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Passive Mechanical Stimulation Regulates Expression of Acetylcholinesterase in Skeletal Muscle Fibers

by

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ABSTRACT

Acetylcholinesterase (AChE) is responsible for the rapid hydrolysis of acetylcholine into acetic acid and choline thus allowing precise temporal control of muscle contraction. The expression of AChE in skeletal muscle is strongly influenced by both nerve-evoked electrical activity and trophic factors. In recent years however, several lines of evidence have emerged indicating that mechanical forces influence the growth and differentiation of a variety of tissues including cardiac and skeletal muscles. In the present study, we therefore tested the hypothesis that AChE expression is modulated by mechanical stimulation by using two distinct, yet complementary approaches.

In a first series of experiments, primary cultures of myotubes were subjected to repeated cycles of stretch/relaxation for 5 min, 30 min, 3 hr and 24 hr using the Flexercell FX-2000 strain unit. Total AChE activity increased by 42% over control levels at 24 hr. This increase reflected a general elevation in the activity of all AChE molecular forms as opposed to a preferential increase in a specific molecular form. Tetrodotoxin (TTX) did not prevent the increase in AChE expression whereas nifedipine partially blocked it. Mechanical stimulation of cultured clonal cells preferentially containing fast or slow myotubes further revealed that the increase in expression was not selective for a specific fiber type since AChE levels increased similarly in both fast and slow myotubes. Quantitative RT-PCR analysis showed that these changes in enzyme expression were paralleled by increases in the levels of AChE mRNA thereby suggesting the involvement of pretranslational regulatory mechanisms. Analysis of AChE secretion also showed a

70% increase in the amount of G_4 present in the media of 24 hr-mechanically stimulated myotubes. Since the AChE promoter contains consensus binding sites for transcription factors whose expression is known to be sensitive to mechanical stimulation in other cell types (Egr-1 and SSRE), we thus additionally examined the effects of mechanical stimulation on Egr-1 mRNA levels under these conditions. Egr-1 mRNAs increased by more than 300% after 30 min of mechanical stimulation. The time course of expression of Egr-1 mRNAs in response to mechanical stimulation is coherent with those previously found in other cell types, thus reaffirming the validity of this experimental model.

In a second series of experiments, we further tested our hypothesis *in vivo* by examining AChE expression in denervated rat hemidiaphragm muscle. The diaphragm represents a unique model since the denervated hemidiaphragm muscle is subjected to repeated cycles of stretch/relaxation due to rhythmical contractions of the intact contralateral hemidiaphragm. Levels of AChE transcripts and enzyme activity were markedly affected by sectioning the left phrenic nerve. In these muscles, there was a rapid and pronounced decrease in AChE expression which occurred as early as two days after denervation. Five days following sectioning, however, AChE activity and mRNA levels began to increase and appeared to plateau between 10 to 20 days post-surgery. The time course of recovery coincides in fact, with the return of normal breathing patterns following thoracic surgery. These results are therefore coherent with the notion that passive mechanical stretching of muscle fibers increases expression of AChE *in vivo*.

Results from these studies indicate that in addition to neural activation and trophic factors, passive mechanical forces modulate the expression of AChE in skeletal muscle

fibers. Since in tissue cultured myotubes TTX did not prevent the increase in AChE expression, it appears that the effects of mechanical stimulation are independent of electrical activity and further indicates the use of an alternate signalling pathway. The results therefore show for the first time, that passive mechanical forces modulate expression of a synaptic protein in skeletal muscle fibers. As such, these results fit well with converging lines of evidence indicating that the imposition of a mechanical stimulus dramatically affects expression of several muscle genes encoding specialized contractile proteins.

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ABBREVIATIONS

A forms	Asymmetric acetylcholinesterase molecular forms
AChE	Acetylcholinesterase
Ca^{++}	Calcium
cDNA	Complementary deoxyribonucleic acid
CGRP	Calcitonin gene-related peptide
CNTF	Ciliary neurotrophic factor
DBS	Donor bovine serum
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
E	Exon
EDL	Extensor digitorum longus
Egr-1	Early growth response gene
G forms	Globular acetylcholinesterase molecular forms
GPI	Glycophosphatidylinositol
H	Hydrophobic
HSB	High salt buffer
IEG	Immediate early gene
MEM	Minimal essential media
Na^{+}	Sodium

ABBREVIATIONS (cont.)

P	Hydrophobic tail structural subunit (G_4)
PEEP	Positive end-expiratory pressure
Q	Collagen tail structural subunit (asymmetric forms)
R	Read-through
RNA	Ribonucleic acid
RT-PCR	Reverse transcription-polymerase chain reaction
SSRE	Shear stress response element
T	Tail
TE	Trypsin and EDTA media
TTX	Tetrodotoxin

CHAPTER 1

INTRODUCTION

In 1914, Dale first suggested the existence of an enzyme which could degrade the esters of choline and played a role in neurotransmission within the autonomic and somatic nervous system. This enzyme, acetylcholinesterase (AChE), was subsequently demonstrated to fulfill a role in cholinergic transmission in the central and peripheral nervous systems through the rapid hydrolysis of acetylcholine into acetic acid and choline (Loewi and Navratil, 1926). Although AChE was discovered over 70 years ago, it is still today an intensely studied enzyme not only because it is an essential component of cholinergic synapses but also, because it represents an excellent marker of cellular differentiation. Furthermore, the fact that AChE may be involved in several pathological conditions such as Alzheimer's disease and leukemia has undoubtedly contributed to make AChE one of the most thoroughly studied enzymes to date (see Massoulié et al., 1993).

1.1 MOLECULAR FORMS OF ACETYLCHOLINESTERASE

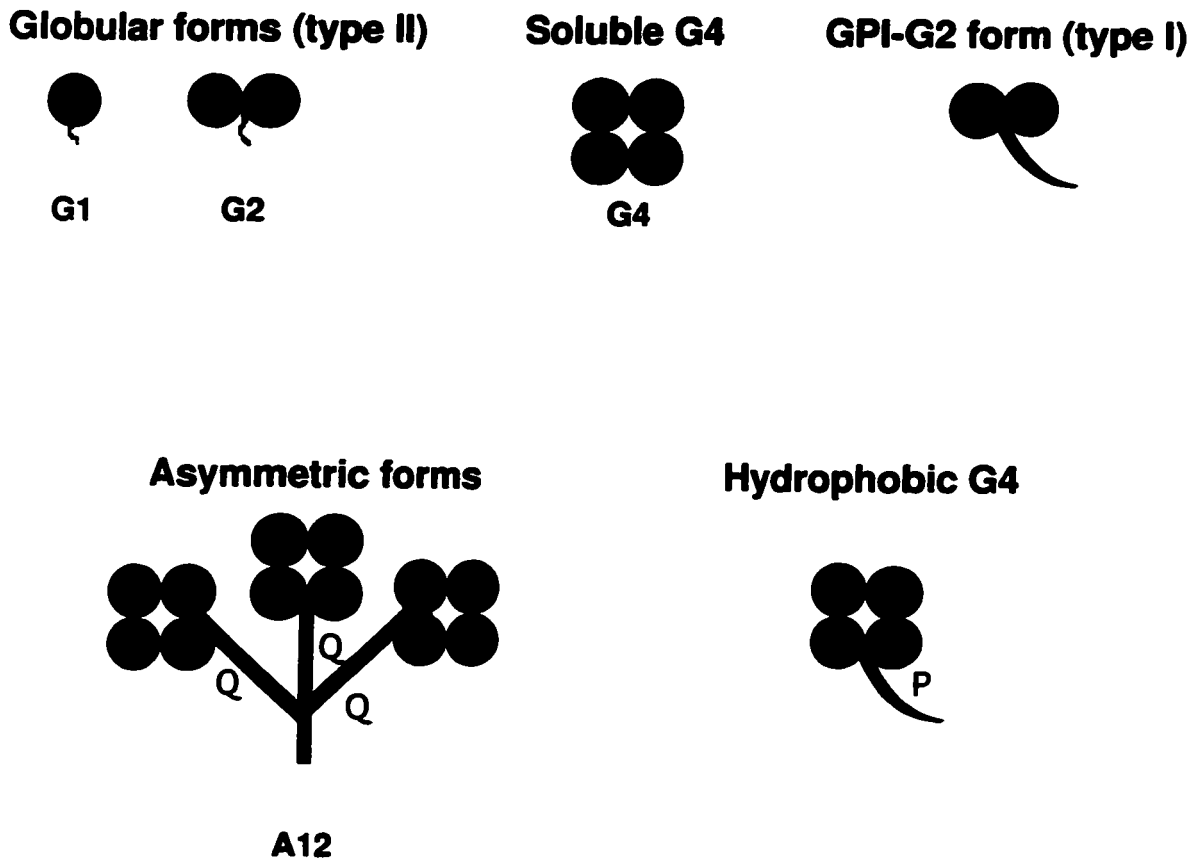
Acetylcholinesterase exists as a family of molecular forms consisting of monomers or oligomers which diverge structurally from one another due to alternate splicing of the gene and post-translational modifications. AChE molecular forms can be further found associated with specialized structural subunits. Although the molecular forms may differ in their structure, the catalytic activity (with the identical number of active sites) is identical (Vigny et al., 1978).

The globular forms are structurally the most basic forms of AChE. Monomers (G_1) can associate with each other through disulfide bonds producing homomeric dimers (G_2)

Figure 1

STRUCTURES OF THE MAJOR FORMS OF ACETYLCHOLINESTERASE IN VERTEBRATES

Two types of globular AChE enzymes have been identified. Type I G_2^a AChE possesses a glycophosphatidylinositol (GPI) membrane anchor, while the type II G_1 and G_2 AChE forms contain a different, as yet unknown anchor. The hydrophobic-tailed G_4 AChE is bound to the membrane via a 20 kDa non-catalytic subunit designated as P. The soluble G_4 molecules lack this structural subunit. In the asymmetric form A_{12}, three catalytic tetramers are linked via disulfide-bonds to a collagenic triple helical tail designated as Q.


Globular forms (type II)
Soluble G4
GPI-G2 form (type I)
G1
G2
G4
Asymmetric forms
Hydrophobic G4
Q
Q
Q
P
A12

and tetramers (G_4; see Figure 1). The globular forms can be further sub-divided into type I and type II AChE molecular forms based on their association with a structural subunit. Type I AChE dimers have been shown to bind a glycophosphatidylinositol (GPI) tail which allows for their insertion in the cell membrane (Bon et al., 1988; Rotundo, 1988). Type II G_1 and G_2 forms are expressed exclusively in adult nerve and muscle from mammals (Li et al., 1991, 1993B; Legay et al., 1993). There is some evidence suggesting that these forms are subject to a post-translational palmitoylation (Roberts et al., 1988; Randall, 1994) which may allow these molecular forms to associate with membranes although most are found as soluble subunits within the cytoplasm. As with the monomers and dimers, AChE tetramers are also subject to a sub-classification based on the presence of a structural subunit. The amphiphilic G_4 (G_4^a) is the predominant tetramer expressed in adult muscle cells and neurons. This molecular form is also referred to as the hydrophobic-tailed AChE form due to its association with a 20 kDa hydrophobic structural subunit designated as P (Inestrosa et al., 1987). In contrast, non-amphiphilic tetrameric forms (G_4^{na}) lack structural elements and are found at low levels in the cytoplasm of adult muscle fibers.

Asymmetric forms of AChE (A forms; see Figure 1) are characterized by the presence of a collagen tail which is formed by the helical association of three collagenic subunits designated as Q (Krejci et al., 1991). Each of the Q subunits can in turn attach to one, two, or three catalytic tetramers resulting in the asymmetric forms A_4, A_8 and A_{12}, respectively (see Massoulié and Bon, 1982, for a review on nomenclature).

Despite the rich polymorphism displayed by AChE, there is a tissue-specific

expression pattern that likely meets the functional demands placed upon the variety of cells expressing AChE. For example, adult mammalian haematopoietic cells are the only cell type expressing the type I GPI-linked dimer bound to the cell membrane and exposed to the extracellular environment (Gibney and Taylor, 1990; Duval et al., 1992). In contrast, muscle and brain display a more complex pattern expressing various levels of A_{12}, A_8, A_4, G_4, G_2 and G_1. Furthermore, the localization of the individual molecular forms is equally diverse. In skeletal muscle fibers for example, the asymmetric forms are found almost exclusively at the neuromuscular junction (Hall, 1973; Younkin et al., 1982; Fernandez et al., 1984) while the globular forms, although concentrated at the synapse, are found throughout the cell (Brimijoin, 1983). The exception to this is the G_4^s form of AChE whose expression is localized within the perijunctional area (Gisiger and Stephen, 1988). The factors influencing the tissue-specific expression as well as the selective localization are currently unclear. It is thought however, that the different physical properties of the molecular forms influence their localization and hence their function in adult cells (Vigny et al., 1978).

Although AChE is primarily found at cholinergic synapses, several authors have suggested that AChE may play other important physiological functions in addition to its well-described role in terminating neurotransmission (Greenfield, 1984; 1991; Appleyard, 1992). Although the current evidence is fragmentary, it is thought that AChE possesses several properties that affect neuronal function including alterations of membrane properties, enhancement of amino acid transmission and hydrolysis of peptides (Appleyard, 1992). Some of these functions have in fact been linked to the homology of

AChE to other known proteins. For example, AChE is homologous to neurotactin, a membrane protein of the *Drosophilia* nervous system that confers heterophilic adhesive properties to neurons (Barthalay et al., 1990). Since the cholinesterase-like motif of this protein constitutes the extracellular domain, it is believed that it is involved in these interactions (Barthalay et al., 1990; see also Massoulié et al., 1993). AChE has also been postulated to function during early stages of development by promoting neurite outgrowth (Layer, 1990) and haematopoietic cell proliferation (Soreq et al., 1994). Many questions remain however, regarding the putative non-classical function of AChE. Nonetheless, the evidence accumulated to date suggests that AChE plays additional roles distinct from its well-described function in neurotransmission. Its presence in plants and bacteria further reinforces this concept (Rama-Sastry and Sadavongvidad, 1979).

1.2 CELL BIOLOGY OF ACETYLCHOLINESTERASE

AChE molecules are first synthesized as globular molecular forms at the level of the rough endoplasmic reticulum where the signal peptide is cleaved after passage into the endoplasmic reticulum lumen (Rotundo and Fambrough, 1980). The globular forms are initially synthesized as inactive precursors of which ~80% become part of a rapidly-turning over pool degraded within an hour of synthesis by an unknown proteolytic process (Rotundo, 1988). It should be noted, however, that to date this inactive pool has only been characterized in chick skeletal muscle and brain (Rotundo, 1988; Chatel et al., 1994) and its existence in mammalian cells is still questioned (Hammond et al., 1988). Once the molecules become catalytically active, their main function appears to act as a precursor

pool for the assembly of the more complex forms of AChE. The asymmetric forms are assembled through the addition of the collagenic Q subunit to globular tetramers (Krejci et al., 1991) approximately 90 minutes later in the trans-Golgi apparatus (Rotundo, 1984; 1988). In contrast, the details of the assembly of the hydrophobic-tailed tetramer are still rudimentary. It is postulated however, that the assembly of the tetramers with the 20 kDa hydrophobic P subunit (Gennari et al., 1987; Inestrosa et al., 1987; Fuentes et al., 1988; Fuentes and Inestrosa, 1988) also occurs in the trans-Golgi apparatus in a manner similar to that of the asymmetric forms (Massoulié et al., 1993). The assembly of the asymmetric and hydrophobic-tailed molecular forms likely occurs via a default pathway that depends on the availability of the structural subunits. For example, when the Q subunit is more readily available than the P subunit, asymmetric forms are synthesized. The availability of the Q and P structural subunits are likely regulated by extrinsic factors (see Massoulié et al., 1993). This is discussed further in the context of AChE plasticity in section 1.5.

The Golgi apparatus does not only serve as the site of assembly of the asymmetric and hydrophobic-tailed forms. AChE molecular forms are also heavily glycosylated gaining about 10-15% of its molecular weight in carbohydrates in the Golgi. The functional significance of this glycosylation is currently unclear since it has been shown to be necessary for the acquisition of catalytic activity in chick (Rotundo, 1988) but not in human AChE (Velan et al., 1993). After glycosylation and upon exiting the Golgi apparatus, the enzymes are sorted and targeted to their respective and distinct cellular locations. The mechanisms underlying this sequence of events in skeletal muscle fibers remain vague. However, it has been shown that clathrin-coated vesicles participate in the

externalization of asymmetric and hydrophobic-tailed forms in rat muscle (Hodges-Sovola et al., 1989). In addition, the G_2^a form of AChE carries a specific glycan which may be responsible for its targeting to the nerve terminal in neurons (Massoulié, et al., 1993). Furthermore, colchicine, which inhibits polymerization of microtubules, blocks the secretion of AChE molecules and results in a cytoplasmic build-up of the enzyme in neurons. This suggests that microtubules may be involved in delivering the active enzyme to the cell surface (Lucas and Kreutzberg, 1985).

1.3 MOLECULAR BIOLOGY OF ACETYLCHOLINESTERASE

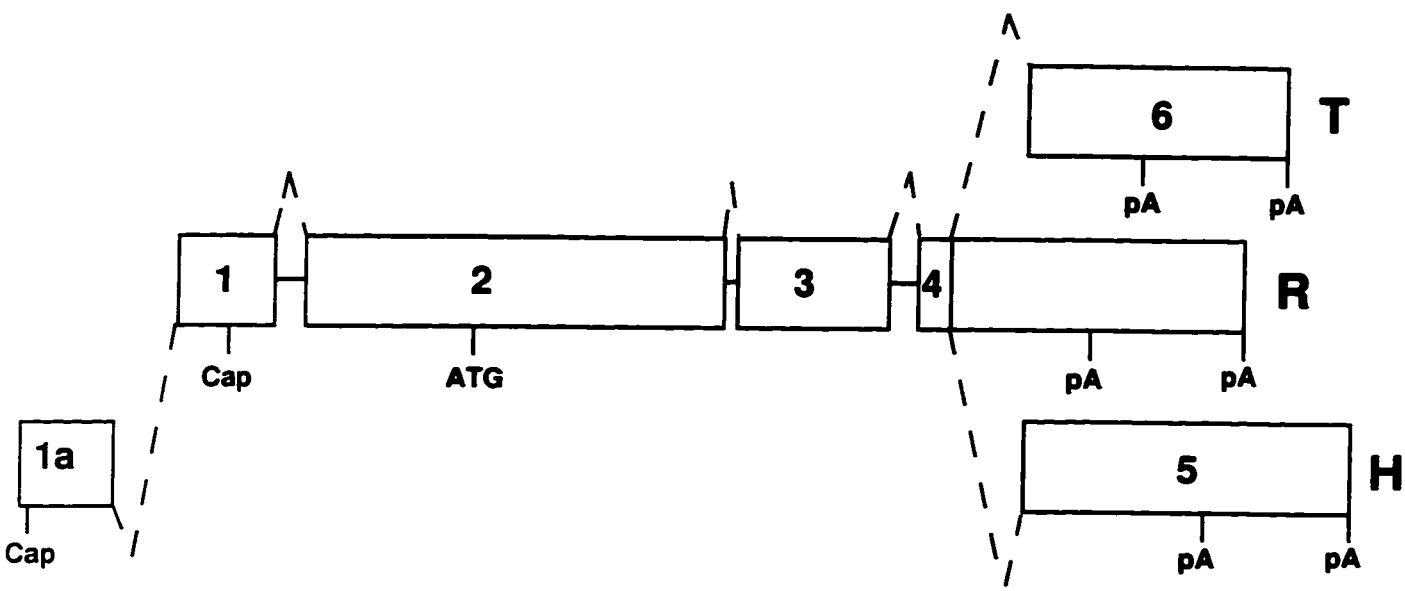
Acetylcholinesterase is encoded by a single gene in all vertebrate species studied to date (Rotundo et al., 1988; Sikorav , 1988; Shumacher et al., 1988; Rachinsky et al., 1990; Li et al., 1991; Getman et al., 1992; Chan et al., 1996). The AChE gene is located on the long arm of chromosome 7, at 7q22 in humans (Getman et al., 1992) and on chromosome 5 in the mouse (Rachinsky et al., 1992). In mammals, the multiple molecular forms of AChE are generated through the alternate splicing of six exons. Within the 4.5-4.7 kb mammalian gene (Li et al., 1991), there are three constant exons (E2, E3, and E4) which encode the common catalytic domain of the enzyme. The region 3' of exon 4 is subject to tissue-specific alternative splicing which generates the various mRNAs encoding the different molecular forms (see Figure 2). The lack of splicing at the 3' end of exon 4 results in the R, or read-through transcript. The enzyme translated from this mRNA is a hydrophilic, soluble monomer which lacks any hydrophobic stretch suitable for membrane insertion (Li et al., 1993A). To date, this transcript has not been detected in adult skeletal

Figure 2

ALTERNATIVE SPLICING OF THE MAMMALIAN ACETYLCHOLINESTERASE GENE

All AChE molecular forms arise from a common gene through alternate splicing. The common coding region of AChE is comprised of the invariant exons two and three. Alternate splicing from exon four to exon six results in the T transcript, while splicing from exon four to exon five gives rise to the H mRNA. A lack of splicing, or reading through at exon four generates the R transcript. The transcriptional start site (Cap), translational start site (ATG) and polyadenylation signals (pA) are indicated.

(modified from Taylor and Radic, 1994)


6
T
pA
pA
1
2
3
4
R
Cap
ATG
pA
pA
1a
5
H
Cap
pA
pA

muscle fibers (Legay et al., 1995). A second transcript is generated through splicing of exon 4 to exon 5 resulting in a peptide with a hydrophobic sequence at its carboxyl terminus (Li et al., 1991). This H, or hydrophobic subunit encodes an enzyme in which the terminal hydrophobic sequence provides a signal for cleaving this peptide prior to leaving the endoplasmic reticulum with the concomitant addition of a GPI tail (Li et al., 1991; Duval et al., 1992). These amphiphilic GPI dimers are found exclusively in cells of haematopoietic origin in adult mammals (Rachinsky et al., 1990; Li et al., 1991; 1993B). The third, and only AChE mRNA expressed in adult mammalian muscle and brain is the T, or tailed subunit. Splicing from exon 4 to exon 6 gives rise to this mRNA encoding a catalytic subunit having hydrophilic properties and an important cysteine residue near the carboxyl terminus (Li et al., 1991; 1993B; Legay et al., 1993). T transcripts of 2.4 and 3.2 kb are the predominant forms found in adult muscle and brain and their lengths vary due to the use of alternate polyadenylation start sites (see Li et al., 1991; 1993B). These transcripts are functionally identical except for the different lengths of the poly A tail which may contribute to mRNA stability (Taylor et al., 1991; Legay et al., 1993). This subunit can form homomeric, soluble dimers and tetramers by forming disulfide bonds with identical AChE catalytic subunits, or form the heteromeric AChE species through the association of homomeric tetramers with collagenic (Q subunit) or hydrophobic (P subunit) structural subunits. The H and T subunits therefore account for all known species of AChE expressed in cells (Duval et al., 1992).

Of particular interest to any studies examining the expression of a protein or gene are the factors which regulate transcription. In this context, several groups have

succeeded in isolating the 5' regulatory regions that correspond to AChE promoters. The AChE gene contains two CAP sites (Li et al., 1991; 1993A; Getman et al., 1995) and is extremely GC rich (Soreq et al., 1990; Getman et al., 1995; Chan et al., 1996). The 5' flanking region of the gene contains various consensus binding sites for transcription factors including Sp1, Egr-1, AP-2, Shear-Stress Response Element (SSRE), as well as numerous E-boxes (Figure 3; Aziz-Aloya et al., 1993; Li et al., 1993A; Getman et al., 1995; Mutero et al., 1995; Chan et al., 1996). The functional significance of these regulatory sites is discussed in the section on the contribution of these consensus sites in regulating AChE expression (section 1.7).

1.4 DEVELOPMENTAL EXPRESSION AND DISTRIBUTION OF ACETYLCHOLINESTERASE

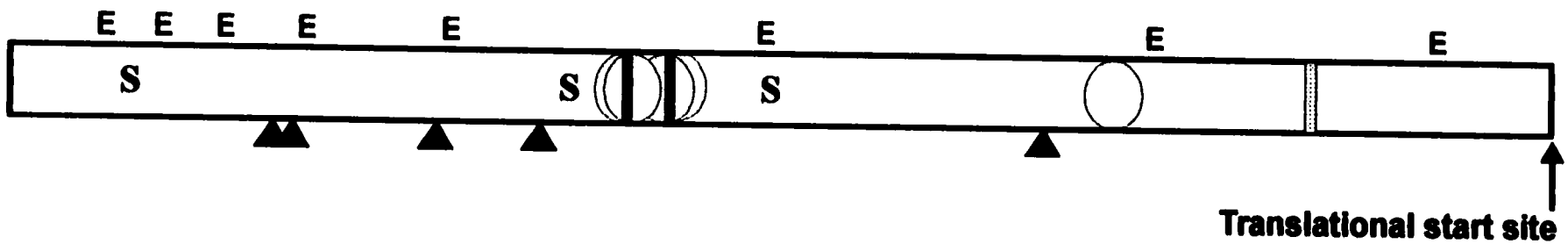
Acetylcholinesterase is already detectable, albeit at low levels, in mononuclear myogenic satellite cells derived from human skeletal muscle (Grubic et al., 1995). These levels of expression remain low prior to the fusion of myoblasts into myotubes after which a large increase in enzyme activity is observed in response to an unknown myogenic signal (Wilson et al., 1973; Rieger et al., 1980; Toutant and Massoulié, 1988). Vigny et al. (1976) reported that prior to day 14-15 of gestation in rat, AChE molecular forms consist primarily of globular enzymes after which the asymmetric forms begin to appear. Since motor endplates are formed during the last stage of development (Tello, 1917), the asymmetric forms therefore appear prior to the formation of neuromuscular contacts in rat hindlimb and diaphragm muscles (Kelly and Zacks, 1969; Bennet and Pettigrew, 1974). These asymmetric forms are initially present along the entire length of rat embryonic and

Figure 3

PROMOTER ELEMENTS OF THE RAT ACETYLCHOLINESTERASE GENE

Schematic representation of the consensus binding sequences for myogenic factors (E box), as well as for Egr-1, AP2 and Sp1. Consensus sequences for shear-stress response elements (S) and TATA box are also shown. The translational start sequence is indicated.

(from Chan et al., 1996, submitted)



S SSRE

E E-boxes

TATA box

Sp1 binding site

AP-2 binding site

EGR-1 binding site

100 bp

neo-natal muscles (Sketel and Brzin, 1980; Koenig and Rieger, 1981). The nerve contact subsequently appears to leave instructions leading to the selective accumulation of AChE at the endplate region (Koenig and Vigny, 1978; Weinberg and Hall, 1979; Lomo and Slater, 1980). This accumulation in fact, continues during post-natal development for up to 28 days after which AChE levels drop and adult concentrations are essentially reached (Vigny et al., 1976; Fernandez and Seiter, 1984). It has been suggested that the selective accumulation of AChE within the junctional region is due to localized expression since the enzyme displays a gaussian distribution pattern from the neuromuscular junction (Gisiger and Stephens, 1988). These findings however, hold true only for the globular AChE forms (G_1^a, G_2^a,[type II] and G_4^{na}) which are mostly intracellular since asymmetric (A_{12}, A_8) and hydrophobic-tailed (G_4^a) forms are found at the neuromuscular junction due to their association with synaptic structures (Hall, 1973; Younkin et al., 1982, Fernandez et al., 1984; Gisiger and Stephens, 1988). An exception to this is the slow soleus muscle which retains significant proportions of A_{12} and A_8 in extrajunctional regions (Sketelj et al., 1992). The reason for this difference in expression between muscle types is presently unknown and may represent an intrinsic property of muscle.

The variable distribution and expression of AChE enzyme observed during development may be directly linked to the expression of AChE mRNAs. For example, the ubiquitous expression of AChE enzyme observed along developing multinucleated myotubes coincides with transcription of AChE mRNAs that occurs asynchronously and independently in myotube nuclei (Grubic et al., 1995; see also Jasmin et al., 1993). Initially, all three AChE transcripts (R, H and T) are expressed in embryonic muscles

(Legay et al., 1995). However, by day 14 of embryonic development in the mouse, the R and H transcripts decline and are not detectable in adult muscle (Legay et al., 1995). The functional significance of the R and H transcripts in developing muscle remains unknown. These transcripts may serve a specific function during development or they may represent immature splicing events (Legay et al., 1995). The production of H and R transcripts appears to reflect a more primitive state of muscle evolution since they are also expressed in the mouse C_2-C_{12} cell line (Li et al., 1993B).

At the onset of synaptogenesis, the mechanisms modulating AChE levels and localization are altered dramatically. Expression of AChE and its mRNA become restricted to the junctional area; a selective pattern of expression which is retained throughout adulthood (Jasmin et al., 1993; Michel et al., 1994; Legay et al., 1995; see also Grubic et al., 1995). The synapse-specific localization of AChE mRNAs may be achieved by three different mechanisms (as suggested in Jasmin et al., 1993): 1) Transcription of the AChE gene is not restricted to nuclei in the junctional regions and newly transcribed mRNAs are transported to the junctional area; 2) AChE mRNAs are selectively stabilized at the neuromuscular junction; and 3) there is selective expression of the AChE gene in junctional nuclei.

