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**Role of Neural Factors in the Regulation of Acetylcholinesterase
Expression in Mammalian Skeletal Muscle Cells**

 Céline Boudreau-Larivière

A thesis submitted to the School of Graduate Studies and Research, University of Ottawa,
in partial fulfilment of the requirements for the degree of Doctor of Philosophy.
Department of Cellular and Molecular Medicine, Faculty of Medicine.



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ABSTRACT

Neural signals modulate acetylcholinesterase (AChE) expression in skeletal muscle cells. However, the cellular and molecular events mediating these effects are poorly understood. The mechanisms by which nerve-evoked electrical activity and nerve-derived trophic factors regulate the expression of AChE along muscle fibers was therefore elucidated.

The contribution of nerve-evoked activity versus intrinsic properties of muscle fibers was assessed for their role in dictating the distinct patterns of AChE expression displayed by fast and slow muscles. We demonstrate that nerve-evoked electrical impulses play a key role in regulating AChE synthesis in these distinct muscle types. We also demonstrate that myogenic precursor cells from fast and slow muscle fibers generate myotubes that display similar patterns of AChE expression. Together, these findings suggest that nerve-evoked electrical activity rather than intrinsic properties of muscle cells is the primary regulator of AChE expression in fast and slow muscles.

The importance of transcriptional versus post-transcriptional mechanisms in mediating the activity-dependent regulation of AChE was also determined. We demonstrate that higher levels of AChE mRNA observed in fast versus slow muscles is due to post-transcriptional events. We also show that reductions of AChE mRNA seen in mature denervated muscles cannot be accounted for by a decrease in the rate of AChE gene transcription. Denervation-induced reductions in the levels of AChE mRNA were less pronounced in muscles of developing animals. This attenuated decrease in AChE transcript abundance may be due to a transient enhancement in the transcriptional activity of the AChE gene observed in denervated muscles of developing rats. Transcriptional as well as post-transcriptional

mechanisms are therefore important in mediating the activity-dependent regulation of AChE in developing muscles whereas post-transcriptional events are the primary mediators controlling AChE expression in mature muscles.

Finally, the activity of several rat AChE promoter fragments in synaptic versus extrasynaptic regions of muscle fibers was assessed to determine whether enhanced transcription of the AChE gene in synaptic nuclei contributes to the accumulation of AChE transcripts at the neuromuscular synapse. Our findings indicate that synapse-specific activity of the AChE gene contributes to localized expression of the enzyme and its mRNA at the neuromuscular junction. Furthermore, we observed that calcitonin gene-related peptide (CGRP) and ciliary neurotrophic factor (CNTF) downregulate AChE expression. The suppressive effects of CGRP and CNTF on AChE expression suggest that a combination of activating and inhibiting signalling cascades may function in concert to regulate the abundance of AChE transcripts at synaptic sites.

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LIST OF ABBREVIATIONS

A; asymmetric forms
ACh; acetylcholine
AChE; acetylcholinesterase
AChR; acetylcholine receptor
AP2; activator protein
ARIA; acetylcholine receptor inducing activity
AURE; AU-rich element
BCA; bicinchoninic acid assay
 β -gal; β -galactosidase
CAT; chloramphenicol acetyltransferase
CGRP; calcitonin gene-related peptide
CTL; control
CNTF; ciliary neurotrophic factor
CNTFR; ciliary neurotrophic factor receptor
ColQ; collagenic structural subunit
CPM; counts per minute
DBS; donor bovine serum
DEN; denervated
DEPC; diethyl pyrocarbonate
DFP; diisopropyl fluorophosphate
DMEM; Dulbecco's modified Eagle's medium
E; DNA binding site for myogenic factors
EDL; extensor digitorum longus
Egr1; early growth response
EMG; electromyograph
EMSA; electrophoretic mobility shift assay
FRAP; functional rat acetylcholinesterase promoter
G; globular forms
GAPDH; glyceraldehyde-3-phosphate dehydrogenase
GPI; glycosphosphatidyl inositol
GRAP; giant rat acetylcholinesterase promoter
H; hydrophobic transcript/catalytic peptide
Inr; initiator element
iso-OMPA; tetraisopropylpyrophosphoramidate
kDa; kilodalton
LacZ; β -galactosidase gene
MEL; murine erythroleukemia
mRNA; messenger RNA
N; N-box
NF κ B; nuclear factor κ B
nls; nuclear localization signal
NRAP; N box containing rat acetylcholinesterase promoter

P; hydrophobic structural subunit
PBS; phosphate buffered saline
PCR; polymerase chain reaction
pEF-BOS; plasmid elongation factor-BOS
PN; post-natal
PRAD; proline rich attachment domain
RAP; rat acetylcholinesterase promoter
RER; rough endoplasmic reticulum
R; readthrough transcript
rRNA; ribosomal RNA
RT; reverse transcription
SDS-PAGE; sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM; standard error of the mean
SK; bluescript
SOL; soleus
STTX; tetrodotoxin-inactivated and stimulated via the sciatic nerve
T; tail transcript/catalytic peptide
TA; tibialis anterior
TAS; superficial tibialis anterior
TFIID; transcription factor IID
TID; 3-(trifluoromethyl)-3-(m-iodophenyl)diazirine
TK; thymidine kinase
TTX; tetrodotoxin
UTR; untranslated region

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THESIS FORMAT

In accordance with the guidelines established by the School of Graduate Studies, the present thesis is a compilation of published or submitted journal articles preceded by a general review of the relevant literature (Chapter I) and followed by an overall discussion (Chapter VIII). As such, Chapters II to VII each have an abstract, introduction, materials and methods, results and discussion. In addition, the status of each manuscript is indicated on the title page at the beginning of Chapters II to VII. Permission to reproduce published articles and articles in press was granted by the copyright holder (i.e. publisher). All references are found in Chapter X. For manuscripts with multiple authors, the contributions of other authors are detailed on the title page of each Chapter.

CHAPTER I

REVIEW OF THE LITERATURE

The process of synaptic transmission at cholinergic synapses begins with the release of acetylcholine (ACh) from the presynaptic nerve terminal, followed by diffusion of the neurotransmitter across the synaptic cleft and binding to acetylcholine receptors (AChR) situated within the postsynaptic membrane. Termination of cholinergic neurotransmission is then achieved by AChE, a serine hydrolase responsible for rapidly eliminating ACh. Interestingly, Sir Henry Dale, more than 80 years ago, postulated the existence of a neurotransmitter hydrolysing enzyme at the neuromuscular junction that we now know to be AChE (Dale, 1914). The physiological role of AChE in both central and peripheral nervous systems is absolutely vital. AChE has therefore been the focus of numerous studies aimed at elucidating its function, structure, assembly, as well as the cellular and molecular mechanisms governing its expression in a variety of tissues.

(A) Mechanisms of Action of AChE

Hydrolysis of ACh by AChE results in the production of choline and acetate. There are three amino acid residues implicated in the catalytic triad of the active site of the enzyme, namely serine, histidine and glutamic acid (reviewed in Taylor, 1991; Massoulié et al., 1993). Furthermore, the active site is composed of an esteratic subsite responsible for the hydrolysis of the ester bond as well as an anionic subsite which serves as a recognition site for binding the choline portion of ACh (Silman and Sussman, 1998). The mechanisms of action of AChE

have been shown to involve an acyl-enzyme intermediate that is subsequently deacylated with a water molecule thereby yielding the catalytic products of ACh (choline and acetate) as well as the active enzyme (Froede and Wilson, 1984; Quinn, 1987; Taylor, 1991; Silman and Sussman, 1998). Elucidation of the three-dimensional structure of Torpedo AChE (Sussman et al., 1991) has also revealed that the active site is situated at the bottom of a gorge which has been suggested to permit maximal ACh-AChE interaction thereby accounting for the high catalytic power of the enzyme. A peripheral anionic site is located at the rim of the gorge and has been shown to be involved in substrate inhibition (Radic et al., 1991).

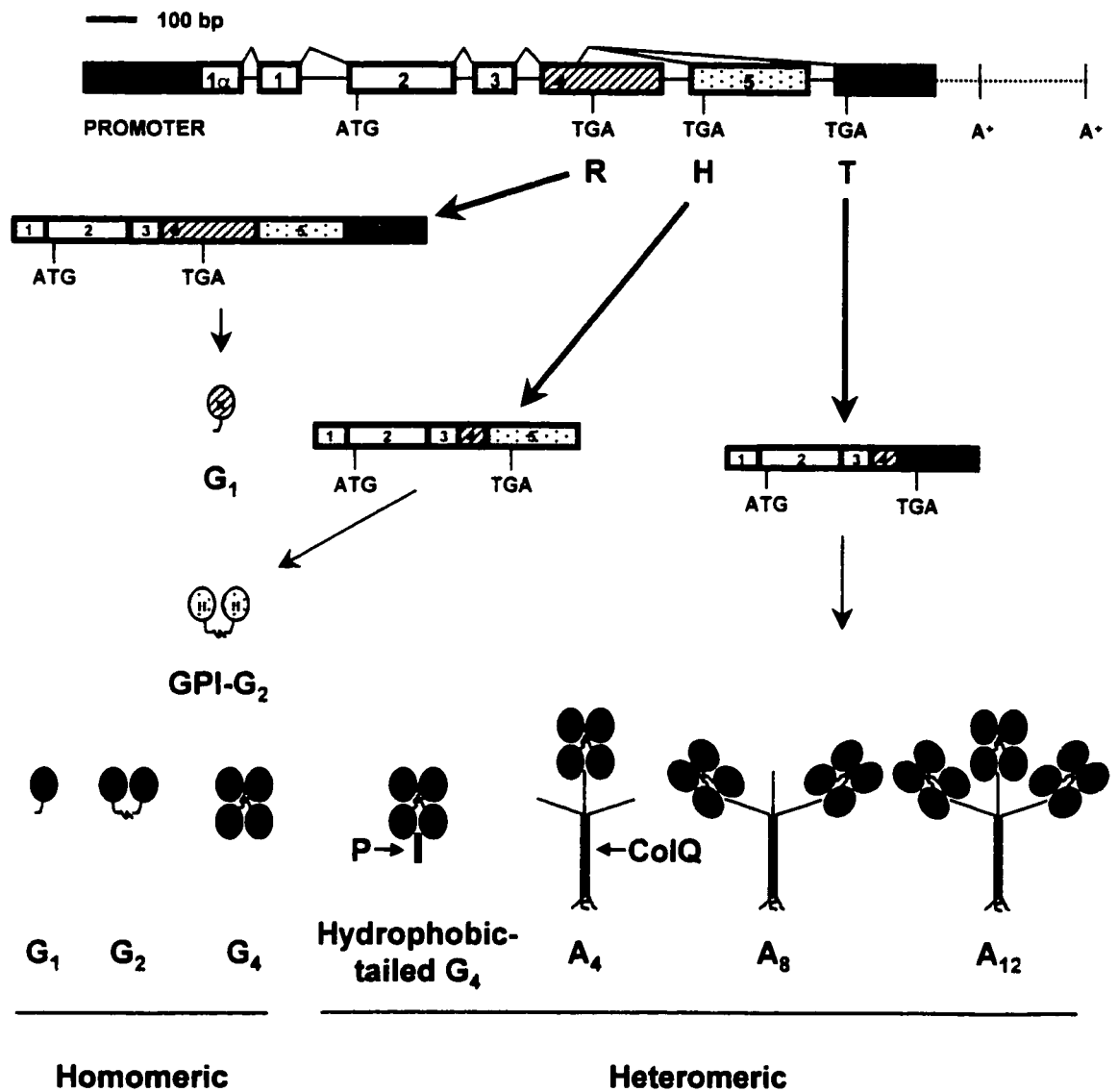
AChE is the target of insecticides as well as nerve agents which act to inhibit the catalytic activity of this enzyme (Quinn, 1987). For example, organophosphates such as eserine inhibit AChE by interacting with the active-site serine and are therefore toxic. AChE inhibitors however, can also serve as therapeutic agents by increasing the action of ACh. For instance, administration of the reversible AChE inhibitor edrophonium in myasthenia gravis, increases the availability of ACh to enhance interactions of this neurotransmitter with AChRs which are deficient in these muscles (Millard and Broomfield, 1995; Henze, 1996; Lindner et al., 1997). Similarly, cholinergic deficiencies associated with Alzheimer's disease can be partially remedied by cholinesterase inhibitors such as tacrine and donepezil which artificially increase ACh levels thereby improving cognition (Giacobini, 1998). Research in this area is ongoing in order to develop less toxic second generation inhibitors.

(B) AChE Molecular Forms and Gene Structure

AChE exists as a complex family of molecular forms that are classified as either homomeric or heteromeric forms. Homomeric species, also referred to as globular forms, exist as either monomers (G_1), dimers (G_2), tetramers (G_4) or glycosylphosphatidyl inositol (GPI)-linked dimers (Figure 1.1). Heteromeric molecular forms are characterized by the assembly of several catalytic subunits with structural proteins through disulfide bonds (reviewed in Silman and Futerman, 1987). Within the heteromeric group, asymmetric molecular forms encompass multiple sets of catalytic subunits (either 4, 8, or 12; A_4 , A_8 , A_{12}) attached to a triple helical collagenic-like structural subunit termed ColQ (Krejci et al., 1991; Krejci et al., 1997; Donger et al., 1998; Ohno et al., 1998) (Figure 1.1). Another member of the heteromeric class exists as a tetramer linked to a 20 kDa hydrophobic anchoring subunit designated as the P subunit (Inestrosa et al., 1987; Gennari et al., 1987). These multiple molecular forms can be simultaneously extracted from tissues using high-salt, non-denaturing detergent buffers and can be separated from one another on sucrose gradients due to their varying sedimentation coefficients reflecting differences in their quaternary structures and molecular weights (reviewed in Massoulié and Bon, 1982; Brimijoin, 1983; Massoulié and Toutant, 1988).

The polymorphism of AChE molecules is attributed in part to the structure of the AChE gene encoding the catalytic subunits. In mouse and human, a single AChE gene with an open reading frame of 5 kb (Li et al., 1991), gives rise to a 68-80 kDa protein product (Rachinsky et al., 1990; Getman et al., 1992) (Figure 1.1). Similarly, in other vertebrates such as *Torpedo marmorata* (Sikorav et al., 1987), rat (Chan et al., 1999) bovine (Mendelson et

Figure 1.1 Schematic representation of the AChE gene displaying the promoter, the exons (boxes), the introns (lines) and the two polyadenylation signals within the 3'-untranslated region (dotted line). Shown are the three known alternatively spliced transcripts (R, H and T) as well as the various molecular forms obtained from each transcript. The translation start codon (ATG), as indicated for each transcript, is located near the beginning of exon 2 whereas the stop codon (TGA) is localized within exon 4 for the R (hatched box), exon 5 for the H (stippled box), and exon 6 for the T transcript (shaded box). Heteromeric molecular forms are generated by the association of T catalytic peptides with the hydrophobic (P) or collagenic (ColQ) structural subunits.



al., 1998) and chick (Rotundo et al., 1988), AChE is also encoded by a single gene. Alternative splicing of this gene yields several different AChE transcripts all comprising untranslated exon 1, as well as exons 2, 3 and a portion of exon 4 that encode the signal peptide and the catalytic domain (Figure 1.1) (Schumacher et al., 1988; Rachinsky et al., 1990). Variable C-terminal peptides are generated by splicing of exon 4 to either exon 5 or 6 to produce the H (hydrophobic) or T (tail) transcript, respectively. An R (readthrough) transcript, encoded by all exons, is synthesized without alternative splicing of the gene. In addition, following exon 6 are two polyadenylation signals within the 3'-untranslated region that result, in the case of the T transcript for example, in the production of two transcripts of 2.4 and 3.2 kb (Figure 1.1) (Li et al., 1991; Legay et al., 1993b).

Translation of T, H and R transcripts as well as post-translational modifications of these various peptide chains give rise to the various molecular forms in vertebrates. All heteromeric and most homomeric AChE forms encompass T catalytic peptides which contain critical cysteine residues necessary for oligomerization of catalytic peptides and for attachment of collagenic or hydrophobic structural subunits via disulfide bonds (Lockridge et al., 1979; Roberts et al., 1991; Velan et al., 1991; Heider and Brodbeck, 1992). Catalytic subunits generated from the H transcript, in contrast, contain a signal for cleavage and attachment of a GPI molecule within the carboxyl-terminus thereby yielding GPI-linked homomeric dimers. Finally, the R transcript produces catalytic subunits giving rise only to monomeric globular forms since these peptides lack cysteine residues important for subunit assembly (Li et al., 1993b; Kerem et al., 1993).

1. AChE structural subunits

A critical component of all asymmetric molecular forms of AChE is the collagenic structural subunit (Col Q) which allows binding of these heteromeric species to the extracellular matrix (Figure 1.1). Cloning and elucidation of the primary structure of the collagenic structural subunit from Torpedo (Krejci et al., 1991), mouse, rat (Krejci et al., 1997) and human (Donger et al., 1998; Ohno et al., 1998) revealed a high degree of homology between the rodent and human primary sequences (89%) whereas the Torpedo sequence showed a 52% and 58% identity with the rat and human homologues, respectively (Donger et al., 1998; Ohno et al., 1998). Collagenic structural subunits from these species encompass a proline-rich N-terminal region, a collagen-containing central domain as well as a C-terminal segment rich in proline and cysteine residues. Transfection experiments using cultured COS cells, have in fact demonstrated that the presence of a proline-rich attachment domain (PRAD) within the N-terminal sequence of the collagenic tail, is required for associating catalytic T peptides with this particular anchoring subunit (Duval et al., 1992; Bon et al., 1997).

An additional unspliced readthrough transcript containing the PRAD domain without the collagenic region was detected during the cloning of the rodent collagenic tail which thus raised the possibility that both collagenic and hydrophobic structural (P subunit) subunits are encoded by a single gene (Krejci et al., 1997). Co-transfection of constructs containing cDNAs encoding the readthrough clone and the rat T catalytic subunit in COS cells or *Xenopus* oocytes, failed however, to generate membrane-associated tetramers (Krejci et al.,

1997). Taken together, these experiments indicate that two separate genes likely encode Col Q and P structural subunits that associate with AChE catalytic subunits.

Although many advances have been made in elucidating the composition and function of the collagenic structural subunit (Krejci et al., 1991; Krejci et al., 1997; Donger et al., 1998; Ohno et al., 1998), our understanding of the 20 kDa hydrophobic tail is still rudimentary (Figure 1.1). Using brain caudate nuclei enriched with heteromeric G₄, the hydrophobic tail was identified and found to interact with radio-labelled 3-(trifluoromethyl)-3-(m-iodophenyl)diazirine (TID), a reagent that is highly selective for the membrane-binding domains of several proteins including the AChE GPI-dimer (Inestrosa et al., 1987; Gennari et al., 1987). G₄ hydrophobic tail-linked molecules were later found to associate with membranes in a manner distinct from that of the GPI-dimers as evidenced by the absence of phosphatidyl-inositol (PI)-glycolipid domains in the hydrophobic tail (Inestrosa and Perelman, 1990). The 20 kDa structural subunit was also shown to be peptidic in nature since it was sensitive to proteases (Fuentes et al., 1988) and was found to contain fatty acids likely involved in linking the enzyme to the membrane (reviewed in Fernandez et al., 1996). These structural characterizations of the hydrophobic tail provide a solid framework that will enable further elucidation of specialized protein domains as well as the eventual cloning of the P subunit cDNA.

2. Characterization of the AChE promoter region

The promoter region of the AChE gene has been recently cloned from a variety of species in order to further characterize the structure of the AChE gene. Analysis of the 5'

regulatory sequences from mouse (Li et al., 1991; Li et al., 1993a), human (Ben Aziz-Aloya et al., 1993a, b; Getman et al., 1995) and rat (Chan et al., 1999) indicates that the AChE promoter is enriched in guanosine and cytosine residues and contains several conserved DNA binding sites which include E boxes, Egr1, Sp1 and AP2 binding sites. In addition, several putative DNA binding sites which include a CArG box and a NF κ B site are also present within the first intron of the rat gene (Chan et al., 1999). Sequence analysis of the rat and mouse promoter fragments also revealed the presence of N box motifs recently shown to be important in the synapse-specific expression of other synaptic proteins (Duclert et al., 1993; Koike et al., 1995; Duclert et al., 1996; Gramolini et al., 1997; Gramolini et al., 1998). Of particular interest is the observation that AChE promoter fragments so far cloned lack a TATA box within the vicinity of the transcription start site. Studies of the human promoter region demonstrate that a dominant transcription start site is utilized for transcription initiation (Getman et al., 1995). Detailed analysis of the mouse AChE promoter fragment, in comparison, revealed that a major cap site encompassing 20 bp is employed to initiate transcription of the AChE gene in muscle and erythroid cells maintained in culture whereas another cap site further upstream is utilized preferentially in brain to generate AChE mRNA (Li et al., 1993a). This apparent alternative splicing of AChE mRNA within the 5'-end of the gene likely reflects yet another level of regulation that remains to be fully characterized.

(C) Synthesis and Assembly of AChE Molecular Forms

The synthesis and assembly of AChE molecular forms involves multiple steps which

have been characterized for peptides generated from the T transcript, the most abundantly expressed of all AChE mRNA (see section C). The process begins with the production of G_1 monomers, the translation product of the T transcript of the AChE gene (reviewed in Chatel et al., 1994; Massoulié et al., 1996). Several lines of evidence indicate that globular monomers serve as precursor molecules for the subsequent formation of the more complex forms (Wilson and Walker, 1974; Lazar et al., 1984; Rotundo, 1984). Notably, studies investigating the recovery of AChE molecular forms in various cultured cells following treatment with specific AChE irreversible inhibitors indicate that low molecular weight G_1 monomers are the first AChE molecular forms to reappear followed sequentially by G_2 , G_3 , then asymmetric forms (Wilson and Walker, 1974; Gisiger, Vigny, 1977; Koenig and Vigny, 1978; Rotundo, 1984; Brockman et al., 1986). Globular monomers were also found to be synthesized much more rapidly than G_4 tetramers in heavy isotope-labeling studies designed to examine the metabolic turnover of the enzyme (Lazar et al., 1984). Together, these findings indicate that G_1 monomers, which is the earliest synthesized molecular form, is also a precursor for the synthesis of larger AChE forms.

The subcellular compartments where oligomerization of AChE molecular forms takes place have also been identified. Inhibiting vesicular export from the rough endoplasmic reticulum (RER) of human embryonic kidney cells transfected with an expression construct containing the human AChE coding sequence, did not impair production of dimers thereby suggesting that some oligomerization already takes place within the RER (Kerem et al., 1993). Other studies took advantage of the fact that AChE is a glycoprotein containing several asparagine-linked oligosaccharides (Velan et al., 1993) that undergo distinct processing within the RER as well as the proximal and distal compartments of the Golgi apparatus (Rotundo,

1984; Rotundo, 1988; Kerem et al., 1993). Examination of the lectin binding patterns of newly synthesized AChE molecules, which allows specific detection of terminal carbohydrates processed in these subcellular compartments, indicates that the precursor G_1 pool is first detected within the RER and that the more complex asymmetric forms are assembled within the Golgi apparatus (Rotundo, 1984). Regardless of whether molecular forms are homomeric or heteromeric, all catalytically active forms destined for secretion or for attachment to the cellular membrane eventually transit the Golgi apparatus where they acquire complex sugars before being exported outside the cell (Rotundo, 1988). Although glycosylation does not appear to influence AChE catalytic activity, it is likely important for stabilizing the enzyme (Velan et al., 1993).

Pulse labeling studies of AChE polypeptides in cultured chick myotubes have revealed that a large fraction of newly synthesized AChE molecules are catalytically inactive and rapidly degraded (Rotundo, 1988). These inactive AChE molecules, composed predominantly of monomers and dimers, represent a separate pool of enzyme subunits that do not serve as precursors for the assembly of more complex forms (Rotundo, 1988). In fact, these forms do not appear to transit the Golgi apparatus as evidenced by their sensitivity to endoglycosidase H which removes sugar moieties added within the RER but not those processed within the Golgi apparatus (Rotundo, 1988). Instead, this inactive pool is sorted early during the biosynthetic pathway and targeted for degradation in a non-lysosomal compartment (Rotundo et al., 1989). The existence of an inactive pool of AChE molecules has also been reported in chick brain tissue by comparing levels of immunoreactive protein corresponding to active and inactive AChE using specific antibodies recognizing enzymatically active or inactive forms of the enzyme, respectively (Chatel et al., 1993). The proportion of inactive AChE molecules

in brain and muscle tissues of chicks were also found to vary according to the stage of development (Anselmet et al., 1994; Chatel et al., 1994). For instance, the proportion of the inactive pool was reduced in brain regions from newborn versus embryonic animals whereas in muscle, the relative levels of inactive molecules increased in newborn versus embryonic chicks (Anselmet et al., 1994; Chatel et al., 1994). However, previous studies of rat brain and muscle as well as human brain and red blood cells have failed to identify an inactive pool of AChE in these species (Brimijoin et al., 1987; Hammond and Brimijoin, 1988) though a recent study of TrkA-deficient mice has revealed the existence of such an inactive pool in the adrenal medulla (Schober et al., 1997). Although it is not clear why inactive AChE molecules are detected specifically in avian tissues, it is possible that the mechanisms of protein folding involving molecular chaperones such as heat shock proteins may differ between species (Eichler et al., 1991; Eichler and Silman, 1995).

(D) Tissue Distribution of AChE Molecular Forms

The relative proportion of each major AChE molecular form varies from one tissue-type to another and has been most extensively studied in excitable cells. The overwhelming majority of AChE molecular forms synthesized in neural tissue are the globular forms. In the brain (Rieger and Vigny, 1976; Grassi et al., 1982; Gennari and Brodbeck, 1985; Legay et al., 1993b) as well as in motoneurons (Di Giamberardino and Couraud, 1978; Fernandez et al., 1979; Gisiger and Stephens, 1982b; Gisiger and Stephens, 1984) and sympathetic ganglia of

the autonomic nervous system (Gisiger and Vigny, 1977; Gisiger et al., 1978), tetrameric globular forms are the most highly expressed. Asymmetric forms, although minimally synthesized in neural tissue (Gisiger et al., 1978; Fernandez et al., 1979), are proportionately more abundant in skeletal muscle which also synthesizes membrane-linked tetramers and globular dimers and monomers (Hall, 1973; Gisiger and Stephens, 1983; Groswald and Dettbarn, 1983). Cardiac muscle, similarly, has also been shown to express collagen-tailed AChE as well as globular monomers and tetramers (Skau and Brimijoin, 1980). Muscle tissue is therefore capable of expressing a full range of AChE molecular forms.

Co-transfection of COS cells with expression constructs encoding the rat T subunit and the Torpedo collagenic structural subunit results in the synthesis of both asymmetric and globular molecular forms that are comparable to those observed in muscle thereby suggesting that AChE molecular forms generated in excitable tissues originate from the T catalytic subunit (Legay et al., 1993b). Consistent with this finding, Northern blotting, RNase protection assays as well as reverse transcription-polymerase chain reaction (RT-PCR) using RNA extracted from mammalian brain and muscle tissues have demonstrated that mRNA encoding the T catalytic subunit is by far the most highly expressed splice variant in these tissues (Li et al., 1991; Li et al., 1993b; Legay et al., 1993b; Legay et al., 1995; Cresnar et al., 1994; Michel et al., 1994). However, mRNAs for the R and the H catalytic subunits have also been shown to be expressed in embryonic rat diaphragm and leg muscles (Legay et al., 1995). The physiological relevance for the transient expression of the R and H transcripts in developing muscles of embryos is not known. However, it has been hypothesized that factors originating from innervating motoneurons may be necessary to establish the restricted expression pattern of T transcripts in adult skeletal muscle (Legay et al., 1995). Putative

splicing factors that directly or indirectly regulate the preferential expression of the T transcript in adult muscle have yet to be identified and characterized. In this context however, recent evidence suggest that the binding of putative muscle specific splicing factors to intronic sequences of the AChE gene direct splicing of exon 4 to exon 6 giving rise to AChE T transcripts (Luo et al., 1998).

Although AChE molecules are predominantly expressed in neural and muscle cells, the enzyme is also synthesized in non-excitabile tissues. In mammals notably, GPI-dimers are exclusively expressed in mature erythrocytes (Futerman et al., 1985; Roberts et al., 1987; Toutant et al., 1989) as well as in embryonic liver (Legay et al., 1993a). In accordance with these findings, transcripts encoding the H catalytic subunit have been detected in mouse erythroid cells and in rat embryonic liver (Li et al., 1991; Li et al., 1993b; Legay et al., 1993a). Interestingly, differentiating erythroid cells in culture rather than expressing GPI-dimers, synthesize monomeric forms of the enzyme generated from T and R transcripts (Li et al., 1991; Li et al., 1993b; Chan et al., 1998). These findings suggest that production of GPI-anchored dimers, characteristic of mature erythroid cells, depends on developmentally-activated splicing events required to generate H transcripts (Chan et al., 1998).

(E) AChE Expression in Developing Skeletal Muscle

In the early stages of muscle differentiation, the primary AChE molecular forms expressed are the globular forms, particularly the monomers and tetramers (Vigny et al., 1976; Lyles et al., 1979; Haynes et al., 1984; Cisson et al., 1981; reviewed in Massoulié and Bon,

1982; Toutant and Massoulié, 1988). Thereafter, synthesis of asymmetric forms is considerably enhanced by the establishment of nerve-muscle contacts. Specifically, analysis of leg muscles from rodent and avian species demonstrates that expression of asymmetric AChE is upregulated around embryonic day E14-16 corresponding to the time period of early nerve-muscle interactions (Vigny et al., 1976; Koenig and Vigny, 1978; Toutant et al., 1983; reviewed in Dennis, 1981). Innervation of muscle fibers also alters the distribution of the asymmetric forms along the length of the muscle fiber by inducing their focalization at the sites of nerve-muscle contact (Kelly and Zacks, 1969; Bennett and Pettigrew, 1974). In particular, asymmetric forms in neonatal rat muscle are present in non-endplate regions of muscle fibers whereas they become more restricted to endplate segments as muscles undergo post-natal maturation (Sketelj and Brzin, 1980; Koenig and Rieger, 1981). Furthermore, accumulation of AChE molecules at synaptic sites during embryonic development also coincides temporally with aggregation of AChE mRNAs encoding the T catalytic subunit at synaptic sites of mouse skeletal muscle fibers (Legay et al., 1995). Taken together, these findings indicate that neuromuscular interactions regulate the synthesis as well as the distribution of collagen-tailed forms of AChE in muscle.

Accumulation of AChE transcripts and molecules at synaptic sites requires signals from the motoneuron. Indeed, the motoneuron exerts long-lasting effects on the distribution of asymmetric forms of AChE in muscle fibers since ectopic reinnervation of denervated adult muscle induces the appearance of asymmetric A₁₂ not only at newly formed ectopic endplates but also at original endplates (Vigny et al., 1976; Weinberg and Hall, 1979). Modifications of the extracellular basal lamina by motoneuronal signals have been demonstrated to play a critical role in aggregating AChE molecules (McMahan et al., 1978; Weinberg and Hall,

1979). In this context, agrin a glycoprotein released by the motoneuron, has been shown to induce the aggregation of several extracellular, plasma membrane as well as cytoplasmic components at the neuromuscular junction (Godfrey et al., 1984; Wallace et al., 1985; Wallace, 1986; Wallace, 1989; McMahan, 1990). In particular, agrin induces the formation of AChE clusters in culture without affecting the biosynthesis of the enzyme (Wallace, 1989). Aggregation of AChE molecules may also be dependent on the prior or parallel agrin-induced clustering of AChR as recently reported (De La Porte et al., 1998). Collectively, these lines of evidence indicate that focalization of asymmetric AChE at synaptic sites of muscle is nerve-mediated.

In primary cultures of myotubes, expression of asymmetric forms has been shown to be dependent on myotube contraction since blocking spontaneous myofiber contraction by application of tetrodotoxin, a sodium channel blocker, significantly compromises the production of collagen-tailed AChE molecular forms (Rieger et al., 1980; Brockman et al., 1984; Rubin, 1985; Fernandez-Valle, Rotundo, 1989). Further analyses of cultured muscle cells have in fact revealed that ionic fluxes occurring during myofiber contraction play a critical role in regulating expression of asymmetric AChE. In particular, increased intracellular levels of Na^+ by application of veratridine, a Na^+ channel agonist, or elevations of Ca^{+2} levels using a Ca^{+2} ionophore, both lead to enhanced expression of asymmetric forms (De La Porte et al., 1984; Brockman et al., 1984; Rubin, 1985; Fernandez-Valle and Rotundo, 1989; Vallette and Massoulié, 1991). Consistent with these findings, nifedipine treatment of cultured myotubes, which blocks L-type Ca^{+2} channels, results in reduced levels of A_{12} as well as globular dimers (Decker and Berman, 1990). Ionic fluxes triggered by membrane depolarization appear therefore to regulate AChE expression in muscle cells by mechanisms

that have yet to be fully elucidated.

The underlying molecular events that control AChE expression during myotube formation have begun to be explored. Notably, increases in AChE activity that occur during myoblast to myotube differentiation in culture (Inestrosa et al., 1983) have been shown to be paralleled by a rise in the abundance of AChE transcripts (Fuentes and Taylor, 1993; Grubic et al., 1995). Furthermore, analysis of the transcriptional activity of the AChE gene in myoblast versus myotubes reveals that the rise in AChE transcripts occurring during differentiation is not due to increased AChE gene transcription but results primarily from increased AChE mRNA stability (Fuentes and Taylor, 1993; Li et al., 1993a). Interestingly, Ca^{2+} -mediated events have been reported to regulate AChE transcript stability during differentiation of myoblast to myotubes (Luo et al., 1994; Luo et al., 1996). Taken together, these data suggest that post-transcriptional mechanisms are involved in regulating AChE expression during myotube differentiation and that ionic fluxes are implicated in modulating the stability of AChE transcripts as well as the synthesis of AChE molecular forms in developing muscle.

(F) Synaptic Accumulation of AChE at the Neuromuscular Junction

AChE accumulation and maintenance within the postsynaptic region of the neuromuscular junction are thought to result from a combination of regulatory mechanisms which lead to the aggregation of AChE transcripts and the subsequent localized translation and anchoring of AChE molecules at the endplate.

1. AChE mRNA Localization at the Neuromuscular Junction

In a series of recent studies, the cellular distribution of AChE transcripts in muscle fibers was analysed in order to compare the relative abundance of AChE transcripts in junctional versus extrajunctional regions. Using the RT-PCR assay, levels of AChE mRNA measured from junctional segments of adult avian and rat myofibers were shown to be significantly higher than those observed from extrajunctional muscle fiber segments (Jasmin et al., 1993; Michel et al., 1994). Similarly, adult rat skeletal muscles processed for in situ hybridization displayed more intense AChE mRNA staining within the postsynaptic muscle fiber compartments (Michel et al., 1994). Focalization of AChE mRNA at the site of nerve-muscle contacts was also shown in cocultures of human myotubes and rat motor neurons (Grubic et al., 1995). In this latter study, AChE mRNA positive nuclei were initially found throughout the length of the myotube but upon innervation and myotube contraction, AChE mRNAs became preferentially localized to nuclei in junctional regions. Taken together, these findings demonstrate that accumulation of AChE mRNAs within endplate regions likely contributes significantly to the selective aggregation of AChE molecules at the neuromuscular junction. The molecular mechanisms responsible for this focalized expression however have not been fully explored.

2. Local translation of AChE mRNA at the neuromuscular junction

Findings obtained from cultured myotubes, suggest that once transcribed, AChE

transcripts remain near the nucleus of origin where they are locally translated (Rotundo, 1990; Tsim et al., 1992). Once synthesized, AChE catalytic subunits are then assembled into oligomers and targeted to the segment of the sarcolemma overlying the myonuclei of origin (Rossi and Rotundo, 1992). Localized translation of AChE mRNA and targeting of AChE molecules as seen in myotubes has therefore been hypothesized to play a significant role in concentrating AChE molecules selectively within the postsynaptic compartment of the neuromuscular junction (Rossi and Rotundo, 1992). Interestingly, previous histological studies have shown that organelles such the Golgi apparatus and microtubules displaying specialized features and known to be involved in the processing and targeting of peptides, are associated with myonuclei located within the postsynaptic region of the neuromuscular junction (Jasmin et al., 1989; Jasmin et al., 1990; Jasmin et al., 1995). The presence of such specialized structures within subsynaptic regions of the muscle fiber suggests therefore that these structures may participate in the compartmentalized expression of AChE at synaptic sites.

3. Role of heparan sulfate proteoglycans (HSPG) in anchoring AChE at the neuromuscular junction

Studies by McMahan and colleagues (1978) have shown that AChE activity was detectable within the extracellular matrix of frog muscle following degeneration of both pre and postsynaptic compartments. These results demonstrated unequivocally that at least some AChE molecules are attached to the basal lamina. In this context, the role of extracellular matrix protein in junctional protein accumulation has been actively pursued (Anderson and

Fambrough, 1983; Sanes et al., 1986) In particular, HSPGs are important components of the synaptic basal lamina where their aggregation has been shown, in culture, to be mediated by agrin through increased biosynthesis (Wallace, 1989). Interestingly, anchoring of A₁₂ AChE to the synaptic basal lamina has been proposed to occur via interactions between HSPGs and heparin binding domains within the collagenic structural subunit (Deprez and Inestrosa, 1995) which can be interrupted with heparin or heparan sulfate (Inestrosa et al., 1982; Brandan et al., 1985; Brandan and Inestrosa, 1986; Rotundo et al., 1997). In addition, the presence of specific binding sites for asymmetric AChE within the synaptic basal lamina has been proposed based on studies of cultured muscle cells. More precisely, purified quail collagen-tailed AChE was shown to bind specifically to synaptic sites of frog muscle fibers (Rotundo et al., 1997). Furthermore, heparin treatment was found to inhibit binding of quail collagen-tailed AChE to these synaptic regions therefore indicating that interactions between HSPGs and asymmetric AChE likely occur. These findings imply that HSPGs play an important role in anchoring asymmetric forms of AChE to the synaptic basal lamina.

Although HSPGs are likely candidates involved in anchoring asymmetric AChE molecules (Rotundo et al., 1997; Casanueva et al., 1998a), additional evidence suggests that other components of the extracellular matrix may also be implicated in this anchorage. For instance, although most of the AChE enzyme is extracted with high-salt and non-denaturing detergent buffers, a small proportion of the enzyme remains attached to the basal lamina and can only be fully extracted upon collagenase treatment (Rossi and Rotundo, 1993; Rossi and Rotundo, 1996; Casanueva et al., 1998a). In this context, novel putative basal lamina receptors for asymmetric AChE have been recently identified using an overlay assay comprised of basal lamina preparations isolated from Torpedo electric organ and purified

asymmetric AChE molecules (Casanueva et al., 1998b). These experiments led to the identification of two additional basal lamina components besides HSPGs which specifically bind asymmetric AChE. These include collagenous polypeptides of 140 kDa and a doublet of 195-215 kDa which share homologies with collagens type IV and V (Casanueva et al., 1998b). It is interesting to note that collagen α IV isoforms have been shown to be highly focalized within the synaptic basal lamina (Taylor, 1991; Massoulié et al., 1993). It is therefore probable that HSPGs and collagenous polypeptides represent two distinct basal lamina constituents that bind asymmetric AChE molecules within synaptic regions.

(G) AChE Expression in Fast- versus Slow-twitch Skeletal Muscle

The phenotype of fast and slow contracting skeletal muscles differs substantially with respect to contractile and metabolic properties (reviewed in Pette and Vrbova, 1992). Whereas fast muscles, typically express fast contractile protein isoforms and high levels of glycolytic enzymes, slow muscles characteristically express slow contractile protein isoforms and an elevated level of oxidative enzymes. These and other phenotypic differences between fast and slow muscles are regulated in large part by the pattern of neural activity dictated by the innervating motoneuron (reviewed in Pette and Vrbova, 1985; Swynghedauw, 1986; Booth and Thomason, 1991; Pette and Vrbova, 1992; Kraus et al., 1994; Booth et al., 1998; Gundersen, 1998).

1. AChE Activity and Molecular Form Profiles

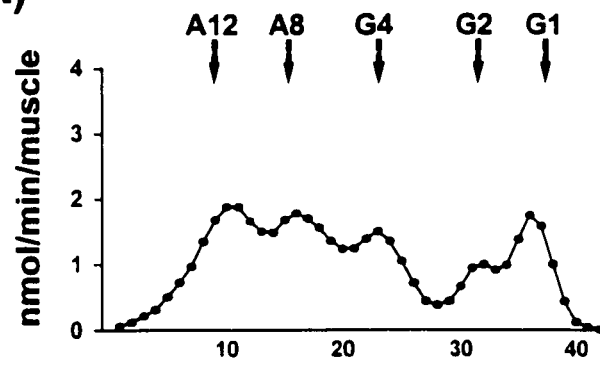
The overall production of AChE in muscle varies according to whether a muscle displays fast- or slow-twitch properties. For instance, AChE enzyme activity is considerably higher in rodent fast versus slow muscles (Gisiger and Stephens, 1982b; Groswald and Dettbarn, 1983). Consistent with this observation, AChE transcripts encoding the T catalytic peptide are significantly more abundant in fast versus slow muscles (Cresnar et al., 1994; Michel et al., 1994; Sketelj et al., 1998). Whether these differences in AChE mRNA levels are due to greater rates of AChE gene transcription in fast versus slow muscles remain to be determined.

The relative proportions of the various molecular forms synthesized in skeletal muscles also differ substantially between fast and slow muscles. Whereas fast muscles express relatively high amounts of tetramers and monomers and lower levels of asymmetric forms, slow muscles display proportionately more A_{12} and A_8 asymmetric forms and less tetramers and monomers (Figure 1.2). Enhanced expression of asymmetric forms in slow muscles may in fact reflect the higher levels of collagenic structural subunits synthesized in slow versus fast muscles (Legay et al., 1998). Asymmetric forms are also localized almost exclusively at the site of nerve-muscle contacts in fast muscles whereas in slow muscles, asymmetric forms are detected in extrajunctional regions of muscle fibers (Sketelj et al., 1991). This observation is consistent with recent immunohistochemical and in situ hybridization studies documenting focalized synaptic expression of the collagenic structural subunit in fast muscles and synaptic as well as extrasynaptic expression of the collagenic tail in slow muscles (Legay et al., 1998).

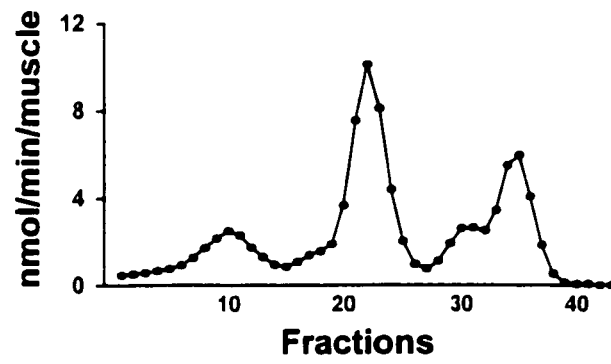
Marked differences in total AChE activity as well as in the molecular form distribution

Figure 1.2 Representative examples of AChE molecular form profiles obtained from the sedimentation analysis of rat slow soleus (A) and fast EDL (B) skeletal muscle extracts. Note the different Y axis scales for each profile reflecting the higher total AChE activity for the fast skeletal muscle. Also note the relatively higher levels of G_4 in the fast- versus slow-contracting muscle as well as the proportionately more abundant levels of asymmetric forms in the slow muscle.

(A)



(B)



between fast and slow-contracting muscles have also been noted for motoneurons innervating these muscles types. In particular, motoneurons innervating fast rodent extensor digitorum longus muscles, compared to slow soleus motor nerves, display greater AChE activity and are characterized by elevated levels of G₄ (Gisiger and Stephens, 1982a, b; Gisiger and Stephens, 1984). Muscles and their innervating motoneurons therefore appear to exhibit similar patterns of AChE expression that are correlated with their fast or slow contractile properties.

2. Intrinsic Regulation of AChE Expression in Fast versus Slow Muscles

Neural activity is recognized as being largely responsible for differences in AChE expression observed between mature fast and slow muscles (reviewed in Massoulié et al., 1993; see section G). However, intrinsic properties of the two muscle types appears to also contribute to these variations. For example, slow and fast muscles from post-natal rats, already display prominent differences in their molecular form profiles (Sketelj et al., 1991) despite receiving, at this developmental stage, similar impulse patterns delivered by their respective innervating motoneurons (Navarrete and Vrbova, 1983). Furthermore, when slow and fast rat hindlimb muscles are forced to undergo regeneration in situ in the presence or absence of innervation or at a different functional site by transplantation, regenerated muscles display AChE molecular form profiles resembling those observed in the original slow or fast muscle (Sketelj et al., 1991; Dolenc et al., 1994). These latter data have been interpreted to suggest that myogenic precursors or satellite cells, which undergo proliferation and differentiation during the process of muscle regeneration (Mauro, 1961; reviewed in Campion,

1984; Schultz and McCormick, 1994), are intrinsically programmed to express either slow or fast AChE profiles independent of the presence of the motor nerve (Sketelj et al., 1991; Dolenc et al., 1994). The relative contribution of innervation versus intrinsic factors in controlling AChE expression in skeletal muscle remains controversial and needs to be clearly established if the ultimate goal is to understand all the events contributing to the regulation of AChE in slow and fast muscles.

