verify that the cDNAs used in our experiments resulted in correctly processed AChE, we examined the molecular forms expressed in myotubes under the various transfection conditions by velocity sedimentation of myotube homogenates (cell extracts) and media. Transfection of cultures with the muscle-specific $AChE_T$ cDNA resulted in the production of G_1 and G_2 forms that were retained in the sarcoplasm and G_4 molecules that were assembled intracellularly and efficiently secreted into the media. Although the tetramers produced were most likely soluble, a fraction of this G_4 pool could represent the assembly of catalytically active tetramers with endogenous hydrophobic proteins such as the 20 kDa subunit found in membrane-bound tetramers identified in muscle (see review in Fernandez et al., 1996). This observation is in agreement with the primary structure of the T transcript which contains cysteine residues in the C-terminal exon (exon 6) that allow for association with other catalytic subunits to form homo-oligomers similar to the globular forms we observed (Li et al., 1991; Velan et al., 1991).

Transfection of myotubes with the cDNA encoding the H transcript, $AChE_H$, resulted exclusively in the production of high levels of dimers. PI-PLC treatment of these cell extracts in velocity sedimentation experiments confirmed that these dimers indeed contain GPI-anchors. Furthermore, immunofluorescence labelling of AChE in these transfected myotubes, using the rabbit A63 polyclonal antibody, showed that a large proportion of the enzyme was anchored to the surface. These experiments confirmed therefore, that the AChE molecules produced in myotubes transfected with $AChE_H$ are in fact correctly modified to form GPI-anchored dimers that are typically found in erythroid cells.

Interestingly, transfection of myotubes with $AChE_H$ resulted in much higher activity than transfection with $AChE_T$. This is in contrast with transfected COS cells in which $AChE_H$ resulted in similar activity levels as $AChE_T$ (Legay et al., 1993). Because both cDNAs are driven by the same constitutive promoter, it is unlikely that the difference in activity levels was due to differences in transcription. Furthermore, metabolic labelling experiments revealed that both constructs are translated at the same rate in COS cells (C. Legay, personal communication). Hence, post-translational regulatory mechanisms conferred by the GPI-anchor signal in the protein product of the $AChE_H$ cDNA may result in a more stable molecule that became efficiently inserted in the sarcolemma of the transfected myotubes. Furthermore, the product of $AChE_T$ was likely degraded at a higher rate since T subunits were predominantly retained in the cytoplasm where they may have been more accessible to proteases. This latter hypothesis is supported by experiments which showed that 70-80% of newly synthesized AChE molecules are degraded in tissue-cultured muscle cells before they exit the endoplasmic reticulum (Rotundo, 1988).

The $AChE_{RH}$ construct contains all the components necessary for expression of both the R and H subunits since it includes the readthrough extension sequence of intron 4 and all of exon 5. Total AChE activity in myotubes transfected with $AChE_{RH}$ was similar to that of myotubes transfected with $AChE_T$. However, a larger proportion of activity was found in the secreted fraction of $AChE_{RH}$-transfected myotubes. In fact, approximately 50% of the total activity resulting from $AChE_{RH}$ was secreted as compared to the 35% seen with $AChE_T$. In addition, analysis of the molecular forms produced in myotubes transfected with this construct showed that a soluble monomer was expressed and secreted. This correlates well with transfection of COS cells or Xenopus embryos with an AChE cDNA encoding an mRNA containing an extension of exon 4 without splicing to a downstream exon which resulted exclusively in production of hydrophilic monomers secreted into the media (Li et al., 1993; Seidman et al., 1995). This is also in agreement with studies using recombinant human AChE in which mutation of the T exon resulted exclusively in secretion of monomeric AChE molecules (Kerem et al., 1993)

Myotubes transfected with $AChE_{RH}$ resulted in a concomitant expression of a minor proportion of cell-associated dimers. Velocity sedimentation analysis of PI-PLC treated cell extracts showed these dimers to be GPI-anchored G_2 molecules originating from the H transcript. Interestingly, Seidman and colleagues (1995) performed similar experiments and only detected soluble monomers predominantly secreted into the medium. In contrast to our experiments, they observed no GPI-anchored dimers in myotubes transfected with a DNA construct similar to $AChE_{RH}$. Nevertheless, our experiments show that the $AChE_{RH}$ cDNA is indeed able to express both H and R subunits in myotubes through the translation of alternatively spliced mRNAs.