Although there is fragmentary evidence supporting the first and third hypothesis (see Jasmin et al., 1989; 1990; Rotundo, 1990; Rossi and Rotuno, 1992; Tsim et al.,1992; Aziz-Aloya et al., 1993; Seidman et al., 1995), our current understanding of the factors governing AChE gene expression suggests that the second putative mechanism likely accounts for the synapse-specific expression of AChE mRNA. Fuentes and Taylor (1993)

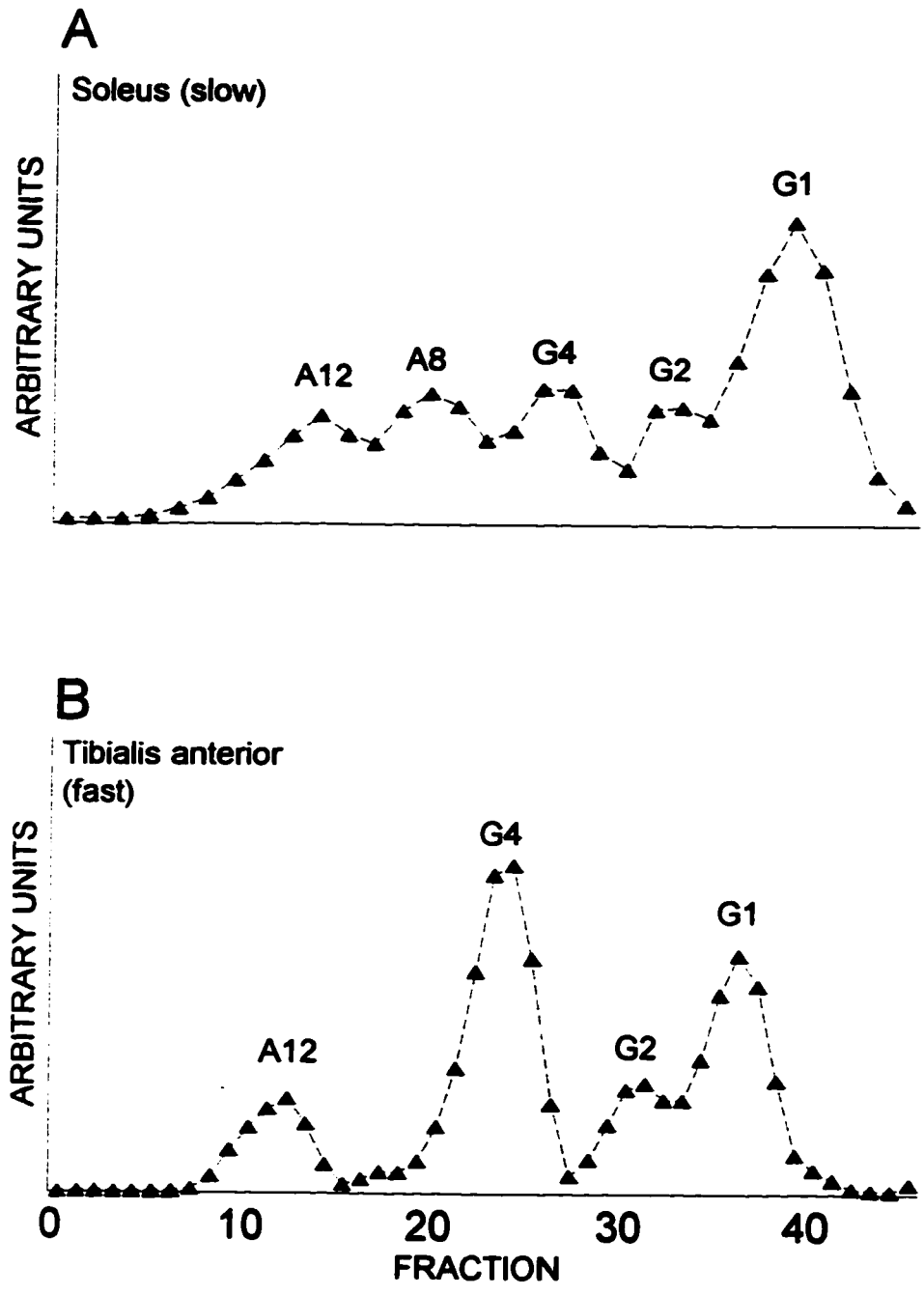
performed nuclear run-on analysis on nuclei isolated from C_2-C_{12} cells at different stages of development. Transcription rates were found to remain similar throughout differentiation suggesting that the accumulation of mRNAs in myotubes was due to enhanced stability of existing transcripts as opposed to an increase in transcription. The authors also found that cycloheximide treatment had no affect on the transcription rate of the AChE gene but that it induced a 20-fold increase in the levels of AChE mRNA. It is therefore likely that stabilization of AChE mRNA levels was increased by inhibiting the synthesis of components which influence the degradation rate of AChE transcripts (Fuentes and Taylor, 1993). These *in vitro* studies were recently reinforced *in vivo* in adult skeletal muscles. Chan et al. (1996) found using nuclear run-on assays that the large decrease in AChE transcripts which accompany denervation of skeletal muscles occurred independently of transcription and was thus due to a destabilization of AChE mRNA. These findings collectively suggest that stability of existing transcripts could alone account for the compartmentalized expression and activity-dependent regulation of AChE observed *in vivo* (Chan et al., 1996).

Significant differences in the pattern and distribution of AChE molecular forms exist between slow and fast skeletal muscles (Gisiger and Stephens, 1982). Asymmetric forms are more abundant in slow muscle fibers since they continue to be expressed extrajunctionally (Sketelj et al., 1991; Crne-Finderle et al., 1995). Furthermore, slow muscles, such as the soleus, express a more complex pattern of AChE molecular forms characterized by significant levels of A_8 and relatively low levels of G_4 (Figure 4). In contrast, fast muscles display a prominent peak of G_4 and lack any notable quantities of

Figure 4

ACETYLCHOLINESTERASE MOLECULAR FORM PROFILES OF FAST AND SLOW RAT SKELETAL MUSCLES

Aliquots of homogenates from slow soleus and fast tibialis anterior muscles were separated by velocity sedimentation on sucrose gradients and AChE enzyme activity analyzed spectrophotometrically. Note that the soleus profile (A) is more complex displaying peaks of A_{12}, A_8, G_4, G_2 and G_1. The tibialis anterior profile (B) lacks significant amounts of A_8 but has a large peak of the G_4 tetramer.


A
Soleus (slow)
ARBITRARY UNITS
A12
A8
G4
G2
G1
B
Tibialis anterior
(fast)
ARBITRARY UNITS
A12
G4
G2
G1
0
10
20
30
40
FRACTION

the A_8 molecular form. The observed differences in AChE expression in slow and fast skeletal muscles are established immediately after birth (Sketelj et al., 1991) before the institution of the specific neural stimulation patterns which are known to exist between these muscle types (Navarette and Vrbova, 1983). Nerve-derived electrical activity has in fact been shown not to be involved in initiating the characteristic fast or slow AChE profiles in skeletal muscle during the early stages of development in regenerating muscles (Dolenc et al., 1994). This implies that intrinsic differences exist in muscle progenitor cells giving rise to fast and slow muscles. Beyond the first three weeks of post-natal development however, the intrinsic expression pattern in fast muscles is subject to the effects of differential stimulation patterns (see section 1.5.2). Taken together, these results indicate that muscle satellite cells may have an intrinsic expression pattern of AChE during the early stages of muscle differentiation. After this initial period, extrinsic influences such as stimulation patterns, fine-tune the intrinsic pattern to modify it accordingly (Dolenc et al., 1994).

Several studies however, contend that patterns of AChE expression in slow and fast muscles may not be intrinsic but that a basic common profile exists for all muscle types (Boudreau-Larivière et al., 1996A). Following muscle paralysis induced with either tetrodotoxin (TTX) or botulinum toxin, AChE molecular form profiles of fast-twitch muscles were transformed into a profile typical of the slow-twitch soleus (Boudreau-Larivière et al., 1996A; see also Sketelj et al., 1993A). The transformation of the fast profile into a slow one was due to a combination of two events: a general decrease in all AChE molecular forms except A_8, concomitant with a preferential reduction in the G_4 content. Furthermore,

analysis of the AChE content in primary cultures of myotubes derived from slow or fast muscles indicated that both expressed a molecular form pattern resembling that of slow muscle (Boudreau-Larivière et al., 1996A). Differences in AChE expression between adult fast and slow muscles, previously thought to be intrinsic, may therefore be strictly due to the distinct pattern of nerve-derived electrical activity they receive.

1.5 PLASTICITY OF ACETYLCHOLINESTERASE IN RESPONSE TO VARYING LEVELS OF NEUROMUSCULAR ACTIVITY

1.5.1 Decreased neuromuscular activity

Paralysis induced via surgical denervation produces a rapid decrease of up to 60% of total muscle AChE activity within 7 days which reflects an almost total loss of the asymmetric forms (Hall, 1973; Vigny et al., 1976; Butler et al., 1978; Davey et al., 1978; Fernandez et al., 1979; Collins and Younkin, 1982; Lomo et al., 1985; Decker and Berman, 1990A; Cresnar et al., 1994; Boudreau-Larivière, 1996A). This selective loss of asymmetric molecular forms of AChE is probably due to the local action of proteases (Fernandez and Duell, 1980). The exception to this rapid decrease in AChE levels is during short-term denervation experiments (24-60 hr) where a transient increase in the levels of G_4 (~40%) occurs (Gregory et al., 1989). The authors attributed this transient increase in the levels of G_4 to supranormal levels of acetylcholine which are released from motor neurons immediately following denervation (Stanley and Drackman, 1986).

The decreased levels of AChE enzyme observed after denervation are likely due to reduced levels of AChE mRNAs. Indeed, accumulation of AChE transcripts has also

been demonstrated to be tightly coupled to the presence of nerve terminals. Denervation causes a rapid decrease in AChE mRNA levels (Cresnar et al., 1994; Michel et al., 1994) which results primarily from destabilization of existing transcripts as opposed to a transcriptional inactivation (Chan et al., 1996).

The adaptative response of AChE to the effects of denervation is different depending on the muscle fiber type. Although AChE activity decreases rapidly in both rat slow and fast muscles, the slow soleus is able to maintain approximately 25% of its AChE activity after two weeks of denervation while the fast extensor digitorum longus (EDL) is unable to maintain similar levels (Lomo et al., 1985; Michel et al., 1994; Boudreau-Larivière et al., 1996A). Although the exact reason for this is currently unknown, it is thought that the different stimulation pattern the muscle receives while innervated contributes to AChE plasticity and sensitivity to denervation.

Muscle paralysis achieved through TTX or botulinum toxin treatments also results in a reduced expression of both AChE transcripts (Michel et al., 1994, Cresnar et al., 1994) and enzyme levels (Stromblad; 1960; Drackman, 1972; Butler et al., 1978; Sketelj et al., 1993; Cresnar et al., 1994; Boudreau-Larivière et al., 1996A). These decreases are less pronounced than those induced by denervation. These results suggest therefore, that although nerve-evoked electrical activity per se appears to be a key regulator of AChE expression, the integrity of the synapse and/or the release of putative trophic factors contribute to the maintenance of AChE levels (Michel et al., 1994). Although the exact nerve-derived factors contributing to AChE expression in muscle have yet to be identified, recent studies have shown that previously identified trophic factors can modulate AChE

expression in skeletal muscle fibers. The contribution of trophic factors to the expression of AChE in skeletal muscle fibers are discussed in more detail in section 1.6.

1.5.2 Increased neuromuscular activity

Exercise training programs result in significant increases in enzyme activity in mammalian skeletal muscle (Crockett et al., 1976; Gardiner et al., 1982) . More recent studies, however, have taken into account that AChE exists as a family of molecular forms whose selective expression may depend on the functional demands placed upon the muscles. For example, various exercise regimes including walking (Fernandez and Donoso, 1988), running (Jasmin and Gisiger, 1990; Gisiger et al., 1994) and swimming (Gisiger et al., 1991) induce an increase in total AChE activity which in fast muscles is manifested primarily by a prominent elevation in the levels of G_4. In contrast, the slow soleus shows no changes in G_4 and may even display a slight decrease in total enzyme activity (Fernandez and Donoso, 1988; Gisiger et al., 1994; Sveistrup et al., 1995). Increases in the G_4 content are highly correlated to the amount of exercise performed by the animal (Gisiger et al., 1994). Furthermore, variations in the levels of G_4 have been shown to be tightly coupled to the type of superimposed neuromuscular activation. For example, phasic activity increases the G_4 content of fast muscles whereas tonic activation reduces it (Jasmin and Gisiger, 1990). Finally, the increase in the G_4 content described after exercise is due to an increased expression of G_4^a at the junction while the levels of the extrajunctional enzyme remain unchanged (Fernandez and Donoso, 1988; Gisiger et al., 1993). Since G_4^a associates with a structural subunit (P), it is possible to envisage

that the regulation of this synaptic enzyme is, at least partially, governed by the availability of this structural subunit (Sveistrup et al., 1995; see also Massoulié et al., 1993).

The dependence of G_4 on phasic electrical activity has been further demonstrated recently by a series of electrical stimulation studies. As previously discussed, TTX-induced paralysis of both fast and slow muscles results in a basic molecular form pattern which resembles that of a slow muscle. Boudreau-Larivière and colleagues (1996A) found that the fast AChE molecular form profile of a TTX-inactivated EDL muscle could be restored by superimposing on this muscle a phasic high-frequency stimulation pattern. Furthermore, restoration of high G_4 levels in stimulated but TTX-inactivated EDL muscle was seemingly at the expense of asymmetric forms suggesting that the regulatory mechanisms may lie at the level of the assembly of G_4 and asymmetric forms (Boudreau-Larivière et al., 1996A).

The functional significance of the G_4 plasticity in response to varying levels of electrical activity is not known. Gisiger and Stephens (1988) described a perijunctional compartment of G_4, the amount of which is high or low depending on whether the muscle is fast or slow. Because of this localization, it is also reasonable to postulate that G_4 is responsible for hydrolysis of acetylcholine diffusing out of the synaptic cleft (Fernandez and Donoso, 1988; Jasmin and Gisiger, 1990). Since these G_4 levels rise during periods of heightened electrical impulses, it seems likely to envisage that this pool of G_4 may participate in the regulation of endplate excitability by eliminating an excess of acetylcholine molecules in the synaptic cleft thereby avoiding desensitization of the acetylcholine receptor (Jasmin and Gisiger, 1990). Furthermore, it has been recently

found that G_4 levels increase significantly (>40%) between 4 to 8 hr after a single exercise or stimulation session indicating that AChE may be among the first motor endplate components to adapt to increases in neuromuscular activity (Boudreau-Larivière et al., 1996B; Fernandez and Hodges-Savola, 1996). This rapid adaptative response of G_4 further supports the notion that this molecular form plays a role in governing endplate excitability distinct altogether from that of A_{12} (Gisiger and Stephens, 1983; 1988; Fernandez and Donoso, 1988; Jasmin and Gisiger, 1990; Fernandez and Hodges-Savola, 1996).

Exercise represents an active model of increased neuromuscular activity. This enhancement can also be accomplished through a tonic activity model known as compensatory hypertrophy which is achieved via tenotomy of a muscles' functional synergists (Gardiner et al., 1986; Roy et al., 1991). This tonic model of neuromuscular activity results in an upregulation of total AChE enzyme activity in the overloaded, functionally intact muscle (Guth et al., 1964; 1966; Granbacher, 1971; Synder et al., 1973; Gardiner et al., 1986, Jasmin et al., 1991; Sveistrup et al., 1995). In contrast to exercise training programs, compensatory hypertrophy fails to elicit a selective increase in G_4. Instead, all molecular forms are increased with the overall molecular form profile shifting toward a slow muscle pattern (Jasmin et al., 1991; Sveistrup et al., 1995). The mechanisms underlying this adaptive response remains currently unknown. However, because compensatory hypertrophy elicits a response different from that of exercise training, distinct post-translational mechanisms may be selectively activated under each condition and in turn regulate the assembly of AChE molecular forms in the two models

(Sveistrup et al., 1995).

In contrast to the known effects of exercise and compensatory hypertrophy on AChE enzyme levels, the effects of these models of enhanced activity on AChE mRNA levels are less well-understood. Chronic increases of neuromuscular activity by way of voluntary wheel-running or compensatory hypertrophy result in significant elevations of AChE mRNAs in rat hindlimb muscles (Sveistrup et al., 1995). These results suggest that increases in AChE enzyme levels involves pretranslational regulatory mechanisms (Sveistrup et al., 1995). Together with the demonstration that AChE transcripts accumulates selectively within the junctional region of skeletal muscle fibers (Jasmin et al., 1993; Michel et al., 1994), these findings indicate that enhanced neuromuscular activation following either exercise or compensatory hypertrophy leads to increased expression of AChE mRNAs within the synaptic region of muscle fibers.

1.6 EFFECTS OF NERVE-DERIVED TROPHIC FACTORS ON ACETYLCHOLINESTERASE EXPRESSION

Nerve-derived trophic factors have been demonstrated to influence the phenotype of skeletal muscle fibers. For example, trophic factors have been shown to be capable of reversing the atrophy normally associated with muscle denervation (Davis and Heinicke, 1984). The notion that AChE regulation may be sensitive to trophic regulation was first demonstrated by Drackman in 1972 and, more recently, confirmed by Decker and Berman (1990B). In these studies, it was found that denervation of skeletal muscle not only decreased AChE enzyme levels, but also led to significant reductions in the contralateral muscle. In addition, blockade of axonal transport using colchicine results in decreased

levels of AChE in skeletal muscle fibers (Fernandez and Inestrosa, 1976) while total AChE activity in denervated muscle fibers maintained in culture increases upon treatment with soluble nerve extracts (Davey et al., 1979; Fernandez et al., 1980). Finally, TTX superfusion on the sciatic nerve, which inhibits electrical stimulation from neurons but does not block axonal transport, results in reductions of AChE activity and transcript levels that are less pronounced in comparison to those induced by denervation (Michel et al., 1994; Boudreau-Larivière et al., 1996B). In this context, researchers are searching for nerve-derived trophic factors which influence expression of AChE in skeletal muscle fibers. One route currently being followed is to analyze the effects of specific nerve-derived trophic factors on AChE expression.

The ciliary neurotrophic factor (CNTF) is a nerve-derived compound which has been shown to prevent the atrophy normally associated with skeletal muscle denervation (Helgren et al., 1994). In a recent study, daily administration of CNTF was unable to increase AChE transcript levels in denervated rat skeletal muscles (Boudreau-Larivière et al., 1996B). In fact, it was found that CNTF inhibited AChE expression without modifying the molecular form patterns in both intact and denervated skeletal muscles (Boudreau-Larivière et al., 1996B). Since mRNAs coding for the receptor for CNTF were found to be distributed homogeneously throughout the muscle fiber, the authors speculated that this trophic factor plays a role in down-regulating AChE expression in extrajunctional regions of muscle fibers.

Calcitonin gene-related peptide (CGRP) is a nerve-derived trophic factor known to contribute to the regulation of the acetylcholine receptor in vertebrates (New and

Mudge, 1986). Nerve-derived CGRP was recently found to also affect AChE expression. In these studies, it was observed that CGRP treatment decreased AChE enzyme levels in skeletal muscles by specifically preventing the increases in the levels of G_4 normally occurring in short-term denervated and exercise-trained muscles (Hodges-Savola and Fernandez, 1995; Fernandez and Hodges-Savola, 1996).

These findings collectively illustrate that nerve-derived trophic factors such as CNTF and CGRP may participate in the regulation of AChE in skeletal muscle fibers. Furthermore, they demonstrate that trophic factors may regulate AChE expression by modulating levels of both AChE enzyme and transcripts.

1.7 CONTRIBUTION OF PROMOTER REGULATORY SEQUENCES IN THE MODULATION OF ACETYLCHOLINESTERASE EXPRESSION

The mechanisms by which AChE expression is regulated at the level of transcription are currently vague. In recent years however, a number of putative regulatory sites that may play a role in modulating gene expression have been identified within the AChE promoter (see section 1.3). Although many sites have been identified, a number have been shown to be insignificant in modulating AChE expression including a TATA or CAAT box (Aziz-Aloya et al., 1993; Chan et al., 1996) and MyoD sites (Berri et al., 1995; Mutero et al., 1995). It should be noted however, that the majority of these studies have been carried out *in vitro* using promoter-reporter gene constructs. The situation *in vivo* may be significantly different.

In contrast, numerous sites which may play a role in modulating AChE expression in skeletal muscle fibers have been identified. The mammalian AChE gene has been

found to contain two CAP sites, both of which are capable of driving transcription, although the downstream one is used more frequently (Li et al., 1993). Although the significance of having two transcriptional start sites is unclear, the importance of this region was nonetheless recently demonstrated by Getman et al. (1995). Using an AChE promoter-luciferase reporter gene construct, Getman and colleagues (1995) found that mutating three bases within the initiator sequence resulted in the transcriptional rate dropping by 98% in human tetratocarcinoma cells. These CAP sites may therefore contribute to the efficiency of transcription in mammalian cells (Sikorav et al., 1987; Getman et al., 1995).

Of further interest to our studies is the influence of consensus sequences for SSRE and Egr-1. SSRE is a sequence that has been shown to be essential in genes which respond to mechanical stimulation in vascular endothelial cells (Resnick et al., 1993). Furthermore, the expression of Egr-1 is known to increase in response to mechanical stimuli in other cell types (Akai et al., 1994; Yamazaki et al., 1995). The current role played by these factors in AChE expression in muscle is unknown. In fact, no studies have examined the significance of the SSRE sequences in skeletal muscles while a single study has shown that the Egr-1 binding sequence is critical for AChE expression in mouse C_2-C_{12} cells (Li et al., 1993A).

Mutations in the Sp-1 sites have been shown to cause a significant decrease in promoter-reporter gene activity in mouse cells (Mutero et al., 1995). In fact, intact Sp-1 sites have been shown to be essential for human AChE promoter-reporter gene expression in fibroblasts and tetratocarcinoma cells (Aziz-Aloya et al., 1993; Getman et al., 1995). In contrast, the role of AP-2 sites still remains unclear. In mouse fibroblast cells, mutation

of these sites has been shown to down-regulate reporter gene expression (Mutero et al., 1995) while AP-2 has been demonstrated to act as a repressor in both mouse and human cancer cells (Getman et al., 1995). These sites may additionally play an indirect role in modulating AChE gene expression since Egr-1 and Sp-1 may compete for binding sites on overlapping sequences.

The use of alternate consensus sequences to regulate expression of gene products is a strategy commonly used. The tissue-specific expression patterns, developmental modulation and plasticity of AChE likely involves use of alternate promoter regulatory sequences. An inherent problem in many of the previous studies is that AChE promoter-reporter gene constructs have been typically expressed in cultured non-muscle cells. It is therefore plausible that other muscle-specific factors may influence AChE expression in concert with identified consensus sequences. In this context, only two groups have succeeded in performing expression studies *in vivo* using AChE promoter-reporter gene constructs. The laboratory of Hermona Soreq demonstrated that a short promoter sequence was sufficient for expression of human AChE in *Xenopus* embryos and further demonstrated that the gene product could be targeted appropriately within the embryos (see Aziz-Aloya et al., 1993; Seidman et al., 1995). Chan et al. (1996) recently succeeded in expressing a rat AChE promoter-reporter construct in rat diaphragm and tibialis anterior muscles. Preliminary studies indicated that the promoter fragment does not confer synapse-specific expression suggesting that post-transcriptional mechanisms account for the synapse-specific accumulation of AChE mRNA (Chan et al., 1996; see also section 1.4). Therefore, mutation studies using these types of model system would be clearly

more relevant than previous experiments performed with cultured fibroblasts or cancerous cells.

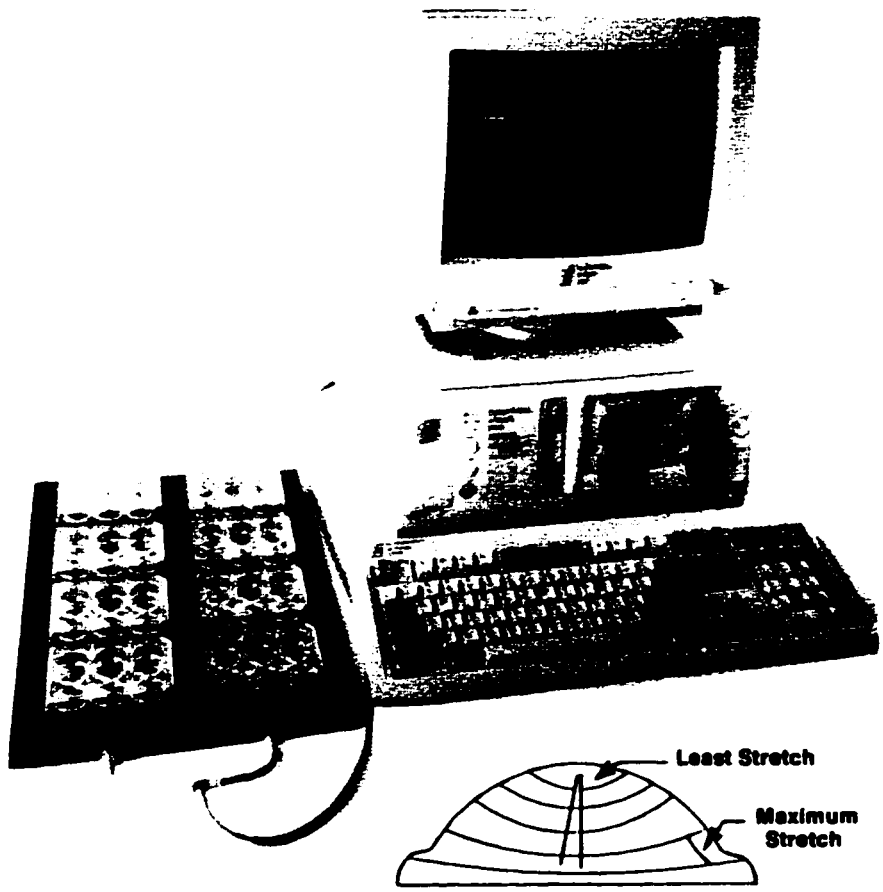
1.8 *IN VITRO* MECHANICAL STIMULATION OF CULTURED CELLS

It has been known for many years that mechanical forces modulate the growth and differentiation of a number of tissues including bone, lung and cardiac muscle (Vandenburgh, 1992; Inger, 1993). Progress in understanding the mechanisms by which these mechanogenic transduction processes occur at the cellular level has led to the development of various *in vitro* mechanical stimulation systems. Although they may differ in the basic mechanisms by which they work, they all allow cells to be grown on flexible bottom-tissue culture plates which can then be subjected to mechanical stimulation. In one of these systems, the Flexercell FX-2000 strain unit (see Figure 5), cells can be grown on flexible-bottom well plates and stimulation is achieved by deflecting the substratum downward with a vacuum while still in a proper CO_2 environment. The percent stretch of the substratum has been shown to be a non-linear function of the degree of vacuum applied (Banes et al., 1995), but a linear relationship exists between the percent of substratum stretch and the percent of longitudinal myofiber stretch (Vandenburgh, 1987; 1988). The stimulation cycle is highly reproducible since the percent stimulation is monitored by computer and it can be adjusted to compensate for external variables such as a weaker vacuum. The pattern of stimulation is programmed into the computer and can be tailored to mimic the conditions the cells might encounter *in vivo*. For example, cyclic stimulation patterns can be programmed to reproduce beating hearts, inhaling lungs and

Figure 5

FLEXERCELL MODEL FX-2000 MECHANICAL STRAIN UNIT

Cultured cells were grown on flexible bottom culture plates and subjected to a programmed mechanical stimulation protocol. The baseplate onto which the culture plates are positioned was connected to the computer via a small vacuum hose. The extent and duration of the mechanical stimulation was programmed into, and controlled by the computer and the Flexercell software. The surface deformation (inverted) of the flexible substratum is shown.


Least Stretch
Maximum Stretch

pulsating blood vessels (Vandenburgh, 1992). Skeletal muscle fibers can be subjected to either high intensity/low frequency or low intensity/high frequency stimulations, duplicating the effects of weightlifting or endurance exercises (Vandenburgh, 1992). The metabolic effects of these types of exercise *in vivo* leads to cell hypertrophy or to increases in the oxidative capacity of muscle fibers. Similar types of alterations have been reproduced *in vitro* in cultured skeletal muscle myotubes using a mechanical cell stimulator (Hatfaludy et al., 1989; Vandenburgh et al., 1990) demonstrating the validity of these model systems as valuable tools in understanding how physical forces modulate expression of specific gene products.

1.8.1 Mechanical stimulation of cardiac myocytes

The diversity of cells which can be studied using mechanical cell stimulators is limited only by the availability of specific cells. Although a wide variety of cell types have previously been subjected to mechanical forces, a significant amount of research focused on cardiac myocytes. Cardiac muscle cells, by their very nature, are subject to repeated cycles of mechanical stimulation. Although cardiac cellular hypertrophy is an adaptive process to the increased workload (Morgan et al., 1987), it is also at the root of the most complicated cardiovascular disorders (Yamazaki et al., 1995). Mechanical overload increases protein synthesis in isolated hearts (Kira et al., 1984), as does a stretch stimulus in cardiac myocytes (Mann et al., 1989; Komuro et al., 1991). Furthermore, many of the same biochemical and cellular responses which occur *in vivo* have been identified in cultured cardiac myocytes in response to mechanical stimulation (see Komuro and Yazaki,

1993; Vandenburgh et al., 1995; Yamazaki et al., 1995).

Over the last several years, a number of genes that respond to mechanical stress have been identified in cardiomyocytes. Among these is a group referred to as immediate early genes (IEG) whose transcription is activated rapidly and transiently by extracellular stimulation. The IEG include c-fos, c-myc, c-jun and Egr-1 and their tightly controlled expression suggests that their protein products play a regulatory role in the cellular response to external stimuli (Yamazaki et al., 1995). Although many of the biochemical and transcriptional events subsequent to mechanical stimulation have been clarified in cardiocytes (see for example Sadoshima and Izumo, 1993), the signalling mechanisms in many other cell types remain unknown.

1.8.2 Mechanical stimulation of skeletal muscle cultures

Skeletal muscle use is a major determinant in regulating growth and protein turnover. *In vivo,* an increased workload leads to cell growth and hypertrophy, whereas disuse leads to atrophy. More recent *in vitro* experiments indicate that cultured myotubes are also sensitive to the effects of mechanical stimulation since alterations in a number of cellular components including contractile proteins (Loughna et al., 1990), proteins required for cell growth and hypertrophy (Vandenburgh and Kaufman, 1979; 1981; Hatfuldy et al., 1989; Vandenburgh et al., 1989; 1990; 1991; Chromiak and Vandenburgh; 1992), prostaglandins (Vandenburgh et al., 1990; 1991; 1995), phospholipases (Vandenburgh et al., 1993), insulin-like growth factor-1 (Perrone et al., 1995), and cyclooxygenase (Vandenburgh et al., 1995), have been reported.