(H) Activity-Dependent Expression of AChE in Skeletal Muscle

1. Adaptations to Neuromuscular Inactivation

AChE enzyme activity as well as the molecular form profiles of fast and slow muscles are governed extensively by the firing pattern of their constituent motor units. Inhibition of neural electrical activity either surgically by severing the nerve or chemically by using neurotoxins such as tetrodotoxin (TTX) or botulinum toxin results in dramatic reductions in AChE enzyme activity levels in both fast and slow muscles of mouse and rat (Drachman, 1972; Butler et al., 1978; Cresnar et al., 1994; Michel et al., 1994). Other disuse models such as limb immobilization and hindlimb suspension also result in reduced AChE activity in fast contracting muscles of rat (Guth, 1969; Snyder et al., 1973; Gardiner et al., 1982). Increases in AChE activity however, have been reported for soleus muscles subjected to 2 to 3 weeks of hindlimb suspension (Gupta et al., 1985) as this model of disuse is known to shift the contractile properties of soleus muscles from slow to fast (Reiser et al., 1987; Pierotti et al., 1990). It is interesting to note, in addition, that in other species such as rabbit, chick and

guinea pig, muscle inactivation leads to an increase in AChE enzyme activity suggesting diversity in the response of different species to denervation (Tennyson et al., 1977; Sketelj et al., 1978; Silman et al., 1979; Bacou et al., 1982; Lai et al., 1986). The mechanisms underlying this species-specific response have yet to be elucidated.

AChE molecular form profiles of skeletal muscle fibers undergo significant alterations in response to reduced neuromuscular activation. Denervation for instance, dramatically downregulates endplate-associated collagenic-tailed AChE (Hall, 1973; Vigny et al., 1976; Fernandez et al., 1979; Cresnar et al., 1994). Increased proteolytic activity at the neuromuscular junction following denervation is thought to account significantly for this pronounced reduction in endplate-associated A_{12} since administering protease inhibitors to denervated muscles significantly delays the loss of asymmetric AChE activity at synaptic sites (Fernandez and Duell, 1980). Surprisingly, partially denervated soleus muscles of rat display increased extrajunctional expression of A_{12} (Crme-Finderle et al., 1995). This latter finding may be the result of increased expression of the collagenic structural subunit within non-endplate region since synthesis of asymmetric AChE is dependent on the availability of the collagenic tail (Duval et al., 1992; Legay et al., 1993b). Denervation also substantially reduces expression of all globular forms of AChE in rat muscles (Hall, 1973; Vigny et al., 1976; Collins and Younkin, 1982; Cresnar et al., 1994) though briefly after denervation, transient and selective increases in the level of the G_4 molecular form have been documented for fast muscles (Gregory et al., 1989). Collectively, these findings indicate that reduced neural activation significantly compromises AChE expression in skeletal muscles which is manifested by reduced AChE activity and downregulation of all AChE molecular forms.

More recently, studies of the molecular mechanisms mediating the activity-dependent

expression of the AChE gene revealed that reductions in AChE activity in both fast and slow muscles, in response to denervation or TTX-induced paralysis, are accompanied by a parallel decline in AChE mRNA (Cresnar et al., 1994; Michel et al., 1994). It remains to be determined, however, whether modifications in the transcriptional activity of the AChE gene account for this decrease in AChE transcript levels. In this context, several studies performed on the α , β , γ , and δ subunits of the AChR have clearly demonstrated that muscle activity also regulates expression of these subunit genes (reviewed in Hall and Sanes, 1993; Duclert, Changeux, 1995). Denervation has been shown not only to dramatically increase mRNA levels encoding the α , β , γ , and δ AChR subunits (Merlie et al., 1984; Goldman et al., 1985; Goldman et al., 1988; Goldman and Staple, 1989; Witzemann et al., 1991; Adams et al., 1995) but also to significantly upregulate the rate of transcription of their respective genes as directly assessed by nuclear run on assays (Tsay and Schmidt, 1989), in situ hybridization using genomic probes (Fontaine and Changeux, 1989) and in vivo analyses of cloned promoter regions (Merlie and Kornhauser, 1989; Salmon and Changeux, 1992; Simon et al., 1992; Gundersen et al., 1993; Bessereau et al., 1994; Merlie et al., 1994; Tang et al., 1994; Walke et al., 1996; Bessereau et al., 1998). It is therefore conceivable that alterations in the transcriptional activity of the AChE gene may also account for changes in AChE transcript abundance in muscles in response to reduced neuromuscular activation. However, post-transcriptional events may also be implicated, since several lines of evidence suggest that mechanisms regulating AChE mRNA stability play an important role in controlling AChE expression in cultured myogenic (Fuentes and Taylor, 1993), neural (Coleman and Taylor, 1996) and hematopoietic (Chan et al., 1998) cells.

2. Adaptations to Enhanced Neuromuscular Activation

Increased neuromuscular activity using either forced or voluntary exercise regimens or through surgical intervention, in the case of compensatory hypertrophy, lead to elevations in the levels of AChE activity particularly in fast contracting muscles (Snyder et al., 1973; Crockett et al., 1976; Gardiner et al., 1982; Fernandez and Donoso, 1988; Jasmin and Gisiger, 1990; Gisiger et al., 1994; Sveistrup et al., 1995). Enhanced neuromuscular activation is also associated with concomitant elevations in the mRNA levels encoding the T catalytic subunit in both fast and slow contracting muscles (Sveistrup et al., 1995). However, it is not yet known whether increased neural activity induces AChE gene transcription or stabilization of existing AChE transcripts.

The relative abundance of the various molecular forms of AChE in skeletal muscles undergoes remarkable adaptations to elevations in neural activity. Voluntary or enforced training programs involving wheel cage running, treadmill walking or running, and swimming have convincingly shown that the G_4 content in fast muscles is selectively modified whereas the A_{12} levels undergo only minor alterations (Fernandez and Donoso, 1988; Jasmin and Gisiger, 1990; Jasmin et al., 1991; Gisiger et al., 1991; Gisiger et al., 1994; Sveistrup et al., 1995). For instance, 12 weeks of treadmill running increases the levels of G_4 in fast contracting ankle flexors by more than 50% whereas in fast ankle extensors levels of G_4 are reduced by approximately 40% (Jasmin and Gisiger, 1990). Conversely, fast muscles of both ankle extensors and flexors display significant elevations in their G_4 levels following swimming (Gisiger et al., 1991). Whereas the fast extensor muscles are activated in a primarily tonic fashion during running training, these muscles are subjected to dynamic-like activity during

swimming exercise. Together, these findings demonstrate that fast muscles adapt their G_4 levels to altered neuromuscular activity by increasing or decreasing the G_4 content according to whether muscles are exposed to dynamic or tonic activity respectively.

The extent of the G_4 adaptation has been shown to be extensive in fast muscles of animals subjected to voluntary wheel cage running (Gisiger et al., 1994). Indeed, a 400% increase in the G_4 content of these fast muscles was reported in this latter study. In addition, the magnitude of these elevations in G_4 levels were found to be positively correlated with the number of wheel cage revolutions indicating that the level of muscle exertion dictates the extent of the G_4 adaptation (Gisiger et al., 1994). Furthermore, many studies have indicated that the relative proportions of the various molecular forms in slow soleus muscles, conversely, remain relatively intact when these muscles are subjected to enhanced neuromuscular activation protocols (Jasmin and Gisiger, 1990; Gisiger et al., 1994; Sveistrup et al., 1995). One exception however is the 30 to 45% reduction in the levels of A_{12} reported for soleus muscles of rats trained using the voluntary wheel cage running model (Gisiger et al., 1994; Sveistrup et al., 1995). Given that significant levels of asymmetric forms are normally expressed in the extrajunctional regions of rat soleus muscles (Sketelj et al., 1991), it is possible to envisage that increased neuromuscular activation may downregulate the synthesis of asymmetric forms in non-endplate regions thereby enhancing the focalization of these forms within the postsynaptic segment of muscle fibers resulting in an overall reduction in the levels of A_{12} .

Fast hindlimb muscles exposed to increased functional demands imposed following ablation of synergist muscles display particular AChE molecular form adaptations that differ from those reported using other models of enhanced neuromuscular activity as described

above. For instance, rat plantaris or medial gastrocnemius muscles undergoing compensatory hypertrophy adapt their AChE molecular form distribution by a general increase in the levels of all molecular forms (Jasmin et al., 1991; Sveistrup et al., 1995). For medial gastrocnemius muscles, levels of the A₈, G₁ and G₂ forms increase the most (Jasmin et al., 1991) whereas in plantaris muscles, the A₈ and A₁₂ content were most significantly enhanced (Sveistrup et al., 1995). These overloaded fast muscles in fact exhibited AChE molecular form profiles resembling those normally expressed by slow contracting muscles. Interestingly, when overloaded muscles are in addition subjected to running training, the G₄ levels are further induced (Jasmin et al., 1991). Since overloaded muscles must undertake a significant antigravity role upon ablation of synergist postural muscles (Gardiner et al., 1986) and since compensatory overload increases the proportion of slow twitch fibers in the target fast muscles (Roy et al., 1985), these data suggest that overloaded fast muscles are activated tonically. These studies, taken together, further demonstrate that dynamic versus tonic neuromuscular activation elicit specific adaptations in the levels of AChE molecular forms in skeletal muscles. Indeed, dynamic activity appears to selectively induce G₄ levels in fast muscles whereas tonic activity increases the levels of all molecular forms with a slight preferential upregulation of the asymmetric forms.

The role of dynamic versus tonic activation in regulating AChE molecular form profiles has also been studied using electrical stimulation whereby hindlimb muscles are activated either directly or indirectly (via the nerve) with a specific pattern of electrical activity (Lomo et al., 1985; Sketelj et al., 1997; Sketelj et al., 1998). Results from these studies are variable given that (1) stimulated muscles were either denervated (Lomo et al., 1985) or left intact (Sketelj et al., 1997; Sketelj et al., 1998) and that (2) the electrical stimulation

paradigms were of different durations and patterns. For instance, denervated soleus muscles directly stimulated with a chronic fast high frequency pattern of activation (100 Hz) for 2 to 21 days, expressed an AChE molecular form profile characterized by a prominent peak of G₁ and equivalent amounts of A₁₂ and G₄ thereby exhibiting some features of a typical fast AChE profile (Lomo et al., 1985). Low frequency stimulation of denervated EDL muscles in this same study did not shift the molecular form profile to that typically expressed in slow muscles (Lomo et al., 1985). However, a fast to slow transformation in the AChE molecular form profile was reported when low frequency activation was superimposed onto the normal activity of intact EDL muscles (Sketelj et al., 1997; Sketelj et al., 1998). These discrepancies may be due to the varying activation paradigms or may suggest that the effects of low frequency activation on the distribution of AChE molecular forms within fast muscles depends on the state of innervation of stimulated muscles (i.e. denervated versus intact). Although slow muscles appear to undergo some transformations of their molecular form profiles in response to high frequency activation (Lomo et al., 1985), based on other models of altered usage, the adaptability of these slow muscles is generally less extensive than that observed in fast muscles.

(I) Neurotrophic Regulation of AChE Expression

Although the amount and pattern of nerve-derived electrical activity markedly influences AChE expression in skeletal muscles, there are several lines of evidence demonstrating that nerve-derived trophic molecules also play a significant role in this

regulation. Chronic application of TTX on to the sciatic nerve, which inhibits electrical activity of muscle fibers without compromising axoplasmic flow or nerve-muscle contacts, leads to less pronounced reductions of AChE activity and transcript levels in comparison to those induced by denervation (Butler et al., 1978; Michel et al., 1994). Similarly, selective disruption of axoplasmic flow with colchicine application on peripheral nerves decreases AChE activity in target muscle fibers (Fernandez and Inestrosa, 1976). In addition, organ cultured denervated EDL muscles treated with extracts from the brain, sciatic nerve or spinal cord display higher levels of AChE activity than untreated cultures (Davey et al., 1979). Finally, several studies have also shown that denervation-induced reductions in endplate associated AChE develop sooner when the innervating nerve is sectioned close to rather than at a distance from the muscle (Davey and Younkin, 1978; Fernandez et al., 1979; Ranish et al., 1980). These studies, taken together, suggest that neurogenic molecules and/or simply the nerve-muscle contact (Choi et al., 1997), significantly modulate AChE expression in muscles. The identity of specific neurotrophic molecules and their effects on AChE expression, however, still remain to be investigated.

1. Nerve-derived trophic molecules

(a) Calcitonin gene-related peptide (CGRP)

CGRP is a 37 amino acid neuropeptide synthesized by spinal cord motoneurons (Gibson et al., 1984; Sala et al., 1995) and transported to nerve terminals via axonal transport (Kashihara et al., 1989; Fernandez and Hodges-Savola, 1994) where it is released from the

nerve in response to electrical impulses (Uchida et al., 1990; Sala et al., 1995). Until recently, most studies have characterized the role of CGRP in regulating expression of the AChR (reviewed in Changeux et al., 1992; Duclert and Changeux, 1995). For example, CGRP has been shown to increase the number of cell surface AChR as well as the levels of mRNA encoding the α -subunit of the AChR by 1.5- and 3-fold respectively (New and Mudge, 1986; Fontaine et al., 1986; Fontaine et al., 1987; Osterlund et al., 1989). Given that CGRP influences expression of the AChR, CGRP has also been studied as a potential regulator of AChE synthesis in skeletal muscle.

Following short-term denervation (24-48hrs) or after 2 days of exercise training, concentrations of CGRP in nerve terminals measured from endplate samples of rat muscles are reduced by 20 to 40 % (Hodges-Savola and Fernandez, 1995; Fernandez and Hodges-Savola, 1996). Interestingly, these decreases in CGRP were found to coincide with the concomitant elevation in the levels of G_4 AChE activity (Hodges-Savola and Fernandez, 1995; Fernandez and Hodges-Savola, 1996). It was therefore hypothesized that CGRP downregulates G_4 activity levels. In this context, subcutaneous injections of CGRP, were found to partially block the transient induction of G_4 observed following short-term denervation (Hodges-Savola and Fernandez, 1995). Similarly, G_4 levels in muscles from exercised rats treated with CGRP were shown to be lower than those measured from exercised animals treated with PBS (Fernandez and Hodges-Savola, 1996). Additional experiments also suggest that the effects of CGRP on AChE activity are indeed mediated through CGRP receptors (Hodges-Savola and Fernandez, 1995) which are concentrated at the neuromuscular junction (Popper and Micevych, 1989).

More recently, the molecular mechanisms mediating the CGRP-dependent regulation of AChE expression have been assessed in vitro. In cultured chick myotubes, the levels of AChE mRNA increased 3-fold with CGRP application (Choi et al., 1996), and this adaptation was shown to be dependent on the adenylate cyclase/cAMP signalling pathway (Choi et al., 1998) as previously described in the studies of the effects of CGRP on AChR expression (Laufer and Changeux, 1989). This elevation in AChE transcript levels was accompanied by a 1.7 fold increase in AChE protein levels as assessed by Western blot analysis but was not paralleled by an elevation in AChE enzyme activity (Choi et al., 1996). This latter finding indicated that CGRP appeared to specifically upregulate expression of the inactive pool of the enzyme in chick myotubes. Given these findings, it is reasonable to anticipate that CGRP-mediated AChE regulation in rodent muscle fibers likely differs from that displayed by avian muscles since rodent muscles appear to lack the inactive enzyme pool (Brimijoin et al., 1987; Hammond and Brimijoin, 1988) and given the diversity of the response of these species to muscle denervation (reviewed in Massoulié et al., 1993).

(b) β -endorphin and its derivatives

Cultured myotubes derived from E20 rat hindlimb muscles express particularly high levels of A₁₂ (Haynes et al., 1984). Interestingly, when these cells are maintained in medium previously conditioned by developing spinal cord extracts, the levels A₁₂ were reduced whereas levels of G₁ and G₂ forms were enhanced (Haynes et al., 1984). In the latter study,

β -endorphins were detected in the conditioned medium and were therefore suggested as potential candidate trophic molecules regulating A_{12} activity. In this regard, ventral horn neurons and motoneuron axons have been shown to contain β -endorphin-like immunoreactive peptides in young rats up to 5 weeks of age (Haynes et al., 1982). This transient expression of β -endorphins suggest that they may play a role in regulating the developmental expression of AChE in muscle. The precise action of β -endorphins in inhibiting the activity of the A_{12} molecular form, although not clearly known, appears to be dependent upon the interactions between the catalytic subunit and the collagenic tail. Specifically, removal of the structural subunit from A_{12} with collagenase renders the residual catalytic subunits resistant to the effects of β -endorphins (Haynes et al., 1982).

Glycyl-L-glutamine (Gly-Gln), a dipeptide derived from the proteolytic cleavage of the C-terminal of β -endorphins, has been shown conversely, to stimulate levels of A_{12} and G_4 in cultured embryonic rat and quail muscle fibers by perhaps controlling the conversion of G_1 precursors into more complex AChE molecular forms (Haynes and Smith, 1985; Lotwick et al., 1990). Gly-Gln has also been demonstrated to maintain AChE activity in cat denervated superior cervical ganglia cells which normally display reduced AChE activity in response to axotomy (Koelle et al., 1985; Koelle et al., 1988). Treatment of quail muscle cultures with Gly-Gln does not alter levels of intracellular cAMP thereby suggesting that alternative signal transduction mechanisms are triggered by Gly-Gln to regulate AChE biosynthesis (Lotwick et al., 1990). Notably, it was previously proposed that Gly-Gln instead may influence AChE expression at other levels such as transcription by crossing both cellular and nuclear membranes in combination with a plasma factor (Koelle, 1988). Evidence for such a

mechanism however has yet to be documented.

(c) ACh

The importance of ACh and AChRs interactions in regulating AChE expression in skeletal muscle cells has been demonstrated. In particular, enhanced levels of specific AChE molecular forms, namely the G_4 tetramer, and maintenance of the $G_1 + G_2$ pool of the enzyme have been attributed to AChRs activation by ACh. For instance, nerve impulse conduction blockade by alpha-bungarotoxin, which binds AChRs thereby inhibiting ACh-AChR interactions, attenuated the increase in G_4 levels in rat gracilis muscle subjected to low-intensity treadmill exercise (Fernandez and Hodges-Savola, 1992). Similarly, the transient increase of G_4 levels in short-term denervated muscles is abolished when AChR activation is inhibited by alpha-bungarotoxin treatment (Gregory et al., 1989; Hodges-Savola and Fernandez, 1991). These findings highlight the possible role of ACh-AChR interactions in controlling the synthesis of the G_4 tetramer. The cellular and molecular mechanisms underlying this regulatory pathway however remain to be elucidated.

2. Schwann cell-derived trophic molecules

(a) antigen 6.17

Addition of Schwann cells to nerve-muscle co-cultures has been demonstrated to produce synaptic contacts that display mostly characteristics of mature synapses such as the

disappearance of polyinnervation and the appearance of junctional folds (Koenig et al., 1988; Chapron and Koenig, 1989). A significant portion of the effects of Schwann cells on synapse maturation in vitro has been ascribed to a particular antigen produced by Schwann cells that is recognized by a monoclonal antibody referred to as 6.17. This antigen becomes concentrated at the sites of nerve-muscle contact 2 weeks after birth in rat muscle and is co-localized with AChRs (Koenig et al., 1988). The involvement of antigen 6.17 in synaptic maturation in vitro is further emphasized by the finding that application of the 6.17 antibody attenuates the development of synaptic contacts in the nerve-muscle co-cultures (Chapron and Koenig, 1989). Given the apparent involvement of the Schwann-cell derived antigen in synaptic formation, it was hypothesized that the antigen could also regulate AChE expression at the developing neuromuscular junction.

In a recent study, antigen 6.17 was tested for its potential function in regulating AChE activity in vitro during synapse formation (Chapron et al., 1997). Co-cultures of rat spinal cord cells and rat primary myoblasts display large as well as numerous clusters of AChE activity. Conversely, co-cultures supplemented with Schwann cells, although having fewer AChE patches, possess more highly developed synaptic contacts as evidenced by the appearance of subneural folds. When co-cultures are incubated simultaneously with Schwann cells and the antibody directed against the 6.17 antigen, AChE clusters remain numerous and synaptic contacts resemble those observed in co-cultures alone. Taken together, these findings indicate that antigen 6.17 downregulates AChE expression while it promotes maturation of the neuromuscular junction.

(b) Ciliary neurotrophic factor (CNTF)

CNTF represents an additional Schwann cell-derived trophic molecule that has recently been demonstrated to exhibit myotrophic effects. Specifically, CNTF was shown to attenuate the severity of muscle atrophy in response to hindlimb denervation (Helgren et al, 1994). In addition, subcutaneous injections of CNTF in these animals prevented the decrements in contractile properties that normally result from denervation (Helgren et al., 1994). Given this apparent myoprotective effect of CNTF, it is conceivable that this neuropeptide may also function to prevent the denervation-induced alterations in AChE expression in skeletal muscle.

STATEMENT OF PROBLEM AND OBJECTIVES

Several lines of evidence clearly demonstrate that signals from motoneurons, namely nerve-evoked electrical activity and nerve-derived trophic molecules, play a pivotal role in regulating the patterns of AChE expression in skeletal muscles including the molecular form profiles as well as the spatial distribution of AChE molecules along the muscle fibers. However, our understanding of the cellular and molecular mechanisms mediating the effects of these nerve-derived signals on AChE expression in muscles is still rudimentary. Therefore, the objectives of the present research project are as follows.

- 1. To determine the contribution of innervation versus intrinsic properties of muscle cells in regulating AChE expression in slow- and fast-contracting skeletal muscles (Chapter II and III).**

Mature slow and fast muscles display significant differences in their patterns of AChE expression. Although nerve-evoked electrical activity is regarded as being the primary regulator of AChE expression in slow and fast muscles, other lines of evidence indicate that the distinct AChE profiles displayed by slow and fast muscles is due in part to intrinsic properties of these muscle types. Therefore, the contribution of innervation versus intrinsic features in regulating AChE expression in slow- and fast-contracting muscles will be investigated using cultured myotubes (in vitro) as well as rat muscles (in vivo).

2. To examine the molecular mechanisms regulating the synaptic accumulation of AChE transcripts at the neuromuscular junction of rat skeletal muscle fibers (Chapter IV).

AChE enzyme molecules are enriched within the post-synaptic region of the neuromuscular junction. Synaptic accumulation of transcripts encoding AChE as well as localized translation of the protein within endplate regions of muscle fibers likely account for aggregation of the enzyme within this compartment. However, the contribution of transcriptional versus post-transcriptional mechanisms to localized AChE expression within synaptic regions of muscle fibers remains to be investigated. We will therefore determine whether accumulation of AChE transcripts at endplates involves enhanced transcription of the AChE gene within synaptic myonuclei.

3. To determine the molecular mechanisms mediating the activity-dependent regulation of AChE in rat skeletal muscle (Chapter V).

Nerve-evoked electrical activity plays a pivotal role in regulating AChE expression in skeletal muscles. Although altered neural activation has been shown to control AChE transcript levels in muscle, it is not known whether the impact of neural activity on AChE mRNA abundance is mediated by transcriptional and/or post-transcriptional events. We will therefore determine whether neural-activity modulates AChE gene transcription using the denervation model which most dramatically downregulates AChE transcript levels in muscle.

4. Determine the impact of neurotrophic molecules on AChE expression in skeletal muscle (Chapter VI and VII).

Although several lines of evidence suggest the involvement of nerve-derived trophic molecules in regulating AChE expression in skeletal muscle, knowledge of the precise identity of such molecules as well as the mechanisms by which they regulate AChE expression is lacking. Since the ciliary neurotrophic factor (CNTF) has been previously shown to have myoprotective effects on denervated muscles, we will determine whether CNTF exerts myotrophic effects on AChE expression. Calcitonin-gene-related peptide (CGRP) has also been previously shown to modulate AChE expression in rodent skeletal muscle. However, the molecular events mediating the effects of CGRP remain to be established. We will therefore also determine the impact of CGRP on AChE gene expression in rodent myofibers.

CHAPTER II

Fast and Slow Skeletal Muscles Express a Common Basic Profile of Acetylcholinesterase Molecular Forms

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Dr. R. Michel performed some of the surgeries. Dr. Jasmin contributed to the analysis of AChE molecular forms which was carried out in Dr. Gisiger's laboratory. Mr. D. Hubatsch was involved in culturing satellite cells as well as in the analysis of AChE expression in myotubes.

ABSTRACT

Recent evidence suggests that the high content of acetylcholinesterase (AChE) globular form G_4 characteristic of fast muscles, is controlled by phasic high-frequency activity performed by these muscles. This indicates that inactive, although still innervated fast muscles should be devoid of their characteristic G_4 pool. Accordingly, in the absence of phasic activity both fast and slow muscles should exhibit a common basic profile of AChE molecular forms of the slow type. We first tested this hypothesis by examining the AChE content in cultures of myotubes obtained from the fusion of satellite cells originating from fast and slow muscles. These two cell populations produced AChE molecular form profiles of the slow type characterized by modest levels of G_4 together with an increased proportion of the asymmetric forms A_8 relative to A_{12} . Second, we determined the impact of muscle paralysis on the specific content of AChE molecular forms of adult rat fast and slow muscles. Complete paralysis of hindlimb muscles was achieved by chronic superfusion of tetrodotoxin (TTX) onto the sciatic nerve. Ten days following TTX-inactivation, the distributions of AChE molecular forms of both fast EDL and plantaris muscles were transformed into ones resembling the slow soleus, the latter showing no significant modifications in its AChE profile. Finally, we investigated the impact of nerve-mediated, phasic high-frequency stimulation of TTX-inactivated fast and slow muscles on the content of AChE molecular forms. The stimulation produced a profile of AChE molecular forms similar to that observed in control EDL muscle indicating that phasic activation counteracted the TTX-induced transformation in the distribution of AChE molecular forms in fast EDL muscle. Together, these results are consistent with the proposal that adult fast muscles constitutively express a basic profile of AChE molecular forms of the type displayed by slow muscles, onto which varying levels of G_4 are added according to the amount of phasic activity performed by the muscles.

INTRODUCTION

Acetylcholinesterase (AChE) is a major functional constituent of synapses where it ensures sustained synaptic transmission by hydrolyzing acetylcholine released from nerve terminals. This enzyme displays a marked and complex polymorphism since it exists as a family of molecular forms presenting distinct structural features (reviewed in Taylor, 1991; Massoulié et al., 1993). Briefly, there are 3 globular (monomer G_1 , dimer G_2 and tetramer G_4) and 3 asymmetric forms (A_4 , A_8 and A_{12}) in which 1, 2 or 3 tetramers associate with a collagen-containing tail. These molecular forms also exhibit different solubility properties thus allowing specific AChE molecular forms to be positioned at distinct subcellular locations to fulfill site-specific functions. In skeletal muscle fibers for example, asymmetric AChE forms accumulate at the synaptic basal lamina (Hall, 1973; McMahan et al., 1978) while G_4 tetramers are highly concentrated within the perijunctional compartment (Gisiger and Stephens, 1988). Of special relevance is the marked difference in the distributions of AChE molecular forms expressed by fast- versus slow-contracting muscles. Indeed, whereas fast muscles characteristically exhibit a high G_4 content, slow muscles contain only modest levels of G_4 together with an increased proportion of A_8 relative to A_{12} (Gisiger and Stephens, 1982b; Gisiger and Stephens, 1983). On the other hand, levels of the monomer G_1 and dimer G_2 , 2 molecular forms which are mostly intracellular, are not related to the contractile properties of either fast or slow muscles (Gisiger and Stephens, 1983; Gisiger and Stephens, 1988).

Several recent studies performed in the rat have revealed that the content of AChE

molecular forms is modulated according to the functional demands placed upon muscles. In particular, chronic enhancement of neuromuscular activity achieved by exercise training programs involving daily walking (Fernandez and Donoso, 1988), running (Jasmin and Gisiger, 1990; Gisiger et al., 1994; Sveistrup et al., 1995) and swimming (Gisiger et al., 1991) cause selective increases or decreases in the G_4 content of fast muscles according to whether the activity of the muscles is predominantly phasic or tonic, respectively. These adaptations are very extensive since G_4 increases exceeding 400% (Gisiger et al., 1994; Sveistrup et al., 1995) and decreases by almost half (Jasmin and Gisiger, 1990) have been reported. In addition, it has also been shown that the G_4 content of fast muscles is highly correlated with the amount of phasic activity recently performed (Gisiger et al., 1994) suggesting that G_4 constantly adapts its level to the type and intensity of muscular activity.

In all these studies, the pronounced changes displayed by fast muscles in their content of G_4 were in marked opposition to the lack of adaptation in the other AChE molecular forms and in particular in the synaptic form A_{12} . Taken together with the evidence showing that the G_4 pool is predominantly located around motor endplates (Gisiger and Stephens, 1988), these results strengthened the notion that G_4 and the other molecular forms represent separate compartments of AChE that are subjected to distinct regulatory mechanisms (see Massoulié et al., 1993). The close relationship that exists between G_4 and the amount of phasic high-frequency activity also suggests that inactive, although still innervated fast muscles should be devoid of their characteristic G_4 pool and, therefore, should only contain a minimal amount of tetramer as observed in active slow-twitch muscles. Accordingly, in the absence of phasic activity both fast and slow muscles should exhibit a common basic profile of AChE molecular

forms of the slow type.

In the present study, we tested this hypothesis by using three distinct, yet complementary approaches. First, we determined the distribution of AChE molecular forms intrinsically expressed by cultured myotubes obtained from the fusion of satellite cells originating from fast and slow muscles. Second, we examined the content of AChE molecular forms in paralyzed but still innervated fast and slow muscles. Complete paralysis of hindlimb muscles was induced by chronic superfusion of tetrodotoxin (TTX) onto the sciatic nerve (Michel et al., 1994; Jasmin et al., 1995). As a model of inactivity, TTX-inactivation offers several advantages which include: i) complete reversibility of the drug effects; ii) preservation of the integrity of nerve-muscle contacts; and iii) maintenance of axonal transport (Lavoie et al., 1976). In addition, the morphological changes in TTX-inactivated muscles are limited to reductions in the size of both fibers and motor endplates without concurrent damage (Gardiner et al., 1992; Boudreau and Michel, 1993). This particular paralysis model thus provides a means to examine alterations in the content of AChE molecular forms in mature innervated skeletal muscles that are specifically induced by inactivity. Finally, we determined the impact of daily superimposed nerve-mediated stimulation on the profile of AChE molecular forms in TTX-inactivated fast and slow muscles. To elicit hindlimb muscle contractile activity, we stimulated the sciatic nerve distal to the site of TTX delivery with a pattern of activity mimicking phasic high-frequency activation typically observed in fast contracting skeletal muscles (Hennig and Lomo, 1985).

MATERIALS and METHODS

Animal Care and Surgery

Female Sprague Dawley rats weighing between 180-200 g were obtained from Charles River Laboratories (St-Constant, Québec). Care and treatment of the animals were in accordance with the guidelines presented by the Canadian Council on Animal Care. Surgery was performed under aseptic conditions on animals anesthetized with sodium pentobarbital (35 mg/kg, i.p.).

After one week of acclimation, rats were randomly assigned to one of 4 groups: 1) Control; 2) DEN: Denervated ; 3) TTX: Tetrodotoxin-inactivated; and 4) STTX: TTX-inactivated and stimulated via the sciatic nerve. Left hindlimb muscles of DEN animals were denervated by cutting and removing a 4 mm segment of the sciatic nerve 10 mm distal to the sciatic notch. TTX rats were implanted with a chronic drug delivery system as described in detail elsewhere (Michel et al., 1994; Jasmin et al., 1995). Briefly, a mini-osmotic pump (Alza Corp.; Palo Alto CA) and a silastic tubing-cuff system (Dow Corning Corp.; Midland MI) were loaded with approximately 250 µl of sterile physiological saline containing 350 µg/ml TTX (Sigma; St-Louis MO). The cuff was carefully positioned around the left sciatic nerve distal to the sciatic notch, and sealed with 3.0 surgical silk. The silastic cuff was secured in place by suturing the overlying hamstring musculature with silk. The tubing and pump attachment were led subcutaneously to the thoracic region on the animal's back. The incision was subsequently closed with suture clips and the animal returned to its cage. STTX rats were implanted with a similar TTX delivery system equipped with fine silicone-covered wires

(Biomed wires; Cooner Wire Comp., Chatsworth CA) placed distal to the site of TTX delivery as described earlier (Michel et al., 1996). These fine wires were led to the animal's upper back and secured in place with a plastic mesh sutured subcutaneously such that approximately 2 cm of the distal end of the wires protruded from the closed incision thus allowing for connection to the stimulator.

Efficacy of TTX-inactivation

Animals were checked daily to ensure the completeness of left hindlimb paralysis using as indicators of recovery: i) toe-spreading in response to hindlimb unweighting; ii) the flexor reflex in response to pinching of the foot pad; and iii) plantar flexion in response to forced dorsi-flexion of the ankle. Similarly, the efficacy of the TTX-inactivation was verified before removing the hindlimb muscles by stimulating the sciatic nerve proximal to the TTX cuff with a platinum bipolar electrode and monitoring the functional response of the triceps surae muscle group as previously described (Michel et al., 1994; Jasmin et al., 1995). TTX-inactivated animals included in this study did not demonstrate any of these responses. Moreover, evidence of total inactivity was obtained in an earlier study by *in vivo* assessment of EMG activity of TTX-treated muscles (Spector, 1985). In these muscles, there was continued absence of toe extension reflex and EMG activity for up to 21 days of TTX-inactivation. Finally, in the present study, loss in muscle mass due to TTX-inactivation (EDL 32 %; PL 40 %; SOL 35 %) was identical to that induced by cutting the sciatic nerve (EDL 30 %; PL 38 %; SOL 32 %) in all muscles studied indicating that the entire muscle fiber population had been successfully inactivated.

In a distinct set of experiments, the possibility that partial or total denervation may have occurred as a result of positioning the silastic cuff around the sciatic nerve was also investigated. This was done by implanting a silastic-tubing cuff system around the sciatic nerve and determining the AChE content 10 days later. In these sham-operated animals (n = 3), both soleus and extensor digitorum longus (EDL) muscles displayed peaks of A_{12} and A_8 similar to those observed in control untreated muscles (data not shown), thereby eliminating the possibility that partial or total denervation had occurred as a result of this surgical procedure, more specifically possible injury to the sciatic nerve due to placement of the cuff.

Nerve-Mediated Stimulation of TTX-Inactivated Hindlimb Muscles

Starting the day after cuff implantation and confirmation of left hindlimb paralysis, STTX rats were anesthetized using a mixture of Ketamine (100 mg/ml, Rogar/STB Inc.; London, Ontario) - Rompun (20 mg/ml, Bayvet; Etobicoke, Ontario) in a ratio of 1.6 to 1, respectively (0.09 ml/100g body weight, i.m.). Hindlimb muscles were then positioned such that the ankle and knee joints were secured at right angles. Isometric contractions of hindlimb muscles were elicited via electrical stimulation of the inactivated sciatic nerve at supramaximal voltage (5 - 8 Volts) using a pattern that mimics phasic high-frequency activation of fast contracting muscles (18), i.e. 100 Hz bursts, 1 sec duration every 2 min, 60 min daily, for 7 days starting 24 hr after initiation of TTX-inactivation. We have recently used this painless stimulation procedure to study the effects of nerve-mediated stimulation on expression of succinate dehydrogenase in various compartments of TTX-inactivated hindlimb muscle fibers (Michel et al., 1996).

Cultures of Satellite Cells

Young female Sprague Dawley rats (~50 - 75 g) were anesthetized with sodium pentobarbital (35 mg/kg, i.p.) and the slow soleus and fast tibialis anterior muscles from both hindlimbs were excised quickly under aseptic conditions. Muscles from 6 to 10 animals were typically pooled together for these experiments and they were kept in cold phosphate-buffered saline (PBS) until surgery on all animals was completed. Soleus and tibialis anterior muscles were minced in 8 volumes of minimum essential medium (MEM) containing 15% donor bovine serum (DBS, Cansera; Toronto, Ontario), 1% Penicillin/Streptomycin (Gibco; Burlington, Ontario), 0.1 % fungizone (Gibco) and Collagenase (1.79 mg/g tissue, Sigma) until fragments of approximately 2 mm were obtained. The mixture was then incubated at 37°C for 1.75 hr and subsequently spun at 250 X g for three minutes. The supernatant was discarded and the soft pellet resuspended in MEM (as above) with the exception that dispase (20 mg/g tissue, Boehringer Mannheim; Laval, Quebec) was added instead of collagenase. This mixture was then incubated at 37°C for 45 min. Digested muscle fibers were filtered through a 53 mm nylon filter and the filtrate spun for 10 min at 250 x g. The pellet was resuspended in MEM (with DBS and antibiotics) and satellite cells were plated at a density of 700 cells/mm² on 60 mm dishes coated with Matrigel (Collaborative Biomedical Products; Bedford MA). Cells were kept at 37°C in a water-saturated atmosphere containing 5% CO₂. The medium was changed every other day until cells reached confluency. At this stage, DBS was reduced to 2% to promote differentiation of myoblasts into myotubes. Cells were used for AChE analysis 8 - 10 days after plating.

Extraction of AChE

Following 5 to 10 days of inactivity, rats from the various experimental groups along with their respective controls were anaesthetized. The left soleus and extensor digitorum longus (EDL) as well as plantaris muscles (in the case of 10 day denervation and TTX-inactivation experiments) were rapidly excised, weighed and immediately frozen in liquid nitrogen. They were stored at -80°C until further analysis. Whole frozen muscles were subsequently homogenized in 2.5 ml of a high-salt detergent buffer containing anti-proteolytic agents: 10 mM Tris-HCl, pH 7.0; 10 mM EDTA; 1 M NaCl; 1% Triton X-100; 1 mg/ml bacitracin (Sigma); 25 U/ml aprotinin (Sigma). Homogenization was performed on ice for 2 X 15 sec with a Polytron set at 6. The homogenates were centrifuged (20,000 x g) at 4°C for 15 min and the resulting supernatants kept at -80°C until further analysis.

Cultures of myotubes obtained from the fusion of satellite cells from fast and slow muscles were washed with cold PBS, scraped and homogenized with 200 µl of high-salt detergent buffer per dish (see above).

AChE Enzyme Assay and Velocity Sedimentation Analysis

AChE activity was measured using a modified version of the spectrophotometric method of Ellman et al. (1961) as described previously (Gisiger and Stephens, 1983; Jasmin and Gisiger, 1990). Fifty µl aliquots were incubated in 1 ml of a phosphate buffer solution (pH 7.0) containing 7.5×10^{-4} M acetylthiocholine (Sigma) as the substrate, 5×10^{-4} M dithiobisnitrobenzoic acid (DTNB; Sigma) and 10^{-5} M of the non-specific cholinesterase inhibitor tetraisopropylpyrophosphoramidate (iso-OMPA; Sigma).

Velocity sedimentation analysis of AChE molecular forms was performed as previously described (Gisiger and Stephens, 1983; Jasmin and Gisiger, 1990). For these experiments, 50 - 100 μ l aliquots of the muscle and culture extracts were layered onto 5 - 20% sucrose gradients. Samples were centrifuged in a Beckman SW41 rotor at 281 000 x g for 19 h at 4°C. Approximately 45 fractions were collected from the bottom of the tubes and assayed for AChE activity. Analysis of AChE molecular forms and processing of the raw data were performed as described in detail elsewhere (Gisiger and Stephens, 1983; Jasmin and Gisiger, 1990). The activity of each molecular form, except that of A₄, which represents less than 5% of AChE activity, was calculated from the relative proportion of each molecular form, (as determined by sedimentation analysis) and the total AChE activity per muscle.

Statistical Analysis

Student's t-tests were used to determine whether significant differences existed in the relative content of AChE molecular forms between myotubes obtained from the fusion of satellite cells originating from slow soleus and fast tibialis anterior muscles and to assess the impact of stimulation of TTX-inactivated muscles on the content of AChE molecular forms. A one-way analysis of variance (ANOVA) was performed to evaluate the effect of denervation and TTX on muscle mass, total AChE activity and molecular forms in soleus, EDL and plantaris muscles. Scheffé post-hoc tests were used to locate significant differences between group means. The level of significance was set at $P < 0.05$.

RESULTS

AChE Molecular Forms in Cultures of Satellite Cells from Fast and Slow Muscles

We examined the content of AChE molecular forms in cultures of myotubes derived from the fusion of satellite cells originating from the fast tibialis anterior (TA) and slow soleus muscles. In particular, we determined whether these cultures exhibit the same characteristic differences in AChE content as those displayed in functional fast and slow muscles of adult rats (for example see Figure 2.3, control muscles; and for review, Massoulié et al., 1993). Sedimentation analysis revealed that the myotubes obtained from satellite cells originating from these two muscles produced similar AChE molecular form profiles which were clearly of the slow type (Figure 2.1). Indeed, myotubes from both fast TA and slow soleus satellite cells consistently showed a modest peak of G_4 together with a relatively high content of the asymmetric form A_8 in comparison to A_{12} (Figure 2.1; Table 2.1).

Effects of Muscle Paralysis

In another set of experiments, we examined the impact of 10 days of TTX-inactivation in 3 rat hindlimb muscles (slow soleus, fast EDL and plantaris) and compared the effects to those resulting from 10 days of denervation. We focused on this particular time-point to avoid the transient G_4 increase known to occur within the first 48 hr in short-term inactivated muscles (Gregory et al., 1989). As indicated earlier, (Michel et al., 1994; see also Materials and Methods), loss in muscle mass following TTX-inactivation and denervation was similar ($P > 0.05$; data not shown).

Figure 2.1 Profiles of AChE molecular forms observed in myotubes obtained from the fusion of satellite cells originating from slow soleus (A) and fast tibialis anterior (B) muscles. Shown are representative examples of the relative content of the various AChE molecular forms in these cells, 8-10 days after plating.

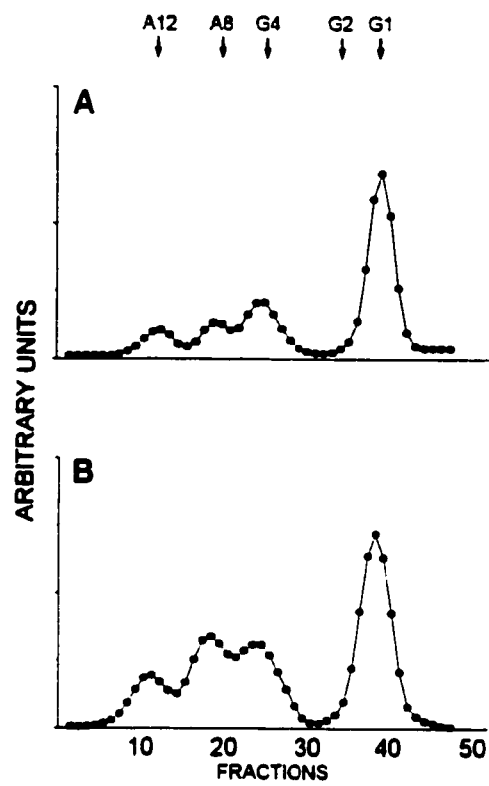


Table 2.1 Relative content in AChE molecular forms in cultured myotubes obtained from the fusion of satellite cells originating from slow soleus (SOL) and fast tibialis anterior (TA) muscles.

	A ₁₂	A ₈	G ₄	G ₁
SOL	9 ± 1	11 ± 4	30 ± 7	50 ± 4
TA	17 ± 8	20 ± 1 *	24 ± 11	39 ± 5 *

Values represent the relative content of each of the four AChE molecular forms present in these cultured cells. Data are expressed as a percentage of total activity. Means ± SD are shown. n = 3; * denote significant differences, P < 0.05.

Total AChE Activity. Following 10 days of chronic superfusion of TTX onto the sciatic nerve, AChE activity was reduced considerably in all 3 muscles with the effect being more pronounced in EDL muscle (Figure 2.2; see also Michel et al., 1994). The effect of TTX-inactivation was indeed severe in the latter muscle since AChE activity was ~ 25% of that seen in control EDL muscle. Plantaris and soleus muscles displayed AChE activity that were 37% and 48% of that measured in their respective controls. In contrast to the changes observed with muscle mass however, these AChE reductions were significantly ($P < 0.05$) less extensive than those observed in denervated EDL and plantaris muscles. Indeed, 10 days following sectioning of the sciatic nerve, AChE activity was reduced to ~ 10% of control in EDL muscle, and to 21% and 34% of control in plantaris and soleus muscles, respectively (Figure 2.2).

Effects of TTX-Inactivation on AChE Molecular Forms in Fast Muscles. The pronounced reduction in AChE activity induced by 10 days of chronic superfusion of TTX onto the sciatic nerve was accompanied by marked changes in the distribution of AChE molecular forms of fast muscles (Figure 2.3; note differences in Y-axis scales between different panels). Levels of G_4 were considerably reduced in TTX-inactivated EDL and plantaris muscles whereas the peak of A_8 relative to A_{12} appeared higher compared to controls (Figure 2.4). The net result of these adaptations in AChE molecular forms was that both fast contracting EDL and plantaris muscles essentially presented profiles typical of control slow-twitch soleus muscle (Figure 2.3). Additional experiments with EDL muscle revealed that the inactivity-induced transition in the profile of AChE molecular forms of fast muscles was already evident

Figure 2.2 Total AChE activity in soleus (SOL), extensor digitorum longus (EDL) and plantaris (PL) muscles after complete hindlimb muscle paralysis. AChE enzyme activity was measured in each of these muscles following 10 days of inactivity induced either by chronic superfusion of TTX onto the sciatic nerve (closed bar) or denervation (hatched bar). Values are means $\pm$ SD expressed as a percent of control values. n = 3 to 4 per data point.