The exclusive expression of $AChE_T$ transcripts in skeletal muscle fibres raises an important question pertaining to the mechanisms involved in this tissue-specific distribution. Specifically, is expression of T a result of differential mRNA stability or is it related to restrictions imposed on RNA splicing by elements in the sequence of the AChE primary transcript? To address this issue, we engineered a plasmid containing a cDNA termed $AChE_{RHT}$, which contains all the invariant exons as well as the extension of intron 4, exon 5, intron 5 and exon 6. This cDNA therefore, expresses a primary transcript that can be spliced to create all three AChE transcripts. When expressed in C_2C_{12} myotubes, $AChE_{RHT}$ resulted in molecular form profiles identical to those seen in myotubes transfected with $AChE_T$, suggesting that only the muscle-specific T forms of AChE were produced. These results favor a model in which a distinct splicing mechanism directed specifically to the T exon exists in muscle cells. Interestingly, the non-coding region upstream of the T exon (intron 5) contains a 100 nucleotide pyrimidine stretch that has already been shown to direct the splicing of exons of other genes that are exclusively expressed in muscle such as β-tropomyosin (Goux-Pelletan et al., 1990). Hence, our experiments suggest that elements present in the sequence immediately 5' to and including exon 6 (the T exon) contribute to the tissue-specific alternative splicing of $AChE_T$ in muscle fibres.

4.2 Assembly of Asymmetric AChE in Cultured Myotubes

Asymmetric forms of AChE, localized at the neuromuscular junction, are critical for the precise control of neuromuscular transmission. These hetero-oligomers are known to

originate predominantly from muscle fibres (Anglister, 1991) where they are assembled in the Golgi apparatus (Rotundo, 1984). To better understand the mechanisms involved in the biosynthesis and targeting of AChE asymmetric forms in skeletal muscle fibres, we co-transfected cDNAs encoding $AChE_T$ and tQ1 into C_2C_{12} muscle cells and analyzed their assembly, secretion and anchoring. Indeed, co-expression of $AChE_T$ and tQ1 resulted in an increase in total AChE activity as compared to myotubes transfected with $AChE_T$ alone. More importantly, the increase occurred in both the cell-associated and secreted fractions, emphasizing the importance of hetero-oligomerization for efficient secretion of AChE into the external media. Our experiments suggested that newly synthesized AChE molecules that normally would have been degraded in the endoplasmic reticulum (Rotundo, 1988) were rescued in the presence of tQ1 subunits.

The increase in total AChE activity observed in co-transfected myotubes, reflected the assembly of asymmetric forms. Velocity sedimentation analysis revealed that this increase was predominantly due to recruitment of A_{12} forms which were not present in myotubes transfected with $AChE_T$ alone. As previously described, these A_{12} molecules represent three tetramers of AChE catalytic subunits, each associated with the N-terminal of one strand of the triple helical collagenic structural subunit via the recently identified stretch of proline residues termed the proline rich attachment domain or PRAD (Bon and Massoulie, 1997). A_{12} molecules were present in both the cell fraction and media. Furthermore, analysis of the media revealed a concomitant decrease in globular forms (G_1 and G_2) as compared to $AChE_T$ transfected myotubes. Hence, the assembly of higher order asymmetric forms results in enhanced secretion of AChE due to recruitment of intracellular monomers and dimers.

Interestingly, recent experiments have shown that non-excitable cells such as the rat basoleukemia (RBL) cell line and COS cells, co-transfected with the same cDNA constructs we have used, resulted in significantly different expression patterns. RBL cells co-transfected with $AChE_T$ and tQ1 produced high levels of A_4 and no A_{12} (Coussen et al., 1995) whereas COS cells only produced a small proportion of A_{12} as compared to other molecular forms (Legay et al., 1993). Secretion of AChE asymmetric forms did not occur in either of these cell lines. These findings suggest that expression of high levels of A_{12} in C_2C_{12} myotubes results from a unique post-translational assembly mechanism, distinct from other cells, that allows for interaction between the collagenic subunits and AChE molecules in muscle fibres.