In a recent study, Jasmin et al. (1992) found that innervated chick anterior latissimus dorsi muscle subjected to stretch-induced hypertrophy demonstrated an increase in AChE expression, suggesting that passive mechanical forces may play an important role in the regulation of AChE in skeletal muscle fibers. In support of this hypothesis, Chan et al. (1996) found the presence of consensus binding sites for SSRE and Egr-1 within the rat AChE promoter whose expression is known to be sensitive to passive mechanical forces in other cell types (Komuro and Yazaki, 1993; Resnick et al., 1993; Akai et al., 1994; Yamazaki et al., 1995).

1.9 OBJECTIVES OF STUDY AND HYPOTHESIS

While it is clear that expression of AChE is significantly modulated by nerve-evoked electrical activity and nerve-derived trophic factors, the possibility that passive mechanical forces associated with repeated cycles of muscle contraction and relaxation influence AChE expression also exists (Jasmin et al., 1995). In the present study, we determined the impact of passive mechanical forces on AChE expression and began to examine the role of putative mechanogenic signalling mechanisms. On the basis of the current literature, we hypothesized that passive mechanical stimulation of rat skeletal muscle fibers increases AChE expression. Since compensatory hypertrophy increases all molecular forms of AChE, we also expected to observe a general elevation in all molecular forms in cultured rat myotubes subjected to mechanical stimulation as opposed to a selective increase in the expression of a specific form.

To test this hypothesis, we employed two models whereby skeletal muscle fibers

are subjected to passive mechanical forces. In the first model, cultures of myotubes were subjected to a cyclic pattern of passive mechanical stimulation using a computerized cell stimulator. The second model makes use of a unique physiological situation whereby the denervated hemidiaphragm muscle undergoes passive mechanical stimulation as a result of the movements of the intact contralateral side during the inspiratory phase of breathing (Zhan et al., 1992; Zhan and Sieck, 1992).

CHAPTER 2

METHODS AND MATERIALS

2.1 ANIMAL CARE AND SURGICAL PROCEDURES

Female Sprague-Dawley rats were used for all studies and were purchased from Charles River Laboratories (St-Constant, P.Q.). Care and treatment of all animals were in accordance with the guidelines presented by the Canadian Council on Animal Care.

2.1.1 Primary cultures of rat myotubes

To obtain primary cultures of rat skeletal myotubes, young rats (~50 g) were anaesthetized with sodium pentobarbital (35 mg/kg, i.p.) and the gastrocnemius, EDL, soleus, plantaris, and tibialis anterior muscles were quickly excised under aseptic conditions. To obtain a large number of satellite cells, the various muscles from 4 to 5 animals were typically pooled together for these experiment to yield an n of 1. Upon excision, all muscles were kept in ice-cold phosphate-buffered saline until surgery on all animals was completed.

2.1.2 Acetylcholinesterase expression during neo-natal development

Pregnant Sprague-Dawley rats (16 to 18 day gestation) were obtained for the studies on the developmental expression of AChE. Pups were removed from this litter at 1, 4, 7, 14 and 21 days after birth (day 1 animals were less than 3 hr-old). Soleus and tibialis anterior muscles were removed under a dissecting microscope and muscles from individual time-points were pooled, frozen in liquid nitrogen and kept at -80 °C until analyzed. The number of soleus and tibialis anterior muscles analyzed per time-point was typically between 14 to 20 and were analyzed as one sample.

2.1.3 *In vivo* denervated hemidiaphragm model

For denervation studies, female Sprague-dawley rats (180 to 200 g) were anaesthetized using halothane in conjunction with a respirator set at 70 strokes/min and a tidal volume of 2.5 ml (5 cm H_2O positive end-expiratory pressure, PEEP). The left side of the animal was shaved and the skin thoroughly cleansed using hibitane followed by betadine. All surgery was performed under aseptic conditions and the instruments were disinfected using a 70% ethanol solution containing 5% Dettol. A 2 cm incision was made in the skin slightly above the 6th rib and the skin was separated from the underlying musculature using blunt-end scissors. After gently separating the muscle fibers of the external abdominal muscle, an intra-thoracic incision was made between the 6th and 7th ribs. The lung volume was then temporarily reduced by removing the PEEP and the phrenic nerve was isolated using a glass rod. A 2 mm segment of the left branch of the phrenic nerve was removed to denervate the hemidiaphragm and prevent its reinnervation. Lung atelactesis was removed by reapplying 5 mm H_20 of PEEP. Pneumothorax was removed and the chest sealed using Prolene 4.0 sutures and rodent autoclips. The animal was ventilated on ambient air until revived. At 2, 5, 10 or 20 days post-denervation, the rats were anaesthetized and the entire diaphragm removed. Hemidiaphragms from sham operated animals were used for control samples as the left and right hemidiaphragms are not of equal side and the right side may have hypertrophied due to the added workload. The hemidiaphragms were separated and frozen immediately in liquid nitrogen and stored at -80 °C until used.

2.2 PRIMARY CULTURES OF SKELETAL MUSCLE MYOTUBES

Freshly dissected muscles were minced in 8 volumes of minimum essential media (MEM, Gibco; Burlington, Ont.) containing 15% donor bovine serum (DBS, Cansera; Toronto, Ont.), 1% penicillin/streptomycin (Gibco), 0.1% fungizone (Gibco), and collagenase (1.79 mg/g tissue, Sigma; St. Louis, MO) 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 3 min at 4 °C. The soft pellet was resuspended in MEM (with 15% DBS and antibiotics) with the exception that dispase, a non-specific protease (20 mg/g tissue, Boehringer Mannheim; Laval, P.Q.) was added instead of collagenase. The mixture was further incubated at 37 °C for 45 min. Digested muscle fibers were filtered through a sterile 53 μM nylon filter and the resulting filtrate was spun at 4 °C for 10 min at 250 x g. The pellet was resuspended in MEM with DBS and antibiotics. Satellite cells thus obtained were plated at a density of 2 x 10^6 cells /well (715 cells/mm^2) on Matrigel (Collaborative Biomedical Products; Bedford, MA) coated Flexcell plates (Flexcell International Corporation; McKeesport, PA). Cells were grown at 37 °C in a water-saturated environment containing 5% CO_2. Medium was changed every other day until cells reached confluence. At this stage, DBS was reduced to 2% to promote differentiation of myoblasts into myotubes. Control cells were grown on identical Flexcell plates to avoid substratum-induced selective expression of proteins. Cells were used for AChE analysis 8 to 10 days after plating.

2.3 MOUSE H-2K^b-tsA58 AND C_2-C_{12} CELL LINES

Mouse H-2K^b-tsA58 (see Jat et al., 1991) clonal fast or slow satellite cells, and C_2-C_{12} cells frozen in MEM:fetal calf serum:dimethylsulphoxide (7:2:1) were obtained as a stock from Dr. D.J. Parry (University of Ottawa). Cells were thawed at 37 °C and pelleted at 350 x g for 10 min at 4 °C. They were subsequently washed 3 x at room temperature in MEM. They were resuspended in a growth media consisting of 10% fetal calf serum (Gibco), 20% horse serum (Gibco), 1% chick embryo extract (Gibco), 1% penicillin/streptomycin and 1% L-glutamate (Gibco). Mouse H-2K^b-tsA58 cells additionally had 20 U/ml of mouse recombinant interferon-γ (Gibco) in the media. Cells were plated at a density of 1 x 10^4 cells /well (20 cells/mm^2) on Matrigel coated Flexcell plates. The growth media was changed every other day until the cells reached confluence. Cells derived from H-2K^b-tsA58 mice have two promoters controlling their rate of proliferation and differentiation. The H-2K^b promoter, which is highly active, is used to direct expression in a broad range of tissues and is further enhanced by interferons. Conferring immortality to these mouse cells is the large tumour antigen gene from the simian virus 40 strain tsA58. This promoter is temperature sensitive and is functional only at 33 °C. Increasing the culture temperature conditions to 37 °C results in a loss of expression of this gene and the satellite cells no longer proliferate but begin to differentiate. Satellite cells from H-2K^b-tsA58 mice were kept at 33 °C in a water-saturated environment containing 5% CO_2 to stimulate proliferation into a confluent monolayer. Satellite cells from C_2-C_{12} cells were grown at 37 °C in a 5% CO_2 water-saturated environment. To promote differentiation into myotubes, growth media from both cell lines was replaced with a differentiation media

consisting of 2% horse serum, 0.5% chick embryo extract, 1% penicillin/streptomycin, 1% L-glutamate in MEM and all myotubes were grown at 37 °C in a 5% CO_2-saturated environment. Myotubes were typically formed within three days after growing in differentiation media and were then used for experiments.

Cell lines were passaged when they were approximately 75% confluent. The media was removed and replaced with MEM containing 0.25% trypsin and 1mM EDTA (TE). The mixture was gently swirled for 1 min observing under a microscope that cells were detaching. After a further two min incubation at 37 °C, the TE solution was neutralized with an equal amount of previously removed growth media. Culture dishes were washed 3 x with MEM. This MEM was then added to the previous cell suspension and the mixture was centrifuged at 350 x g for 10 min at 4 °C. The resultant pellet was gently resuspended in growth media and the cells were replated at the desired density.

2.4 FLEXERCELL FX-2000 PROTOCOL

The set-up of the Flexercell FX-2000 was performed as described by the manufacturer (Flexcell International Corporation). Briefly, the system consists of flexible bottom culture plates, a vacuum baseplate, hoses and a computer system (see Figure 5). The computer controls the extent (% stretch substratum) and duration of the mechanical stimulation achieved via a water-generated vacuum. The actual mechanical stimulation of the cells occurs by creating a vacuum below the flexible bottom wells. This vacuum deflects the substratum downward, increasing the surface area and stretching the cells growing on it. In preliminary experiments, rat myotubes were subjected to stretches of 12,

20 or 24% (substratum) following a cyclic protocol of five, 2 sec on/2 sec off stimulations, followed by a 10 sec rest (stimulations were completed within the 2 sec on period). Cells were mechanically stimulated using this pattern for 5 min, 30 min, 3 hr and 24 hr. In these studies, the largest increase in AChE expression was with a 24% stretch. This value was therefore used in all subsequent experiments.

The fact that the pattern of expression and regulation of AChE in muscle from mouse and rats is identical (Massoulié et al., 1993), allowed us to make us of several culture systems which used cells from either of the rodents (as indicated). Since studies showed that the largest increase in the expression of AChE in primary cultures occurred at the 24 hr time-point, we also mechanically stimulated mouse myotubes (both C_2-C_{12} and H-2K^b-tsA58 cells) for 24 hr only.

2.4.1 Drug treatment and mechanical stimulation

Mechanical stimulation protocols involving the use of pharmaceutical agents were performed as described above. Cultures of rat or mouse myotubes were subjected to 24% mechanical stimulation for 24 hr in the presence of 10 μM TTX (Sigma), a potent sodium (Na^+) channel blocker, or 10 μM nifedipine (Miles Laboratories; Pittsburgh, PA), a blocker of L-type calcium (Ca^{++}) channels.

2.5 ACETYLCHOLINESTERASE ENZYME EXTRACTION AND ANALYSIS

Cultures of myotubes obtained from Flexcell plates were washed with cold PBS and scrapped with 600 μl (6 wells, 25 mm^2/well) of a high-salt buffer (HSB) containing anti-proteolytic agents: 10 mM Tris-HCl (Sigma), pH 7.0; 10 mM EDTA (Sigma); 1 M NaCl

proteolytic agents: 10 mM Tris-HCl (Sigma), pH 7.0; 10 mM EDTA (Sigma); 1 M NaCl (BDH; Toronto, Ont.); 1% Triton X-100 (BDH); 1 mg/ml bacitracin (Sigma); 25 U/ml aprotinin (Boehringer Mannheim). Cells were homogenized on ice for 2 x 15 sec with a Polytron (Kinematica; Littan, Switzerland) set at 1. Pooled soleus and tibialis anterior muscles from neo-natal rats were homogenized 2 x 15 sec in 1.0 ml of extraction buffer with the polytron set at 6. For denervation studies, hemidiaphragm muscles were homogenized in 2.5 ml of extraction buffer for 2 x 15 sec with the polytron set at 6. All homogenates were spun at 20,000 x g at 4 °C for 15 min and the resulting supernatants kept at -80 °C for further 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). Aliquots of 25 or 50 µl from the homogenates were incubated in 1 ml of a phosphate-buffer solution (pH 7.0) containing 7.5 x 10^{-4} M acetylthiocholine iodide (Sigma) as the substrate, 5 x 10^{-4} M dithionitrobenzoic acid (DTNB, Sigma) and 10^{-5} M of the non-specific cholinesterase inhibitor tetraisopropylpyrophosphoramide (iso-OMPA, Sigma). Non-specific hydrolysis was measured at 412 nm in the presence of both iso-OMPA and the AChE specific inhibitor 5-bis(4-allyldimethylammonium phenyl)pentanone dibromide (Sigma). Protein concentration was determined using the Bicinchoninic acid protein assay reagent kit (Pierce; Rockford, Il) as described by the manufacturer.

Velocity sedimentation analysis of AChE molecular forms was performed as previously described (Gisiger and Stephens, 1988; Jasmin and Gisiger, 1990). One

hundred or 150 µl aliquots of the muscle extracts were layered onto 5-20% sucrose gradients prepared in HSB. Samples were centrifuged in a Beckman SW41 rotor at 40,000 rpm for 16 hr at 4 °C. Approximately 45 fractions were collected from the bottom of the tubes and assayed for AChE activity by the method of Ellman et al. (1961). Peaks were assigned different molecular forms based on the nomenclature of Bon et al. (1979) according to their apparent sedimentation coefficients.

2.6 RNA EXTRACTION AND QUANTITATIVE RT-PCR

Total RNA from cultured myotubes was extracted using Trizol reagent (Gibco) as described by the manufacturer. For one Flexcell dish (6 wells, 25 mm^2/well) 500 µl of the Trizol reagent was added. The cells were scrapped and homogenized 2 x 15 sec using a Polytron set at 1. After 5 min at room temperature, 100 µl of chloroform was added and the solution mixed vigorously. After a further 2 min at room temperature, the solution was spun at 12,000 x g for 15 min at 4 °C. The top, aqueous layer was transferred to a fresh tube along with 250 µl of ice-cold isopropanol. RNA was stored in this form at -80 °C until use.

Total RNA from hemidiaphragm muscles was extracted using the phenol-chloroform method previously described by Chomczyski and Sacchi (1987). Hemidiaphragms (~200 mg) were homogenized 2 x 15 seconds in 2 ml of solution D (10 µl/µg tissue; 4 M guanidinium thiocynate; 25 mM sodium citrate, pH 7.0; 0.5% sarcosyl; 0.1 M β-mercaptoethanol) using a Polytron set at 6 (Kinematica). Two hundred µl of sodium acetate, 2 ml phenol, and 400 µl of chloroform:isoamyl alcohol (49:1) were sequentially

added to the resulting homogenate. After a 15 min incubation on ice, the mixture was centrifuged at 4 °C for 15 min at 12,000 x g. The aqueous phase was then transferred to a fresh tube along with 1 ml of isopropanol. The RNA was stored at -80°C until further analyzed.

For RNA precipitation, the isopropanol:RNA mixture was spun at 12,000 x g for 15 min at 4 °C. The resultant pellets were washed 3 x with ice-cold 75% ethanol (Commercial Alcohols Limited; Brampton, Ont.) followed by centrifugation at 12,000 x g for 15 min at 4 °C each time. Final pellets were dried using a Speed-Vac roto-vap (Savant; Framingdale, NY) and resuspended in 20 µl and 100 µl of diethylpyrocarbonate (DEPC; ICN, Montreal, P.Q.) -treated water for culture and hemidiaphragm samples, respectively.

For cultured cells, 2 µl from each sample at each experimental time-point was pooled for analysis. Total RNA concentrations from these pooled samples were measured at 260 nm and they were found to have minimal variance. Control pooled samples were diluted 10-, 50- and 250-fold while samples from the experimental time-points were diluted 100 -fold. Only 2 µl of the diluted RNA was reverse transcribed. Control samples were diluted over a larger range to confirm that the working dilution (100-fold) was within the exponential phase of amplification for the PCR reaction. For whole muscle hemidiaphragm experiments, RNA stocks were diluted 10-, 100-, and 1000-fold, and only 2 µl of diluted RNA was reverse transcribed.

The reverse transcription mixture contained 5 mM $MgCl_2$, 1 x PCR buffer II (50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1 mM dNTPs, 20 U RNAse inhibitor, 50 U murine leukemia virus reverse transcriptase and 2.5 mM random hexamer primers (GeneAmp RNA PCR kit;

Perkin-Elmer Cetus Instruments, Branchburg, NJ). Negative controls consisted of the same mixture with the RNA being replaced with DEPC-treated water. Reverse transcription was performed at 42 °C for 45 min, followed by heating at 99 °C for 5 min to terminate the reaction.

To amplify AChE cDNAs, specific synthetic primers based on the rat AChE cDNA sequence were used (Legay et al. 1993). These 5' (CTGGGTGCGGATCGGT) and 3' (TCACAGGTCTGAGCAGCGTT) primers amplified a 670 bp target sequence in the common coding region of the AChE cDNA. Egr-1 transcripts were selectively amplified using primers based on the rat Egr-1 cDNA (Kendall et al., 1994). These 5' (GAGTTGGGACTGGTAGGTGT) and 3' (GCAACACTTTGTGGCCTGAA) primers amplified a 512 bp target sequence of the Egr-1 cDNA. PCR was performed by adding 5 µl of the reverse transcription mixture to 20 µl of a solution containing 0.625 U Ampli'Taq DNA polymerase, 0.25 µg each of the appropriate 5' and 3' primers, $MgCl_2$ (2 mM) and PCR buffer II (1 x). PCR conditions for AChE consisted of a cycle of denaturation at 94°C for 1 min, and primer annealing and extension at 70°C for 3 min. A final 10 min elongation step at 72°C was added after the last cycle. Cycle number for AChE was typically 42 and 34 for cultured and hemidiaphragm samples, respectively. These cycle numbers were within the linear range of amplification as determined with the dilution curve made from total RNA obtained from control samples. Amplification conditions for Egr-1 transcripts were 94°C for 1 min, 65°C for 1 min and 72°C for 1 min, followed by a 10 min elongation step after the last cycle. The cycle number for these experiments was 38 and was within the linear range of amplification as determined with the dilution curve. The amplification conditions for AChE cDNAs were

were visualized on either a 1% or 1.5% agarose gel containing ethidium bromide. The molecular mass of the PCR products was estimated by comparing product size to a 100 bp ladder marker (Gibco). Quantitative PCR was performed as previously described in detail in Jasmin et al. (1993). Selected cDNAs were amplified using primers which were end-labelled with ^{32}P using a terminal kinase. Radiolabelled PCR products were visualized on 1.5% agrose gels and excised using a scalpel. The counts per minute were determined directly by Cerenkov counting.

After performing quantitative RT-PCR on AChE and Egr-1 mRNAs from pooled samples, individual samples (n=10) obtained at the 24 hr and 30 min time-points were analyzed individually since these time-points represent peak accumulation for these transcripts, respectively (see Figures 22 and 24). Quantitation of the individual bands was performed as described above.

2.7 STATISTICAL ANALYSIS

To evaluate the effects of the experimental conditions an ANOVA or student's t-test was performed. Paired or independent student's t-test were performed to examine the effects of the *in vitro* and *in vivo* experimental conditions, respectively. They were used to strictly compare the effects of mechanical stimulation vs control conditions. Since our hypothesis (see section 1.9) is that mechanical stimulation will increase AChE expression, one tailed tests were used to determine significant differences among control and experimental groups. The level of significance for all tests was set at $P < 0.05$ and the specific tests used are indicated in the figure legends. For some analysis, Fisher post-hoc

tests were used to locate significant differences between group means following analysis of variance (ANOVA's). Data are expressed as mean ± SEM throughout. The profiles of AChE molecular forms displayed in the Figures are representative examples.

CHAPTER 3

RESULTS

To determine whether passive mechanical forces modulate the expression of AChE in rat skeletal muscle fibers, we employed two distinct models. In the first set of experiments, primary cultures of myotubes were subjected to passive mechanical forces using a mechanical cell stimulator. In a second series of studies, we made use of a unique *in vivo* model in which the denervated hemidiaphragm muscle is subjected to passive mechanical stimulation as a result of the contractile activity of the intact contralateral side during breathing cycles.

3.1 *IN VITRO* MECHANICAL STIMULATION OF RAT MYOTUBES

3.1.1 Primary cultures of rat myotubes

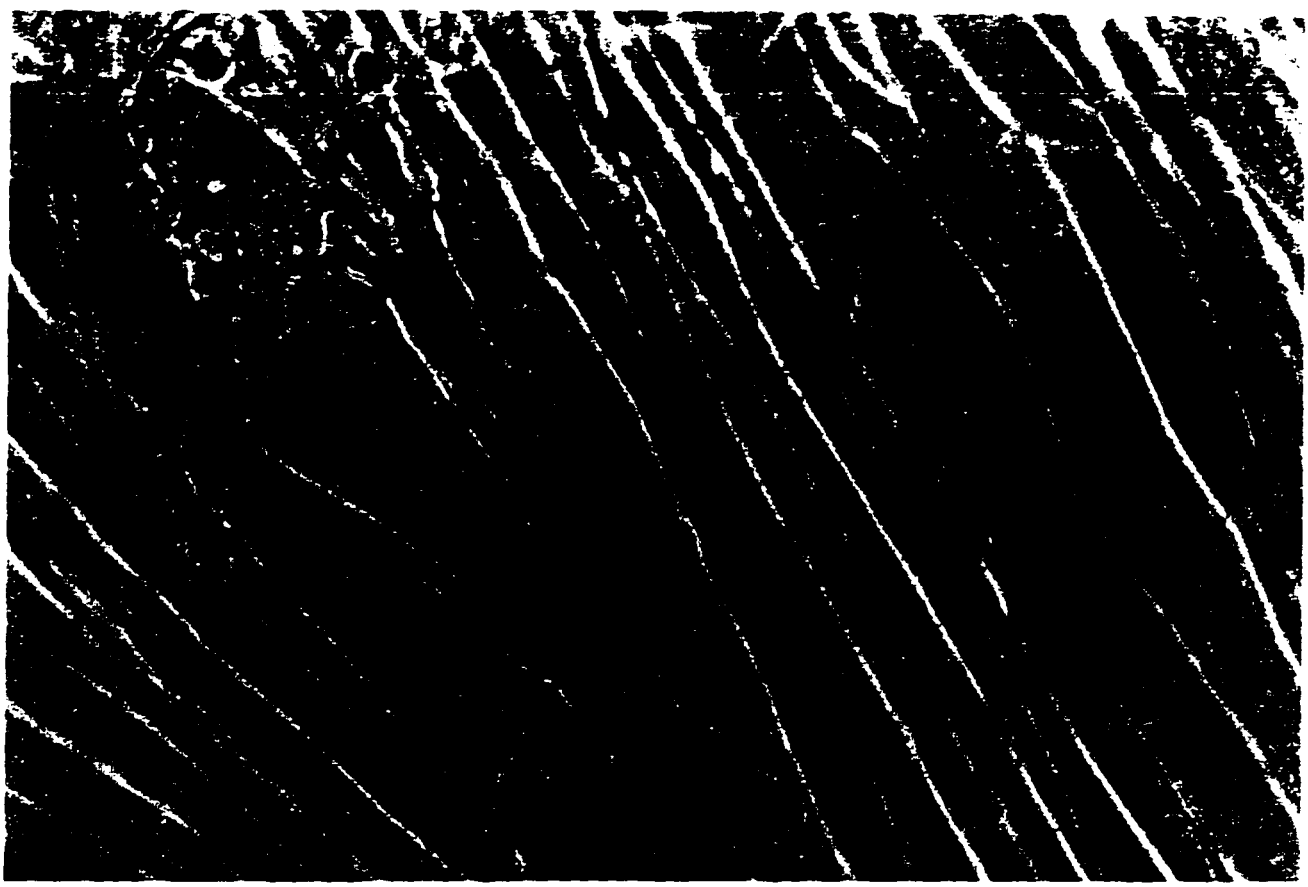
Primary cultures of rat myotubes were confluent typically after three days of being in differentiation media (Figure 6A). Visual analysis of the cultures revealed that two types of myotube cultures were obtained. One had a high percentage of spontaneously contracting myofibers while the other had lacked apparent spontaneous contractile activity. The percent of contracting myofibers as well as the duration of contractile activity differed greatly between cultures. Both culture types were used for all experiments. Analysis of AChE molecular forms expressed by these various culture types revealed important differences. Spontaneously contracting myotube cultures (high percentage of fibers contracting) exhibited a profile of A_{12}, A_8, G_4 and G_1 (Figure 6B) while inactive cultures (low percentage contracting) lacked asymmetric forms and displayed only G_4 and G_1 molecular forms (Figure 6C).

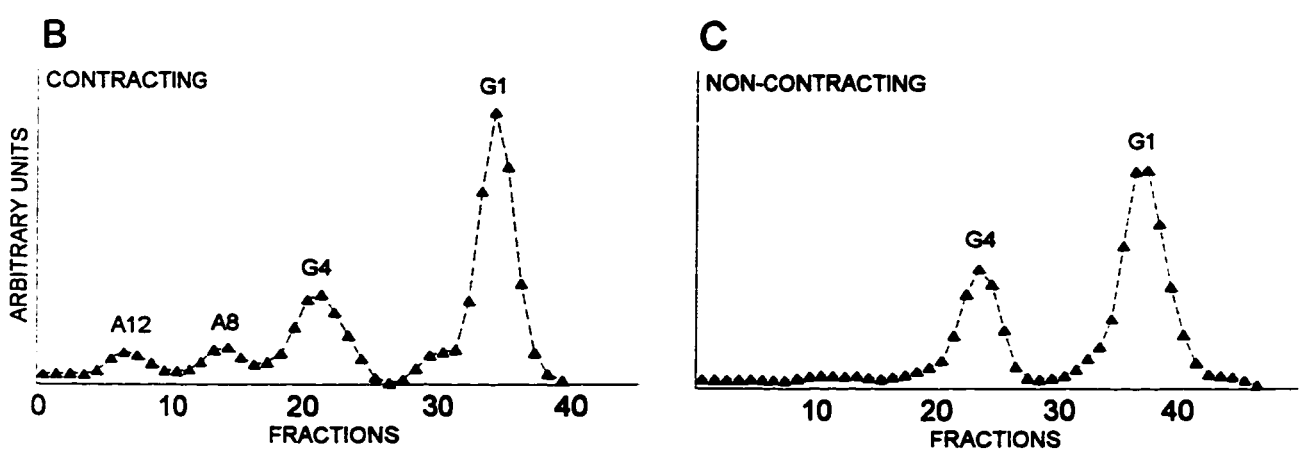
Figure 6

PRIMARY CULTURES OF RAT MYOTUBES

Figure A shows 10 day-old primary cultures of rat myotubes grown on Flexercell culture plates (400 x, phase contrast). Fibroblasts and collagen fill the spaces between the myofibers. Analysis of AChE molecular forms revealed two types of profiles. Contracting cultures displayed a profile of A_{12}, A_8, G_4 and G_1 (B), while non-contracting cultures were devoid of asymmetric forms and only showed the presence of G_4 and G_1 peaks (C).

A




B
CONTRACTING
ARBITRARY UNITS
A12
A8
G4
G1
0
10
20
30
40
FRACTIONS
C
NON-CONTRACTING
G4
G1
10
20
30
40
FRACTIONS

3.1.2 Effects of mechanical stimulation on acetylcholinesterase activity

To evaluate the effects of passive mechanical stimulation on AChE enzyme activity in cultured rat myotubes, cultured muscle cells were subjected to either 12, 20 or 24% mechanical stimulation over a cyclic pattern consisting of five, 2 sec on/2 sec off stimulations followed by a 10 second rest period. Cells were mechanically stimulated using this pattern for 5 min, 30 min, 3 hr and 24 hr. In preliminary experiments, the largest increase in total AChE enzyme activity was observed with 24% of mechanical stimulation and this value was used in all subsequent experiments. The impact of mechanical stimulation on AChE activity was determined spectrophotometrically. Total AChE activity remained unchanged up to 3 hr after initiation of mechanical stimulation (Figure 7). At 24 hr however, there was a large increase in comparison to control levels (42%; $P < 0.05$). Examination of cultured myotubes under a microscope indicated that cell morphology did not change as a result of mechanical stimulation.

In vivo, enhanced phasic neuromuscular activity has been shown to increase AChE expression in muscle by selectively increasing the content of G_4 (see section 1.5.2). To determine if the increase in AChE activity observed in response to mechanical stimulation induced a similar adaptation, aliquots of muscle homogenates were separated by velocity sedimentation on sucrose gradients and AChE activity was analyzed. Control myotube cultures exhibited a molecular form profile consisting of A_{12}, A_8, G_4 and G_1 (Figure 8A) . After 24 hr of mechanical stimulation, a similar AChE pattern was observed indicating that this treatment increased the expression of all molecular forms (Figure 8B). Quantitative analysis revealed that the activity of each molecular form expressed as a percent of total

Figure 7

TOTAL ACETYLCHOLINESTERASE ENZYME ACTIVITY IN PRIMARY CULTURES OF RAT MYOTUBES SUBJECTED TO MECHANICAL STIMULATION

Eight to 10 day-old primary cultures of rat myotubes were subjected to 24% mechanical stimulation and harvested 5 min, 30 min, 3 hr and 24 hr later. Data are expressed as mean ± SEM. Asterisk indicates a significant difference between control (CTL) and experimental samples (paired t-test, $P < 0.05$, $n=10$).