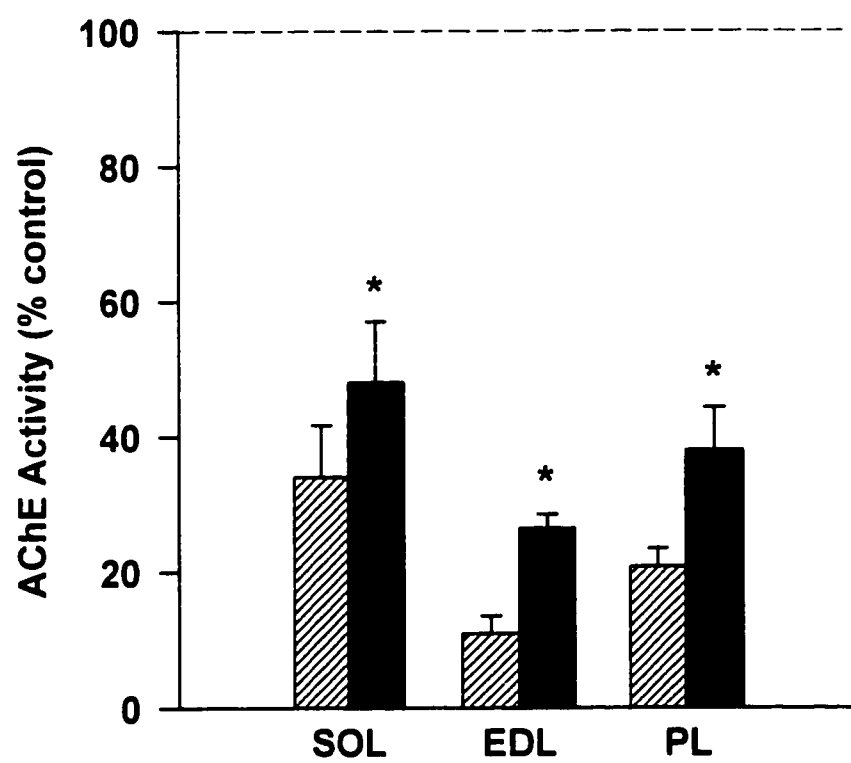


Figure 2.3 AChE molecular form content observed in control (CTL), denervated (DEN) and TTX-inactivated (TTX) rat hindlimb muscles. Shown are representative examples of the average content in AChE molecular forms in slow-twitch soleus (SOL) as well as in fast-twitch EDL and plantaris (PL) muscles. The distributions of the molecular forms are expressed as activity per muscle and were computed from the overall AChE activity per muscle and the sedimentation profiles. Note differences in Y-axis scales.

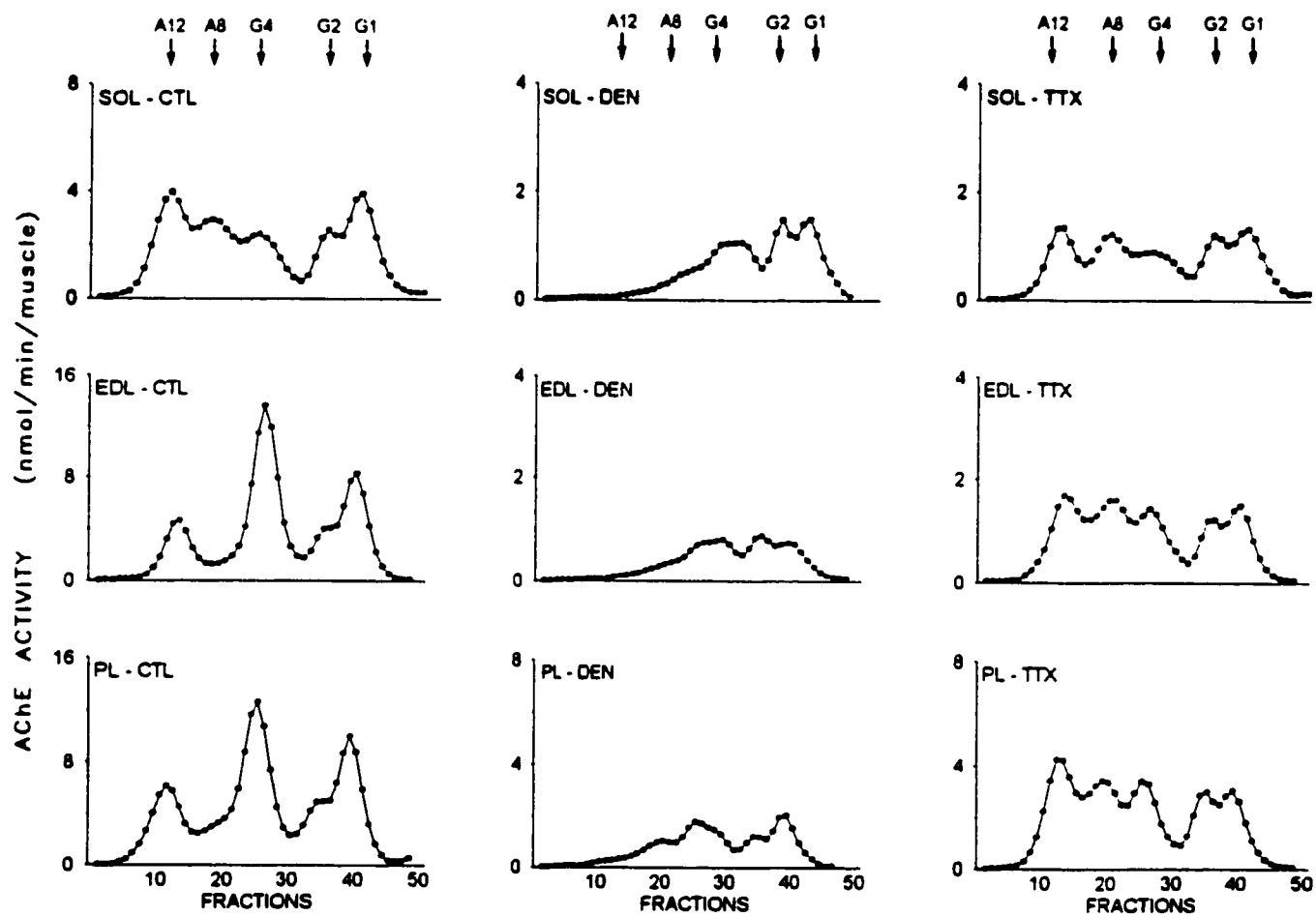
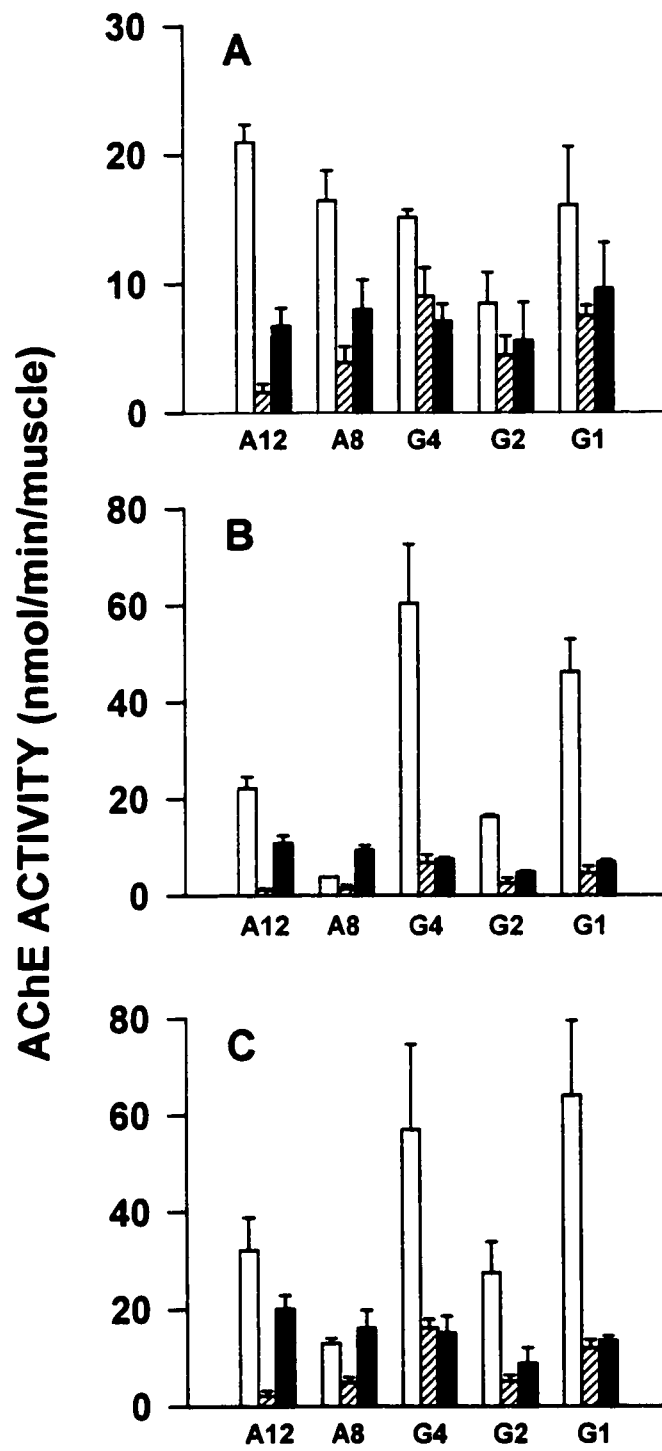


Figure 2.4 Activity per muscle of the various AChE molecular forms in control (CTL; n = 3; open bar), denervated (DEN; n = 3; hatched bar) and TTX-inactivated (TTX; n = 4; closed bar) slow-twitch soleus (A) as well as fast-twitch EDL (B) and plantaris (C) muscles. The activity of each AChE molecular form per muscle was calculated from the total AChE activity and the relative proportions of the molecular forms obtained from the sedimentation analyses. Means $\pm$ SD are presented.



following 5 days of TTX-inactivation and that it was complete after 7 - 8 days of inactivity (data not shown).

Effects of TTX-Inactivation on AChE Molecular Forms in Slow Muscles. In contrast to the marked changes seen in fast muscles, the profile of AChE molecular forms in slow soleus muscle paralyzed for 10 days was essentially unaffected when compared to controls; all molecular forms were reduced by approximately 50% (Figure 2.4). Thus, the sedimentation profile of the inactivated slow muscle displayed significant peaks of A_{12} and A_8 as well as moderate levels of G_4 , as observed in control soleus muscle (Figure 2.3). The decrease in the activity of the different AChE molecular forms following TTX-inactivation developed progressively over the 10 day period. After 5 days of TTX superfusion onto the sciatic nerve, the amount of AChE molecular forms was reduced by approximately 25% (data not shown). As observed for EDL muscle, the content in AChE molecular forms of soleus muscle after 7 - 8 days of inactivity was similar to that seen after 10 days of TTX-inactivation.

Effects of Denervation on AChE Molecular Forms. In our experiments, we used the denervation model strictly to stress potential differences between the effects of TTX-inactivation and denervation on muscle AChE content. Ten days after sectioning of the sciatic nerve, the profiles of AChE molecular forms in soleus, EDL and plantaris muscles were also altered but in a manner clearly distinct from that produced following TTX-inactivation (Figure 2.3). In particular, levels of the asymmetric form A_{12} were severely decreased in all muscles studied (Figure 2.4). In denervated EDL muscle for example, A_{12} activity was reduced by

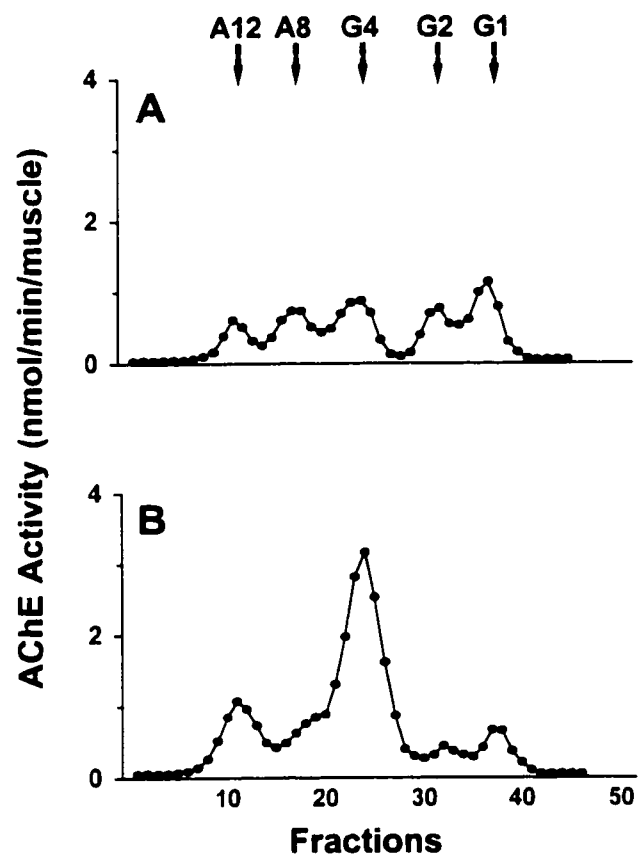
more than 95% in comparison to control muscles (1.08 ± 0.30 vs 22.20 ± 2.46 nmol/min/muscle; mean $\pm$ SD; $n = 3$). The effects of denervation on the other AChE molecular forms (for example G_4) were less pronounced thereby indicating a preferential impact of denervation on the asymmetric form A_{12} (Figure 2.3 and 2.4). The marked decrease in total AChE enzyme activity and the pronounced reduction in the levels of A_{12} that we observed following denervation correspond well with other published reports (for review see Massoulié et al., 1993).

Nerve-Mediated Stimulation of TTX-Inactivated Hindlimb Muscles

In a final set of experiments, we investigated the effects of nerve-mediated activation of TTX-inactivated fast EDL and slow soleus muscles on the content of AChE molecular forms. For these studies, we stimulated the sciatic nerve distal to the site of TTX delivery with a pattern mimicking phasic high-frequency activation typical of fast-contracting muscles (100 Hz bursts, 1 s duration every 2 min for 1 hour per day; see Hennig, Lomo, 1985). Since the transformation in the distribution of AChE molecular forms of fast muscles is complete following 7 - 8 days of TTX-inactivation, we focused on this particular time point to determine the impact of superimposed nerve-mediated phasic high-frequency activity.

AChE Molecular Form Profiles in Stimulated TTX-Inactivated Fast Muscles. Total AChE activity in stimulated TTX-inactivated (STTX) EDL muscle was slightly higher (11%) in comparison to TTX-inactivated muscles. In contrast, the stimulation paradigm markedly affected expression of G_4 and to a lesser extent, that of A_8 (Figure 2.5). In comparison to

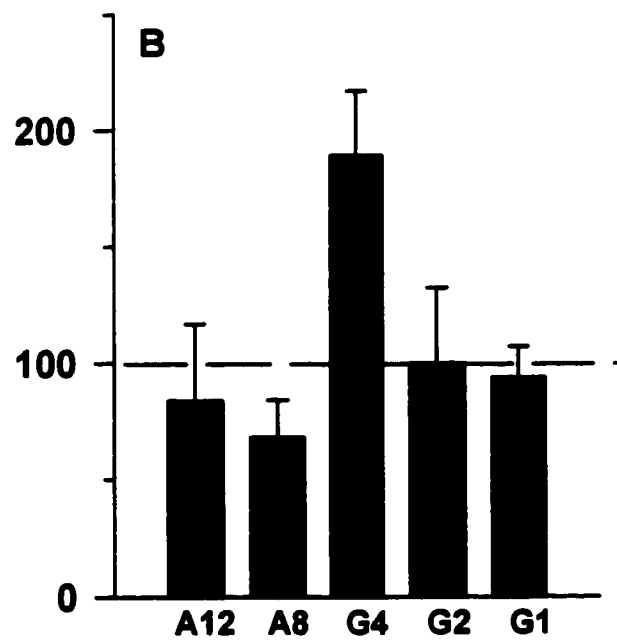
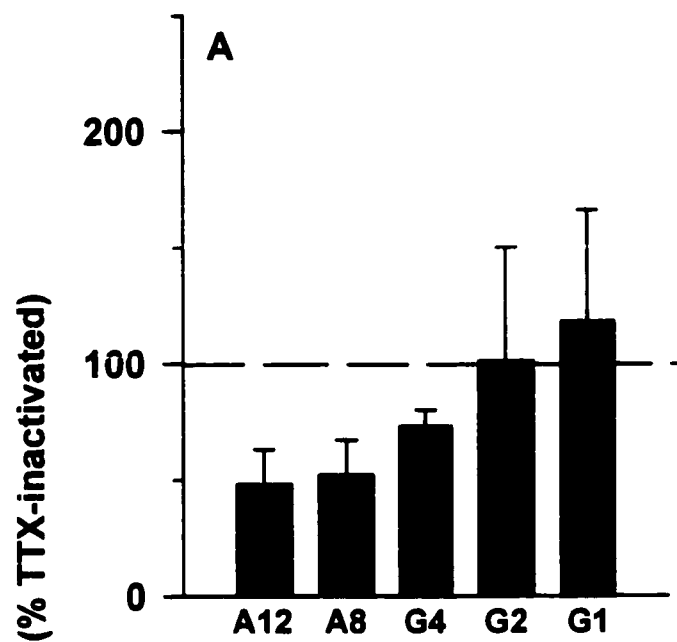
Figure 2.5 AChE molecular form content in TTX-inactivated soleus (A) and EDL (B) muscles stimulated for 7 days with a nerve-mediated phasic high-frequency activity pattern (STTX) (See Materials and Methods). Shown are representative examples of the average content in AChE molecular forms in SOL and EDL muscles obtained from the same animal. The molecular form profiles are expressed as activity per muscle and were computed from the overall AChE activity per muscle and the sedimentation profiles.



TTX-inactivated EDL muscles, levels of G_4 in STTX EDL muscles were 89% higher ($P < 0.05$) whereas the amount of A_8 was reduced by 32% ($P < 0.05$; Figure 2.6). As a result, STTX EDL muscles showed a high content of G_4 together with a modest peak of A_8 so that the A_8/A_{12} ratio resembled that typically observed in control fast muscles. Therefore, 1 hr of nerve-mediated phasic activation per day was sufficient to counteract the transformation in the AChE molecular form profile seen in TTX-inactivated EDL muscles (compare Figures 2.3 and 2.5). Hence, our stimulation protocol was able to maintain an AChE profile typically observed in EDL muscle of control (see Figure 2.3) and moderately exercised animals (Gisiger et al., 1994).

AChE Molecular Form Profiles in Stimulated TTX-Inactivated Slow Muscles. Stimulated TTX-inactivated soleus muscle displayed a 23% decrease in total AChE activity in comparison to their TTX-inactivated counterparts. As observed for EDL muscle, the distribution of AChE molecular forms was also significantly altered in STTX soleus muscle (Figure 2.5). In this case however, nerve-mediated phasic activation of TTX-inactivated soleus muscle led to a pronounced and selective reduction in the content of asymmetric forms which were further diminished by approximately 50% ($P < 0.05$) (Figure 2.6). In striking contrast to the marked effect of phasic activation on the content of G_4 in fast muscle, superimposed high frequency stimulation failed to affect expression of G_4 in TTX-inactivated soleus muscle (Figure 2.6).

Figure 2.6 Activity per muscle of AChE molecular forms in stimulated (STTX) soleus (A) and EDL (B) muscles. Values are means $\pm$ SD expressed as a percent of TTX values. n = 3 to 4 per data point.



DISCUSSION

We examined whether fast muscles contain two distinct compartments of AChE subjected to separate regulations, namely, a basic profile of AChE molecular forms common to fast and slow muscles, and an additional pool of G_4 which exists in fast muscles only as far as they exhibit significant activity of the phasic type. The complementary experimental approaches used in the present study provided strong evidence supporting our hypothesis.

Basic Profile of AChE Molecular Forms Common to Fast and Slow Muscles

After blockade of action potential propagation with TTX, AChE molecular form profiles of both predominantly fast EDL and plantaris muscles were essentially transformed into ones resembling the AChE content typical of rodent slow-twitch soleus muscle as defined earlier (Gisiger and Stephens, 1982a; Gisiger and Stephens, 1983). The transformation of AChE profiles in fast muscles resulted mainly from a combination of two changes: a preferential reduction in the G_4 content accompanied by a general decrease in all molecular forms except A_8 . Conversely, under these conditions, the AChE molecular form profile of soleus muscle showed no significant modification since all molecular forms showed a similar ~50% decrease. Chronic superfusion of TTX onto the sciatic nerve has the distinct advantages of silencing motor nerves while maintaining the integrity of nerve-muscle contacts as well as the transport of various substances along axons (Lavoie et al., 1976). Thus, in contrast to the alterations seen in several murine neuromuscular diseases (Skau and Brimijoin, 1981; Gisiger and Stephens, 1983; Lindenbaum and Livett, 1983; Rieger et al., 1983; Yeakley

et al., 1987; Oliver et al., 1992), the AChE changes displayed by fast muscles in response to TTX-inactivation can be ascribed to inactivity per se. Interestingly, the AChE profiles obtained recently by Sketelj and colleagues (Sketelj et al., 1993) from EDL muscle paralyzed by botulinum toxin A, which blocks the quantal release of acetylcholine from nerve terminals, appeared to show a similar transformation towards the slow type, strengthening the view of a causal relationship between inactivity and the expression of a basic AChE profile of the slow type in fast muscles. In the case of denervation however, the peculiar and marked reduction in asymmetric forms has been related to proteolysis developing at the denervated endplate (Fernandez and Duell, 1980). As for the cause of the general AChE decrease accompanying paralysis, there are indications that atrophy of the neuromuscular synapses is an important factor. Indeed, we have observed that, in addition to lowering AChE activity, TTX-inactivation also significantly reduces the size of endplates (Boudreau and Michel, 1993). Conversely, compensatory hypertrophy leads simultaneously to both a general increase in all AChE molecular forms (Jasmin et al., 1991; Sveistrup et al., 1995) and enlargements of the neuromuscular junctions (Granbacher, 1971). This suggests that the size of endplates represents one parameter affecting the expression of the basic AChE profile.

The results yielded by the culture of satellite cells obtained from fast and slow muscles extend our concept of a basic AChE content by suggesting that this AChE profile constitutes an intrinsic property of skeletal muscle cells expressed constitutively and independent of their anatomical and functional state as well as their contractile properties. Indeed, a similar type of AChE molecular form profile was expressed by myotubes obtained from the fusion of satellite cells from fast and slow muscles. Both populations of cultured cells showed modest

levels of G_4 with a relatively high content of A_8 typical of slow contracting muscles. Our results however, are at variance with data recently obtained from regeneration and cross-transplantation experiments according to which satellite cells of fast and slow muscles express distinct AChE profiles (Sketelj and Brzin, 1991; Dolenc et al., 1994). Such a discrepancy is not too surprising considering the complexity of the regeneration and transplantation models which in contrast to cultures of satellite cells, involve an intricate sequence of events, particularly reinsertion and reinnervation of the regenerating muscle fibers. Therefore, our results obtained with: i) an *in vitro* system involving cultures of satellite cells, coupled with ii) an *in vivo* model which eliminates neuromuscular activity while preserving the integrity of nerve-muscle contacts, support the proposal that fast and slow muscles express a common basic profile of AChE molecular forms of the slow type.

Dependence of the G_4 Pool of Fast Muscles on Phasic Activity

Preservation of the integrity of nerve-muscle contacts provided by the TTX-inactivity model allowed us to stimulate hindlimb muscles trans-synaptically in order to test another aspect of our hypothesis, namely that phasic high-frequency activation regulates expression of the additional pool of G_4 in fast muscles. To this end, we superimposed nerve-mediated phasic activation on TTX-inactivated EDL muscle using a pattern mimicking the daily activity of this muscle in sedentary rats (Hennig and Lomo, 1985). As a result of this stimulation protocol, EDL muscle exhibited an AChE profile characterized by a high G_4 peak as observed in fast muscles of control (see Figure 2 in Jasmin and Gisiger, 1990) and moderately exercised animals (see Figure 5 in Gisiger et al., 1994). However, 1 hr per day of stimulation did not

maintain the level of total AChE activity displayed by control muscles. In addition, activation of TTX-inactivated EDL muscle induced a reduction in the content of asymmetric forms as compared to TTX-inactivated muscles thereby suggesting that maintenance of high G_4 levels may have occurred at the expense of the asymmetric forms. This indicates that the mechanisms involved in this regulation may operate at the level of assembly of G_4 and asymmetric forms.

Stimulation of TTX-inactivated soleus muscle on the other hand led to a selective decrease in the content of asymmetric forms as compared to TTX-inactivated muscle without affecting the G_4 content. Interestingly, these adaptations in AChE molecular forms reproduced those exhibited by soleus muscles in response to voluntary wheel running (Gisiger et al., 1994; Sveistrup et al., 1995). Taken together, these results obtained with two different experimental systems emphasize the fact that fast and slow muscles respond differentially to enhanced phasic neuromuscular activation. Although the exact mechanism underlying the adaptive changes in AChE molecular forms in fast and slow muscles subjected to phasic activation is still elusive, the divergent responses observed under these conditions suggest that signalling events linking membrane depolarization to changes in specific AChE molecular forms may differ in fast versus slow muscles.

In our experiments, the AChE content of soleus muscle activated by a phasic pattern of stimulation did not exhibit a shift toward a fast type profile as reported by Lomo et al. (1985) in denervated soleus muscle subjected to chronic direct phasic stimulation. The discrepancy may be reconciled if one considers the significant dissimilarities in the models of inactivation and stimulation used in the two studies. The difference bearing the most

significance is most likely the use of denervated muscles versus our use of TTX-inactivated muscles. While both models of inactivity induce muscle paralysis, the TTX model ensures synaptic integrity which is important given that G_4 adaptations depend on synaptic events (Hodges-Savola and Fernandez, 1991). Alternatively, the continuous high-frequency stimulation protocol used by Lomo et al. (1985) may have led to a general adaptation in the soleus muscle conferring it fast contractile properties. Such dramatic transformation in phenotype could improve the ability of soleus muscles to respond and adapt to high frequency stimuli by displaying for example an increase in G_4 levels. These facts emphasize the important role of slow contractile properties in the lack of G_4 adaptation observed in innervated soleus muscle subjected enhanced activity.

Dual Compartments of AChE in Fast Muscles

The present study provides significant evidence indicating that in mature fast muscles, AChE is present as two separate compartments. One, representing the basic content of AChE, is concentrated at the neuromuscular junction where it appears to be expressed constitutively in an amount depending primarily, though not exclusively, on the state of innervation of the muscle including the size of the endplates. The second, which constitutes the G_4 pool present in fast muscles subjected to activity of the phasic type, is positioned around the endplates within the perijunctional area as previously shown (Gisiger and Stephens, 1988; see also Massoulié et al., 1993). This additional pool of G_4 may be expressed intermittently according to the functional demands placed upon fast muscles.

The compartment containing the basic AChE profile is the main cholinesterase component of the G_4 -poor muscles such as slow muscles as well as those fast muscles exhibiting predominantly a tonic pattern of neuromuscular activity (Jasmin and Gisiger, 1990; Jasmin et al., 1991; Sveistrup et al., 1995). Since these muscles are capable of performing sustained levels of activity, the basic compartment thus appears to fulfill by itself the classic role ascribed to muscle AChE, namely, the rapid hydrolysis of acetylcholine to maintain neuromuscular transmission. The constitutive mode of expression suggested by our results for this basic junctional AChE compartment is consistent with the concept established previously (Salpeter and Eldefrawi, 1973; Rosenberry, 1979; Anglister et al., 1994) that junctional AChE is present in an amount exceeding what is required for normal functioning of the neuromuscular synapse. Therefore, in contrast to the G_4 pool, the basic AChE content does not have to adapt to variations in the level of activity superimposed upon the muscle.

In turn, the additional G_4 pool present only in fast muscles subjected to phasic high-frequency activity does not significantly contribute to the classical role of AChE. Therefore, one has to postulate that this G_4 pool fulfills a peculiar function clearly distinct from that of junctional AChE. Although this role still remains elusive, it would nonetheless appear essential given the very large G_4 adaptations which have been reported in fast muscles subjected to high levels of phasic activity (Gisiger et al., 1994; Sveistrup et al., 1995). In a recent model, we have proposed that the G_4 pool contributes to the maintenance of muscle excitability by maintaining low acetylcholine background concentrations during activities involving high-frequency trans-synaptic activation thus preventing receptor desensitization (Jasmin and Gisiger, 1990; Gisiger et al., 1991). In this context, it is interesting to note that

in the central nervous system, G_4 also seems to play peculiar non-classical roles such as in neuromodulation and neuronal signalling (see for example Greenfield, 1991; Greenfield, 1995).

CHAPTER III

Myotubes Originating From Single Fast and Slow Satellite Cells Display Similar Patterns of Acetylcholinesterase Expression

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Manuscript written by C. Boudreau-Larivière and Dr. B. Jasmin

Isolation of single satellite cells from slow and fast muscle fibers was carried out by Dr. D. Parry.

ABSTRACT

Slow- and fast-contracting skeletal muscles of both rats and mice display significant differences in their patterns of acetylcholinesterase (AChE) expression. Although neural influences account for a large proportion of these differences, intrinsic variations between fast and slow myogenic precursor cells have been implicated. In the present study, we have capitalized on the use of Immorto transgenic mice to obtain single myogenic precursor cells isolated from either slow or fast muscle fibers and determined whether these cells generated myotubes that produced distinct patterns of AChE expression as observed in vivo between slow and fast muscles. These two myotube populations displayed similar cell-associated and secreted AChE enzyme activity as well as comparable levels of AChE transcripts. Both myotube populations also express nearly identical molecular form profiles. By contrast, AChE activity and transcript levels were ~2- and 5-fold greater in fast skeletal muscles as compared to slow ones. Together, these findings indicate that differences in AChE expression between fast and slow muscles are not due to inherent differences in myogenic precursor cells, thereby suggesting that other factors, such as innervation, play a predominant role in establishing the distinct patterns of AChE expression in these muscle types.

INTRODUCTION

Acetylcholinesterase (AChE) is a key constituent of central and peripheral cholinergic synapses where it hydrolyzes acetylcholine released by nerve terminals thereby ensuring efficient neurotransmission. The enzyme exists as a family of several molecular forms expressed in a variety of tissues at specific subcellular compartments (for example see Massoulié et al., 1993). Specifically, homomeric forms include globular monomers (G_1), dimers (G_2) and tetramers (G_4). Heteromeric forms, on the other hand, encompass asymmetric forms composed of either one (A_4) two (A_8) or three (A_{12}) tetramers associated with a collagenic structural subunit (Krejci et al., 1997) as well as a membrane-linked G_4 tetramer attached to a hydrophobic anchor (Fernandez and Hodges-Savola, 1996). In skeletal muscle, the asymmetric forms accumulate within the synaptic basal lamina of the neuromuscular junction (McMahan et al., 1978), whereas tetramers are concentrated within the perijunctional region of nerve-muscle contacts (Gisiger and Stephens, 1988).

Several studies have shown that the pattern of AChE expression differs significantly between slow- and fast-contracting muscles. For instance, total AChE activity is greater in fast versus slow muscles of rat and mouse (Gisiger and Stephens, 1983; Groswald and Dettbarn, 1983). Analysis of the molecular form distributions in fast and slow muscles also revealed that the relative content of G_4 is considerably higher in fast muscles whereas the levels of asymmetric forms are proportionately greater in slow muscles (Gisiger and Stephens, 1983; Groswald and Dettbarn, 1983). Recent studies further demonstrated that the levels of

transcripts encoding AChE are higher in fast- versus slow-contracting muscles (Cresnar et al., 1994; Michel et al., 1994; Sketelj et al., 1998).

Although neural activity is recognized as being responsible for a large proportion of these differences in AChE expression between mature fast and slow muscles (see for instance Jasmin and Gisiger, 1990 and refs therein), intrinsic properties of the two muscle types appears to also contribute to these variations. For example, soleus (SOL) and extensor digitorum longus (EDL) muscles from post-natal rats, already display prominent differences in their molecular form profiles (Sketelj et al., 1991) despite receiving, at this developmental stage, similar impulse patterns delivered by their respective innervating motoneurons (Navarrete and Vrbova, 1983). Furthermore, when slow and fast rat hindlimb muscles are forced to undergo regeneration in situ in the presence or absence of innervation or at a different functional site by transplantation, regenerated muscles display AChE molecular form profiles resembling those observed in the original slow or fast muscle (Sketelj et al., 1991; Dolenc et al., 1994). These latter data have been interpreted to suggest that myogenic precursors or satellite cells, which undergo proliferation and differentiation during the process of muscle regeneration (reviewed in Schultz and McCormick, 1994), are intrinsically programmed to express either slow or fast AChE profiles independent of the presence of the motor nerve (Sketelj et al., 1991; Dolenc et al., 1994).

In the present study, we further examined the contribution of intrinsic factors in determining the patterns of AChE expression in fast versus slow muscles of small rodents. Specifically, we assessed the profiles of AChE expression in myotubes derived from single myogenic satellite cell clones isolated from either slow or fast muscle fibers excised from H-

2K^b-tsA58 (Immorto) transgenic mice. These transgenic mice harbor a temperature sensitive SV40 large-T-antigen gene (tsA58) controlled by the activity of an interferon-gamma (IFN- γ) sensitive promoter (H-2K^b) which elicits indefinite growth of the cells under permissive conditions (Jat et al., 1991). Under non-permissive conditions, these cells are capable of undergoing normal myogenic differentiation (Morgan et al., 1994). Accordingly, we took advantage of these mice to obtain pure populations of myotubes derived from a single myogenic cell isolated from either a slow or fast fiber. The pattern of AChE expression from these two myotube populations was therefore determined and compared to the profiles displayed by slow and skeletal fast muscles.

MATERIALS and METHODS

Animal Care and Surgery. Female Sprague-Dawley rats weighing ~ 250 g were obtained from Charles River Laboratories (St-Constant, Québec). Rats were euthanized using CO₂ and the EDL and SOL muscles were excised, frozen in liquid nitrogen and stored at -80°C until further analysis. Immorto mice (H-2K^b-tsA58 transgenic mice; see Jat et al., 1991) were originally obtained from Charles River Laboratories, and they were subsequently bred in the University of Ottawa Animal Care Facility. Transgenic mice were euthanized by barbiturate overdose and the superficial portion of the tibialis anterior (TAS) muscle and the entire SOL muscle which contain fast and slow muscle fibers respectively (Zardini and Parry, 1994), were carefully excised to minimize muscle fiber damage. Care and treatment of the animals were in accordance with the guidelines established by the Canadian Council on Animal Care.

Clonal Myogenic Cell Cultures. TAS and SOL muscles from 6 to 8 week-old Immorto mice were rinsed briefly in sterile phosphate-buffered saline (PBS) and digested at 35°C for 1.5 to 2 hours in a plastic petridish containing type I collagenase (Sigma; St-Louis, MO) in Dulbecco's modified Eagles medium (DMEM; Gibco BRL, Burlington, ON). Single muscle fibers from TAS and SOL muscles were then isolated as previously described (Rosenblatt et al., 1995). Briefly, following muscle digestion, single muscle fibers were dissociated by repeated trituration in DMEM with fire-polished-tip Pasteur pipettes. Single intact muscle fibers were transferred to Matrigel- (Collaborative Biomedical Products, Bedford, MA) coated 24-well tissue culture plates (1 fiber/well) and given 3 min to attach before adding

plating medium consisting of 10% horse serum (HS) and 0.5% chick embryo extract in DMEM. After approximately 3 days in culture, satellite cells displaying myogenic morphology detached from the single muscle fibers. Single myogenic cells were isolated in one of two ways. In the first procedure, the fiber was removed from the well as soon as a single satellite cell had detached and at this point, the plating medium was then replaced with growth medium containing 10% fetal bovine serum (FBS), 20% HS, 1% chick embryo extract and 2% L-glutamine in DMEM. Myogenic cells were grown at 33°C in 5% CO₂ in the presence of mouse recombinant IFN γ (20 U/ ml; Gibco). If several satellite cells detached, a second procedure was used. The fiber was removed and the satellite cells were induced to proliferate in growth medium. The cells were then trypsinized and plated at low density in Matrigel-coated 35 mm tissue culture plates from which single myogenic cells were subsequently isolated with cloning cylinders. Regardless of the isolation procedure, when cells were near confluency, they were passaged and plated on a 6 x 35 mm well plate. Upon confluency, cells were incubated at 37°C in 5% CO₂ in fusion medium #1 containing 5% HS for 24 hours. Fusion medium #1 was then replaced with a second fusion media (#2) containing 2% HS for a total of 4 days (see Rosenblatt et al., 1995).

Extraction of AChE from Cultured Myotubes and Mature Muscles. Cultures of myotubes (4 x 35 mm wells) obtained from the fusion of myogenic cells from fast and slow muscles were washed with cold PBS, scraped and homogenized in 1 ml of a high-salt detergent buffer containing anti-proteolytic agents: 10 mM Tris-HCl, pH 7.0; 10 mM EDTA; 1 M NaCl; 1% Triton X-100; 1 mg/ml bacitracin (Sigma); 25 U/ml aprotinin (Sigma). Whole SOL and EDL

muscles, conversely, were homogenized in 2 ml of extraction buffer. Homogenization was performed on ice for 30 sec with a Polytron set at low speed. The homogenates were centrifuged (20,000 x g) at 4°C for 15 min and the supernatants were collected and frozen at -80°C. To assess AChE activity secreted from myotubes derived from fast and slow myogenic cells, diisopropyl fluorophosphate (DFP; Sigma)-treated HS was used to prepare fusion media #2. Endogenous serum AChE was inactivated by incubating the HS with DFP for 48 hours and then allowing the DFP to degrade for 10 days. Samples of fusion media #2 which had been incubated with myotubes for 48 hours prior to harvesting were collected and spun at 250 x g to remove cell debris. Supernatants were kept at -80°C until further analysis.

AChE Enzyme Assay and Velocity Sedimentation Analysis. AChE activity was measured using a modified version of the spectrophotometric method of Ellman et al. (1961) as described previously (Gisiger and Stephens, 1983; Jasmin and Gisiger, 1990). Fifty µl aliquots were incubated in 1 ml of a phosphate buffer solution (pH 7.0) containing 7.5×10^{-4} M acetylthiocholine (Sigma) as the substrate and 5×10^{-4} M dithiobisnitrobenzoic acid (DTNB; Sigma). Non-specific hydrolysis was determined by measuring substrate hydrolysis in the presence of both tetraisopropylpyrophosphoramidate (iso-OMPA; Sigma) and the AChE specific inhibitor 5-bis(4-allyldimethylammonium phenyl)pentanone dibromide (BW284c51; Sigma).

Velocity sedimentation analysis of AChE molecular forms was performed as previously described (Gisiger and Stephens, 1983; Jasmin and Gisiger, 1990). For these experiments, 100 µl aliquots were layered onto 5 - 20% sucrose gradients. Samples were

centrifuged in a Beckman SW41 rotor at 281 000 x g for 16 h at 4°C. Approximately 45 fractions were collected from the bottom of the tubes and assayed for AChE activity. Analysis of AChE molecular forms and processing of the raw data were performed as described in detail elsewhere (Gisiger and Stephens, 1983; Jasmin and Gisiger, 1990).

RNA Extraction and Reverse Transcription - Polymerase Chain Reaction. Total RNA was isolated from cultured myotubes using 1 ml of Trizol (Gibco) per 35 mm plate whereas 1 ml of Trizol per 100 mg of rat muscle tissue was used to extract total RNA from muscle. Myotubes were scraped from the plates and disrupted by pipetting repeatedly up and down for 30 sec. Muscles were homogenized with a Polytron set at maximum speed twice for 15 sec. Following addition of chloroform, the solution was mixed vigorously and spun at 12,000 g for 15 min at 4°C. The aqueous layer was then transferred to a fresh tube and an appropriate amount of isopropanol was added. For RNA precipitation, the samples were spun and the resulting pellets washed with 70% ethanol. Pellets were then briefly air-dried and they were resuspended in RNase-free water. All samples were stored at -80°C until use.

For reverse-transcription and polymerase-chain reaction (RT-PCR) analysis, all RNA samples were adjusted to a final concentration of 80 ng/ml. Two µl of each RNA sample were reverse transcribed at 42°C for 45 min followed by 5 min at 99°C as previously described in detail elsewhere (Jasmin et al., 1993; Michel et al., 1994; Boudreau-Larivière et al., 1996). Negative controls consisted of the same RT mixture in which sample RNA was replaced by the corresponding volume of RNase-free water.

cDNAs encoding AChE and S12 ribosomal RNA (rRNA) were amplified using PCR as described in detail elsewhere (Jasmin et al., 1993; Michel et al., 1994; Boudreau-Larivière et al., 1996). Primers for AChE (5': CTGGGGTGCGGATCGGTGTACCCC; 3': TCACAGGTCTGAGCAGCGTTCCTG) and rRNA (5'-GGAAGGCATAGCTGCTGG, 3'CCTCGATGACATCCTTGG)(internal control for RT-PCR experiments) were synthesized on the basis of available sequences (Legay et al., 1993b; Forster et al., 1993). Cycle parameters for AChE included denaturation for 1 min at 94°C followed by primer annealing and extension at 70°C for 3 min. Primer annealing and extension for rRNA were 54°C for 1 min and 72°C for 2 min, respectively. In each experiment, the last cycle was followed by a 10 min elongation step at 72°C. The PCR products were visualized on 1% ethidium bromide-stained agarose gels. Quantitation of the PCR products was performed by separating PCR products in agarose gels containing the fluorescent dye VistraGreen (Amersham; Arlington Heights, IL) and the labeling intensity of the PCR product, which is linearly related to the amount of DNA, was quantitated using a Storm phosphorImager and analyzed with the ImageQuant software program (Molecular Dynamics, Inc.; Sunnyvale, CA). All values obtained for AChE were corrected according to their corresponding level of rRNA present in the sample.

All RT-PCR experiments aimed at determining the relative abundance of AChE mRNA in fast and slow muscles as well as in cultured myotubes derived from fast and slow myogenic cells were performed using cycle numbers that lay within the linear phase of amplification (Jasmin et al., 1993; Michel et al., 1994; Chan et al., 1998). The cycle numbers were 37 for AChE and 28 for rRNA. RT-PCR conditions (primer concentrations, input RNA,

choice of RT primer, cycling conditions) were initially optimized, and they were identical for all samples. Appropriate precautions (use of sterile filtered tips and gloves) were taken to avoid contamination and RNA degradation. Samples, including the negative controls (RNase-free water) were always prepared using the same master mixes of RT and PCR reagents and they were run in parallel. In all experiments, PCR products were never detected in these negative controls.

Nuclear Run-on Assays. Nuclear run-on assays were performed as described elsewhere (Chan et al., 1998; Boudreau-Larivière and Jasmin, 1999). Nuclei were obtained by homogenizing ~1 g of frozen EDL or SOL muscle (8 to 9 muscles) in 10 volumes of lysis buffer made of 0.3 M sucrose, 60 mM KCl, 15 mM NaCl, 15 mM HEPES, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 0.15 mM spermine, 0.5 mM spermidine, 10 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride. Typically, we obtained approximately 4×10^6 nuclei per g of muscle tissue. Following centrifugation, pellets were resuspended in 1 ml of lysis buffer containing 0.05% Nonidet P-40 for further homogenization. Nuclei were then sedimented at $500 \times g$ and resuspended in transcription buffer containing 0.6 M $(\text{NH}_4)_2\text{SO}_4$, 0.4 M Tris, pH 7.9, 0.2 M MgCl_2 , 0.2 M MnCl_2 , 1 M NaCl, 100 mM EDTA, pH 8, 0.02 M phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 10 mM creatine phosphate, 1 mM each of GTP, ATP and CTP, 5% glycerol, 50 U of RNase inhibitor (Promega; Madison WI) and 200 mCi of $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ to a final volume of 200 μl . RNA was transcribed at 28°C for 30 min. Following RQ1 DNase (Promega) treatment, labelled RNA was isolated using Trizol and hybridized for 48 hours with 10 μg of genomic DNA, linearized AChE (2 Kb) and β -actin (2

Kb) cDNAs as well as the empty plasmids pEF-BOS (AChE) and SK (actin) immobilized on Genescreen Plus nylon membrane (DuPont). Following hybridization, membranes were washed thoroughly (1 x SSC, 0.1% SDS) at 42°C, and exposed for autoradiography at -80°C for 2 to 5 days with intensifying screens. The intensity of the signals was quantified using a Storm PhosphorImager (Molecular Dynamics). The signals corresponding to AChE were standardized relative to the β -actin signal. Hybridization signals were below detectable levels for SK and pEF-BOS empty plasmids.

Statistical Analysis. Student's *t*-tests were performed to determine whether the differences between group means were significant. The level of significance was set at $P < 0.05$. The data are expressed as means $\pm$ SE throughout.

RESULTS

AChE Expression in Mature Slow- and Fast-Contracting Hindlimb Muscles.

Determination of AChE activity in mature slow SOL and fast EDL muscles, which are muscles of similar size, revealed that EDL muscles contained significantly higher enzyme activity as previously reported (Figure 3.1; $P < 0.05$; see Gisiger and Stephens, 1983; Groswald and Dettbarn, 1983; Boudreau-Larivière et al., 1997). In agreement with previous studies (see for example Jasmin and Gisiger, 1990), significant differences between the proportions of the various AChE molecular forms were also observed in SOL and EDL muscles (Figure 3.2). Notably, asymmetric forms accounted for a greater proportion of total AChE activity in SOL muscles (~50 % compared to 20 % in EDL) whereas globular forms, particularly G_4 , were relatively more abundant in EDL muscles (G_4 content ~40 % compared to 20 % in SOL) (see also Table 3.1).