As previously described, AChE asymmetric forms secreted from skeletal muscle fibres become attached to the synaptic extracellular matrix (ECM) (Bon et al., 1978). Since 30% of the total AChE observed in myotubes co-transfected with $AChE_T$ and tQ1 was secreted into the media, we expected a large quantity of asymmetric forms to become anchored in the ECM. Immunofluorescence labelling of myotubes co-transfected with the $AChE_T$ and tQ1 using the A63 anti-AChE polyclonal antibody showed punctate staining around the periphery of individual myotubes that was not visible in myotubes transfected with $AChE_T$ alone. Although a large fraction of the overexpressed AChE was retained in the sarcoplasm of co-transfected myotubes, the surface immunofluorescence pattern suggested that asymmetric forms were efficiently incorporated into the basal lamina. Association of AChE collagenic-tailed forms to the muscle basal lamina has recently been attributed to interaction of the C-terminal of the collagenic subunit to the heparan-sulfate proteoglycan (HSPG) perlecan (Casanueva et al., 1998; Peng et al., 1999). C_2C_{12} cells

are known to produce heparan sulfate proteoglycans (Ferns et al., 1993) thus facilitating the attachment of asymmetric forms to the basal lamina.

C_2C_{12} cells co-transfected with $AChE_T$ and the Q_H/N_C chimeric structural subunit resulted in a dramatic increase in activity similar to those co-transfected with the wild-type tQ1 subunit. These myotubes produced high levels of predominantly cell-associated tetramers. Immunofluorescence experiments using the A63 anti-AChE antibody revealed intense surface labelling, confirming that the tetramers produced did assemble with the Q_H/N_C subunit and were efficiently incorporated into the sarcolemma via GPI-anchors. These findings are in sharp contrast to transfection of $AChE_T$ alone which resulted in no surface labelling. The presence of tetramers and absence of A_{12} in these co-transfected myotubes is in accordance with the sequence of the Q_H/N_C subunit which contains the PRAD necessary for association with catalytic tetramers (Bon et al., 1997), but lacks the collagenic domain containing cysteine residues deemed necessary for triple helical formation and thus A_{12} assembly (Krejci et al., 1991; Krejci et al., 1997). Some activity was detected in the secreted fraction of myotubes co-transfected with $AChE_T$ and Q_H/N_C comparable to that observed in the media of cells transfected with $AChE_T$ alone. Velocity sedimentation analysis revealed that the AChE secreted from these myotubes consisted mainly of tetramers. These molecules may have represented Q_H/N_C associated tetramers that did not bind to the sarcolemma due to a saturation of insertion sites or to inefficient processing of GPI-anchors. These findings show that association with the N-terminal PRAD is sufficient to increase AChE levels in skeletal muscle cells.

The dramatic increase in AChE activity observed in co-transfected myotubes raises an important question as to the source of this AChE. An inactive pool of AChE has been

suggested to occur in muscle fibres and brain cells (Anselmet et al., 1994; Rotundo, 1988; Schober et al., 1997). Hence, the possibility that co-transfection of $AChE_T$ with tQ1 or Q_N/H_C subunits resulted in recruitment of inactive or rapidly degraded endogenous AChE precursor molecules already present in C_2C_{12} myotubes into active hetero-oligomers, exists. However, transfection of C_2C_{12} muscle cells with tQ1 or Q_N/H_C subunits alone did not result in an induction of AChE expression or assembly of A_{12}. In fact, AChE activity remained similar to control myotubes. This finding argues against the possibility that the increased AChE activity was a result of recruitment of an endogenous pool of inactive enzymes that became activated after association with structural subunits.

Association of AChE with the collagenic structural subunit by the interaction of the PRAD of tQ1 with exon 6 of the catalytic subunit has been well established (Bon et al., 1997; Bon and Massoulie, 1997; Krejci et al., 1997). Assembly of collagenic subunits with each other to form a triple helix has been suggested to be a function of the proline- and cysteine-rich domains in the C-terminal of the tQ1 subunit (see Figure 5) (Krejci et al., 1991). This process is believed to proceed from the C-terminal to the N-terminal of the mature collagenic subunit (Prockop and Kivirikko, 1995). Hence the C-terminal region of tQ1 may play an important role in secretion and attachment of asymmetric forms to the synaptic basal lamina. To further dissect the mechanisms involved in this association, we generated tQ1 subunits, truncated to varying lengths from their C-terminals and co-transfected them with $AChE_T$ into C_2C_{12} cells.