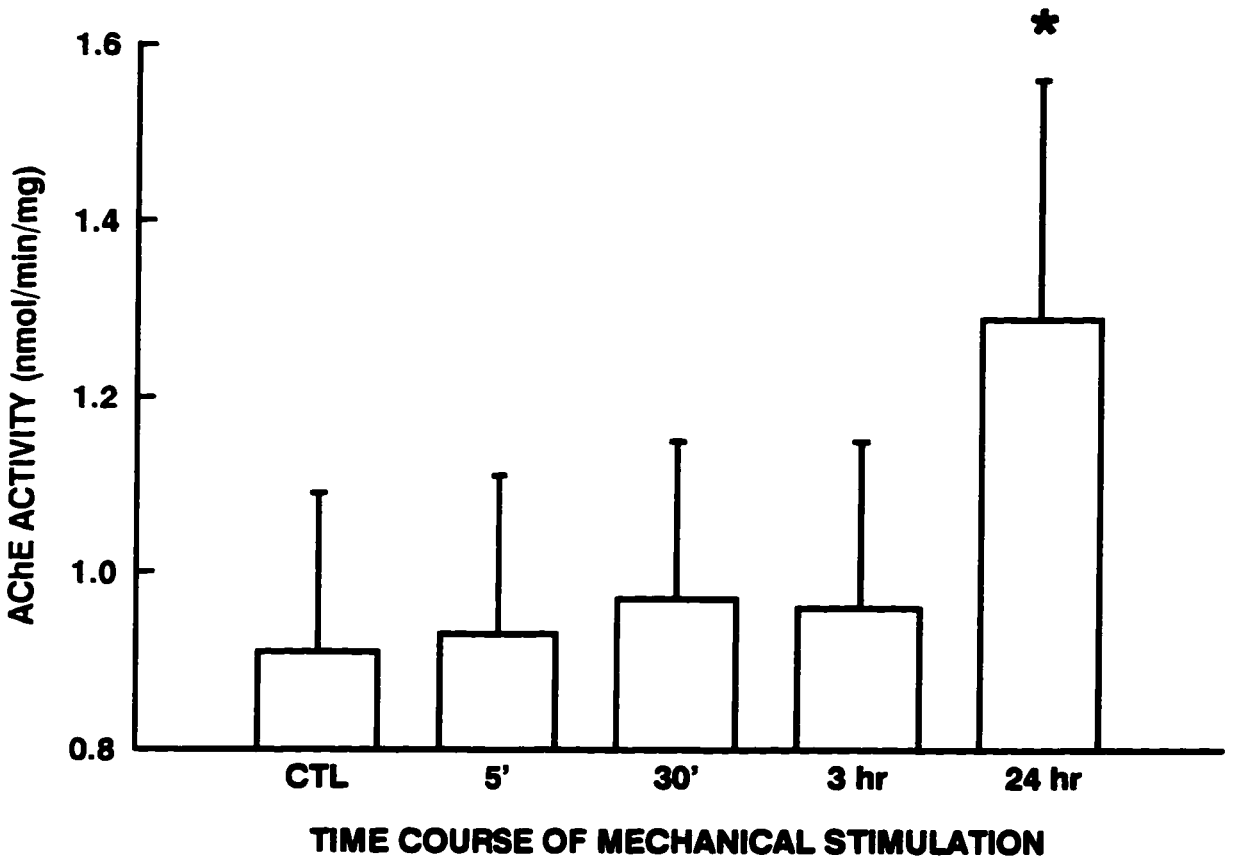
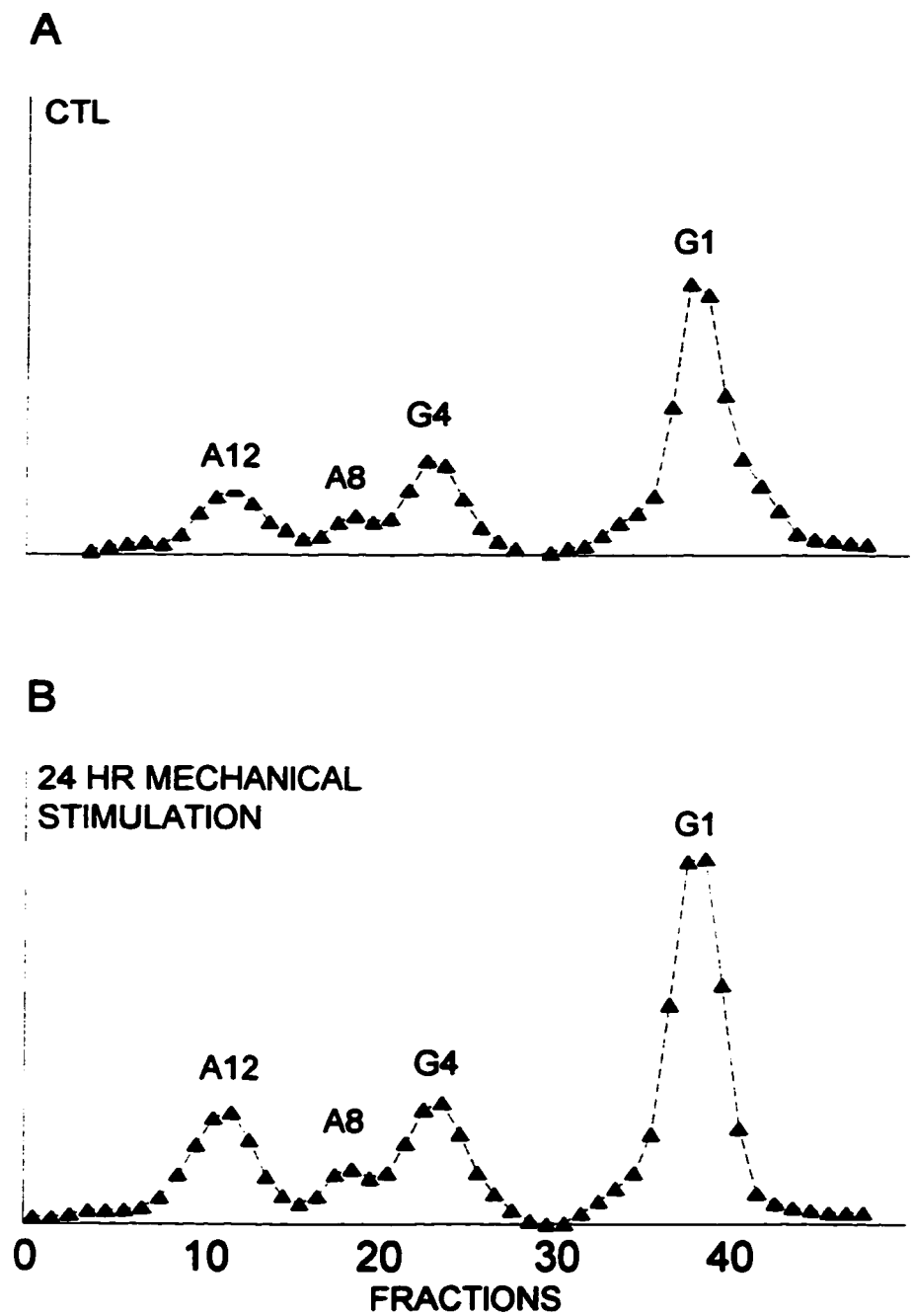

1.6
1.4
1.2
1.0
0.8
AChE ACTIVITY (nmol/min/mg)
*
CTL
5'
30'
3 hr
24 hr
TIME COURSE OF MECHANICAL STIMULATION

Figure 8

ACETYLCHOLINESTERASE MOLECULAR FORMS IN RAT MYOTUBES SUBJECTED TO MECHANICAL STIMULATION

Primary cultures of rat myotubes were mechanically stimulated for 24 hr and harvested along with their controls (CTL). AChE molecular forms were identified according to the nomenclature of Bon et al. (1982) on the basis of their apparent sedimentation coefficients (Gisiger and Stephens, 1983). Note that the relative ratios of the molecular forms in myotubes subjected to mechanical stimulation (B) did not change in comparison to control cultures (A).


A
CTL
G1
G4
A12
A8
B
24 HR MECHANICAL
STIMULATION
G1
A12
G4
A8
0
10
20
30
40
FRACTIONS

activity, did not change significantly thereby confirming that mechanical stimulation led to an elevation in all AChE molecular forms as opposed to a preferential increase in a specific form (Figure 9)

3.1.3 Effects of mechanical stimulation on acetylcholinesterase secretion

Under control conditions, cultured myotubes secrete G_4 into the culture media (see for example Rubin et al., 1985). Investigators routinely grow myotube cultures in defined media when analyzing secreted AChE due to high background enzyme activity found in the serum. In our studies however, DBS was used instead of the more common horse serum which has considerable inherent AChE activity and is often replaced in defined media (Walker and Wilson, 1976; Rubin et al., 1985). Analysis of the media incubated for 24 hr without cells revealed negligible AChE activity (<10%) indicating that the use of defined media was not necessary. Spectrophotometric analysis of AChE in media collected from mechanically stimulated myotubes revealed a 70% increase in enzyme activity (Figure 10A; $P < 0.05$). To determine whether passive mechanical forces altered the pattern of AChE molecular form secreted, an aliquot of the media was separated by velocity sedimentation on sucrose gradients and analyzed. Figure 10B shows that, as expected, only the G_4 form of AChE was present in the media of control myotubes. Media collected from myotubes mechanically stimulated also contained only the G_4 form of AChE although the amount had increased (Figure 10C).

Figure 9

RELATIVE CONTENT OF ACETYLCHOLINESTERASE MOLECULAR FORMS IN PRIMARY CULTURES SUBJECTED TO MECHANICAL STIMULATION

Values represent the relative content of A_{12}, A_8, G_4 and G_1 present in primary cultures of rat myotubes before (CTL, control) and after 24 hr of mechanical stimulation (MS). Data are expressed as a percentage of total AChE activity. Means ± SEM are shown. (paired t-test, n=3)

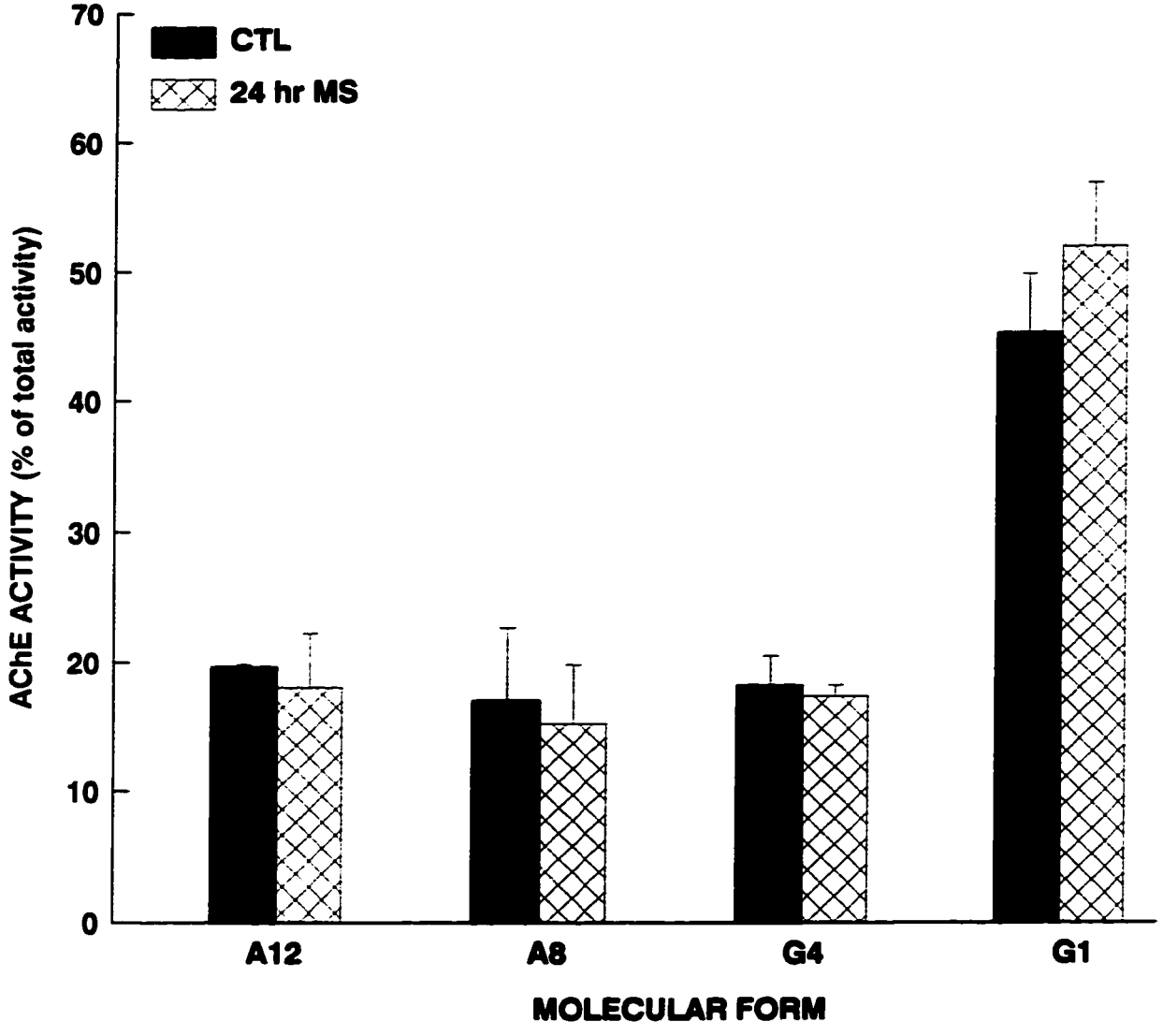
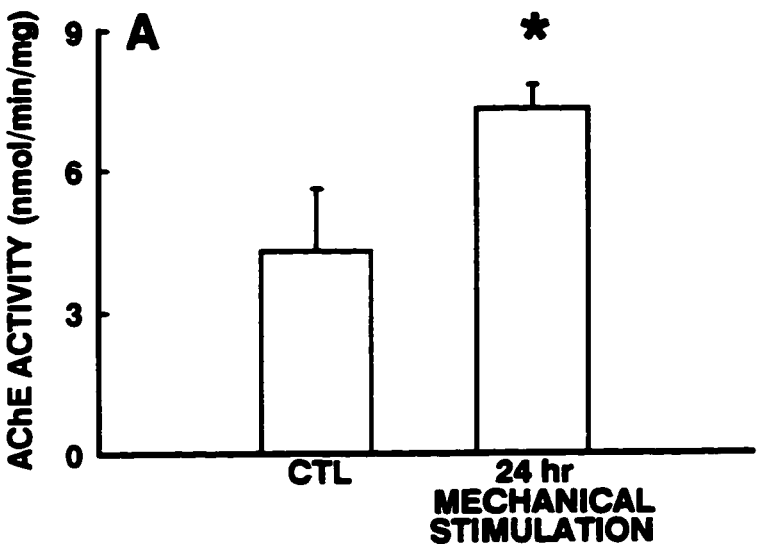
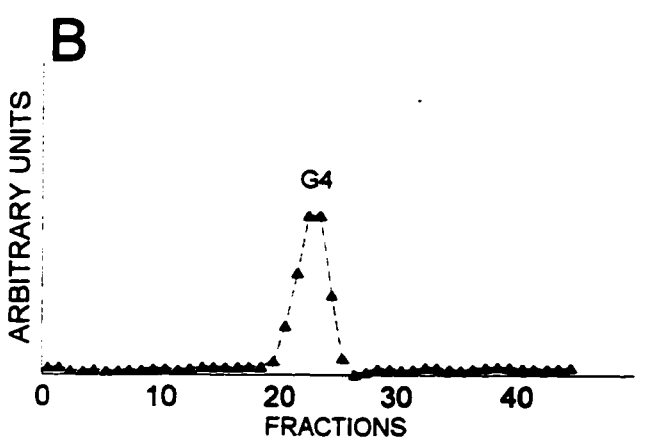

CTL
24 hr MS
ACHE ACTIVITY (% of total activity)
70
60
50
40
30
20
10
0
A12
A8
G4
G1
MOLECULAR FORM

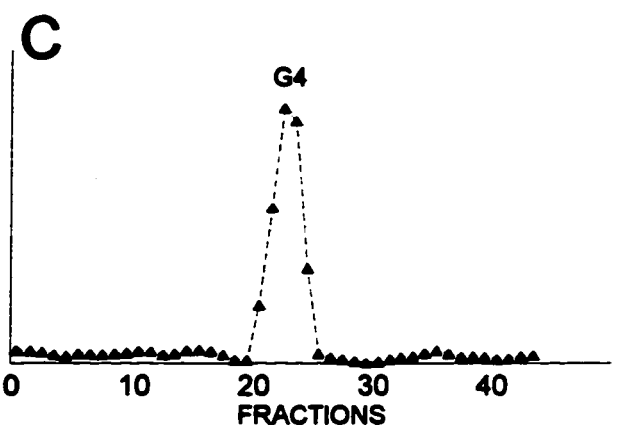
Figure 10

EFFECTS OF MECHANICAL STIMULATION ON ACETYLCHOLINESTERASE SECRETION IN CULTURED RAT MYOTUBES

Media was collected from rat myotube cultures mechanically stimulated for 24 hr. A depicts total AChE activity in media from control (CTL) and mechanically stimulated myotubes. B shows that only the G_4 molecular form of AChE was present in the media of control myotubes. Following mechanical stimulation, the pattern of molecular forms secreted remained unchanged (C). Values in A are expressed as mean ± SEM. Asterisk indicates a significant difference between control and experimental groups. (paired t-test, $P < 0.05$, n=3)


A
*
AChE ACTIVITY (nmol/min/mg)
9
6
3
0
CTL
24 hr
MECHANICAL
STIMULATION


B
ARBITRARY UNITS
G4
0
10
20
30
40
FRACTIONS


C
G4
0
10
20
30
40
FRACTIONS

3.1.4 Potential signalling pathways

The regulation of AChE in cultured myotubes has been shown to be sensitive to electrical activity (Fernandez-Valle and Rotundo, 1989). The role of electrical activity in AChE expression in response to passive mechanical forces was thus determined by mechanically stimulating myotubes in the presence of 10 μM TTX, a potent Na^+ channel blocker. In mouse C_2-C_{12} myotubes, 24 hr of passive mechanical forces increased AChE activity by 92% in comparison to control values (see Figure 11A). Mechanically stimulating myotubes in the presence of TTX resulted in a 72% increase in AChE activity indicating that TTX was unable to block the AChE increase in response to mechanical stimulation (Figure 11A). In these C_2-C_{12} cultures, secretion of AChE into the media was also increased by 70% in response to mechanical stimulation and TTX treatment did not prevent this adaptation (Figure 11B).

Since TTX is known to affect selectively the expression of asymmetric forms in cultured myotubes (Fernandez-Valle and Rotundo, 1989), we also determined whether the upregulation in enzyme activity following mechanical stimulation was reflected by changes in specific AChE molecular forms. Cultures of rat myotubes which lacked spontaneous contractile activity were devoid of asymmetric forms (Figure 12A, see also Figure 6C). TTX, therefore, had no effect on their molecular form profiles (Figure 12B) and mechanical stimulation of the myotubes in the presence of TTX did not induce the appearance of asymmetric forms of AChE (Figure 12C). Analysis of the activity of each molecular form expressed as a percentage of total AChE activity, was unchanged following mechanical stimulation illustrating that mechanical stimulation in the presence of TTX did not

Figure 11

EFFECTS OF TETRODOTOXIN ON ACETYLCHOLINESTERASE EXPRESSION IN MECHANICALLY STIMULATED MYOTUBES

Mouse C_2-C_{12} myotubes were subjected to mechanical stimulation (MS) for 24 hr in the presence of 10 μM tetrodotoxin (TTX). AChE activity associated with the cells (A) and media (B) is shown. AChE activity is expressed as mean ± SEM. Asterisk indicates a significant difference between control and experimental groups. (ANOVA, Fisher post-hoc, $P < 0.05$, n=5) CTL; control.

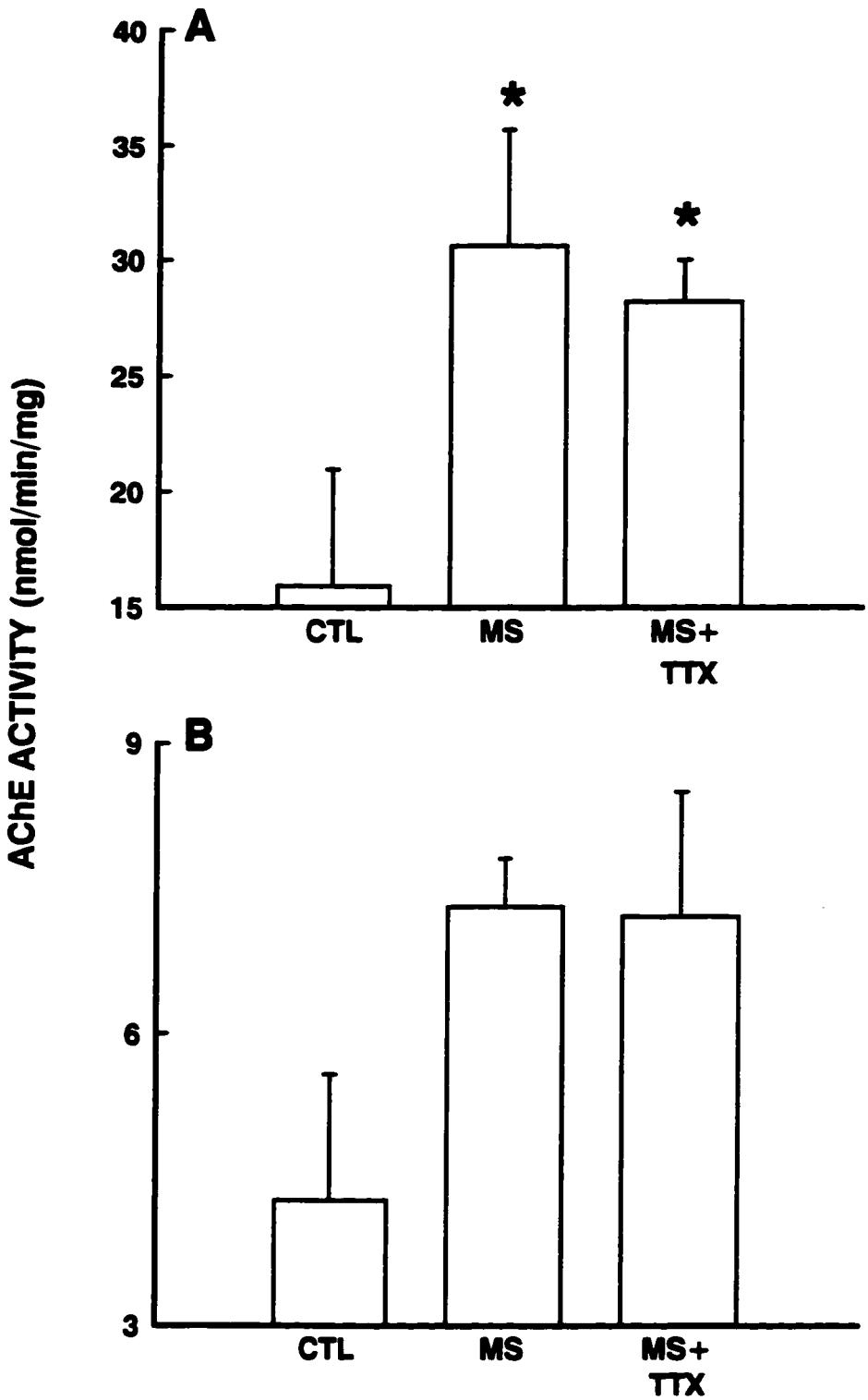
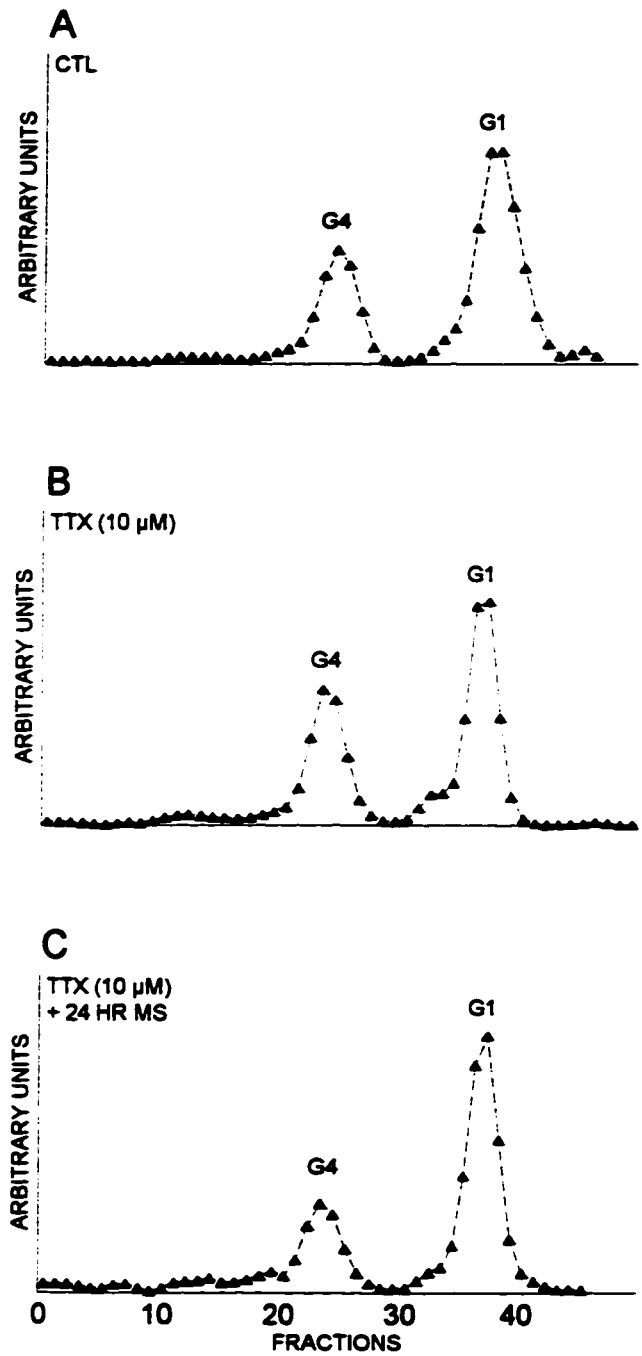

A
40
35
30
25
20
15
*
*
CTL
MS
MS+
TTX
B
9
6
3
CTL
MS
MS+
TTX
AChE ACTIVITY (nmol/min/mg)

Figure 12

EFFECTS OF TETRODOTOXIN TREATMENT AND MECHANICAL STIMULATION ON ACETYLCHOLINESTERASE MOLECULAR FORMS IN NON-CONTRACTING RAT MYOTUBES

Molecular form profiles of non-contracting myotubes treated with 10 μM tetrodotoxin (TTX) with (C) or without (B) mechanical stimulation (MS) for 24 hr. Note that the AChE molecular form profiles did not change in comparison to controls (CTL; A).


A
CTL
ARBITRARY UNITS
G1
G4
B
TTX (10 µM)
ARBITRARY UNITS
G1
G4
C
TTX (10 µM)
+ 24 HR MS
ARBITRARY UNITS
G1
G4
0
10
20
30
40
FRACTIONS

selectively affect expression of a specific AChE molecular form (Figure 13). These observations were confirmed in cultures containing spontaneously contracting rat myotubes. In these cells, 10 μM TTX decreased the expression of asymmetric forms as expected (Figure 14A and B, see Fernandez-Valle and Rotundo, 1989). Mechanically stimulating myotubes in the presence of TTX did not induce the reappearance, or upregulation of the asymmetric forms (Figure 14C) further indicating that the regulation of these forms is independent of mechanical stimulation (see also Figure 15).

Extracellular Ca^{++} has also been shown to play a critical role in regulating AChE in cultured myotubes (see for example Rubin, 1985). Calcium, in particular, has been shown during myogenesis to contribute to the regulation of AChE via L-type Ca^{++} channels (Luo et al., 1994). Using nifedipine, a specific blocker of the L-type channel, we determined whether extracellular Ca^{++} entering through these channels was involved in the AChE response to mechanical stimulation. Mechanical forces increased AChE activity by 28% over control levels in these experiments. Nifedipine was able to block 43% of this increase (Figure 16).