We next examined whether fast EDL and slow SOL muscles exhibited differences in the pattern of expression of the AChE gene. We initially determined the relative amount of AChE transcripts in SOL and EDL muscles by RT-PCR. As shown in Figure 3.3, AChE mRNA levels, normalized to rRNA, were found to be approximately 5-fold greater ($P < 0.05$) in EDL muscles as compared to SOL muscles. Given these findings, we assessed whether these differences in AChE mRNA content between fast- and slow-contracting muscles could be attributed to enhanced transcriptional activity of the AChE gene in fast muscles. To address this issue, we performed nuclear run-on assays using nuclei isolated from SOL and EDL muscles. Representative hybridization signals of newly synthesized AChE mRNA,

Figure 3.1 Comparison of total AChE activity in control SOL and EDL muscles from adult rats. Asterisk denotes a significant difference at $P < 0.05$. $n = 4$ per group.

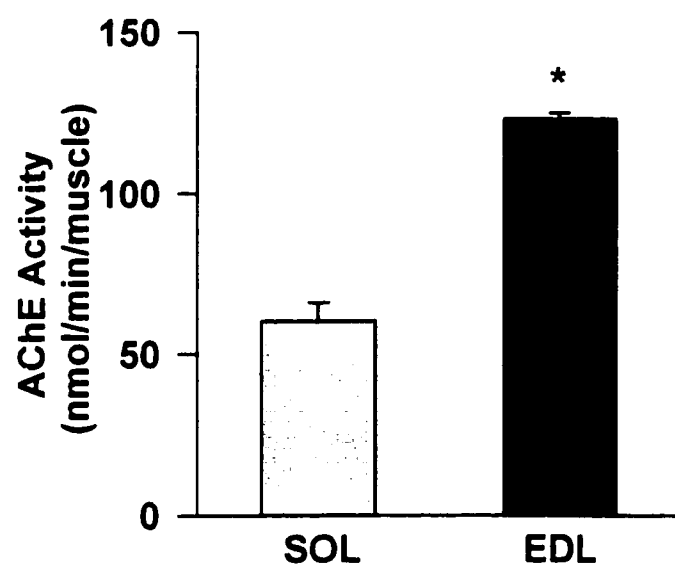
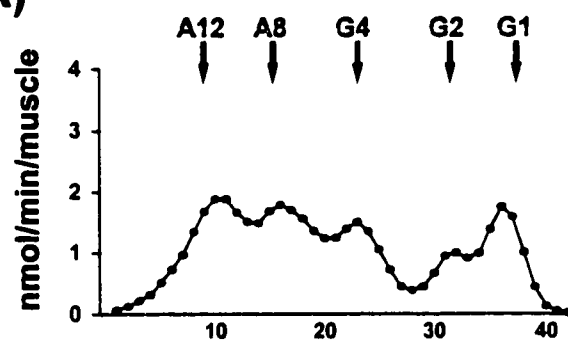


Figure 3.2 Profiles of AChE molecular forms observed in adult SOL (A) and EDL (B) rat muscles. Shown are representative examples. Note the different Y-axis scales.

(A)



(B)

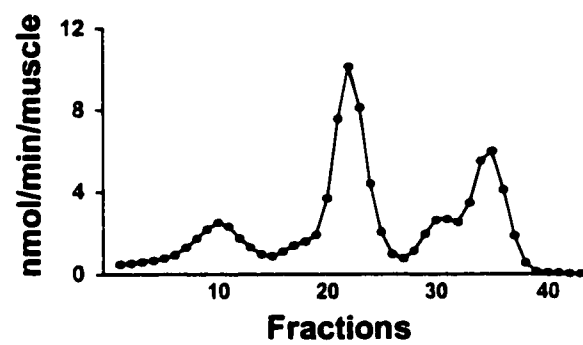


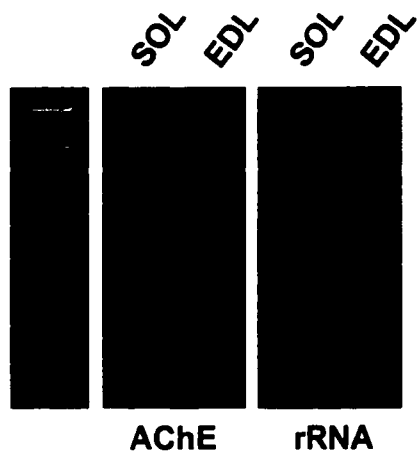
Table 3.1 Distribution of AChE molecular forms in adult SOL and EDL muscles as well as in myotubes derived from the proliferation and fusion of single clonal SOL and TAS myogenic cells.

	A ₁₂	A ₈	G ₄	G ₂	G ₁
<i>In vivo</i>					
adult SOL	27.1 ± 2.3 ^a	23.6 ± 1.3 ^{ab}	18.2 ± 1.7	10.5 ± 1.2	18.9 ± 1.6 ^b
adult EDL	15.9 ± 0.9	4.2 ± 1.0	38.9 ± 2.0 ^{ab}	9.7 ± 0.9	29.9 ± 3.6 ^{ab}
<i>In vitro</i>					
SOL cell-associated	17.7 ± 3.3	2.4 ± 1.5	20.4 ± 1.9	9.6 ± 1.6	49.9 ± 2.3
TAS cell-associated	17.5 ± 4.5	7.4 ± 1.1	22.2 ± 3.2	10.4 ± 1.2	42.6 ± 0.9
SOL secreted	n. d.	n. d.	49.7 ± 0.5	n. d.	44.6 ± 1.2
TAS secreted	n. d.	n. d.	47.8 ± 1.6	n. d.	46.7 ± 2.5

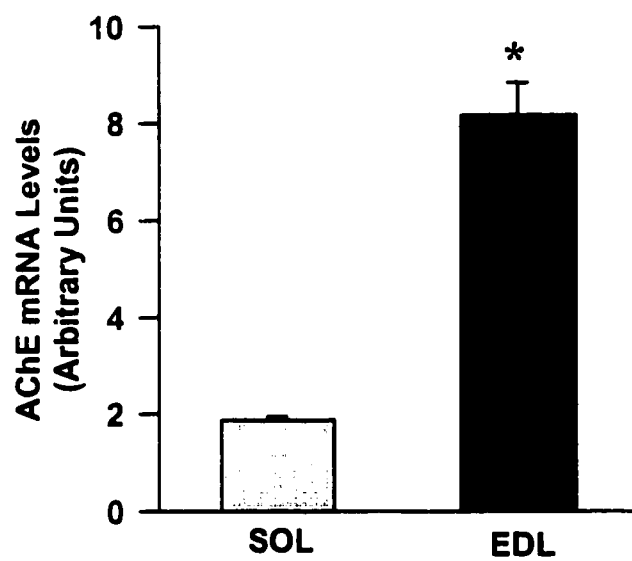
Data are percentages of total AChE activity. Means ± SE are shown (n = 3). ^a; denotes significant differences between adult SOL and EDL muscles. ^b; denotes significant differences between in vivo and in vitro cell-associated. n.d.; not detected.

Figure 3.3 Determination of AChE transcript levels in slow SOL and fast EDL muscles. (A) is a representative example of an ethidium bromide-stained agarose gel displaying RT-PCR products for AChE and S12 rRNA obtained from the amplification of reverse transcribed RNA extracted from SOL and EDL muscles. (B) Quantitative analysis of AChE mRNA levels in SOL and EDL muscles standardized using corresponding rRNA signals. Asterisk denotes a significant difference at $P < 0.05$. $n = 6$ per group.

(A)



(B)



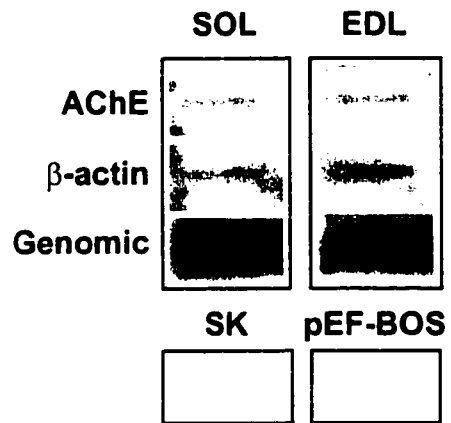
reflecting transcriptional activity of the AChE gene, are shown in Figure 3.4A. Quantitative analysis revealed that nuclei isolated from both SOL and EDL muscles transcribed the AChE gene at approximately the same rate (Figure 3.4B; $P > 0.05$).

AChE Expression in Myotubes Obtained from Clonal Satellite Cells Isolated From Slow and Fast Muscle Fibers. Given that fast-contracting muscles display significantly higher levels of AChE activity and transcripts as well as distinct molecular form profiles compared to their slow-twitch counterparts, we determined whether myogenic precursor cells originating from fast and slow muscle fibers are intrinsically programmed to express distinct AChE profiles. To achieve this objective, SOL and TAS muscles were excised from adult Immorto transgenic mice (see Materials and Methods) and single myogenic cells were isolated from slow SOL and fast TAS muscle fibers. We took advantage of the fact that single clonal myogenic cells can be isolated directly from slow and fast muscle fibers and subsequently grown to give rise to a pure population of myoblasts capable of undergoing normal myogenic differentiation in culture (Morgan et al., 1994). This approach therefore, enabled us to analyze the pattern of AChE expression in two separate populations of myotubes.

We first measured total AChE activity in myotube cultures obtained from single myogenic cell clones derived from slow SOL and fast TAS muscle fibers. Figure 3.5 shows that cell-associated (A) and secreted (B) AChE activity were nearly identical for both myotube populations ($P > 0.05$). AChE specific activity (per mg of protein) for both cell-associated

Figure 3.4 Transcription of the AChE gene in slow and fast hindlimb muscles. (A) shows representative run-on assays performed with nuclei isolated from SOL and EDL muscles. Also shown are examples of background hybridization signals obtained with empty plasmids for actin (SK) and AChE (pEF-BOS). (B) is the quantitative analysis of AChE gene transcription in SOL and EDL muscles standardized using β -actin (actin). Data were obtained from three separate experiments using 8 to 9 SOL and EDL muscles per experiment.

(A)



(B)

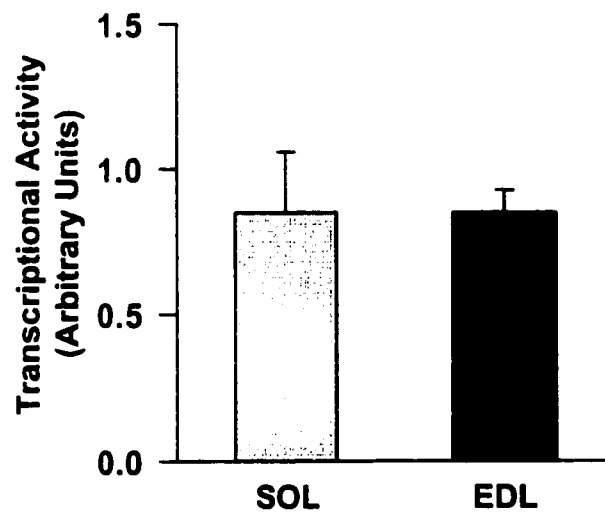
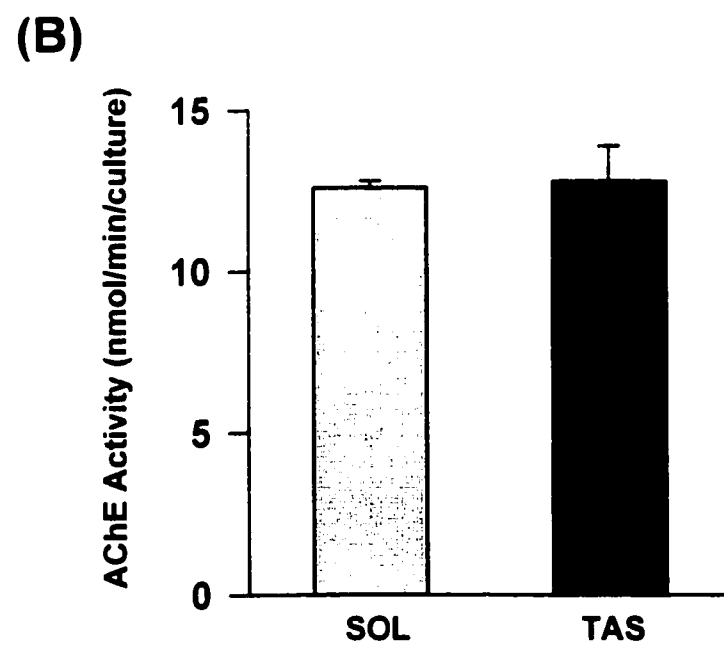
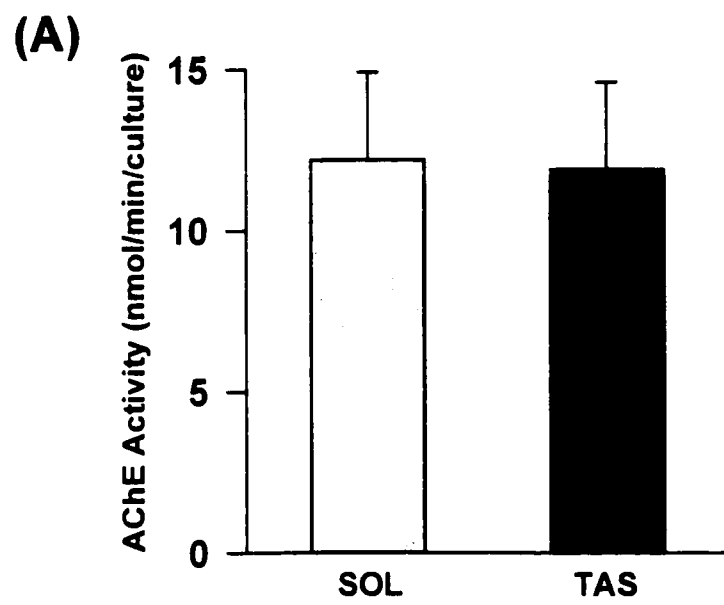


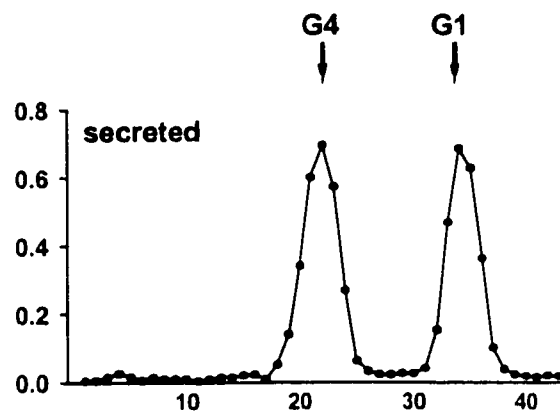
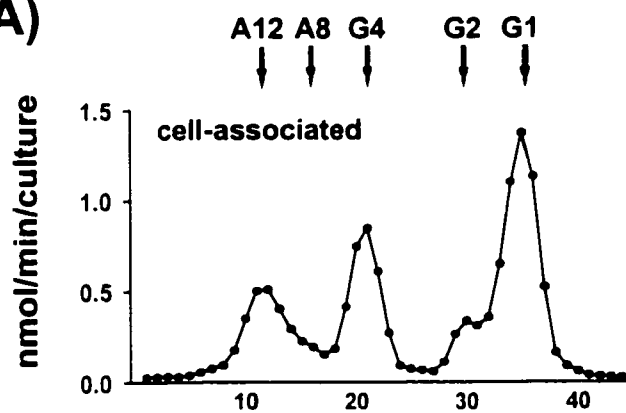
Figure 3.5 Total AChE activity from cultured myotubes derived from the fusion of myoblasts obtained from the proliferation of single myogenic cell clones isolated from slow SOL and fast TAS muscle fibers from Immorto mice. (A) and (B) show cell-associated and secreted AChE activity per culture, respectively. n = 6 per group



and secreted enzyme was also found to be similar for both sets of cultures. Analysis of AChE molecular form profiles using myotube extracts revealed that asymmetric forms, particularly A_{12} , were readily detected in these myotubes along with G_4 , G_2 and a high level of G_1 (Figure 3.6). Furthermore, the relative proportions of cell-associated molecular forms in myotubes derived from myogenic cells isolated from SOL (Figure 3.6A) and TAS (Figure 3.6B) muscle fibers showed the same distribution (Table 3.1). In the fusion media, G_4 and G_1 were the only detectable forms of the enzyme with comparable levels for both myotube populations (Figure 3.6; Table 3.1). Consistent with these AChE activity data, we did not observe differences in the abundance of AChE transcripts between these two myotube populations (Figure 3.7, $P > 0.05$).

Figure 3.6 AChE molecular form profiles observed in cultured myotubes and media. Shown are representative examples of the average content in AChE molecular forms for cell-associated and secreted enzyme obtained from myotubes derived from single myogenic cells isolated from slow SOL (A) and fast TAS (B) muscle fibers.

(A)



(B)

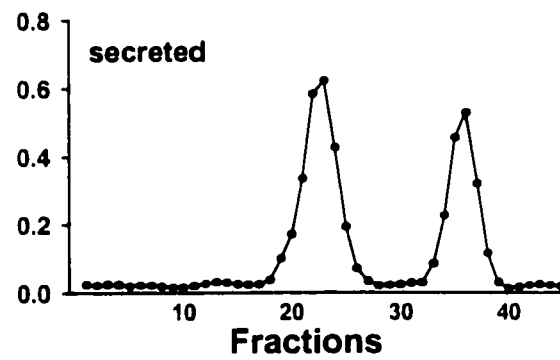
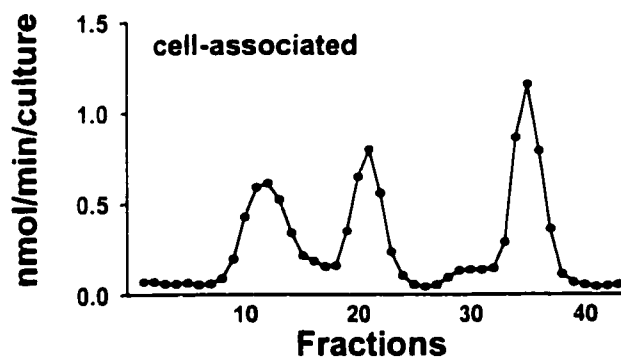
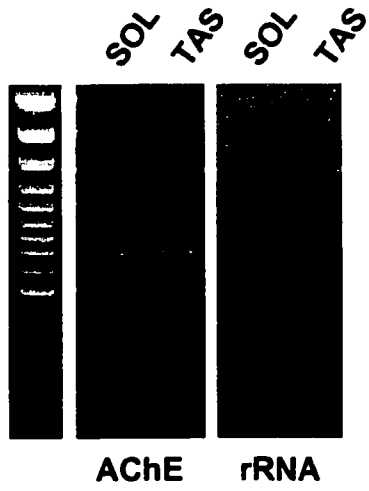
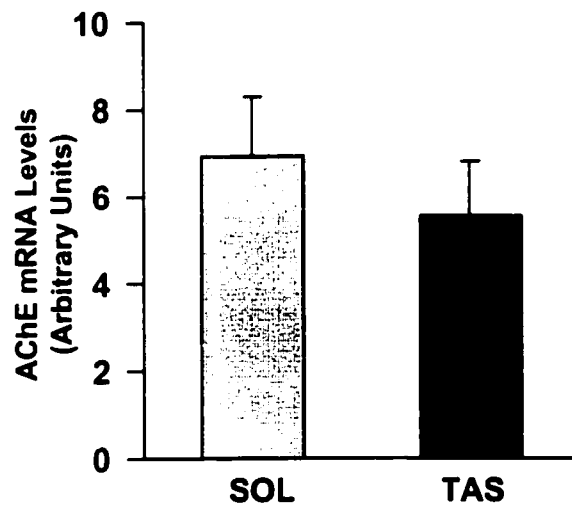


Figure 3.7 AChE mRNA levels determined from cultured myotubes obtained from single myogenic cells derived from slow SOL and fast TAS muscle fibers. (A) is an ethidium bromide-stained agarose gel displaying RT-PCR products for AChE and S12 rRNA. (B) is the quantitative analysis of AChE mRNA levels standardized using rRNA signals. n = 6 per group.

(A)



(B)



DISCUSSION

Several lines of evidence clearly demonstrate that motoneuron-derived signals play a pivotal role in regulating AChE expression in skeletal muscle (reviewed in Massoulié et al., 1993). However, the contribution of innervation versus intrinsic factors in controlling AChE expression in skeletal muscle remains controversial and needs to be clearly established if the ultimate goal is to understand all of the events contributing to the regulation of AChE in slow and fast muscles. In the present study, we provide evidence that myotubes derived from a pure population of myogenic precursor cells isolated from slow and fast muscle fibers, exhibit intrinsically similar patterns of AChE expression. Together, these findings indicate that differences in AChE expression between fast and slow muscles are not due to inherent differences in myogenic precursor cells, thereby suggesting that other factors, such as innervation, play a predominant role in establishing the distinct patterns of AChE expression in these muscle types.

Mechanisms Regulating AChE Expression in Slow- and Fast-contracting Muscles In Vivo. Compared to slow skeletal muscles, fast muscles of rats display significantly greater AChE activity which is paralleled by a higher level of AChE mRNA (Cresnar et al., 1994; Michel et al., 1994; Sketelj et al., 1998). In the present study, we show that AChE mRNAs are 5-fold more abundant in fast versus slow muscles thereby confirming recent findings (Sketelj et al., 1998). However, we demonstrate also that AChE activity in fast muscle is only 2-fold higher. The more modest differences in AChE activity between slow and fast muscles

as compared to the larger variations in their AChE mRNA content, suggest therefore that the production of AChE molecules in muscle may also be regulated at the translational or post-translational level. Indeed, evidence for translational control of AChE biosynthesis was reported recently in rats treated with glucocorticoids (Brank et al., 1998). Whereas AChE mRNA levels assessed by Northern blot, remained unchanged in fast muscles of glucocorticoid-treated versus non-treated animals, substantial reductions in the levels of G₁ and G₂ molecular forms were reported indicating that AChE protein synthesis was specifically hindered under these experimental conditions (Brank et al., 1998). Similarly, we have also shown that post-translational events and in particular the association of AChE catalytic subunits with the collagenic structural subunit, may in fact enhance AChE activity in cultured muscle cells through the stabilization of newly synthesized catalytic peptides (Legay et al., 1999). A similar stabilization mechanism may also account for the more modest differences seen in AChE activity between slow and fast muscles since it was recently shown that the collagenic structural subunit is more abundant in slow muscles (Legay et al., 1998).

One issue that had yet to be resolved is whether greater levels of AChE expression in fast muscles are due to enhanced transcription of the AChE gene in this muscle type. In the present investigation, we measured by run-on assays, the levels of newly synthesized AChE mRNAs in nuclei isolated from slow SOL and fast EDL muscles. Our analysis showed that the rate of AChE gene transcription in both muscle types was similar thereby indicating that post-transcriptional mechanisms are important in controlling the levels of AChE transcripts in slow versus fast muscles.

Previous *in vitro* experiments using cultured neural (Coleman and Taylor, 1996), hematopoietic (Chan et al., 1998) and myogenic cells (Fuentes and Taylor, 1993) have provided evidence that enhanced AChE mRNA stability accounts for the increase in AChE expression that occurs during differentiation of these cells. Our current data support and extend these findings by indicating that a similar regulatory mechanism is likely playing a role *in vivo* by controlling the levels of AChE mRNA in slow and fast muscles. Interestingly, post-transcriptional mechanisms have also been postulated to regulate the differential expression of the cytochrome *c* gene in slow and fast muscles (Yan and Booth, 1998). Although the mechanisms that dictate the half-life of AChE transcripts in muscle have yet to be elucidated, it is reasonable to envisage that interactions between trans-acting factors and regions along the mRNA, and in particular in the 3'-untranslated region (3'UTR), are involved (see for review Ross, 1995). Therefore, the abundance of trans-acting factors involved in RNA-protein interactions may differ in slow and fast muscles thereby controlling the longevity of AChE transcripts in these muscle types.

Myotubes Derived from Myogenic Clonal Cells Isolated from Slow or Fast Muscle Fibers Display Similar Patterns of AChE Expression. Findings from previous studies suggest that slow and fast muscle fibers are intrinsically programmed to display distinct patterns of AChE expression. For example, Dolenc and colleagues (1994) showed that slow and fast muscles of adult rats undergoing regeneration due to an ischemic-toxic injury, in the presence or absence of the nerve, display distinct molecular form profiles comparable to those seen in intact slow and fast muscles, respectively (Dolenc et al., 1994). These findings imply that

myogenic precursor cells from slow and fast muscles, which are activated during the regeneration process, are therefore pre-programmed to express distinct AChE molecular form profiles. In our experiments, we were able to obtain clonal populations of myogenic precursor cells from single muscle fibers of the slow SOL and fast TAS muscles which comprise 60% type I/40% type IIA fibers, and 80% type IIB/20% type IIX fibers, respectively (Zardini and Parry, 1994). This enabled therefore, the analysis of myotubes generated from a single myogenic cell isolated from either slow or fast muscle fibers. With this approach, which has been used previously to obtain several conditionally immortal cell lines from a variety of tissues (Jat et al., 1991; Chambers et al., 1993; Groves et al., 1993; Whitehead et al., 1993) including skeletal muscle (Morgan et al., 1994), we demonstrate that myotubes generated from clonal populations of myoblasts obtained from the proliferation of single myogenic cells isolated from slow or fast muscle fibers, display nearly identical patterns of AChE expression (Sketelj et al., 1991; Dolenc et al., 1994). Specifically, these two myotube populations displayed similar total AChE activity, molecular form profiles and transcript levels.

The reasons for these divergent results may be explained if we consider that during regeneration, fusion of satellite cells with pre-existing muscle fibers that survive ischemic-toxic injury, may express AChE molecular form profiles of the host fiber under the influence of mechanisms that override the intrinsic program of these satellite cells (Hughes and Blau, 1992). In addition, satellite cells associated with muscle fibers within the core of the muscle may be damaged during severe ischemic conditions thereby affecting their normal program of AChE expression (Phillips et al., 1987; Schultz et al., 1988). Since interaction with other cell types or molecules influences the phenotype of myogenic cells (Butler et al., 1988; Donoghue

et al., 1992), it is also possible that in situ regeneration of muscle fibers within a previously deposited basal lamina may induce these cells to express an AChE profile reminiscent of that expressed in previously existing fibers. In this context, basal lamina-associated molecules such as agrin and ARIA, which are known to regulate the expression of genes encoding synaptic proteins in muscle (for review see Burden, 1998), may continue to exert their effects within regenerating muscles. Given the above caveats, similar intrinsic regulation of AChE expression in myogenic precursor cells from slow and fast muscle fibers, as observed in the present investigation, may have been masked in previous studies using the regeneration model (Sketelj et al., 1991; Dolenc et al., 1994).

Contribution of Neural versus Intrinsic Factors in Regulating AChE Molecular Form Profiles in Slow- and Fast-contracting Muscles. In a recent study, we showed that inactivated though still innervated slow and fast hindlimb muscles expressed a common molecular form profile resembling that expressed in slow muscles (Boudreau-Larivière et al., 1997). Our present findings provide evidence that myogenic precursor cells from slow or fast muscle fibers give rise to myotubes that also express comparable AChE molecular form profiles. In addition, the molecular form distributions of these myotubes displayed characteristics intermediate to those seen in slow and fast muscles in vivo. Together, these findings indicate that innervation plays a dominant role in establishing distinct AChE molecular form profiles in slow versus fast muscles in vivo. In this context, it is noteworthy that in the study of Dolenc et al. (1994), regenerating fast muscles eventually altered their AChE profiles

to a slow type following prolonged reinnervation by the soleus nerve further highlighting the important contribution of innervation (Dolenc et al., 1994).

Previous studies have shown that SOL and EDL muscles from post-natal rats, already display prominent differences in their molecular form profiles (Sketelj et al., 1991) despite receiving at this developmental stage, similar high-frequency impulse patterns delivered by their respective innervating motoneurons (Navarrete and Vrbova, 1983). Based on these findings, it has been suggested that early post-natal slow and fast muscles are pre-programmed to express distinct AChE molecular form profiles independently of neural activation. However, an alternative explanation for these observations relates to the findings that slow muscles are known to be largely insensitive to phasic, high-frequency activity whereas fast muscles readily adapt to this activity pattern as previously reported (Jasmin and Gisiger, 1990; Boudreau-Larivière et al., 1997). Thus, the distinct AChE profiles seen in neo-natal fast and slow muscles likely reflect: i) the adaptation of a basic AChE profile to high-frequency activity in fast muscle; and ii) the refractiveness of slow muscles to this type of neuromuscular activation.

The availability of structural subunits such as the collagenic and hydrophobic tails, are believed to dictate the relative proportions of asymmetric forms and hydrophobic-tailed tetramers synthesized in cells (Legay et al., 1993b; Legay et al., 1999; Krejci et al., 1997). Since in the current study, we found that fast and slow myotube populations expressed similar levels of AChE activity as well as comparable proportions of asymmetric and G₄ forms, it is reasonable to postulate that AChE-associated structural subunits are also expressed at similar levels within these myotubes. Transformation of AChE molecular form profiles from those

expressed in myotubes into those observed in adult slow or fast muscles may therefore also be dependent on parallel modifications in the synthesis of the structural subunits. Accordingly, levels of the collagenic as well as the hydrophobic structural subunits may be highly sensitive to innervation and specific patterns of electrical activity (i.e. tonic versus phasic) which in turn, could dictate the molecular form distributions observed in slow- and fast-contracting muscles (Sveistrup et al., 1995). Future studies aimed at determining the impact of neural signals on the expression of these structural subunits should prove useful for our understanding of additional biosynthetic events underlying the plasticity of AChE expression in muscle.

Perspectives. The phenotype of skeletal muscles is strongly influenced by nerve-derived signals, although intrinsic properties of the muscles may also determine its characteristics. In the present investigation, we demonstrate that intrinsic factors do not account for the distinct patterns of AChE expression displayed by fast- and slow-contracting skeletal muscles. Rather, extrinsic factors such as motoneuron-derived signals, i.e., electrical activity and trophic factors, as well as mechanical loading appear as key determinants governing expression of this enzyme via posttranscriptional regulatory mechanisms. In this context, our findings indicate that these extrinsic factors likely initiate signaling cascades that culminate 1) in the modulation of AChE mRNA stability and 2) in the assembly of AChE catalytic subunits with available structural subunits.

CHAPTER IV

Molecular Mechanisms Underlying Activity-linked Alterations in Acetylcholinesterase mRNAs in Developing versus Adult Skeletal Muscles

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Cloning of the rat AChE promoter and preparation of the promoter-reporter construct was carried out by Dr. R. Chan.

ABSTRACT

In skeletal muscle fibers, levels of acetylcholinesterase (AChE) mRNA are highly sensitive to nerve-evoked electrical activity yet, the molecular mechanisms underlying this plasticity remain to be established. Here, we demonstrate that in comparison to the sharp increase in the levels of transcripts encoding the α -subunit of the acetylcholine receptor (AChR), denervation of adult muscle induces a dramatic (up to 90%) and rapid (within 24 hrs) decrease in the abundance of AChE mRNAs. Northern blot analysis also revealed that the two predominant species of AChE T transcripts are affected similarly by muscle denervation. By contrast, denervation of muscles from 14 day-old rats led to a significantly less pronounced reduction ($P < 0.05$; 50% of control) in the expression of AChE mRNAs. Nuclear run-on assays revealed that the transcriptional activity of the AChE gene remained essentially unchanged in adult denervated muscles whereas it displayed a ~2- to 3-fold increase ($P < 0.05$) in denervated muscles from 2 to 14 day-old rats. Direct plasmid injection of a rat AChE promoter-reporter gene construct into control and denervated muscles from both post-natal and adult rats further confirmed these observations. Taken together, these data suggest that in denervated adult skeletal muscle, the abundance of AChE transcripts is primarily controlled via post-transcriptional regulatory mechanisms whereas in neo- and post-natal muscles, transcriptional as well as post-transcriptional regulation are both critical events dictating AChE mRNA levels. Accordingly, the activity-linked transcriptional regulation of the AChE gene appears to demonstrate a high level of plasticity during muscle development when maturation of the neuromuscular junctions is still occurring.

INTRODUCTION

Acetylcholinesterase (AChE) plays an essential role at cholinergic synapses of both peripheral and central nervous systems since it rapidly hydrolyzes acetylcholine released from nerve terminals. In vertebrates, several AChE catalytic subunits differing at their C-terminus are produced through alternative splicing of a single gene. Distinct post-translational modifications of these catalytic subunits in turn, generate multiple molecular forms of AChE that are expressed in a variety of tissues and subcellular locations (for reviews see Massoulié et al., 1993; Taylor and Radic, 1994). In skeletal muscle fibers for instance, asymmetric forms of AChE and hydrophobic-tailed tetramers accumulate within the synaptic basal lamina (McMahan et al., 1978) and the perijunctional compartment (Gisiger and Stephens, 1988), respectively, where their expression is known to be markedly influenced by the levels of superimposed neuronal activation.

Despite the wealth of information available on the plasticity of AChE molecular forms confronted with altered levels of neuromuscular activation, our knowledge of the cellular and molecular mechanisms involved in the activity-linked regulation of AChE in muscle is still rudimentary. Several studies have recently begun to explore the molecular basis underlying the activity-linked regulation of AChE in skeletal muscle. Results of these studies have shown for example, that muscle inactivation induced either by surgical denervation or tetrodotoxin application onto the motor nerve, leads to profound reductions in the levels of AChE mRNA in mammalian skeletal muscle fibers (Cresnar et al., 1994; Michel et al., 1994; Sketelj et al., 1998). Conversely, increased neuromuscular activation achieved by exercise training or

compensatory overload, increases significantly the abundance of AChE transcripts (Sveistrup et al., 1995). Although these studies clearly demonstrate that nerve-evoked electrical activity constitutes a key regulator of AChE expression in skeletal muscle, the molecular mechanisms underlying this activity-linked regulation of AChE mRNAs have yet to be fully elucidated. In this context, several levels of regulation including transcriptional as well as post-transcriptional control, may be considered. Results from several laboratories have shown for instance that, in denervated muscle, the increased expression of mRNAs encoding the various acetylcholine receptor (AChR) subunits is caused by a transcriptional activation of their respective genes (Fontaine and Changeux, 1989; Merlie and Kornhauser, 1989; Tsay and Schmidt, 1989; Salmon and Changeux, 1992; Simon et al., 1992; Gundersen et al., 1993; Bessereau et al., 1994; Merlie et al., 1994; Tang et al., 1994; Walke et al., 1996). Alternatively, post-transcriptional regulatory mechanisms appear to play a significant role in the regulation of AChE during differentiation of myogenic (Fuentes and Taylor, 1993; Luo et al., 1994), neuronal (Coleman and Taylor, 1996) and hematopoietic (Chan et al., 1998) cells grown in culture.

In the present study, we have therefore begun to define the molecular mechanisms underlying the activity-linked regulation of AChE in skeletal muscle fibers. Using two distinct yet complementary approaches that reflect the transcriptional activity of the AChE gene, we have assessed the contribution of transcriptional versus post-transcriptional mechanisms in the regulation of AChE mRNA levels in denervated post-natal and adult skeletal muscles.

MATERIALS and METHODS

Animal Care and Surgery

Pregnant Sprague-Dawley rats, female Sprague-Dawley rats weighing ~ 250 g and male and female rat pups (1.5 to 2 weeks of age) were obtained from Charles River Laboratories (St-Constant, Québec). Care and treatment of the animals were in accordance with the guidelines established by the Canadian Council on Animal Care. Hindlimb muscles were denervated by cutting and removing a short segment of the sciatic nerve in the mid-thigh region while the animals were anaesthetized with methoxyflurane. One to 10 days later, animals were anaesthetized with sodium pentobarbital (35mg/kg i.p.) and their hindlimb muscles were dissected, excised and rapidly frozen in liquid nitrogen. All surgical procedures were performed under aseptic conditions.

RNA Extraction and Reverse Transcription - Polymerase Chain Reaction

Total RNA was isolated from rat hindlimb muscles using 1 ml of Trizol (Gibco BRL; Burlington, Ontario) per 100 mg of tissue. Muscles were homogenized with a Polytron set at maximum speed for 2 X 15 sec. Following addition of chloroform, the solution was mixed vigorously and spun at 12,000 x g for 15 min at 4°C. The aqueous layer was then transferred to a fresh tube and an appropriate amount of isopropanol was added. For RNA precipitation, the samples were spun and the resulting pellets washed with 70% ethanol. Pellets were then briefly air-dried and they were resuspended in RNase-free water. All samples were stored at -80°C until use.

For reverse-transcription and polymerase-chain reaction (RT-PCR) analysis, all RNA samples were quantitated using a Pharmacia GeneQuant II RNA/DNA spectrophotometer and the final concentration was adjusted to 80 ng/ μ l. Two μ l of each RNA sample were reverse transcribed at 42°C for 45 min followed by 5 min at 99°C as previously described in detail elsewhere (Jasmin et al., 1993; Michel et al., 1994; Boudreau-Larivière et al., 1996). Negative controls consisted of the same RT mixture in which sample RNA was replaced by 2 μ l of RNase-free water.

cDNAs encoding AChE, AChR α -subunit and ribosomal RNA (rRNA) were amplified using PCR as described in detail elsewhere (Jasmin et al., 1993; Michel et al., 1994; Sveistrup et al., 1995; Boudreau-Larivière et al., 1996). Primers for AChE (5': CTGGGGTGCGGATCGGTGTACCCC; 3': TCACAGGTCTGAGCAGCGTTCCTG), AChR α -subunit (5': GACTATGGAGGAGTGAAAAA; 3': TGGAGGTGGAAGGGATTAGC), and S12 rRNA (5'-GGAAGGCATAGCTGCTGG, 3'CCTCGATGACATCCTTGG) (internal control for RT-PCR experiments) were synthesized on the basis of available sequences (Boulter et al., 1985; Legay et al., 1993b; Forster et al., 1993). Cycle parameters for AChE included denaturation for 1 min at 94°C followed by primer annealing and extension at 70°C for 3 min. For the AChR α -subunit, primer annealing was carried out for 1 min at 60°C and extension was for 1 min at 72°C. Primer annealing and extension for rRNA were 54°C for 1 min and 72°C for 2 min, respectively. In each experiment, the last cycle was followed by a 10 min elongation step at 72°C. The PCR products (AChE, 670 bp; AChR α -subunit, 576 bp; S12 rRNA 368 bp) were visualized on 1% ethidium bromide-stained agarose gels. Quantitation of the PCR products was performed by separating PCR products in agarose gels

containing the fluorescent dye VistraGreen (Amersham; Arlington Heights, IL) and the labeling intensity of the PCR product, which is linearly related to the amount of DNA, was quantitated using a Storm phosphorImager and analyzed with the accompanying ImageQuant software program (Molecular Dynamics, Inc.; Sunnyvale, CA). All values obtained for AChE and the AChR α -subunit were corrected according to their corresponding level of rRNA present in the sample.

All RT-PCR measurements aimed at determining the relative abundance of AChE and AChR α -subunit mRNAs as well as rRNA in control and denervated rat muscles, were performed during the linear phase of amplification (Jasmin et al., 1993; Michel et al., 1994; Hubatsch and Jasmin, 1997). The cycle numbers were typically 37 for AChE, 35 for AChR α -subunit and 28 for rRNA. RT-PCR conditions (primer concentrations, input RNA, choice of RT primer, cycling conditions) were initially optimized and they were identical for all samples. Appropriate precautions (e.g., dedicated areas for sample preparation and analysis, use of sterile filtered tips and gloves) were taken to avoid contamination and RNA degradation. Control and denervated samples as well as negative controls (see above) were prepared using common master mixes containing the same RT and PCR reagents and they were ran in parallel. In all experiments, PCR products were never detected for negative controls.

Northern Blot and Nuclear Run-on Analyses

Poly (A)⁺ RNA extracted using the Oligotex kit (Qiagen; Chatsworth, CA) was used for analysis of AChE mRNA levels by Northern blotting. Samples (3 μ g) obtained from

control and denervated tibialis anterior muscles were first size-fractionated on 1% agarose/0.6 M formaldehyde gels and subsequently transferred onto Genescreen nylon membranes (DuPont; Wilmington, DE). For hybridization, AChE (879-1722 bp) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; full-length) cDNA fragments were labelled with [α 32 P]dCTP using the random prime labeling method.

Nuclear run-on assays were performed using a modified version of a procedure described elsewhere (Ray et al., 1995). Nuclei were obtained by homogenizing ~1 g of frozen control or denervated muscle in 10 volumes of lysis buffer made of 0.3 M sucrose, 60 mM KCl, 15 mM NaCl, 15 mM HEPES, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 0.15 mM spermine, 0.5 mM spermidine, 10 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride. Following centrifugation, pellets were resuspended in 1 ml of lysis buffer containing 0.05% Nonidet P-40 for further homogenization. Nuclei were then sedimented at 500 x g and resuspended in transcription buffer containing 0.6 M $(\text{NH}_4)_2\text{SO}_4$, 0.4 M Tris, pH 7.9, 0.2 M MgCl_2 , 0.2 M MnCl_2 , 1 M NaCl, 100 mM EDTA, pH 8, 0.02 M phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 10 mM creatine phosphate, 1 mM each of GTP, ATP and CTP, 5% glycerol, 50 U of RNase inhibitor (Promega; Madison WI) and 200 μCi of [α - 32 P]UTP to a final volume of 200 μl . RNA was transcribed at 28°C for 30 min. Following RQ1 DNase (Promega) treatment, labelled RNA was isolated using Trizol and hybridized for 48 hr with 10 μg each of linearized rat AChE (2 Kb), AChR α -subunit (1.8 Kb), and β -actin (2 Kb) cDNAs immobilized on Genescreen Plus nylon membrane (DuPont). Following hybridization, membranes were washed thoroughly (1 x SSC, 0.1% SDS) at 42°C, and exposed for autoradiography at -80°C for 2 to 5 days with intensifying screens. The intensity of the signals

was quantified with a Storm PhosphorImager (Molecular Dynamics). The signals corresponding to AChE and AChR α -subunit were standardized relative to the β -actin signal.

Injection of a Rat AChE Promoter-Reporter Gene Construct in Muscle

A 1.9 kb fragment located approximately 600 bp from the translational start site corresponding to the rat AChE promoter (Chan et al., 1999) was subcloned into a LacZ reporter vector (Gundersen et al., 1993). This rat AChE promoter fragment contains 807 bp upstream of the initiator element and displays more than 90 % homology with the mouse AChE promoter region (Getman et al., 1995). In recent studies, we have shown that this rat promoter fragment directs the synapse-specific expression of a reporter gene along multinucleated rat muscle fibers (Chan et al., 1999). Plasmid DNA was prepared using the Qiagen mega-prep procedure and final pellets were resuspended in sterile phosphate-buffered saline to a final concentration of 10 $\mu\text{g}/\mu\text{l}$. In vivo gene transfer into control and denervated tibialis anterior (TA) muscles of rat was performed as described in Gramolini et al. (1997, 1998) using an experimental framework detailed elsewhere (Walke et al., 1996). Briefly, control and denervated TA muscles from either post-natal or adult rats were injected with 50 μg of the rat AChE promoter-reporter gene construct. Seven days after injection, TA muscles were excised and frozen in liquid nitrogen. For post-natal muscles, activity of β -galactosidase was assayed in control and denervated muscles using a luminescent β -galactosidase detection kit (Clontech; Palo Alto, CA). Activity of β -galactosidase was normalized according to a co-injected chloramphenicol acetyltransferase (CAT) plasmid (Promega) under the control of the SV40 promoter. For adult muscles, levels of mRNA encoding β -galactosidase and CAT were

determined by quantitative RT-PCR using sequence specific primers (β -galactosidase, 5' GTGACGGCAGTTATCTGG; 3' ATGATGCTCGTGACGGTT, 506 bp; CAT, 5' TGGCAATGAAAGACGGTGAG; 3' GAAAACGGGGGCGAAGAAGT, 290 bp; 95°C-1 min, 55°C-1 min, 72°C-1 min, 39 cycles) since a previous study clearly showed that expression of β -galactosidase is subjected to post-translational regulation in adult muscle (Gundersen et al., 1993).

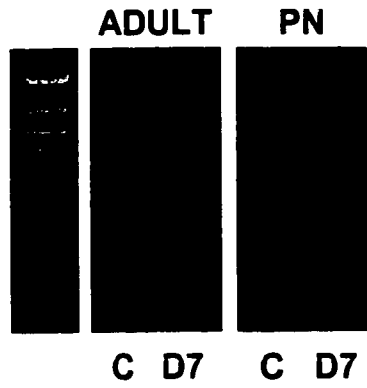
RESULTS

Denervation Affects AChE mRNA Levels Differentially in Adult versus Post-Natal Skeletal Muscles. In a recent study, we showed that AChE mRNA levels drop by ~ 10-fold in adult muscle denervated for 10 days (Cresnar et al., 1994; Michel et al., 1994). In the present investigation, we initially determined the time-course of this response to inactivation by analyzing AChE mRNA levels in muscles denervated from 1 to 7 days. Our quantitative RT-PCR data performed within the linear range of amplification, indicated that AChE mRNA levels in adult muscle decreased markedly and rapidly following denervation. As shown in Figure 4.1B for example, AChE transcript levels were reduced by approximately 90% ($P < 0.05$) in adult muscles denervated for 2 days. Northern blot analysis performed with control and denervated adult muscles revealed that the two predominant mRNA species encoding the AChE catalytic T subunit expressed in muscle (Legay et al., 1993b), decreased to a similar extent over the experimental time-course following denervation (see Figure 4.2). In agreement with previous findings, the levels of mRNA encoding the AChR α -subunit increased significantly ($P < 0.05$) following denervation reaching more than 20-fold in adult muscles denervated for 7 days (Figure 4.1C).