Co-transfection of $AChE_T$ with QC2, a construct that lacks the last 83 amino acids containing the cysteine-rich domain of the C-terminal (identified in Figure 5), did not affect the assembly of asymmetric forms in myotubes as compared to the wild-type tQ1. Similar

to myotubes expressing tQ1, those expressing QC2 produced abundant levels of A_{12} indicating that the C-terminal cysteine-rich region is not essential for the formation of triple helices. Furthermore, similar to the wild-type tQ1 subunit, only asymmetric molecules were secreted. This is consistent with the model of Rotundo (1988) who suggested that muscle cells recruit oligomeric complexes for secretion, while monomeric catalytic subunits remain in the ER and become degraded. AChE monomers may contain an ER retention signal in their C-terminal that is masked when oligomerization of the catalytic subunits occurs resulting in secretion of oligomers (Velan et al., 1994).

Two other tQ1 mutants were also generated: 1) QC1 lacking the entire C-terminal domain including the cysteine- and proline-rich regions; and 2) QN lacking the above regions as well as the entire collagenic domain (see Figure 5). Co-transfection of myotubes with $AChE_T$ and either of these mutants failed to induce the production of A_{12} molecules observed in co-transfections with tQ1 and QC2. However, a marked increase in the amount of secreted tetramers occurred as compared to myotubes tranfected with $AChE_T$ alone. These mutants thus lack important regions, most likely in the proline-rich domain that are essential for triple helix assembly. In this case, AChE tetramers were assembled with a single strand of collagen that were readily secreted into the medium. These experiments therefore suggest that the proline-rich domain, and not the cysteine-rich domain located in the C-terminal of tQ1 is essential for initiating assembly of the triple helix.

Our co-transfection experiments suggest that Q is critical for the proper assembly and maintenance of AChE asymmetric forms in skeletal muscle fibres. Indeed, mutations in the C- terminal of Q have been shown to associate with end-plate AChE deficiency

resulting in congenital myasthenic syndrome (CMS), a rare autosomal recessive disease (Donger et al, 1998). Also inactivation of the COLQ gene in mice resulted in a loss of AChE accumulation that normally occurs at the neuromuscular junction (Feng et al., 1999). Hence, these findings support our experiments in suggesting that the simple availability of structural subunits is sufficient to stabilize and maintain high levels of AChE activity in muscle fibres.

4.3 The Contribution of Post-translational Regulation in Targeting AChE to the Neuromuscular Junction

To date, the mechanisms involved in the accumulation of AChE at the neuromuscular junction remain to be fully understood. AChE transcripts were shown to accumulate in the region of the muscle fibre directly beneath the synapse (Jasmin et al., 1993; Michel et al., 1994). This localization may be attributed to preferential transcription of the AChE gene by nuclei in the synaptic compartment or to increased stabilization of AChE transcripts in this compartment. Currently, no direct evidence exists to support the involvement of post-transcriptional stabilization of AChE transcripts in the synaptic compartment. However, stabilization of AChE mRNA has been shown to cause the increase in AChE expression that occurs during muscle differentiation (Fuentes and Taylor, 1993) as well as neuronal (Coleman and Taylor, 1996) and hematopoeitic cell differentiation (Chan et al., 1998). Hence, the accumulation of AChE mRNA in the synaptic compartment of innervated muscle fibres may be a result of increased transcript stability.

Alternatively, expression of AChE transcipts may specifically be a function of a specialized subset of nuclei, termed "fundamental" nuclei (Ranvier, 1888), located in the synaptic compartment of muscle fibres. Recently, a cis-acting element termed the N-box motif was identified and shown to confer synapse-specific transcription of genes encoding utrophin and several subunits of the AChR in the fundamental nuclei of skeletal muscle fibres (Duclert et al., 1996; Gramolini et al., 1999; Gramolini and Jasmin, 1998; Koike et al., 1995). Expression of reporter genes driven by N-box containing promoter fragments resulted in the synapse-specific localization of the gene products that could be abolished by mutation of the consensus sequence. Furthermore, GABP, a member of the Ets transcription factor family has been shown to bind to the N-box and to modulate its activity (Fromm and Burden, 1998; Gramolini et al., 1999; Schaeffer et al., 1998). Since the AChE gene contains several N-boxes (Chan et al., 1999), one can envisage a model in which this gene is transcriptionally active in the fundamental nuclei of the junctional compartment of muscle fibres due to activation of N-boxes in the AChE promoter by members of the Ets family of transcription factors.