3.1.5 Effects of mechanical stimulation on acetylcholinesterase activity in slow and fast myotubes

In vivo, exercise is known to affect AChE expression differentially in fast or slow skeletal muscles (see Introduction). Primary cultures of rat myotubes consist of a mixture of both fast and slow myofibers and therefore, they do not allow analysis of fiber-type specific effects. For this reason, mouse H-$2K^b$-tsA58 clonal fast or slow myotube cultures were used to examine the effects of mechanical stimulation on AChE expression in fast or

Figure 13

RELATIVE CONTENT OF ACETYLCHOLINESTERASE MOLECULAR FORMS IN TETRODOTOXIN-TREATED NON-CONTRACTING MYOTUBES SUBJECTED TO MECHANICAL STIMULATION

Values represent the relative content of G_4 and G_1 present in primary cultures of rat myotubes before (CTL, control) and after tetrodotoxin (TTX) treatment. The effects of 24 hr of mechanical stimulation (MS) in the presence of TTX are shown. Data are expressed as a percentage of total AChE activity. Means ± SEM are shown. (paired t-test, n=3)

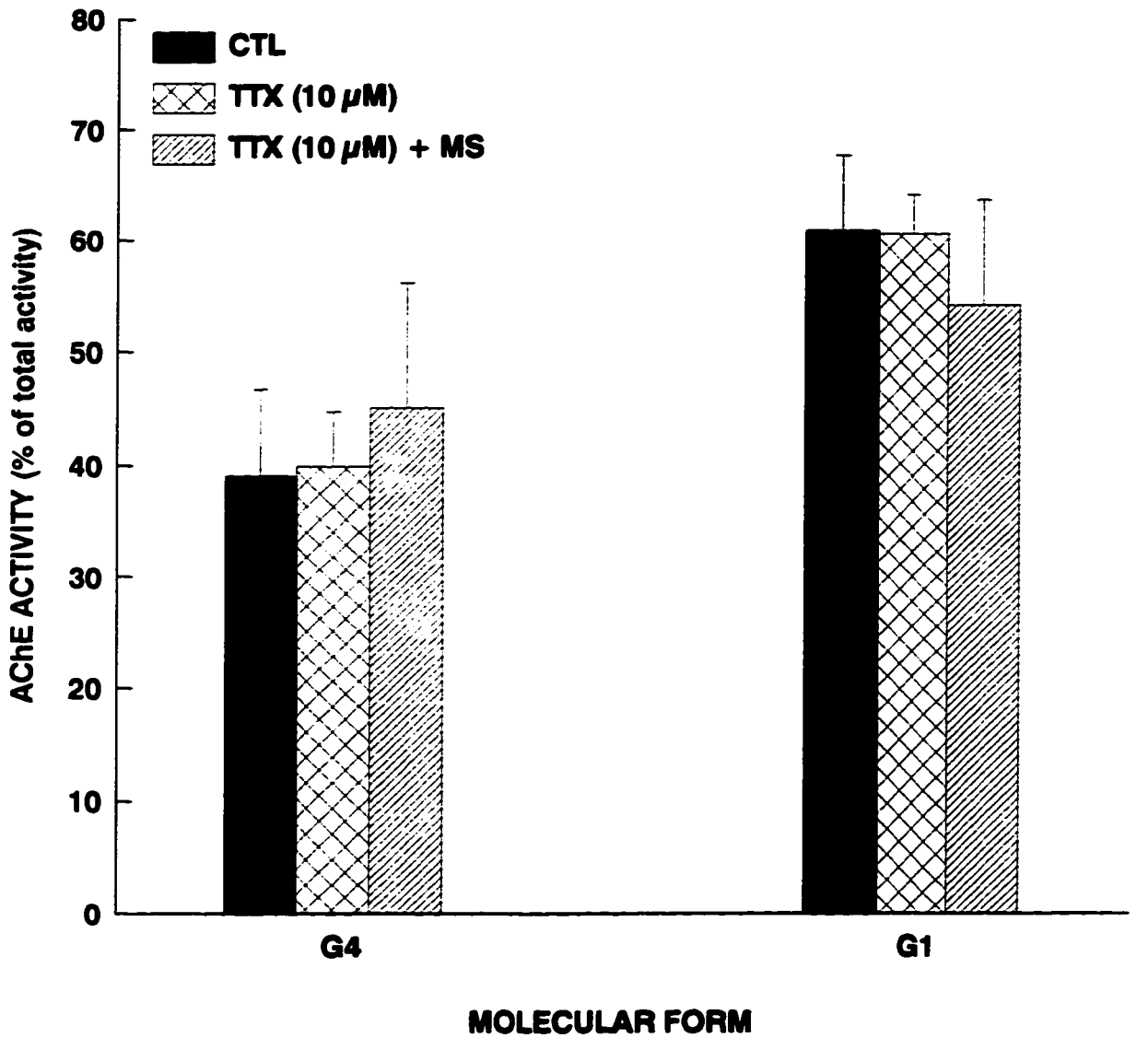

CTL
TTX (10 μM)
TTX (10 μM) + MS
AChE ACTIVITY (% of total activity)
0
10
20
30
40
50
60
70
80
G4
G1
MOLECULAR FORM

Figure 14

EFFECTS OF TETRODOTOXIN TREATMENT AND MECHANICAL STIMULATION ON ACETYLCHOLINESTERASE MOLECULAR FORMS IN SPONTANEOUSLY CONTRACTING RAT MYOTUBES

Molecular form profiles of contracting myotubes treated with 10 μM tetrodotoxin (TTX) with (C) or without (B) mechanical stimulation (MS) for 24 hr. Note that the asymmetric forms present in control cells (CTL, A) are lost due to the TTX treatment (B). These forms did not return to control levels (A) following mechanical stimulation (C).

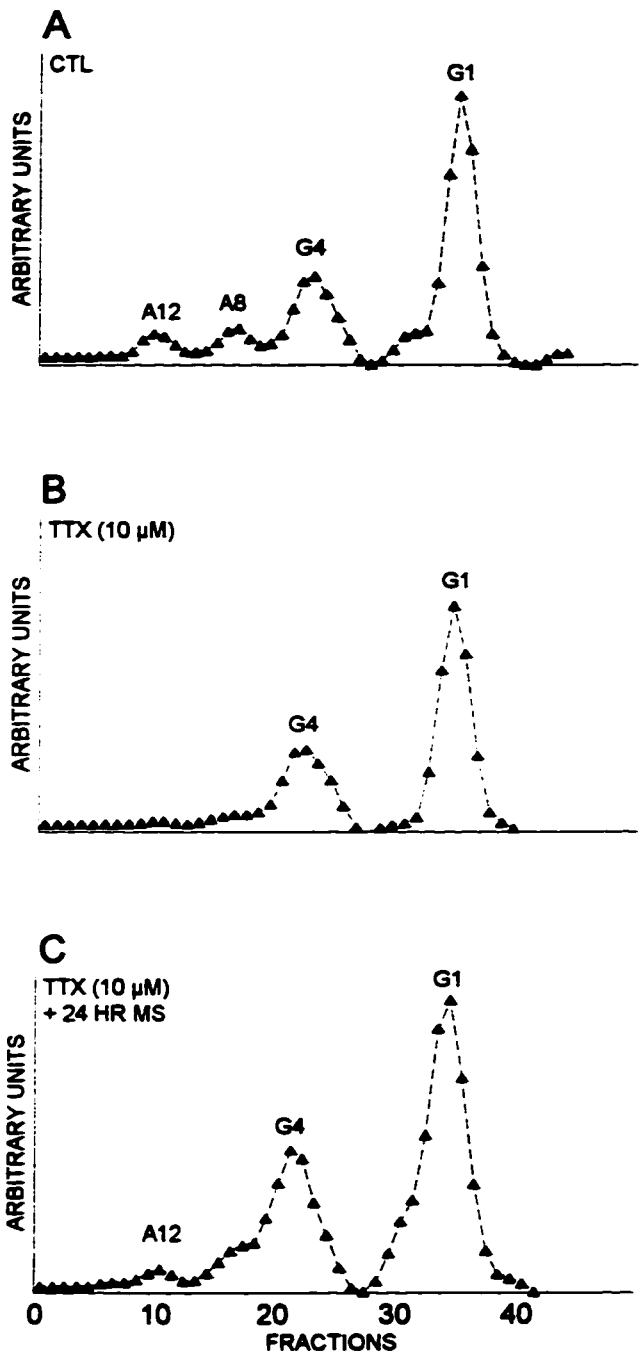

A
CTL
G1
G4
A12
A8
ARBITRARY UNITS
B
TTX (10 µM)
G1
G4
ARBITRARY UNITS
C
TTX (10 µM)
+ 24 HR MS
G1
G4
A12
ARBITRARY UNITS
0
10
20
30
40
FRACTIONS

Figure 15

RELATIVE CONTENT OF ACETYLCHOLINESTERASE MOLECULAR FORMS IN TETRODOTOXIN-TREATED SPONTANEOUSLY CONTRACTING MYOTUBES SUBJECTED TO MECHANICAL STIMULATION

Values represent the relative content of A_{12}, A_8, G_4 and G_1 present in primary cultures of rat myotubes before (CTL, control) and after tetrodotoxin (TTX) treatment. The effects of 24 hr of mechanical stimulation (MS) in the presence of TTX are shown. Data are expressed as a percentage of total AChE activity. (n=1)

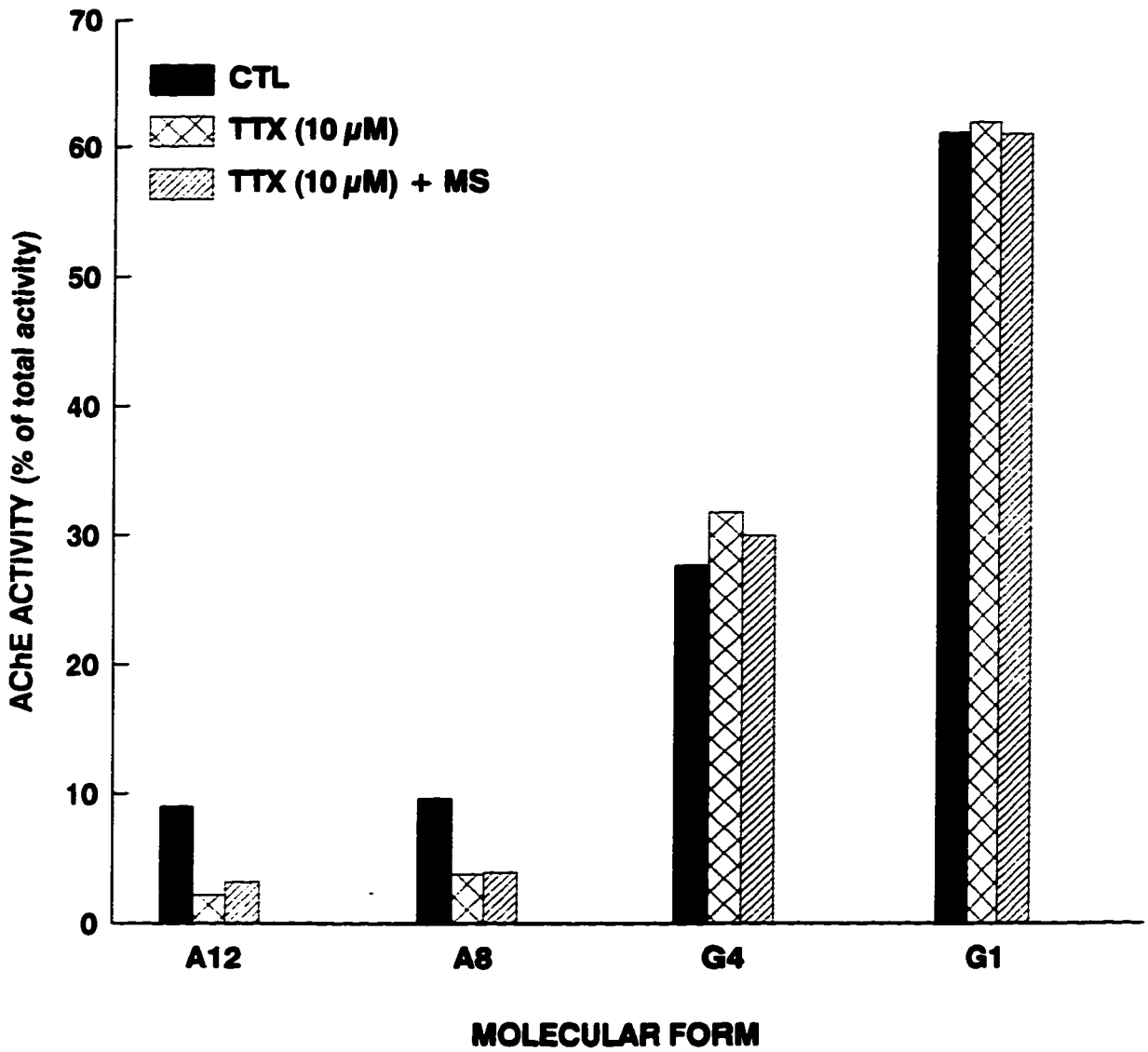
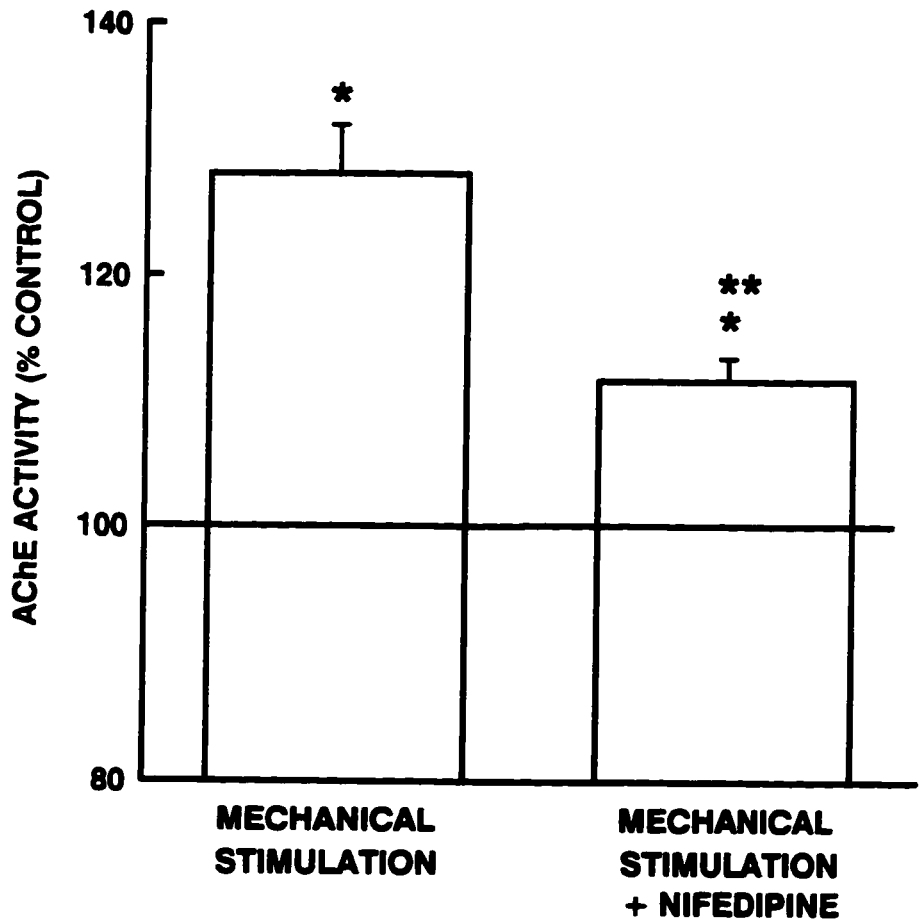

70
60
50
40
30
20
10
0
CTL
TTX (10 μM)
TTX (10 μM) + MS
ACHE ACTIVITY (% of total activity)
A12
A8
G4
G1
MOLECULAR FORM

Figure 16

EFFECTS OF NIFEDIPINE ON ACETYLCHOLINESTERASE ACTIVITY IN RAT MYOTUBES SUBJECTED TO MECHANICAL STIMULATION

Primary cultures of rat myotubes were mechanically stimulated in the presence of 10 μM nifedipine, an L-type calcium channel blocker. Values are expressed as percent of controls ± SEM. Single asterisk indicates a significant difference between experimental and control groups. Double asterisks indicate a significant difference between mechanically stimulated and nifedipine+mechanically stimulated groups. (paired t-test, $P < 0.05$, n=3)


140
120
100
80
ACHE ACTIVITY (% CONTROL)
*
**
*
MECHANICAL STIMULATION
MECHANICAL STIMULATION + NIFEDIPINE

slow muscle fibers. To determine the validity of using these clonal myotube cultures as representative of adult fast and slow muscles, the molecular form profiles of the slow soleus and fast tibialis anterior muscles were examined at various time-points during post-natal development. Figure 17 shows AChE profiles from soleus muscles of rats at 1, 4, 7, 14 and 21 days after birth. Muscles from 1 day-old rats were less than 3 hr old. The adult soleus muscle (Figure 17, bottom right) displayed the AChE profile typical of slow muscles displaying peaks for A_{12}, A_8, G_4 and G_1. This AChE profile was already expressed within hr after birth (Figure 17, top left) and remained unchanged thereafter. Interestingly, high levels of A_{12} were observed in neo-natal muscles up to 21 days. The molecular form profile of the fast tibialis anterior muscle was also analyzed. The adult fast muscle profile characterized by peaks of A_{12}, G_4 and G_1 is shown in Figure 18 (bottom right). As observed with the slow soleus muscle, the fast muscle AChE profile was already evident within hr after birth (Figure 18, top left).

To determine whether passive mechanical stimulation affected AChE expression differentially in fast or slow muscle fibers, cultures of mouse H-2K^b-tsA58 clonal fast or slow myotubes were mechanically stimulated for 24 hr. Spectrophotometric analysis revealed a two-fold increase in AChE activity in slow myotubes after mechanical stimulation (Figure 19A). Examination of molecular form profiles of clonal slow mouse cultures confirmed that the myotubes expressed the slow muscle AChE pattern displaying A_{12}, A_8, G_4 and G_1 peaks (Figure 19B). Sedimentation analysis of the molecular form profiles following mechanical stimulation revealed that the increase in expression was due to a general elevation in all AChE molecular forms rather than an increase in a specific

Figure 17

ACETYLCHOLINESTERASE MOLECULAR FORM PROFILES OF SOLEUS MUSCLES FROM NEO-NATAL RATS

Soleus muscles were removed from rat pups at 1, 4, 7, 14 and 21 days after birth and analyzed for their AChE molecular form content. The slow muscle profile consisting of A_{12}, A_8, G_4 and G_1 was already evident at day 1 (top left) and remained unchanged thereafter.

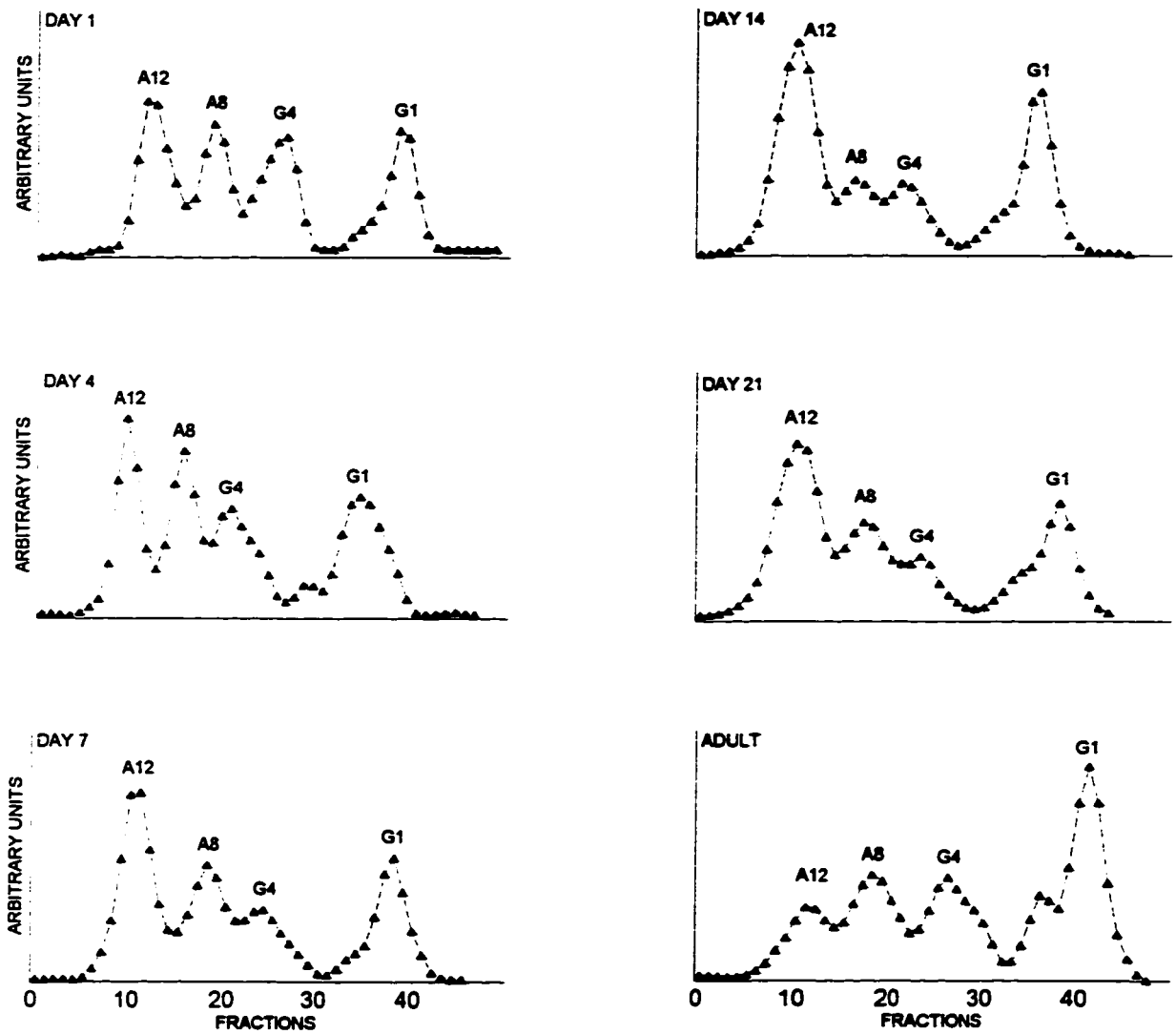

DAY 1
A12
A8
G4
G1
ARBITRARY UNITS
DAY 14
A12
A8
G4
G1
DAY 4
A12
A8
G4
G1
ARBITRARY UNITS
DAY 21
A12
A8
G4
G1
DAY 7
A12
A8
G4
G1
ARBITRARY UNITS
ADULT
A12
A8
G4
G1
0
10
20
30
40
FRACTIONS
0
10
20
30
40
FRACTIONS

Figure 18

ACETYLCHOLINESTERASE MOLECULAR FORM PROFILES OF TIBIALIS ANTERIOR MUSCLES FROM NEO-NATAL RATS

Tibialis anterior muscles were removed from rat pups at 1, 4, 7, 14 and 21 days after birth and analyzed for their AChE molecular form content. The fast muscle profile consisting of A_{12}, G_4 and G_1 was already evident at day 1 and remained unchanged thereafter.

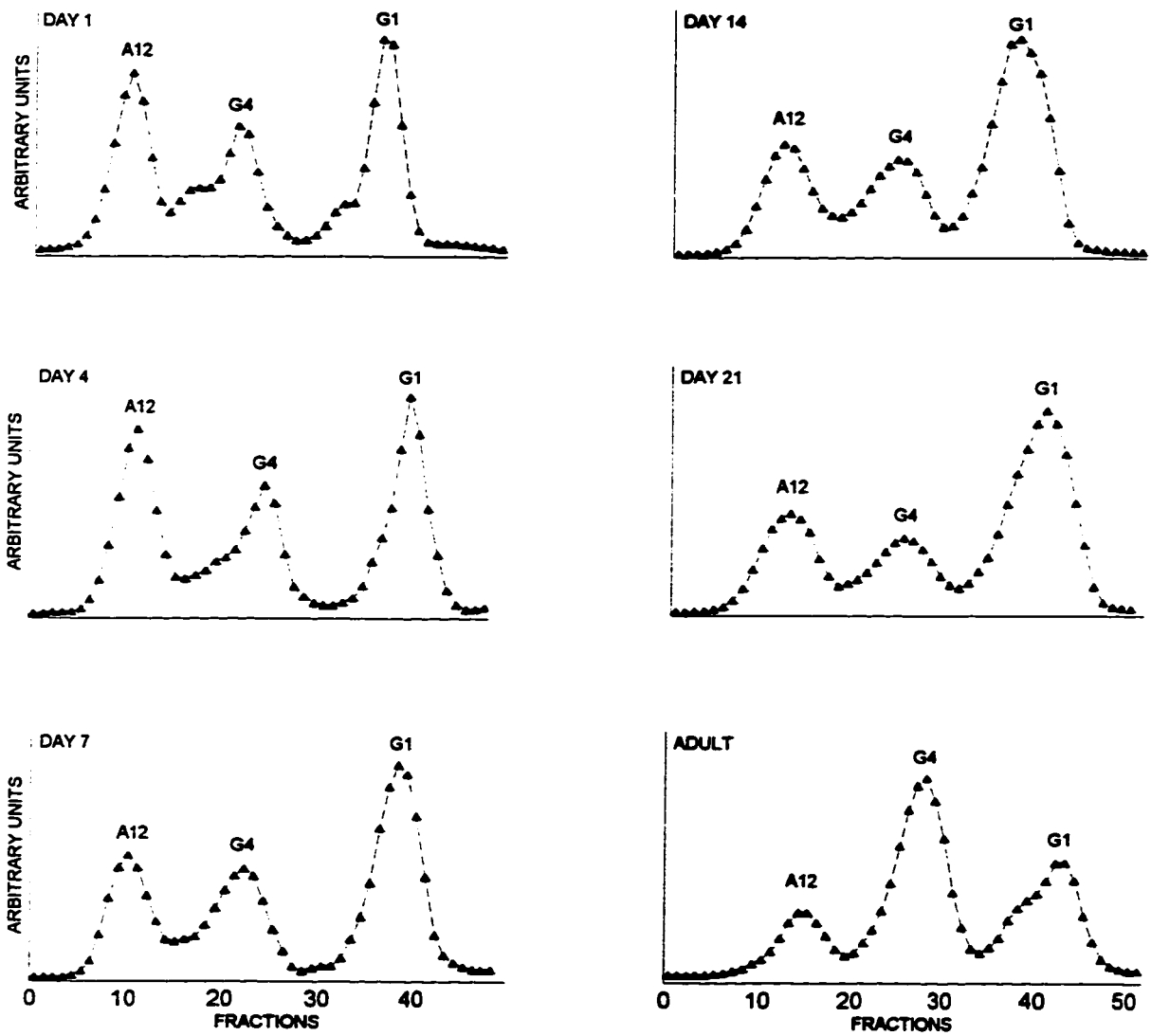
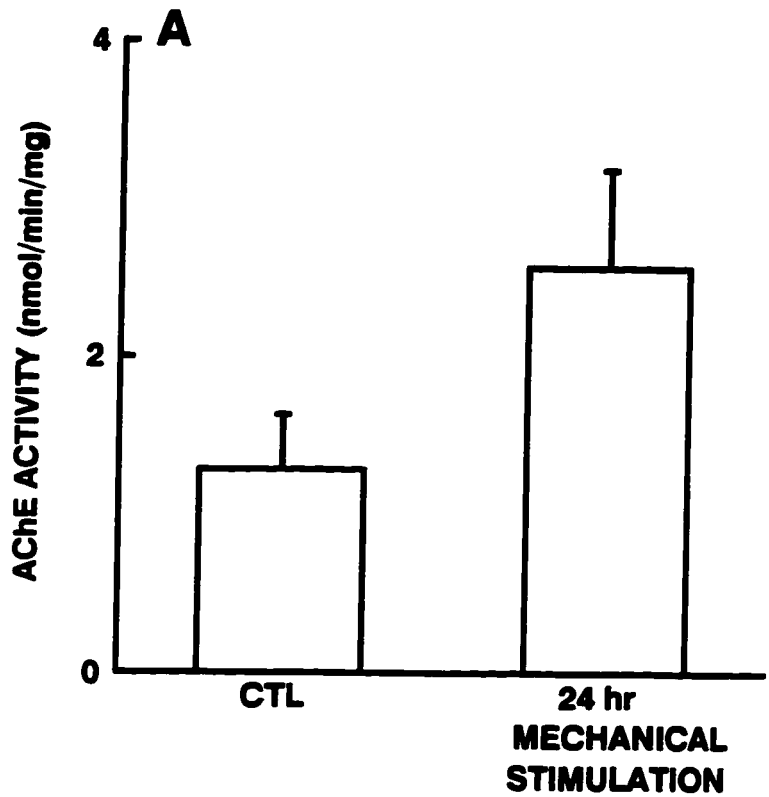
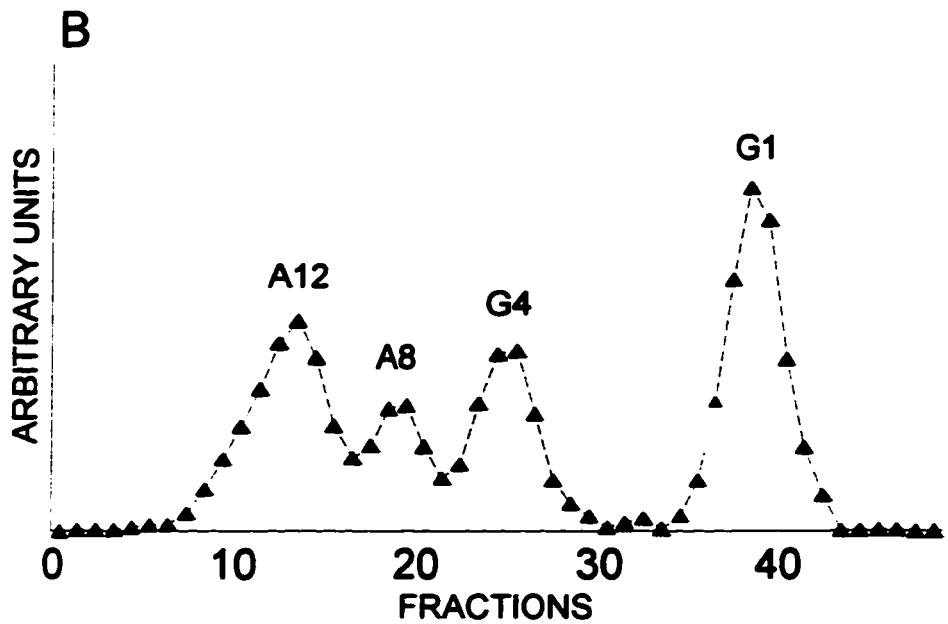

DAY 1
A12
G4
G1
ARBITRARY UNITS
DAY 14
A12
G4
G1
DAY 4
A12
G4
G1
ARBITRARY UNITS
DAY 21
A12
G4
G1
DAY 7
A12
G4
G1
ARBITRARY UNITS
0
10
20
30
40
FRACTIONS
ADULT
A12
G4
G1
0
10
20
30
40
50
FRACTIONS

Figure 19

ACETYLCHOLINESTERASE ACTIVITY IN MOUSE SLOW MYOTUBE CULTURES SUBJECTED TO MECHANICAL STIMULATION

Slow myotube cultures derived from mouse H-2K^b-tsA58 clonal cells were mechanically stimulated for 24 hr. Slow myotubes exhibited an increase in AChE activity in response to mechanical stimulation (A). Analysis of the AChE profile revealed a typical slow muscle pattern displaying peaks of A_{12}, A_8, G_4 and G_1 (B). Values in A are means ± SEM. (CTL, control, paired t-test, n=3)


A
4
2
0
AChE ACTIVITY (nmol/min/mg)
CTL
24 hr
MECHANICAL
STIMULATION


B
ARBITRARY UNITS
A12
A8
G4
G1
0
10
20
30
40
FRACTIONS

form (data not shown) as observed with primary cultures of rat myotubes (see also Figures 8 and 9).