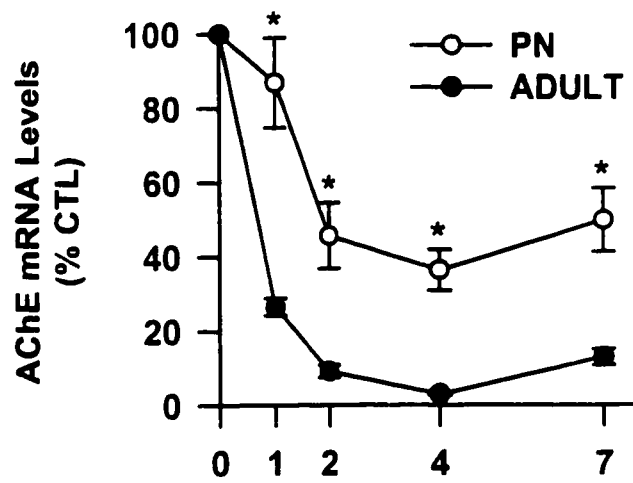
Next, we examined the response of the AChE gene to denervation in post-natal muscles. For these studies, hindlimb muscles from 2 week-old rats were denervated and levels of AChE and AChR α -subunit mRNAs were determined at 1, 2, 4 and 7 days following denervation. The pattern of increased AChR α -subunit mRNA expression in response to

Figure 4.1 Effects of denervation on AChE and AChR α -subunit mRNA levels in hindlimb muscles of adult and post-natal rats. (A) Ethidium bromide-stained gels displaying PCR products for AChE obtained using total RNA extracted from control (C) and 7 day-denervated (D7) muscles of adult and post-natal (PN) rats. (B) and (C) show the levels of AChE and AChR α -subunit transcripts in muscles from adult (closed circles) and post-natal (open circles) rats denervated for 1 to 7 days, respectively. Note the different Y-axis scales in (B) and (C) as well as the dramatic difference in the pattern of expression of AChE and AChR α -subunit mRNAs in response to denervation. Also, the effect of denervation on AChE mRNA levels was significantly different in post-natal versus adult muscles. Data are means $\pm$ SEM expressed as a percent of control levels. Asterisks denote significant differences between adult and post-natal samples at each denervation time point ($P < 0.05$; Student's *t*-test).

(A)



(B)



(C)

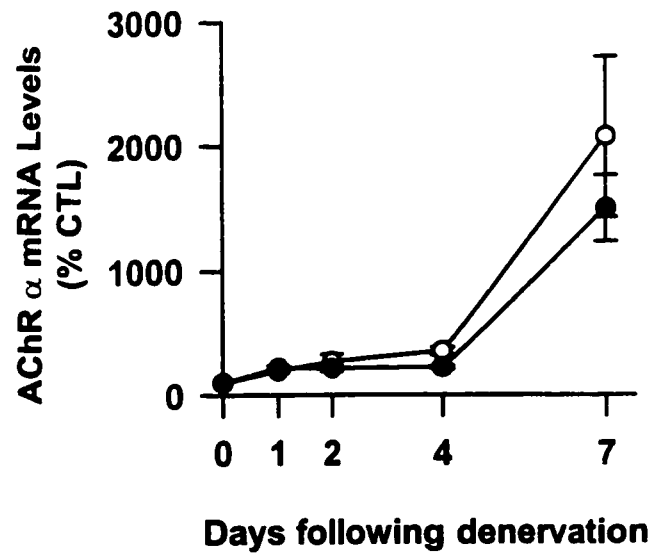
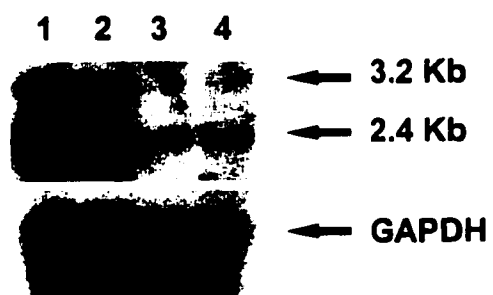


Figure 4.2 Northern blot analysis of the two predominant AChE mRNA species (2.4 and 3.2 kb) encoding the catalytic T subunit in control (lanes 1 and 2) and 7 day-denervated (lanes 3 and 4) rat fast hindlimb muscles. The size of the bands was determined by comparing their positions relative to 18S and 28S rRNAs. Note the marked reduction in the levels of both AChE transcripts in denervated muscles. Similar results were obtained with muscles denervated for 1, 2 and 4 days. In these experiments, levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA served as an internal standard.



denervation was identical ($P > 0.05$) between adult and post-natal rats (Figure 4.1C). However, although AChE transcript levels in post-natal muscles were reduced following denervation, they displayed nonetheless, a significantly smaller decrease ($P < 0.05$) than that seen in adult denervated muscles (Figure 4.1B). For example, the abundance of AChE mRNAs in denervated muscles from post-natal rats was near control levels after 1 day of denervation (i.e., decreased by only 14%) whereas it was reduced by more than 70% in adult denervated muscles. In addition, the effects of denervation on AChE transcript levels in post-natal rats were significantly ($P < 0.05$) less pronounced throughout the time-course of the experiment since they remained at approximately 50% of control values.

Molecular Mechanisms Underlying the Changes in AChE mRNA Levels in Adult versus Post-Natal Denervated Muscles. To determine whether alterations in the transcriptional activity of the AChE gene could account for the observed changes in mRNA levels following denervation, we first performed run-on assays with nuclei from control and denervated muscles excised from adult and post-natal rats. In agreement with previous studies that have shown enhanced transcription of AChR subunit genes in denervated muscles from post-natal (Fontaine and Changeux, 1989; Merlie and Kornhauser, 1989; Tsay and Schmidt, 1989; Gundersen et al., 1993; Bessereau et al., 1994; Walke et al., 1996) and adult (Gundersen et al., 1993; Merlie et al., 1994) animals, we also observed an increase in the rate of transcription of the α -subunit gene in denervated muscles (for example, see Figure 4.4). In these experiments, transcription of the AChE gene was also increased 2-3-fold ($P < 0.05$) in 2-day denervated post-natal muscles (Figure 4.3A and 4.4). This increase was transient however,

Figure 4.3 Transcription of the AChE gene in rat hindlimb muscles. (A) and (B) are representative run-on assays performed with nuclei isolated from control (CTL) and denervated (DEN) muscles from post-natal (2-day denervated) and adult (7-day denervated) muscles, respectively. β -actin (actin) served as the internal standard. Also shown in (C), are examples of the background hybridization signals obtained with empty plasmids for actin (SK) and AChE (pEF-BOS).

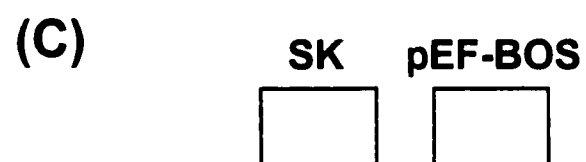
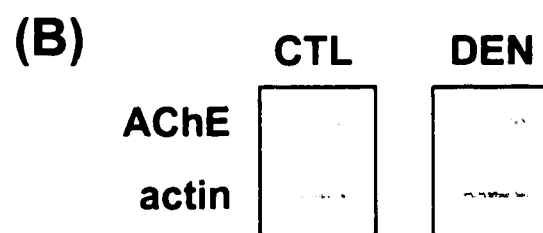
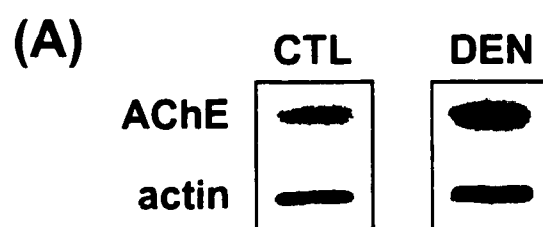
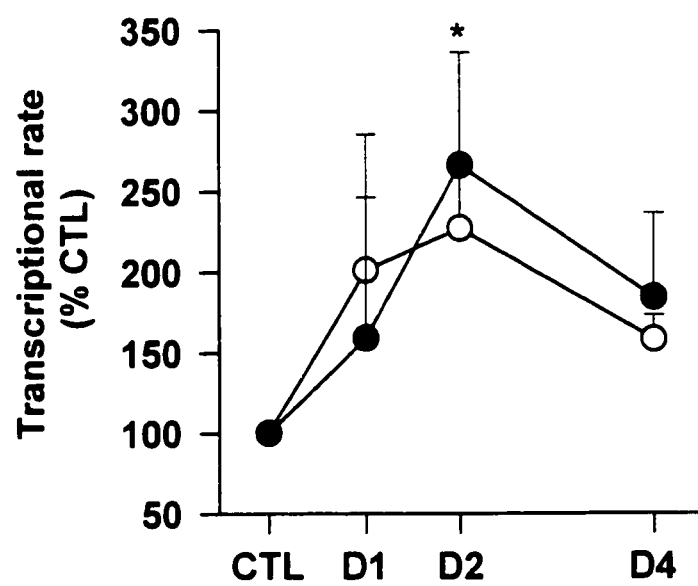


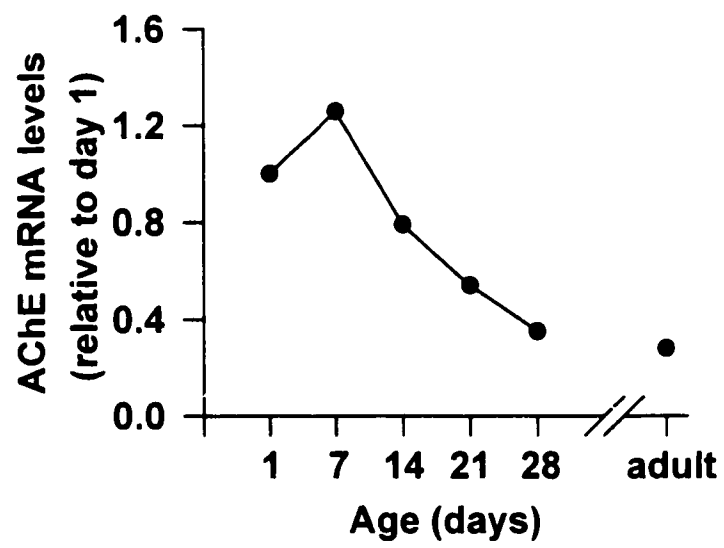
Figure 4.4 Quantitative analysis of AChE and AChR α -subunit transcriptional activity obtained with control (CTL) and 1, 2 and 4 day-denervated (D1 to D4) post-natal muscles as assessed by nuclear run-on assays. Note the expected increase in the transcriptional activity of the AChR α -subunit gene in denervated muscle (see Tsay and Schmidt, 1989) as well as the parallel and transient increase in the transcriptional rate for both the AChE (open circles) and AChR α -subunit (closed circles) genes. Similar results were obtained with control and denervated muscles from neo-natal (2 to 3 day-old) muscles. β -actin served as the internal standard. Pools of 8 muscles were used for each control and denervation time point. Data are mean $\pm$ SEM from three separate experiments. Asterisk denotes significant difference from control for AChE and AChR α -subunit ($P < 0.05$; Student's *t*-test).



since the rate of transcription of the AChE gene returned towards control levels by day 4. Interestingly, the pattern of expression of the AChE gene in these denervated post-natal muscles resembled that seen for the AChR α -subunit gene (see Figure 4.4 and see also Tsay and Schmidt, 1989). Additional experiments performed with hindlimb muscles obtained from neonatal rat pups (2 to 3 day-old) denervated for 3 days showed a similar 2-3-fold increase in the transcriptional activity of the AChE gene in response to denervation. Conversely, no alteration in the rate of transcription of the AChE gene was observed in denervated muscles from adult animals (Figure 4.3B; range of 95-129% of control for day-1 to -7 denervated muscle) despite, as expected, an increase in the transcriptional activity of the MyoD gene (see Huang et al., 1993). In these experiments, we also noted that the transcriptional activity of the AChE and β -actin genes were higher in muscles from post-natal versus adult rats (see Figure 4.3). These data are entirely coherent with the observations that AChE mRNA levels are 3- to 4-fold higher in developing muscles compared to adult (Figure 4.5), and with the previous findings which showed that transcription of the β -actin gene indeed, decreases during post-natal muscle development (Cox and Buckingham, 1992).

We further assessed the expression of the rat AChE gene by directly injecting into control and denervated muscles, a rat AChE promoter-LacZ reporter gene construct (Chan et al., 1999). This approach has been used recently to examine the response of various AChR subunit genes to muscle denervation (Bessereau et al., 1994; Walke et al., 1996; Bessereau et al., 1998). As performed by Gundersen et al. (1993), we monitored the activity and the mRNA levels of β -galactosidase in post-natal and adult muscles, respectively, since enhanced proteolysis of the enzyme in adult denervated muscle renders determination of β -galactosidase

Figure 4.5 AChE mRNA levels in hindlimb muscles from post-natal and adult rats. Quantitation of AChE mRNA abundance at neo-natal day 1, post-natal day 7, 14, 21 and 28, and in adult muscles as assessed by RT-PCR. Data are presented as a percent of the levels seen in neo-natal day 1 muscles (pools of 5 muscles/ per data point).



activity unreliable. In excellent agreement with our nuclear run-on data (Figures 4.3 and 4.4), the activity of β -galactosidase was increased by approximately 3-fold ($P < 0.05$) in denervated post-natal muscles (Figure 4.6) whereas no significant change ($P > 0.05$) in its mRNA level was detected in adult muscle (Figure 4.7).

Figure 4.6 Activity of β -galactosidase following direct plasmid injection of a rat AChE promoter-reporter gene construct in control (CTL) and denervated (DEN) post-natal muscles. Note the 3-fold increase in β -galactosidase activity in denervated muscle. The magnitude of the increase is similar to that seen by nuclear run-on assays (see Figures 4.3 and 4.4). Data are means $\pm$ SEM (n = 5). Asterisk denotes a significant differences ($P < 0.05$; Student's *t*-test). β -galactosidase activity values are normalized to the activity of a co-injected CAT plasmid.

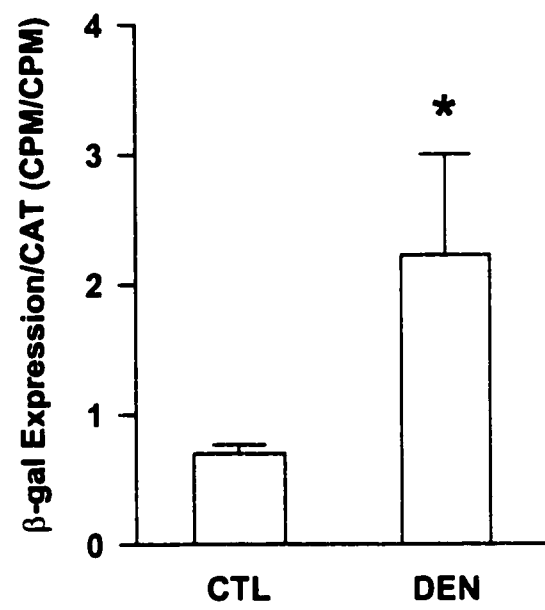
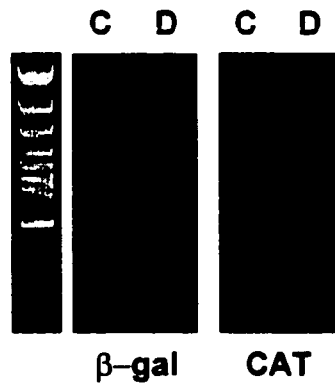
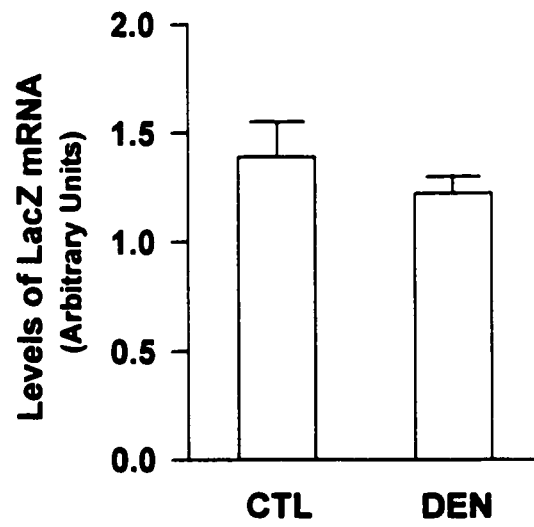


Figure 4.7 Levels of LacZ mRNA following direct plasmid injection of a rat AChE promoter-reporter gene construct in control and denervated adult muscles. (A) shows a representative example of an ethidium bromide-stained gel displaying PCR products for LacZ (β -gal) and CAT obtained from control (C) and denervated (D) adult muscles. (B) represents the quantitative analysis of LacZ PCR products normalized to the co-injected CAT plasmid obtained from control (CTL) and denervated (DEN) adult muscles. Note that LacZ mRNA levels from control and denervated samples are similar ($P > 0.05$) thus supporting results obtained using nuclear run-on assays (see Figure 4.3). Data are means $\pm$ SEM ($n = 9$).

(A)



(B)



DISCUSSION

Recent studies have shown that in vivo, the levels of AChE mRNA in skeletal muscle can display significant changes in response to alterations in the amount and pattern of neuromuscular activation (Cresnar et al., 1994; Michel et al., 1994; Sveistrup et al., 1995; Sketelj et al., 1998). In the present study, we have therefore examined the contribution of transcriptional versus post-transcriptional regulatory mechanisms in the activity-linked regulation of AChE transcripts in adult and post-natal skeletal muscles. Since our previous work showed that denervation of adult muscles induces large reductions in AChE mRNA levels (Michel et al., 1994), we chose, in the present study, to focus on this particular model to elucidate the nature of the underlying molecular mechanisms. Our results obtained using two complementary experimental approaches, indicate that post-transcriptional regulation appears as the primary mechanism controlling the levels of transcripts encoding AChE in adult skeletal muscle whereas in neo- and post-natal muscle tissues, expression of AChE mRNA appears regulated at both transcriptional and post-transcriptional levels.

Regulation of AChE Gene Expression in Adult Muscle

Several reports have demonstrated unequivocally the important role of neural electrical activation in regulating AChE enzyme activity in skeletal muscle (for review see Massoulié et al., 1993). For instance, muscle denervation in rodents leads to a substantial reduction in AChE enzymatic activity (Drachman, 1972; Butler et al., 1978; Fernandez and Duell, 1980; Michel et al., 1994; Boudreau-Larivière et al., 1997). In a recent study, we showed that 10

days of denervation resulted in a 10-fold reduction in enzyme activity which was accompanied by an equally pronounced decrease in AChE transcript levels (Cresnar et al., 1994; Michel et al., 1994). Our current results confirm and extend these initial findings on AChE mRNA levels in muscle. Indeed, we observed that the decrease in AChE mRNA levels following abolition of neuromuscular activity is extremely rapid since the amount of transcripts is reduced by approximately 90% within the first 48 hrs following denervation. In addition, we show by Northern blot analysis, that the two predominant mRNA species encoding the T catalytic subunit expressed in adult muscle (Legay et al., 1993b), are reduced to a similar extent and in parallel, in denervated muscle. As previously shown by others and in the present study, this effect of denervation stands in sharp contrast to the marked increase that occurs in the levels of the AChR α -subunit transcript in denervated muscle (for example see Merlie et al., 1984; Goldman et al., 1985; Goldman et al., 1988; Witzemann et al., 1991) thereby confirming that the regulation of these transcripts is clearly distinct in adult mammalian muscle.

The pronounced reduction in the levels of AChE mRNA could have resulted from alterations in the rate of mRNA synthesis and/or degradation. To examine this issue, we performed nuclear run-on assays as well as direct injections of a rat AChE promoter-reporter gene construct. Our complementary analyses revealed that the 10-fold reduction in AChE mRNA levels in adult muscle is not accompanied by parallel modification in the rate of AChE gene expression. Given that transcriptional control appears as the primary mechanism regulating AChR α -subunit mRNA levels in denervated muscle (see Fontaine and Changeux, 1989; Tsay and Schmidt, 1989), our data further highlight the discordance between the regulatory mechanisms controlling expression of the genes encoding AChE and the AChR α -

subunit. Taken together, our results are therefore coherent with the notion that in adult skeletal muscle, the activity-linked regulation of AChE mRNA levels occurs via post-transcriptional regulatory mechanisms.

Since alterations in message stability appeared as a key post-transcriptional event accounting for the modifications in the relative abundance of AChE transcripts in adult skeletal muscle, we attempted to measure in separate experiments, the stability of AChE mRNAs in control versus denervated adult muscles by treating animals with the transcriptional inhibitor actinomycin D, as previously described (Neville et al., 1992; Connor et al., 1996). Under these conditions however, estimation of the half-life for AChE mRNAs in both control and denervated muscles proved difficult given that animals did not survive beyond 8 hrs following transcription inhibition. Furthermore, AChE mRNA levels remained relatively stable within this time period making the necessary extrapolations to obtain half-life measurements unreliable (see also discussion in Yan and Booth, 1998). One possibility that could account for this latter observation is that transcriptional blockade induced by actinomycin D treatment may have inhibited the denervation-induced synthesis of specific RNA binding proteins that normally act to destabilize AChE mRNA. Interestingly, this mechanism has previously been postulated to play a role during transcription inhibition in myogenic cells growing in culture (Fuentes and Taylor, 1993).

Although the mechanisms responsible for the longevity of mRNAs in cells are largely undefined, specific mRNA *cis*-elements and *trans*-acting factors which appear to modify the degradation rate, have been identified (for reviews see Greenberg and Belasco, 1993; Sachs, 1993; Ross, 1995). For instance, the poly(A)⁺ tail of mRNAs appears to protect transcripts

from degradation whereas AU-rich elements (AURE) located within the 3' untranslated region (3'UTR) have been linked to mRNA destabilisation. The mRNA half-life is also dependent on interactions with trans-acting regulatory factors whose abundance or activity can be altered in response to changes in the cellular environment. In a recent study for example, an increase in the levels of transcripts encoding cytochrome *c* in rat muscles subjected to continuous low frequency stimulation, coincided with a reduction in the levels of RNA-protein interactions in the 3'UTR of the cytochrome *c* mRNA (Yan et al., 1996). It will therefore be interesting to determine therefore whether the large decreases in AChE mRNA levels seen in denervated muscles are accompanied by alterations in the pattern and/or abundance of RNA-protein interactions.

Regulation of AChE Gene Expression in Post-Natal Muscle

In contrast to the nearly identical response of AChR α -subunit transcripts to denervation in post-natal and adult muscles, the pattern of expression of AChE mRNAs following denervation differed significantly in developing versus mature muscles. Specifically, AChE mRNA levels in adult muscles were rapidly and markedly affected following denervation since they decreased by ~ 90% within the first 48 hrs. In denervated post-natal muscles however, the reduction was clearly not as pronounced and, accordingly, levels of AChE transcripts were approximately 50% of those seen in control muscles even after 7 days of denervation. Interestingly and in agreement with our findings, previous studies have shown in denervated post-natal muscles, an increase in AChE activity in extrasynaptic compartments of muscle fibers (Lubinska and Zelena, 1966).

Our nuclear run-on assays have revealed that the transcriptional activity of the AChE gene is increased in denervated post-natal muscle. Furthermore, direct plasmid injection of a rat AChE promoter-reporter gene construct into muscles from post-natal animals also showed a 3-fold increase in β -galactosidase activity thereby confirming the direction as well as the magnitude of the transcription induction as determined by nuclear run-on assays. Although the rat AChE promoter fragment tested likely does not contain all of the regulatory elements present in the endogenous gene, we are confident that most of the essential cis-elements that mediate the activity-linked regulation of the AChE gene are found in the NRAP promoter fragment given the similar rates of transcriptional induction observed by nuclear run-on and plasmid injection assays. This elevation in AChE gene transcription likely accounts for the attenuated reduction in AChE mRNA levels observed following denervation of post-natal muscle. However, although transcriptional activation of the AChE gene occurs in denervated post-natal muscle, our experiments also suggest that post-transcriptional control is implicated in post-natal rats given the observed reduction in AChE transcript levels. Taken together with the finding obtained with adult muscles, these data indicate that the activity-linked expression of AChE mRNAs in muscle is subjected to developmental influences and that distinct molecular mechanisms operate at specific stages of muscle fiber maturation.

The reason for the differential response of the gene encoding AChE seen in neo- and post-natal versus adult muscles remains unclear. Based on our experiments using a rat AChE promoter-reporter gene construct however, it appears that alterations in the relative abundance of trans-acting factors in developing versus adult muscles, which ultimately regulate the transcriptional activity of the AChE gene, account for the observed differences. In this

context, age-associated changes in the basic mechanisms regulating transcription have been observed in a variety of experimental systems (see Hsieh et al., 1998 and references therein). It would therefore be important in future studies to identify the specific 5' DNA regulatory elements within the AChE gene that mediate this age-dependent, activity-linked transcriptional regulation.

One attractive possibility to explain the different levels of transcription factors targeting genes encoding synaptic proteins in developing versus adult muscles is that their relative abundance may depend on the level of maturity of the postsynaptic apparatus. Indeed, ultrastructural studies have shown that although exploratory motor axons reach the surface of developing myotubes at approximately E13-14, full differentiation of the presynaptic nerve terminals and of the postsynaptic membrane of the neuromuscular junction requires several weeks and occurs not only during embryonic development but also in neo-natal and post-natal muscles (Kullberg et al., 1977). For example, polyinnervation remains for 2 to 3 weeks post-natally before it is eliminated thereby indicating that the establishment of the mature neuromuscular junction occurs only once this process of synapse elimination has subsided (Dennis, 1981). On the basis of our current results and those of others (Fontaine and Changeux, 1989; Tsay and Schmidt, 1989; Piette et al., 1993; Legay et al., 1995), it appears that during synaptogenesis and maturation of the neuromuscular junction, the activity-linked regulation of the AChE gene, involves transcriptional and post-transcriptional regulatory mechanisms and that in mature muscle, regulation of this synaptic protein is achieved primarily via post-transcriptional events. Accordingly, it appears therefore that the activity-linked

transcriptional regulation of the AChE gene demonstrates a high level of plasticity during muscle development when maturation of neuromuscular junctions is still occurring.

CHAPTER V

An Intronic Enhancer Containing an N-box Motif is Required for Synapse- and Tissue-Specific Expression of the Acetylcholinesterase Gene in Skeletal Muscle Fibers

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Mutant promoter constructs were prepared by R. Chan and L. Angus. EMSAs were carried out by L. Angus. Hematopoietic cell culture experiments were conducted by Dr. R. Chan. F. Mankal was involved in the direct gene transfer experiments.

ABSTRACT

mRNAs encoding acetylcholinesterase (AChE) are highly concentrated within the postsynaptic sarcoplasm of adult skeletal muscle fibers where their expression is markedly influenced by nerve-evoked electrical activity. To determine whether transcriptional regulatory mechanisms account for the synaptic accumulation of AChE transcripts at the mammalian neuromuscular synapse, we cloned a 5.3 kb DNA fragment that contained the 5' regulatory region of the rat AChE gene and generated several constructs in which AChE promoter fragments were placed upstream of the reporter gene LacZ and a nuclear localization signal (nlsLacZ). Using a recently described transient expression assay system in intact skeletal muscle, we show that this AChE promoter fragment directs the synapse-specific expression of the reporter gene. Deletion analysis revealed that a 499 bp fragment located in the first intron of the AChE gene is essential for expression in muscle fibers. Further analysis showed that sequences contained within this intronic fragment were: i) functionally independent of position and orientation; and ii) inactive in hematopoietic cells. Disruption of an N-box motif located within this DNA fragment reduced by more than 80% the expression of the reporter gene in muscle fibers. In contrast, mutation of an adjacent CArG element had no effect on nlsLacZ expression. Taken together, these results indicate that a muscle-specific enhancer is present within the first intron of the AChE gene and that an intronic N-box is essential for the regulation of AChE along multinucleated skeletal muscle fibers.

INTRODUCTION

Acetylcholinesterase (AChE) is an essential synaptic component in the nervous system since it is responsible for the rapid hydrolysis of acetylcholine released from nerve terminals. Although a single gene encodes AChE, alternative splicing as well as distinct processing of the catalytic subunits account for the multiplicity of molecular forms expressed at specific subcellular locations in muscle, neuronal and hematopoietic cells (Massoulié et al., 1993; Taylor and Radic, 1994). In skeletal muscle, AChE accumulates at the neuromuscular synapse where its expression is known to be markedly influenced by the levels of superimposed neuronal activation (Boudreau-Larivière et al., 1997; Sketelj et al., 1998 and references therein).

Despite the wealth of information available on the plasticity of AChE confronted with altered levels of neuromuscular activation, our knowledge of the cellular and molecular mechanisms involved in the localization and activity-linked regulation of AChE in muscle is still rudimentary. Several recent studies have begun to explore the molecular basis underlying the accumulation of AChE at both avian and mammalian neuromuscular synapses. Results of these studies have shown that AChE mRNAs are ~10-fold more abundant in synaptic versus extrasynaptic regions of muscle cells (Jasmin et al., 1993; Michel et al., 1994; Legay et al., 1995) yet, the molecular events responsible for this compartmentalized expression of AChE transcripts remain to be elucidated. In this context, several levels of regulation including transcriptional as well as post-transcriptional, may be considered. For instance, several recent studies have shown that post-transcriptional mechanisms operating at the level of transcript

stability, play a significant role in the regulation of AChE in differentiating myogenic (Fuentes and Taylor, 1993), neuronal (Coleman and Taylor, 1996) and hematopoietic (Chan et al., 1998) cells maintained in culture. Alternatively, results from several laboratories have shown that the synaptic accumulation of mRNAs encoding the various acetylcholine receptor (AChR) subunits within the postsynaptic sarcoplasm results primarily from localized gene transcription occurring in junctional myonuclei (see Hall and Sanes, 1993; Duclert and Changeux, 1995; Burden, 1998).

In the present study, we have thus examined whether local transcriptional activation of the AChE gene contributes to the synaptic accumulation of AChE transcripts within the postsynaptic sarcoplasm. Specifically, we determined the transcriptional activity and pattern of expression of several rat AChE promoter-reporter gene constructs along multinucleated muscle fibers *in vivo*. To this end, we employed a transient expression assay system in intact skeletal muscle which has recently proven useful to study the activity of several AChR promoters in synaptic versus extrasynaptic compartments of muscle cells (see Duclert et al., 1993; Koike et al., 1995; Duclert et al., 1996; Walke et al., 1996; Sapru et al., 1998a; Schaeffer et al., 1998).

MATERIALS and METHODS

Screening of a Rat Genomic Library. Genomic DNA from rat kidneys was partially digested with MboI and size-fractionated by ultracentrifugation on a continuous 10-40% sucrose gradient. DNA fragments between 9 to 23 kilobases were ligated to lambda DASH II/BamHI vector and packaged using a lambda DASH II/Gigapack II Cloning kit (Stratagene). Approximately 1.6 million plaques were then screened with a ³²P-labelled rat AChE cDNA corresponding to the common coding region (879-1722 bp) (Legay et al., 1993b). A positive clone containing a ~9 kb insert was further purified. PstI digestion of this clone yielded a 950 bp fragment which hybridized to the 5' end of the rat AChE cDNA. This clone was then sequenced and designated as the rat AChE promoter (RAP) on the basis of its homology to both mouse and human AChE promoters (Figure 5.1). RAP was subsequently used as a probe to identify a 5.3 kb fragment, designated as the giant rat AChE promoter (GRAP), resulting from NcoI-SspI digestion of the initial ~9 kb genomic clone. Sequences were obtained using both the dideoxynucleotide chain termination method with Sequenase Version 2.0 (United States Biochemical) and the Dye-Deoxy Termination cycle sequencing (Applied Biosystems).

Reporter Constructs and In Vivo Analyses of Promoter-Reporter Gene Expression. Rat AChE promoter fragments (Figure 5.3) were subcloned into a LacZ reporter vector containing a nuclear localization signal (nls) (Gundersen et al., 1993). Mutagenesis was performed using the Altered Sites II in vitro mutagenesis system (Promega). To prepare the thymidine kinase (TK)-LacZ constructs containing an intronic region from the rat AChE gene, a fragment from

the first intron was excised using *Apa*I and *Sac*I and subcloned into bluescript SK. This DNA fragment was subsequently excised using *Kpn*I and subcloned into pGEM 7Z. The orientation of the fragment was assessed using *Eco*R1 and *Fsp*I, and the intronic fragment was subcloned in either orientation upstream of the basic pTK-LacZ vector (Clontech).

Plasmid DNA was prepared using the Qiagen mega-prep procedure and final pellets were resuspended in sterile phosphate-buffered saline to a final concentration of 2-4 µg/µl. In vivo gene transfer into tibialis anterior (TA) muscles of mice was performed as described previously (Duclert et al., 1993; Koike et al., 1995; Duclert et al., 1996; Gramolini et al., 1997; Gramolini et al., 1998). Seven to 14 days later, injected muscles were excised and frozen in isopentane pre-cooled with liquid nitrogen. Cryostat tissue sections were processed histochemically for the simultaneous demonstration of β -galactosidase (β -gal) and AChE. The position of blue myonuclei indicative of AChE promoter activity, was determined and compared with the presence of neuromuscular junctions using a procedure established recently (Koike et al., 1995; Gramolini et al., 1997; Gramolini et al., 1998).

To determine biochemically the activity of various promoter-reporter gene constructs in vivo, 20 µl of a DNA mixture containing the appropriate AChE or TK construct and a plasmid encoding chloramphenicol acetyltransferase (CAT) driven by the constitutive SV40 promoter (used to monitor the efficiency of transduction), were injected into TA muscles. Muscles were excised seven days later and frozen in liquid nitrogen. Whole muscles were subsequently homogenized in a reporter lysis buffer (Promega) and the activity of β -gal and CAT was determined by luminescence (Clontech) and biochemical assays (Promega), respectively, using available kits. The β -gal values obtained with the promoter constructs

were first corrected by subtracting the basal activity derived from a promoterless nlsLacZ plasmid, which gave values similar to those seen with non-injected muscles. The resulting β -gal activity was then normalized to CAT levels.

Transfection of Rat AChE Promoter-Reporter Gene Constructs in Cultured Cells. Friend murine erythroleukemia (MEL) cells were grown as described (Chan et al., 1998), and transfected using Superfect reagents (Qiagen). Forty-eight hrs later, expression of β -gal driven by AChE promoter fragments was determined and compared to a constitutively expressed CAT plasmid. C2 muscle cells were grown and transfected as described (Gramolini et al., 1998).

Nuclear Extract and Electrophoretic Mobility Shift Assays. Muscle nuclei were collected as described previously (Ray et al., 1995) with the exception that an additional purification step was performed by resuspending the nuclei in 27% Percoll and centrifugation at 29,000 x g for 15 min at 4 °C (Hahn and Cox, 1990). Nuclear proteins were extracted for 45 min on ice in a high-salt buffer containing 20 mM Hepes-KOH (pH 7.9), 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% glycerol, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM spermidine, 0.15 mM spermine, and 5 μ g/ml of aprotinin, leupeptin and pepstatin (Dignam et al., 1983). After centrifugation, the supernatant was diluted to reduce NaCl concentration to 150 mM and stored at -80 °C.

For electrophoretic mobility shift assays (EMSA), synthetic oligonucleotides were:

5' CCTCGGGGTTCCGGAATTTCCAC 3' (sense) and

5'GTGGAAATTCCGGAACCCCGAGG3' (antisense) for the promoter N-box; 5'CTGGAGAAGCCGGAACCTACAGCAG3' (sense) and 5'CTGCTGTAGTTCCGGCTTCTCCAG3' (antisense) for the intronic N-box at position +755 bp ; 5'CCGGAGCTCCCGGAACACAGACGTC3' (sense) and 5'GACGTCTGTGTTCCGGGAGCTCCGG3' (antisense) for the intronic N-box at position +823 bp; and 5'CTGCGACCCTAATTAGGGTCCCTA3' (sense) and 5'TAGGGACCCTAATTAGGGTCGCAG3' (antisense) for the CARG-box. Annealed oligonucleotides probes were labelled with T4 polynucleotide kinase and [γ - 32 P]ATP. The binding reaction mixture included 0.2 ng labelled probes, 1.5-5 μ g of poly (dI-dC) and 4 μ g of nuclear extract. Polyacrylamide gel electrophoresis and exposure to Kodak XAR-5 films were performed as described (Rosner et al., 1990). For competition assays, a 250-fold molar excess of unlabelled oligonucleotides was added to the binding reaction mixture prior to the addition of the labelled probes. Finally, antibodies to GABP α and β (provided by Dr. S. McNight, Tularik Inc.) were used in supershift assays.

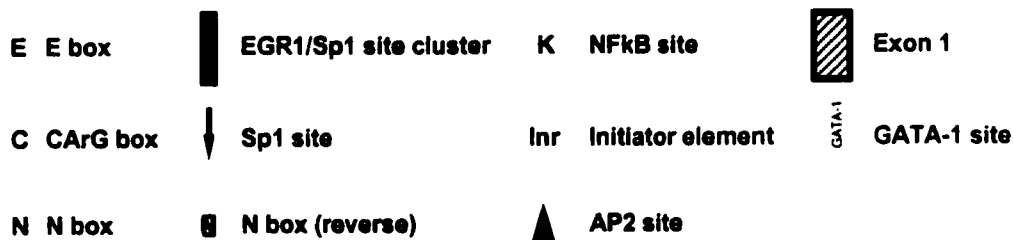
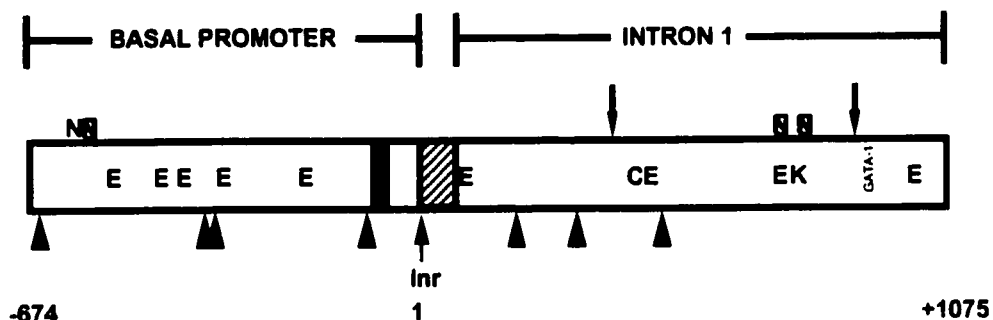
RESULTS

Sequence of the rat AChE promoter. We isolated a 5.3 kb DNA fragment from a rat genomic library using the 5' end of the rat AChE cDNA. Sequence analyses of this DNA fragment revealed that it is extremely GC-rich and TATA-less. As previously reported for mouse and human AChE promoters (Ben Aziz-Aloya et al., 1993b; Li et al., 1993a), this 5' DNA fragment contains an initiator element (Inr) (Figure 5.1). Since the transcriptional activation of TATA-less promoters is effected through the binding of TFIID at the Inr (Smale and Baltimore, 1989; Pugh and Tjian, 1991) thereby providing the primary transcriptional start site in this type of promoter (Li et al., 1993a; Kollmar et al., 1994), we assigned the first nucleotide of the Inr in this DNA fragment as +1 (Figure 5.1). Alignment of the region from -384 to +134 with the corresponding regions of the mouse (Li et al., 1993a) and human (Ben Aziz-Aloya et al., 1993b) promoters showed an overall identity of 99.5% and 69.5%, respectively (Figure 5.1, upper panel). On this basis, we designated this DNA fragment as GRAP (Giant Rat AChE promoter).

Further analyses of a smaller DNA fragment termed NRAP (see below), revealed the presence of several consensus sequences for DNA-binding proteins (Figure 5.1, lower panel). In addition to an EGR-1/Sp1 cluster, there are multiple E-boxes as well as a CArG element. Moreover, there are also binding sites for AP2, NF κ B and GATA-1. In view of the recent evidence showing the crucial role of an N-box motif (TTCCGG) in the synapse-specific expression of AChR subunit (Koike et al., 1995; Duclert et al., 1996; Walke et al., 1996) and utrophin (Gramolini et al., 1998) genes, it is noteworthy that there are four N-boxes present

Figure 5.1 5' regulatory region of the rat AChE gene. (A) shows the nucleotide sequence of the rat AChE promoter aligned with corresponding regions from the mouse and human genes. The first nucleotide in the initiator element (Inr; underlined) is designated as +1. Note that the sequence in the rectangular box (+5 to +75) exhibits more than 97% identity with exon 1 in the mouse and human AChE genes. Arrows indicate EGR-1/Spl clusters; oval shows an AP2 site; and hatched regions indicate E-boxes. (B) is a schematic representation of the Inr and other consensus sites for DNA binding proteins present in a larger promoter fragment as well as in the first intron of the gene.

Rat	aagtatt aggcctctgatct ttctgga ttagagctgtcag tgtgtccttccgtccgtgaaag tcggtctgt	-314
Mouse	aaatatt aggcctctgatct ttctgga ttagagctgtcag tgtgtccttccgtccgtgaaagccggtctgt	
Human	taacttc accagcctcgagcgagaccgacaggtgtccaagtgtgtccgtccgtctgtgaaag tcgtccctg	
Rat	ctgtgac ttgtcaccgcagg agactgt cgctcggtgaaagggtgtctggtgtctccgcaaggctgcggtcc....g	-243
Mouse	ctgtgac ttgtcaccgcagg agactgt cgctcggtgaaagggtgtctggtgtctccgcaaggctgcggtcc....g	
Human	cgtttgt ctgtgcc tgtcac tgcata ggtcgt ccgtctg cgctgt .ctgtctccacgggtctctgtccgtctg	
Rat	tctgtcagctgggtgtctgcccaccgctgggggtctgcgggtc tgtgggtctgtctg	-185
Mouse	tctgtcagctgggtgtctgcccaccgctgggggtctgcgggtc tgtcggtctgtctg	
Human	tctgtcagctgggtgtctactcccgctgcaggtctttgggtcgctgtctactgcgctctgtctgtcggtcgg	
Rat	aatccac cactgga gtgtgt ctgggcc cccgt ccccggggtctcgggacttgacggggagggagagggaggt	-110
Mouse	aatctac cactgga gtgtgt ctgggt cccgt ccccggggtctcgggacttgaa .agggagggaggaggagggaggt	
Human	cgtctgg cactgga gtgct ctgggcc cgggg tctccgggtctcggc tccaaggaggagagagaggaggaggt	
Rat	ggcagcc cggggga gcgggg agggcg ggggcg gagacagtggg cgggcgggggcgcgtgcggcccg	-41
Mouse	ggcagcc cggggga gcgggg agggcg ggggcg gagacagtggg cgggcgggggcgcgtgcggcccg	
Human	ggccacc cgggaga gcgggg agggcg ggggcg gagacagtggg cgggcgggggcgcgtgcggcccg	
Rat	aggggtgtgtgcggggggccgga .gggcgggtgtcactgtcggtcagcctgcgcgggggaacattggcgctc	+34
Mouse	aggggtgtgtgcggggggccgga .gggcgggtgtcactgtcggtcagcctgcgcgggggaacattggcgctc	
Human	a .gggtgtgtgcggggggccgga .gggcgggtgtcactgtcggtcagcctgcgcgggggaacattggcgctc	
Rat	cagctcc cggcgcg gccga cccggcc cggcttggcgccctcaggtcagctcccaaccacccgggacccctcca	+109
Mouse	cagctcc cggcgcg gccga cccggcc cggcttggcgccctcaggtcagctcccaaccacccgggacccctcca	
Human	cagctcc cggcgcg gccga cccggcc cggcttggcgccctcaggtcagctcccaaccacccgggacccctcca	
Rat	ccggttcgagaccaatccgctttct	+134
Mouse	ccgagtcgggaccaatccgaatccc	
Human	 ttaaggcccggtc	



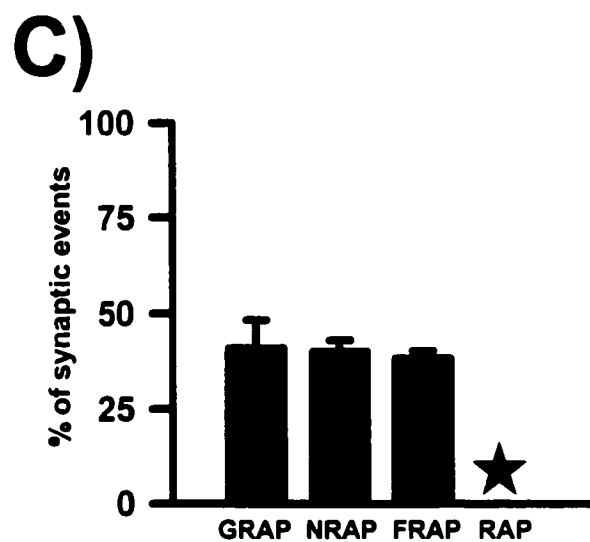
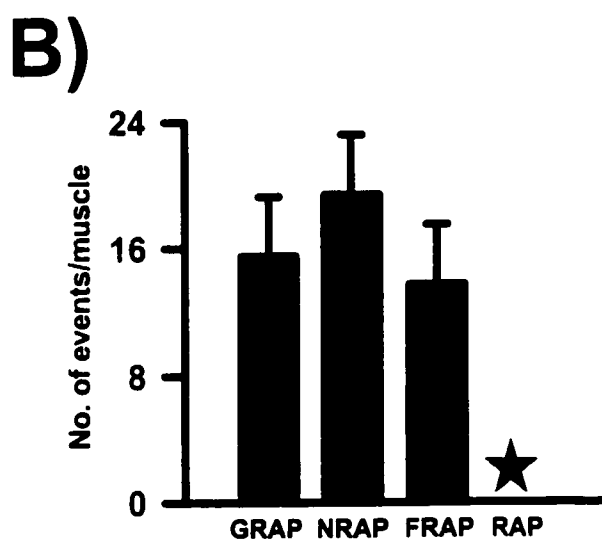
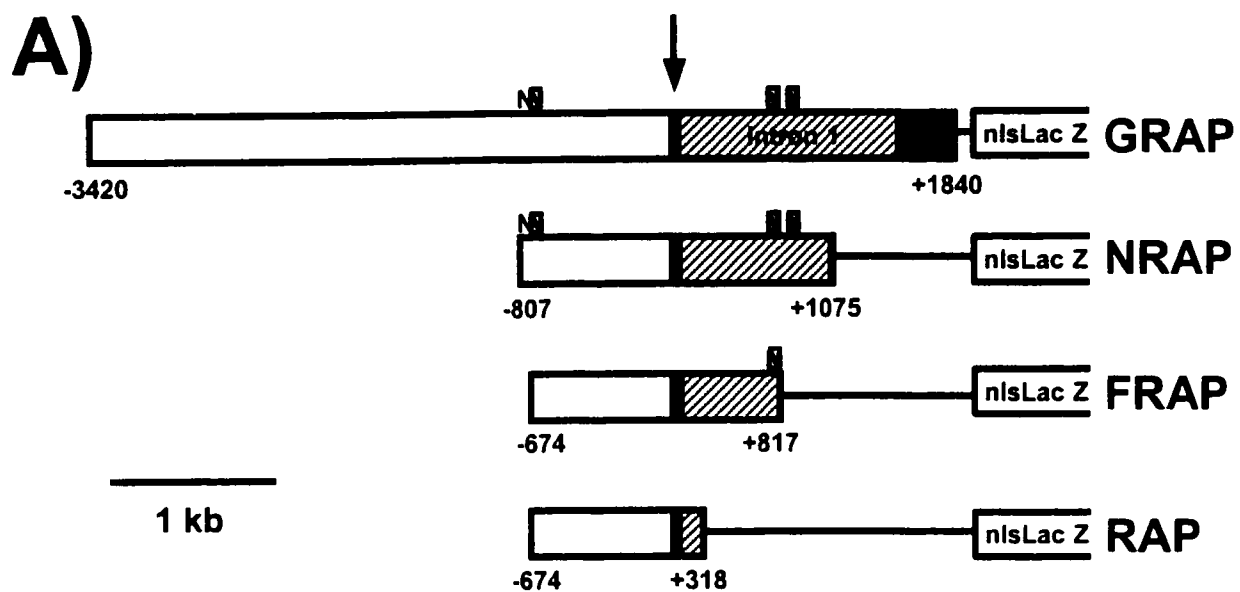
in NRAP (N-box containing RAP) with three being in a reverse orientation. Interestingly, several of the putative sites for DNA binding proteins including two N-boxes, are located in intron 1 of the rat AChE gene.