Although there is compelling evidence to indicate that transcriptional mechanisms confer synapse-specific accumulation of AChE, some evidence exists suggesting that post-translational mechanisms may also be involved in this synapse-specific localization. Most notably, markers of the Golgi apparatus are only detected in association with nuclei in the subneural compartment of adult skeletal muscle fibres (Jasmin et al., 1989; Antony et al., 1992; Jasmin et al., 1995). A complete lack of two important markers of the Golgi apparatus, TGN38 and α-mannosidase II in innervated muscle fibres, suggests a biochemical differentiation of the subneural Golgi apparatus in muscle fibres (Jasmin et

al., 1995). In addition, synapse-specific carbohydrates are associated with AChE at the neuromuscular junction, correlating with the localization of acetylgalactosaminyl transferase, an enzyme involved in protein glycosylation (Scott et al., 1988; Scott et al., 1990). The synaptic compartment has also been shown to contain a distinct set of stable microtubles (Jasmin et al., 1990) and AChE containing clathrin coated vesicles (Hodges-Savola et al., 1989; Rotundo et al., 1989). These specializations suggest that the secretory pathway, including the Golgi apparatus plays an important role in the biosynthesis, assembly and distribution of AChE molecular forms in skeletal muscle fibes.

Hence, we hypothesized that post-translational regulatory mechanisms play a crucial role in ensuring the proper assembly and targeting of AChE to the basal lamina directly above the post-synaptic membrane of the muscle fibre. To this end, we employed in vivo gene transfer techniques to introduce cDNAs encoding AChE and the collagenic structural subunit into rodent hindlimb muscles and examined the localization of the final gene products by AChE histochemistry and immunofluorescence.

Our hypothesis led us to believe that co-injection of these cDNAs would result in expression of exogenous AChE exclusively in the basal lamina spanning the synaptic compartment of muscle fibres. The key assumption in these experiments was that use of the pEF-BOS constitutive promoter instead of the native AChE and collagenic structural subunit promoters, would bypass the important contribution of transcriptional regulation. This allowed us to determine whether or not the specialized Golgi apparatus plays a major role in the assembly of AChE for targeting to the neuromuscular junction in vivo.

We initially co-injected cDNAs encoding $AChE_T$ and Q_H/N_C into rodent hindlimb muscles, since this combination gave us the highest level of surface labelling in C_2C_{12}

myotubes. Co-injection of these cDNAs resulted in intense AChE histochemical staining in the periphery of muscle fibres that had taken up the plasmids. This peripheral staining pattern could be attributed to efficient targeting of GPI-anchored AChE tetramers assembled by the Q_H/N_C subunit and anchored in the sarcolemma of the muscle fibres. Localization of the exogenously expressed enzyme was not limited to the synaptic compartment of the muscle fibre indicating that although the GPI-anchored tetramers were correctly assembled and processed, the presence of a subneural Golgi apparatus was not sufficient to target AChE to the neuromuscular synapse.

Co-injection of cDNAs encoding rat $AChE_T$ and the wild-type *Torpedo* tQ1 subunit into rodent hindlimb muscles also resulted in expression of AChE in the region of plasmid uptake. The increased AChE most likely represents A_{12} since these asymmetric form were predominantly assembled in similarly co-transfected myotubes. As in the Q_H/N_C experiments, activity was not limited to the junctional compartment of muscle fibres. AChE activity and immunofluorescence were detected in both junctional and extrajunctional compartments despite the lack of an intact and normally organized Golgi apparatus in the extrajunctional compartment (Jasmin et al., 1989; Jasmin et al., 1995).

The importance of the collagenic structural subunit in maintaining high levels of AChE is emphasized by the fact that we could only detect expression of AChE when both constructs were co-injected into adult muscle fibres. This finding mirrors the results we obtained in cultured myotubes in which co-transfection of $AChE_T$ with tQ1 resulted in a marked increase in AChE activity as compared to $AChE_T$ alone. Injection of $AChE_T$ alone in vivo, did not result in detectable AChE activity or immunofluorescence in TA muscles, likely due to degradation of these molecules in the endoplasmic reticulum (Rotundo, 1988).