Fast myotubes exhibited a similar increase in AChE activity over control levels after 24 hr of mechanical stimulation (Figure 20A). Analysis of the molecular form profile revealed peaks of A_{12}, G_4 and G_1, indicative of a fast muscle pattern of AChE expression (Figure 20B). As observed for slow myotubes, the increase in AChE levels following mechanical stimulation was due to a general elevation in all AChE molecular forms (data not shown).

3.1.6 Effects of mechanical stimulation on acetylcholinesterase and Egr-1 mRNA levels

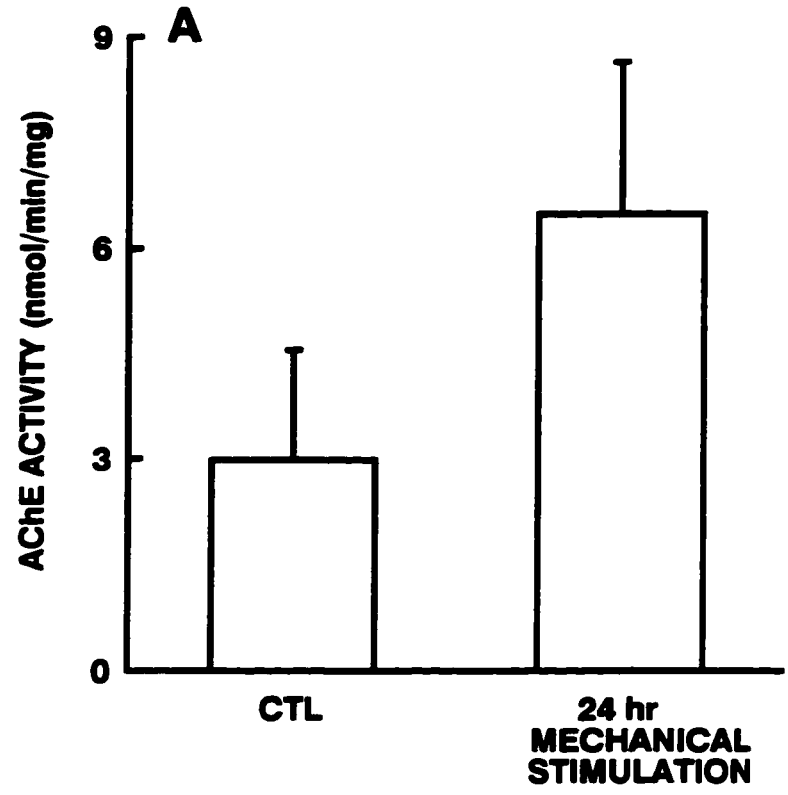
To determine the effects of mechanical stimulation on levels of AChE transcripts, quantitative RT-PCR was performed. Selective amplification of AChE cDNAs produced the expected band of 670 bp (Figures 21A and 22A). To confirm that the PCR conditions for AChE were within the linear range of amplification, control RNA was serially diluted 10 x, 50 x and 250 x. Quantitation of AChE RT-PCR products confirmed the linear relationship between log cpm vs RNA concentration under these conditions (Figure 21B).

Quantitation of AChE PCR products from the pooled samples over the experimental time course is shown in Figure 22. Transcript levels were found to increase progressively over the time course of the experiment (Figure 22A and B). To determine whether pooled samples from the 24 hr time-point (see Methods) were significantly different from control levels, RT-PCR was performed on individual samples under identical conditions. Quantitation of individual samples revealed a significant 3.5-fold increase over control

Figure 20

ACETYLCHOLINESTERASE ACTIVITY IN MOUSE FAST MYOTUBE CULTURES SUBJECTED TO MECHANICAL STIMULATION

Fast myotube cultures derived from H-2K^b-tsA58 clonal cells were mechanically stimulated for 24 hr. Fast myotubes exhibited an increase in AChE activity in response to mechanical stimulation (A). Analysis of the AChE profile revealed a typical fast muscle profile displaying peaks of A_{12}, G_4 and G_1 (B). Values in A are means ± SEM. (CTL, control, paired t-test, n=3)


A
9
6
3
0
AChE ACTIVITY (nmol/min/mg)
CTL
24 hr
MECHANICAL
STIMULATION

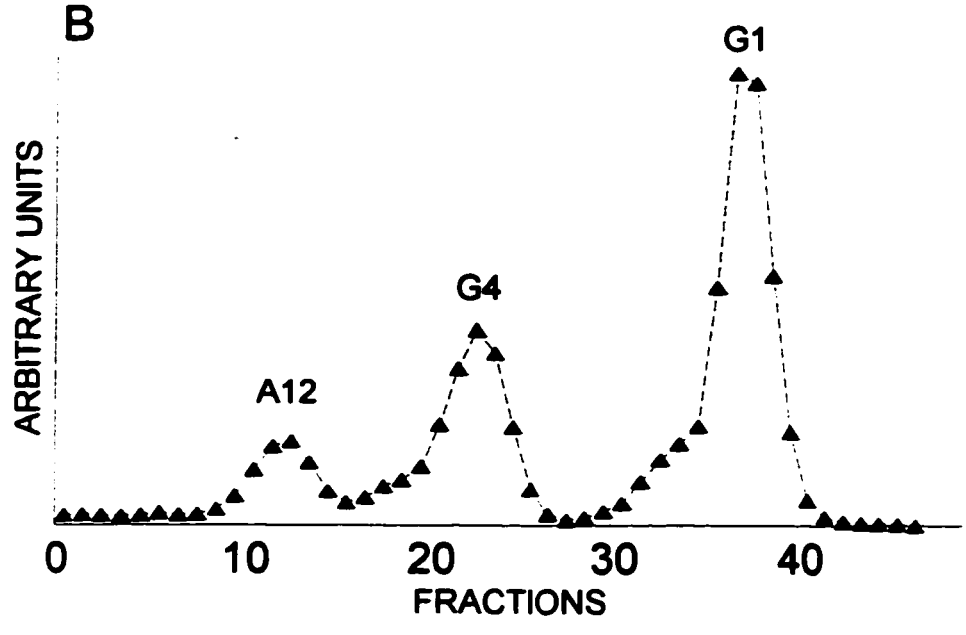
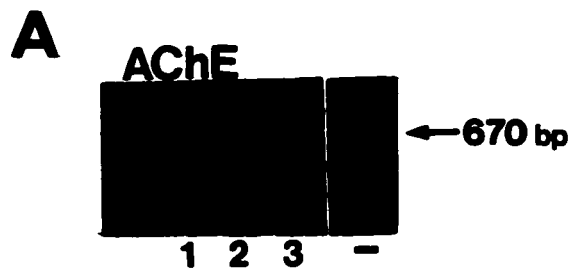

B
G1
G4
A12
ARBITRARY UNITS
0
10
20
30
40
FRACTIONS

Figure 21

QUANTITATIVE ANALYSIS OF ACETYLCHOLINESTERASE mRNA LEVELS IN RAT MYOTUBES

RT-PCR of AChE mRNAs from control samples produced the expected product size of 670 bp (A). To confirm that the PCR was within the linear phase of amplification for these experiments, control RNA was serially diluted (A, lanes 1-3) 10 x, 50 x and 250 x, reverse transcribed and amplified for AChE cDNAs over 42 cycles. Quantitation of these PCR products plotted on a logarithmic scale reveals a linear relationship between concentration of RNA and CPM under these conditions (B). Negative control lane is denoted as - .


A
AChE
670 bp
1 2 3 –

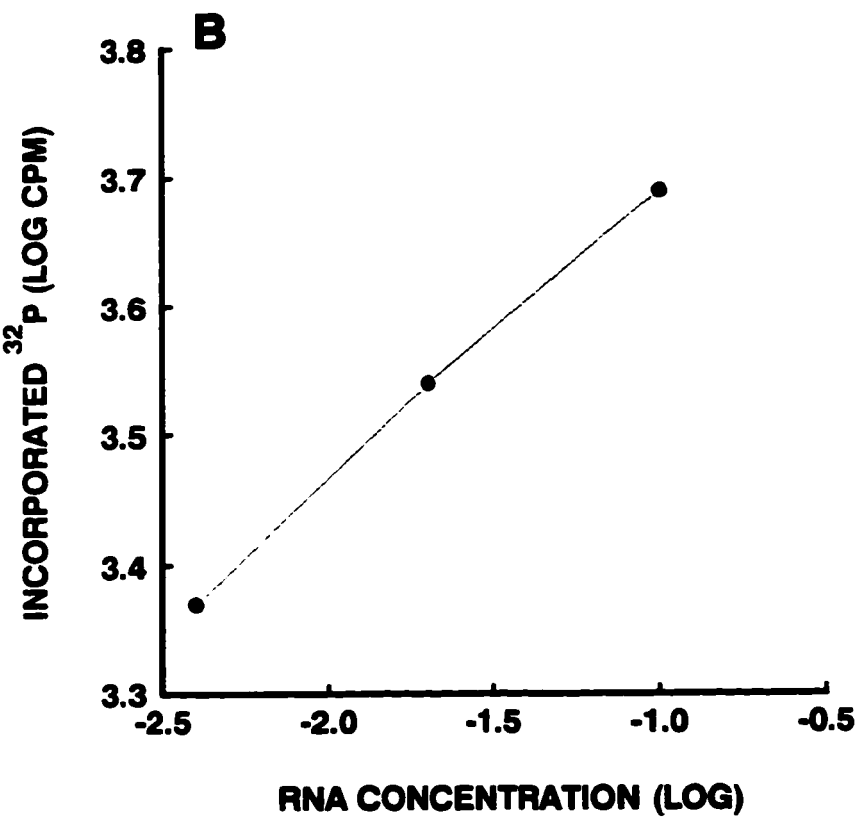
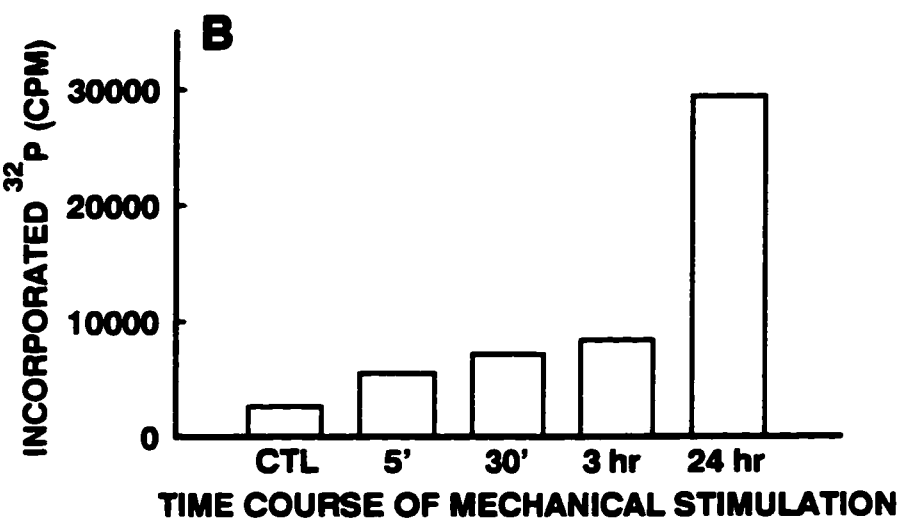

B
3.8
3.7
3.6
3.5
3.4
3.3
INCORPORATED 32P (LOG CPM)
-2.5
-2.0
-1.5
-1.0
-0.5
RNA CONCENTRATION (LOG)

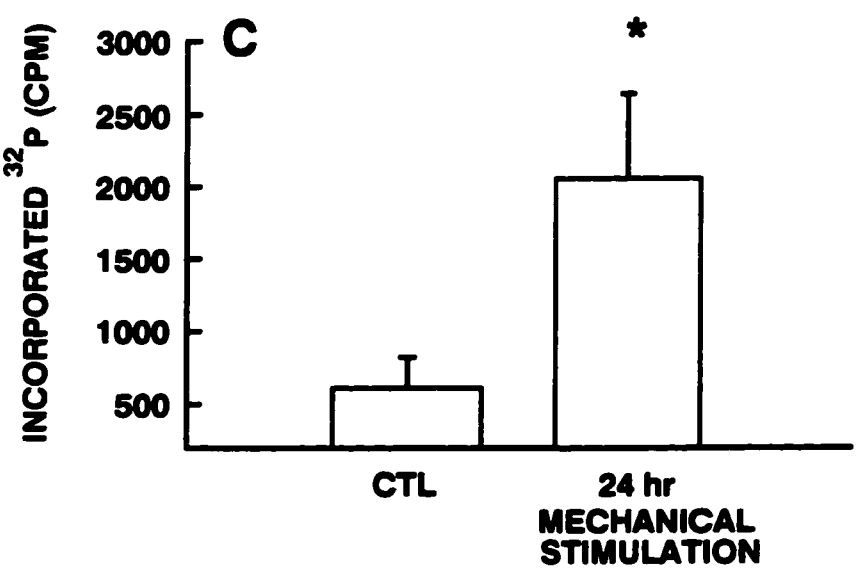
Figure 22

QUANTITATION OF ACETYLCHOLINESTERASE mRNA LEVELS IN RAT MYOTUBES SUBJECTED TO MECHANICAL STIMULATION

RT-PCR of AChE mRNAs from myotubes mechanically stimulated are shown in A (control, 5 min, 30 min, 3 hr and 24 hr; lanes 1 to 5, respectively). Quantitation of PCR products over the time course revealed maximal accumulation of AChE transcripts at 24 hr (B). Quantitation of individual control and 24 hr samples are shown in C. Asterisk indicates a significant difference between control (CTL) and experimental groups. (paired t-test, $P < 0.05$, $n=10$)


A
AChE
670bp
1 2 3 4 5 –


B
INCORPORATED 32P (CPM)
30000
20000
10000
0
CTL
5'
30'
3 hr
24 hr
TIME COURSE OF MECHANICAL STIMULATION


C
*
INCORPORATED 32P (CPM)
3000
2500
2000
1500
1000
500
CTL
24 hr
MECHANICAL STIMULATION

levels at 24 hr (Figure 22C; $P < 0.05$).

Egr-1 mRNA levels are known to increase in response to mechanical stress in other cell types (see for example Kumoro and Yamazaki, 1993). Since the rat AChE promoter has binding sites for Egr-1 trans-activating factors (Chan et al., 1996), we determined whether this immediate-early gene played a putative role in the modulation of AChE expression. Selective amplification of Egr-1 cDNAs produced the expected band of 512 bp (see Figure 23A and 24A). As shown in Figure 23, the PCR conditions for Egr-1 were within the linear range of amplification under these experimental conditions as indicated by the straight line between cpm vs RNA concentration. Levels of Egr-1 mRNAs from mechanically stimulated cells were found to increase rapidly peaking at 30 min and returning towards control levels thereafter (Figure 24A and B). RT-PCR was performed on individual samples from control and the 30 min time-point to determine statistical significance. Quantitation of individual samples revealed a significant 2.3-fold increase over control levels at 30 min (Figure 24C).

3.2 *IN VIVO* MECHANICAL STIMULATION USING THE DENERVATED HEMIDIAPHRAGM MODEL

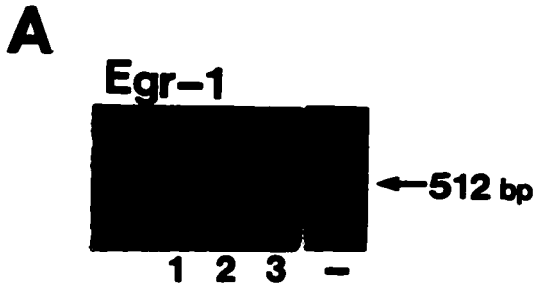
3.2.1 Hemidiaphragm hypertrophy and RNA content

To verify that denervated hemidiaphragm muscles underwent passive mechanical forces, we measured the changes in muscle mass and RNA content since these parameters have been shown to transiently increase following denervation of hemidiaphragms (Sola and Martin, 1953; Stewart, 1955; Stewart and Martin, 1956; Buse et

Figure 23

QUANTITATIVE ANALYSIS OF Egr-1 mRNA LEVELS IN RAT MYOTUBES

RT-PCR of Egr-1 mRNAs from control samples produced the expected product size of 512 bp (A). To confirm that the PCR was within the linear phase of amplification for these experiments, control RNA was serially diluted (A, lanes 1-3) 10 x, 50 x and 250 x, reverse transcribed and amplified for Egr-1 cDNAs over 39 cycles. Quantitation of these PCR products reveals a linear relationship between concentration of RNA and CPM under these conditions (B). Negative control lane is denoted as - .


A
Egr-1
512 bp
1 2 3 –

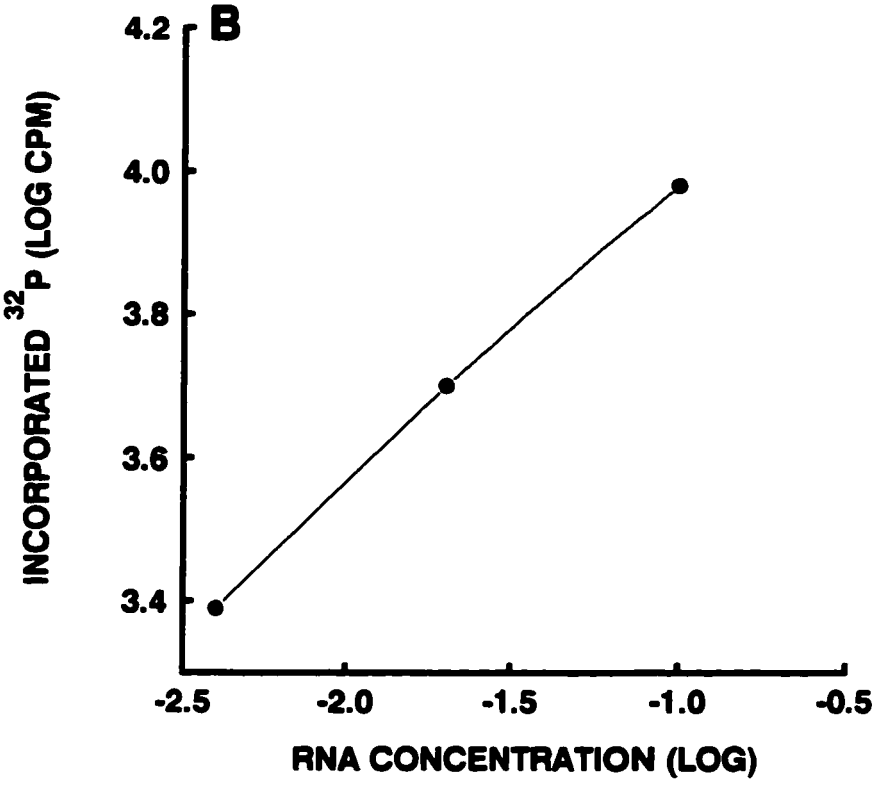
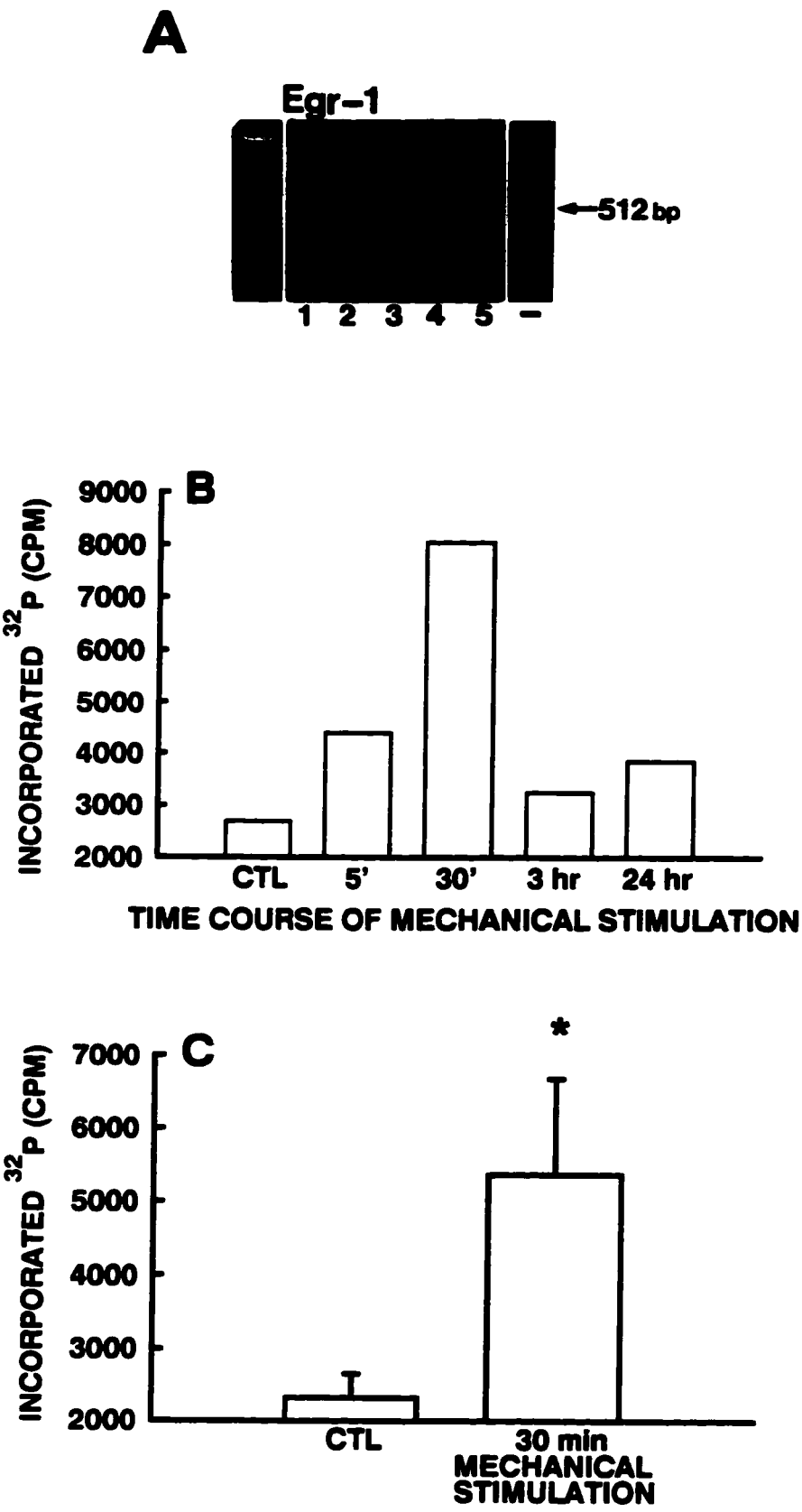

B
4.2
4.0
3.8
3.6
3.4
INCORPORATED 32P (LOG CPM)
-2.5
-2.0
-1.5
-1.0
-0.5
RNA CONCENTRATION (LOG)

Figure 24

LEVELS OF Egr-1 TRANSCRIPTS IN RAT MYOTUBES SUBJECTED TO MECHANICAL STIMULATION

RT-PCR of Egr-1 mRNAs from myotubes mechanically stimulated are shown in A (control, 5 min, 30 min, 3 hr and 24 hr; lanes 1 to 5, respectively). Quantitation of these PCR products revealed a maximal accumulation of transcripts at 30 min (B). Quantitation of individual control and 30 min samples are shown in C. Asterisk indicates a significant difference between control (CTL) and experimental groups. (paired t-test, $P < 0.05$, $n=10$)


A
Egr-1
512 bp
1 2 3 4 5 –
B
INCORPORATED ^{32}P (CPM)
9000
8000
7000
6000
5000
4000
3000
2000
CTL
5'
30'
3 hr
24 hr
TIME COURSE OF MECHANICAL STIMULATION
C
*
INCORPORATED ^{32}P (CPM)
7000
6000
5000
4000
3000
2000
CTL
30 min
MECHANICAL STIMULATION

al., 1965; Feng and Lu, 1965; Gutmann et al., 1966; Manchester and Harris, 1968; Yellin, 1974; Collins and Younkin, 1982; Mamatas and Oja, 1987) . Denervated hemidiaphragms were excised at 2, 5, 10 and 20 days post-denervation. Under normal conditions, denervation results in a rapid and prominent atrophy of skeletal muscle (see section 1.5.1). Under these experimental conditions however, hemidiaphragms exhibited a transient hypertrophy which peaked at day 10 with a 30% increase over control levels (see Figure 25).

Total RNA content from denervated hemidiaphragms was determined spectrophotometrically at 260 nm (see Figure 26). Hemidiaphragms exhibited an increase from control levels peaking at day 5 and returning towards control levels by day 10. By 20 days post-denervation, RNA concentrations were 5% below control levels.

3.2.2 Total acetylcholinesterase activity and molecular forms

AChE enzyme levels in denervated hemidiaphragm muscles were analyzed (Figure 27). AChE activity was found to drop dramatically by day 5 with enzyme levels decreasing by 63% from control levels ($P < 0.05$). By 10 days post-denervation however, AChE levels began to increase and recovered to 67% of controls. This recovery was essentially complete by day 20 with AChE levels recovering an additional 4 % to reach 71% of control levels. The AChE levels seen at day 20 post-surgery was not significantly different as compared to enzyme activity measured in control muscles ($P < 0.05$).