Expression of AChE Promoter-Reporter Gene Constructs in Muscle. To investigate the functional competence of the cloned DNA fragment in driving transcription in vivo, we first transduced mouse TA muscle fibers with plasmids containing various AChE promoter fragments linked to nlsLacZ (see schematic representation in Figure 5.3A) using a procedure recently developed (see for example Duclert et al., 1993; Koike et al., 1995; Duclert et al., 1996; Walke et al., 1996; Gramolini et al., 1997; Gramolini et al., 1998; Sapru et al., 1998a; Schaeffer et al., 1998). Histochemical analysis for detection of β -gal activity in transduced muscle fibers showed the presence of distinct blue myonuclei (Figure 5.2). To assess whether GRAP conferred synapse-specific expression to the reporter gene in muscle fibers, the position of blue myonuclei was determined and compared to the presence of neuromuscular junctions identified by AChE histochemistry. Quantitative analysis revealed that GRAP was capable of directing the preferential expression of the reporter gene in synaptic compartments of muscle fibers since approximately 40% of all the events were synaptic (Figure 5.3). This percentage of synaptic events fits nicely with those recently obtained for the AChR δ and ϵ subunit promoters (Koike et al., 1995; Duclert et al., 1996) thereby suggesting that local activation of the AChE gene contributes to the enrichment of AChE transcripts within the postsynaptic sarcoplasm of muscle fibers. By contrast, expression of β -gal was more homogeneous along muscle fibers following direct injection of a plasmid containing nlsLacZ driven by the

Figure 5.2 Expression of AChE promoter-reporter gene constructs in synaptic compartments of mouse TA muscle fibers. Shown are cryostat sections stained histochemically for the simultaneous demonstration of β -gal (blue staining) and AChE (brown staining) activity. Note that the presence of blue nuclei coincides with the occurrence of neuromuscular junctions in A, B and C reflecting AChE promoter activity within junctional myonuclei. D and E are examples of promoter-reporter activity in extrajunctional nuclei. Bar = 75 μ m.



Figure 5.3 (A) is a schematic representation of several AChE promoter-reporter gene constructs used for the in vivo studies. Note that the four N-boxes (N) found in GRAP are retained in NRAP whereas only the first intronic N-box at position +755 bp, is still present in FRAP (shaded N-boxes indicate those that are in reverse orientation). Arrow points to the first nucleotide of the Inr. (B and C) show the total number of events and the percentage of synaptic events seen in TA muscles following injection of the different constructs, respectively. Note that the pattern of expression of the reporter gene is unchanged despite large deletions of the 5' and 3' regions in the original 5.3 kb fragment (compare GRAP with NRAP and FRAP). However, note that deletion of an additional 499 bp intronic region abolished AChE promoter activity (compare FRAP vs RAP). Star indicates that no expression was detected. A minimum of 10 muscles were analyzed per construct. Mean $\pm$ SEM are shown.



constitutive CMV promoter since, as expected (see Koike et al., 1995; Duclert et al., 1996; Gramolini et al., 1997; Gramolini et al., 1998), only ~12% of all events were synaptic.

Intron 1 is Essential for AChE Gene Expression in Muscle. To delineate DNA elements responsible for AChE expression in muscle, we deleted the bulk of the 5' and 3' ends in GRAP up to the region containing the N-boxes in the promoter and intronic regions (see NRAP in Figure 5.3), and injected the NRAP-nlsLacZ construct into TA muscles. Histochemical staining showed that the pattern of β -gal expression in transduced fibers was similar to that seen following injections of plasmids containing GRAP-nlsLacZ indicating that NRAP is sufficient to drive the preferential synaptic expression of the reporter gene in muscle. To further characterize the DNA regulatory elements that confer synaptic expression, we deleted additional regions in NRAP. As shown in Figure 5.3, removal of 133 nt (-807 to -674 bp) and 258 nt (+817 to +1075 bp) encompassing respectively the two N-boxes in the promoter region and one N-box in the first intron (see FRAP; functional RAP in Figure 5.3) thereby leaving only one N-box intact in the intronic region, did not markedly affect the pattern of expression of the reporter gene (Figure 5.3). However, further deletion from +318 to +817 in intron 1, completely abolished β -gal expression in muscle fibers (RAP in Figure 5.3) suggesting that this intronic region is essential for expression of the AChE gene in muscle.

To determine whether this intronic fragment is involved in enhancing preferentially the expression of the AChE gene in muscle, we next transfected hematopoietic and myogenic cells grown in culture. For these experiments, we compared the activity of FRAP- and RAP-nlsLacZ constructs in MEL versus C2 cells. In striking contrast to our findings obtained with

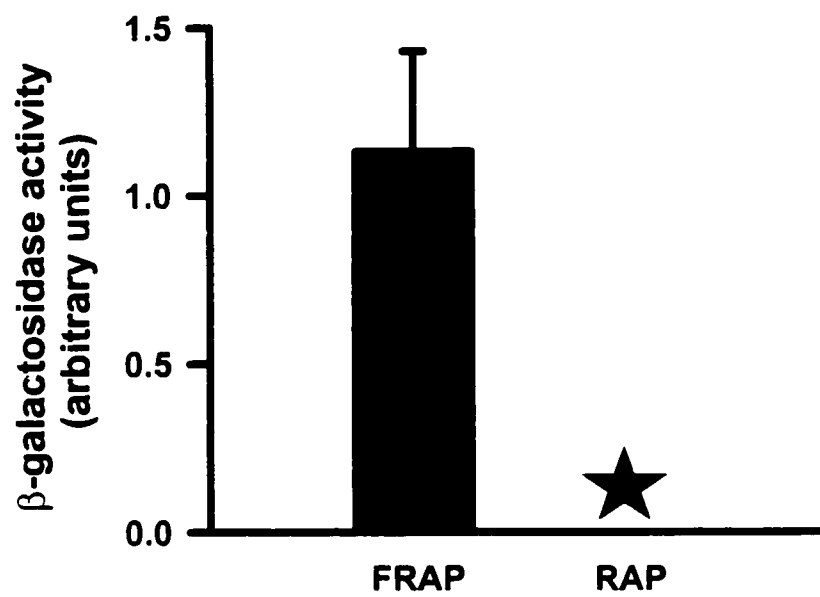
muscle fibers in vivo (Figure 5.3) and myotubes in culture (Figure 5.4A), we observed that both constructs had a similar transcriptional activity in hematopoietic cells (Figure 5.4B) indicating that the additional intronic region present in FRAP is distinctively involved in muscle-specific expression of the AChE gene.

In subsequent experiments, we examined whether the intronic fragment located between +318 and +817 could act as an enhancer of transcription. To this end, we engineered promoter-reporter gene constructs in which this DNA fragment was placed 5' of the TK promoter fused to LacZ. Direct injections of these constructs into mouse muscle indicated that the basal TK promoter induced a low level of reporter gene expression in transduced fibers (Figure 5.5). However, we noted a large and significant increase ($P < 0.001$) in β -gal activity when these constructs contained the AChE intronic region from +318 to +817 in either orientation.

Role of the Intronic N-box in AChE Gene Expression. Since the expression studies indicated that important regulatory elements are contained within intron 1 of the AChE gene, ie between +318 to +817 bp, we next examined the role of specific DNA consensus elements known to be critical for expression of a subset of genes in skeletal muscle. Initially, we determined whether the CArG-box is functionally important for expression of the AChE gene by mutating its core consensus sequence (see Figure 5.6A and B) and by comparing expression of the nlsLacZ reporter gene driven by either NRAP or its mutated counterpart mC-NRAP, following direct gene transfer experiments performed in TA muscles. Despite the loss of binding affinity for nuclear proteins, the mC-NRAP promoter fragment led to a level

Figure 5.4 Expression of AChE promoter-reporter gene constructs in myogenic and hematopoietic cells grown in culture. C2 and MEL cells were transfected with plasmids containing the reporter gene nlsLacZ and the AChE promoter fragment FRAP or RAP. Note that in contrast to myogenic cells (A; see also Figure 5.3), both constructs were equally active in hematopoietic cells (B). In these assays (3 independent experiments performed in triplicate), transfection efficiency was monitored by determining the expression of a constitutively expressed CAT plasmid. Star indicates that no expression was detected. Mean $\pm$ SEM are shown.

A)



B)

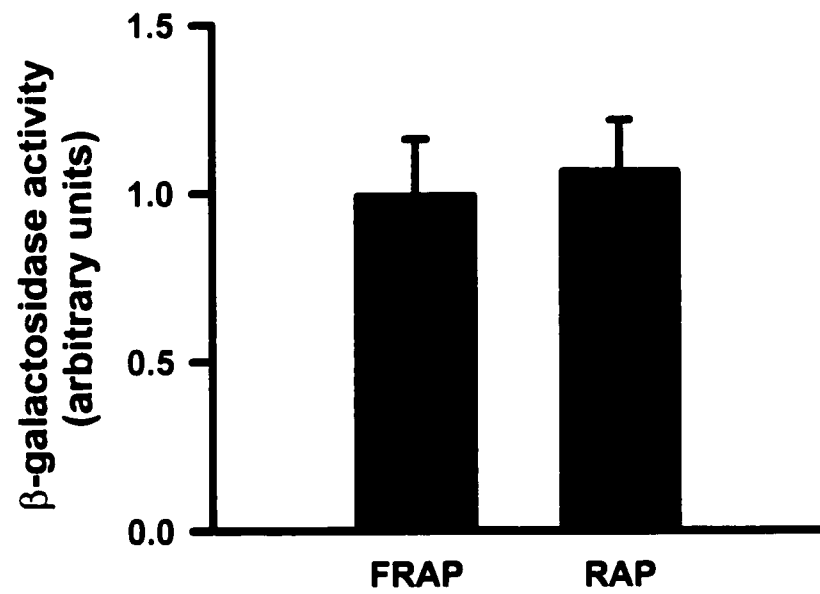


Figure 5.5 Expression of β -gal in TA muscle fibers following injection of a plasmid containing the reporter gene LacZ with and without the intronic fragment placed in either orientation (r=reverse) upstream of the thymidine kinase (TK) promoter. Note the significant ($P < 0.001$; ANOVA) ~ 6-fold induction in β -gal activity with the presence of the intronic DNA fragment in either orientation. A minimum of 12 muscles per construct were injected, and expression of β -gal was normalized to the activity of a co-injected CAT plasmid used to monitor transduction efficiency. Mean $\pm$ SEM are shown.

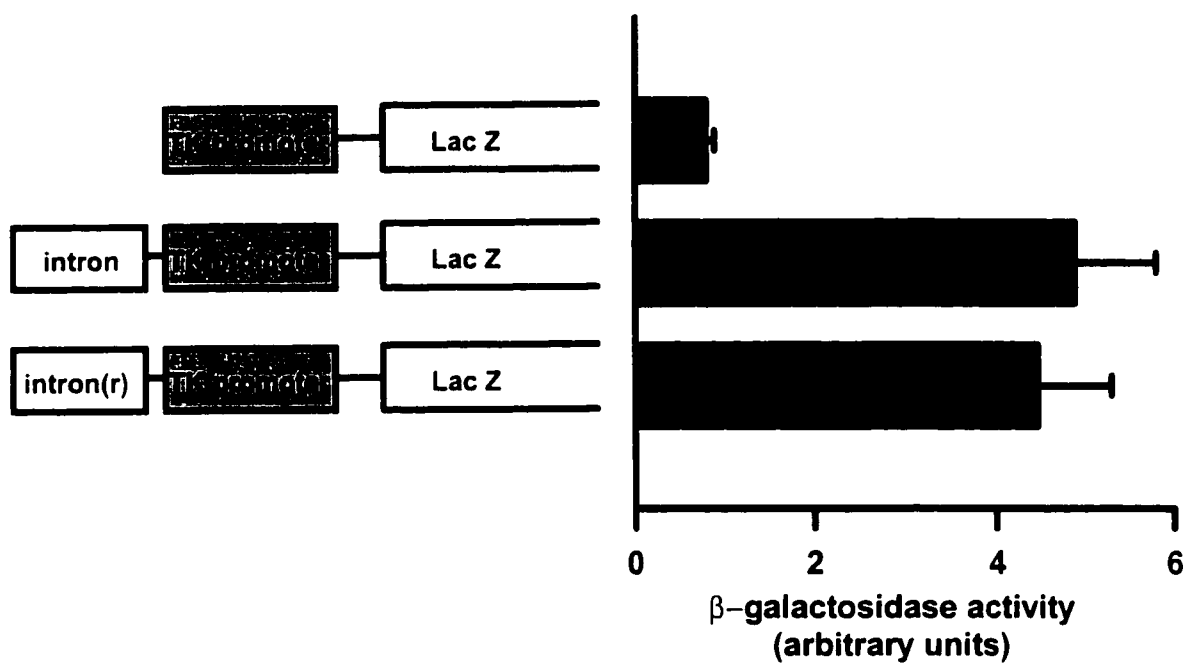
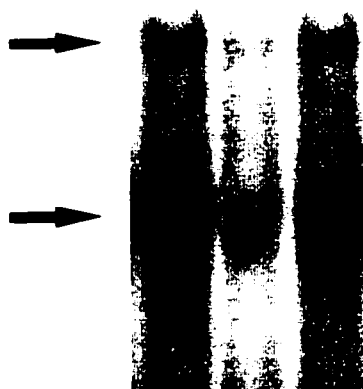


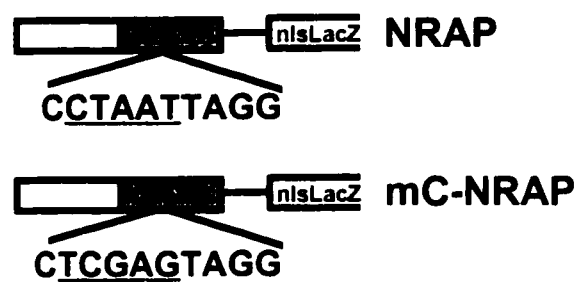
Figure 5.6 Disruption of the CArG element does not affect expression of AChE promoter-reporter gene constructs in TA muscle. (A) shows an EMSA using radiolabelled oligonucleotides containing the CArG-box. Note the presence of two major DNA-protein complexes (arrows) using muscle nuclear extracts that were specifically competed by a 250-fold molar excess of the wildtype (WT) but not the mutant oligonucleotides. (B) is a schematic representation of the nucleotides that were mutated (underlined) in the core region of the CArG element in NRAP to generate the mutant CArG-NRAP promoter fragment (mC-NRAP). As shown in panel (A), this mutation resulted in a failure to compete for specific DNA-protein complexes. (C) shows expression of β -gal in TA muscles injected with reporter plasmids containing either NRAP or mC-NRAP. Note that disruption of this DNA regulatory element did not affect significantly ($P > 0.05$; Student's *t*-test) expression of the reporter gene. A minimum of 10 muscles were injected per construct, and expression of β -gal was normalized to the activity of a co-injected CAT plasmid used to monitor transduction efficiency. Mean $\pm$ SEM are shown.

A)

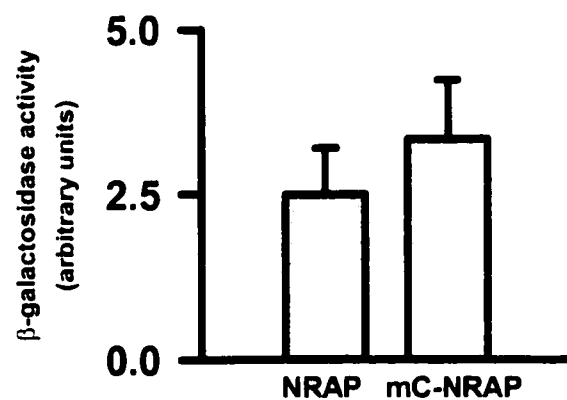
CArG
 extract
 + WT oligo
 + mutant oligo



B)



C)



of β -gal expression comparable to that seen with the wildtype promoter ($P > 0.05$; Figure 5.6C) suggesting that the CArG-box is dispensable for AChE gene expression in skeletal muscle.

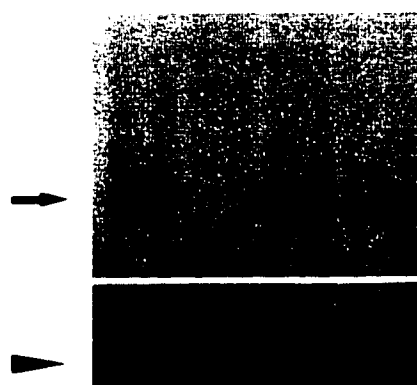
Our sequencing data also showed that there are four N-boxes in the AChE gene: two are palindromically located at -694 and -692 bp from the Inr while the others are located in intron 1 at positions +755 and +823 bp (Figure 5.1B). To determine whether these DNA regulatory sites are capable of binding protein factors, EMSA were performed using extracts of purified muscle nuclei. In experiments where labelled oligonucleotides containing the N-box located at +755 bp in the first intron were used, a single DNA-protein complex was observed (see N int-1 in Figure 5.7A). This N-box protein complex was specific since it could be competed by a 250-fold molar excess of unlabelled oligonucleotides. In addition, mutation of the N-box consensus sequence as shown in Figure 5.7C, functionally abolished its protein binding affinity as indicated by the inability of mutant oligonucleotides to compete effectively with the wildtype for the formation of this protein complex (Figure 5.7A). Interestingly, we detected considerably less protein binding when oligonucleotides containing the N-box at positions -694, -692 and +823 bp were used (for example compare N int-1 vs N prom in Figure 5.7A). These observations are in fact entirely consistent with our in vivo functional studies showing that the transcriptional activity of the various AChE promoter fragments is unaffected by deletions of these three N-boxes (see Figure 5.3). In supershift assays, the binding activity to the N-box motif was shown to involve GABP α and β (Figure 5.7B).

Based on our promoter analysis and EMSA, we next examined whether this intronic N-box is essential for the regulation of the AChE gene in muscle. To this end, we directly

Figure 5.7 Disruption of the N-box motif reduces drastically expression of AChE promoter-reporter gene constructs in TA muscle. (A) shows an EMSA using radiolabelled oligonucleotides containing the N-box motif. Note that one specific DNA-protein complex (arrow) was formed using a 24-bp oligonucleotide encompassing the first intronic N-box at position + 755 bp (N int-1). This protein complex was competed by a 250-fold molar excess of unlabelled wildtype oligonucleotides (WT oligo). Mutation of the core sequence as shown in (C), abolished its protein binding capacity as indicated by the inability of the mutant oligonucleotides to compete for specific protein complex. Note also that oligonucleotides containing the two palindromic N-box motifs located in the promoter region (N prom) displayed a weaker affinity for specific protein complexes. Arrow head indicates the amount of unbound radioactive oligonucleotides present in each sample. (B) shows that the protein complex was supershifted (white arrow) by an additional incubation with antibodies against either GABP α and β . (C) is a schematic representation of the nucleotides that were mutated (underlined) in the core region of the first intronic N-box motif at position +755 bp in NRAP to generate the mutant N-box-NRAP promoter fragment (mN-NRAP). As shown in panel (A), this mutation resulted in the failure to compete for specific DNA-protein complexes. (D) shows expression of β -gal in TA muscles injected with reporter plasmids containing either NRAP or mN-NRAP. Note that disruption of this DNA regulatory element essentially abolished ($P < 0.001$; Student's *t*-test) expression of the reporter gene indicating that the N-box is involved in enhancing expression of AChE in muscle. Expression of β -gal was normalized to the activity of a co-injected CAT plasmid used to monitor transduction efficiency. (E) reveals that the percentage of synaptic events was also significantly reduced ($P < 0.005$; Student's *t*-test) in muscles injected with reporter plasmids containing mN-NRAP. Mean $\pm$ SE are shown; minimum of 10 muscles were injected per construct.

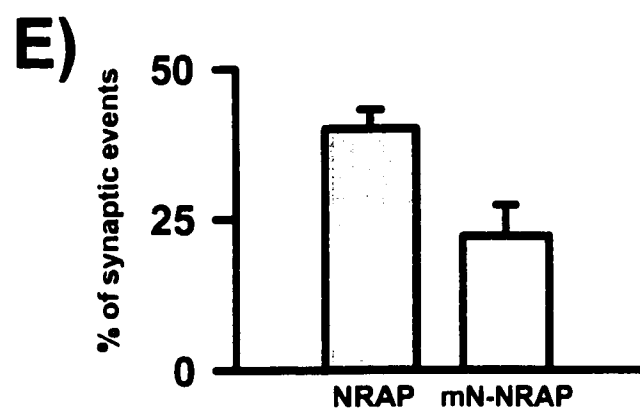
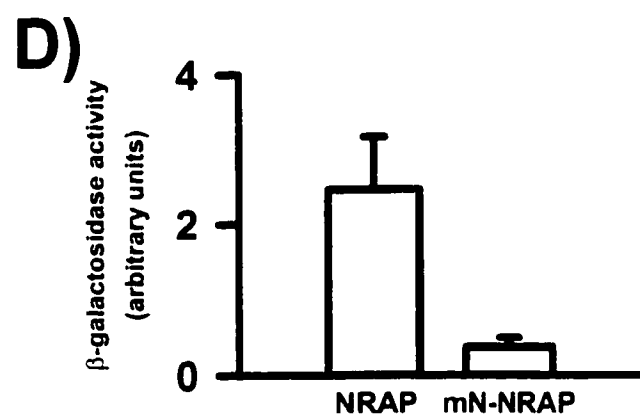
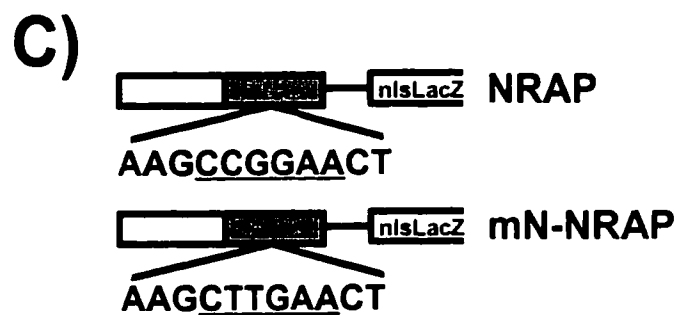
A) N-box

N int-1
+ WT oligo
+ mutant oligo
N prom



B)

N int-1
+ GABP
+ GABP
+ serum



injected into TA muscle, a plasmid containing the reporter gene nlsLacZ driven by the NRAP promoter fragment mutated within the intronic -box at position +755 bp (mN-NRAP in Figure 5.7C). In comparison to the wild-type AChE promoter fragment NRAP, mN-NRAP lost more than 80% of its transcriptional activity ($P < 0.001$; Figure 5.7D) indicating therefore that this intronic -box plays a critical role in the expression of AChE in muscle. Along with this dramatic reduction in the level of expression, we also observed a significant decrease in the percentage of synaptic events (Figure 5.7E). By contrast, mutation of this intronic -box did not alter expression of the reporter gene in MEL cells.

DISCUSSION

In the present study, we report the isolation of a 5.3 kb DNA fragment located in the 5' region of the rat AChE gene as well as its functional characterization in skeletal muscle. Alignment of this DNA fragment with available human and murine promoter elements (Ben Aziz-Aloya et al., 1993b; Li et al., 1993a) revealed a significant degree of sequence identity as exemplified by the presence of highly conserved DNA binding sites for transcription factors. Additional in vivo experiments confirmed that this DNA fragment functions indeed as a promoter since it induces expression of a reporter gene in muscle fibers. Although DNA fragments corresponding to the promoter region of the mouse and human AChE genes have been studied previously in cultured cells (Li et al., 1993a; Getman et al., 1995; Mutero et al., 1995), *X. laevis* embryos (Ben Aziz-Aloya et al., 1993a) and transgenic mice (Beeri et al., 1995), our data are the first to show that an AChE promoter fragment confers transgene expression in muscle fibers in vivo.

Previous studies have shown that, similar to the transcripts encoding the various AChR subunits, AChE mRNAs are approximately 10-fold more abundant in synaptic versus extrasynaptic compartments of muscle fibers (Jasmin et al., 1993; Michel et al., 1994; Legay et al., 1995). However, in contrast to the progress made recently in our understanding of the mechanisms underlying the expression of AChR mRNAs at the neuromuscular synapse, there is currently no information concerning the molecular events responsible for maintaining a high concentration of AChE transcripts within the postsynaptic sarcoplasm of muscle fibers. Based on the compartmentalized transcriptional activation of AChR subunit genes within synaptic myonuclei (for reviews see Hall and Sanes, 1993; Duclert and Changeux, 1995; Burden,

1998), it may be argued that enhanced transcription of the AChE gene within these nuclei also accounts for the accumulation of AChE transcripts within the postsynaptic sarcoplasm. Using a transient transfection assay system recently employed to study AChR subunit (Duclert et al., 1993; Koike et al., 1995; Duclert et al., 1996; Walke et al., 1996; Sapru et al., 1998a; Schaeffer et al., 1998) and utrophin promoters (Gramolini et al., 1997; Gramolini et al., 1998) in intact muscle fibers, we show here that a DNA fragment located in the 5' region of the AChE gene leads to the preferential expression of a reporter gene in synaptic compartments of muscle fibers. Taken together, these data indicate therefore that local activation of genes is a general mechanism employed by muscle fibers to ensure sufficient quantities and appropriate location of postsynaptic membrane proteins along muscle fibers.

Our deletion studies have led to the identification of a region located in the first intron which appears critical for expression of the AChE gene in muscle cells. Interestingly, sequence comparison between this DNA fragment and the respective intronic region from the mouse gene (P. Taylor and S. Camp, personal communication) revealed a striking similarity (93%). Both sequences are extremely rich in DNA motifs previously shown to be important for expression of several muscle genes such as CArG- and N-boxes. In agreement with our current data, Taylor and colleagues (Luo et al., 1998) have also recently observed that indeed, intron 1 is necessary for expression of AChE gene constructs in C2 cells. In our experiments, we further showed that this intronic DNA fragment failed to enhance expression of a reporter gene in hematopoietic cells. Moreover, it significantly increased in muscle, the expression of LacZ driven by the heterologous TK promoter when positioned in either orientation. Together with the observation that this element is functional at the 5' end of the heterologous promoter as well as in its native downstream location in the AChE gene, these results strongly

suggest that this intronic DNA fragment acts as an enhancer in an orientation- and position-independent manner in addition to being tissue-specific.

Recent studies have identified a 6-bp sequence termed an N-box, which is critical for the synapse-specific expression of AChR subunit genes (Koike et al., 1995; Duclert et al., 1996; Sapru et al., 1998a) as well as the utrophin gene (Gramolini et al., 1998). Interestingly, there are four N-boxes within the 5' regulatory region of the rat AChE gene. However, our deletion and mutation analyses revealed that only the N-box located in the first intron at position + 755 bp was essential for expression of the AChE gene in muscle fibers. These functional data are in fact entirely consistent with our EMSA showing that, although all N-boxes in the AChE gene appear to bind specifically the same protein complex, the latter intronic N-box clearly displays the highest binding activity. Since all N-box oligonucleotides used in our EMSA have the same 6-bp core element, these results further indicate that protein binding affinity of the N-box is dependent upon flanking sequences. Taken together, these data indicate that the N-box motif may not only regulate expression of several genes from an upstream position (Koike et al., 1995; Duclert et al., 1996; Gramolini et al., 1998), but that it can also function as a muscle-specific enhancer in a downstream location.

Based on the various studies that have examined so far the role of the N-box motif in the regulation of genes encoding synaptic proteins, it is becoming apparent that this DNA regulatory element can in fact act as an enhancer and/or repressor in synaptic versus extrasynaptic compartments of muscle fibers. Transcription factors belonging to the Ets family (Janknecht and Nordheim, 1993; Wasylyk et al., 1993) are currently becoming recognized as important regulators of AChR gene expression given their ability to bind the N-box motif. For example, GABP and Ets-2 were shown recently to transactivate in tissue

culture experiments, AChR subunit promoters (Sapru et al., 1998a; Schaeffer et al., 1998). Additional studies have also highlighted the contribution of the Erp and Sap1a in the repression of the ϵ subunit promoter (Sapru et al., 1998b) thereby indicating that the complement of Ets factors expressed along muscle fibers can in fact mediate the transcriptional activation or repression of genes encoding synaptic proteins in synaptic versus extrasynaptic regions of muscle fibers. Since the N-box motif is present in the promoter (see Koike et al., 1995; Gramolini and Jasmin, 1998) as well as intronic regions (present study) of several genes encoding synaptic proteins, it appears therefore that Ets-related transcription factors represent key determinants mediating the development and maintenance of the postsynaptic apparatus since they may lead ultimately to the coordinate activation of this subset of genes within synaptic myonuclei.

CHAPTER VI

Ciliary Neurotrophic Factor: Regulation of Acetylcholinesterase in Skeletal Muscle and Distribution of Messenger RNA Encoding its Receptor in Synaptic versus Extrasynaptic Compartments

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and Bernard J. Jasmin

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Manuscript written by C. Boudreau-Larivière and Dr. B. Jasmin

Soleus muscles from CNTF-treated rats were provided by Regeneron Pharmaceuticals Inc. (Tarrytown, NY, U.S.A.) through Dr. D. Parry. Dr. H. Sveistrup completed some of the RT-PCR experiments. Isolation of junctional and extrajunctional regions of muscle fibers was carried out by Dr. B. Jasmin.

ABSTRACT

Several recent studies have shown that the ciliary neurotrophic factor exerts myotrophic effects in addition to its well-characterized neurotrophic actions on various neuronal populations. Since expression of acetylcholinesterase in skeletal muscle has been shown to be regulated by putative yet unknown nerve-derived trophic factors, we tested the hypothesis that the ciliary neurotrophic factor is a neurotrophic agent capable of influencing expression of acetylcholinesterase in adult rat skeletal muscle *in vivo*. To this end, we first determined the impact of daily ciliary neurotrophic factor administration on expression of acetylcholinesterase in both intact and denervated rat soleus muscles. Results of our experiments indicate that although chronic administration of ciliary neurotrophic factor partially counteracted the atrophic response of soleus muscles to surgical denervation thus confirming its myotrophic effects, it failed to either increase acetylcholinesterase expression in intact muscles or prevent the decrease normally occurring in 7-day denervated muscles. In fact, acetylcholinesterase messenger RNA and enzyme levels were further reduced by ciliary neurotrophic factor treatment in both intact and denervated muscles without significant modifications in the pattern of acetylcholinesterase molecular forms. Conversely, transcript levels of the ϵ subunit of the acetylcholine receptor in intact and denervated soleus muscles treated with the ciliary neurotrophic factor were similar to those observed in their respective counterparts from vehicle-treated animals. In addition, we also determined whether transcripts encoding the receptor for the ciliary neurotrophic factor selectively accumulate in junctional domains of rat skeletal muscle fibres. In contrast to the preferential localization of transcripts encoding acetylcholinesterase and the ϵ subunit of the acetylcholine receptor within the

postsynaptic sarcoplasm, messenger RNAs for the ciliary neurotrophic factor receptor appeared homogeneously distributed between junctional and extrajunctional compartments of both diaphragm and extensor digitorum longus muscle fibres as also observed for dystrophin transcripts.

These data show that the ciliary neurotrophic factor exerts an inhibitory influence on expression of acetylcholinesterase in muscle fibres. Furthermore, the lack of an effect on expression of the ϵ acetylcholine receptor transcripts indicates that treatment with ciliary neurotrophic factor does not lead to general adaptations in the expression of all synaptic proteins. Given the distribution of transcripts encoding the ciliary neurotrophic factor receptor along multinucleated muscle fibres, we propose a model whereby the ciliary neurotrophic factor contributes to the maintenance of low levels of enzyme activity in extrajunctional regions of muscle fibres by acting as a repressor of acetylcholinesterase expression that functions directly or indirectly via a pretranslational regulatory mechanism. Accordingly, these results further highlight the complexity of the regulatory mechanisms presiding over acetylcholinesterase expression in vivo.

INTRODUCTION

Acetylcholinesterase (AChE) is responsible for inactivation of acetylcholine at cholinergic synapses of both central and peripheral nervous systems (for reviews see Taylor, 1991; Massoulié et al., 1993). This enzyme displays a rich polymorphism since it exists as a family of molecular forms that may be classified as either homomeric or heteromeric on the basis of their association with specialized structural subunits. Although a single gene encodes AChE (Schumacher et al., 1986; Sikorav et al., 1987; Rotundo et al., 1988; Maulet et al., 1990; Rachinsky et al., 1990; Li et al., 1991; Legay et al., 1993b) alternative splicing as well as distinct processing of the peptide chains account for the multiplicity of AChE molecular forms expressed in a variety of tissues and subcellular locations. In skeletal muscle fibres for example, asymmetric collagen-tailed AChE forms and G_4 tetramer accumulate within the synaptic basal lamina (Hall, 1973) and perijunctional compartment (Gisiger and Stephens, 1988), respectively.

Expression of AChE in muscle is known to be markedly influenced by the amount and pattern of superimposed neuronal activation (see for review Massoulié et al., 1993). Several lines of evidence however, also suggest that putative nerve-derived trophic factors modulate expression of AChE. For instance, selective disruption of axonal transport with colchicine application on peripheral nerves decreases AChE activity in muscle fibres (Fernandez and Donoso, 1988) while total AChE activity in denervated muscle fibres maintained in culture increases upon treatment with soluble nerve extracts (Fernandez and Inestrosa, 1976; Davey et al., 1979). In addition, chronic application of tetrodotoxin onto the sciatic nerve which

inhibits electrical activity of muscle fibres without compromising axoplasmic transport (Lavoie et al., 1976), leads to less pronounced reductions in AChE activity and transcript levels in comparison to those induced by denervation (Butler et al., 1978; Jasmin et al., 1993; Michel et al., 1994). Taken together, these studies indicate that the release of nerve-derived trophic factors contribute significantly to the regulation of AChE expression in skeletal muscle fibres in vivo.

The ciliary neurotrophic factor (CNTF) is a neural cytokine originally characterized for its sparing effects on embryonic neurons of chick ciliary ganglia (Adler et al., 1979; Barbin et al., 1984). Since then, several studies have demonstrated the survival promoting effects of CNTF on various neuronal populations in both central and peripheral nervous systems (Sendtner et al., 1990; Oppenheim et al., 1991; Sendtner et al., 1992; Gurney et al., 1992; English and Schwart, 1995) as well as its capacity to stimulate conversion of cultured rat sympathetic neurons from noradrenergic to cholinergic phenotypes (Saadat et al., 1989; Kalberg et al., 1993; Lewis et al., 1994; Zurn and Wernig, 1994). Together, these studies have led to the suggestion that CNTF may in fact have pluripotent effects in the nervous system. Since expression of the CNTF-binding subunit referred to as CNTFR α (Davis et al., 1991; Ip et al., 1993) is not limited to neurons and glia but also occurs in skeletal muscle (Ip et al., 1993), CNTF may therefore also act as a myotrophic agent. This was recently demonstrated by Helgren and colleagues (Helgren et al., 1994) for example, who showed that daily CNTF injections partially counteracted several morphological and functional changes normally occurring in denervated muscle (see also Forger et al., 1993; Michel et al., 1994).

Based on these observations, we hypothesized that CNTF modulates expression of AChE within mammalian skeletal muscle fibres. In the present studies, we tested this by determining the impact of chronic CNTF treatment on expression of AChE molecular forms and mRNA levels in intact and 7-day denervated rat soleus muscles. In addition, we examined the distribution of transcripts encoding CNTFR α along muscle fibres in attempts to determine whether CNTF mediates its effects on muscle via the postsynaptic membrane of the neuromuscular junction.

MATERIALS and METHODS

Denervation and Ciliary Neurotrophic Factor Treatment

Right hindlimb muscles from young male adult rats were denervated by severing the sciatic nerve and removing a 5 mm segment while sham-operations were performed on the contralateral side. Rats were then placed randomly into two groups. One group of denervated rats were injected daily with a vehicle solution made of phosphate-buffered saline containing bovine serum albumin (1 mg/ml). The second group of denervated animals received daily injections of phosphate-buffered saline containing recombinant rat CNTF or human CNTF (0.3 mg/kg body weight) with similar results. All injections were given subcutaneously. Seven days later, both left and right soleus muscles from vehicle and CNTF groups were excised, quickly weighed and then frozen in liquid nitrogen. Muscles were stored at -80°C until further analyzed.

Dissection of Junctional and Extrajunctional Regions of Rat Diaphragm and Extensor Digitorum Longus Muscle Fibres

For these experiments, diaphragm and extensor digitorum longus (EDL) muscles were excised and secured onto a paraffin bed at resting length. Junctional regions of hemidiaphragm muscle fibres were dissected by cutting the central portion of the muscle where essentially all motor endplates are localized. An extrajunctional segment of similar size was also obtained for each muscle by cutting a strip ~ 5 mm away from the junctional segment to avoid possible contamination by postsynaptic membrane domains. For EDL muscles, junctional regions were identified by AChE histochemistry (Karnovsky and Roots, 1964).

After one hour of staining, small bundles containing 10-15 muscle fibres were carefully microdissected from the surrounding connective tissue under a stereomicroscope (see Jasmin et al., 1993; Michel et al., 1994). Junctional and extrajunctional segments of similar sizes were cut and placed into separate tubes. Total RNA from all junctional and extrajunctional samples were immediately extracted as described below.

RNA Isolation

The acid guanidinium phenol chloroform procedure of Chomczynski and Sacchi (1987) was used to isolate total RNA from whole muscles as well as from junctional and extrajunctional segments. For soleus whole muscle experiments, muscles were immersed in 10 volumes of denaturing solution D and quickly homogenized with a Polytron (Kinematica, Littan, Switzerland) set at maximum speed twice for 15 s. Microdissected samples from hemidiaphragm and EDL muscles were homogenized in 500 µl of solution D. After three washes, RNA pellets were stored at -20°C in 75% ethanol.

Quantitative Reverse Transcription and Polymerase Chain Reaction

RNA pellets from soleus and diaphragm muscles were redissolved into 100 µl of RNase-free water. From this RNA stock, 10-, 100-, 1000-, and 10000-fold serial dilutions were prepared and only 2 µl of these were used for reverse transcription (RT). Reverse transcription was carried out in 5 mM MgCl₂, 1X PCR buffer II (50 mM KCl, 10 mM Tris-HCl [pH 8.3]), 1 mM dNTPs, 20 U RNase inhibitor, 50 U Moloney murine leukemia virus reverse transcriptase, and 2.5 mM random hexamer primers (GeneAmp RNA PCR kit; Perkin-

Elmer Cetus Instruments, Norwalk, CT). Junctional and extrajunctional RNA pellets from EDL muscles were directly redissolved in 20 μ l of RT mixture. Reverse transcription was carried out at 42°C for 45 min. Negative control consisted of RT mixtures in which the RNA sample was replaced with 2 μ l of RNase-free water.

Amplification of cDNAs encoding AChE, the ϵ subunit of the acetylcholine receptor (AChR ϵ), dystrophin and CNTFR α was performed using the polymerase chain reaction (PCR) as described in detail elsewhere (Jasmin et al., 1993; Michel et al., 1994). Primers were synthesized on the basis of available sequences for rat AChE (Legay et al., 1993b), AChR ϵ (Criado et al., 1988), CNTFR α (Ip et al., 1993) and mouse dystrophin (Tanaka and Ozawa, 1990; Ho-Kim and Rogers, 1992). The sequences of the various primers used in this study are given in Table 6.1. PCR was performed by adding 5 μ l of RT to 20 μ l of a mixture consisting of 0.625 U AmpliTaq DNA polymerase, 0.25 μ g of each 5' and 3' primer of interest, MgCl₂ (2 mM final concentration) and PCR buffer II (1X final concentration). PCR samples were covered with a drop of mineral oil before being placed in a DNA thermal cycler (Perkin-Elmer Corp.). For all target cDNAs, cycle parameters included denaturation at 94°C for 1 min. For both AChE and dystrophin, primer annealing and extension was carried out at 70°C for 3 min. Annealing temperature was 60°C and 55°C for AChR ϵ and CNTFR α , respectively, followed by a 1 min extension at 72°C for both sequences. A final elongation period lasting 10 min at 72°C followed the last cycle. Cycle number varied between 37 and 50 for whole muscles and 40 to 84 for junctional and extrajunctional samples. PCR products were visualized by loading 10 μ l of the reaction mixtures onto 1-1.5% agarose gels containing

Table 6.1 Primer Sequences

Target	5' Primer	3' Primer	Product Size
cDNA			(bp)
AChE	CTGGGGTGCGGATC GGT	TCACAGGTCTGAGC AGCGTT	670
AChR ϵ	TGGTGCTACTCGCTT ACTT	ATGCCTCTGTCCCTC AAAC	428
DYS	TGAAATAATGGAGG AGAGACTCGG	GCAGGCCATTCTC TTTCAGGAAA	314
CNTR α	ATCCCAATACCTTC AAT	TACTCCTTCCCAGCA TAG	419

ethidium bromide. Product size was estimated using the 100-bp ladder (Gibco BRL, Gaithersburg, MD).

Quantitative PCR experiments under non-competitive conditions were performed since in the present studies, we were primarily interested in comparing the relative abundance of the different mRNAs in muscles subjected to CNTF treatment as well as in junctional and extrajunctional samples. These were performed as discussed elsewhere (Jasmin et al., 1993; Michel et al., 1994). Briefly, 2×10^6 cpm of $\gamma^{32}\text{P}$ (ATP)-end labelled primers were added to the PCR reaction mixture. After amplification, 10 μl aliquots of the PCR reactions were Cerenkov counted and separated by gel electrophoresis on 1.5% agarose gels. The gels were then photographed, the appropriate gel bands excised and the amount of radioactivity determined by Cerenkov counting. The counts per minute were adjusted for the amount of sample loaded per gel lane and background activity. These final counts per minute are proportional to the relative abundance of the different transcripts in the various samples (see Jasmin et al., 1993; Michel et al., 1994).

Extraction and Analysis of Acetylcholinesterase Enzyme Activity

Whole frozen soleus muscles were homogenized 2×15 sec on ice in 2.5 ml of a high-salt buffer solution containing 2.5 mg/ml of aprotinin and 1 mg/ml of bacitracin as antiproteolytic agents. The homogenates were centrifuged at low speed ($20,000 \times g$) at 4°C for 15 min. Aliquots of the supernatants were kept at -80°C .

AChE activity was determined using the spectrophotometric method of Ellman *et al* (1961) as described elsewhere (Gisiger and Stephens, 1988; Jasmin and Gisiger, 1990). The

activity was measured in the presence of 10^{-5} M of the nonspecific cholinesterase inhibitor tetraisopropylpyrophosphoramidate (iso-OMPA). Nonspecific hydrolysis was determined by measuring substrate hydrolysis in the presence of both iso-OMPA and the AChE specific inhibitor 5-bis(4-allyldimethylammonium phenyl) pentanone dibromide (BW284c51).

Sedimentation analyses of AChE molecular forms were performed according to Jasmin and Gisiger (1990). Briefly, 100 μ l aliquots of the muscle extracts were loaded onto 5-20% sucrose gradients and centrifuged at 4°C for 16 h at 281 000 $\times g$ in a SW41 rotor. Approximately 45 fractions were collected from the bottom of each gradient and assayed for AChE activity. AChE molecular forms were identified based on the nomenclature of Bon et al. (1979).