During the course of these studies, we obtained a cDNA encoding the rat collagenic structural subunit termed COLQ (Krejci et al., 1997). This provided us with an ideal opportunity to examine expression and targeting of AChE in muscle fibres using gene sequences originating from the same species. We were thus able to further elucidate the involvement of post-translational regulatory mechanisms in the targeting of asymmetric AChE by eliminating any potential species-specific differences between the coding sequences of the rat AChE and *Torpedo* collagenic structural subunit. However, expression of $AChE_T$ and COLQ was not limited to the synaptic compartment also suggesting that biosynthesis of AChE does not rely on an intact Golgi apparatus. Identical to co-injection with the *Torpedo* tQ1 subunit, AChE was localized predominantly at the site of plasmid uptake providing further evidence that the specialized synaptic secretory pathway alone, does not play an important role in the assembly and localization of asymmetric AChE at the neuromuscular synapse.

The observation that AChE was localized to the periphery of TA muscle fibres suggests that the asymmetric forms were efficiently secreted and anchored to receptors in the basal lamina surrounding the entire surface of skeletal muscle fibres. Interestingly our results are in contrast to the molecular parking lot hypothesis of Rotundo and colleagues (1997). This group has shown that frog muscle fibres incubated with purified quail asymmetric AChE resulted in attachment of the quail molecules exclusively in the synaptic basal lamina suggesting that receptors for AChE asymmetric forms are exclusively found in the synaptic compartment. Furthermore, they identified perlecan, a heparan sulfate proteoglycan, highly accumulated in the extracellular matrix at the neuromuscular junction, as the acceptor for the collagenic structural subunit (Peng et al.,

1999). Our results clearly show that asymmetric forms of AChE could be targeted to regions of the basal lamina outside the junctional compartment, suggesting that other receptors for AChE must be present extrajunctionally.

4.4 Conclusions

In the present study, factors influencing the expression of AChE in a C2C12 myogenic cell line as well as in tibialis anterior muscles in vivo, were examined by transfection of plasmids encoding AChE catalytic and structural subunits. Our goal was to determine the mechanisms involved in the preferential accumulation of AChE asymmetric forms in the synaptic basal lamina of skeletal muscle fibres. The results obtained in this study have led to three main conclusions: 1) Exclusive expression of $AChE_T$ in mature muscle cells is governed by specific alternative splicing events; 2) availability of the collagenic structural subunit limits the production of physiologically stable forms of AChE; and 3) processing and assembly of AChE globular and asymmetric forms can occur in extrajunctional regions of muscle fibres and does not require the specialized junctional Golgi apparatus.

Thus, we envisage a model in which accumulation of AChE in the synaptic basal lamina of skeletal muscle is regulated by several complex mechanisms. Our experiments showed that accumulation of T transcripts is due to muscle-specific alternative splicing events involving elements present 5' to and including the T exon which favour this splicing

pathway. Furthermore, these T transcripts accumulate in the synaptic compartment of the muscle fibre (Jasmin et al., 1993; Michel et al., 1994) because of a combination of several mechanisms including preferential expression of AChE in the fundamental nuclei beneath synaptic contacts (Chan et al., 1999). Transcript accumulation is the most important factor in the preferential targeting of AChE to the synaptic compartment. This is evident in our experiments which showed that AChE could be expressed in extrajunctional regions of the muscle fibre depending on the efficiency of plasmid uptake and thus transcript availability, despite the lack of Golgi apparatus in these regions (Jasmin et al., 1995; Jasmin et al., 1989). Hence, transcriptional regulation ensures that abundant levels of AChE T transcripts are localized in the synaptic compartment such that protein synthesis will also be limited to this compartment. Since a large fraction of AChE is degraded in muscle fibres (Rotundo, 1988), structural subunits such as the collagen tail or the P subunit are critical in stabilizing the enzyme to prevent degradation. This allows for the maintenance of abundant enzyme levels in the junctional compartment, as shown in our experiments in which AChE levels increase dramatically in the presence of the collagenic structural subunit. The interaction with structural subunits not only stabilizes AChE but also provides a signal for secretion of these molecules, likely by masking a retention signal in the C-terminal of the enzyme (Velan et al., 1994). Lastly, once AChE is secreted into the synaptic cleft, the enzymes are trapped in the basal lamina via interaction with perlecan, and other extracellular matrix components concentrated in this compartment (Peng et al., 1999). Because perlecan is associated with both the post-synaptic membrane and cytoskeleton (Henry and Campbell, 1996), it can direct migration of AChE in the synaptic region by lateral shuttling (Kidokoro and Brass, 1985). These mechanisms thus ensure

that high levels of AChE are available in the synaptic basal lamina for precise control of neuromuscular synaptic transmission.

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