Analysis of AChE molecular forms from 5 and 30 day-denervated hemidiaphragm muscles is shown in Figure 28. The control hemidiaphragm muscles displayed a slow

Figure 25

MASS OF RAT HEMIDIAPHRAGM MUSCLE AFTER DENERVATION

Entire diaphragm muscles were removed and hemidiaphragms dissected and weighed at 2, 5, 10 and 20 days post-denervation. The number of samples per experimental time-point were from CTL to 20 day, 5, 2, 4, 5 and 4, respectively. Asterisk indicates a significant difference between control and experimental groups. Mean ± SEM are shown. (independent t-test, $P < 0.05$)

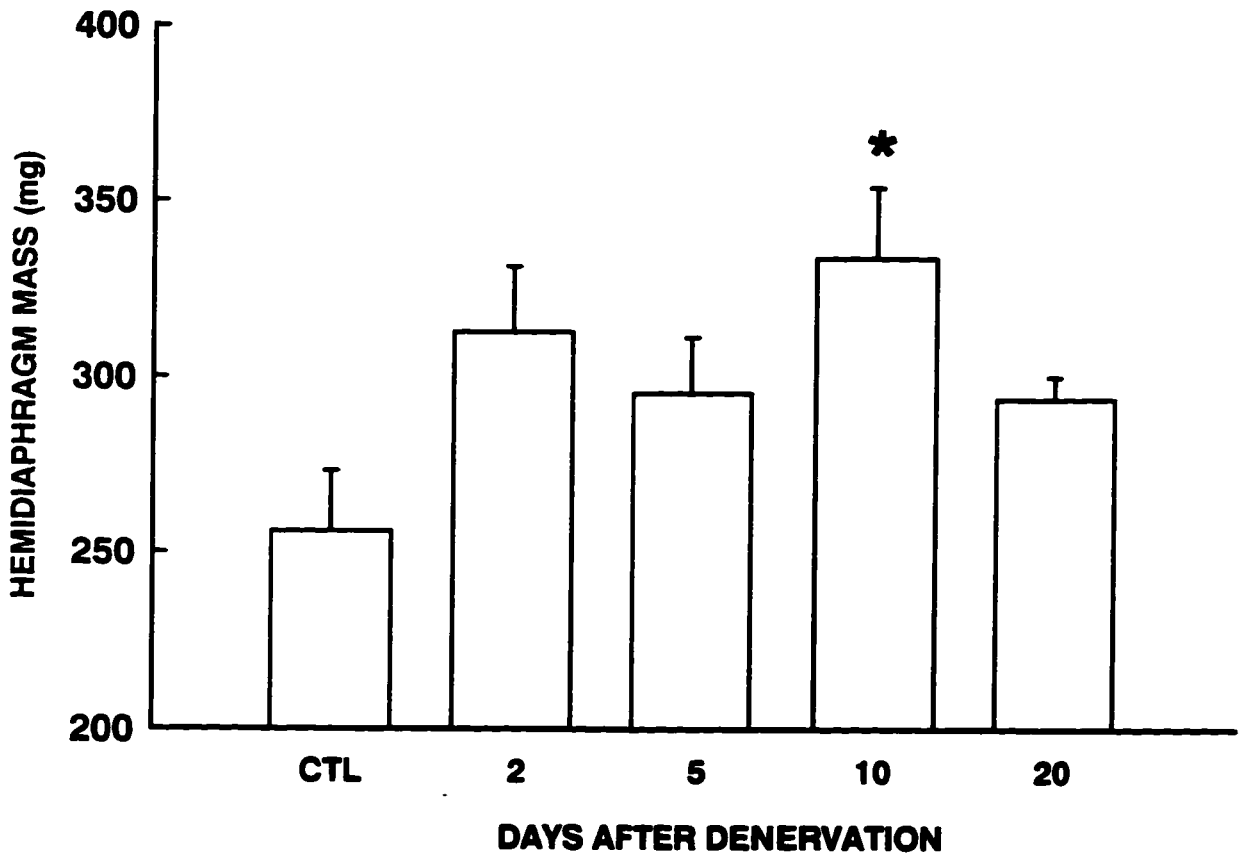

400
350
300
250
200
HEMIDIAPHRAGM MASS (mg)
*
CTL
2
5
10
20
DAYS AFTER DENERVATION

Figure 26

TOTAL RNA CONCENTRATION IN DENERVATED RAT HEMIDIAPHRAGM MUSCLE

Total RNA from denervated rat hemidiaphragm muscles was extracted and concentrations determined at 260 nm. The number of samples per experimental time-point were from CTL to 20 day, 5, 2, 4, 5 and 4, respectively. Mean ± SEM are shown. (independent t-test)

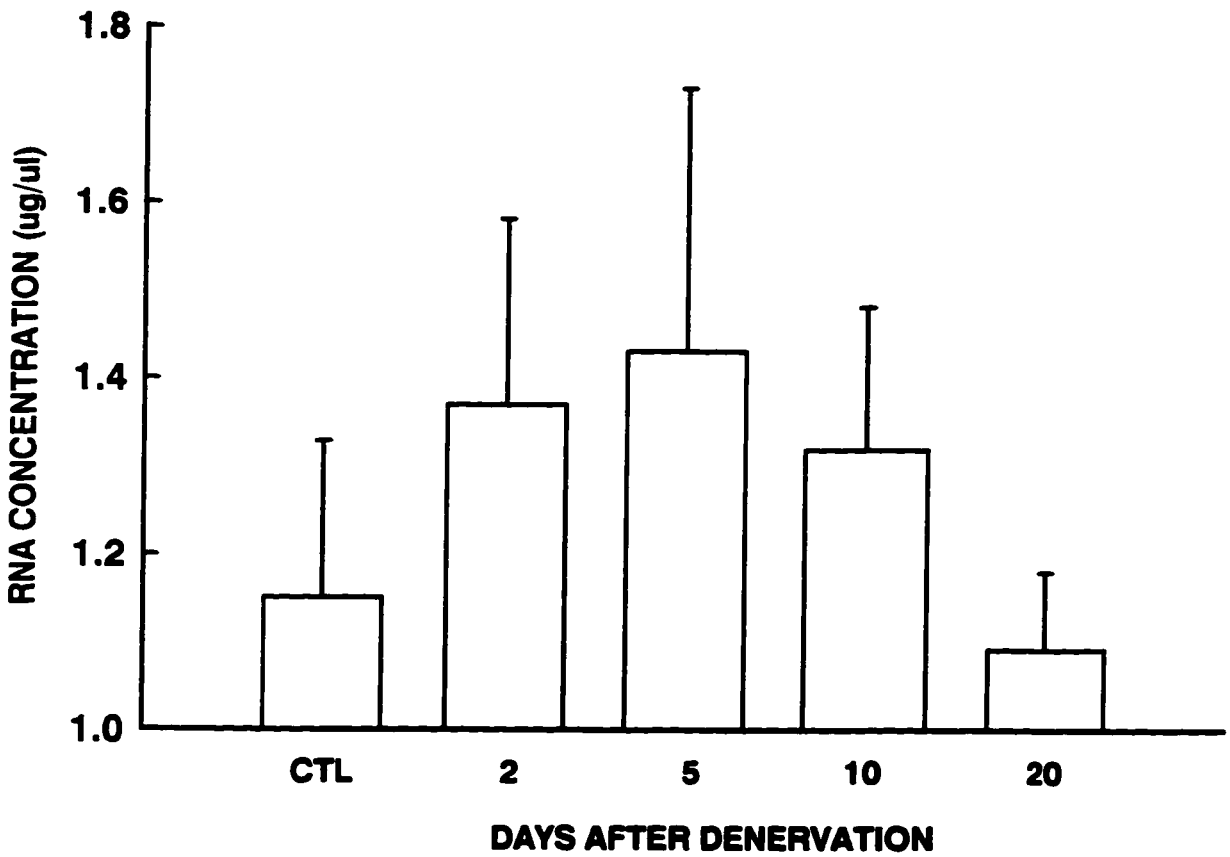

1.8
1.6
1.4
1.2
1.0
RNA CONCENTRATION (ug/ul)
CTL
2
5
10
20
DAYS AFTER DENERVATION

Figure 27

TOTAL ACETYLCHOLINESTERASE ACTIVITY IN DENERVATED HEMIDIAPHRAGM MUSCLE

Hemidiaphragm muscles were analyzed for AChE activity at 5, 10 and 20 days post-denervation. Values are expressed as mean ± SEM. The number of samples per experimental time-point were from CTL to 20 day, 3, 5, 3 and 3, respectively. Asterisk indicates a significant difference between control (CTL) and experimental groups. Double asterisks indicate a significant difference between 5 day post-denervation and sample groups. (ANOVA, Fisher post-hoc, $P < 0.05$)

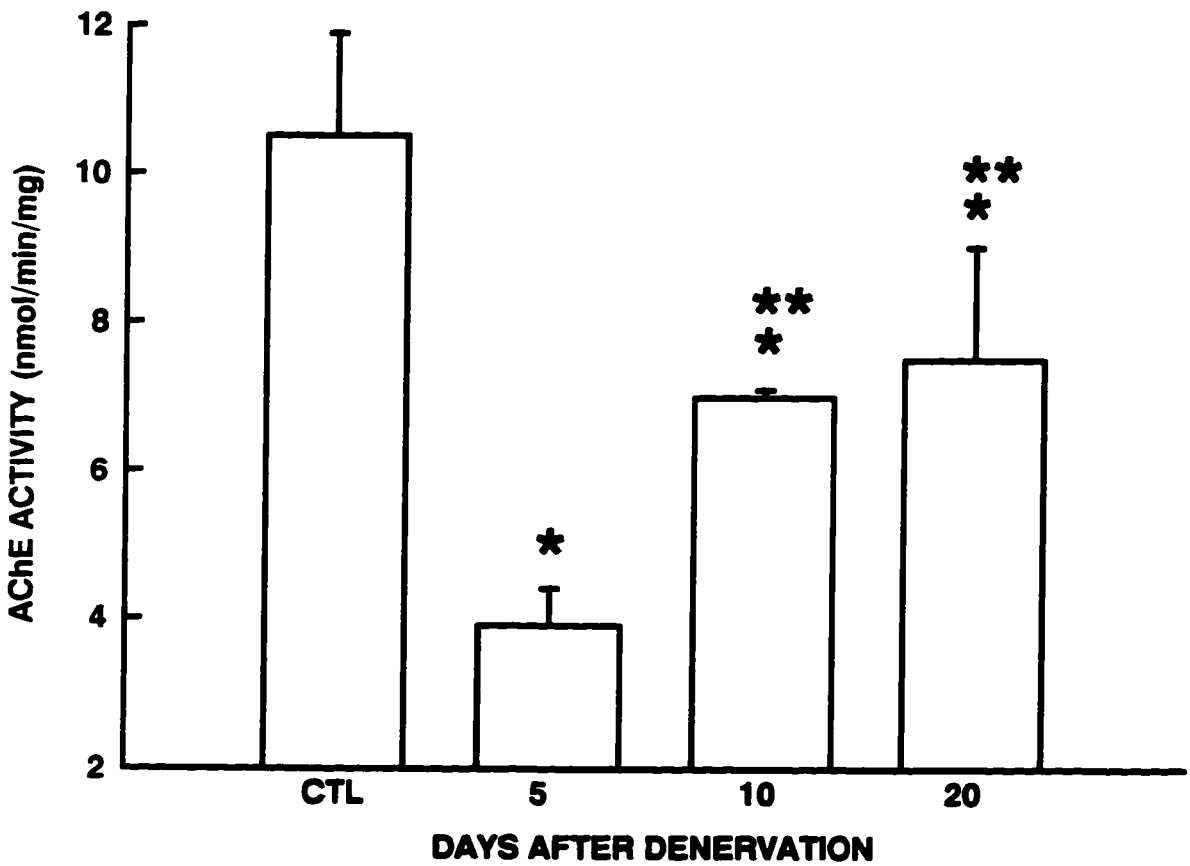
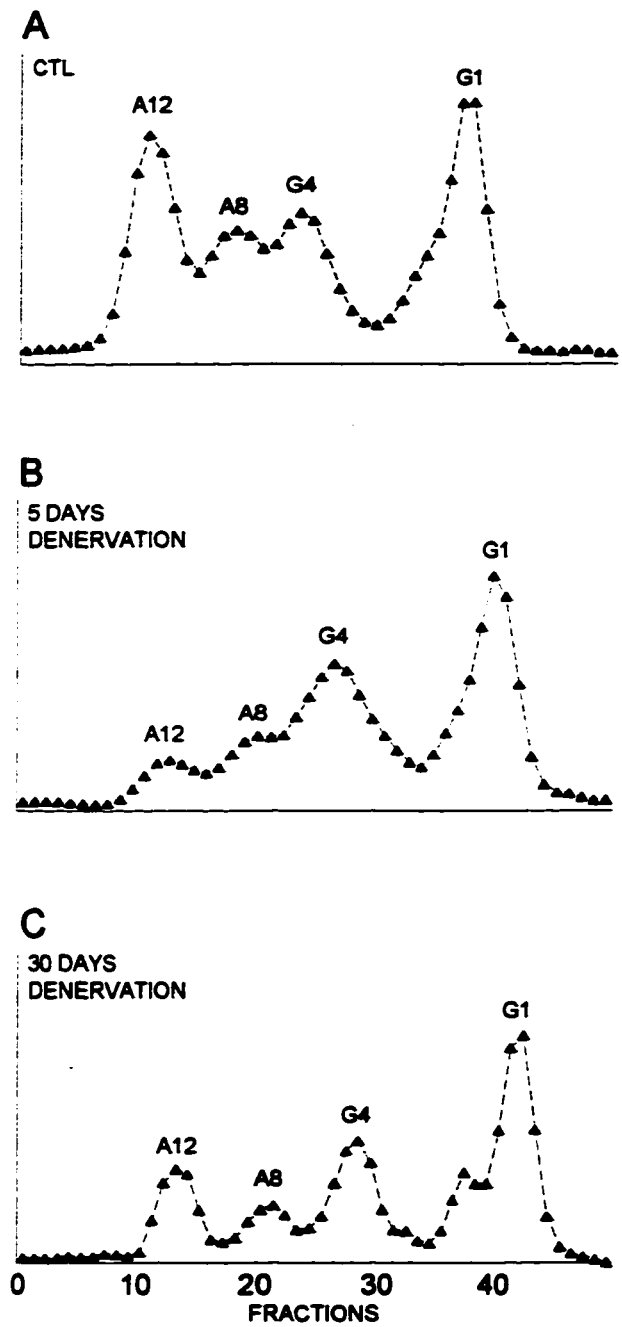

12
10
8
6
4
2
AChE ACTIVITY (nmol/min/mg)
*
**
*
**
*
CTL
5
10
20
DAYS AFTER DENERVATION

Figure 28

ACETYLCHOLINESTERASE MOLECULAR FORM PROFILES OF DENERVATED HEMIDIAPHRAGM MUSCLE

Aliquots of hemidiaphragm homogenates from control (CTL), 5 and 30 day-denervated hemidiaphragms were separated by velocity sedimentation on sucrose gradients and analyzed spectrophotometrically. The AChE molecular forms were identified according to the nomenclature of Bon et al. (1982) on the basis of their apparent sedimentation coefficients.


A
CTL
A12
A8
G4
G1
B
5 DAYS
DENERVATION
A12
A8
G4
G1
C
30 DAYS
DENERVATION
A12
A8
G4
G1
0
10
20
30
40
FRACTIONS

muscle profile consisting of the molecular forms A_{12}, A_8, G_4 and G_1 (Figure 28A). After 5 days of denervation however, the asymmetric forms were dramatically reduced while globular forms remained relatively unaffected (Figure 28B). By 30 days post-denervation, a significant recovery of the A_{12} forms was observed (vs 5 day, Figure 29), although the activity levels were still significantly lower than controls. In contrast, the A_8 molecular form showed no recovery after denervation (Figure 29). Denervation did not induce a selective downregulation of the globular forms, rather both G_4 and G_1 increased their relative content after 5 days of denervation and remained significantly higher than control levels at 30 days (Figure 29).

3.2.3 Acetylcholinesterase transcript levels in denervated hemidiaphragm muscles

To determine the effects of denervation on AChE mRNA levels in hemidiaphragm muscles, we employed quantitative RT-PCR. Selective amplification of AChE cDNAs produced a single band of the expected size of 670 bp (Figure 30). Quantitation of AChE transcripts over the experimental time course revealed a progressive decrease which by day 5 had dropped by 37% (Figure 31). By day 10 however, AChE transcripts began to increase, reaching 67% of control levels. By day 20, AChE transcript levels had reached 78% of pre-denervation levels. These AChE mRNA levels were not different from those observed in control hemidiaphragm muscles (see Figure 31).

Figure 29

RELATIVE CONTENT OF ACETYLCHOLINESTERASE MOLECULAR FORMS IN DENERVATED HEMIDIAPHRAGM MUSCLE

Values represent the relative content of A_{12}, A_8, G_4 and G_1 present in control (CTL), 5 and 30 days denervated hemidiaphragm. Data are expressed as a percentage of total activity. Means ± SEM are shown. Single asterisk indicates a significant difference between control and 5 or 30 day denervated groups. Double asterisks indicate a significant difference between 5 and 30 day denervated groups. (independent t-test, $P < 0.05$, n=3)

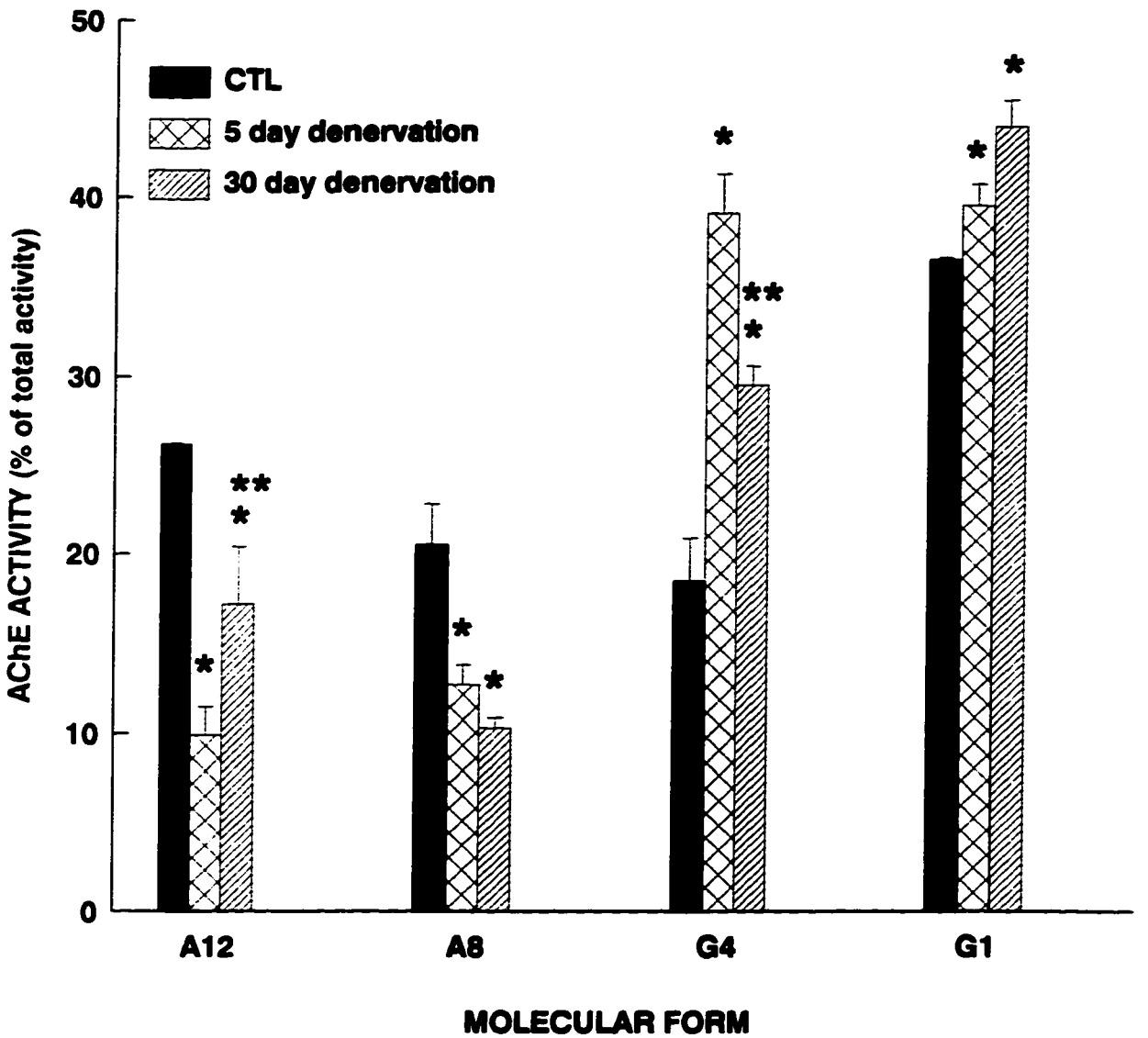

50
40
30
20
10
0
CTL
5 day denervation
30 day denervation
AChE ACTIVITY (% of total activity)
A12
A8
G4
G1
MOLECULAR FORM

Figure 30

ACETYLCHOLINESTERASE TRANSCRIPT LEVELS IN DENERVATED HEMIDIAPHRAGM MUSCLE

Total RNA was extracted from hemidiaphragms at 2, 5, 10 and 20 days post-denervation. RT-PCR of AChE mRNA produced a band of the expected size of 670 bp. The number of samples per experimental time-point were from control (C) to 20 day, 5, 2, 4, 5 and 4, respectively.

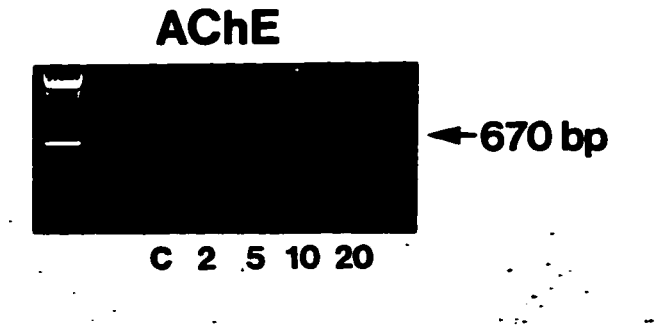
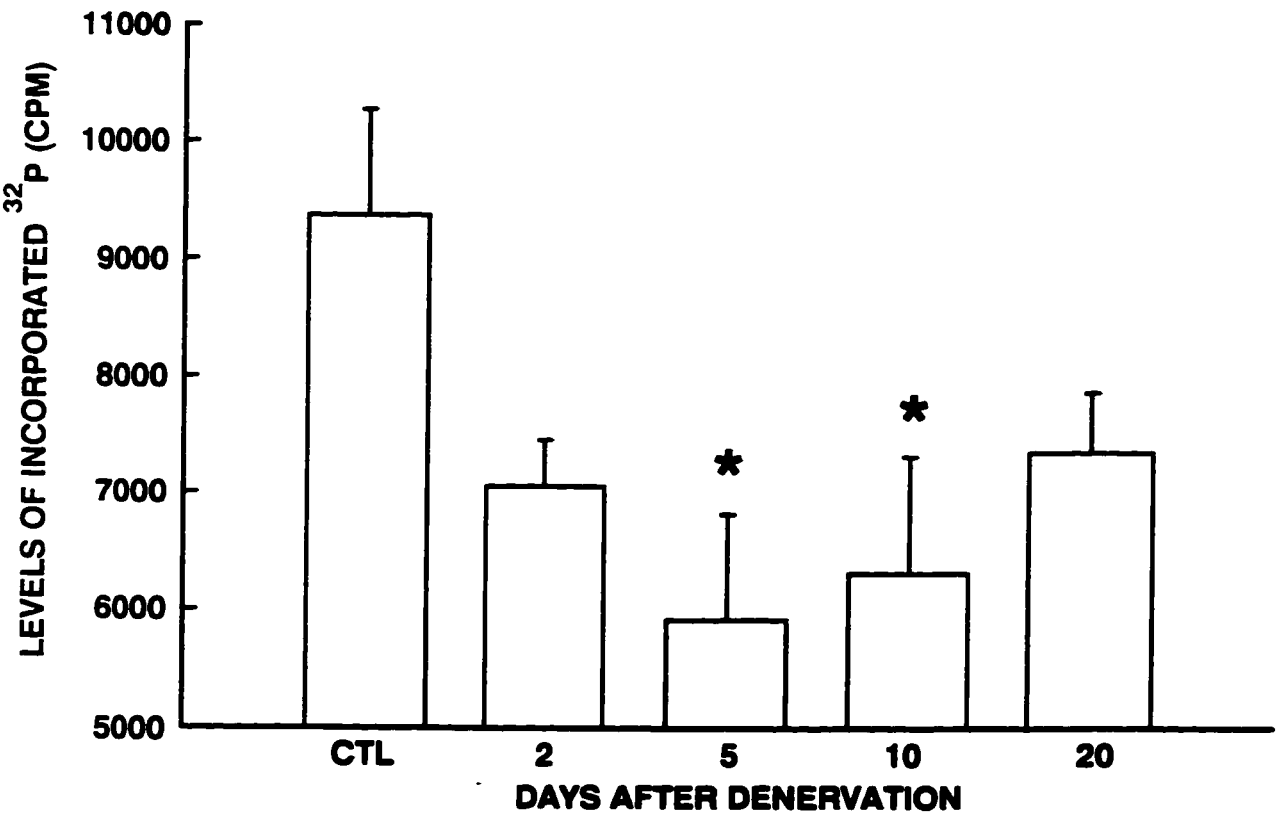

AChE
670 bp
C 2 5 10 20

Figure 31

QUANTITATION OF ACETYLCHOLINESTERASE mRNA LEVELS IN DENERVATED HEMIDIAPHRAGM MUSCLE

Quantitation of AChE transcript levels from hemidiaphragms at 2, 5, 10 and 20 days post-denervation are shown. The number of samples per experimental time-points were from control (CTL) to 20 day, 5, 2, 4, 5 and 4, respectively. Asterisks indicate a significant difference between control and experimental groups. (independent t-test, $P < 0.05$)


11000
10000
9000
8000
7000
6000
5000
LEVELS OF INCORPORATED ^{32}P (CPM)
CTL
2
5
10
20
DAYS AFTER DENERVATION
*
*

CHAPTER 4

DISCUSSION

The expression of AChE in muscle fibers is known to be sensitive to nerve-derived electrical activity and trophic factors (see sections 1.5 & 1.6). In recent years however, several lines of evidence indicate that mechanical forces influence the growth and differentiation of a number of tissues including skeletal muscle. Using *in vitro* and *in vivo* models, we tested the hypothesis that mechanical stimulation influences AChE expression in skeletal muscle fibers.

The effects of mechanical stimulation on AChE expression in cultured myotubes were first examined using a mechanical cell stimulator. In these studies, it was found that expression of AChE enzyme and mRNA was significantly elevated after 24 hr of mechanical stimulation. Interestingly, enzyme levels did not change significantly until this time-point whereas the amount of transcripts increased steadily over the time course of stimulation peaking at 24 hr. The elevation in enzyme activity observed in myotube cultures was reflected by a general elevation in all AChE molecular forms as opposed to an increase in a specific form. Preliminary examination of putative signalling pathways revealed that the mechanogenic mechanism is independent of electrical activity but involves extracellular Ca^{++} influx. Furthermore, it is possible that the immediate early gene Egr-1 plays a role since its mRNA levels increased transiently in response to mechanical stimulation.

In addition to these studies, we also tested this hypothesis *in vivo* by examining the effects of mechanogenic forces on denervated hemidiaphragm muscle. Passive mechanical forces associated with rhythmical contractions of the intact contralateral side were found to reverse the normal physiological effects of denervation. For example,

muscle mass and total RNA levels did not progressively decrease in response to denervation. Instead, they exhibited a transient increase which returned towards control levels 20 days after denervation. Additionally, AChE enzyme and transcript levels were found to return toward control levels after initially decreasing rapidly in response to denervation. These recovery profiles coincide in fact, with the return of normal breathing patterns after thoracic surgery and results from the imposition of passive mechanical forces on the denervated hemidiaphragm muscles during inspiratory movements.

4.1 *IN VITRO* MECHANICAL STIMULATION OF MYOTUBE CULTURES

Primary cultures of rat myotubes typically exhibited varying levels of spontaneous contractile activity after forming confluent myotubes. The reason for this variation between primary cultures is currently unknown. Previous studies have shown that AChE levels are higher in contracting than in non-contracting myotubes and hence, a culture with 90% contracting fibers will have a higher basal level of AChE activity than a culture with 50% of its fibers contracting (Brockman et al., 1984). This results in large variations in AChE activity between control cultures (see for example Figure 7). This variation is therefore an intrinsic characteristic of rat primary cultures.

Analysis of AChE molecular forms in myotube cultures revealed two distinct profiles. Cultures which were spontaneously contracting contained significant levels of A_{12}, A_8, G_4 and G_1. In contrast, non-contracting cultures lacked asymmetric forms but displayed the G_4 and G_1 forms. It was impossible to predict after plating whether myotubes would be contracting.

4.1.1 Effects of mechanical stimulation on acetylcholinesterase

Myotube cultures were mechanically stimulated following a pattern of high intensity (24%) stretch/relaxation with minimal rest to mimic the stretch a muscle fiber may be subjected to *in vivo* during an exercise session (Vandenburgh, 1992). Although this type of stimulation has been shown to induce cell damage, as based on elevated release of creatine kinase into the culture medium (Vandenburgh et al., 1989), the damage is temporary with cell responses returning toward control levels within hr after the onset of stimulation (Vandenburgh et al., 1989). No significant changes in the levels of AChE were found after 3 hr of mechanical stimulation. However, a burst of expression was detected after 24 hr with AChE enzyme levels increasing by 42% over control levels.

In vivo, skeletal muscles respond to increasing levels of neuromuscular activity such as during exercise, by elevating levels of AChE enzyme (Crockett et al., 1976; Gardiner et al., 1982). This elevation is primarily due to an increase in the expression of the G_4 molecular form of AChE in fast skeletal muscles provided that the recruitment pattern of the muscle is phasic rather than tonic (Fernandez and Donoso, 1988; Jasmin and Gisiger, 1990; Gisiger et al., 1991; 1994). To determine the nature of the AChE increase in response to mechanical stimulation, AChE molecular form profiles from the cultures were examined. Analysis of myotube extracts revealed that the increase in AChE enzyme levels was due to an elevation in all molecular forms as opposed to an increase in a specific one. This type of general elevation of all molecular forms resembles that seen in response to compensatory hypertrophy (Jasmin et al., 1991; Sveistrup et al., 1995) and confirm our hypothesis. This suggests that the adaptative response of skeletal muscle fibers to

mechanical stimulation may be similar to those seen in hypertrophied muscles. Indeed, cultured rat myotubes react to mechanical stimulation by displaying cell growth and hypertrophy (Vandenburgh and Kaufman, 1979; 1981; Hatfuldy et al., 1989; Vandenburgh et al., 1989; 1990; 1991); an adaptative response previously observed *in vivo* in muscle undergoing compensatory hypertrophy (see for example Goldspink et al., 1995 and references therein).

The general increase in AChE molecular forms observed in response to mechanical stimulation may be partially attributed to the mechanical stimulation pattern chosen as well as to the muscle fiber types present in the culture. It is known that *in vivo*, AChE regulation in fast muscle is particularly sensitive to the pattern of electrical activity. For example, fast muscle fibers were shown to increase total AChE enzyme levels in response to both slow (10 Hz) and fast (100 Hz) stimulation patterns (Lomo et al., 1985; Boudreau-Larivière et al., 1996A). However, the G_4 content only increased in muscles receiving a fast stimulation pattern (Boudreau-Larivière et al., 1996A). Taken together, these studies emphasize the necessity of a phasic stimulation pattern in the selective adaptative response of G_4 in fast skeletal muscle fibers. Given that this modulation only occurs within narrow physiological parameters, it is plausible, and somewhat predictable, that the mechanical stimulation pattern would not elicit a similar selective increase in G_4 in cultured myotubes.

The delayed expression of AChE in response to mechanical stimulation has been observed for other cellular constituents. For example, prostaglandins levels have been shown to increase in cultured myotubes in response to mechanical stimulation.

Prostaglandin E_2 levels however, rapidly increased and peaked within a 5 hr-period while prostaglandin $F_{2\alpha}$ levels increased slowly, although continuously, over 50 hr (Vandenburgh et al., 1991). The delay is therefore unlikely related to the forementioned transient damage to cells incurred during mechanical stimulation, but may be caused by the mechanogenic signalling pathways involved in the expression of AChE. For example, a muscle cell may respond in a rapid (milliseconds), intermediate (minutes), or longer (hours-days) fashion to an external mechanical signal (Banes et al., 1995). The 3 hr-minimum delay that was observed for AChE expression suggests that the signalling pathway likely involves pre-translational regulatory mechanisms (Vandenburgh, 1992; Banes et al., 1995). This, however, does not preclude rapid mechanical activation of ion channels (ie Ca^{++}) or pathways involving the activation of kinases since these, in turn, can stimulate expression of proteins involved in regulating transcription such as NF-κB and Egr-1 (Banes et al., 1995).

4.1.2 Acetylcholinesterase expression in clonal fast and slow myotubes subjected to mechanical stimulation

Primary cultures of rat myotubes consist of both fast and slow muscle fibers since the satellite cells originated from a mixture of slow and fast muscles. As forementioned, different skeletal muscle fiber types respond to superimposed electrical activity with distinct AChE adaptations. Although the exact mechanisms underlying the adaptative changes in fast and slow fibers to varied stimulation patterns are currently unknown, the divergent responses suggest that alternate signalling pathways exist between fiber types (Boudreau-Larivière et al., 1996A). We therefore separated the effects of mechanical

stimulation on fast vs slow muscles using clonal muscle cell lines derived from H-2K^b-tsA58 mice.