Statistical Analysis

The impact of CNTF treatment on muscle mass was assessed using one-tailed unpaired Student's t-tests since we expected CNTF to counteract the atrophic response normally observed in denervated soleus muscles. Paired Student's t-tests were used to determine the effect of CNTF treatment on AChE expression.

RESULTS

Effects of Ciliary Neurotrophic Factor on Acetylcholinesterase Expression

In a first series of studies, we examined whether daily administration of CNTF influenced expression of AChE in rat muscle fibres. The success of the CNTF injection protocol was ascertained by examining the mass of denervated rat soleus muscles. As reported earlier using the same experimental paradigm (Helgren et al., 1994), CNTF treatment partially counteracted the denervation-induced atrophy normally observed in rat soleus muscles. The extent of muscle atrophy in denervated CNTF-treated animals (25%) was significantly less ($P < 0.05$) than in denervated vehicle-treated soleus muscles (40%). These data therefore confirm the myotrophic effect of CNTF.

To test whether CNTF treatment affected AChE expression, we compared levels of AChE transcripts in intact soleus muscles obtained from vehicle- and CNTF-treated rats. Quantitative RT-PCR analysis showed that daily administration of CNTF did not increase AChE mRNA levels in intact muscles (Figures 6.1 and 6.2). In fact, AChE transcript levels in intact CNTF-treated muscles appeared lower ($P = 0.188$) than those observed in intact vehicle-treated muscles. In addition, since recent studies have shown that denervation reduces the abundance of transcripts encoding AChE (Cresnar et al., 1994; Michel et al., 1994), we also determined whether CNTF treatment could attenuate this profound change in gene expression. In vehicle-treated animals, AChE transcript levels were reduced by ~ 65% ($P < 0.05$) in 7-day denervated soleus muscles as compared to contralateral control muscles (Figure 6.2). In agreement with the data obtained with intact muscles, a greater decrease (~ 80%; P

Figure 6.1 Effect of chronic CNTF treatment on levels of AChE mRNA in rat muscles. Shown is a representative example of an ethidium bromide-stained agarose gel of AChE PCR products for control (C) and 7-day denervated (D) soleus muscles obtained from vehicle (VEH)- and CNTF-treated animals.

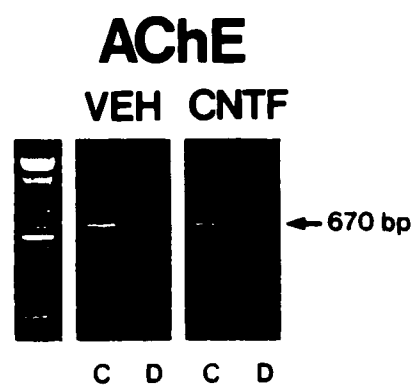
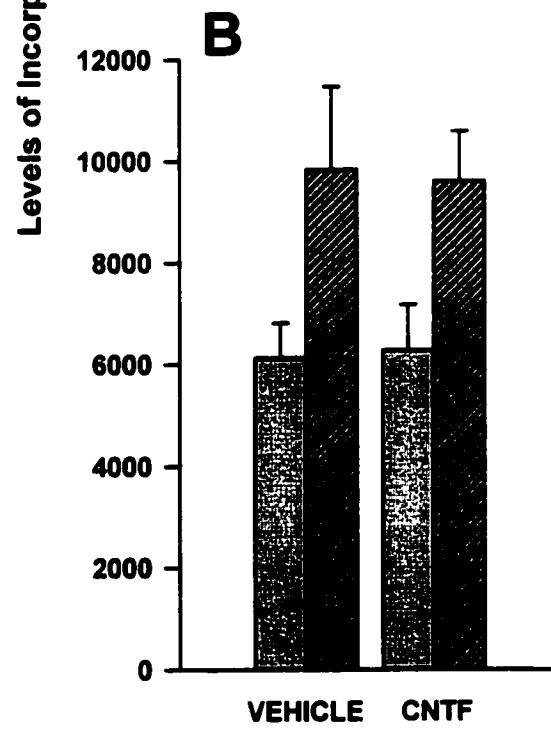
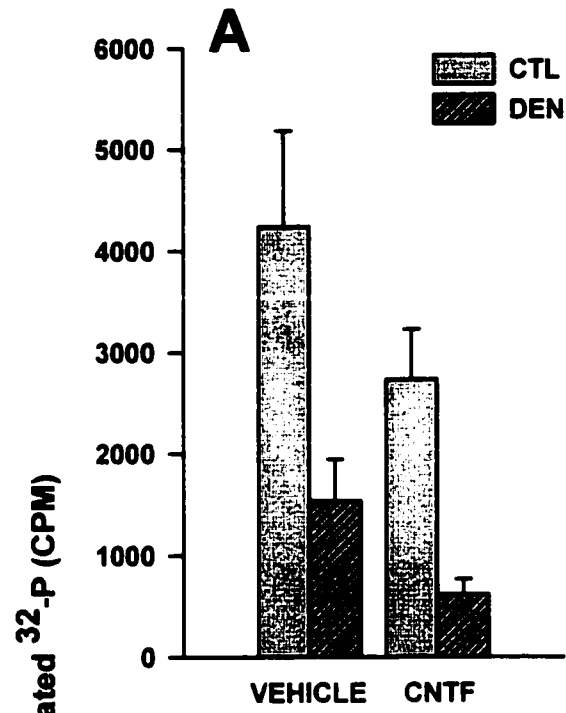


Figure 6.2 Quantitation of AChE (A) and AChR ϵ (B) mRNA levels in control (CTL) and denervated (DEN) soleus muscles obtained from vehicle- and CNTF-treated animals. Note the decreases in the relative amount of AChE transcripts in muscles following daily administration of CNTF. AChR ϵ mRNA levels on the other hand, appear insensitive to the CNTF treatment. Means $\pm$ SEM are presented; $n = 6$ animals per group.



< 0.05) in AChE transcript levels was observed in CNTF-treated denervated muscles. Comparisons of intact and denervated sides revealed that the ratios were considerably different for vehicle- (3.6) versus CNTF-treated (5.6) animals. These latter data further highlight the greater decrease in AChE transcripts in denervated soleus muscles following CNTF administration.

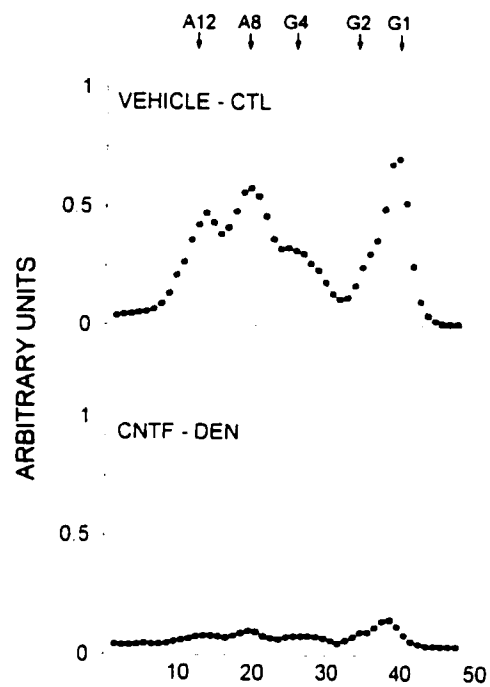
We also determined the impact of CNTF treatment on expression of the ϵ subunit of the acetylcholine receptor (AChR ϵ). Levels of AChR ϵ mRNAs were modestly increased in 7-day denervated rat soleus muscles (Figure 6.2). In contrast to the findings with AChE, daily administration of CNTF did not influence ($P > 0.05$) expression of these transcripts thereby indicating that the effects observed on AChE may not be extended to expression of all synaptic proteins.

Since these results indicated that CNTF treatment induced a decrease in expression of AChE transcripts in intact muscles and a further reduction of AChE mRNA in denervated muscles, we determined whether these changes are paralleled by a similar effect on AChE activity. Denervation of skeletal muscle is known to induce pronounced decreases in total AChE activity that include prominent reductions in the levels of asymmetric forms (see for review Massoulié et al., 1993). In vehicle-treated rats, total AChE activity was reduced by more than 60% in 7-day denervated soleus muscles as compared to contralateral muscles (Table 6.2). As expected, these changes were accompanied by a large decrease in the content of asymmetric forms. Chronic treatment with CNTF did not have a sparing effect on either total AChE activity (Table 6.2) or expression of the various molecular forms (Figure 6.3). In agreement with the data on expression of AChE mRNAs, it appeared that CNTF treatment

Table 6.2. Total AChE activity in control (CTL) and 7-day denervated (DEN) soleus muscle from vehicle- and CNTF-treated animals. Means $\pm$ SEM are presented (n = 4).

Vehicle		CNTF	
CTL	DEN	CTL	DEN
60.8 $\pm$ 16.8	22.3 $\pm$ 7.0	78.2 $\pm$ 16.6	11.0 $\pm$ 1.8

Figure 6.3 AChE molecular form content observed in control muscles from vehicle-treated animals and in 7-day denervated muscles from CNTF-treated rats. Shown are representative examples of the average contents of AChE molecular forms in soleus muscles. The pattern of AChE molecular forms seen in denervated soleus muscles from CNTF-treated animals was similar to that observed in denervated muscles from vehicle-treated rats.



further reduced enzyme activity by an additional 25% in comparison to vehicle-treated animals.

Distribution of the Receptor for the Ciliary Neurotrophic Factor Along Muscle Fibres

On the basis of these results which indicate that CNTF treatment leads to further decreases in expression of AChE, we determined whether transcripts encoding CNTFR α selectively accumulate in junctional domains of rat skeletal muscle fibres. This was achieved by comparing CNTFR α mRNA levels in both junctional and extrajunctional compartments obtained from diaphragm and microdissected pooled fibres from EDL muscles using quantitative RT-PCR. Levels of CNTFR α transcripts were compared to those for dystrophin, AChR ϵ and AChE measured in the same junctional and extrajunctional samples. In diaphragm muscle fibres, the abundance of CNTFR α transcripts appeared relatively equal in the two muscle fibre compartments (Figures 6.4 and 6.5). As expected, levels of dystrophin mRNAs were also similar in junctional and extrajunctional regions (see Michel et al., 1994) whereas transcripts encoding AChR ϵ (Figure 6.5) and AChE (data not shown) selectively accumulated within the junctional sarcoplasm as previously shown (see for example Witzemann et al., 1991; Michel et al., 1994). Similarly, transcripts for CNTFR α appeared homogeneously distributed along pooled fibres of EDL muscles whereas AChR ϵ mRNAs were ~ 20-fold more abundant in junctional versus extrajunctional compartments (Figure 6.5). In these same EDL muscle fibre samples, dystrophin transcripts were evenly distributed between junctional and extrajunctional areas (data not shown).

Figure 6.4 Comparison of AChR ϵ , dystrophin (DYS) and CNTFR α mRNA levels. Shown are representative examples of ethidium bromide-stained agarose gels of PCR products in junctional (J) and extrajunctional (E) regions of diaphragm muscle fibres.

AChR ϵ



J E

DYS



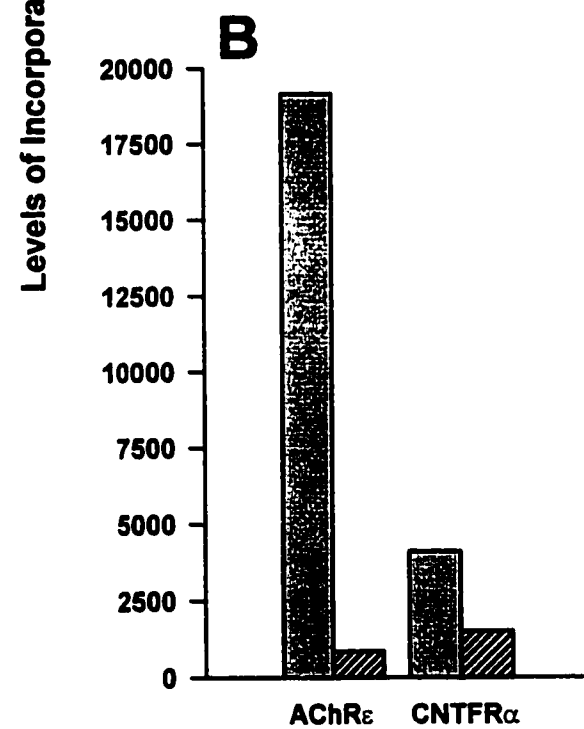
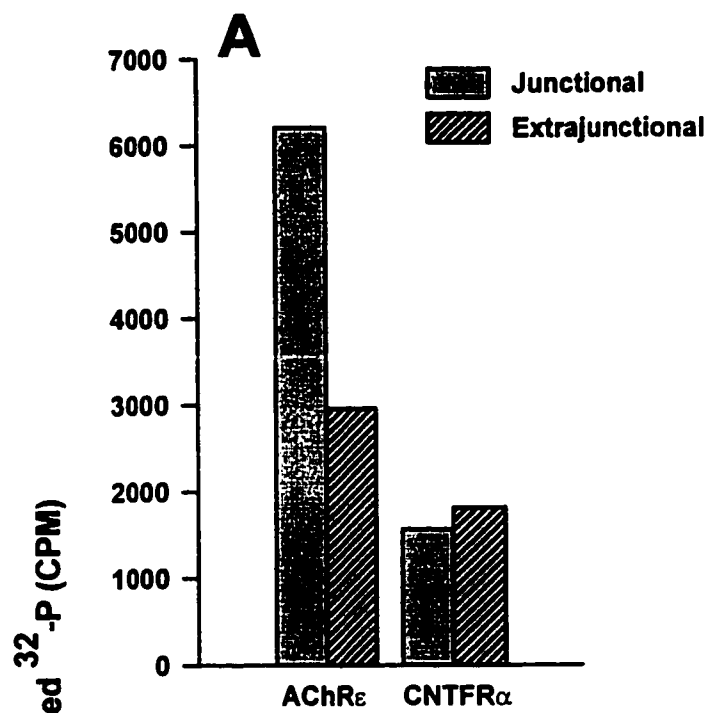
J E

CNTFR α



J E

Figure 6.5 Quantitation of AChR ϵ and CNTFR α mRNA levels in junctional versus extrajunctional regions of diaphragm (A) and EDL (B) muscle fibres. Note the selective accumulation of transcripts encoding AChR ϵ in the junctional regions of both muscles. By comparison, CNTFR α mRNAs appear relatively equal between the two muscle compartments. Results for diaphragm muscles were obtained by pooling total RNA extracted from junctional and extrajunctional regions obtained from 6 different hemidiaphragm muscles. For EDL muscle fibres, results were obtained by pooling total RNA extracted from 9 microdissected junctional and extrajunctional bundles. For more details, see Materials and Methods.



DISCUSSION

The objectives of the present investigation were two fold. First, we wished to determine whether CNTF is a potential nerve-derived trophic factor involved in regulating expression of AChE *in vivo*. In addition, we examined whether the effects of CNTF on synaptic proteins are mediated by a selective accumulation of CNTFR α in the junctional area of muscle fibres. Thus, we determined the impact of daily administration of CNTF on expression of AChE in intact and 7-day denervated rat soleus muscles, and examined the relative abundance of CNTFR α mRNAs within junctional versus extrajunctional areas along skeletal muscle fibres and compared these results to those obtained with both synaptic and non-synaptic transcripts. Our results confirm that CNTF is a myotrophic agent by demonstrating its sparing effect on denervation-induced muscle atrophy (see Helgren et al., 1994). Moreover, we show that CNTF exerts an inhibitory influence on expression of AChE, an essential component of the neuromuscular synapse.

Expression of AChE in skeletal muscle fibres is known to be significantly influenced by the levels of neuromuscular activation. For example, AChE activity (Drachman, 1972; Hall, 1973; Butler et al., 1978; Boudreau-Larivière et al., 1997) as well as mRNA levels (Cresnar et al., 1994; Michel et al., 1994) are both reduced dramatically by abolition of electrical activity via surgical denervation. In contrast, chronic enhancement of neuromuscular activation achieved with exercise training programs and compensatory hypertrophy (Fernandez and Donoso, 1988; Jasmin and Gisiger, 1990; Gisiger et al., 1991; Jasmin et al., 1991; Gisiger et al., 1994; Sveistrup et al., 1995) lead to prominent and selective increases in the levels of

specific AChE molecular forms that are accompanied by concomitant increases in the levels of AChE transcripts (Sveistrup et al., 1995).

Several lines of evidence also indicate that in addition to electrical activity, nerve-derived myotrophic factors contribute to the regulation of AChE within skeletal muscle fibres (Drachman, 1972; Fernandez and Inestrosa, 1976; Butler et al., 1978; Davey et al., 1979; Fernandez et al., 1980; Michel et al., 1994; Boudreau-Larivière et al., 1997). Since CNTFR α is expressed in skeletal muscle fibres (Davis et al., 1991; Ip et al., 1993) and CNTF was recently shown to act as a myotrophic agent (Forger et al., 1993; Helgren et al., 1994; Michel et al., 1996), we hypothesized that CNTF may be involved in regulating AChE expression in muscle. For instance, CNTF treatment may have increased levels of AChE mRNA in intact soleus muscles and attenuated the reduction in this transcript that occurs upon denervation (see Cresnar et al., 1994; Michel et al., 1994). Results of our experiments however indicate that although chronic administration of CNTF partially counteracted the atrophic response of soleus muscles to surgical denervation, it failed to either increase AChE expression in intact muscles or prevent the decrease in 7-day denervated muscles. We expected that under similar CNTF-treatment conditions shown to attenuate the morphological and functional alterations occurring in denervated muscle fibres (Helgren et al., 1994), we would also observe a myotrophic effect on AChE expression. In fact, our observations indicate that CNTF treatment further down-regulates expression of both AChE transcripts and enzyme levels without affecting the pattern of molecular forms. Therefore, CNTF appears to act as a repressor of AChE enzyme expression operating directly or indirectly via a pretranslational regulatory mechanism. In this context, it is noteworthy that interleukin-6, a member of the

same family of neurotrophic cytokines as CNTF, induces a decrease in AChE activity in rat brain (Clarençon et al., 1995).

In attempts to determine whether these effects of CNTF were specific for AChE, we also determined levels of transcripts encoding the ϵ subunit of AChR in vehicle- and CNTF-treated muscles. We chose this particular subunit since its expression is known to be largely regulated by neurotrophic factors (Brenner et al., 1990; Martinou and Merlie, 1991; Gundersen et al., 1993; Kues et al., 1995). As reported previously (Witzemann et al., 1991), denervation slightly increased levels of AChR ϵ mRNAs in rat soleus muscles. Since the magnitude of the increase was similar for both vehicle- and CNTF-treated animals, it appears that the effects of CNTF may not be extended to expression of all synaptic proteins. Under these conditions, the lack of a CNTF effect on AChR ϵ further highlights the discoordinate nature of the regulatory mechanisms involved in controlling expression of the genes encoding AChE and AChR (see for example Michel et al., 1994). Nonetheless, it will be important in future studies to determine the impact of CNTF administration on expression of other synapse-specific proteins.

The effect of CNTF on expression of a synaptic protein led us to speculate that CNTFR α is not homogeneously distributed along multinucleated muscle fibres but rather, that the level of this receptor is higher within the postsynaptic membrane domain where it could influence locally AChE expression. This hypothesis was particularly attractive since CNTF is known to be synthesized by both Schwann cells (Friedman et al., 1992; but see Lee et al., 1995) and motor neurons (Seniuk-Tatton et al., 1995). In attempts to provide information on this issue, we determined whether transcripts encoding CNTFR α selectively accumulate within

the postsynaptic membrane domain of muscle fibres. Our results indicate that CNTFR α transcripts are evenly distributed between junctional and extrajunctional regions with no compelling evidence for a preferential accumulation within the postsynaptic sarcoplasm. These results are in marked contrast with those obtained for transcripts encoding AChE and the ϵ subunit of AChR which, as confirmed by our experiments, are more abundant within the junctional sarcoplasm. In fact, the distribution of CNTFR α mRNAs resembled that of dystrophin transcripts, a protein known to be expressed along the entire length of muscle fibres (see for reviews Ahn and Kunkel, 1993; Matsumura and Campbell, 1994). Although we did not examine the distribution of CNTFR α per se, we expect that the level of this receptor is also homogeneously distributed along muscle fibres given that in muscle, the homogeneous distributions of specific proteins such as Na⁺/K⁺-ATPase, actin, myosin and dystrophin coincide with localization of their respective transcripts (Fontaine et al., 1988; Goldman and Staple, 1989; Jasmin et al., 1993; Michel et al., 1994; Moscoso et al., 1995).

In addition to interacting with CNTFR α , CNTF requires the presence of the leukemia inhibitory factor-binding protein (Gearing et al., 1991) and gp130 (Hibi et al., 1990) to mediate its effects. The binding of CNTF to its receptor thus initiates a complex cascade of cellular events that culminate in the phosphorylation of various substrates including the STAT family of transcription factors (see for example Curtis and DiStefano, 1994). If we assume that all components of this signalling pathway are present in both junctional and extrajunctional regions, our results suggest therefore that muscle fibres can respond to CNTF, or a closely related yet unknown molecule that utilizes also CNTFR α (DeChiara et al., 1995), throughout their entire length. This hypothesis fits well with the results of several recent

studies showing that systemic injections of CNTF lead to generalized adaptations of core constituents of muscle fibres including for instance, contractile proteins (Helgren et al., 1994) and metabolic enzymes (Michel et al., 1996). However, in an in vivo situation where CNTF is not exogenously supplied, the functional significance of the distribution of CNTFR α along multinucleated muscle fibres is unclear especially since CNTF may only be found near synaptic sites (Friedman et al., 1992; Lee et al., 1995; Seniuk-Tatton et al., 1995).

During embryonic development, AChE enzyme activity as well as mRNAs are present throughout the length of non-innervated myotubes (Grubic et al., 1995). Upon innervation however, AChE becomes enriched within synaptic areas although enzyme activity can still be detected in extrajunctional regions. The compartmentalization of AChE activity occurs as a result of a progressive restriction in the expression of transcripts encoding AChE within the postsynaptic membrane domain (Jasmin et al., 1993; Michel et al., 1994; Grubic et al., 1995; Legay et al., 1995). Recent studies have shown that the maintenance of the synaptic accumulation of AChE transcripts depends largely on superimposed neuronal activation as well as on anterograde neurotrophic factors (Michel et al., 1994; Sveistrup et al., 1995). What limits expression of AChE transcripts in extrajunctional regions however is currently unknown. Given the findings of the present study showing that CNTF exerts an inhibitory influence on AChE expression, it is possible that CNTF may represent a factor that regulates AChE levels in extrajunctional regions. In this model, CNTF may be released in an activity-dependent manner from either Schwann cells (Friedman et al., 1992; Lee et al., 1995) or motor axons (Seniuk-Tatton et al., 1995) and bind to extrajunctional CNTFR α thereby triggering the cascade of cellular events leading to a down-regulation of AChE levels. The recently

postulated CNTF-like ligand of unknown origin may participate in this regulatory mechanism by acting also on CNTFR α (DeChiara et al., 1995). Conversely, within the junctional region of muscle fibres, the effects of CNTF on the synaptic accumulation of AChE mRNA and enzyme may be overridden by a combination of factors involving both nerve-evoked electrical activity and nerve-derived trophic agents acting as activators of AChE expression. As such, this model provides an experimental framework for further studies on the role of CNTF in the regulation of AChE and, in particular, for identifying the signalling pathway linking activation of CNTFR α to changes in expression of AChE in muscle.

In a recent study, Hodges-Savola and Fernandez (1995) showed that injections of calcitonin gene-related peptide (CGRP), an agent known to significantly modulate expression of AChR in muscle (New and Mudge, 1986), prevented the increase in AChE tetramer G₄ that normally occurs in short-term denervated muscle (Gregory et al., 1989). Interestingly, these results which demonstrate that CGRP treatment down-regulates AChE expression in muscle, are in good agreement with our present findings using CNTF. Thus, results of these studies provide for the first time evidence for the role of specific nerve-derived myotrophic factors contributing to the regulation of AChE in muscle. Taken together with the observations that superimposed neuronal activation and other nerve-derived yet unidentified trophic factors increase AChE levels, these results suggest that the overall regulation of AChE in muscle is inextricably linked to electrical activity as well as to various myotrophic factors that can act as repressors or activators. Accordingly, these findings further highlight the complexity of the regulatory mechanisms controlling AChE expression in skeletal muscle fibres *in vivo*.

CHAPTER VII

CGRP Decreases Expression of Acetylcholinesterase in Mammalian Myotubes

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Manuscript written by C. Boudreau-Larivière and Dr. B. Jasmin

All experiments carried out by C. Boudreau-Larivière

ABSTRACT

Nerve-derived trophic factors are known to modulate expression of acetylcholinesterase (AChE) in skeletal muscle fibers yet, the precise identity of these factors remains elusive. In the present study, we treated mouse C2 myotubes with CGRP. Compared to non-treated myotubes, cell-associated AChE activity levels were decreased by ~60% after 48 hrs of treatment. A parallel reduction in AChE total protein levels was also observed as determined by Western blot analysis. The reduction in AChE activity was due to a decrease in the levels of the G₁ molecular form and to an elimination of G₄. By contrast, levels of secreted AChE remained unchanged following CGRP treatment. Finally, the overall decrease in AChE activity was accompanied by a reduction in AChE transcripts which could not be attributed to changes in the transcriptional rate of the AChE gene.

INTRODUCTION

Several lines of evidence indicate that interactions between motoneurons and their target muscle fibers regulate expression of acetylcholinesterase (AChE) in muscle tissue (for review see Massoulié et al., 1993). Although previous studies have shown that nerve-evoked electrical activity is a key regulator of AChE in muscle, it appears that nerve-derived trophic factors also play a significant role. In earlier studies for instance, nerve extracts applied to cultures of denervated muscle fibers were shown to maintain total AChE activity (Davey et al., 1979) whereas disruption of axonal transport with colchicine reduced AChE activity within muscle fibers (Fernandez and Inestrosa, 1976). In more recent reports, application of tetrodotoxin onto the sciatic nerve which abolishes nerve action potentials without affecting axonal transport, was shown to lead to lesser reductions in both AChE activity and transcript levels as compared to the effects induced by denervation (Michel et al., 1994). Taken together, these findings demonstrate the importance of nerve-derived molecules in directing AChE expression at the neuromuscular junction. Nonetheless, our current knowledge regarding the identity of specific nerve-derived trophic factors regulating AChE expression in muscle as well as the mechanisms by which they act is currently lacking.

Numerous studies focusing on the acetylcholine receptor (AChR) have also highlighted the role of both nerve-derived electrical activity and trophic substances in regulating expression of the genes encoding the various AChR subunits in synaptic versus extrasynaptic compartments of muscle fibers (for reviews see Hall and Sanes, 1993; Duclert and Changeux, 1995). In particular, the calcitonin gene-related peptide (CGRP) has been shown to affect

markedly expression of AChR in cultured myotubes (New and Mudge, 1986; Fontaine et al., 1987; Osterlund et al., 1989; for review see Duclert and Changeux, 1995). We therefore hypothesized in the present study, that this factor may also regulate expression of AChE in mammalian muscle fibers particularly since it was demonstrated recently that CGRP increased AChE mRNA expression in chick myotubes (Choi et al., 1996; Choi et al., 1998).

MATERIALS and METHODS

C2 Cell Cultures and CGRP Treatment

C2 cells were plated on Matrigel- (Collaborative Biomedical Products, Bedford, MA) coated 60 mm culture dishes and grown at 37°C in Dulbecco's modified Eagle's medium (DMEM) (Life Sciences/Gibco; Burlington, ON) containing 10% fetal bovine serum and L-glutamine. When myoblasts became confluent, the growth media was replaced with differentiation media containing low serum. Five days after plating, myotubes were treated with rat CGRP (Sigma, St-Louis, MO) or an equal volume of sterile vehicle solution for 48 hrs. Since similar effects on AChE expression were observed with CGRP concentrations of 0.01, 0.05, 0.1 and 1 μ M, we chose to treat cultured myotubes with 0.1 μ M because several previous studies have in fact used this particular dose /see for example (Fontaine et al., 1987; Choi et al., 1996). Following the first 24 hrs, the media was collected and replaced with fresh differentiation media containing either CGRP or vehicle solution.

To assess AChE activity secreted from vehicle- and CGRP-treated myotubes, diisopropyl fluorophosphate (DFP; Sigma)-treated horse serum was used to prepare the differentiation media. Endogenous serum AChE was inactivated by incubating the horse serum with DFP for 48 hrs then allowing the DFP to degrade for 10 days.

Extraction and Analysis of Acetylcholinesterase Enzyme Activity

Vehicle- and CGRP-treated myotubes were homogenized for 30 sec with a Polytron set at low speed in 1 ml of high-salt detergent buffer containing anti-proteolytic agents: 10

mM tris (hydroxymethyl)-aminomethane (Tris) HCl (pH 7.0), 10 mM EDTA, 1 M NaCl, 1% Triton X-100, 2.5 mg/ml aprotinin and 1 mg/ml bacitracin (Jasmin and Gisiger, 1990). Samples were then centrifuged at 20 000 x g for 15 min. The media was collected after each 24 hr-period of vehicle or CGRP treatment. Samples were spun at 20 000 x g to remove cell debris. Following centrifugation, the supernatants obtained from the cell extracts and media were stored at -80°C.

Total AChE activity was determined using the spectrophotometric method of Ellman et al. (1961) as described elsewhere (Gisiger and Stephens, 1988; Jasmin and Gisiger, 1990). The activity was measured in the presence of the non-specific cholinesterase inhibitor tetraisopropylpyrophosphoramidate (iso-OMPA). The total amount of protein present in the extracts was determined by the bicinchoninic acid assay (BCA; Pierce Laboratories). AChE molecular form profiles were determined by sedimentation analysis according to Jasmin and Gisiger (1990). Creatine kinase activity was assayed using a commercially available kit (Sigma).

Western Blot Analysis

Protein extracts from cultured myotubes were denatured at 100°C for 5 min in a buffer containing 0.5 % sodium dodecyl sulfate (SDS), 0.5 % Triton, 1mM phenylmethylsulfonyl fluoride, aprotinin (0.2 U/ml), 0.01 M Tris-HCl (pH 8.0), 0.14 M NaCl and 0.025 % NaN₃. Samples were separated by SDS-PAGE in a mini-PROTEAN II Cell apparatus (BioRad, Richmond, CA). After separation, proteins were electroblotted onto a PVDF membrane (Schleicher & Schuell, Keen, NH) and the membrane was then incubated with a mouse

monoclonal AChE antibody (Transduction Laboratories; Lexington, KY; 1:2500 dilution) for 1 hr followed by a second incubation with a horse radish peroxidase-conjugated secondary antibody for 30 min (1:3000 dilution). The band corresponding to AChE (68 kDa) was visualized by chemiluminescence using an ECL kit (NEN Life Science Products, Boston, MA) and its intensity was quantitated with the ImageQuant software program (Molecular Dynamics, Inc.; Sunnyvale, CA).

Reverse Transcription and Polymerase Chain Reaction

Total RNA was isolated from myotubes using 2 ml of Trizol (Gibco) per dish. Following isopropanol precipitation, the final pellets were washed with 70% ethanol, air-dried and resuspended in RNase-free water. RNA samples were adjusted to a final concentration of 80 ng/ μ l using a GeneQuant II RNA/DNA spectrophotometer (Pharmacia). Reverse transcription (RT) of 2 μ l of each RNA sample was carried out as previously described (Michel et al., 1994). Negative control samples were also prepared by substituting the 2 μ l of input RNA by the same volume of RNase-free water.

cDNAs encoding the AChE T subunit and ribosomal RNA (rRNA) were amplified using the polymerase chain reaction (PCR) as described in detail elsewhere (Jasmin et al., 1993; Michel et al., 1994). Primers for mouse AChE (5'-CTGGGGTGCGGATCGGTGTACCCC, cDNA nucleotides 1175-1198; 3'-TCACAGGTCTGAGCAGCGCTCCTG, cDNA nucleotides 1821-1844) and S12 rRNA (5'-GGAAGGCATAGCTGCTGG, cDNA nucleotides 65-82; 3'-CCTCGATGACATCCTTGG, cDNA nucleotides 415-432) were synthesized on the basis of available sequences (Forster et

al., 1993; Legay et al., 1995). For these experiments, several precautions were taken to minimize sample contamination and mRNA degradation (see Hubatsch and Jasmin, 1997) and to ensure that all measurements were taken within the linear phase of amplification (see Jasmin et al., 1993; Michel et al., 1994; Svestrup et al., 1995). Following amplification (AChE, 35 cycles; rRNA, 28 cycles), PCR reaction products were visualized on a 1% ethidium bromide-stained agarose gel. Quantitative PCR experiments were performed by separating PCR products on a Vistragreen (Amersham; Arlington Heights, IL) -stained agarose gel and by scanning the gel with a Storm PhosphorImager (Molecular Dynamics, Inc.; Sunnyvale, CA). The intensity of the AChE bands was determined and standardized according to the rRNA PCR products using the accompanying ImageQuant software program.

Nuclear Run-On Analysis

Nuclear run-on assays were performed using a procedure described elsewhere (Ray et al., 1995). Following in vitro transcription and RQ1 DNase (Promega) treatment, labelled RNA was isolated using Trizol and hybridized for 48 hr at 42°C with 10 µg of linearized AChE (2 Kb) and β -actin (2 Kb) cDNAs and genomic DNA immobilized on Genescreen Plus nylon membrane (DuPont). Following hybridization, membranes were washed thoroughly (1 X SSC, 0.1% SDS) at 42°C, and exposed for autoradiography. The intensity of the signals was quantified with a Storm PhosphorImager (Molecular Dynamics). The signals corresponding to AChE were standardized relative to the β -actin signal.

Statistical Analysis

Paired Student's *t*-tests were performed to determine the impact of CGRP treatment on AChE expression. The level of significance was set at $P < 0.05$. Data are expressed as means $\pm$ SE throughout. The pattern of AChE molecular forms displayed in Figures 7.1 and 7.3 are representative examples.

RESULTS

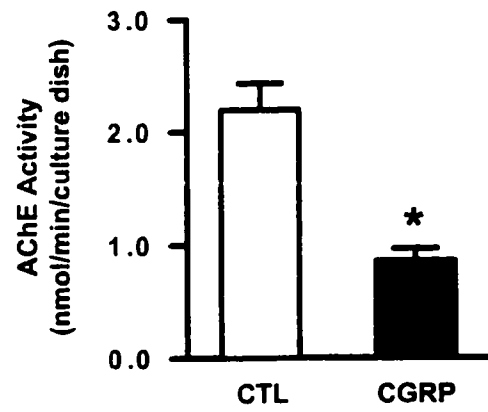
As shown in Figure 7.1A, total AChE activity expressed per culture dish was reduced by approximately 60% ($P < 0.05$) after CGRP treatment. AChE activity expressed per mg of extracted proteins was also reduced to the same extent following CGRP application. Similarly, primary cultures of mouse myotubes treated with CGRP also displayed a significant reduction in AChE expression (data not shown). Consistent with these activity data, Western blot analysis of extracts from vehicle- and CGRP-treated myotubes also revealed a significant reduction (CTL = 9244 ± 296 , $n = 3$; CGRP = 5037 ± 407 , $n = 3$; arbitrary units; $P < 0.05$) in AChE protein levels (Figure 7.2).

Velocity sedimentation analysis of extracts obtained from vehicle- and CGRP-treated myotubes revealed that the distribution of AChE molecular forms was altered following CGRP treatment (Figure 7.1B). Consistent with a recent report (Luo et al., 1998), we observed in control myotubes, an elevated level of G_1 as well as a significant peak of G_4 . By contrast, levels of G_1 were reduced considerably following CGRP treatment whereas the amount of tetramer was virtually eliminated (Figure 7.1C). This latter effect of CGRP on G_4 levels appeared as a specific downregulation since even prolonged incubation (up to 24 hr) of extracts from CGRP-treated myotubes with Ellman's assay buffer failed to yield detectable G_4 peaks.

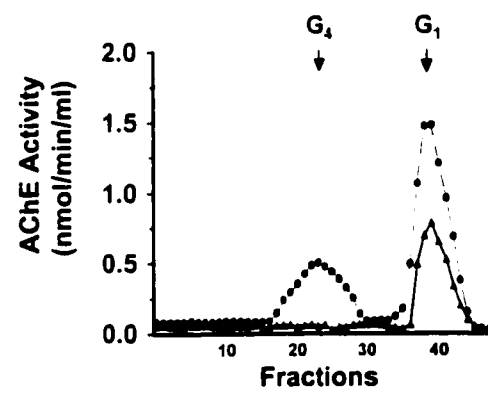
We next determined whether AChE activity and molecular forms in the media were also affected by CGRP. Samples of differentiation media collected after the first and second 24 hr-treatment period were therefore assayed. Interestingly, total activity of the secreted

Figure 7.1 Effects of CGRP on cell-associated AChE activity and molecular forms in myotubes. (A) analysis of AChE activity per culture dish measured from protein extracts isolated from vehicle- (control; CTL, n = 7 independent cell cultures) and CGRP-treated (CGRP, n = 7 independent cell cultures) cultured myotubes. (B) representative examples of AChE molecular form profiles obtained from vehicle- (circles) and CGRP-treated (triangles) myotubes. (C) activity of AChE molecular forms in vehicle-control (open bars) and CGRP-treated (closed bars) myotubes. Asterisks denote significant differences ($P < 0.05$).

(A)



(B)



(C)

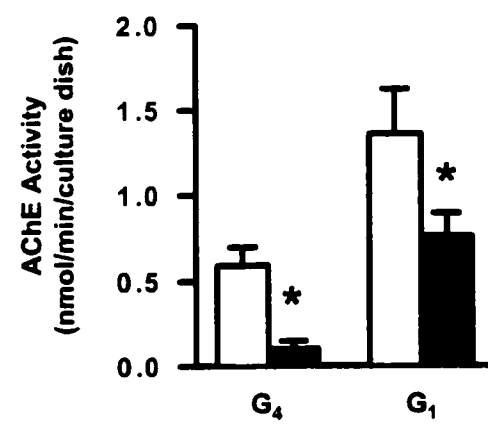
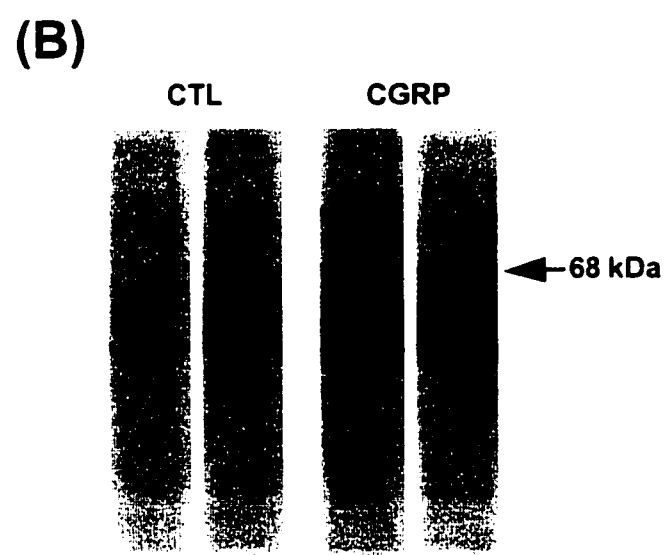
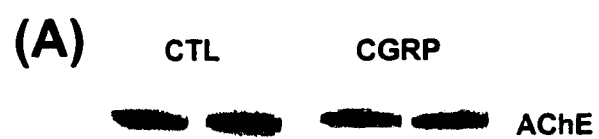


Figure 7.2 Effects of CGRP on AChE protein levels as determined by Western blot analysis. (A) shows representative examples of the immunoreactive 68 kDa AChE protein from vehicle-control (CTL) and CGRP-treated myotube extracts. (B) represents the PVDF membranes stained with Ponceau S which reveals that similar levels of proteins were loaded in each lane.

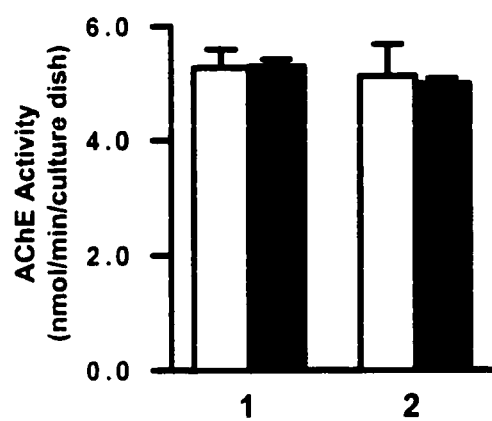


enzyme remained unaltered in CGRP-treated myotubes in comparison to vehicle-treated cells (Figure 7.3A; $P > 0.05$). As previously observed (see for instance Rubin et al., 1985), a large proportion of secreted AChE was contributed by the globular tetramer, and CGRP did not modify this pattern of molecular form secretion (Figure 7.3B and C). When total AChE activity was tabulated per culture dish, thus accounting for both cell-associated and secreted enzyme, we found that CGRP treatment downregulated AChE activity by approximately 20%. Creatine kinase levels, used as a marker of differentiation, were found to be nearly identical in vehicle- versus CGRP-treated myotubes (CTL = $5.07 \pm .97$, $n = 6$; CGRP = 5.32 ± 1.29 , $n = 7$; arbitrary units; $P > 0.05$) thereby ruling out the possibility that the observed reductions in AChE levels in response to CGRP were due to differences in the stage of myotube differentiation.

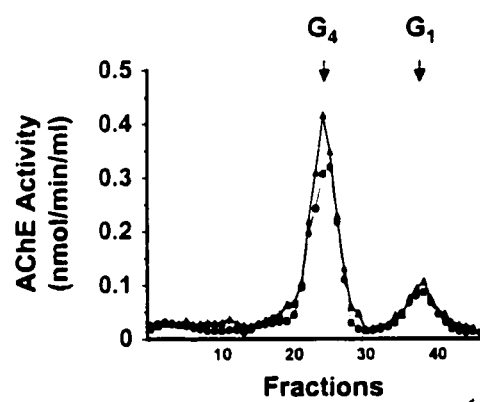
Finally, we also examined whether the decrease in AChE observed following CGRP treatment was accompanied by a concomitant reduction in the abundance of its mRNA. As shown in Figure 7.4A and B, AChE transcript levels were reduced by approximately 30% ($P < 0.05$) following CGRP treatment. This decrease in AChE transcript levels stands therefore, in good agreement with the overall reduction in AChE enzymatic activity (cell-associated plus secreted; see above). Additional experiments revealed that this reduction in AChE mRNA levels was not due to detectable changes in the transcriptional activity of the AChE gene (Figure 7.4C; CGRP $108\% \pm 13$ of control; $n = 3$; $P > 0.05$) suggesting that CGRP likely exerts its effects at the post-transcriptional level.

Figure 7.3 Effects of CGRP on AChE activity and molecular forms secreted in the media. (A) analysis of total secreted AChE activity following the first (1) and second 24 hr (2) period of vehicle (open bars; n = 4 independent cell cultures) or CGRP (closed bars; n = 4 independent cell cultures) treatment. (B) representative examples of molecular form profiles obtained from the media of vehicle- (circles) and CGRP- (triangles) treated myotubes. (C) activity of AChE molecular forms in vehicle- (open bars) and CGRP-treated (closed bars) myotubes.

(A)



(B)



(C)

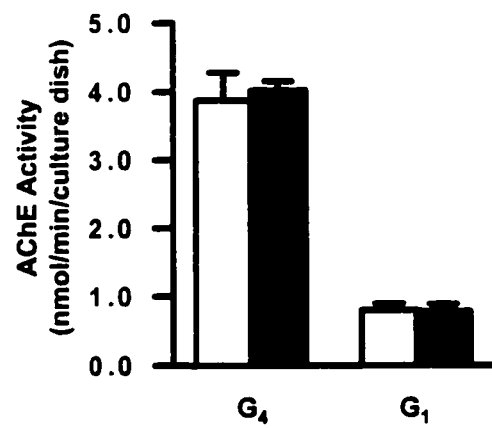
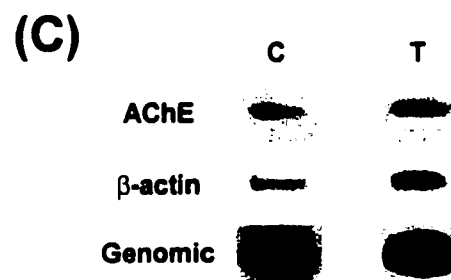
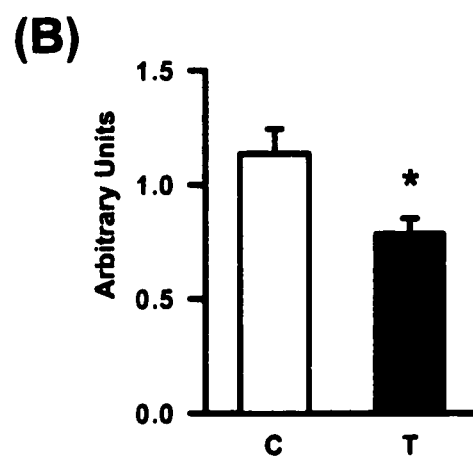
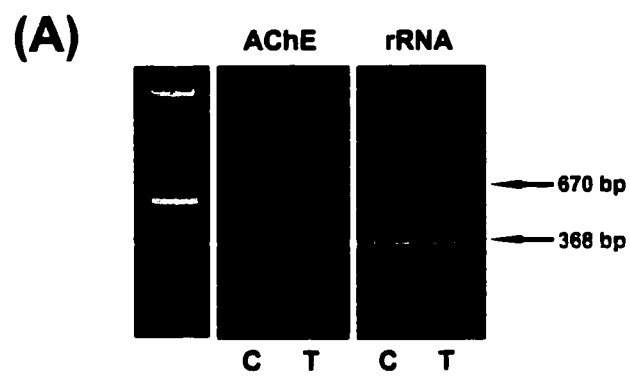


Figure 7.4 Effects of CGRP on AChE mRNA levels in cultured mouse myotubes. (A) examples of ethidium bromide-stained agarose gels displaying AChE and rRNA PCR products from vehicle- (control; C) and CGRP-treated (T) samples. Left lane is the 100 bp molecular mass marker (Gibco, BRL). (B) Quantitation of AChE mRNA levels in vehicle- (control, C; n = 5 independent cell cultures) and CGRP-treated (T; n = 5 independent cell cultures) myotubes standardized to rRNA. Asterisk denotes a significant difference ($P < 0.05$). (C) effects of CGRP on the transcriptional rate of the AChE gene in cultured myotubes. This representative run-on assay was performed with nuclei isolated from vehicle- (control; C) and CGRP-treated (T) cultured myotubes. See text for quantitation.