To determine the validity of using clonal fast and slow cells to represent *in vivo* adult muscle fibers, we initially examined the molecular form profiles of fast tibialis anterior and slow soleus muscles from rats at various times after birth. The pattern of AChE molecular forms in both fast and slow muscles was already established within hours after birth. The slow soleus muscle exhibited a profile consisting of A_{12}, A_8, G_4 and G_1, while the fast tibialis anterior lacked A_8 and displayed a larger proportion of G_4. These results are coherent with those found in previous studies (Dettbarn et al., 1985; Sketlj et al., 1991) and indicate that the regulation of AChE in slow and fast muscle fibers is already instituted at birth even before the establishment of distinct neural stimulation patterns between fast and slow muscles (Narvarette and Vroba, 1983). The differences in molecular form profiles therefore, originate in myogenic precursor cells destined to form fast or slow muscle fibers (Dolenc et al., 1994). It was interesting to note however, that despite the fact that the distinct AChE patterns were established by day 1, levels of asymmetric forms in both fiber types remained higher than in adult muscles up to 21 days after birth. Over-expression of total AChE enzyme levels during post-natal development has been reported previously by Vigny et al. (1976) and Fernandez and Seiter (1984) who showed that AChE levels are initially high at birth but decrease progressively to reach adult concentrations by day 28. The authors were unaware however, that this was primarily due to elevated levels of the asymmetric forms of AChE.

After determining that the distinct patterns of AChE in fast and slow muscles were already established at birth, we examined the molecular form profiles of fast and slow clonal cells derived from H-2K^b-tsA58 mice to determine whether these satellite cells also displayed intrinsic differences in their AChE molecular form profile. Sedimentation analysis of slow myotube cultures revealed a pattern of A_{12}, A_8, G_4 and G_1 with the characteristic proportions of slow muscle thereby confirming that these cultured myotubes exhibited a slow profile of AChE. In contrast, fast myotube cultures lacked significant amounts of A_8 and had levels of G_4 typical of fast muscle. The AChE profiles of these myotubes originating from satellite cells obtained from fast or slow muscles therefore reflect their fiber type of origin. These findings are in contrast to those reported by Boudreau-Larivière et al. (1996A) who showed the existence of a common basic AChE molecular form profile of the slow muscle type in primary cultures of myotubes derived from fast and slow muscles. Although the primary cultures were obtained from predominantly slow and fast muscles, the possibility that the cultures were not pure fast and slow fibers exists. Furthermore, it is also possible that slow and fast satellite cells do not proliferate at the same rate. Therefore, even the smallest contamination by cells from one or the other fiber type could have a significant impact. The H-2K^b-tsA58 cells, in contrast, are clonal cell lines and these concerns are not relevant. Our conclusion showing that the characteristic profile of AChE molecular forms seen in fast and slow muscles is intrinsically pre-programmed in satellite cells extend previous results which demonstrated, based on the pattern of myosin heavy chain expression, that the phenotype of satellite cells is indicative of the type of fiber from which they are derived (Rosenblatt et al., 1996).

After establishing the validity of the fast and slow clonal cell lines, we examined the effects of mechanical stimulation on these two cell populations. Mechanical stimulation of slow myotubes resulted in a marked 2-fold increase in AChE expression which was also reflected by a general elevation in all molecular forms. Cultures of fast myotubes responded to mechanical stimulation in a similar manner. Fast mouse myotube cultures increased expression of total AChE activity by more than 200%. Mechanical stimulation did not affect the expression of a particular molecular form in these fast myotubes since all molecular forms increased proportionally. Therefore, on the basis of these studies, both fast and slow muscle fibers appear to respond similarly to the effects of mechanical stimulation.

An interesting observation is that these cells exhibited a 2-fold increase in AChE expression whereas primary cultures increased by only 42%. This result is not entirely surprising if one considers the differences in the culture conditions. Primary cultures consist of a mixture of myotubes and fibroblasts. In fact, fibroblasts have been shown to play an essential role in studying the effects of mechanical stimulation in a culture system (see Vandenburgh et al., 1991). Individual muscle fibers are connected together by an extensive array of connective tissue consisting of collagen and other extracellular matrix components secreted by fibroblasts in the culture (Hatfuldy et al., 1989). This extracellular matrix permits the maintenance of contractile myotubes under tension and permits the transmission of externally applied forces to muscle cells (Vandenburgh et al., 1991). Furthermore, it allows the myotubes to develop the structural integrity necessary to withstand repetitive mechanical stimulations without rupturing (Vandenburgh et al., 1988).

It is therefore possible that the extracellular matrix dampened the effects of mechanical stimulation thereby shielding primary cultured myotubes from the mechanogenic stress. The H-2K^b-tsA58 cell cultures lack fibroblasts, and may therefore be subjected to a greater and more intense mechanical stimulation.

4.1.3 Modulation of acetylcholinesterase secretion in response to mechanical stimulation

Under control conditions, cultures of rat myotubes are know to secrete significant amounts of AChE into the medium (Rubin, 1985; Brockman and Younkin, 1986; Bursztajn et al., 1991). In response to mechanical stimulation, this amount increased by 70%. Although our current knowledge regarding protein secretion in response to mechanical stimulation is still limited, increases in the secretion of insulin-like growth factor-1 and insulin-like growth factor binding proteins have been observed in mechanically stimulated skeletal muscle cells (Perrone et al., 1995). Since these proteins are potent mitogens involved in stimulating skeletal muscle growth (for a review see Florini et al., 1991), these studies suggest that mechanical stimulation may additionally act on cells indirectly through paracrine and autocrine mechanisms. This is especially relevant since some of these proteins are concentrated in the vicinity of the neuromuscular junction. In addition, insulin-like growth factors appear to influence muscle development and may also be involved in the sprouting reaction within the neuromuscular system (see Caroni et al., 1994). It is therefore possible that secreted AChE may have an unidentified autocrine or paracrine role in response to mechanogenic forces.

Closer examination of the media from mechanically stimulated cells revealed that the increase in AChE activity was due entirely to an elevated secretion of G_4. These findings are coherent with previous studies indicating that G_4 is the predominant molecular form secreted in the media of rat skeletal muscle fibers (Carter and Brimijoin, 1981; Rubin, 1985). It is unlikely that the increase in AChE levels in the media is due to enhanced degradation of the enzyme. If proteases became more active as a result of mechanical stimulation, a variety of AChE molecular forms would be present in the media as a result of the breakdown of predominant membrane bound forms A_{12} and G_4. Since this is not the case, the elevation in enzyme activity in the media likely reflects an increase in active secretion.

4.1.4 Effects of sodium channel blockade on acetylcholinesterase expression

In vivo, electrical activity has been shown to modulate AChE expression in skeletal muscle fibers by influencing primarily the levels of the asymmetric and G_4 molecular forms (for example see Hall, 1973; Lomo et al., 1985; Boudreau-Larivière et al., 1996A). The electrical component however, consists of a series of consecutive events and therefore it cannot be examined in a simplistic manner. For example, muscle depolarization leads to the opening of voltage-sensitive ion channels, ion influx and efflux, followed by the mechanical component of muscle contraction. To understand the significance of these events, each must be studied separately. Using TTX, a potent Na^+ channel blocker, we uncoupled the effects of electrical activity from those due to mechanical stimulation in cultures of myotubes. TTX was unable to block the increase in AChE expression following mechanical stimulation thereby indicating that the regulatory mechanism involved in the

increased expression of AChE are independent of electrical events. Similarly, TTX treatment had no affect on AChE secretion in mechanically stimulated cultures since AChE levels increased by 67%. TTX treatment of chick skeletal myotubes has, in fact, been shown previously to have no effect of the rate of AChE secretion or on the species of molecular forms secreted (Bursztajn et al., 1991).

The inability of TTX to block the upregulation of AChE enzyme expression following mechanical stimulation suggests indeed that a non-electrical pathway modulates AChE expression in response to mechanogenic forces. These results are consistent with previous findings. For example, TTX has been shown to be unable to block the expression of a variety of molecules in cultured skeletal myotubes subjected to mechanical stimulation including prostaglandins and cyclooxygenase (Vandenburgh et al., 1991; 1995), phospholipase A_2, C and D (Vandenburgh et al., 1993) as well as other proteins required for cell growth and hypertrophy (Hatfaludy et al., 1989; Vandenburgh et al., 1989; 1991). Since TTX treatment of myotubes has been shown previously to decrease selectively the levels of asymmetric forms of AChE (Fernandez-Valle and Rotundo, 1989), we determined whether expression of the asymmetric forms of AChE was also dependent on mechanogenic activity by mechanically stimulating myotubes in the presence of TTX. TTX had no effect on the AChE molecular form profile of non-contracting cultures since they have no asymmetric forms. Mechanical stimulation was unable to induce their reappearance. In contracting myotube cultures, TTX, as expected, had the effect of diminishing the levels of the asymmetric forms (see Fernandez-Valle and Rotundo, 1989). Asymmetric forms did not return upon mechanical stimulation. These results clearly

demonstrate that the regulation of the asymmetric forms is not dependent on passive mechanogenic forces but is only sensitive to electrical activity and the ionic events associated with it. The mechanical stimulation must therefore regulate selectively AChE expression at the level of the globular forms. These conclusions are coherent with results from previous studies which showed that the levels of the asymmetric forms in cultured dysgenic myotubes depends largely on electrical activity per se (see Powell et al., 1986). Total AChE levels in these myogenic cells were found to be normal and to have no apparent deficiency in the content of asymmetric forms (Powell et al., 1986) despite the absence of contractile activity (Powell and Fambrough, 1973). Taken together, these results demonstrate therefore that membrane electrical activity is the key regulator dictating expression of asymmetric forms in muscle.

4.1.5 Involvement of calcium in the acetylcholinesterase response to mechanical stimulation

The influence of Ca^{++} on AChE expression was discovered indirectly through a series of *in vitro* experiments performed by several different investigators. Rieger et al. (1980) determined the effects of electrical activity on AChE expression and demonstrated that TTX treatment led to a pronounced decrease in AChE levels (see also above). To further understand the contribution of Na^{+} channels on AChE regulation, De la Porte and colleges (1984) studied the effects of veratridine, a drug that maintains the Na^{+} channels in an open state. In these experiments, myotube cultures were initially treated with TTX in order to duplicate the effects previously reported by Rieger and colleagues (1980). Subsequent veratridine treatment however, increased AChE enzyme levels dramatically

by selectively upregulating the asymmetric forms (De la Porte et al., 1984). Although both veratridine and elevated extracellular potassium depolarize the cell membrane, only veratridine increased AChE levels in myotubes (Rubin, 1985). These studies therefore indicate that it is the actual Na^+ ion influx and not the depolarization of the membrane per se which affects AChE expression (Rubin, 1985). Knowing that intracellular levels of Ca^{++} underwent a transient increase during muscle contraction, Rubin (1985) speculated that Ca^{++} also regulates AChE expression. Indeed, treatment of TTX-inactivated myotubes with the Ca^{++} ionophore A23187 resulted in an increase in AChE enzyme levels which paralleled that seen with veratridine. Additionally, Bursztajn et al. (1991) found that elevated extracellular Ca^{++} could induce the same effects as that of A23187. The authors further demonstrated that elevated cytoplasmic Ca^{++} levels not only increased the expression of intracellular forms of AChE, but it also increased the secretion of AChE thereby suggesting the existence of a concomitant regulatory mechanism.

Further studies have indicated that L-type Ca^{++} channels play a significant role in the regulation of cellular AChE. Blocking L-type Ca^{++} channels results in a significant decrease in intracellular Ca^{++} levels following depolarization not only by blocking extracellular Ca^{++} entry but also by preventing release from the sarcoplasmic reticulum (Rios and Brum, 1987). Decker and Berman (1990B) demonstrated that blocking these channels with nifedipine resulted in a ~50% decrease in the levels of AChE. Furthermore, they showed that these reductions were incompatible with accelerated protein degradation, enhanced enzyme secretion, or alterations in assembly or processing. The reductions were thought to indicate that nifedipine induced a reduction in AChE biosynthesis since

cell surface or extracellular population of the enzyme were unaffected (Decker and Berman, 1990B). Further experiments have extended the notion that altered levels of intracellular Ca^{++} affect AChE biosynthesis. During myogenesis for example, AChE enzyme levels have been shown to undergo large increases due to stabilization of normally rapidly turning over AChE mRNAs (Fuentes and Taylor, 1993). This selective mRNA stabilization was subsequently found to be a Ca^{++} mediated process involving entry through L-type channels (Luo et al., 1994;1996).

Cells subjected to mechanical stimulation respond to mechanogenic forces by exhibiting a variety of changes including a rise in intracellular Ca^{++} levels (Akai et al., 1994; Yamazaki et al., 1995). This rise in intracellular Ca^{++} occurs quickly, increasing by 50% within 10 min after the onset of stimulation (Akai et al., 1994). Since levels of intracellular Ca^{++} have been shown to be a major modulator of AChE expression, we determined whether Ca^{++} was involved in the signalling pathway. L-type channels were blocked using 10 μM nifedipine and myotubes were mechanically stimulated for 24 hr. Nifedepine was able to partially block (~50%) the increase seen in response to mechanical stimulation. These results suggest that Ca^{++}, indeed, plays a role in modulating AChE expression in response to mechanogenic forces. They additionally demonstrate that although Ca^{++} is involved, it is not the sole signalling pathway since the AChE response was only partially abolished. Additional Ca^{++} influx from other sources other than the L-type channels may thus also participate in the adaptive changes of AChE in response to mechanical stimulation.

An interesting observation is that TTX was unable to block the increase in AChE associated with mechanical stimulation whereas nifedipine partially did. Since the L-type channels are voltage-dependent, one would expect that they remained closed in myotubes treated with TTX thereby resulting in an effect similar to that induced by nifedipine. Our observations however, suggest that L-type channels may nonetheless be activated during mechanical stimulation. This may be due to the mechanogenic forces altering the structure of the channels or generating local ionic currents.

There are three potential sources of Ca^{++} influx during action potentials and muscle contraction: flux through ion channels, release from the sarcoplasmic reticulum, and the Na^{+}/Ca^{++} exchanger (Rubin, 1985). The voltage sensitive L-type Ca^{++} channels have been shown in our studies as well as in others (Decker and Berman, 1990B) to play only a partial role in modulating AChE expression. These studies indicate therefore, that the L-type channels are not the only Ca^{++} entry pathway involved in the AChE response to mechanical stimulation. Membrane depolarization per se, was proven insignificant using elevated potassium (Rubin, 1985) leaving the increased intracellular Na^{+} concentrations as part of the potential signalling pathway in response to mechanogenic forces. In this context, it is known that the Na^{+}/Ca^{++} exchanger reverses its normal Ca^{++} export role during periods of high intracellular Na^{+} concentration in smooth muscle (Gillespie et al., 1992) and other excitable tissues (Lehning et al., 1996 and references therein). This may therefore result in a significant Ca^{++} influx triggering the increased expression of AChE (Rubin, 1985).

A further putative source of Ca^{++} influx could occur through mechano-sensitive ion channels. Mechano-sensitive ion channels have been observed by single-channel recordings in all cells studied to date (Morris, 1990). These channels are linked to the cytoskeleton (Sachs, 1987) and have been shown to be extremely efficient, being capable of passing ions at a rate of 10^5 to 10^6 per second (Sigyrdson et al., 1992 and references therein). These channels may in fact play a significant role in the initial response to mechanotransduction pathway in cells. For example, once activated, these stretch-sensitive channels could change the intracellular Ca^{++} concentration by 20-fold in just milliseconds and may thus be the fastest mechanogenic signalling mechanism (Banes et al., 1995).

Mechano-sensitive ion channels have been suggested as the transduction mechanism between load and protein synthesis in cardiocytes (Kumoro and Yazaki, 1993). These channels allow the passage of a number of ions including Ca^{++}, in response to mechanical stimulation in cardiac cells (Yamazaki et al., 1995). Mechanical stimulation of cardiocytes causes a rapid induction of a number of second messenger pathways including tyrosine kinases, mitogen-activated protein kinases, and protein kinase C (Sadoshima et al., 1992; Kumoro and Yazaki, 1993). These pathways ultimately appear to converge with a resultant induction of immediate-early genes including Egr-1 (Sadoshima et al., 1992; Kumoro and Yazaki, 1993; Yamazaki et al., 1995). Unfortunately, there exists no specific stretch-channel blockers. However, blocking these stretch-sensitive channels non-specifically using gadolinium has been shown to have no effect on the stretch-induced upregulation of immediate early genes in myocytes (Kumoro and

Mouse skeletal muscle fibers have also been shown to contain channels which are sensitive to mechanical forces (Franco and Lansman, 1990A,B). There is evidence which suggests that there are both stretch activating (opening) and inactivating (closing) channels in myotubes (Franco and Landsman, 1990A). To date however, these channels have only been characterized in mouse cultured cells and no studies have shown their existence in adult muscle cells *in vivo*. The lack of a specific stretch-activated Ca^{++} channel blocker prevented us from analyzing their contribution in the overall response of AChE to mechanical stimulation.

4.1.6 Altered levels of acetylcholinesterase mRNAs in response to mechanical stimulation

Mechanical stimulation of cultured cells evokes an increased level of Ca^{++} influx which may signal the increase in expression of AChE. The time course of expression suggests that the mechanism is pretranslational rather than translational (Banes et al., 1995). In this context, we thus examined the levels of AChE transcripts in response to mechanical stimulation. Levels of AChE mRNAs were found to accumulate progressively over the experimental time course reaching a significant 2.3-fold increase over control levels at 24 hr. This accumulation of AChE mRNAs may occur via two mechanisms that are not mutually exclusive. First, there may be a decrease in the rate of degradation of AChE transcripts. Alternatively, the rate of transcription of the AChE gene may be significantly increased. During myogenesis however, AChE mRNA levels have been shown to be regulated by affecting primarily the stability of the message rather than through transcriptional activation of the AChE gene (Fuentes and Taylor, 1993). It is therefore possible to envision that mechanical stimulation of myotubes increased AChE

expression by increasing the half-life of existing transcripts. These results however, do not preclude the involvement of other post-translational regulatory controls such as transcript elongation, polyadenylation and post-transcriptional protein modifications. There is however, no literature to date supporting the notion that these mechanisms play a role in the expression of AChE.

4.1.7 Potential involvement of Egr-1 in the acetylcholinesterase response to mechanical stimulation

An alternate regulatory pathway in response to mechanical stimulation could involve transcription factors and, as already mentioned, the subsequent transcriptional activation of the AChE gene. The expression of many immediate-early genes such as Egr-1, have been shown to be sensitive to the effects of mechanical stimulation (for reviews see Komuro and Yazaki, 1993; Banes et al., 1995; Yamazaki et al., 1995). Furthermore, a recent study has demonstrated that passive stretch can modulate the expression of myogenic regulatory transcription factors in skeletal muscle fibers indicating that this may be a potential mechanism to regulate gene expression (Loughna and Brownson, 1996). Since the rat AChE promoter has a number of binding sites for Egr-1 as well as E-boxes (Chan et al., 1996), it is possible that these trans-activating factors influence AChE expression in response to mechanical stimulation.

In the present study, we therefore examined the effects of mechanical stimulation on Egr-1 mRNA levels. Following cyclic mechanical stimulation, Egr-1 mRNA levels increased rapidly peaking 30 min after the onset of mechanical stimulation. The time course and the magnitude of the Egr-1 mRNA accumulation was similar to that reported

by others in cultured cardiac and kidney mesangial cells using the Flexercell apparatus thus validating our experimental system using cultured myotubes (Sadoshima and Izumo; 1993; Akai et al., 1994). This transient increase in Egr-1 transcripts suggests that they may play a role in modulating AChE gene transcription. Indeed, Li et al. (1993) reported that mutation of this consensus sequence on the AChE promoter results in a marked loss of reporter gene activity in cultured muscle cells. Furthermore, it has been reported that intracellular Ca^{++} levels rise prior to the increase in Egr-1 transcripts (Sadoshima and Izumo; 1993; Akai et al., 1994) suggesting that multiple regulatory mechanisms may be involved in regulating the expression of AChE in response to mechanical stimulation.

Taken together, results from these studies suggest therefore that mechanical stimulation increases Ca^{++} influx and that the long-term effects may occur through pretranslational regulatory mechanisms involving transcriptional activation of the AChE gene or stabilization of existing AChE transcripts Literature to date, however, suggests that the rate of transcription of the AChE gene remains largely unaffected by extrinsic factors with the regulatory mechanism operating at the level of transcript stability (Luo et al., 1994; Chan et al., 1996).

4.2 *IN VIVO* DENERVATED HEMIDIAPHRAGM MODEL

The effects of denervation on AChE expression in skeletal muscle are well documented (Hall, 1973; Vigny et al., 1976; Butler et al., 1978; Davey et al., 1978; Fernandez et al., 1979; Collins and Younkin, 1982; Lomo et al., 1985; Decker and Berman, 1990A; Cresnar et al., 1994; Michel et al., 1994; Boudreau-Larivière, 1996A,B). In most cases, the denervated muscle becomes inactive and is not subjected to the constant cyclical

mechanogenic forces normally experienced during contractile activity. Furthermore, it has been known for many years that tension is a major regulator of skeletal muscle growth and hypertrophy (Vandenburgh, 1992). In this context, the diaphragm represents a unique model. In fact, denervation does not isolate this muscle from mechanogenic forces since the denervated hemidiaphragm is subjected to passive mechanical forces as a result of the contractions of the intact contralateral hemidiaphragm and intercostal muscles during the inspiratory movements of the animal (Zhan et al., 1992).

4.2.1 Validity of the denervated hemidiaphragm as a model of mechanical stimulation

The validity of the denervated hemidiaphragm model to investigate the effects of passive mechanical forces was demonstrated by experiments designed to remove the mechanical forces imposed on the denervated hemidiaphragm. Denervation of the hemidiaphragm results in a temporary hypertrophy of the denervated muscle (Sola and Martin, 1953; Stewart, 1955; Stewart and Martin, 1956; Buse et al., 1965; Feng and Lu, 1965; Gutmann et al., 1966; Manchester and Harris, 1968; Yellin, 1974; Collins and Younkin, 1982; Mamatas and Oja, 1987). Feng and Lu (1965) showed that this transient hypertrophy of the denervated hemidiaphragm could be prevented by a bilateral denervation of the diaphragm. In these experiments, the mechanogenic forces derived from intercostal muscles were insufficient to maintain or increase the mass of the hemidiaphragm muscle. Similar results were found by Gutmann et al. (1966) who, in an elegant experiment, eased the tension imposed on the denervated hemidiaphragm by cutting the ribs supporting the denervated hemidiaphragm. More recently, the denervated hemidiaphragm was shown unequivocally to undergo passive mechanical stretch. Using sonomicrometry, it was demonstrated that

muscle fibers of the denervated hemidiaphragm muscle lengthen passively during the inspiratory-related activation of the intact contralateral side (Zhan et al., 1992). Taken together, these results demonstrate that the denervated hemidiaphragm undergoes passive mechanical stimulation in response to the normal contractile activity of the intact contralateral hemidiaphragm and intercostal muscles. We therefore tested *in vivo* our hypothesis that passive mechanical stimulation of rat skeletal muscle fibers influences AChE expression by denervating the rat hemidiaphragm and examining the effects of repeated cycles of mechanogenic forces on AChE expression. Analysis of the mass of denervated hemidiaphragms and their total RNA content revealed that both parameters underwent a transient increase over the experimental time course. These results are coherent with those previously found by others (see Sola and Martin, 1953; Stewart, 1955; Stewart and Martin, 1956; Buse et al., 1965; Feng and Lu, 1965; Gutmann et al., 1966; Manchester and Harris, 1968; Yellin, 1974; Collins and Younkin, 1982; Mamatas and Oja, 1987) thereby demonstrating that the technical aspects of our study were valid.

4.2.2 Consequence of hemidiaphragm denervation on acetylcholinesterase expression

The effects of denervation on AChE expression in hemidiaphragm muscles were thus examined. AChE enzyme levels were found to drop dramatically at day 5 post-denervation; a response typical of denervated skeletal muscles. By day 10 however, AChE enzyme levels returned towards control levels coherent with previously reported results for both AChE enzyme levels (Collins and Younkin, 1982) and cholinesterase levels (Gutmann et al., 1966). By contrast, Gutmann and colleagues (1966) failed to find a similar magnitude in the recovery of cholinesterase levels in denervated hemidiaphragm muscles. However, they compared

AChE levels in denervated hemidiaphragm to those in the intact contralateral side which may have hypertrophied due to the increased load (Sola and Martin, 1953). The recovery may thus have been masked somewhat by the lack of an appropriate control.

Denervation of skeletal muscle results in a reduced expression of AChE by affecting primarily the levels of the asymmetric forms (Hall, 1973). To determine if the recovery of AChE was due to a selective increase of a particular molecular form, the profiles of hemidiaphragms from day 5 and 30 were examined. Denervation dramatically reduced the levels of asymmetric forms with the content of globular forms remaining close to controls. Although by 30 days post-surgery, there was a recovery in total AChE enzyme levels, the asymmetric forms failed to recover to pre-surgery levels suggesting that the mechanogenic factors capable of increasing total AChE levels are insufficient to elevate expression of the asymmetric forms to control levels. The results from this study therefore illustrate that *in vivo* and *in vitro* mechanical stimulation cannot induce the expression of the asymmetric forms of AChE.

4.2.3 Effects of denervation on acetylcholinesterase mRNA levels in hemidiaphragm muscle

Expression AChE mRNAs is known to be sensitive to the effects of nerve-evoked electrical activity and trophic factors (Michel et al., 1994). To analyze the effects of passive mechanical stimulation, we examined levels of AChE transcripts in denervated hemidiaphragm muscles by quantitative RT-PCR. AChE transcript levels decreased rapidly reaching 60% of control levels by day 5 post-denervation. This response is coherent with the effects of denervation previously seen in hindlimb muscles (Michel et al.,

1994). However, by day 10 and 20, levels of AChE mRNA began to increase steadily returning towards control levels. This effect has never previously been reported. These results demonstrate therefore that the mechanism regulating the recovery of AChE activity affects, in addition, AChE mRNA levels. Furthermore, they are in agreement with those obtained in cultured myotubes subjected to mechanical stimulation since the increase in enzyme levels closely matched that of the transcripts suggesting that the mechanogenic regulatory mechanisms operate at the pretranslational level.

4.2.4 Return to normal breathing patterns after thoracic surgery

Interestingly, the recovery of AChE expression following denervation began ~5 days post-surgery. This delay is possibly linked to the physiological effects associated with thoracic surgery. Indeed, normal breathing patterns do not return following thoracic surgery for ~10 days (Dureuil et al., 1986; Easton et al., 1989; Torres et al., 1989). These results suggest therefore that the mechanical stimulation required to increase AChE expression does not reach threshold values until shallow breathing patterns are replaced with normal ones thereby leading to the imposition of passive mechanical stimulation on the denervated hemidiaphragm muscles.

4.3 FUNCTIONAL IMPLICATIONS OF MECHANICAL STIMULATION ON ACETYLCHOLINESTERASE EXPRESSION

Adult skeletal muscle fibers are dependent on the influence of motoneurons to modulate their AChE content. Altering AChE expression in response to passive mechanical stimulation however, confers an additional mechanism, independent of the nerve, by which AChE levels can be modified. A recent study has in fact demonstrated

that the neuromuscular synapse undergoes intense mechanical distortions during cycles of muscle contraction and relaxation (Santo Neto et al., 1996). Given that AChE expression in this region is sensitive to extrinsic factors, mechanical stimulation may therefore represent an additional regulatory mechanism.

It is plausible that this regulatory pathway has more important ramifications during embryogenesis and myogenesis since during differentiation of skeletal muscle, developing fibers are likely subjected to the effects of passive mechanical forces as a result of the growth of the bones (Stewart, 1972). Thus, AChE expression may be initially at basal levels due to intrinsic signals within the myogenic cells. However, in response to bone-evoked passive mechanical stimulation, AChE expression may increase in anticipation of the maturation process preparing myofibers for the arrival of exploratory axons. Finally, after neural contact and synapse formation, nerve-evoked electrical activity and nerve-derived trophic factors fine-tune the intrinsic muscle expression pattern yielding the levels and molecular form patterns seen in adult skeletal muscle.

4.4 PERSPECTIVES AND FUTURE STUDIES

Although these studies clearly indicate that mechanical stimulation modulates AChE expression in skeletal muscle fibers, future work needs to address the specific signalling pathways involved in the process of mechanotransduction.

Our studies indicate that the increase in AChE expression likely occurs via a pretranslational regulatory mechanism. In this context, it is possible that mRNA stability and/or transcriptional activation of the gene could account for the increased expression.

does not preclude the possibility that AChE transcripts are being stabilized. This second possibility may be elucidated by measuring the half-life of AChE mRNAs and determine whether it changes in response to mechanical stimulation.

If AChE increases transcription following mechanical stimulation one would next determine the factors modulating these changes. The AChE gene has a number of regulatory elements which may play a role in modulating its expression. Three however, are of particular interest since their expression is known to be sensitive to the effects of mechanical stimulation. These trans-activating factors, Egr-1, MyoD and SSRE, may activate the AChE gene by binding to their respective consensus sequences thereby increasing the transcriptional rate. Determining the contribution of these sites may be achieved by expression studies *in vitro* using AChE promoter-reporter gene constructs in which these binding sites have been mutated.

Mechano-sensitive ion channels remain currently an attractive mechanism for signalling mechanogenic events in skeletal muscle fibers. The future availability of ion specific channel blockers would allow the contribution of these putative signalling pathways to the overall response of AChE to be elucidated. Studies in other cell types have suggested that the effects of mechanical forces may be mediated through an autocrine or paracrine mechanism (see Kumoro et al., 1991; Banes et al., 1996). Treating control myotube cultures with conditioned media from mechanically stimulated myotubes would determine whether this mechanism plays a role in our system.

Finally, several studies in skeletal muscle fibers have shown that mechanical stimulation modulates the expression of a number of proteins associated with cell growth

Finally, several studies in skeletal muscle fibers have shown that mechanical stimulation modulates the expression of a number of proteins associated with cell growth and hypertrophy. This study is the first to demonstrate directly that the expression of a synaptic protein is modulated in response to mechanical forces. In this context, it would be also of interest to determine whether the expression of other synaptic proteins is altered in response to mechanical stimulation.

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