DISCUSSION

CGRP is a neuropeptide expressed by spinal cord motor neurons that has received considerable attention recently for its potential function at the neuromuscular junction (for review see Duclert and Changeux, 1995). Until now however, the focus of most studies has been to characterize the role of CGRP in the regulation of AChR expression. For instance, CGRP has been shown to increase the number of cell surface AChR by approximately 1.5 fold (New and Mudge, 1986). In addition, CGRP selectively increases the level of transcripts encoding the AChR α -subunit by approximately 3-fold (Fontaine et al., 1987; Osterlund et al., 1989). In the present investigation, we demonstrate that CGRP also exerts trophic effects on AChE expression by reducing enzyme activity as well as the abundance of AChE transcripts in mouse myotubes.

Modulations in AChE enzyme activity has been shown previously to correlate well with changes in AChE immunoreactivity in mammalian tissue. For instance, analysis of rat denervated muscle revealed a parallel reduction in both AChE activity and AChE protein levels (Brimijoin et al., 1987). Consistent with these findings, our data show a similar reduction in AChE activity and AChE protein levels in CGRP-treated cultured myotubes. Similarly, correlations between modifications in AChE enzyme activity and mRNA levels have also been reported. Following denervation of the rat extensor digitorum longus muscle for example, AChE activity is downregulated by 90% while AChE transcripts undergo a 10-fold reduction (Michel et al., 1994; see also Sveistrup et al., 1995). For these reasons, we hypothesized that the decrease in total AChE enzyme activity seen in response to CGRP

treatment would be paralleled by a similar reduction in AChE transcript levels. Our findings support such a correlation.

In attempts to gain insights into the molecular mechanisms underlying these reductions in AChE mRNA levels, we performed run-on assays with nuclei isolated from control and CGRP-treated myotubes. Within the range of sensitivity of this assay, we failed to observe any significant change in the rate of transcription of the AChE gene with CGRP treatment. Although there is evidence indicating that CGRP regulates transcription of different genes via the cAMP pathway, the contribution of this signalling cascade in the regulation of mRNA stability has also been documented (Oddis et al., 1995; Danner et al., 1998; Tian et al., 1998). Our results in fact, fit nicely with the recent observations showing that stability of existing transcripts is the primary mechanism by which AChE mRNA levels are controlled in differentiating myotubes (Fuentes and Taylor, 1993), neurons (Coleman and Taylor, 1996) and hematopoietic cells (Chan et al., 1998) maintained in culture. Taken together, these data indicate therefore, that CGRP likely exerts its effects on AChE expression at the post-transcriptional level.

Analysis of AChE molecular forms further indicated that CGRP exerts its effects on the pattern of molecular forms expressed by these cultured myotubes. More specifically, CGRP significantly reduced the levels of G_3 in agreement with recent *in vivo* studies (Hodges-Savola and Fernandez, 1995; Fernandez and Hodges-Savola, 1996). Since CGRP also reduced the amount of G_1 , the absence of G_4 could therefore imply that CGRP simply downregulates translation of G_1 catalytic precursors necessary for the assembly of the tetramer. Analysis of the secreted forms of the enzyme however, clearly demonstrates that

CGRP-treated myotubes are still capable of assembling tetramers destined for secretion thereby indicating that the reduction of the cell-associated G_4 pool represents a specific effect.

Previous studies have provided compelling evidence that in skeletal muscle, levels of G_4 are regulated independently from the other molecular forms (Fernandez and Donoso, 1988; Jasmin and Gisiger, 1990; Boudreau-Larivière et al., 1997). These studies have demonstrated in fast muscle, the presence of a separate pool of G_4 which can be increased substantially in response to high-frequency neuromuscular activation. Such specific regulation has in turn, led to the suggestion that the AChE tetramer fulfils a function altogether distinct from that assumed by the synaptic forms of AChE (see for instance Jasmin and Gisiger, 1990). Specifically, it has been suggested that G_4 may be responsible for the hydrolysis of excess acetylcholine released during high-frequency activation thereby preventing desensitization of the AChR. Interestingly, CGRP has previously been shown to directly increase the rate of desensitization of the AChR by modulating its phosphorylation status (Mulle et al., 1988; Miles et al., 1989). CGRP may therefore exert its effects both directly and indirectly by phosphorylating AChR and reducing G_4 levels, respectively, in order to ultimately increase the rate of AChR desensitization thereby modifying the efficacy of neurotransmission. Although the physiological relevance of increased AChR desensitization may be unclear, it appears reasonable to envisage that desensitization at the neuromuscular junction may provide a myoprotective mechanism necessary during periods of elevated phasic neuromuscular activity. In a recent study, the effects of CGRP on AChE expression were also examined in cultured chick myotubes (Choi et al., 1996; Choi et al., 1998). In this latter study, levels of AChE transcripts increased approximately 3-fold following CGRP treatment with no change in AChE

activity. These data which stand in contrast to the results of the present investigation, are not entirely unexpected especially if we consider the dramatic differences already reported concerning the effects of muscle denervation on AChE expression in rodent versus chick. Specifically, whereas AChE activity levels are reduced substantially in denervated rat muscle, enzyme activity in avian muscle appears to increase (Massoulié et al., 1993). Taken together with our current results, these findings provide further evidence that AChE levels in muscle are differentially regulated in response to the influence of neural factors in small rodents versus avian species.

CHAPTER VIII

GENERAL DISCUSSION

The objective of the present study was to determine the impact of nerve-derived signals on the cellular and molecular mechanisms underlying the expression and distribution of AChE in muscle. Using a combination of *in vitro* and *in vivo* approaches, we demonstrate that the synthesis of AChE in fast versus slow muscles is highly dependent on the pattern of nerve-evoked electrical activity rather than intrinsic properties of these muscle types. We also provide evidence that post-transcriptional events mediate nerve activity-dependent as well as neurotrophic regulation of AChE expression in muscle. Furthermore, our findings indicate that enrichment of AChE at the neuromuscular junction is due in part to enhanced transcriptional activity of the AChE gene in synaptic nuclei.

(A) AChE Expression During Muscle Differentiation and Maturation

AChE activity is detectable as early as day 9 of gestation in presumptive myoblasts of rodents (Tennyson et al., 1971). During the following days of gestation, AChE activity increases and is primarily localized intracellularly within the endoplasmic reticulum and near the nucleus (Tennyson et al., 1973). In mononuclear myogenic cells grown in culture, AChE activity as well as AChE mRNA are discernable and are both found to be induced upon fusion of myoblasts (Rieger et al., 1980; Inestrosa et al., 1983; Fuentes and Taylor, 1993; Grubic et

al., 1995). At these early stages of muscle differentiation, the primary AChE molecular forms expressed are the globular forms, particularly the monomers and tetramers (Figure 8.1) (Vigny et al., 1976; Inestrosa et al., 1983; Haynes et al., 1984; reviewed in Massoulié and Bon, 1982; Toutant and Massoulié, 1988).

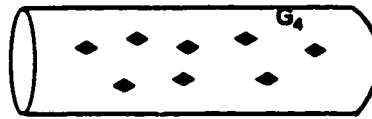
During the period of muscle innervation, which occurs between days 13 and 18 of gestation in small rodents (Bennett and Pettigrew, 1974; Dennis et al., 1981), endplate potentials of sufficient magnitude to initiate muscle contractions are detectable (Dennis et al., 1981). Interestingly, this time period temporally correlates with the appearance of asymmetric forms of AChE (Vigny et al., 1976) which have been shown in cultured myotubes, to be dependent on spontaneous or nerve-dependent muscle contractions (Rieger et al., 1980; Inestrosa et al., 1983; Brockman et al., 1984; Rubin, 1985; Fernandez-Valle and Rotundo, 1989). Asymmetric forms, in particular A_{12} , are at first present along the whole length of muscle fibers but become progressively restricted within the endplate region beginning at day 17 of gestation (Bennett and Pettigrew, 1974; Bevan and Steinbach, 1977) during the early phase of basal lamina deposition (Figure 8.1) (Dennis, 1981). At day 16 of embryonic development, clusters of AChRs coinciding with sites of nerve-muscle contact are observed (Bevan and Steinbach, 1977; Braithwaite and Harris, 1979) thereby supporting the proposal that accumulation of AChE at these sites is dependent on the prior aggregation of AChRs (De La Porte et al., 1998).

At birth, both fast and slow skeletal muscles display similar AChE molecular form profiles resembling in part those observed in mature slow muscles (Sketelj et al., 1991). Shortly thereafter, the molecular form profile expressed by early post-natal slow muscles is

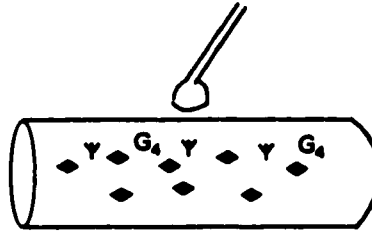
Figure 8.1 Schematic representation of AChE expression during embryonic (E) and post-natal (PN) development. Globular molecular forms are expressed in presumptive myoblast cells (E9) as well as very early during myotube formation, whereas the synthesis of asymmetric forms coincides with the onset of muscle innervation (~E15). The dotted line represents the basal lamina. Note that asymmetric forms become progressively restricted to the endplate region beginning late in embryonic life and continuing during post-natal development. At birth (PN 0-7), slow and fast muscles display similar AChE molecular form profiles whereas later during post-natal development (PN 14-28), these muscle types express distinct profiles characterized by greater levels of globular forms in fast fibers as well as the presence of asymmetric forms in extrasynaptic regions of slow fibers.



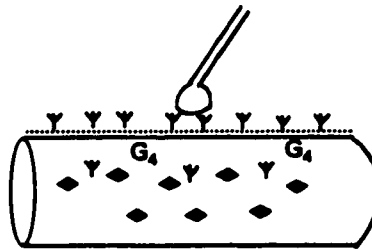
E 9



E 13



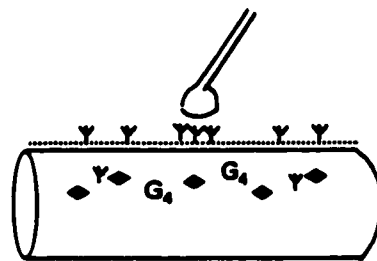
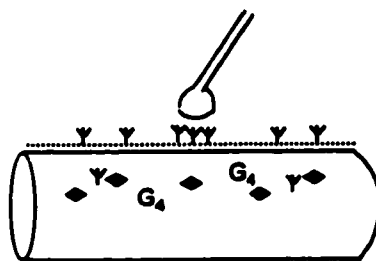
E 15



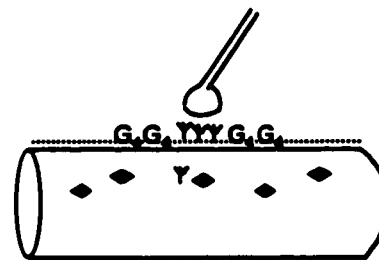
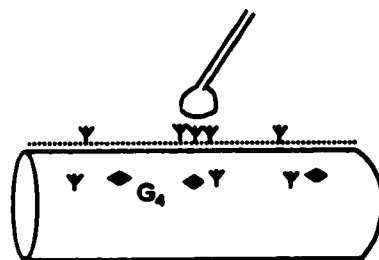
E 18

SLOW

FAST



PN 0-7



PN 14-28

◆ Globular
G₁ + G₂

Υ Asymmetric
A₁₂

nearly identical to that obtained from adult muscles of the same type characterized by proportionately high levels of asymmetric forms (Sketelj et al., 1991). In contrast, AChE molecular form profiles of fast muscles undergo profound alterations in their distribution during the second and third week of post-natal development before ultimately acquiring a mature profile (Vigny et al., 1976; Sketelj et al., 1991). Notably, the level of globular forms is increased whereas the proportion of asymmetric forms declines (Vigny et al., 1976; Koenig and Rieger, 1981; Sketelj et al., 1991). With maturation the activity of asymmetric forms in non-endplate regions of muscle fibers is also reduced in both fast and slow muscles but remains relatively high in slow muscles. Indeed, in slow muscle fibers, asymmetric forms account for approximately 40% of extrajunctional AChE whereas these complex forms represent less than 5% of AChE activity in non-endplate fast fibers (Figure 8.1) (Crme-Finderle et al., 1995). This latter observation may be explained by considering that the expression of the collagenic structural subunit appears to be as prominent in junctional and extrajunctional regions of slow muscle fibers whereas its expression is endplate-specific in fast fibers (Legay et al., 1998). In mature animals, fast muscles therefore differ from their slow counterparts by displaying higher total AChE activity, proportionately greater levels of globular forms as well as more focalized synaptic expression of asymmetric forms.

Although nerve-evoked electrical activity is regarded as a key regulator of AChE expression in mature fast and slow muscles, other lines of evidence suggest that the distinct patterns of AChE expression displayed by these muscle types is also attributable to intrinsic properties of the muscles (Sketelj et al., 1991; Dolenc et al., 1994). In Chapters II and III, we therefore assessed the role of nerve activity versus intrinsic muscle fiber properties in the

regulation of AChE synthesis in fast and slow-contracting muscles. Our findings (Chapter II) indicate that upon selective inhibition of neural activity, fast and slow muscles from adult animals express a common basic AChE molecular form profile in fact resembling that of newborn fast and slow muscles (Sketelj et al., 1991). Similarly, our data (Chapter III) demonstrate that myogenic precursor cells from either fast or slow muscle fibers give rise to myotubes displaying nearly identical levels of total AChE activity as well as AChE molecular form profiles. These findings, taken together, suggest that the patterns of AChE expression characteristic of mature fast and slow muscles are not attributable to intrinsic properties of these muscles but rather to nerve-evoked electrical activity.

The pattern of neural activation plays a significant role in regulating AChE molecular form profiles expressed in fast and slow muscles. In particular, it is apparent from our studies, that phasic high-frequency electrical impulses control the levels of G_4 in fast-contracting muscles. Notably, nerve-mediated phasic impulses were shown to counteract the effects of inactivation such that prominent levels of G_4 were maintained in fast muscles (Chapter II). Accordingly, it is conceivable that nerve impulse patterns may dictate the proportions of the various molecular forms in fast and slow muscles in part by regulating the synthesis of the collagenic and hydrophobic structural subunits (Sveistrup et al., 1995). Phasic activation of fast muscles may, for example, induce expression of the hydrophobic structural subunit thereby promoting the assembly of hydrophobic G_4 whereas production of the collagenic tail may be more dependent on tonic activity characteristic of slow muscles thereby favoring the synthesis of asymmetric AChE. In this context, expression of the collagenic structural subunit has in fact been demonstrated to be proportionately greater in slow versus fast muscles (Legay

et al., 1998). The pattern of neural activity may therefore be involved in fine tuning the levels of expression of the structural subunits thereby ultimately regulating AChE molecular form profiles in fast and slow muscles. Future studies aimed at elucidating the mechanisms regulating expression of these anchoring subunits will therefore be necessary to address this issue.

The degree of plasticity of AChE molecular forms in mature fast and slow muscles, appears to be less pronounced in response to altered patterns of neuromuscular activation. For example, fast muscles activated with tonic low-frequency stimulation express comparable AChE activity levels as their slow counterparts yet remain devoid of asymmetric forms in non-endplate regions (Sketelj et al., 1997). Similarly, our findings (Chapter III) and those of others (Jasmin and Gisiger, 1990; Sveistrup et al., 1995; Sketelj et al., 1997) indicate that AChE molecular form profiles of slow muscles are refractory to phasic activation patterns. Consequently, these findings imply that alterations in the molecular form profile of mature muscles occur within an "*adaptive range*" (Westgaard, Lomo, 1988) that is less extensive in slow than in fast muscles. These findings indicate that phasic and tonic activity are critical in the establishment of AChE molecular form profiles displayed by fast and slow muscles and that these activation patterns are capable of modulating these profiles within a limited range in mature muscles.

The levels of AChE activity displayed by slow and fast skeletal muscles, as well as their adaptation to altered neuromuscular activation, are reflective of the ability of endplates to respond to specific activation patterns thereby allowing sustained neuromuscular transmission. Indeed, the levels of AChE activity have been correlated with the frequency of synaptic

transmission such that the lower levels of AChE activity in slow muscles account for the slow time course of endplate potentials in this muscle type (Magazanik et al., 1979). Similarly, the endplates of fast muscle fibers are equipped with higher levels of AChE activity that ensures efficient hydrolysis of the greater number of ACh molecules secreted as a result of high frequency impulses delivered by their innervating motoneuron. The relative AChE content of slow and fast muscles is therefore responsive to the frequency of neuromuscular activation which in turn allows these muscle types to be activated either tonically (slow) or phasically (fast).

(B) Importance of Post-transcriptional Mechanisms in Regulating AChE Expression in Muscle

Nerve-evoked electrical activity is an important regulator of AChE expression in skeletal muscle (reviewed Massoulié et al., 1993). Findings presented in this thesis provide insights into the molecular mechanisms that mediate the effects of nerve activity. In particular, we demonstrate that higher AChE activity in mature fast versus slow muscles, is paralleled by greater levels of AChE mRNA in fast muscles as compared to slow (Chapter III; see also (Cresnar et al., 1994; Michel et al., 1994; Sketelj et al., 1998). Furthermore, analysis of the rates of AChE gene transcription using nuclei isolated from fast and slow muscles, revealed similar levels of AChE gene activity. These findings therefore suggest that the pattern of neural activation modulates AChE expression via post-transcriptional mechanisms. Interestingly, AChE mRNA levels have been shown to be significantly downregulated in intact

fast muscles electrically stimulated with tonic low-frequency activity thereby further indicating the importance of the activation pattern in regulating AChE expression (Sketelj et al., 1998). Using the denervation model, we provide further evidence that post-transcriptional events are key regulators of AChE synthesis in muscles. We observed that the dramatic reduction in AChE mRNA abundance in mature muscles following denervation is not due to reduced transcriptional activity of the AChE gene as assessed using two approaches, namely nuclear run-on assays and direct plasmid injection of an AChE promoter-reporter construct (Chapter IV). These observations strongly suggest that AChE transcript longevity is likely shortened in the denervated muscle thereby reducing AChE mRNA levels. In this context, preliminary findings from our laboratory suggest that incubating in vitro transcribed AChE cRNA with protein extracts from denervated muscles increases the rate of AChE RNA degradation compared to protein extracts from control muscles (Chan and Jasmin, unpublished observations). Expression of trans-acting factors involved in RNA-protein interactions may be dependent not only on electrical stimulation per se but also on the actual pattern of neuromuscular activation. The nerve may therefore control the relative abundance of these putative factors in fast- and slow-contracting muscles, in turn dictating AChE transcript longevity as well as the abundance of AChE mRNA in these muscle types.

AChE mRNA stability may be regulated by muscle contractile activity via modulations in the intracellular Ca^{+2} levels. Ca^{+2} influxes through voltage-sensitive L-type Ca^{+2} channels as well as ryanodine-sensitive Ca^{+2} channels have in fact been shown to be necessary for stabilizing AChE transcripts in cultured myotubes during the differentiation process (Luo et al., 1994; Luo et al., 1996). This Ca^{+2} -induced signalling cascade may in addition represent

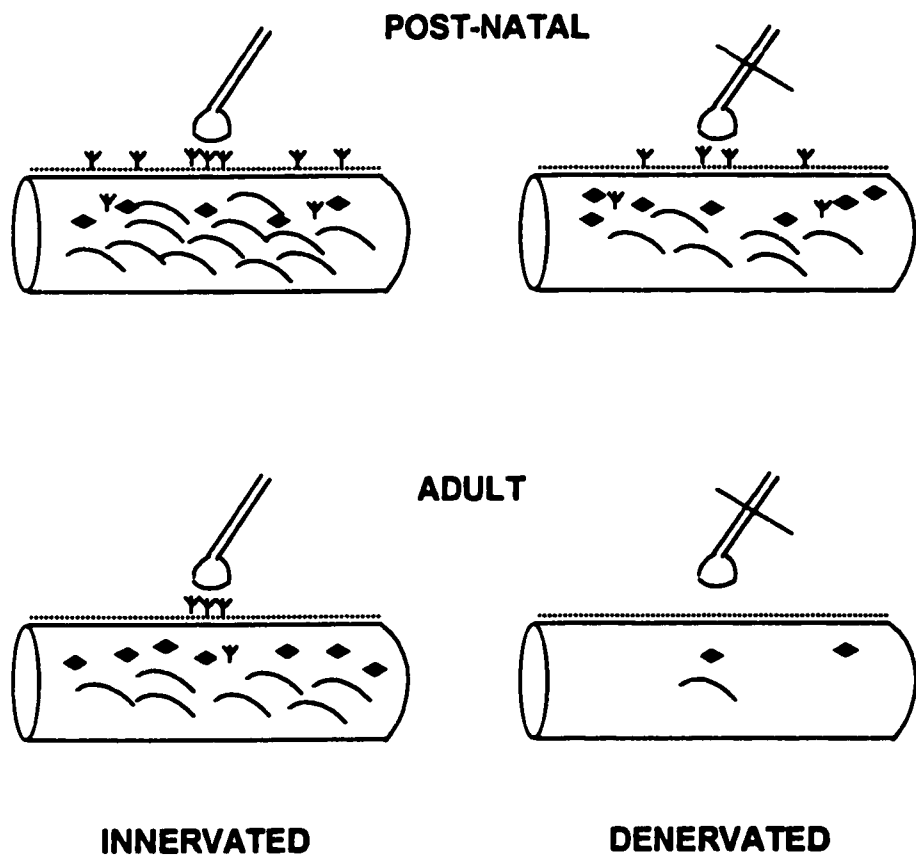
a common regulatory pathway involved in controlling post-transcriptional expression of other genes as recently reported for the AChR ϵ -subunit (Adams and Goldman, 1998). Ca^{+2} -mediated signals therefore play an important role in stabilizing AChE mRNAs during myogenesis and may also influence AChE message stability in muscles in vivo by regulating the synthesis and/or stability of RNA-binding proteins. Collectively, these findings provide a strong basis for future efforts aimed at identifying (i) mRNA binding factors which control AChE message stability, (ii) specific sites along the AChE transcript involved in governing message stability as well as (iii) mechanisms that regulate expression of these putative mRNA-binding proteins.

(1) Age-dependent regulation of the AChE gene

Denervation of mature skeletal muscle of small rodents dramatically downregulates AChE activity and profoundly reduces levels of asymmetric forms (Hall, 1973; Vigny et al., 1976; Fernandez et al., 1979; Weinberg and Hall, 1979; Chapter II). Denervation of muscles from neonatal and early post-natal rats and mice however, reveals that developing muscles respond in a markedly different manner. Specifically, these denervated muscles acquire the ability of forming several new patches of AChE activity in non-endplate regions of muscle fibers and continue to display elevated levels of asymmetric forms (Figure 8.2) (Lubinska and Zelena, 1966; Gautron et al., 1983; Yeakley et al., 1987).

Consistent with this differential impact of denervation on AChE synthesis in developing muscles, we provide evidence that muscle denervation induces different responses in AChE

Figure 8.2 Age-dependent AChE expression in response to denervation. Asymmetric forms persist in denervated post-natal muscles whereas these complex forms are virtually eliminated in mature muscles. In addition, new patches of globular forms appear in extrajunctional regions of denervated post-natal muscles while pronounced reductions of globular forms are observed in adult muscles. In innervated post-natal muscles, the basal levels of AChE mRNA are higher than those observed in mature muscles. Note also that the relative reduction in AChE mRNA is less pronounced in post-natal versus adult muscles following denervation.



◆ Globular $G_1 + G_2$ Y Asymmetric A_{12} ~ AChE mRNA

gene expression in young versus adult muscles. For instance, reductions in the abundance of AChE transcripts were clearly not as pronounced in denervated muscles of developing post-natal rats compared to adult rats (Chapter IV). Furthermore, our data suggest that increased AChE gene transcription in denervated post-natal rats is likely involved in attenuating the denervation response (Chapter IV) (Figure 8.2). In this context, preliminary EMSA results obtained in our laboratory using a DNA oligo corresponding to the first intronic N-box of the rat AChE promoter, revealed increased protein binding activity upon incubation with muscle extracts from denervated post-natal muscles compared to age-matched controls (Angus and Jasmin, unpublished observation). In contrast no change in binding activity was observed with protein extracts from adult denervated versus control muscles (Angus and Jasmin, unpublished observation). Although the reasons for this apparent age-dependent response are unclear, it is possible to envisage that developing fibers may retain some residual innervated features compared to adult muscles following denervation (Yeakley et al., 1987). Differentiation of the junctional membrane in developing fibers, for example, has been shown to proceed for sometime in the absence of innervation (Dennis, 1981). Collectively, these findings illustrate that nerve-derived signals exert their influence at different levels of the AChE biosynthetic pathway depending on the stage of maturation of the muscle. Whereas post-transcriptional mechanisms are the dominant regulators mediating activity-dependent expression of the AChE gene in adult muscle, transcriptional as well as post-transcriptional mechanisms are contributors to the regulation of AChE synthesis in developing muscles (Chapter IV).

Synapse formation, including elimination of polyinnervation, requires up to three weeks after birth for its completion (Dennis, 1981). During this time, the spatial and temporal

coordinate expression of several junctional components has been proposed to be necessary for ensuring proper growth and maturation of the developing synapse (Merlie and Sanes, 1986; Klarsfeld, 1987). In this context, our data (Chapter IV), together with those obtained from Witzemann et al. (1989), show that transcripts encoding AChE and AChR α -subunit undergo a similar pattern of down-regulation during post-natal development. Although AChE and AChR α -subunit mRNA levels are differentially affected by muscle denervation (i.e. AChE mRNA decreases, AChR α -subunit mRNA increases), our nuclear run-on experiments performed using myonuclei isolated from control and denervated neo- and post-natal muscles revealed a similar 3-fold increase in both AChE and AChR α -subunit gene transcription. Furthermore, transcripts encoding AChE and AChR α -subunit have been found to accumulate under newly formed synaptic contacts at the same developmental stage of the embryo (Piette et al., 1993; Legay et al., 1995). These findings therefore provide evidence that AChE and AChR α -subunit genes have common patterns of spatial and temporal expression during synapse formation and maturation and that transcriptional plasticity of these genes may play a critical role during the establishment of the mature neuromuscular junction.

(C) Mechanisms Underlying Expression of AChE mRNA at the Neuromuscular Junction

AChE molecules and in particular A₁₂, are highly concentrated at synaptic sites of adult muscle fibers (Hall, 1973). Accumulation of AChE transcripts within the post-synaptic region

of muscle has been demonstrated and therefore regarded as an important event leading to the aggregation of the enzyme at sites of nerve-muscle contact (Jasmin et al., 1993; Michel et al., 1994). Although the mechanisms responsible for the focalization of AChE mRNA within synaptic regions were not known, we anticipated that localized transcription of the AChE gene in synaptic nuclei may contribute to this process since genes encoding other synaptic proteins, such as AChR subunits, were shown to be preferentially active within these specialized regions of muscle fibers (Klarsfeld et al., 1991; Sanes et al., 1991; Simon et al., 1992; Duclert et al., 1993; Koike et al., 1995; Duclert et al., 1996).

Indeed, experiments described in Chapter V demonstrate that a DNA fragment corresponding to the 5' region of the rat AChE gene displays enhanced activity in synaptic regions of muscle fibers as evidenced by the preferential expression of a reporter gene within subsynaptic nuclei. In particular, the extent of synapse-specific events was 40% using a 3.4 kb segment of the AChE promoter. In contrast, however, a 2.2 kb DNA fragment corresponding to the AChR ϵ -subunit was able to drive significantly greater levels of synapse-specific gene expression (75 to 80%) (Duclert et al., 1993). These findings indicate that although enhanced transcriptional activity of the AChE gene at the synapse contributes to AChE mRNA aggregation at sites of nerve-muscle contact, additional regulatory events such as post-transcriptional mechanisms are likely involved.

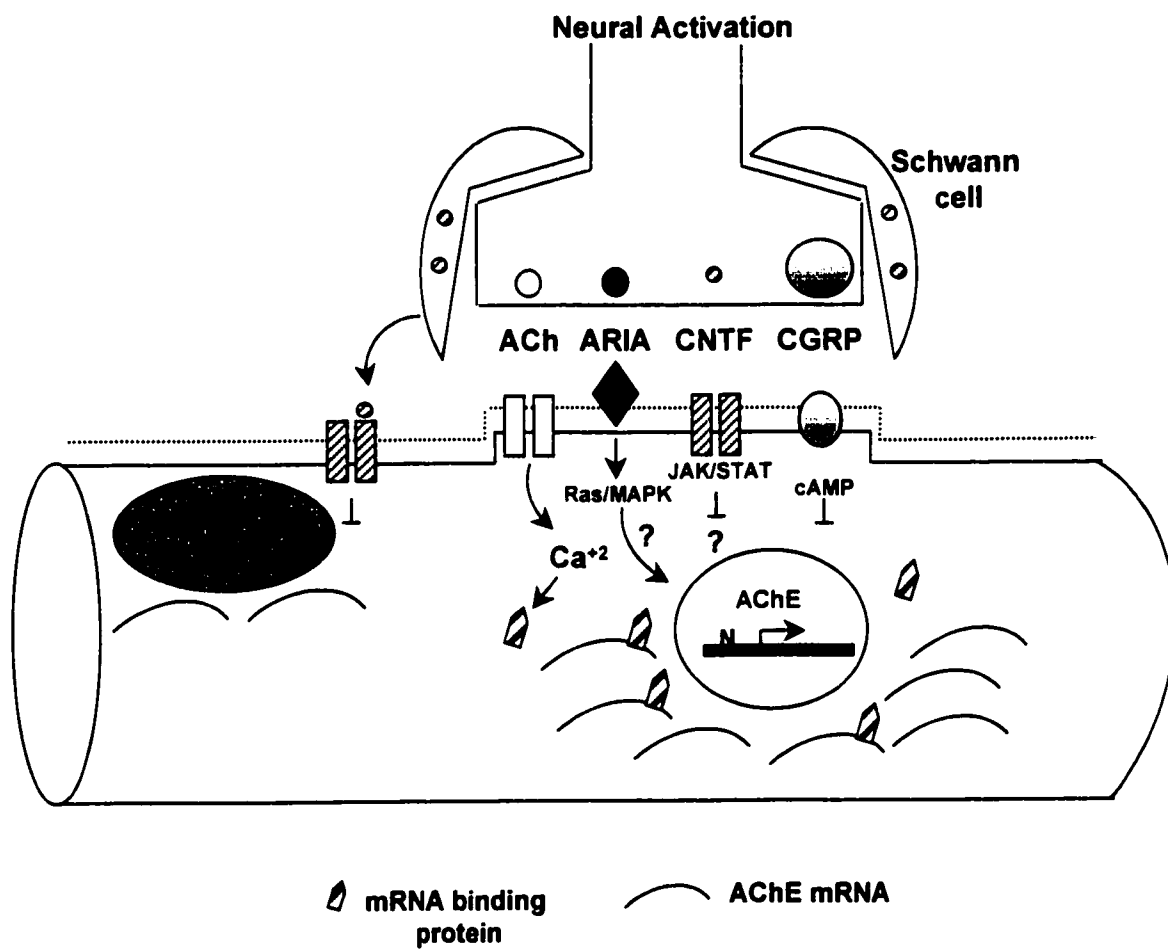
The neuromuscular junction represents less than 0.1% of the entire muscle fiber surface yet displays a 10-fold enrichment of AChE transcripts compared to non-endplate regions of the fiber (Jasmin et al., 1993; Michel et al., 1994). Since this pronounced accumulation of AChE mRNA is proposed to occur in part via post-transcriptional regulatory

mechanisms, it is conceivable that mRNA stabilizing proteins may also be preferentially expressed at the junction in response to nerve-derived signals (Figure 8.3). Furthermore, these mRNA-binding proteins may be similar or distinct from those involved in mediating activity-dependent AChE mRNA stabilization. Additional studies aimed at identifying such mRNA binding proteins as well as their distribution along muscle fibers will therefore contribute to our understanding of synapse-specific AChE expression.

Signals delivered by the nerve terminal are the most obvious candidates involved in enhancing and regulating AChE expression at the transcriptional and post-transcriptional level. In this context, several lines of evidence indicate that neuregulins, for example, promote expression of AChR subunit genes at the endplate via a Ras/MAP kinase phosphorylation cascade that ultimately targets Ets transcription factors which activate gene transcription through interactions with N-box motifs (Fromm and Burden, 1998; Sapru et al., 1998a; Schaeffer et al., 1998). Contrary to AChR subunit genes, however, there is no compelling evidence suggesting that neuregulin enhances AChE expression at least in cultured cells (Usdin and Fischbach, 1986; Pun and Tsim, 1995). Transcriptional regulation by Ets factors is known to be mediated through synergistic activity with transcription factors bound to neighbouring cis-elements (reviewed in Janknecht and Nordheim, 1993; Wasylyk et al., 1993). It is therefore conceivable that N-box binding proteins activated via the neuregulin signalling cascade, in combination with transcription factors bound to nearby cis-sites, are required for enhanced transcription of the AChE gene in synaptic nuclei. In this context, the contribution of an E box element located near the N box motif within intron 1, is currently being

Figure 8.3 Proposed molecular mechanisms regulating synapse-specific AChE expression.

Depolarization of the muscle membrane by cholinergic neurotransmission increases intracellular Ca^{+2} which may preferentially stabilize AChE mRNA in part by enhancing the synthesis of mRNA-binding proteins. ARIA (neuregulin) released by the nerve terminal initiates a phosphorylation cascade which may increase AChE gene transcription in synaptic nuclei through synergistic activity of N-box binding transcription factors with other trans-acting factors bound to nearby cis-acting elements. CGRP, contained within large dense-cored vesicles at nerve terminals, downregulates AChE expression via post-transcriptional mechanisms likely via the adenylate/cyclase signalling pathway. CNTF, synthesized by motor neurons and Schwann cells, suppresses AChE expression via mechanisms that remain to be determined but likely include transcriptional and/or post-transcriptional events dependent on the JAK/STAT signalling cascade. CNTF may influence AChE expression in both synaptic and extrasynaptic compartments since the CNTFR α -subunit of the receptor is expressed equally in endplate and non-endplate regions.



investigated for its potential involvement in regulating AChE gene transcription possibly in concert with the N box (Figure 8.3) (Angus and Jasmin, unpublished observations).

Aggregation of AChE mRNA within synaptic regions of muscle fibers may also be envisaged to occur through the activation of inductive and suppressive regulatory pathways mediated by transcriptional and post-transcriptional events. Our analysis of two presynaptically-derived molecules, namely CGRP (Chapter VI) and CNTF (Chapter VII), revealed that both trophic molecules downregulate AChE expression in muscle cells. In addition, CGRP was found to exert its suppressive effects on AChE synthesis via post-transcriptional regulatory mechanisms. Although the identification of trophic molecules involved in activating AChE expression remain to be identified, it is conceivable that accumulation of AChE transcripts at synaptic sites may be achieved by a combination of stimulating and repressive signals working in concert to fine-tune the levels of AChE mRNA at the endplate via both transcriptional and post-transcriptional mechanisms.

CHAPTER IX

CONCLUSIONS

The aim of the present thesis was to determine the impact of nerve-derived signals on AChE expression in skeletal muscles. Findings from this set of studies, in the context of our current knowledge of AChE biosynthesis and localization in muscle, may be summarised as follows.

1) Myotubes generated from myogenic precursor cells isolated from fast and slow muscle fibers express nearly identical patterns of AChE expression. The distinct AChE expression paradigms displayed by fast and slow-contracting muscles is therefore due primarily to the pattern of nerve-evoked activity, namely phasic versus tonic, generated by their respective innervating motoneurons. The activation pattern dictates the molecular form distribution displayed by these muscle types by regulating the synthesis of AChE catalytic peptides and probably that of the collagenic and hydrophobic structural subunits.

2) In mature muscles, the degree of plasticity of molecular forms is dependent on muscle type such that fast muscles exhibit pronounced though not complete transformations of the AChE profile in response to altered activity whereas slow muscles display limited adaptations.

3) AChE gene transcription in mature fast and slow muscles is similar thereby indicating that post-transcriptional mechanisms regulating AChE mRNA stability, likely account for the greater levels of AChE mRNA in fast muscles. Furthermore, the dramatic reductions in AChE mRNA observed in denervated muscles is not paralleled by a decrease in AChE gene activity thereby providing additional evidence that post-transcriptional events are important regulators of AChE expression in muscle. In this context, Ca^{2+} -dependent pathways may be regarded as potential signalling cascades modulating AChE transcript longevity in muscles.

4) Nerve-mediated regulation of AChE expression varies according to the stage of muscle maturation. Whereas transcriptional and post-transcriptional events control synthesis of the enzyme in developing muscles, post-transcriptional mechanisms appear as the sole regulators of AChE expression once muscles have reached maturity. Identification of transcription factors and mRNA binding proteins mediating these age-dependent regulatory events will considerably enhance our knowledge of the cellular and molecular mechanisms governing developmentally-controlled AChE expression.

5) Accumulation of AChE molecules within innervated regions of the muscle fiber is achieved in part by synapse-specific induction of the AChE gene. Post-transcriptional events, however, appear also to significantly contribute to endplate clustering of the enzyme. mRNA-binding proteins implicated in AChE transcript stabilization may therefore be envisaged to accumulate preferentially within endplate regions.

6) The precise identity of nerve-derived molecules involved in synapse-specific expression of AChE remain to be clearly elucidated. Our analysis of two presynaptically-derived molecules, namely CGRP and CNTF, indicate that both molecules downregulate AChE expression in muscle cells. These findings suggest that optimal levels of AChE transcripts at synaptic sites may be achieved by a combination of repressive and inductive pathways that function in parallel, ultimately adjusting AChE mRNA concentrations at the neuromuscular junction.

CHAPTER X

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CHAPTER XI

BIBLIOGRAPHY

Awards and Scholarships

External Awards:

- 1996-1997: Bourse Georgette Lemoyne, Canadian Federation of University Women
- 1994-1996: Natural Sciences and Engineering Research Council of Canada (NSERC) Post-graduate scholarship (PGS-B)
- 1992-1994: NSERC Post-graduate scholarship (PGS-A)*
- 1990-1992: NSERC Studentship*
- 1990-1992: Helen Byrnes Memorial Scholarship, Association of University Women*

University Awards:

- 1996-1997: William T. McEachern Scholarship from the University of Toronto
- 1994-1997: University of Ottawa Excellence Scholarship
- 1992-1993: Ambassador of France Award*
- 1991-1992: International Nickel Company (INCO) Bilingual Scholarship*

Other:

- 1996: American Physiological Society Graduate Student Award (poster presentation)
- 1993: Canadian Society for Exercise Physiology (CSEP) Student Award (research paper)*

Asterisk indicates award received while attending Laurentian University.

Publications

(i) Abstracts

- Boudreau-Larivière, C., Chan, R.Y.Y., and Jasmin, B.J.** (1998) Activity-linked regulation of acetylcholinesterase mRNA levels involves distinct molecular mechanisms in developing versus adult skeletal muscle. *Sixth International Conference on Cholinesterases and Related Proteins*, p. 128.
- Chan, R.Y.Y., **Boudreau-Larivière, C., Mankal, F.A., Angus, L., Krupa, A. M. and Jasmin, B.J.** (1998) Expression of the acetylcholinesterase gene in skeletal muscle fibers: role of intronic sequences. *Sixth International Conference on Cholinesterases and Related Proteins*, p. 119.
- Jasmin, B.J., Chan, R.Y.Y., **Boudreau-Larivière, C., Angus, L., Mankal, F.A., and Krupa, A. M.** (1998) Molecular mechanisms underlying the synapse-specific expression and activity-linked regulation of acetylcholinesterase at the neuromuscular junction: role of intronic sequences. *J. Physiol. (Paris)*, 92: 442.
- Boudreau-Larivière, C. and Jasmin, B.J.** (1997) Trophic regulation of acetylcholinesterase expression in cultured mouse myotubes. *Soc. for Neurosci.*, 23: 32.
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- Boudreau-Larivière, C., Chan, R.Y.Y., Mankal, F.A., and Jasmin, B.J.** (1996) Innervation-independent transcriptional regulation of the acetylcholinesterase gene in adult skeletal muscle fibers. *Soc. for Neurosci.*, 22: 1100.
- Boudreau-Larivière, C. and Jasmin, B.J.** (1995) Differential regulation of acetylcholinesterase in slow and fast muscles by nerve-mediated electrical activation. *Mol. Biol. Cell*, 6:250a.
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- Boudreau-Larivière, C. and Michel, R.N.** (1994) Rat plantaris motor unit characteristics after prolonged tetrodotoxin-induced paralysis and periodic short-term neural activation. *Can. J. App. Physiol.* 19, supplement: 7P.*

- Boudreau, C. and Michel, R.N.** (1993) Counteraction of the short-term effects of complete disuse of rat plantaris by sciatic nerve stimulation. *Can. J. App. Physiol.* 18:400P.*
- Megoney, L., Michel, R., **Boudreau, C.**, Tan, M., Prasad, M. and Bonen, A. (1993) Comparison of denervation and tetrodotoxin (TTX) on GLUT4 expression in rat hindlimb muscles. *Can. J. App. Physiol.* 18:421P.*
- Michel, R.N. and **Boudreau, C.** (1992) Functional axon sprouting of tetrodotoxin-inactivated rat plantaris motor units after partial denervation of the muscle. *Soc. for Neurosci.* 18:218.*

(ii) Book Chapters and Reviews

- Jasmin, B.J., **Boudreau-Larivière, C.**, Adatia, F.A., Angus, L., Chan, R.Y.Y., Kotlarewsky, M., Krupa, A.M., and Mankal, F.A. (1998) Molecular mechanisms controlling the synapse-specific expression and activity-linked regulation of acetylcholinesterase in skeletal muscle fibers. In *Structure and Function of Cholinesterases and Related Proteins*. B.P. Doctor, P. Taylor, D.M. Quinn, R.L. Rotundo, and M.K. Gentry (eds), Plenum Press, New York, p. 45-50.
- Jasmin, B.J., Gramolini, A.O., Adatia, F.A., Angus, L., **Boudreau-Larivière, C.**, Chan, R.Y.Y., Krupa, A.M., Lunde, J.A., Mankal, F.A., Wu, J. (1998) Nerve-derived trophic factors and DNA elements controlling expression of genes encoding synaptic proteins in skeletal muscle fibers. *Can. J. Appl. Physiol.* 23:366-376.
- Jasmin, B.J., **Boudreau-Larivière, C.**, Chan, R., Hubatsch, D.A. and Sveistrup, H. 1995. Neuromuscular factors influencing acetylcholinesterase gene expression in skeletal muscle fibres. In: *Enzymes of the Cholinesterase Family*. A.S. Balasubramanian, B.P. Doctor, P. Taylor, and D.M. Quinn (Eds), Plenum Publishing Corp., New York, p. 261-267.

(iii) Peer-Reviewed Articles

- Boudreau-Larivière, C.**, Chan, R.Y.Y., and Jasmin, B.J. Activity-linked regulation of acetylcholinesterase mRNAs involves distinct molecular mechanisms in developing versus adult skeletal muscles. Submitted.

Boudreau-Larivière, C., Parry, D.J., and Jasmin, B.J. Myotubes originating from single fast and slow satellite cells display similar patterns of AChE expression. *Am. J. Physiol. (Reg. Int. Comp. Physiol.)*, in press.

Chan, R.Y.Y., **Boudreau-Larivière, C.**, Angus, L., Mankal, F.A., and Jasmin, B.J. (1999). An intronic enhancer containing an N-box motif is required for synapse- and tissue-specific expression of the acetylcholinesterase gene in skeletal muscle fibers. *Proc. Natl. Acad. Sci. USA*, 96: 4627-4632.

Boudreau-Larivière, C. and Jasmin, B. J. (1999) CGRP decreases expression of acetylcholinesterase mammalian myotubes. *FEBS Lett.*, 444: 22-26.

Boudreau-Larivière, C., Gisiger, V., Michel, R.N., Hubatsch, D. A., and Jasmin, B. J. (1997) Fast and slow skeletal muscle express a common basic profile of acetylcholinesterase molecular forms. *Am. J. Physiol. (Cell Physiol.)*, 272: C68-C76.

Boudreau-Larivière, C., Sveistrup, H., Parry, D., and Jasmin, B. J. (1996) Ciliary neurotrophic factor: regulation of acetylcholinesterase in rat skeletal muscle and distribution of messenger RNA encoding its receptor in synaptic versus extrasynaptic compartments. *Neurosci.*, 73: 613-622.

Megeney, L., Michel, R., **Boudreau, C.**, Tan, M., Prasad, M. and Bonen, A. (1995) Regulation of muscle glucose transport and GLUT-4 by nerve-derived factors and activity-related processes. *Am. J. Physiol. (Reg. Int. Comp. Physiol.)*, 38: R1148-R1153.*

Asterisk denotes publication resulting from studies completed while attending Laurentian University.